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Preface

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The impetus for a special issue on Human Impact and Chemical Ecology originated from a talk by David Wood at the 25th Anniversary Meeting of the International Society of Chemical Ecology held at Pennsylvania State University in 2008. In Professor Wood's historical review of the field of chemical ecology, he touched on many themes. They included reminders that: we would celebrate the 50th anniversary (in 2009) of the discovery of the first insect pheromone (the silk moth) by Butenandt and colleagues and the first use of the term "pheromone" by Karlson and Lüscher in the same year; the Journal of Chemical Ecology would celebrate its 35th Anniversary in 2010; from its origins in insect semiochemicals, Chemical Ecology has advanced into new frontiers as exemplified by previous specialized issues of the Journal devoted to such topics as Allelopathy (2000), Aquatic Chemical Ecology (2002), Molecular Chemical Ecology (2004), Mammalian Chemical Ecology (2006), and Olfactory Ecology (2008); and finally and importantly that the discoveries made under the broad umbrella of Chemical Ecology must continue to serve and benefit human survival.

Via an exchange of cocktail napkins during and after David's address, Wilhelm Boland and I came up with a working list identifying current topics of focus in Chemical Ecology that directly impact human well-being and survival. The subsequently seven solicited review papers gathered here cover a broad spectrum of topics that range from the challenges of global change, invasive species, and pollution, to disease vectors. Among the four selected contributed papers are two that relate to plant chemistry (global change and invasives), one to pheromone disruption, and one to the protective aspects of geophagy.

We could not address all aspects of human impact within our various sub-disciplines. Many facets that likely deserve review, but are missing, include obvious ones: benthic and pelagic effects in aquatic systems, such as coral reef deterioration and red tides; diminishing populations of beneficial insects, such as honey bees; and accelerated geographic speciation and extinctions.

Notwithstanding, the topics included here ought to give one pause to reflect on: our collective global concerns and challenges; the achieved modest successes in combating many of our self-created problems; and the hope that the future will be better for our efforts. We express our thanks both to the authors of the invited review articles, all of whom claimed it to be a difficult but rewarding task, as well as to the authors of the contributed papers. We hope that readers of the Journal will find the issue enlightening, and provoking, and that perhaps it may serve to focus future research.

John T. Romeo
Editor, JCE

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REVIEW ARTICLE

Impacts of Elevated Atmospheric CO_2 and O_3 on Forests: Phytochemistry, Trophic Interactions, and Ecosystem Dynamics

Richard L. Lindroth

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Abstract Prominent among the many factors now affecting the sustainability of forest ecosystems are anthropogenically-generated carbon dioxide (CO_2) and ozone (O_3). CO_2 is the substrate for photosynthesis and thus can accelerate tree growth, whereas O_3 is a highly reactive oxygen species and interferes with basic physiological functions. This review summarizes the impacts of CO_2 and O_3 on tree chemical composition and highlights the consequences thereof for trophic interactions and ecosystem dynamics. CO_2 and O_3 influence phytochemical composition by altering substrate availability and biochemical/physiological processes such as photosynthesis and defense signaling pathways. Growth of trees under enriched CO_2 generally leads to an increase in the C/N ratio, due to a decline in foliar nitrogen and concomitant increases in carbohydrates and phenolics. Terpenoid levels generally are not affected by atmospheric CO_2 concentration. O_3 triggers up-regulation of antioxidant defense pathways, leading to the production of simple phenolics and flavonoids (more so in angiosperms than gymnosperms). Tannins levels generally are unaffected, while terpenoids exhibit variable responses. In combination, CO_2 and O_3 exert both additive and interactive effects on tree chemical composition. CO_2-and O_3-mediated changes in plant chemistry influence host selection, individual performance (development, growth, reproduction), and population densities of herbivores (primarily phytophagous insects) and soil invertebrates. These changes can effect shifts in the amount and temporal pattern of forest canopy damage and organic substrate deposition. Decomposition rates of leaf litter produced under elevated CO_2 and O_3 may or may not be altered, and can respond to both the independent and interactive effects of the pollutants. Overall, however, CO_2 and O_3 effects on decomposition will be influenced more by their impacts on the quantity, rather than quality, of litter produced. A prominent theme to emerge from this and related reviews is that the effects of elevated CO_2 and O_3 on plant chemistry and ecological interactions are highly context- and species-specific, thus frustrating attempts to identify general, global patterns. Many of the interactions that govern above- and below-ground community and ecosystem processes are chemically mediated, ultimately influencing terrestrial carbon sequestration and feeding back to influence atmospheric composition. Thus, the discipline of chemical ecology is fundamentally important for elucidating the impacts of humans on the health and sustainability of forest ecosystems. Future research should seek to increase the diversity of natural products, species, and biomes studied; incorporate long-term, multi-factor experiments; and employ a comprehensive “genes to ecosystems” perspective that couples genetic/genomic tools with the approaches of evolutionary and ecosystem ecology.

Keywords CO_2 · Decomposition · Ecosystem processes · Forest · Global change · Herbivory · Human impacts · Nutrient cycling · Ozone · Phenolics · Phytochemistry · Plant-insect interactions · Tannins · Terpenoids

Introduction

Forest ecosystems have played roles of unparalleled importance in the evolution and ecology of life on Earth. Currently, forests cover ~30% of the earth’s land surface, store ~45% of terrestrial carbon, and contribute ~50% of

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R. L. Lindroth (✉)
Department of Entomology, University of Wisconsin,
Madison, WI 53706, USA
e-mail: lindroth@entomology.wisc.edu

terrestrial net primary production (Bonan 2008). They provide vital ecological, economic and social services, including regulation of biogeochemical cycles, production of food and fiber, preservation of biodiversity, governing of climate systems, and buffering against natural and anthropogenic disasters (Hassan et al. 2005; Millenium Ecosystem Assessment 2005).

Earth's forests are now subject to a veritable Pandora's Box of environmental evils, the magnitude and diversity of which have never occurred previously in human history. Worldwide, climate and land use changes, invasive species, pest outbreaks, and pollution are exacting an increasing toll on the health, biological diversity, and sustainability of forests (Laurence and Peres 2006; Hari and Kulmala 2008; Kurz et al. 2008; Raffa et al. 2008; van Mantgem et al. 2009). Against this backdrop of massive and seemingly intractably complex socio-environmental ills, the question arises: "of what relevance is chemical ecology?"

In a word, plenty. Naturally-produced chemicals, particularly phytochemicals, govern many, if not most, of the interactions that characterize community and ecosystem function. They *respond to* global environmental change; they *perpetuate*, via interaction networks, the consequences of global change; and they *feed back* to influence future global change. Consider, for example, the carbon cycle (Fig. 1). Enriched atmospheric CO_2 influences plant physiology, with consequences for plant production and chemical composition. Plant chemical composition in turn influences trophic interactions and decomposition, which ultimately feed back to affect atmospheric CO_2 concentrations (Peñuelas and Estiarte 1998; Beedlow et al. 2004; Lindroth and Dearing 2005). Or, consider the isoprenoids and related biogenic volatile organic carbons (BVOCs) released in large quantities by some species of trees (e.g., eucalypts, poplars, and conifers). These compounds are precursors for the formation of tropospheric ozone (O_3), which in turn negatively impacts plant production (Fig. 1) and human health (Fowler et al. 1999; Lerdau and Gray 2003; Monson 2003; Laothawornkitkul et al. 2009). An understanding of the roles of natural products as responders to, and effectors of, global change will improve our ability to predict ecosystem responses to future change, and inform management decisions with respect to mitigation and adaptation strategies.

Analogous in some respects to intestinal villi, forest trees provide structurally expansive and heterogeneous surfaces through which the exchange of chemicals between the atmosphere and biosphere is effected. The same properties enhance exposure of forest trees to gaseous pollutants. Two such gases, CO_2 and O_3, are considered the most important and ubiquitous, affecting forests worldwide (Saxe et al. 1998; Fowler et al. 1999; Felzer et al. 2004; Karnosky et al. 2005, 2007). Independent of their function as greenhouse gases, CO_2 and O_3 are directly and indirectly affecting forest ecosystems now, with increasingly pronounced impacts predicted for the future (Fig. 2). (Note that although both CO_2 and O_3 occur naturally, they are appropriately considered "pollutants" because of their anthropogenic production and effects on living systems.)

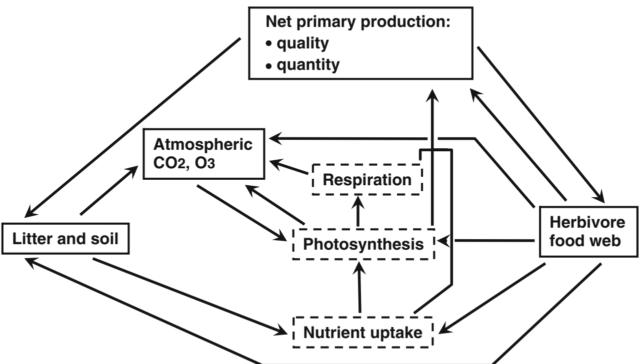


Fig. 1 Carbon cycling and storage in forest ecosystems. Solid boxes represent major pools; dashed boxes represent major plant physiological processes (adapted from Lindroth and Dearing 2005)

The purpose of this review is to assess the impacts of enriched atmospheric CO_2 and O_3, independently and interactively, on the chemical ecology of forest ecosystems. Several recent reviews provide an assessment of the effects of CO_2 and O_3 on plant chemistry; that information will be summarized and updated here. I will then explore the consequences of CO_2- and O_3-mediated changes in tree chemistry for trophic interactions (plant—animal—natural enemy) and ecosystem dynamics (rates of plant damage, organic substrate deposition and decomposition) (Fig. 1). I will not detail the links between ozone and plant volatiles, as those have been covered in several very recent reviews, including elsewhere in this issue (Laothawornkitkul et al. 2009; Pinto et al. 2010; Yuan et al. 2009). Finally, although CO_2 and O_3 are both greenhouse gases, the effects of climate change per se on ecological interactions (e.g., Bale et al. 2002; Parmesan 2006; Berggren et al. 2009; Dukes et al. 2009; Pelini et al. 2009) are beyond the scope of this review.

Temporal Trends in Concentrations of CO_2 and O_3

Concentrations of atmospheric CO_2 now approach 386 ppm, higher than at any time in the last 26 million years (Pearson and Palmer 2000) and 40% higher than at the dawn of the industrial revolution. Emissions scenarios predict a range of substantially elevated concentrations by the end of this century, from a low of 550 ppm to a high of 900 ppm (Karl et al. 2009). Over the last several decades, combustion of fossil fuels, coupled with a smaller contribution from manufacture of cement, have accounted for

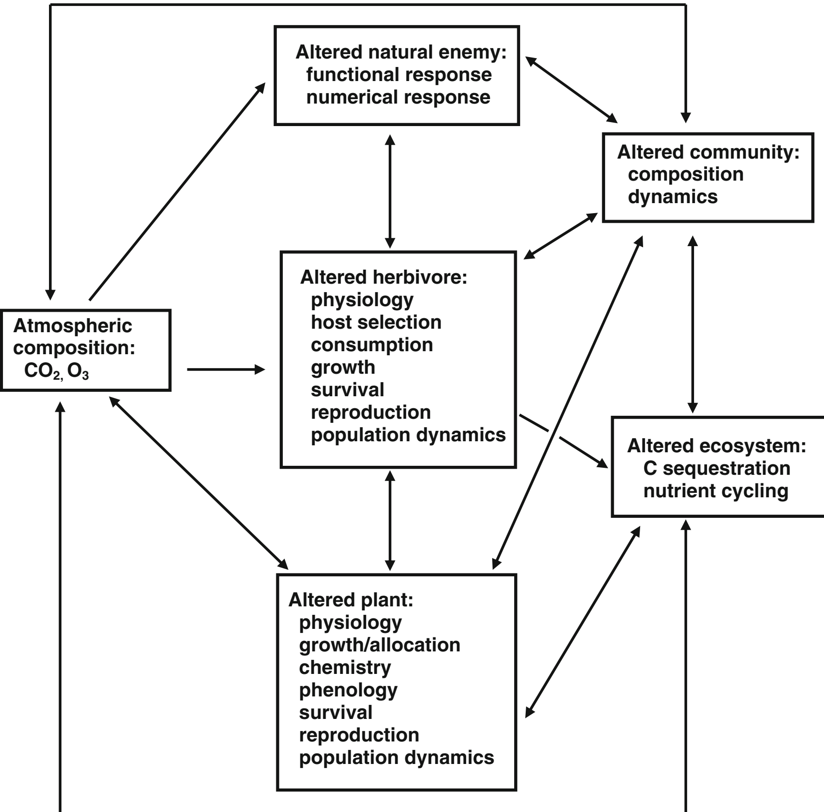


Fig. 2 Complex of interacting factors that feed forward and feed back to influence food webs, community and ecosystem structure and function, and atmospheric composition

approximately 80% of human-caused emissions. The remaining 20% has resulted from changes in land use, primarily deforestation (Denman et al. 2007; Karl et al. 2009).

Since the evolution and spread of large, vascular plants, about 380 MYA, forests have played prominent, regulating roles with respect to atmospheric CO_2 concentrations. They were largely responsible for the massive drawdown of atmospheric CO_2 (peaking at upwards of 5,000 ppm) in the late Devonian and Carboniferous Periods. Forests exerted two major effects on the global carbon cycle, both of which reduced atmospheric CO_2 concentrations (Berner 2005). First, they produced massive amounts of lignified tissue that was refractory to decomposition. This organic material, upon sedimentary burial, led to the formation of enormous coal deposits. Second, large and deep root systems greatly accelerated nutrient uptake from rock substrates, enhancing the weathering of Ca-Mg silicate minerals. Ca and Mg cations, in the presence of carbonic acid (derived from atmospheric CO_2 and water), formed Ca-Mg carbonates, which precipitated in ocean sediments to form limestone and dolomite.

Tropospheric ozone is recognized as the most damaging and widespread pollutant affecting agricultural and forest ecosystems in North America and Europe (Chameides et al. 1994; Ollinger et al. 1997; Skärby et al. 1998; Fuhrer and Booker 2003; Ashmore 2005; Karnosky et al. 2007; Wittig et al. 2009). Concentrations of O_3 have increased an average of 38% (range of 20–50%) since the pre-industrial age (Denman et al. 2007). Although O_3 is a regional pollutant, large areas of the earth already are impacted, background levels are steadily increasing, and 60% of the world's forests are expected to be affected detrimentally by the end of this century (Fowler et al. 1999; Vingarzan 2004; Denman et al. 2007).

O_3 is a secondary pollutant, produced via the catalytic oxidation of hydrocarbons (e.g., VOCs) by OH radicals, in the presence of nitrogen oxides and sunlight (Wennberg and Dabdub 2008). Because O_3 is highly reactive, it persists for short periods (hours to several weeks) in the atmosphere. Rapid degradation, coupled with substantial spatial and temporal variation in production, contribute to highly variable distribution of this pollutant. Regional VOC and NO_x emission control policies are expected to reduce peak O_3 levels in North American and Europe, whereas in Asia and other regions, emissions of these precursors are increasing (Ashmore 2005). Moreover, background levels of O_3 (not attributable to local origin) are increasing steadily worldwide (Vingarzan 2004).

Plant-derived, biogenic VOCs can be important contributors to the formation of O_3 (Sharkey and Lerdau 1999; Lerdau and Gray 2003; Laothawornkitkul et al. 2009; Yuan et al. 2009). When BVOCs such as isoprene, monoterpenes or methylbutenol are oxidized in the presence of NO_x,

substantial amounts of O_3 can be produced. Indeed, large inputs of BVOCs may handicap efforts to reduce ozone pollution through controlling emissions of anthropogenic VOCs. Moreover, ecological interactions may amplify ozone production. A modeling study by Litvak et al. (1999) indicated that even modest (10%) damage by defoliating insects in coniferous forests is sufficient to increase local concentrations of O_3.

In contrast, in the presence of low NO_x concentrations, BVOCs are oxidized by O_3, leading to a decrease in atmospheric O_3 and a concomitant increase in secondary organic aerosols (Laothawornkitkul et al. 2009). Thus, forest ecosystems can serve as effective quenchers of anthropogenic O_3, but in the process contribute to atmospheric aerosol loading that influences climate. Kurpius and Goldstein (2003) documented that for a *Pinus ponderosa* forest during summer, 51% of atmospheric O_3 is broken down via gas phase chemistry (reactions with BVOCs), 30% is lost to stomatal uptake, and 19% is lost to surface deposition.

Effects of CO_2 and O_3 on Plant Physiology and Growth

Carbon Dioxide

As the substrate for photosynthesis, and ultimate source of all carbon fixed via primary production, atmospheric CO_2 strongly influences plant physiology and growth. Photosynthesis is catalyzed by Rubisco (ribulose-1,5-bisphosphate carboxylase oxygenase), which fixes carbon to form carbohydrates. In C3 plants, including most forest tree species, enriched CO_2 atmospheres increase the intracellular CO_2:O_2 ratio, thus reducing photorespiration (oxygenation) and increasing photosynthesis (carboxylation). Carbohydrates then are transported throughout the plant and subsequently converted to various carbon-containing compounds required for plant metabolism, structure, storage and defense (Fig. 3).

The effects of elevated atmospheric CO_2 on tree physiology and growth have been covered in numerous reviews (e.g., McGuire et al. 1995; Saxe et al. 1998; Long et al. 2004; Ainsworth and Long 2005; Norby et al. 2005; Körner 2006; Leakey et al. 2009). In brief, N use efficiency improves as the N allocated to Rubisco (the largest single pool of N in leaves) can be re-allocated to other metabolic processes. Stomatal conductance generally declines, decreasing transpiration and improving water use efficiency. Enhanced photosynthesis, coupled with improved nitrogen and water use efficiencies, often accelerates plant growth, i.e., the “fertilizer effect” of enriched CO_2 (Beedlow et al. 2004). The growth stimulation afforded by enriched CO_2 is especially strong in young forest stands, characterized by abundant soil resources and open canopies. In contrast, studies in mature stands, where tree growth is tightly coupled to soil nutrients and canopies are closed, indicate smaller to zero growth enhancement (Asshoff et al. 2006; Körner 2006).

A key, unresolved issue in global change biology is to what extent improved tree growth can be sustained into the future, affording greater carbon sequestration and thereby mitigating climate change. To date, open-air FACE studies generally indicate that accelerated rates of photosynthesis and growth are sustained over the short to medium term and across a broad range of productivity (Long et al. 2004; Norby et al. 2005; Huang et al. 2007; but see Körner et al. 2005 for a contrary example with mature trees). Still, the duration of these studies is short compared with the lifespan of trees. The Progressive Nitrogen Limitation (PNL) hypothesis (Luo et al. 2004) posits that CO_2-enhanced growth and carbon sequestration will diminish over time: as storage of N in plant biomass and soil organic matter increases, available soil N becomes limiting to growth. If not nitrogen, then a host of other factors (climate change, fire, insects, ozone, etc.) is predicted to constrain the fertilization effect of atmospheric CO_2 enrichment (Beedlow et al. 2004; Huang et al. 2007). For example, Cole et al. (2009) reported that atmospheric CO_2 enrichment over the last 50 years has increased growth rates of natural stands of *Populus tremuloides* by an astonishing 53%, but enhancement is diminished in periods of low precipitation. Finally, elevated atmospheric CO_2 may, via climate change impacts, ultimately lead to reduced carbon sequestration at the landscape level. In Canada, for example, extensive areas of forest recently have transitioned from CO_2 sinks to CO_2 sources, due to insect outbreaks and fires (Kurz et al. 2008).

Ozone

Reactions of tropospheric O_3 with plant chemistry can occur in gaseous, solid, or liquid phases (Fuhrer and Booker 2003). Gaseous reactions with plant VOCs are described by Pinto et al. (2010). Solid phase reactions may occur, for example, with plant cuticular components. The most important reactions for plants, however, occur in the liquid phase, after ozone has entered through the stomata and diffused into the aqueous media of interstellar spaces (Fuhrer and Booker 2003).

O_3 elicits a cascade of damaging physiological consequences in plants, described in detail in numerous recent reviews (e.g., Andersen 2003; Fuhrer and Booker 2003; Ashmore 2005; Kangasjärvi et al. 2005; Wittig et al. 2007, 2009). Because it is so highly reactive, O_3 is minimally distributed following dissolution into intercellular fluids. It reacts with lipid and protein components of cell walls and plasma membranes, leading to the formation of aldehydes, peroxides and assorted reactive oxygen species (ROS). These products

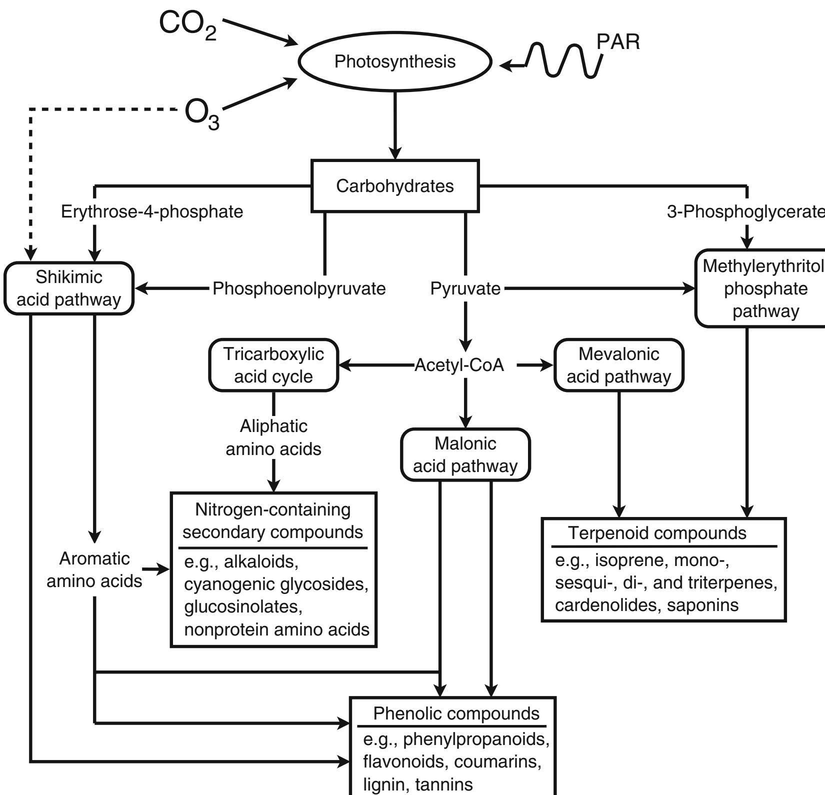


Fig. 3 Biosynthetic pathways for the production of the major classes of plant secondary compounds (adapted from Taiz and Zeiger 2002). Dashed lines indicate a control (signal transduction) pathway. PAR = photosynthetically active radiation

can then cause cell death, or activate various transduction pathways for defense responses, such as stomatal closure, production of anti-oxidants such as ascorbate and phenolics, and programmed cell death (a component of the hypersensitive response) (Fuhrer and Booker 2003; Valkama et al. 2007; Fig. 3). Reduced stomatal conductance, compromised photosynthesis, consumption of carbohydrates in localized tissue repair, and inhibition of phloem transport at the leaf level combine to decrease the supply of carbohydrates throughout the plant (Andersen 2003; Wittig et al. 2007, 2009). As a consequence, senescence is accelerated and whole-plant growth is inhibited.

A recent, comprehensive meta-analysis by Wittig et al. (2009) evaluated the effects of O_3 on the growth of trees representative of northern temperate and boreal forests. They found that, relative to preindustrial levels, current levels of tropospheric O_3 account for an average 7% reduction in tree biomass growth. Moreover, as O_3 levels continue to rise, further decreases of 11% and 17% are predicted for 2050 and 2100, respectively.

Understanding of the interactive effects of CO_2 and O_3 on tree growth is critically important to our ability to predict the efficacy of forests to serve as global carbon sinks in the future (Beedlow et al. 2004). A modeling study by Sitch et al. (2007) indicated that tropospheric O_3 may reduce terrestrial carbon sinks, and thereby effect indirect radiative forcing of climate change. This conclusion was subsequently corroborated by the work of Wittig et al. (2009), which indicated increasingly pronounced reductions in tree growth under elevated concentrations of O_3 in the future. To date, only one experiment has addressed the long-term (10+ years) effects of independent and combined CO_2 and O_3 treatments on forest trees. Karnosky et al. (2005) reported that at Aspen FACE (Wisconsin, U.S.A.), O_3 reduced the growth-enhancement effect of CO_2, although responses differed among tree species.

Effects of CO_2 and O_3 on Phytochemistry

CO_2 and O_3 directly and indirectly influence carbon assimilation and nutrient (e.g., N) acquisition, and thereby the pools and fluxes of key precursors for the production of secondary metabolites (Fig. 3). They also may influence the signal transduction pathways that govern gene expression and subsequent synthesis of secondary compounds. Not surprisingly then, growth under CO_2- or O_3-enriched atmospheres typically alters the chemical composition of forest trees.

Numerous reviews have addressed the effects of CO_2 and O_3 on plant chemistry (Kangasjärvi et al. 1994; Peñuelas et al. 1997; Bezemer and Jones 1998; Cotrufo et al. 1998a; Koricheva et al. 1998a; Peñuelas and Estiarte 1998; Norby et al. 2001; Zvereva and Kozlov 2006; Stiling and Cornelissen 2007; Valkama et al. 2007; Bidart-Bouzat and Imeh-

Nathaniel 2008; Wittig et al. 2009). Unless noted otherwise, the general trends for forest trees, summarized below, are drawn from that comprehensive set of reviews. Specific studies will be mentioned to illustrate particular patterns or highlight recent findings.

To date, research on the effects of CO_2 and O_3 on forest trees has been restricted to a small set of primarily temperate species. Most commonly studied are species of *Acer*, *Betula*, *Fagus*, *Pinus*, *Populus*, and *Quercus*. Although these are prominent, if not "foundation," genera, a major challenge to the global change research community is to broaden the taxonomic and geographic representation of trees, especially tropical species.

Carbon Dioxide

Enriched atmospheric CO_2 produces generally consistent effects on concentrations of primary metabolites in trees (Cotrufo et al. 1998a; Koricheva et al. 1998a; Saxe et al. 1998; Stiling and Cornelissen 2007). Foliar concentrations of nitrogen typically decline (on average, 14–17% relative to ambient CO_2), due to reductions in Rubisco concentrations and dilution by accumulating carbohydrates. Levels of simple sugars may or may not increase, whereas levels of starch generally increase substantially.

Studies of the effects of CO_2 on secondary metabolites in trees have been restricted to "carbon-based" compounds, particularly phenolics and terpenoids. Consequently, this research often has been framed in the context of source-sink balance hypotheses [e.g., carbon-nutrient balance hypothesis (Bryant et al. 1983), growth-differentiation balance hypothesis (Herms and Mattson 1992)]. These hypotheses predict that elevated CO_2 will increase plant concentrations of carbon-based secondary compounds, especially if soil nutrient availability is limiting to growth (Peñuelas and Estiarte 1998).

In general, results have not been as readily predictable as the hypotheses would suggest (reviewed in Koricheva et al. 1998a; Peñuelas and Estiarte 1998; Zvereva and Kozlov 2006; Stiling and Cornelissen 2007; Bidart-Bouzat and Imeh-Nathaniel 2008). Moreover, the shikimic acid pathway, leading to production of phenolics, is more strongly influenced by CO_2 levels than are the mevalonic acid and methylerythritol phosphate pathways, leading to production of terpenoids (Fig. 3). Enriched atmospheric CO_2 typically, but not invariably, increases tannin concentrations. Common, but less consistent, are increases in simple phenolics, including phenolic acids and glycosides (e.g., salicylates). Growth under elevated CO_2 also tends to increase lignin concentrations in tree leaves (Coûteaux et al. 1999; Norby et al. 2001), although more recent results, from long-term FACE studies, have shown no such effects (Finzi et al. 2001; Parsons et al. 2008; Liu et al. 2009). Overall, effects of CO_2 on terpenoid concentrations are minimal; although a few studies have revealed increases, most have found no change or decreases in concentrations.

The C/N ratio is a widely recognized, general index of overall tissue quality, relevant to both herbivory and decomposition. Enriched CO_2 atmospheres almost invariably increase C/N ratios in tree foliage (Coûteaux et al. 1999), due to a combination of decreased N concentrations and increased carbohydrate and phenolic concentrations.

Aside from such general indices, however, empirical studies have frustrated attempts to develop general, predictive models of the impacts of CO_2 on plant secondary chemistry. In short, a host of interacting factors have, over evolutionary time, combined to determine the chemical profiles of trees, and the effects of any one environmental factor can be masked by the direct or interactive effects of others.

One of the major confounding factors is taxon-specificity. Phytochemical responses of trees to CO_2 enrichment differ at the level of class (e.g., gymnosperm vs. angiosperm), species, and genotype (Julkunen-Tiitto et al. 1993; Lindroth et al. 2001, 2002; Zvereva and Kozlov 2006; Stiling and Cornelissen 2007; Bidart-Bouzat and Imeh-Nathaniel 2008). These differences represent taxonomic variation in evolutionary strategies that optimize solutions for the conflicting demands of carbon allocation toward growth, reproduction, and defense. For example, Cseke et al. (2009) recently compared leaf transcription profiles, physiology, and biochemistry between CO_2-responsive and unresponsive clones of *Populus tremuloides* grown at Aspen FACE. They found that the CO_2-responsive clone partitions carbon into pathways associated with "active" defense and stress responses, carbohydrate synthesis, and subsequent growth, whereas the unresponsive clone partitions carbon into pathways associated with cell wall compounds such as lignin, derived from the shikimic acid pathway.

Interactions with abiotic environmental factors also influence chemical responses of trees to CO_2. The availability of key resources (e.g., nutrients, water, light) can strongly shape plant chemical responses to CO_2, but, again, the magnitude and even direction of responses differ among chemical constituents and tree species (e.g., Lavola and Julkunen-Tiitto 1994; Kinney et al. 1997; McDonald et al. 1999; Booker and Maier 2001; Coley et al. 2002; Koike et al. 2006). Of particular importance in the context of global environmental change are potential interactions between atmospheric CO_2 and temperature. To that end, Zvereva and Kozlov (2006) conducted a meta-analysis of studies that assessed the simultaneous elevation of CO_2 and temperature on plant quality. Again, results were compound- and taxon-specific. Thus, responses to enriched CO_2 may be: 1) independent of temperature (e.g., foliar nitrogen and phenolics in Angiosperms); 2) offset by elevated temperature

(e.g., carbohydrates; foliar terpenes in gymnosperms); or 3) apparent only with elevated temperature (e.g., foliar nitrogen in gymnosperms, phenolics, and terpenoids in woody tissues). More recently, Veteli et al. (2007) reported for three tree species (*Betula* and *Salix*) that carbon allocation to phenolics was increased under elevated CO_2, decreased under elevated temperature, and unchanged under a combination of elevated CO_2 and temperature.

Interactions with biotic agents also may influence chemical responses of trees to CO_2, although much less research has been conducted with biotic than with abiotic factors. Herbivory frequently elicits induced chemical changes in trees, particularly in levels of phenolics (Nykänen and Koricheva 2004). Thus, enriched CO_2 atmospheres have been predicted to enhance induced chemical responses. Evidence to date, however, suggests that CO_2 does not modify induced chemical responses to defoliation in trees, although only a few studies have been conducted (Lindroth and Kinney 1998; Roth et al. 1998; Agrell et al. 1999; Rossi et al. 2004; Hall et al. 2005; Huttunen et al. 2008).

A final factor complicating attempts to draw general conclusions about the effects of CO_2 on tree chemistry is ontogeny (genetically-determined developmental changes). Many species of trees exhibit strong ontogenetic shifts in chemical composition (e.g., Bryant and Julkunen-Tiitto 1995; Donaldson et al. 2006), which reflect shifting physiological demands and defense strategies as trees mature (Boege and Marquis 2005). How ontogeny may interact with CO_2 to affect chemical profiles in trees is unknown. Such interactions may be one reason, however, for why chemical responses of plants in long-term FACE studies appear to be less pronounced than in short-term pot studies (Ainsworth and Long 2005; Lindroth, unpublished data).

Ozone

Elevated levels of tropospheric O_3 can modify plant chemical composition via several mechanisms. By disrupting photosynthesis, O_3 inhibits production of carbohydrates and the flow of precursors into pathways of primary and secondary metabolism (Fig. 3). Ozone also functions as a general abiotic elicitor of defense signaling pathways, particularly of phenolic compounds (Kangasjärvi et al. 1994; Heath 2008; Betz et al. 2009a). The two mechanisms may, of course, interact, as reductions in carbohydrate precursors for the shikimic acid pathway may constrain induced defensive responses. Moreover, these mechanisms may play out differently over different time scales, resulting in varying responses to acute versus chronic ozone stress.

The effects of O_3 on levels of primary metabolites vary among compounds and tree species. Levels of nutrients (e.g., N, P, K, Ca) may increase, decrease, or not change in response to O_3 fumigation. Levels of simple sugars generally are unaffected, whereas levels of starch typically decline in angiosperms, but not gymnosperms.

As is true for CO_2, studies of the effects of O_3 on secondary compounds in trees have been restricted almost entirely to phenolics and terpenoids. In general, elevated O_3 leads to increases in concentrations of phenolic acids and flavonoids in angiosperms but not in gymnosperms, and no overall change in concentrations of tannins. Foliar lignin concentrations also are generally unresponsive to O_3 fumigation (Boerner and Rebbeck 1995; Booker et al. 1996; Liu et al. 2005; Oksanen et al. 2005). Effects of O_3 on terpenoid levels have been studied in only a few tree species, and results are species-specific: increases in *Pinus sylvestris* and *Populus* species, and no change in *Picea abies* (Valkama et al. 2007; Blande et al. 2007). Moreover, the magnitude of response varies among types of terpenes, with diterpenes responding more strongly to O_3 than mono- or sesquiterpenes.

That O_3 elicits increases in the levels of some carbon-based secondary metabolites without corresponding increases in carbohydrates runs counter to the predictions of source-sink balance hypotheses (e.g., Bryant et al. 1983), and may be explained by the fact that ozone triggers up-regulation of antioxidant defense systems linked to the shikimic acid pathway (Valkama et al. 2007). Ozone causes oxidative damage, coupled to the proliferation of oxygen radicals. Gene transcription and activity of numerous enzymes in the shikimic acid pathway are elevated in response to O_3 exposure, leading to the production of antioxidants such as flavonoids and other simple phenolics, and shifts in the monomeric composition of lignin (Kangasjärvi et al. 1994; Heath 2008; Betz et al. 2009a, b). Indeed, ozone-induced phenolics have been considered potential bioindicators of O_3 stress in plants under natural conditions (Sager et al. 2005; Bidart-Bouzat and Imeh-Nathaniel 2008).

Ozone exposure also influences the amount and chemical composition (e.g., alkyl esters and fatty acids vs. alkanes and alkanols) of cuticular waxes in tree leaves (Karnosky et al. 2002; Kontunen-Soppela et al. 2007; Percy et al. 2009). These changes in turn determine the physical structure of the cuticular surface, with repercussions for leaf surface properties such as wettability.

In contrast to a large body of research with CO_2, little work has addressed how O_3 pollution interacts with other environmental factors to shape plant chemical profiles. Lindroth et al. (1993a) evaluated the effects of elevated O_3 and light intensity on several foliar constituents of hybrid poplar and sugar maple (*Acer saccharum*), and found that O_3 and light interacted to affect only concentrations of N in maple. Given that forest ecosystems are exposed to

multiple, simultaneous stress factors (e.g., climate change, insect outbreaks) in addition to increasing O_3 damage, and that some of those stressors induce the same chemical defense pathway as does O_3, such research is sorely needed.

Interactions Between CO_2 and O_3

The environmental factor that has been investigated most thoroughly in combination with O_3 pollution is CO_2. Enriched CO_2 may reduce ozone damage to trees both by decreasing stomatal conductance (and consequent oxidative stress) and by increasing carbohydrate pools for the synthesis of antioxidant compounds. The recent meta-analysis by Valkama et al. (2007) evaluated the effects of O_3, and O_3 combined with CO_2, on foliar chemistry of 22 species of trees. They found that enriched CO_2 can offset O_3-induced chemical changes, but their analysis did not differentiate between simple additive, versus interactive, effects of CO_2 and O_3.

Indeed, CO_2 and O_3 exert both independent (additive) and interactive effects on tree chemical profiles. Elevated O_3 concentrations exacerbated the CO_2-mediated reductions in N concentrations in *Betula papyrifera* (Kopper et al. 2001) and *Populus tremuloides* (Holton et al. 2003), but offset reductions in N in *Betula pendula* (significant $CO_2 \times O_3$ interactions). Peltonen et al. (2005) assessed the effects of CO_2 and O_3 on over thirty simple phenolics in *B. pendula*. They identified interactive effects in approximately three-quarters, and independent effects in one-quarter, of the cases. The interactive effects revealed that O_3-mediated induction of phenolics disappeared under elevated CO_2. Tannin levels also are influenced by both additive and interactive effects of CO_2 and O_3. Condensed tannin concentrations responded to the additive, independent effects of CO_2 and O_3 in *Fagus crenata* and various species of *Betula* (Kopper et al. 2001; Peltonen et al. 2005; Karonen et al. 2006), but to the interactive effects of CO_2 and O_3 in *Populus tremuloides* (Holton et al. 2003). Similarly, the effects of CO_2 and O_3 on monoterpenes and sesquiterpenes in *Pinus sylvestris* sometimes were additive and sometimes interactive (Sallas et al. 2001). In short, whether CO_2 and O_3 function in an additive or interactive manner depends on the particular chemical constituent and tree species studied.

Effects of CO_2 and O_3 on Trophic Interactions

Plant chemical composition is a strong ecological and evolutionary driver of trophic interactions, particularly between plants and herbivores. Given that green plants and phytophagous insects comprise nearly half of Earth's recognized species (Strong et al. 1984), it is not surprising that these taxa occupy the vast majority of all studies on plant-herbivore interactions. Several recent reviews have addressed the effects of CO_2 and O_3 on plant-insect interactions (Zvereva and Kozlov 2006; Stiling and Cornelissen 2007; Valkama et al. 2007; Bidart-Bouzat and Imeh-Nathaniel 2008). Composite results will be summarized below and supplemented with additional, relevant studies of tree-feeding herbivores.

Carbon Dioxide

Little evidence exists to suggest that elevated CO_2, upwards of 1,000 ppm predicted for this century, will directly affect insects (Coviella and Trumble 1999). Work by Stange (1997) showed that atmospheric CO_2 concentrations influence host location by *Cactoblastis cactorum*, but no similar work has been done with forest insects. Rather, the major effect of enriched CO_2 on herbivores will be indirect, mediated via changes in plant quality (Fig. 2). Overall, elevated CO_2 reduces the quality of tree foliage as food, due to decreases in nitrogen (an index of protein) and minerals, and increases in carbohydrates and phenolics. These changes typically, but not always, alter the preference and reduce the performance of herbivores.

CO_2 concentration influences selection of plants by insects and mammals. Elevated CO_2 reduced leafminer oviposition on *Populus tremuloides* (Kopper and Lindroth 2003a), but increased birch aphid oviposition on one of two clones of *B. pendula* (Peltonen et al. 2006). The few feeding studies conducted to date suggest that preferences of insects for ambient versus elevated CO_2 foliage are not easily predicted, as shifts in both directions, and no change, have been reported (Traw et al. 1996; Kuokkanen et al. 2003; Agrell et al. 2005; Knepp et al. 2007). Elevated CO_2 decreased the palatability of winter-dormant *Betula* species to hares and rabbits (Mattson et al. 2004), but not to voles (Kuokkanen et al. 2004). Of greater relevance in the universally CO_2-enriched world of the future, however, is whether enrichment alters relative preferences of herbivores for various host species. Minimal evidence to date suggests that such is the case. For example, Agrell et al. (2005) demonstrated that forest tent caterpillars switch relative preferences between *Populus tremuloides* and *Betula papyrifera*, as well as between *P. tremuloides* genotypes, grown in high CO_2 environments.

Elevated CO_2 also influences the individual performance of herbivores, although the effects are highly species-specific for both trees and insects. Among the more uniform (though still variable) of responses is food consumption. In general, insects increase both consumption rates, and total consumption, on CO_2-enriched foliage, ostensibly as a means to compensate for reduced N concentrations. Compensatory

feeding responses may be constrained, however, by the simultaneous consumption of higher concentrations of secondary metabolites such as phenolics. Approximate digestibility of food typically is not significantly affected, but conversion efficiencies of digested food into biomass generally decline. Little is known about how CO_2 environment affects insect detoxication capacities; a study with gypsy moths revealed enhanced oxidase, reductase, and esterase activities in larvae fed CO_2-enriched *P. tremuloides*, but not *Acer saccharum* (Lindroth et al. 1993b). Development times usually are prolonged, while growth rates and final pupal and adult weights decline or are unaffected, but rarely improve. Effects on reproduction have rarely been investigated. Lindroth et al. (1997) found no effect of CO_2 on the number and mass of eggs produced by gypsy moths, whereas Awmack et al. (2004) reported deceased fecundity in aphids on *B. papyrifera* under elevated CO_2.

Species-specificity in terms of insect herbivore responses to enriched CO_2 may be linked to differences among feeding guilds (e.g., chewers, miners, sap-feeders, seed-feeders). Bezemer and Jones (1998) identified such guild effects for plants and phytophagous insects in general. Too few studies have been conducted with tree-feeding insects to evaluate differences among guilds. However, the minimal, if not positive effects of CO_2 on tree-feeding aphids (Docherty et al. 1997; Awmack et al. 2004), in contrast to effects on chewing insects, suggest that guild-specific responses to CO_2 may exist for forest insects.

Just as interactions with various environmental factors influence the effects of CO_2 on plant chemistry, so may they modulate effects on herbivores. For example, the effects of elevated CO_2 on herbivore performance can be ameliorated or exacerbated by the availability of light, water, and soil nutrients (e.g., Kinney et al. 1997; Lawler et al. 1997; Roth et al. 1997; Hättenschwiler and Schafellner 1999; Agrell et al. 2000). Potential interactions with climate warming are of particular interest, as warm temperatures generally affect insects in a manner opposite that of CO_2, i.e., accelerated development and enhanced growth and reproductive performance (Bale et al. 2002). Indeed, Zvereva and Kozlov (2006) conclude from their recent meta-analysis that temperature increases are likely to mitigate predicted negative effects of enriched CO_2 on insects, and emphasize the need for future research to address the two factors in parallel.

In addition to the bottom-up effects of altered plant chemistry, elevated CO_2 may influence the top-down effects of natural enemies (predators, parasitoids, and pathogens) on insect herbivores (Fig. 2). Enriched CO_2 could affect natural enemies by altering the behavioral or physiological defenses of prey, modifying the quality of prey (e.g., insect size, concentrations of plant-derived toxins), or by altering the efficacy of host location and disease transmission. Few studies have addressed these possibilities, however, especially with forest systems. Elevated atmospheric CO_2 dramatically diminished the pheromone-mediated, predator escape behavior of aphids on *Populus tremuloides* (Mondor et al. 2004). In contrast, two studies have found the bottom-up effects of CO_2 on parasitoid performance (survivorship, development, and growth) to be minimal to nil (Roth and Lindroth 1995; Holton et al. 2003). The virulence of pathogens such as nuclearpolyhedrosis virus (NPV) is linked to host plant chemistry [e.g., tannins (Foster et al. 1992)], so CO_2-enriched atmospheres may influence the dynamics of disease outbreaks. CO_2-induced increases in condensed tannins in *P. tremuloides* did not, however, alter the susceptibility of gypsy moths to NPV (Lindroth et al. 1997). At this time insufficient information is available to predict whether the bottom-up effects of CO_2 on natural enemies will in general be buffered, amplified, or, more likely, context- and species-specific.

The impacts of elevated CO_2 concentrations on forest insects at the levels of populations and communities will be determined by a combination of bottom-up, top-down, and abiotic factors (Fig. 2). Historically, inferences about population-level responses have been drawn from studies of individual insects, but such conclusions can be misleading (Awmack et al. 2004). To date, however, few studies have addressed the impacts of elevated CO_2 at higher levels of organization. Several studies with tree-feeding aphids have shown no population-level effects (Docherty et al. 1997; Awmack et al. 2004), while another revealed positive effects of enriched CO_2 on aphid population size (Percy et al. 2002). In contrast, Stiling et al. (1999, 2002, 2003, 2009) found reduced densities (number per leaf) of herbivores, especially leafminers and leaftiers, in several woody species of a scrub-oak community exposed to elevated CO_2. Reduced herbivore densities were attributed to higher rates of both plant- and parasitoid-induced mortality, and were offset in several *Quercus* species by increased foliar production under high CO_2 (Stiling et al. 2009). Monitoring of aspen blotch leafminers (*Phyllonorycter tremuloidiella*) at Aspen-FACE indicated that elevated CO_2 roughly doubled plant-mediated mortality, substantially reduced predator-mediated mortality, and minimally influenced parasitoid-mediated mortality (Fig. 4).

Even fewer studies have addressed the effects of enriched CO_2 on speciose insect communities. Following 4 years of pan-trapping at Aspen FACE, Hillstrom and Lindroth (2008) reported that elevated CO_2 reduced abundance of phloem-feeding herbivores and increased abundance of chewing herbivores, although results were only marginally significant. Enriched CO_2 increased numbers of figitid and ichneumonid parasitoids, but not of braconid or chalcid parasitoids. Insect community composition differed between ambient and elevated CO_2 plots in 3 out of 4 years. In the most comprehensive study to

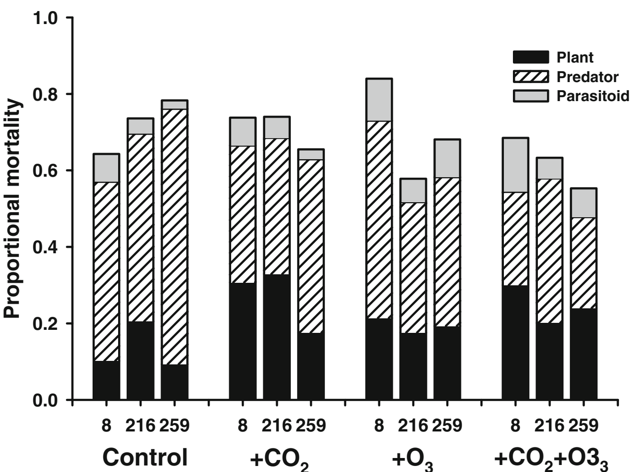


Fig. 4 Effects of CO_2 and O_3, in isolation and combination, on plant-, predator-, and parasitoid-induced mortality of aspen blotch leafminers (*Phyllonorycter tremuloidiella*). Data reveal major sources of mortality, prior to adult emergence, for miners on *Populus tremuloides*. Numerals below bars indicate aspen genotypes. Observations recorded in July 2001 at the Aspen FACE research site (Awmack and Lindroth, unpublished data)

date for forest canopy insects, Hillstrom and Lindroth (unpublished data) visually monitored insects on *Populus tremuloides* and *Betula papyrifera* over 3 years at Aspen FACE, cataloging over 36,000 insects from nearly 300 species. Overall, enriched CO_2 tended to increase the abundance of phloem-feeders and decrease the abundance of chewers and gallers on *P. tremuloides*, although effects were variable among species and over time. Few consistent effects on abundance were found, however, for insects on *B. papyrifera*. Elevated CO_2 did not affect species diversity of canopy insects, except for insects on *P. tremuloides* in 1 year. Elevated CO_2 also did not influence insect community composition, except for insects on *B. papyrifera* in 1 year.

Ozone

Increasing evidence suggests that tropospheric ozone pollution may affect directly insects. Gate et al. (1995) found that under elevated O_3, searching efficiency of a hymenopteran parasitoid, as well as the proportion of hosts parasitized, declined. O_3 likely interacts with the complex of BVOCs that function as "infochemicals" to govern key interactions between insects and both their hosts (plant or insect) and natural enemies (predators and parasitoids) (Laothawornkitkul et al. 2009; Yuan et al. 2009; Pinto et al. 2010, this volume). Chemical degradation or transformation of BVOCs by O_3 is likely to increasingly disrupt olfactory cues in atmospheres of the future.

Selection of plants as oviposition or food hosts is influenced by O_3 concentrations. O_3-treated foliage reduced leaf beetle oviposition onto *Populus deltoides* (Jones and Coleman 1988) and leafminer oviposition onto *Populus tremuloides* (Kopper and Lindroth 2003a). Few feeding studies have compared the preferences of insects for fumigated vs. nonfumigated foliage, and those indicate a range of responses. Leaf beetles preferred O_3-treated *P. deltoides* (Jones and Coleman 1988) and common leaf weevils preferred O_3-treated hybrid aspen (Freiwald et al. 2008), whereas forest tent caterpillars preferred untreated *P. tremuloides* (Agrell et al. 2005). Forest tent caterpillars greatly increased their preference for *Betula papyrifera*, relative to *P. tremuloides*, under elevated O_3, and also shifted their preferences among *P. tremuloides* genotypes (Agrell et al. 2005).

O_3 fumigation changes the individual performance of phytophagous insects, although, as for CO_2, effects are highly species-specific. The meta-analysis by Valkama et al. (2007) summarized results for 22 species of trees and ten species of insects. Food consumption rates typically are unaffected, as are survival and reproduction rates. Development times usually are reduced, and pupal masses increased, under elevated O_3. For example, Kopper and Lindroth (2003b) reported that for forest tent caterpillars reared on high-O_3 *Populus tremuloides*, development times decreased an average of 14% and pupal weights increased an average of 31% compared with insects on control trees. Relative growth rates of chewing, but not sucking, insects generally increase under elevated O_3. On average, insect fecundity is not affected by O_3, although Awmack et al. (2004) reported decreased fecundity for aphids on *Betula papyrifera*.

Under natural conditions, interactions between trees and herbivores likely are influenced by multiple, interacting stressors, including O_3 (Koricheva et al. 1998b). With the exception of interactions with CO_2 (addressed below), however, exceedingly little is known about how O_3 may interact with other environmental factors to influence trophic interactions. Lindroth et al. (1993a) reported that gypsy moth larval performance was influenced by the independent, but not interactive, effects of O_3 and light intensity. With the likelihood of increased pest damage to forests under future scenarios of global environmental change, assessments of the combined effects of ozone and other stressors should be a high priority.

A growing body of literature reveals that O_3 pollution likely will affect interactions between tree-feeding insects and their natural enemies. As indicated above and described elsewhere in detail (Yuan et al. 2009; Pinto et al. 2010), O_3 may directly affect the behavior and fitness of predators and parasitoids. Alternatively, elevated O_3 may alter the behavioral or physiological defenses of prey, or their quality as food. For example, Mondor et al. (2004) reported that at Aspen FACE, high O_3 environments markedly

enhanced predator escape behaviors of aphids on *Populus tremuloides*. Also at Aspen FACE, O_3 fumigation reduced survivorship of the parasitoid *Compsilura concinnata* (Holton et al. 2003). Decreased fitness was consistent with improved performance of its forest tent caterpillar host, which in turn was linked to decreased levels of phenolic glycosides in *P. tremuloides*.

Despite accumulating evidence for impacts of O_3 at the level of individual insect performance, little work has addressed impacts at the level of populations. Awmack et al. (2004) found that O_3 fumigation at Aspen FACE increased population densities of aphids on *Betula papyrifera* when protected from natural enemies, but not when unprotected. Also at Aspen FACE, elevated O_3 levels tended to increase plant-mediated mortality, reduce predator-mediated mortality. and increase parasitoid-mediated mortality of aspen blotch leafminers (*Phyllonorycter tremuloidiella*), although magnitudes of effects varied among insects on different aspen genotypes (Fig. 4).

At the level of insect communities, pan-trapping at Aspen FACE revealed positive effects of elevated O_3 on abundance of some phloem-feeding insects, and strong suppressive effects on a diversity of parasitoids (Hillstrom and Lindroth 2008). O_3 influenced the community composition of insects captured by traps in 2 out of 4 years. In our more recent visual censuses of canopy insects at Aspen FACE, we found that O_3 fumigation increased abundances of several species of chewing insects, but decreased numbers of several phloem-feeders, on *Populus tremuloides* (Hillstrom and Lindroth, unpublished data). O_3 did not, however, consistently affect abundances of insects on *Betula papyrifera*. O_3 also had generally little impact on the species diversity and community composition of canopy insects on several tree species, and across multiple years, at Aspen FACE.

Interactions Between CO_2 and O_3

Valkama et al. (2007) sought to differentiate the effects of O_3 alone, versus O_3 in combination with CO_2, on the performance of tree-feeding insects. Their meta-analysis revealed that many of the positive effects of O_3 on insect performance, especially for leaf chewers, are negated with simultaneous exposure to enriched CO_2. Their review did not, however, differentiate between simple additive, versus truly interactive, effects of CO_2 and O_3 on insects.

Indeed, relatively few studies have been designed to detect potential interactive effects of CO_2 and O_3 on forest insect performance, and those have shown mixed results. Several studies (Awmack et al. 2004; Peltonen et al. 2006) have assessed the independent and combined effects of CO_2 and O_3 on feeding, growth, and reproductive performance of aphids, and revealed no significant interactions. Similarly, no significant interactions were observed with respect to CO_2 and O_3 effects on leafminers on *Populus tremuloides* (Kopper and Lindroth 2003a), whitemarked tussock moths feeding on *Betula papyrifera* (Kopper et al. 2001), or forest tent caterpillar feeding preferences for *P. tremuloides* and *B. papyrifera* (Agrell et al. 2005). In contrast, CO_2 and O_3 interacted to influence development and growth of forest tent caterpillars on *P. tremuloides*: O_3-mediated enhancements in performance were greater at ambient than elevated CO_2. In short, the limited evidence to date suggests that the combined effects of CO_2 and O_3 on insect herbivores will be primarily additive, although the standard caveat of context- and species-specificity applies.

Effects of CO_2 and O_3 on Ecosystem Dynamics

At the level of forest ecosystems, plant chemistry plays a host of important roles, the most prominent of which arguably is regulation of the transfer of fixed carbon to herbivores and decomposers—the dominant pathways of material flow (Fig. 1). Accumulating evidence suggests that elevated concentrations of CO_2 and O_3 will modify the complex of physiological and ecological interactions that determine rates of plant damage, organic substrate deposition, and nutrient cycling. In short, the effects of CO_2 and O_3 on plant chemistry will in turn influence the sequestration and cycling of carbon and nutrients in forest ecosystems.

Forest Canopy Damage

Phytophagous insects generally remove 2–15% of primary production in temperate deciduous forests (Cebrian 1999; Cyr and Pace 1993; Cebrian and Lartigue 2004), although during outbreaks, nearly 100% can be consumed (e.g., Donaldson and Lindroth 2008). Canopy damage rates are a function of the host preferences, individual consumption rates, population densities, and community composition of herbivorous insects, all of which can be influenced by levels of atmospheric CO_2 and O_3.

The effects of CO_2 on loss of primary production to herbivores have been explored in only a few forest systems. In a Florida scrub oak community, enriched CO_2 decreased the frequency of damage to *Quercus myrtifolia* (Stiling et al. 2002). Later work in the same system documented a decline in the frequency of damage by 4 of 6 insect feeding groups, across three *Quercus* species, under elevated CO_2 (Hall et al. 2005). Working with four understory tree species at the FACTS-1 FACE site (loblolly pine—hardwood forest), Hamilton et al. (2004) reported declines of 10–46% in foliar damage rates in high CO_2 plots. These declines were driven largely, however, by the

response of one species (*Ulmus alata*); damage rates in other species were not statistically significant from those of control trees. Knepp et al. (2005) followed up that work by censusing foliar damage rates in twelve species of potted saplings at FACTS-1 FACE. Enriched CO_2 reduced damage rates across all species in 2001, but not in 2002–2003. In the most comprehensive study to date, Couture, Meehan, and Lindroth (unpublished data) evaluated damage rates on leaves collected from *Populus tremuloides* and *Betula papyrifera* over 3 years at Aspen FACE. Elevated CO_2 concentrations did not markedly affect herbivory rates in 1 year, but increased damage rates by 2–3-fold in 2 years of the study. Overall, these studies indicate that canopy damage rates are likely to change under CO_2 concentrations of the future. But, consistent with effects on individual insects, the magnitude and direction of change will be both species-specific and temporally variable.

To date, no published study has evaluated the effects of elevated tropospheric O_3 on community-wide, canopy damage in forest ecosystems. Our work at Aspen FACE (Couture, Meehan and Lindroth, unpublished data) revealed that high levels of O_3 reduced damage to both *Populus tremuloides* and *Betula papyrifera* in 2 of 3 years.

Calculations of losses to primary production based on leaf areas damaged by herbivores may significantly underestimate true reductions in production. Aldea et al. (2006) showed that in a variety of deciduous tree species, chewing damage caused modest and localized suppression of photosynthetic efficiency in adjacent, undamaged leaf tissue, whereas galling damage caused large and extensive suppression. The authors suggest that the spatial propagation of reduced photosynthesis may be influenced by enriched CO_2, but did not observe such effects in their study. More recently, however, P. Nabity and M. Hillstrom (pers. comm.) found that elevated CO_2 decreased the propagation of reduced photosynthesis caused by insects feeding on *Populus tremuloides* and *Betula papyrifera* at Aspen FACE.

Organic Substrate Deposition

Herbivorous insects mediate the transfer of organic materials from the forest canopy to the forest floor by depositing frass, greenfall, and insect biomass, and altering the timing and amounts of leachate througfall and leaf litterfall. These processes have received little attention in the context of global change research.

We recently evaluated the effects of fumigation treatments at Aspen FACE on the quality of frass produced by whitemarked tussock moth larvae feeding on *Populus tremuloides*, and the effects of both frass and greenfall on soil microbial respiration and nitrogen leaching (Hillstrom, Meehan, Kelly and Lindroth, unpublished data). Insect frass had slightly decreased N levels in high O_3 environments, and increased tannin levels in high CO_2 environments. However, neither frass nor greenfall quality affected microbial respiration (CO_2 efflux) or nitrogen (NO_3^-) leaching in soil microcosms. In contrast, the quantity of frass and greenfall added to microcosms markedly affected both respiration and N leaching, under all atmospheric conditions. Thus, insects may affect carbon and nitrogen mineralization in forests of the future more through their effects on the quantity (and timing), than quality, of substrate deposition.

Indeed, atmospheric CO_2 and O_3 concentrations appear to influence rates of insect-mediated organic substrate deposition in forests. At Aspen FACE, rates of combined frass and greenfall deposition were 35% higher in elevated vs. ambient CO_2 stands, and 13% lower in elevated vs. ambient O_3 stands, over three field seasons (Meehan, Couture, Bennett and Lindroth, unpublished data).

Finally, insect feeding and atmospheric composition both may alter the timing of litterfall in forest ecosystems. In general, feeding damage accelerates leaf abscission, whereas enriched CO_2 may either accelerate (Stiling et al. 2002) or decelerate (Karnosky et al. 2003) abscission rates. How herbivory and atmospheric chemistry may interact to influence the magnitude and timing of leaf abscission is unknown.

Soil Invertebrates

Soil invertebrates play important roles in the nutrient cycling dynamics of forest ecosystems, from the comminution and microbial inoculation of plant litter to the vertical transport of organic matter through soil horizons. These processes are governed both by the quality and quantity of litterfall, which in turn are influenced by atmospheric composition (Fig. 1).

Several studies have evaluated the effects of leaf litter from elevated CO_2 plots on feeding preferences of isopods. Using short-term (3 day) feeding choice studies, Cotrufo et al. (1998b) found that isopods (*Oniscus asellus*) consumed 16% less high-CO_2 litter than ambient-CO_2 litter from *Fraxinus excelsior*. Subsequently, Cotrufo et al. (2005a) fed isopods (*Porcellio* sp.) for 2 weeks on litter from six tree species grown under ambient and elevated CO_2, and found no difference in palatability for five species, and increased preference for high-CO_2 litter for one species. Using no-choice feeding studies and two species of isopods, Hättenschwiler et al. (1999) found increased consumption of high-CO_2 beech litter, but no effect of CO_2 on consumption of spruce litter. Hättenschwiler and Bretscher (2001) reported that growth of trees under elevated CO_2 altered the relative preference of *O. asellus* for different litter species—increasing preference for one, decreasing

preference for another, and not affecting yet another. Thus, as is the case for insects feeding on green leaves, the consequences of CO_2 for detritus-feeding invertebrates are species-specific.

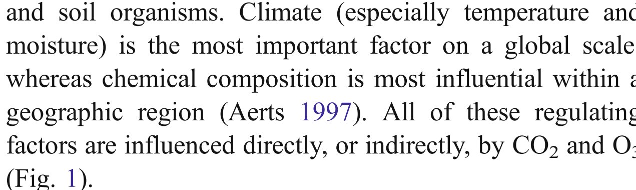
Additional studies have assessed the effects of leaf litter from elevated CO_2 or O_3 environments on fitness indices and population growth of soil invertebrates. Individual growth of juvenile earthworms (*Lumbricus terrestris*) was reduced when fed high-CO_2 or high-O_3 birch (*Betula pendula*) litter (Kasurinen et al. 2007), and high-CO_2 (but not high-O_3) aspen (*P. tremuloides*) litter (Meehan, Crossley and Lindroth, unpublished data). Growth and mortality rates of isopods, however, generally have been shown not to be affected by CO_2 or O_3 treatments (Hättenschwiler and Bretscher 2001; Kasurinen et al. 2007). Population growth of collembola (*Sinella curviseta*) decreased when reared on high-CO_2 aspen litter, but increased when reared on high-O_3 aspen litter (Meehan, Crossley and Lindroth, unpublished data).

Several studies have evaluated the effects of CO_2 and O_3 on soil invertebrate communities under field conditions. Hansen et al. (2001) investigated the effects of elevated CO_2 concentrations on soil microarthropods at the FACTS-1 FACE site. Over the first 19 months of CO_2 treatment, total microathropod abundance declined by 34% in high-CO_2 plots, relative to ambient plots. The decline was driven by changes in abundance of oribatid mites, and was attributed to factors other then changes in litter quality. Haimi et al. (2005) censused populations of enchytraeids, mites and collembola in soil samples surrounding chambered *Pinus sylvestris* trees. Elevated CO_2 concentrations contributed to low numbers of acaridid mites, but did not significantly affect other soil fauna. Loranger et al. (2004) assessed responses of soil invertebrates to CO_2 and O_3 enrichment at Aspen FACE. They found that numbers of collembola and Oribatid mites, but not various other soil animals, declined in elevated CO_2 plots. Mites, but not other fauna, also decreased in high O_3 plots.

In summary, CO_2- and O_3-mediated changes in litter quality are likely to influence the performance of soil invertebrates of forests in the future. Some evidence exists to suggest that reduced litter quality will negatively affect the fitness of soil animals. In turn, their roles in nutrient cycling and soil carbon sequestration are likely to be altered. Overall, however, as noted by Couteaûx and Bolger (2000), no general patterns of response have yet emerged, likely due to the species- and context-specific nature of these interactions.

Litter Decomposition

Rates of litter decomposition and attendant nutrient mineralization are governed primarily by climate, litter chemistry,



and soil organisms. Climate (especially temperature and moisture) is the most important factor on a global scale, whereas chemical composition is most influential within a geographic region (Aerts 1997). All of these regulating factors are influenced directly, or indirectly, by CO_2 and O_3 (Fig. 1).

Litter quality is determined by carbon availability (which ranges from labile sugars to recalcitrant lignin), mineral content, and chemical modifiers such as tannins (Swift et al. 1979; Anderson 1991). Tannins and related phenolic constituents alter decomposition by complexing with other litter components (e.g., amino acids) and/or by inhibiting the degradative activity of enzymes and soil fauna (Horner et al. 1988; Anderson 1991; Coûteaux et al. 1995). For similar litter types in common environments, nitrogen, lignin, and tannin concentrations appear to act as “a hierarchical series of controls” (Anderson 1991). In high quality, readily decomposable litters, tannins may be rate retardants, whereas in low quality, slowly decomposable litters, lignin concentration, or lignin: N ratio typically determine decomposition rates (Anderson 1991). Any factor that markedly affects the nitrogen or C-based secondary metabolite concentration of litter also will affect decomposition, and consequently, nutrient availability (Chapin 1991).

The “litter quality” hypothesis, introduced by Strain and Bazzaz (1983), proposed that CO_2 enrichment will decrease plant nitrogen (relative to carbon) concentrations, and thereby reduce litter decomposition and soil N availability. Progressive soil nitrogen limitation is anticipated to eventually feed back to reduce plant production (Luo et al. 2004; Johnson 2006). Thus, CO_2-mediated changes in the chemical quality of litter have been of key interest with respect to understanding the cycling and storage of carbon in forest ecosystems. Indeed, litter from trees grown under elevated CO_2 characteristically exhibits reduced N concentrations and increased lignin concentrations and C:N ratios (Ceulemans et al. 1999; Norby et al. 2001).

To date, however, studies of the effects of elevated CO_2 on litter decomposition (measured as mass loss or microbial respiration) have not demonstrated a consistent direction of response. A comprehensive meta-analysis by Norby et al. (2001), including data for 33 woody species, showed that data do not support the “litter quality” hypothesis of reduced decomposition rates for litter produced under elevated CO_2. Decomposition studies published since that review have been increasingly realistic, employing more long-term and field-based approaches with open-top chambers or FACE sites. Still, a consistent pattern has yet to emerge. Several studies have documented reduced decomposition of leaf litter produced under elevated CO_2. For example, litter from three *Populus* species grown under elevated CO_2 at POPFACE exhibited reduced decomposi-

tion rates (Cotrufo et al. 2005b), as did litter from *Betula papyrifera* and *Populus tremuloides* from young (sapling) forest stands grown under elevated CO_2 at Aspen FACE (Parsons et al. 2004, 2008). In contrast, CO_2 growth environment did not alter subsequent litter decomposition rates for *Quercus myrtifolia* in a scrub oak community (Hall et al. 2006), for five tree species at the FACTS-1 FACE site (Finzi et al. 2001), or for *B papyrifera* and *P. tremuloides* in older, closed-canopy stands at Aspen FACE (Liu et al. 2009). Diverse responses among experiments have been attributed to a number of factors, including soil fertility, decomposition environment, CO_2 exposure mechanism, study duration, stand development, and, genotype- and species-specific effects (Norby et al. 2001; Kasurinen et al. 2006; Hall et al. 2006; Liu et al. 2009).

Relatively little research has investigated the effects of atmospheric O_3 on litter quality and decomposition. Growth of three species of deciduous tree seedlings (Boerner and Rebbeck 1995; Scherzer et al. 1998) and two species of *Pinus* seedlings and saplings (Scherzer et al. 1998; Kainulainen et al. 2003) under elevated O_3 did not influence subsequent decomposition rates of leaf litter. Findlay et al. (1996), however, found that cottonwood leaves produced under high O_3 levels exhibited reduced decomposition rates (in aquatic microcosms), which were linked to increased phenolic concentrations. Similarly, Kasurinen et al. (2006) observed reduced decomposition in *Betula pendula* leaves produced under high O_3 concentrations. Parsons et al. (2008) found that early in stand development at Aspen FACE, growth under elevated O_3 reduced 2-year decomposition rates of *Populus tremuloides* litter, independent of CO_2 treatment. In contrast, elevated O_3 accelerated decomposition of *Betula papyrifera* litter from ambient CO_2 treatments, but decreased decomposition of litter from enriched CO_2 treatments (a significant $O_3 \times CO_2$ interaction). Later work at Aspen FACE, however, showed that elevated O_3 decreased the decomposition of *P. tremuloides* and *B. papyrifera* litter from closed-canopy stands during only the first year of a 2.6-year study (Liu et al. 2009). As is the case for atmospheric CO_2, the effects of elevated O_3 on litter decomposition appear to be species-, age-, and context-specific.

Although too early for definitive conclusions, the developing scientific consensus is that atmospheric CO_2 and O_3 will affect litter decomposition and subsequent nutrient cycling more through their effects on the quantity, than quality, of foliage produced, and by shifting the species composition of forests (Finzi and Schlesinger 2002; Cotrufo et al. 2005b; Liu et al. 2007, 2009; Hillstrom, Meehan, Kelly and Lindroth, unpublished data). For example, soil carbon sequestration at Aspen FACE appears to be more closely related to CO_2- and O_3-mediated changes in input quantity and species composition than to qualitative changes in litter chemical composition (Loya et al. 2003; Liu et al. 2007, 2009).

Conclusions and Recommendations for Future Research

Human-induced changes in the levels of atmospheric CO_2 and O_3 clearly influence the quantity and quality of primary production. In turn, changes in plant chemical composition cascade through above- and below-ground ecological interactions (e.g., herbivory, decomposition) to alter ecosystem structure and function, ultimately feeding back to affect terrestrial carbon sequestration and atmospheric composition. Many of these interactions are governed by processes that are fundamentally chemical in nature (double entendre intended). Thus, chemical ecologists have much to offer in terms of elucidating the mechanisms that integrate biological organization—from genomes to ecosystems—in a globally changing world.

An admittedly frustrating theme to emerge from this and other recent reviews (e.g., Stiling and Cornelissen 2007; Bidart-Bouzat and Imeh-Nathaniel 2008; Tylianakis et al. 2008) of global environmental change is that the effects of any single change factor on any particular ecological interaction are highly variable. Responses tend to be species- and context-specific, and thus appear idiosyncratic. Global change science must advance considerably further before predictions about impacts on natural systems can be made with accuracy and precision. The work of chemical ecologists should be central to such efforts: it will provide mechanistic insight into ecosystem function and provide data critical to the accurate parameterization of global change and risk assessment models.

Below I provide a non-exhaustive list of some of the more pressing needs for the contributions of chemical ecologists toward furthering our understanding of the effects of CO_2 and O_3 on forest ecosystems. Funding of large, long-term, multi-investigator projects is essential to the success of these efforts.

1. Increase the diversity of natural products studied. Our understanding of the effects of global change on the phytochemistry of forest ecosystems is based on a few classes of chemical compounds, primarily phenolics and terpenoids. Almost nothing is known about effects on other classes of secondary metabolites important to at least some tree species, e.g., alkaloids, cyanogenic glycosides, nonprotein amino acids, coumarins, and iridoid glycosides.
2. Broaden the representation of species and biomes investigated. To date, research has focused on a narrow range of deciduous and coniferous tree species and biomes, and mostly lepidopteran herbivores. Both

tropical and boreal forests are critically important to the global carbon cycle, and poorly represented in global change research. Future studies should emphasize foundation tree species (Ellison et al. 2005) and functional groups (e.g., insect borers, miners) and taxa (e.g., mammals) known to be important to the health and sustainability of forest ecosystems. The coniferous forests and irruptive bark beetles of western North America are a prime example (Raffa et al. 2008). In a similar vein, future research should address non-exploitative ecological interactions, such as mutualistic associations of plants with pollinators and mycorrhizae.

3. Emphasize simultaneous investigation of multiple global change drivers. As documented in this review and others (e.g., Tylianakis et al. 2008), the effects of any particular global change factor on ecological interactions are context-dependent. Multiple drivers should be investigated simultaneously to more realistically approximate future environments and to provide a diversity of conditions under which to test the effects of individual drivers (Tylianakis et al. 2008). Altered temperature and precipitation are arguably the most important additional factors to evaluate in the context of CO_2 and O_3 effects on forests.
4. Conduct long-term experiments. The majority of studies published to date have been executed over short time frames (up to several years), with only a few approaching 10 years—considerably less than the lifespan of forest trees. Some ecosystem processes (e.g., soil nutrient depletion and carbon storage) require assessment over the course of a decade or longer, logistical and funding constraints nothwithstanding. Long-term experiments also could allow for studies of evolutionary responses of insects to CO_2- and O_3-mediated changes in tree chemistry, which are conspicuously absent in the published literature and important to understanding the effects of global change on forest ecosystems.
5. Employ “genes to ecosystems” research perspectives. “Genes to ecosystems” research has emerged as a new disciplinary frontier in genetics and ecology (Whitham et al. 2006). It combines genetic/genomic tools with the approaches of evolutionary ecology to understand the functional significance of genes in ecological context and the dynamics of ecosystems in evolutionary context. The discipline of chemical ecology can play a key role in elucidating the “metabolomes” that link gene expression to ecosystem processes in the context of global environmental change.

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REVIEW ARTICLE

Plant Volatile Organic Compounds (VOCs) in Ozone (O_3) Polluted Atmospheres: The Ecological Effects

Delia M. Pinto · James D. Blande · Silvia R. Souza · Anne-Marja Nerg · Jarmo K. Holopainen

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Abstract Tropospheric ozone (O_3) is an important secondary air pollutant formed as a result of photochemical reactions between primary pollutants, such as nitrogen oxides (NO_x), and volatile organic compounds (VOCs). O_3 concentrations in the lower atmosphere (troposphere) are predicted to continue increasing as a result of anthropogenic activity, which will impact strongly on wild and cultivated plants. O_3 affects photosynthesis and induces the development of visible foliar injuries, which are the result of genetically controlled programmed cell death. It also activates many plant defense responses, including the emission of phytogenic VOCs. Plant emitted VOCs play a role in many eco-physiological functions. Besides protecting the plant from abiotic stresses (high temperatures and oxidative stress) and biotic stressors (competing plants, micro- and macroorganisms), they drive multitrophic interactions between plants, herbivores and their natural enemies e.g., predators and parasitoids as well as interactions between plants (plant-to-plant communication). In addition, VOCs have an important role in atmospheric chemistry. They are O_3 precursors, but at the same time are readily oxidized by O_3, thus resulting in a series of new compounds that include secondary organic aerosols (SOAs). Here, we review the effects of O_3 on plants and their VOC emissions. We also review the state of current knowledge on the effects of ozone on ecological interactions based on VOC signaling, and propose further research directions.

Keywords Ozone · Volatile organic compounds · Trophic interactions · Infochemicals

University of Kuopio will be University of Eastern Finland (@uef.fi) from January 1st, 2010.

D. M. Pinto
Plant Production Research/Plant Protection Unit,
MTT Agrifood Research Finland,
R-Building,
31600 Jokioinen, Finland

J. D. Blande · A.-M. Nerg · J. K. Holopainen (✉)
Department of Environmental Science, University of Kuopio,
P.O. Box 1627, FIN-70211 Kuopio, Finland
e-mail: Jarmo.Holopainen@uku.fi

S. R. Souza
Department of Ecology, Botanic Institute,
P.O. Box 3005,
01061-970 São Paulo, SP, Brazil

Introduction

Since the pre-industrial era, atmospheric O_3 concentration has increased. When globally averaged, this amounts to a 30% rise (IPCC 2007). Recent estimates (Sitch et al. 2007; The Royal Society 2008) project a continuous rise in tropospheric O_3 levels as a result of increases in anthropogenic precursor emissions. It has been estimated that O_3 concentrations increase by between 0.5 and 2% per year (Vingarzan 2004), and that by 2100 the mean monthly 24-h concentration will be above 40 ppb over most of the Earth, and above 70 ppb over some regions (Sitch et al. 2007). Currently, O_3 is the most important pollutant in rural areas (Ashmore 2005) due to long-range transport from polluted sites (IPCC 2007; Vingarzan 2004). Both acute and chronic exposures have a strong negative impact on plants. While acute exposure results in visible symptoms that reduce the economic value of crops (see Ashmore 2005, for a review), chronic exposure affects physiological processes with or without visible symptoms (Long and Naidu 2002), which results in decreased crop yields (see Ashmore 2005, for a review). In

turn, plant-mediated effects can impact on herbivore performance (Kopper and Lindroth 2003; Awmack et al. 2004) and behavior (Jones and Coleman 1988; Jackson et al. 1999; Agrell et al. 2005) as well as the performance and abundance of higher trophic levels e.g., predators and parasitoids of herbivores (Percy et al. 2002; Holton et al. 2003).

During recent years, there has been growing interest in assessing the atmospheric and ecological effects of O_3 on VOC emissions. In a process driven by positive feedback, O_3 exposure enhances the emission of VOCs to the atmosphere (Llusià et al. 2002), which in turn increases atmospheric O_3 concentrations, since VOCs take part in photochemical reactions that lead to O_3 formation (Atkinson and Arey 2003). This positive feedback may be enhanced by increasing temperatures. In field exposure experiments, very moderate temperature increases have been shown to significantly enhance phytogenic VOC emissions, and in particular green leaf volatiles (Hartikainen et al. 2009). Earlier research suggested isoprenoids (mostly isoprene and monoterpenes) act as scavengers of radical oxygen species (ROS) or membrane stabilizers under high temperature stress and thus improve plant thermotolerance (Peñuelas and Llusià 2003; Sharkey et al. 2008).

VOCs are also prone to ozonolysis (Fig. 1), a process that results in the formation of secondary organic aerosols (SOAs) (Seinfeld and Pandis 2006; Laothawornkitkul et al. 2009). From an ecological point of view, both increased emission and destruction of VOCs by O_3 may affect several of their multiple functions (Lerdau and Slobodkin 2002; Holopainen 2004; Yuan et al. 2009). VOCs protect plants from biotic stressors such as fungi and bacteria through antifungal and antimicrobial properties and may repel generalist herbivores (see Kesselmeier and Staudt 1999; Dudareva et al. 2006; Laothawornkitkul et al. 2009 for a review). They also inhibit seed germination and growth of neighboring plant species (Romagni et al. 2000) and thus decrease competition. In addition, VOCs attract pollinators to flowers, animal seed dispersers to odorous fruits (Dudareva et al. 2006), herbivores to host plants (Schoonhoven et al. 2006), and herbivore-induced VOCs mediate the orientation of micro- and macro-organisms from higher trophic levels towards their host or prey (Dicke 1999, for a review; Rasmann et al. 2005). Plant VOCs also are important in within-plant signaling as well as in plant-to-plant signaling (Karban et al. 2003; Frost et al. 2007; Heil and Silva Bueno 2007). As VOCs have significant ecological relevance, we review how O_3 affects their emission and subsequent ecological interactions. Based on current knowledge of O_3 effects on plant biochemistry and physiology and atmospheric chemistry, we identify the potential impact on ecological interactions based on VOCs and propose prospective areas for further research.

O_3 in the Atmosphere: The Photochemical Smog Formation Process

The photochemical formation of O_3 in the troposphere begins with the oxidation of nitric oxide (NO) to nitrogen dioxide (NO_2) under sun light, and then proceeds through reactions 1 and 2 ($O(^3P)$ indicates the ground–state

Fig. 1 The ozonolyzis of alkenes proceeds via an initial O_3 addition to the C–C double bond(s) of alkenes, followed by degradation of the resulting ozonide (**1**), which in turn may decompose and form the Criegee intermediate (**2**) and carbonyl (**3**). The energy-rich Criegee biradical (**2**) can be stabilized by collision, and thus, may react with water leading to carboxylic acid (**4**) formation

energy-rich biradical

oxygen). In unpolluted air, some NO undergoes oxidation leading to NO_2 regeneration (reaction 3). However, in polluted air, this cycle can be broken down by the action of organic (RO_2•) or hydro-peroxy (HO_2) radicals. The free radicals that oxidize NO to NO_2 (reactions 4 and 5) are formed during photo-oxidation of biogenic (from biological processes in both marine and terrestrial environments Fuentes et al. 2000) and anthropogenic VOCs excluding carbon monoxide (CO) and carbon dioxide (CO_2) (Finlayson-Pitts and Pitts 2000).

$$NO_2 + hv \rightarrow NO + O(^3P) \tag{1}$$

$$O(^3P) + O_2 + M \rightarrow O_3 + M \quad (M = \text{air, inert particle}) \tag{2}$$

$$O_3 + NO \rightarrow NO_2 + O_2 \tag{3}$$

$$RO_2\bullet + NO \rightarrow RO\bullet + NO_2 \tag{4}$$

$$HO_2\bullet + NO \rightarrow OH\bullet + NO_2 \tag{5}$$

The best way to understand O_3 production is to consider the oxidation of methane (CH_4), structurally the simplest VOC. The CH_4 oxidation cycle serves as a simplified model for the chemistry of the unpolluted troposphere (reactions 6–12). In the polluted atmosphere, the fundamental chemical principles are the same (Baird and Michael 2005). The oxidation of VOCs is triggered by hydroxyl radicals (OH•), and results in several alkyl peroxy (RO_2•) and hydroperoxy (HO_2•) radicals, which lead to the conversion of NO to NO_2, and hence, promotes O_3 accumulation and the efficient regeneration of the OH•. In turn these can react with other VOCs. The primary source of OH• in the atmosphere is the reaction of electronically excited O atoms, O ($_1$D), a result of O_3 photolysis, with water vapor. The reaction of electronically excited nitrogen oxide (NO_2*) with water is also an important source of OH•. The NO_2* breaks the O–H bond in water, leading to OH• and HONO formation (Li et al. 2009). In polluted areas, the photolysis of aldehydes (e.g., HCHO), nitrous acid (HONO) and hydrogen peroxide (H_2O_2) also can be significant sources of OH• or HO_2• that can rapidly be converted to OH• (Seinfeld and Pandis 2006).

$$CH_4 + OH\bullet \rightarrow CH_3\bullet + H_2O \tag{6}$$

$$CH_3\bullet + O_2 \rightarrow CH_3OO\bullet \tag{7}$$

$$CH_3OO\bullet + NO \rightarrow CH_3O\bullet + NO_2 \tag{8}$$

$$CH_3O\bullet + O_2 \rightarrow H_2CO + HO_2\bullet \tag{9}$$

$$H_2CO \rightarrow H\bullet + HCO\bullet \tag{10}$$

$$H\bullet + O_2 \rightarrow HO_2\bullet \tag{11}$$

$$HCO\bullet + O_2 \rightarrow CO + HO_2\bullet \tag{12}$$

The most important classes of compounds in the photochemical process, including O_3 formation, are alkanes, alkenes, aromatic hydrocarbons, carbonyl compounds (e.g., aldehydes and ketones), alcohols, organic peroxides, and halogenated organic compounds (e.g., alkyl halides). In urban areas, compounds representing all VOC classes are important for O_3 formation, but especially significant are the alkenes such as propene and butenes. In rural vegetated areas, biogenic non-methane VOCs emitted by plants tend to be the most important source (Guenther 1999). The major alkenes emitted by vegetation are isoprene (2-methyl-1,3-butadiene) and monoterpenes ($C_{10}H_{16}$) (Atkinson and Arey 2003).

The photochemical process is driven by NO_x (NO plus NO_2) levels, reactivity of VOCs as well as the amount of OH• in the atmosphere (Seinfeld and Pandis 2006; Wennberg and Dabdub 2009). At low NO_x levels, the net production of O_3 increases with increasing NO_x. However, at high NO_x levels, there is a net destruction of O_3 through reactions with NO. In addition, there is a transition stage in which O_3 shows only a weak dependence on NO_x levels (Seinfeld and Pandis 2006). Thus, the efficiency of O_3 production per unit of NO_x oxidized is generally highest in areas where NO_x levels are low, and it decreases with increasing NO_x concentration. Therefore, in urban areas, the ozone level is directly related to traffic, reaching higher values when truck traffic is lower due to NO_x level drops (Wennberg and Dabdub 2009).

O_3 Effects on VOCs: From the Cell to the Environment

O_3 can affect the biosynthesis and/or emission of VOCs by impacting the biochemistry and physiology of the plant (Rao et al. 2000; Long and Naidu 2002), as well as by reacting with plant surfaces (Fruekilde et al. 1998) and probably by altering their physico-chemical properties (Müller and Riederer 2005). It also can affect VOCs in the leaf boundary layer and in the atmosphere (Loreto et al. 2001; Pinto et al. 2007a, b, c) (Table 1), where VOCs become infochemicals for an array of micro- and macro-

Table 1 Ozone effects on different functional levels, which could affect voc-based signaling

Primary effect	Secondary effect	Related potential ecological outcome
Activation of defense genes and biochemical cascades (Baier et al. 2005; Kangasjärvi et al. 2005; Heath 2008).	Cross-effects of signaling transduction pathways (Rao et al. 2000; Pieterse et al. 2001). Effects on JA- and SA-induced defense responses (Pieterse et al. 2001); decreased/increased/altered VOC emissions (Rao et al. 2000; Blande et al. 2007).	Disruption of the activation of induced defenses, including induced emissions of VOCs.
Decreased photosynthesis (Long and Naidu 2002).	Less carbon for primary production (Sitch et al. 2007). Decreased/altered VOC emissions (Gouinguené and Turlings 2002).	Decreased olfactory cues for herbivores and natural enemies.
Formation of plant cellular injuries (Kangasjärvi et al. 2005).	Induction of VOCs (Heiden et al. 2003); increased/ altered VOC emissions (Heiden et al. 1999, 2003; Vuorinen et al. 2004a).	Alter the orientation of herbivores (increase or decrease herbivory). Alter plant–plant communication Disrupt induced indirect defenses.
Reaction with plant surfaces and leaf boundary layer (Holopainen 2004).	Alteration of the physico-chemical properties of plant surfaces (Müller and Riederer 2005); degradation of VOCs or O_3 quenching (Loreto et al. 2001); formation of new volatile compounds (Calogirou et al. 1999); decreased/altered VOC emissions (Loreto et al. 2004).	Altered herbivory. Effects on herbivore and parasitoid foraging
Reaction of VOCs in the atmosphere (Atkinson and Arey 2003)	Degradation of emitted VOCs (Atkinson and Arey 2003), formation of new volatile compounds (Calogirou et al. 1999), SOA formation (van Reken et al. 2006); decreased/altered VOC emissions (Pinto et al. 2007a, b, c; McFrederick et al. 2008), altered plant surface properties due to SOA (Cape 2008).	Reduced or altered signals to plants, pollinators, herbivores and carnivores (Pinto et al. 2007b; Himanen et al. 2009)

organisms. Quantitative or qualitative alterations to VOC profiles as a result of O_3 exposure may affect interactions between plants and pollinators, herbivores and natural enemies of herbivores (Gate et al. 1995).

Signal Transduction Pathways and Plant Defense Responses to O_3 Exposure

The development of visible symptoms following acute O_3 exposure is the result of a genetically controlled programmed cell death, which resembles the response occurring during incompatible plant-pathogen interactions (hypersensitive response, HR) (Fig. 2). In a recent study, Overmyer et al. (2008) used a range of Arabidopsis mutants to investigate the complexity of O_3 sensitivity, finding multiple phenotypic traits to be involved and to interact. In brief, O_3 gains its entrance to the leaf tissues through stomata. There is an initial increase in Ca^{2+} flux into the cytosol, and accumulation of ROS in the apoplasm. Different ROS are formed as a result of O_3 degradation, but hydrogen peroxide (H_2O_2) seems to be the major signaling molecule for the development of HR. However, there is evidence that superoxide anion radicals (O_2^-) and reactive nitrogen species (peroxynitrite, $ONOO^-$) also may act as signaling molecules. The initiation, development and containment of programmed cell death is controlled by the plant hormones salicylic acid (SA), ethylene (ET), and jasmonic acid (JA), respectively (for a review see Baier et al. 2005; Kangasjärvi et al. 2005). During the development of the HR, there is up and down regulation of many genes. Among others, O_3 induces the expression of genes that encode glutathione S-transferase, pathogenesis related proteins (PR- proteins), phenylalanine ammonia-lyase (PAL), and L-ascorbate peroxidase, which are all involved in defense responses (Baier et al. 2005; Heath 2008). The role of phytohormones in induced plant defenses is well known. SA and JA are particularly important signaling molecules in the activation of defense responses, including the induced emission of VOC after herbivore attack (Rao et al. 2000; Arimura et al. 2005 for a review). In general, the SA-pathway is activated mainly in response to microbial pathogens and is related to programmed cell death, whereas the JA-pathway is activated to a greater extent upon herbivore attack (Pieterse et al. 2001). There is increasing evidence, however, that sucking insects including aphids (Zhu and Park 2005), whiteflies (Kempema et al. 2007), and mirid bugs (Frati et al. 2009) induce methyl salicylate (MeSA) emissions. For instance, silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring) nymphs elicit the activation of SA defense. In addition, they seem to be able to suppress the JA pathway (Kempema et al. 2007), which plays a major role in the induced production of plant VOCs (Arimura et al. 2008). There also are examples of microbial pathogens that activate JA-mediated plant defense (Pieterse et al. 2001 for a review). Cross-talk between signaling pathways is thought to be crucial for the

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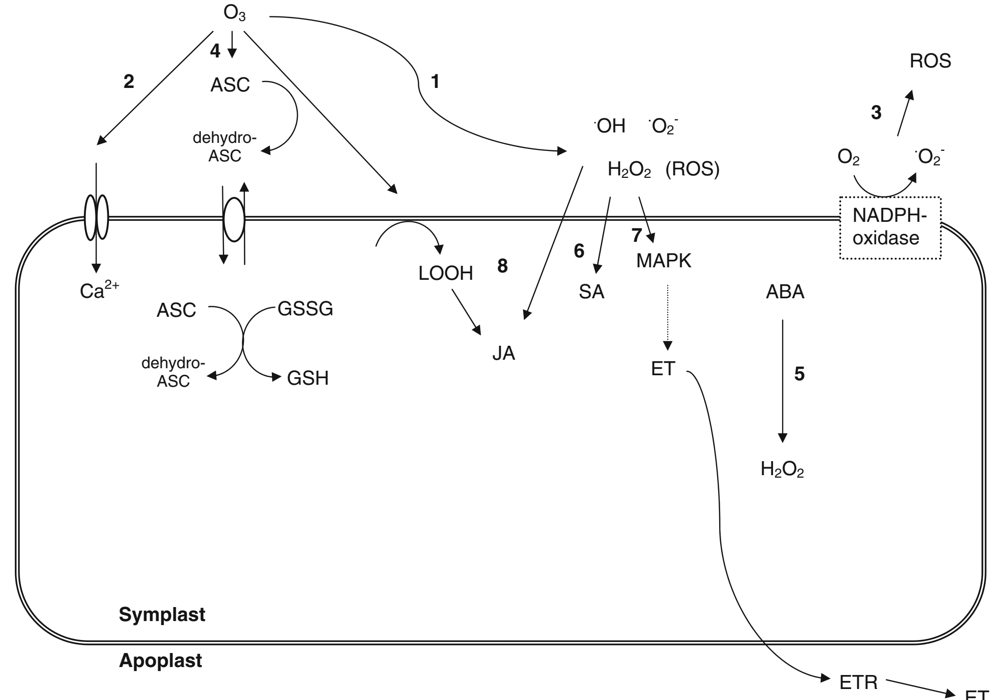


Fig. 2 O_3 degradation in the apoplasm results in the formation of reactive oxygen species (ROS) (**1**). There is an early increase in Ca^{2+} that leads to the activation of a mitogen-activated protein kinase (MAPK) and NADPH oxidase (**2**). This enzyme is responsible for the further production of ROS, and the spreading of the signal to other cells (**3**). A second peak of Ca^{2+} is concurrent with the expression of glutathione S-transferase. Antioxidants such as ascorbic acid (ASC) scavenge O_3, and its regeneration at the expense of glutathione also triggers changes in metabolism and gene expression (**4**). Abscisic acid (ABA)-induced closure of the stomata is the result of Ca^{2+} influx activation in the guard cells. ABA also can result in the production of H_2O_2, which in turn inactivates a protein phosphatase that inhibits ABA-induced stomata opening (**5**). The synthesis of salicylic acid (SA) is induced. SA is involved in the initiation of cell death (**6**). Ethylene (ET) is synthesized and is responsible for the propagation of the lesion (**7**). Jasmonic acid (JA) is synthesized from peroxidation of lipids (LOOH) in the membrane (**8**). The three hormones are involved in the expression of genes and further defense responses (Baier et al. 2005; Kangasjärvi et al. 2005)

optimal response of plants under different threats. Signaling pathways can affect each other in a positive manner; however, negative effects have been observed for plants under simultaneous attack by herbivores and fungal infection (Schoonhoven et al. 2006 for a review).

O_3 exposure elicits similar plant responses to fungal infection (Rao et al. 2000), and at biochemical and genetic levels there is overlap and interaction with signaling pathways involved in responses to different stresses (Heath 2008). Thus, the cross-talk between phytohormone-mediated signaling pathways may affect the induction of plant defense responses to biotic and oxidative stress under simultaneous herbivore attack and O_3 exposure. There are no studies that have strictly assessed the interaction between O_3- and herbivore-induced signaling pathways, but interactions between O_3 and herbivory have been observed in the emission of plant VOCs. Blande et al. (2007) reported increased emissions of some monoterpenes (α-pinene and/or β-pinene), and higher total monoterpene emissions from hybrid aspens (*Populus tremula* × *tremuloides* Michx.) grown under moderately elevated O_3 and infested by *Phyllobius pyri* L. compared to plants grown in ambient O_3 concentrations. In addition, Pinto et al. (2007a) found significant increases of MeSA in the headspace of Lima bean (*Phaseolus lunatus* L.) plants damaged by *Tetranychus urticae* Koch in O_3-enriched atmospheres (60 and 120 ppb) compared to damaged plants in filtered air. However, the mechanisms behind the interaction remain unknown.

O_3 Effects on Plant Photosynthesis: Less Carbon for Primary Production and VOC Biosynthesis

The starting point for the biosynthesis of VOCs is photosynthesis. Carbohydrates produced by photosynthesis are used mainly for primary plant metabolism, e.g., proteins, carbohydrates, and lipids involved in vital physiological functions, but they also are allocated to secondary plant metabolites. Volatile intermediates of primary metabolism, as well as volatile secondary metabolites, include isoprenoids (isoprene and terpenoids), fatty acid derivatives, phenylpropanoids/benzenoids, and other

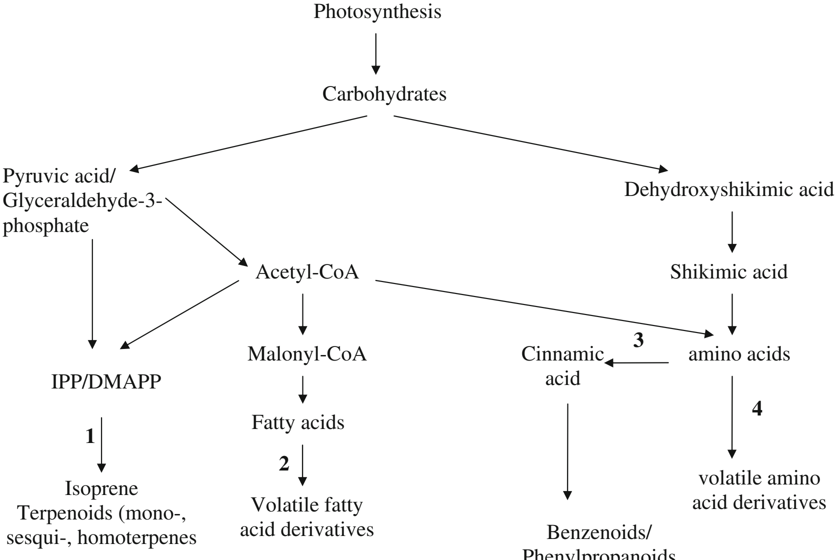


Fig. 3 Isoprenoids are formed from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In the cytosol, IPP is synthesized from 3 molecules of acetyl-CoA (MVA pathway). Two molecules of IPP and one of DMAPP condense to form farnesyl pyrophosphate, precursor of sesquiterpenes. In plastids, IPP is derived from pyruvate and glyceraldehyde-3-phosphate (MEP pathway). Isoprene is synthesized from DMAPP. One molecule of IPP and one of DMAPP condense to form geranyl pyrophosphate, the precursor of monoterpenes, and three of IPP and one molecule of DMAPP condense to form geranylgeranyl pyrophosphate the precursor of diterpenes. Diversity is achieved by terpene synthases. Additional reactions lead to irregular homoterpenes (**1**). Fatty acid derivatives originate in cell membrane-derived fatty acids: linolenic or linoleic acid, which are oxygenated via the LOX pathway. Oxidation results in 9-hydroxyperoxy and 13-hydroxyperoxy derivates of polyenoic fatty acids, which are cleaved resulting in short chain C6 or C9 VOCs (GLVs). Alternatively, they can be metabolized by other enzymes. For example, 13-hydroperoxy-linolenic acid results in jasmonic acid and its volatile ester methyl jasmonate via the octadecanoid pathway (**2**). Phenylpropanoids/benzenoids are formed from L-phenylalanine, synthesized via the shikimic acid pathway. It is converted into (*E*)-cinnamic acid by the L-phenylalanine ammonia-lyase (PAL). In further steps of phenylpropanoid synthesis, (*E*)-cinnamic is converted into hydroxycinnamic acids, aldehydes, and alcohols, which in turn can form VOCs. Benzenoids are formed by the shortening of the (*E*)-cinnamic side chain (**3**). Other amino acids (alanine, valine, leucine, isoleucine, methionine) are precursors for aldehydes, alcohols, esters, acids, and nitrogen and sulfur-containing VOCs (**4**) (Dudareva et al. 2006; Schoonhoven et al. 2006)

volatile compounds derived from amino acids (Dudareva et al. 2006, for a review) (Fig. 3). At the physiological level, O_3 exposure results in a decline in the photosynthetic capacity of plants, alters the assimilate transport capacity from leaves to sinks, and, therefore, alters carbon allocation to storage organs and buds, which may affect the subsequent season in perennial species (Riikonen et al. 2008). Effects on photosynthetic processes explain the reduced concentrations of different types of carbohydrate (from soluble sugars to starch) observed in trees and cultivated species (Jackson et al. 1999; Valkama et al. 2007 for a review). O_3 can damage or inhibit almost every step of photosynthesis from light capture in photosystem II to starch accumulation in chloroplasts and phloem loading (Long and Naidu 2002 and references therein). Chronic exposure to O_3 leads to a reduction in photosynthetic pigments (chlorophyll a, b and carotenoids) (Leitao et al. 2007). It also negatively affects the levels and activity of Rubisco (ribulose-1,5-biphosphate carboxylase/oxygenase), the enzyme that catalyses the first step of net photosynthetic CO_2 assimilation (Long and Naidu 2002 for a review), as well as increasing the activity and quantity of PEPc (phosphoenolpyruvate carboxylase) (Dizengremel et al. 2008), the enzyme that catalyses the carboxylation of phosphoenolpyruvate to form oxaloacetate (Kai et al. 2003 for a review). O_3 exposure also can reduce stomatal conductance (see Fig. 3 for the biochemical processes involved) and the amount of intercellular CO_2 (Black et al. 2007). It has been forecasted that stomatal closure due to ozone exposure will decrease the gross primary production and carbon stocks in plants (Sitch et al. 2007). Although stomatal control has no effect on emission of compounds that diffuse through the leaf cuticle, or emissions from external glands and hairs, it has been suggested that this could affect the emission of newly synthesized compounds (Kesselmeier and Staudt 1999). Since the production of herbivore-induced VOCs correlates with photosynthetic activity (Paré and Tumlinson 1997; Gouinguené and Turlings 2002), adverse effects on the photosynthetic capacity of the plant may also result in a decrease in precursors needed for the biosynthesis of VOCs, including induced compounds (Arimura et al. 2008).

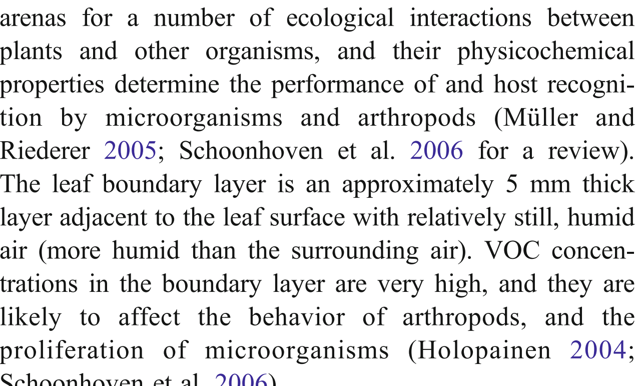
Formation of Plant Cellular and Tissue Injuries: Induction of VOC Emissions

As mentioned above, O_3-induced changes at the biochemical level lead to increased emission of VOCs. During the development of visible symptoms in plants, after acute and in some cases after chronic O_3 exposure, the induced/increased emission of VOCs has been observed as a common response (Rao et al. 2000). In general, O_3 increases VOC emissions from plants (Peñuelas et al. 1999) in a compound specific (Hewitt et al. 1994) manner. O_3-induced VOC blends include compounds that originate from different biosynthetic pathways, some of which are involved in the recruitment of predators and parasitoids, and in plant–plant interactions. For instance, the exposure of tobacco plants (*Nicotiana tabacum* L.) results in the induction of MeSA and an array of sesquiterpenes in both O_3-sensitive and O_3-tolerant cultivars (Heiden et al. 1999; Beauchamp et al. 2005).

The chronic exposure of Mediterranean woody plants (Llusià et al. 2002), *Pinus pinea* L. (Hewitt et al. 1994) and silver birch (*Betula pendula* Roth) (Vuorinen et al. 2005) to moderately elevated O_3 under field conditions resulted in emissions of individual terpenoids that increased, decreased, or remained unaffected. Under controlled conditions, fumigation of 3-year-old *Quercus ilex* L. plants for 5 days and single leaves for 4 h with elevated to extremely elevated O_3 concentrations (100 ppb up to 250 ppb) resulted in increased monoterpene emissions (Loreto et al. 2004). It has been suggested that such increases are involved in anti-oxidative stress responses (Loreto et al. 2004). The homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), commonly emitted after herbivore attack (Karban and Baldwin 1997), also is induced by acute O_3 exposure in Lima bean plants (Vuorinen et al. 2004a).

In addition to isoprenoids, products of the lipoxygenase (LOX) pathway are induced by O_3 (Heiden et al. 1999). Heiden et al. (2003) found a positive relationship between the development of visible symptoms and the emission of LOX products. It is likely that the emission of LOX products is the result of LOX activity as part of the signal cascade that results in the visible symptoms. Lipoxygenase activity and accumulation of linoleic acid has been observed during programmed cell death triggered by O_3 exposure (Rao et al. 2000). Beauchamp et al. (2005) proposed that O_3 induced destruction of the hydroperoxides formed by lipoxygenases leads to the formation of volatile LOX products.

Effects of O_3 on VOCs in the Leaf Boundary Layer

Plant surfaces and the leaf boundary layer are of great importance in chemical ecology. Plant surfaces are the 
arenas for a number of ecological interactions between plants and other organisms, and their physicochemical properties determine the performance of and host recognition by microorganisms and arthropods (Müller and Riederer 2005; Schoonhoven et al. 2006 for a review). The leaf boundary layer is an approximately 5 mm thick layer adjacent to the leaf surface with relatively still, humid air (more humid than the surrounding air). VOC concentrations in the boundary layer are very high, and they are likely to affect the behavior of arthropods, and the proliferation of microorganisms (Holopainen 2004; Schoonhoven et al. 2006).

O_3 may alter VOC concentrations in the leaf boundary layer by affecting the biosynthesis of epicuticular waxes (Percy et al. 1992) or by reacting with them or with VOCs present in the boundary layer (Fruekilde et al. 1998; Loreto et al. 2004). Changes in the biosynthesis of epicuticular waxes may alter the physicochemical properties of the leaf surface (Müller and Riederer 2005), and consequently the emission of VOCs through diffusion. The ozonolysis of epicuticular waxes and VOCs in the leaf boundary layer may result in the production of new compounds from the carbonyl family such as ketones and aldehydes (Fruekilde et al. 1998; Calogirou et al. 1999). These reaction products may act as antagonists of olfactory cues (repellents). New compounds also may be adsorbed to plant surfaces (Müller and Riederer 2005). These likely could prevent oviposition or act as feeding deterrents to insect herbivores, but research on this topic is needed. VOC concentrations in the leaf boundary layer also may be reduced. Monoterpenes, for instance, quench O_3, in a process that has a defensive function against oxidative stress (Loreto et al. 2004). How the reduced concentrations of VOCs in the leaf boundary layer are affected by O_3 episodes, and the consequent effects on, for example, germination of pathogenic fungal spores or foraging activity of small herbivores like thrips, is so far unknown.

Ozonolysis of VOCs in the Atmosphere: Degradation and Secondary Organic Aerosol (SOA) Formation

Many VOCs emitted by plants are very reactive with oxidants such as O_3, hydroxyl radicals, and nitrate radicals in the atmosphere (Atkinson and Arey 2003). In the particular case of O_3, it has been estimated that oxidation of plant VOCs accounts for nearly 50% of the O_3 removal within a forest canopy, whereas stomatal uptake and deposition on surfaces together account for the rest (Kurpius and Goldstein 2003). These figures illustrate the importance of O_3 with regard to degradation of plant VOCs. It has been proposed that O_3 can affect plant–insect interactions through chemical destruction or transformation of olfactory cues (Gate et al. 1995; McFrederick et al. 2008). Indeed, the concentration of the

scent plume is severely affected in the O_3-polluted atmosphere (McFrederick et al. 2008), and differences in the reactivity of individual compounds results in the altered composition of VOC profiles (Pinto et al. 2007a, b, c). In particular, some terpenoids and GLVs are readily oxidized, and their lifetimes in the atmosphere are as short as a few minutes (Atkinson and Arey 2003). Variation in lifetimes is dependent on the number of C–C double bonds or the degree of unsaturation of the compound. Saturated compounds such as 1,8-cineole and camphor are not affected by O_3. At the other extreme, polyunsaturated compounds such as *d*-limonene, β-ocimene, and α-terpinene are significantly affected by O_3 (Calogirou et al. 1996).

The oxidation of terpenes has been reviewed by Calogirou et al. (1999). When oxidized by O_3, they can lead to the formation of a variety of compounds from the carbonyl group (mainly ketones, hydroxyketones, and aldehydes) as well as carboxylic acids (Calogirou et al. 1999; Souza et al. 1999). In brief, O_3 adds to the C–C double bond(s) of terpenes, which results in the formation of a primary unstable ozonide that in turn decomposes to form epoxides as well as an energy-rich biradical (Criegee intermediate, Fig. 1) and a carbonyl (Calogirou et al. 1999). The decomposition of the excited Criegee intermediate also leads to the formation of OH•, which also may have an impact on atmospheric chemistry (Aschmann et al. 2002). OH• also are able to react with terpenes (Atkinson and Arey 2003). A series of reactions leads to stabilized products. The stabilized Criegee intermediates may react with water molecules, thus resulting in the formation of organic acids (Calogirou et al. 1999; Seinfeld and Pandis 2006). Side-reactions also result in the production of aldehydes and H_2O_2 (Calogirou et al. 1999), which can cause direct damage to plants (Sant'Anna et al. 2008 and references therein). Several compounds that result from the reaction of O_3 with common floral VOCs are odorous volatile compounds that may interfere with foraging of bees (McFrederick et al. 2008).

In addition, some products, such as carboxylic acids, are compounds with low volatility that form new particles or condense onto pre-existing ones (gas/phase partitioning) (Seinfeld and Pandis 2006). One factor that influences the potential of VOCs to form SOAs is their chemical structure (Hoffmann et al. 1997; Lee et al. 2006). For instance, compounds with several C–C double bonds, such as limonene, can produce more particles than cyclic terpenes, which in turn can produce more particles than acyclic compounds (Hoffmann et al. 1997). Although the potential of isoprene and monoterpenes for forming SOAs has been the subject of many studies (Hoffmann et al. 1997; Yu et al. 1999; Claeys et al. 2004), the evidence that sesquiterpenes also could be a major source of particles in the atmosphere is accumulating (Bonn and Moortgat 2003; van Reken et al. 2006). These compounds could contribute to the nucleation process through the rapid formation of condensable products (Calogirou et al. 1999; Lee et al. 2006). Although to date no experiments have strictly assessed the effects of SOA on plant-insect interactions, it has been suggested that secondary organic aerosols may affect plant surfaces by deposition, and, therefore, impact interactions of plants with other biotic agents (Cape 2008).

O_3-induced VOCs: Impacts on Herbivores and Predators

O_3-induced VOCs may affect host location by herbivorous insects. O_3 can trigger the emission of individual compounds that make the plant more attractive or repellent to herbivores or higher trophic levels. Jackson et al. (1999) found that O_3 increases the oviposition preference of *Manduca sexta* (L.) on tobacco, which is not explained by O_3 effects on the tobacco leaf chemistry, but according to the authors may owe to changes in VOC emissions. On the other hand, Cannon (1990) found that O_3-induced VOCs from red spruce needles repel spruce budworm larvae. O_3-induced VOCs also may mask herbivore-induced VOCs, alter the emission of signaling compounds and change ratios of compounds in the VOC blend. Moreover, O_3 induces the emission of VOCs, e.g., MeSA (Heiden et al. 1999) and the homoterpene DMNT (Vuorinen et al. 2004a) that are acknowledged olfactory cues for organisms of higher trophic levels (Dicke et al. 1990; De Boer and Dicke 2004). To our knowledge, only one study has tested whether O_3-induced VOC can disrupt induced indirect defense. Vuorinen et al. (2004a) showed that in conditions of filtered air with O_3 removed, the predatory mite *Phytoseiulus persimilis* Athias-Henriot is able to discriminate between VOCs emitted by ozonated Lima bean plants and by ozonated plants simultaneously damaged by its prey, the two spotted mite *T. urticae*. Moreover, it did not discriminate between the ozonated plants and plants grown in filtered air, although O_3 exposure of the plants induced VOC emissions (Vuorinen et al. 2004a).

O_3 Effects on Plant–Plant Signaling

Within-plant and plant–plant signaling mediated by VOCs is a subject that has received significant attention in recent years. However, the results of this signaling and its importance can be subtle. Consequently, debate over its existence has been intense (Baldwin and Schultz 1983; Fowler and Lawton 1985; Karban and Baldwin 1997; Baldwin et al. 2002; Karban et al. 2003). Now, with analyses of gene activation, plant–plant signaling has been demonstrated convincingly (Arimura et al. 2000; Ton et al. 2007). The most commonly reported plant response is priming for more rapid defense against herbivore attack (Ton et al. 2007). However, it seems

fair to say that this signaling is one of the most sensitive processes to occur in nature.

Several chemical compounds from different biosynthetic pathways have been implicated as likely candidates for plant–plant and within-plant signaling. To date, these compounds include the green leaf volatiles [(*E*)-2-hexenal (Arimura et al. 2001; Farag and Paré 2002; Mirabella et al. 2008), (*Z*)-3-hexen-1-ol (Ruther and Kleier 2005), and cis-3-hexenyl acetate (Yan and Wang 2006; Frost et al. 2008)], the terpenes myrcene and blended ocimene volatiles [(*E*)-β-ocimene, (*Z*)-β-ocimene and allo-ocimene) (Godard et al. 2008)], and the phytohormones [methyl jasmonate (Farmer and Ryan 1990; Preston et al. 2001), MeSA (Shulaev et al. 1997), and ethylene (O'Donnell et al. 1996)]. Increases in O_3 and other reactive species in the atmosphere could have a profound impact on this process. A recent laboratory study with Lima bean plants, using extra-floral nectar as a measurable marker of plant–plant communication, indicated that increased O_3 concentration reduces the distance over which this communication can occur (Blande, unpublished). Previous studies have indicated that plant–plant communication provides receiver plants with a tangible defense benefit (Karban et al. 2003; Kost and Heil 2005). Consequently, we may expect plants that receive 'more secure' signals, for example less reactive VOCs or underground signals via root exudates (Guerrieri et al. 2002), to gain advantage under conditions of increasing O_3.

Multitrophic Effects

To date, few laboratory experiments have been conducted to assess whether degradation of VOCs by O_3 can affect tritrophic interactions between plant, herbivore and herbivore natural enemies (Pinto et al. 2007b, c; Himanen et al. 2009). The ozonolysis of major herbivore-inducible VOCs did not affect the orientation of *Cotesia vestalis* (Haliday) (=*Cotesia plutellae*) towards cabbage plants (*Brassica oleraecea*) or oilseed rape (*B. napus* L.) damaged by *Plutella xylostella* L. -(Lepidoptera: Yponomeutidae) (Pinto et al. 2007b; Himanen et al. 2009). The orientation of the predatory mite *P. persimilis* towards herbivore-damaged Lima bean plants also was unaffected (Pinto et al. 2007b). The results of VOC analysis in these studies showed that the most common herbivore-inducible terpenes and GLVs were degraded in O_3-polluted environments, but that there are more atmospherically stable herbivore-induced volatile compounds, such as benzyl cyanide, a nitrile in cabbage, and methyl salicylate in lima bean. It is likely that these stable compounds can provide important long-distance plant-carnivore signals and may be used by natural enemies of herbivores to orientate. However, on insect resistant Bt crops, O_3 episodes may harm the orientation behavior of moth parasitoids. *C. vestalis* prefers the odors of herbivore-induced VOCs in filtered air over those exposed to O_3 and cannot discriminate between VOCs of intact plants and herbivore-damaged plants from a genetically transformed line of oilseed rape in elevated O_3, when feeding damage is substantially reduced (Himanen et al. 2009). Overall, these results suggest that decreased concentrations (McFrederick et al. 2008) or altered ratios of compounds (Pinto et al. 2007a) due to elevated O_3 concentrations, may affect plant–animal interactions.

Further Directions

Plant Biology At the cellular level, research on cross-talk between signal transduction pathways should take into consideration the effects of O_3, as the signaling cascades it elicits overlap with those elicited by biotic stresses (Heath 2008). This is an important focus, and will lead to us gaining insight of how plants simultaneously activate their defense systems against abiotic and biotic stressors. Genetic engineering, for O_3 sensitive mutants for example, is an important tool for obtaining more information about this cross-talk and the possible consequences for plant processes in the field of chemical ecology. It would be interesting to know, for instance, how O_3 induced defense affects further emission of VOCs in an ecological context, in other words, whether O_3 exposure can prime or delay the activation of induced indirect defense.

Ecology To date, few studies have addressed the effects of O_3 on chemical ecology in field conditions (McFrederick et al. 2008; Pinto et al. 2008). There is a need to assess whether herbivory and mutualistic interactions that rely entirely on O_3 reactive compounds can be affected by this pollutant. For example, the effect of O_3 on reactive monoterpenes emitted by coniferous trees, plays an important role in the interaction between trees and bark beetles (Kesselmeier and Staudt 1999). In multitrophic interactions, the orientation of natural enemies under laboratory conditions may be based on herbivore-inducible VOCs that undergo little reaction with O_3 and may act as olfactory cues (Pinto et al. 2007a, b). VOCs are decreased greatly in O_3 polluted atmospheres, and it is important to assess the sustainability of insect chemical communication in the field, particularly in fragmented areas (McFrederick et al. 2008). So far, experiments have been conducted under controlled conditions that do not resemble nature, and therefore, the effects could have been underestimated. Field O_3 enhancement experiments have demonstrated the negative effects of O_3 on the abundance of natural enemies (Percy et al. 2002). Foraging by herbivores, pollinators, and parasitoids occurs over several meters, and therefore, O_3 and other oxidants such as OH, which is also formed during ozonolysis of VOCs, could be affected. In future atmos-

pheres, elevated CO_2 could further reduce VOC emissions from O_3-stressed plants (Vuorinen et al. 2004b; Calfapietra et al. 2008), which may lead to significantly reduced foraging efficiency of parasitoids (Vuorinen et al. 2004b).

Perception EAG and behavioral responses of herbivorous insects to reaction products of induced plant volatiles should be assessed. Typically, parasitoid wasps use their antennae to sense the volatiles in the leaf boundary layer and palpate plant surface chemicals after arrival on a plant infested by a potential host herbivore species. One possible methodology for assessment could be to collect SOA particles formed in a reaction chamber under O_3 exposure, with subsequent offering of inert filter discs to various herbivorous insects. The control discs would consist of condensated plant VOCs collected from a reaction chamber without O_3. Although there is little evidence that O_3 affects plant surfaces and VOCs in the boundary layer, such effects on ecological interactions remain unexplored. There is evidence that plant–fungi interactions can be altered (Percy et al. 2002), but there is a need to assess how O_3-altered boundary layers may affect interactions with arthropods.

Below-Ground Impacts It is unlikely that below-ground interactions can be affected directly by O_3, as the gas will be lost by dry deposition. However, O_3 exposure affects assimilate transport and root/shoot allocation (Grantz et al. 2006). Disturbed carbon allocation may in turn affect the composition and amount of VOCs emitted by roots, and consequently affect interactions between herbivores and natural enemies e.g., entomopathogenic nematodes (Rasmann et al. 2005), or other plants (Nishida et al. 2005).

Reactivity of Pheromones This review is based on the effects of O_3 on biosynthesis, release and atmospheric behavior of phytogenic VOCs that act as infochemicals to other organisms. Functionality of VOCs emitted by arthropods themselves e.g., sexual pheromones, alarm pheromones, and aggregation pheromones in future atmospheres also deserve attention. Particularly long distance sex pheromones of moths (McFrederick et al. 2009), which are comprised of alcohols, ketones, or aldehydes, could be highly vulnerable in O_3-polluted environments.

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Impact of Elevated Levels of Atmospheric CO_2 and Herbivory on Flavonoids of Soybean (*Glycine max* Linnaeus)

Bridget F. O'Neill · Arthur R. Zangerl · Orla Dermody · Damla D. Bilgin · Clare L. Casteel · Jorge A. Zavala · Evan H. DeLucia · May R. Berenbaum

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Abstract Atmospheric levels of carbon dioxide (CO_2) have been increasing steadily over the last century. Plants grown under elevated CO_2 conditions experience physiological changes, particularly in phytochemical content, that can influence their suitability as food for insects. Flavonoids are important plant defense compounds and antioxidants that can have a large effect on leaf palatability and herbivore longevity. In this study, flavonoid content was examined in foliage of soybean (*Glycine max* Linnaeus) grown under ambient and elevated levels of CO_2 and subjected to damage by herbivores in three feeding guilds: leaf skeletonizer (*Popillia japonica* Newman), leaf chewer (*Vanessa cardui* Linnaeus), and phloem feeder (*Aphis glycines* Matsumura). Flavonoid content also was examined in foliage of soybean grown under ambient and elevated levels of O_3 and subjected to damage by the leaf skeletonizer *P. japonica*. The presence of the isoflavones genistein and daidzein and the flavonols quercetin and kaempferol was confirmed in all plants examined, as were their glycosides. All compounds significantly increased in concentration as the growing season progressed. Concentrations of quercetin glycosides were higher in plants grown under elevated levels of CO_2. The majority of compounds in foliage were induced in response to leaf skeletonization damage but remained unchanged in response to non-skeletonizing feeding or phloem-feeding. Most compounds increased in concentration in plants grown under elevated levels of O_3. Insects feeding on *G. max* foliage growing under elevated levels of CO_2 may derive additional antioxidant benefits from their host plants as a consequence of the change in ratios of flavonoid classes. This nutritional benefit could lead to increased herbivore longevity and increased damage to soybean (and perhaps other crop plants) in the future.

Keywords Flavonol · Isoflavone · *Popillia japonica* · *Aphis glycines* · *Vanessa cardui* · Global climate change · Plant-insect interactions

B. F. O'Neill · A. R. Zangerl · M. R. Berenbaum (✉)
Urbana-Champaign, Department of Entomology,
University of Illinois,
320 Morrill Hall, 505 S. Goodwin Avenue,
Urbana, IL 61801, USA
e-mail: maybe@uiuc.edu

O. Dermody
Pioneer Hi-Bred Switzerland S.A.,
DuPont Agriculture and Nutrition,
Via Cantonale/Galleria 3B,
CH-6928 Manno, Switzerland

D. D. Bilgin · E. H. DeLucia · M. R. Berenbaum
Urbana-Champaign, Institute for Genomic Biology,
University of Illinois,
1206 West Gregory Drive,
Urbana, IL 61801, USA

C. L. Casteel · E. H. DeLucia
Urbana-Champaign, Department of Plant Biology,
University of Illinois,
265 Morrill Hall, 505 S. Goodwin Avenue,
Urbana, IL 61801, USA

J. A. Zavala
Consejo Nacional de Investigaciones Cientificas y Technicas,
Catedra de Bioquimica, Facultad de Agronomia,
University of Buenos Aires,
Avenida San Martin 4453,
Buenos Aires, Argentina

Introduction

Atmospheric carbon dioxide (CO_2) levels have risen steadily since the start of the Industrial Revolution, from

280 to 387 ppm today (IPCC 2007). Current levels of atmospheric CO_2 are expected to double within the next 100 years (Ehhalt et al. 2001). Levels of ozone (O_3) also have been increasing in the lower atmosphere over this time period (Ehhalt et al. 2001). Numerous studies have been conducted on how various ecosystems might respond to changing atmospheric levels of these two gases (Kimball et al. 2002; Karnosky 2003; Booker et al. 2009; Lindroth 2010, this issue; Pinto et al. 2010, this issue). SoyFACE (Soybean Free Air gas Concentration Enrichment) is a research site at the University of Illinois, Urbana-Champaign, USA, for the study of the effects of elevated CO_2 and O_3 on a common midwestern agricultural ecosystem, that of *Glycine max* Linnaeus (soybeans). The United States produces 32% of the world's *G. max*, currently a $13 billion industry, and Illinois is one of the largest producers in the country (ASA 2008). Previous research conducted at this site revealed that damage by insect herbivores increased in elevated CO_2 treatments. This increase was associated with higher numbers of Japanese beetles (*Popillia japonica* Newman) found on those plants (Hamilton et al. 2005, Dermody et al. 2008). A subsequent study demonstrated that *P. japonica* fed a diet of *G. max* leaves grown in elevated CO_2 live longer and are more fecund than beetles that consume foliage grown under ambient conditions (O'Neill et al. 2008).

Changes in the antioxidant makeup of foliage under elevated CO_2 may be a factor that contributes to enhanced *P. japonica* longevity. Oxidative stress has been linked to aging, with the long-term build-up of oxidation products in the body leading to diminished cellular function (Harman 1956). This damage is countered by antioxidants, molecules that prevent oxidative damage in cells by quenching oxygen free radicals (Tang et al. 2006). Dietary constituents, including some phytochemicals, with antioxidant properties are associated with enhanced longevity in several insect species (Orr and Sohal 1994). Previous studies on plants grown under elevated CO_2 have determined that antioxidant content increases in fruits (e. g., strawberries, *Fragaria x ananassa* Dutch, Wang et al. 2003) and in foliage (e. g., European silver birch, *Betula pendula* Roth, Saleem et al. 2001). *Glycine max* contains several compounds with antioxidant properties, including flavonoids and isoflavonoids (Lee et al. 2006), but if and how foliar content of these compounds changes when plants are grown under elevated CO_2 has yet to be determined.

Flavonoids and isoflavonoids are biosynthesized via the phenylpropanoid pathway (key enzyme: phenylalanine ammonia-lyase = PAL) and contribute to plant defense against oxidative stressors (Dakora and Phillips 1996), such as pathogens, herbivores, or abiotic factors. Plant wounding induces these compounds (Hagerman and Butler 1991). Individual flavonoids in artificial diets can be detrimental to insect growth by virtue of their prooxidant properties (Ahmad and Pardini 1990). A study by Johnson and Felton (2001), however, indicated that the relative proportions of individual flavonoids in the food source are important in determining biological activity. The total complement of flavonoids can override the prooxidant trend and instead benefit herbivores by providing them with increased antioxidant protection. Quercetin in particular can stimulate feeding and promote herbivore growth (Ruuhola et al. 2001; Saleem et al. 2001). Isoflavonoids can have negative effects on herbivores (Simmonds and Stevenson 2001; Yu et al. 2003) irrespective of composition.

We measured flavonoid content of *G. max* foliage grown under elevated and ambient levels of CO_2 and O_3, singly and in combination. We hypothesized that elevated levels of CO_2, O_3, and herbivore damage will increase the products of the PAL pathway. The PAL pathway requires high levels of carbon to generate its products, including flavonoids (Tsai et al. 2006). Plants grown under elevated CO_2 have a greater C:N ratio than plants grown under ambient levels of CO_2 (Ainsworth et al. 2002), thus increasing the amount of carbon available to be transformed into phenolics. Increased exposure to O_3 is hypothesized to increase flavonoid production in *G. max* tissue and counteract potential oxidative damage. Ground level O_3 is an atmospheric pollutant that cycles seasonally and can have large effects on agricultural systems (Mauzerall and Wang 2001). Ozone is a powerful oxidative stressor, reducing *G. max* yield and increasing the incidence of early senescence (Morgan et al. 2006). Because production of flavonoids is known to vary with types of herbivory (Izaguirre et al. 2007), we measured changes in flavonoid content in response to damage from multiple herbivore feeding guilds (Mewis et al. 2006). Damage to plants grown under elevated levels of CO_2 was inflicted by phloem-feeders (*Aphis glycines* Matsumura, soybean aphid), leaf-chewers (*Vanessa cardui* Linnaeus, painted lady butterfly), and leaf-skeletonizers (*P. japonica*, Japanese beetle). Damage to plants grown under elevated levels of O_3 was inflicted by the Japanese beetle only.

Methods and Materials

Description of Field Site All herbivore feeding and tissue collection took place at the SoyFACE site (South Farms, University of Illinois, Savoy, IL, USA). SoyFACE is an open Free Air gas Concentration Enrichment system that exposes large field plots of *G. max* to elevated CO_2 and elevated O_3, individually or in combination in a full factorial design (Long et al. 2004). Crops at SoyFACE are rotated between corn (*Zea mays*) and *G. max* cv. Pioneer 93B15. Treatment plots (ambient air with 387 μmol mol^{-1}

CO_2, elevated CO_2 with a target of 550 μmol $mol^{-1}CO_2$, addition of 1.2 x ambient levels O_3, and combination of the elevated CO_2 and O_3 treatments) have a diameter of 20 m, cover 350 m^2, and are at least 100 m from any other plots. Treatments were replicated in a randomized block design. There were four plots for each treatment for a total of 16 plots present in the field. Average O_3 concentration during the 2005 season was 63.3 nmol/mol. Average CO_2 concentration during the 2005 season was 552 μmol/mol, during the 2006 season, 550.3 μmol/mol, and during the 2007 season, 553 μmol/mol. Ozone levels in the experimental plots were measured with an O_3 analyzer (model 49C; Thermo Scientific Instruments, Franklin, MA, USA) and the concentration of CO_2 in the plots was measured with an infrared gas analyzer (model SBA-1; PP Systems, Hitchin, UK). As there were no observed effects of feeding on *G. max* foliage grown under elevated O_3 on *P. japonica* longevity at this site, we discontinued measuring changes in flavonoid concentrations in foliage grown under elevated levels of O_3 with *V. cardui* and *A. glycines* feeding damage.

Herbivore Enclosures for P. japonica Adults and V. cardui Larvae Mesh cages for containing study species and excluding non-study species were constructed with 1× 4 mm plastic mesh material covering a PVC-pipe framework. Cages were raised over the course of the season with the height of the plants, with a maximum height of 1 m, 1 m wide, and 1 m deep covering ~30 plants and a total volume of 1 m^3. For *P. japonica* and *V. cardui*, two cages were erected in all elevated CO_2 and ambient plots, at opposite ends of the plot. For *P. japonica*, two cages were also erected in each elevated O_3 plot. *P. japonica* and *V. cardui* experiments were done separately, as natural populations of each species were present in the field at different points during the season.

Collection of P. japonica Emerging *P. japonica* adults were collected from the east side of Meadowbrook Park (Urbana, IL, USA) in the last week of June 2005. Sixty beetles were added to one of the two cages in each plot, 30 individuals on June 29, and 30 individuals on June 30. The second cage was left sealed to prevent any damage to the plants until sampled. Seventy additional beetles were added to cages on July 8, to replace those that had escaped.

Acquisition of V. cardui V. cardui larvae were obtained from Carolina Biological Supply (Burlington, NC, USA). Natural populations at the SoyFACE site were not large enough to use for this experiment, but we observed natural population cycles for the timing of placement of larvae onto the experimental plants. Ten larvae were bagged (1.4 mm mesh bags) over two neighboring trifoliate leaves chosen from the top four trifoliate leaves on each plant on July 14, 2006, with 10 plants bagged in one cage in each ambient and elevated CO_2 plot. The larvae were bagged on the plants in addition to being in the cage, as they were small enough to fit through the mesh of the cage. The second cage was left sealed to prevent any damage from occurring to the plants until they were sampled.

Collection of A. glycines A. glycines adults were obtained from a campus laboratory colony. Fifty aphids were bagged over one trifoliate chosen from the top four trifoliates on each of 10 plants on August 6, 2007, in each ambient plot and on another 10 plants in each elevated CO_2 plot. Plants without visible insect damage were selected. Ten plants were bagged without aphids for control tissue.

Collecting Leaf Tissue from Herbivore-Damaged and Control Plants Damaged and undamaged leaf tissues were sampled 2.5 d and 2 wk after beetles started feeding, 1 and 3 d after *V. cardui* larvae started feeding, and 1, 3, and 7 d after aphids started feeding. Tissue was sampled at multiple time points after herbivore feeding began because flavonoid and isoflavonoid levels are induced over time, and we

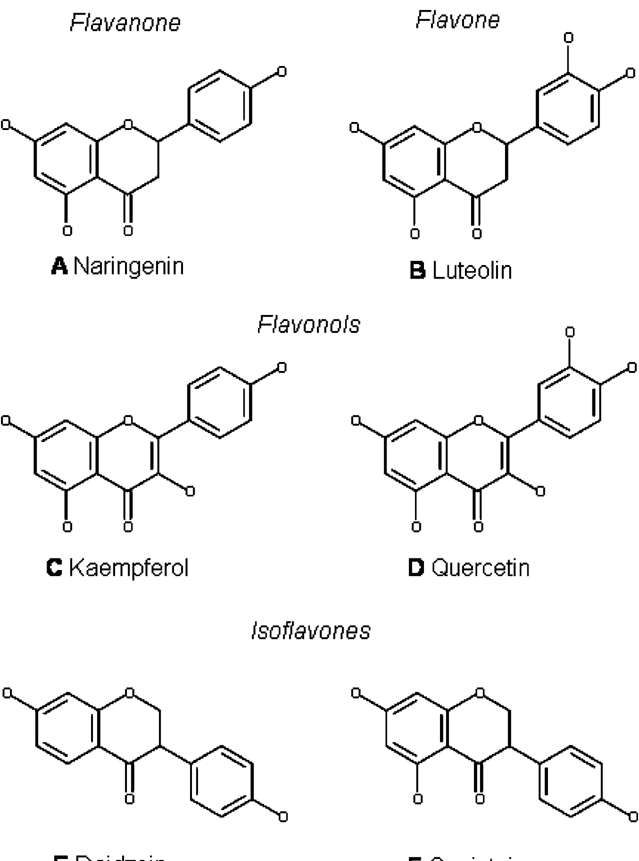

Fig. 1 Structures of the six flavonoid compounds identified in *Glycine max* foliage and phloem. **a**. Naringenin, **b**. Luteolin, **c**. Kaempferol, **d**. Quercetin, **e**. Daidzein, and **f**. Genistein. Figures drawn with SymyxDraw 3.2 (Symyx Solutions, Inc., San Ramon, CA)

wanted to capture their increase and decrease. Tissue always was selected from the top three leaf trifoliates of the sampled plants. On plants with herbivores, leaves exhibiting between 20–40% herbivore damage (as estimated by eye) but that did not have the herbivore currently feeding on them were selected. This allowed us to sample damaged tissue without disrupting feeding. For flavonoid analysis, four leaf disks were punched from one lateral leaflet from 5 plants with a metal cork borer (bore diam ~ 1.5 cm.). Leaf veins and spots of damage (either created mechanically or by herbivores) were avoided when leaves were punched so that equal leaf area was obtained in all disks. Disks from each cage were collected in paper envelopes that were immediately sealed and placed in liquid nitrogen.

Collecting Phloem from Aphid-Damaged and Control Plants A second leaf was selected from the top three leaf trifoliate leaves (again avoiding the trifoliate on which aphids were feeding) for phloem sap collection 1 d, 3 d, and 1 wk after aphids started feeding. Leaves were selected from aphid-damaged and control plants at each time point. Leaves were cut at the petiole, and the petiole was placed immediately in a 5-ml solution of 10 mM EDTA, pH 7 (Walter and DiFonzo 2007). The open tube tops and leaf petioles were Parafilm-wrapped to seal them, and the tubes were left in the dark at 5°C for 24 h. Phloem samples were kept at −80°C until analyzed.

Foliar Flavonoid Analysis for Herbivore-Damaged and Control Samples Six leaf disks (~1.77 cm^2 each) were taken from each sample, ground with a 6-mm glass bead (Fisher Scientific) in a Bead Beater (Wig L Bug, Crescent Dental Mfg. Co., Chicago, IL, USA), and combined with 500 µl methanol. Samples were vortexed and left at room temperature for 1 h. Samples then were sonicated for 1 min and centrifuged at 15,000 rpm for 5 min. Flavonoid compounds were separated on a reverse-phase high-pressure liquid chromatograph (HPLC) (712 WISP, Waters Corporation, USA) using a 250×4.6 mm ID 5 µm Capcell Pak C18 column (AG120, Shiseido Fine Chemicals, Japan) and a diode array detection system that has the capacity to

Table 1 Effects of elevated CO_2 and O_3 level (between-subject effects) on flavonoid amounts in undamaged and beetle-damaged soybean foliage[a]

Flavonoid	CO_2 *P* value	CO_2 *F* value	Ambient/elevated CO_2 mean µg/cm^2 leaf area	Ambient/ elevated CO_2 s.e.	O_3 *P* value	O_3 *F* value	Ambient/elevated O_3 mean µg/cm^2 leaf area	Ambient/ elevated O_3 s.e.	CO_2 x O_3 *P* value	CO_2 x O_3 *F* value
Genistein	0.088	3.440	0.257 0.118	0.053 0.053	**0.027**	**6.340**	**0.093** **0.282**	**0.053** **0.053**	**0.010**	**9.330**
Kaempferol	0.157	2.283	0.419 0.494	0.035 0.035	**0.025**	**6.529**	**0.393** **0.520**	**0.035** **0.035**	0.221	1.670
Kaempferol diglycoside	0.053	4.620	0.223 0.141	0.027 0.027	0.069	3.990	0.144 0.220	0.027 0.027	**0.001**	**17.364**
Kaempferol triglycoside 1	0.469	0.559	0.374 0.308	0.062 0.062	0.556	0.367	0.314 0.367	0.062 0.062	0.060	4.328
Kaempferol triglycoside 2	0.070	3.952	0.405 0.100	0.108 0.108	**0.018**	**7.474**	**0.043** **0.462**	**0.108** **0.108**	**0.030**	**6.079**
Luteolin diglycoside	0.224	1.644	0.130 0.168	0.021 0.021	**0.009**	**9.827**	**0.103** **0.196**	**0.021** **0.021**	0.242	1.513
Naringenin methyl hexose	0.766	0.093	0.140 0.131	0.020 0.020	**0.022**	**6.962**	**0.099** **0.173**	**0.020** **0.020**	**0.019**	**7.336**
Quercetin	0.797	0.069	0.317 0.334	0.045 0.045	**0.046**	**4.941**	**0.254** **0.397**	**0.045** **0.045**	0.191	1.925
Quercetin diglycoside	0.913	0.012	0.871 0.857	0.093 0.093	0.064	4.159	0.730 0.998	0.093 0.093	**0.019**	**7.312**
Quercetin hexose	0.959	0.003	0.223 0.221	0.028 0.028	0.103	3.119	0.187 0.257	0.028 0.028	0.177	2.057
Quercetin triglycoside 1	0.322	1.067	0.460 0.545	0.058 0.058	0.053	4.591	0.415 0.590	0.058 0.058	0.160	2.242
Quercetin triglycoside 2	**0.013**	**8.422**	**0.308** **0.448**	**0.034** **0.034**	**0.013**	**8.442**	**0.308** **0.448**	**0.034** **0.034**	0.943	0.005

[a] Changes in flavonoid amounts across the control and beetle-damage treatments over time were compared in foliage grown under ambient and elevated levels of CO_2 and O_3 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. The repeated measures effects from this analysis are separately reported in Tables 2 and 3.

measure wavelengths in the 210–400 nm range. The mobile phase consisted of 0.05% formic acid in H_20 (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 1 ml/min. The sample injection volume was 10 µl, and components were eluted with the following solvent gradient: from 0–1 min solvent A was 85%; from 1 to 10 min solvent A was decreased to 70%; from 10 to 20 min solvent A was decreased to 62%; from 20 to 25 min solvent A was decreased to 20%; from 25 to 30 min solvent A was maintained at 20%; finally, solvent A was increased to 85% for 5 min. Between each injection, a mixture of 85% solvent A and 15% solvent B was run for 15 min.

A sample containing all flavonoid peaks present in this soybean variety was analyzed by LC/MS (model 2010ev Shimadzu, Kyoto) and diode array detection by employing the same separation and column for quantitative analyses. Sample peak areas were measured at 254 nm. Mass spectra, UV spectra and retention times were used to identify compound peaks in each sample, and commercially available standards were employed to convert peak areas to µg/cm^2 leaf area. The amount of standard injected in g divided by the area of the peak produced was used as the calibration factor. The area of the sample peak multiplied by the calibration factor, then multiplied by the dilution factor (total extract volume divided by the volume injected), and all divided by the total leaf area extracted (number of disks multiplied by the area per disk) was the flavonoid amount in the plant, µg/cm^2 leaf area. Peaks were matched across samples, and concentrations were compared by double repeated measures analysis of variance (*ANOVA*) with a between-subject effect of CO_2 treatment, and a between-subject effect of O_3 treatment in the beetle experiments. Damage and date were considered as repeated measures because matched insect-damaged and control samples were taken from the same CO_2 and O_3 treatment plots for each time point (SPSS 9.0, Chicago, IL, USA).

Phloem Flavonoid Analysis of Aphid-Damaged and Control Samples Phloem samples were dried in a centrifugal evaporator (RC 10.22, Jouan Inc., USA). Five hundred µl of methanol were added to each sample, and samples were analyzed by HPLC using the same protocol as for foliage. Statistical analysis was conducted with the same protocol as used for foliage (SPSS 9.0, Chicago, IL, USA).

Table 2 Effects of beetle-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean µg/cm^2 leaf area	Standard error
Genistein	0.065	4.108	Undamaged	0.162	0.043
			Damaged	0.213	0.036
Kaempferol	0.377	0.842	Undamaged	0.438	0.032
			Damaged	0.475	0.032
Kaempferol diglycoside	**0.005**	**11.625**	Undamaged	**0.148**	**0.024**
			Damaged	**0.217**	**0.019**
Kaempferol triglycoside 1	**0.005**	**11.949**	Undamaged	**0.273**	**0.039**
			Damaged	**0.409**	**0.055**
Kaempferol triglycoside 2	0.995	0.000	Undamaged	0.253	0.103
			Damaged	0.252	0.058
Luteolin diglycoside	0.929	0.008	Undamaged	0.150	0.024
			Damaged	0.148	0.016
Naringenin methyl hexose	**0.025**	**6.540**	Undamaged	**0.100**	**0.019**
			Damaged	**0.171**	**0.020**
Quercetin	0.193	1.905	Undamaged	0.294	0.038
			Damaged	0.357	0.040
Quercetin diglycoside	0.064	4.172	Undamaged	0.776	0.096
			Damaged	0.952	0.056
Quercetin hexose	0.337	1.002	Undamaged	0.242	0.030
			Damaged	0.201	0.026
Quercetin triglycoside 1	0.632	0.241	Undamaged	0.519	0.056
			Damaged	0.486	0.050
Quercetin triglycoside 2	**0.044**	**5.066**	Undamaged	**0.312**	**0.030**
			Damaged	**0.444**	**0.044**

[a] Flavonoid amounts across the two atmospheric treatments over time were compared in control and beetle-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

Results

The isoflavone genistein (Fig. 1f), the flavone luteolin (Fig. 1b), the flavanone naringenin (Fig. 1a), and the flavonols quercetin (Fig. 1d) and kaempferol (Fig. 1c) were identified in all foliage irrespective of damage, as were glycosides of these compounds.

Flavonoid Results for Beetle-Damaged and Control Tissues The only main effect of CO_2 involved a single flavonoid; one of the two quercetin diglycosides increased significantly under elevated CO_2 (Table 1). In contrast, seven (genistein, kaempferol, kaempferol triglycoside, luteolin diglycoside, naringenin methyl hexose, quercetin, and quercetin triglycoside) of the 12 flavonoids increased in plants exposed to elevated ozone (Table 1). Five compounds not belonging to any particular flavonoid class were significantly affected by interactions between CO_2 and O_3. These interactions were of one type; large increases in flavonoids associated with elevated ozone were wholly or largely suppressed in elevated CO_2 treatments (data not shown). There also was a significant interaction between CO_2 and time with kaempferol triglycoside doubling in concentration at ambient CO_2 (from 0.267±s.e. 0.052 to 0.480±s.e. 0.092, μg/cm^2 leaf area) but remaining unchanged at elevated CO_2 (from 0.337±s.e. 0.052 to 0.279±s.e. 0.092, μg/cm^2 leaf area) (d.f.=63, F=5.322, P=0.040).

Beetle damage induced significantly increased concentrations of naringenin methyl hexose, kaempferol diglycoside, kaempferol triglycoside, and quercetin triglycoside (Table 2). Over the course of the sampling period, concentrations of six compounds (genistein, kaempferol, kaempferol diglycoside, naringenin methyl hexose, quercetin, and quercetin triglycoside) increased from the first to the second collection date (Table 3).

Flavonoid Results for Caterpillar-Damaged and Control Tissues There were no significant effects of elevated CO_2 (Table 4) on flavonoid concentration, and no significant interactions between effects of elevated CO_2 and damage by caterpillars. Levels of genistein were significantly higher in foliage damaged by *V. cardui* (Table 5). Quercetin triglycoside decreased in abundance from the first to the second collection date (Table 6).

Table 3 Effects of time (repeated measure) on flavonoid levels in undamaged and beetle-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	**0.021**	**7.015**	First collection	**0.097**	**0.026**
			Second collection	**0.278**	**0.067**
Kaempferol	**< 0.001**	**23.472**	First collection	**0.338**	**0.031**
			Second collection	**0.575**	**0.038**
Kaempferol diglycoside	**0.027**	**6.384**	First collection	**0.114**	**0.034**
			Second collection	**0.250**	**0.032**
Kaempferol triglycoside 1	0.215	1.718	First collection	0.302	0.037
			Second collection	0.379	0.065
Kaempferol triglycoside 2	0.054	4.552	First collection	0.099	0.034
			Second collection	0.407	0.145
Luteolin diglycoside	0.840	0.042	First collection	0.153	0.027
			Second collection	0.145	0.020
Naringenin methyl hexose	**0.029**	**6.189**	First collection	**0.089**	**0.024**
			Second collection	**0.183**	**0.023**
Quercetin	**0.034**	**5.757**	First collection	**0.272**	**0.032**
			Second collection	**0.379**	**0.045**
Quercetin diglycoside	0.719	0.136	First collection	0.840	0.079
			Second collection	0.888	0.105
Quercetin hexose	0.232	1.581	First collection	0.192	0.025
			Second collection	0.252	0.036
Quercetin triglycoside 1	**0.006**	**10.932**	First collection	**0.368**	**0.037**
			Second collection	**0.637**	**0.072**
	0.241	1,524	First collection	0.428	0.040
			Second collection	0.328	0.053

[a] Flavonoid amounts in the control and beetle-damage treatments and across the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

Table 4 Effects of CO_2 (between-subjects effect) on flavonoid levels in undamaged and caterpillar-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean µg/cm² leaf area	Standard error
Genistein	0.904	0.016	Ambient	0.083	0.024
			Elevated	0.122	0.024
Kaempferol diglycoside	0.605	0.297	Ambient	0.157	0.045
			Elevated	0.192	0.045
Kaempferol triglycoside 1	0.467	0.604	Ambient	0.569	0.052
			Elevated	0.627	0.052
Quercetin diglycoside	0.925	0.010	Ambient	1.195	0.091
			Elevated	1.208	0.091
Quercetin hexose	0.582	0.339	Ambient	0.771	0.060
			Elevated	0.721	0.060
Quercetin triglycoside 1	0.910	0.014	Ambient	0.663	0.048
			Elevated	0.671	0.048
Quercetin triglycoside 2	0.690	0.175	Ambient	0.961	0.100
			Elevated	1.021	0.100

[a] Flavonoid amounts over time and across the control and caterpillar-damage treatments were compared in foliage grown under ambient and elevated levels of CO_2 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. The repeated measures effects from this analysis are separately reported in tables 5 and 6.

Flavonoid Results for Aphid-Damaged and Control Foliar Tissues Concentration of only one flavonoid, quercetin, increased under elevated CO_2 conditions (Table 7). Two of the quercetin triglycosides, kaempferol triglycoside, quercetin hexose, and genistein increased from the first to the third collection date, whereas luteolin diglycoside declined in abundance from the first to the third collection date (Table 8). There were no significant effects of aphid damage (Table 9), and no significant interactions between effects of elevated CO_2 and aphid feeding.

Flavonoid Results for Aphid-Damaged and Control Phloem The only confirmed flavonoid in phloem was the isoflavone daidzein (Fig. 1e). This isoflavone increased in concentration from the first to the third collection date (Table 8) and showed a marginally significant increase in concentration in response to aphid damage (Table 9). There were no significant interactions.

Discussion

Elevated CO_2 affected only concentrations of a single flavonoid in *G. max*. Plants grown under elevated amounts of CO_2 have higher quercetin:kaempferol ratios, and quercetin may be acting as an antioxidant for these plants,

Table 5 Effects of caterpillar-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean µg/cm² leaf area	Standard error
Genistein	**0.001**	**36.102**	Undamaged	**0.074**	**0.022**
			Damaged	**0.131**	**0.020**
Kaempferol diglycoside	0.863	0.032	Undamaged	0.179	0.049
			Damaged	0.170	0.027
Kaempferol triglycoside 1	0.937	0.007	Undamaged	0.600	0.051
			Damaged	0.596	0.034
Quercetin diglycoside	0.873	0.028	Undamaged	1.208	0.097
			Damaged	1.294	0.049
Quercetin hexose	0.769	0.094	Undamaged	0.732	0.078
			Damaged	0.760	0.042
Quercetin triglycoside 1	0.825	0.053	Undamaged	0.658	0.057
			Damaged	0.677	0.048
Quercetin triglycoside 2	0.605	0.297	Undamaged	0.945	0.122
			Damaged	1.037	0.097

[a] Flavonoid amounts were compared across atmospheric treatments over time in control and caterpillar-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

Table 6 Effects of collection date (repeated measure) on flavonoid levels in undamaged and caterpillar-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.976	0.001	First collection	0.095	0.022
			Second collection	0.109	0.013
Kaempferol diglycoside	0.453	0.643	First collection	0.204	0.042
			Second collection	0.145	0.054
Kaempferol triglycoside 1	0.560	0.380	First collection	0.612	0.031
			Second collection	0.584	0.053
Quercetin diglycoside	0.244	1.669	First collection	1.263	0.044
			Second collection	1.140	0.104
Quercetin hexose	0.192	2.159	First collection	0.773	0.030
			Second collection	0.719	0.059
Quercetin triglycoside 1	**0.019**	**10.232**	First collection	**0.712**	**0.025**
			Second collection	**0.623**	**0.046**
Quercetin triglycoside 2	0.541	0.420	First collection	1.016	0.091
			Second collection	0.966	0.069

[a] Flavonoid amounts across control and caterpillar-damaged tissues in the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

quenching reactive oxygen species (ROS) (Qiu et al. 2008). Elevated levels of atmospheric CO_2 have been suggested to cause oxidative damage to plants, so additional amounts of antioxidants may well be produced to counter this increased damage (Cheeseman 2006). Ozone, a strong oxidizing agent, was a far more effective inducer of flavonoid production in this study. This inducing effect, however, was almost entirely absent when CO_2 also was elevated. One possible explanation for this interaction is that soybean leaf stomatal conductance is decreased in elevated CO_2 atmospheres, thus reducing ozone fluxes into the leaf and buffering its effects (Bernacchi et al. 2007; Mishra et al. 2008).

The significant increase in quercetin and the non-significant decrease of kaempferol changes the flavonoid ratios found in plants grown under elevated CO_2 and may change the total balance for herbivores also from prooxidant to antioxidant (Galati et al. 2002). Similar shifts in the quercetin:kaempferol ratio have been observed in plants grown under temperature stress (Albert et al. 2009). Quercetin has a higher quenching capability than kaempferol (Rice-Evans et al. 1996), and it was theorized that under temperature stress more quercetin was produced because of this quenching ability (Albert et al. 2009). Similar effects may occur in soybeans growing under

Table 7 Effects of CO_2 (between-subject effect) on flavonoid lvels in undamaged and aphid-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.473	0.587	Ambient	0.163	0.034
			Elevated	0.126	0.034
Quercetin	**0.015**	**11.510**	Ambient	**0.452**	**0.099**
			Elevated	**0.927**	**0.099**
Luteolin diglycoside	0.607	0.295	Ambient	0.290	0.036
			Elevated	0.318	0.036
Kaempferol triglycoside 1	0.260	1.547	Ambient	0.893	0.219
			Elevated	1.278	0.219
Quercetin hexose	0.937	0.007	Ambient	1.220	0.199
			Elevated	1.243	0.199
Quercetin triglycoside 1	0.215	1.923	Ambient	0.831	0.295
			Elevated	1.411	0.295
Quercetin triglycoside 2	0.158	2.593	Ambient	1.335	0.251
			Elevated	1.907	0.251

[a] Flavonoid amounts over time and across the control and aphid-damaged treatments were compared in foliage grown under ambient and elevated levels of CO_2 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. Repeated measures effects from this analysis are reported in Tables 8 and 9.

Table 8 Effects of collection date (repeated measure) on flavonoid levels in undamaged and aphid-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm² leaf area	Standard error
Genistein	**0.028**	**7.880**	First collection	**0.061**	**0.015**
			Third collection	**0.273**	**0.058**
Quercetin	0.800	0.234	First collection	0.762	0.204
			Third collection	0.698	0.118
Luteolin diglycoside	**< 0.001**	**54.017**	First collection	**0.379**	**0.061**
			Third collection	**0.095**	**0.023**
Kaempferol triglycoside 1	**0.001**	**33.137**	First collection	**0.626**	**0.141**
			Third collection	**1.598**	**0.285**
Quercetin hexose	**0.015**	**10.919**	First collection	**0.639**	**0.120**
			Third collection	**2.183**	**0.442**
Quercetin triglycoside 1	**0.019**	**9.742**	First collection	**0.627**	**0.118**
			Third collection	**1.490**	**0.238**
Quercetin triglycoside 2	**0.004**	**20.481**	First collection	**1.020**	**0.199**
			Third collection	**2.403**	**0.397**

[a] Flavonoid amounts across control and aphid-damaged tissue in the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

elevated CO_2 stress. Genistein levels in *G. max* were decreased under elevated CO_2 treatment at the $P=0.1$ level. This decrease in genistein content in plants grown under elevated CO_2 is fortuitous for most herbivores, as genistein reduces most insects' longevity and overall fitness (Piubelli et al. 2005). The multiple changes in *G. max* flavonoid content under elevated CO_2 conditions may contribute to the increased longevity experienced by *P. japonica* on *G. max* foliage grown under elevated carbon dioxide (O'Neill et al. 2008). This hypothesis could be tested by supplementing soybean foliage grown under ambient levels of CO_2 with enough quercetin to equal the quercetin:kaempferol ratio found in foliage grown under elevated levels of CO_2, even with the higher levels of kaempferol found in the foliage grown under ambient levels of CO_2.

The differential flavonoid response of *G. max* to leaf-skeletonization, non-skeletonizing leaf chewing, and phloem sucking feeding damage suggests that the induced defense responses of plants depend on the type of damage inflicted (Felton et al. 1994; Kaplan et al. 2008). Whereas leaf damage in the form of skeletonization induces changes in several flavonoid and isoflavonoid levels, feeding damage by the

Table 9 Effects of aphid-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm² leaf area	Standard error
Genistein	0.237	1.726	Undamaged	0.102	0.028
			Damaged	0.187	0.050
Quercetin	0.333	1.107	Undamaged	0.622	0.105
			Damaged	0.757	0.084
Luteolin diglycoside	0.071	4.786	Undamaged	0.246	0.028
			Damaged	0.362	0.043
Kaempferol triglycoside 1	0.731	0.129	Undamaged	1.048	0.219
			Damaged	1.124	0.149
Quercetin hexose	0.720	0.141	Undamaged	1.184	0.197
			Damaged	1.279	0.180
Quercetin triglycoside 1	0.735	0.126	Undamaged	1.181	0.352
			Damaged	1.061	0.144
Quercetin triglycoside 2	0.550	0.400	Undamaged	1.517	0.257
			Damaged	1.724	0.225

[a] Flavonoid amounts across the two atmospheric treatments over time were compared in undamaged and aphid-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

caterpillar *V. cardui* increased levels of one genistein glycoside, and feeding by the aphid *A. glycines* did not affect production of any compound. The increase in flavonoid content over time is to be expected as these compounds are induced by herbivore damage (Hagerman and Butler 1991), and by increased photooxidative stress, particularly in August when the aphid experiments were conducted (Stark et al. 2008).

The increase in flavonoid content in *G. max* tissue grown under elevated O_3 conditions is also expected as O_3 is a powerful oxidative agent (Sager et al. 2005). This increase is consistent with the results of other studies that have looked at changes in genes involved in the flavonoid biosynthesis pathway in *G. max* foliage in response to elevated O_3 conditions (Bilgin et al. 2008). The increase in content of all flavonoid compounds regardless of class could be utilized by the plant as a general response against adverse environmental conditions (Bilgin et al. 2008). This increase in content of all flavonoids in *G. max* foliage grown under elevated O_3 leaves the quercetin:kaempferol balance unchanged, helping to explain the lack of an effect on longevity of *P. japonica* feeding on this foliage (O'Neill et al. 2008) and contributing to the lack of an effect on herbivory on plants grown under elevated levels of O_3 at this research site (Dermody et al. 2008).

In a separate study, tissue from plants in the beetle treatments from this experiment was examined *via* microarray to investigate transcriptional responses to elevated CO_2 and leaf skeletonization (Casteel et al. 2008). Transcriptional changes involved in flavonoid and isoflavonoid biosynthesis supported our findings that abundance increased after beetle damage (e.g., putative flavonol synthase, 2′-hydroxydihydrodaidzein reductase, isoflavone reductase-like protein), with or without elevated CO_2, and increased flavonol biosynthesis (e.g., flavonol synthase-like oxidoreductase) under elevated CO_2 conditions (Casteel et al. 2008).

These changes in defense compound/antioxidant concentrations were examined in the context of damage inflicted by only a single herbivore; impacts of different types of damage inflicted simultaneously have yet to be determined. Given the multiplicity of responses that depend on the nature of feeding damage, the prediction of future impacts of global atmospheric changes on the soybean agroecosystem will be difficult. The changes in the quercetin: kaempferol ratio of *G. max* foliage under elevated CO_2 though, suggests that the benefits of *G. max* foliage to select herbivores may be great.

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REVIEW ARTICLE

Heavy Metal Pollutants and Chemical Ecology: Exploring New Frontiers

Robert S. Boyd

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Abstract Heavy metals are an important class of pollutants with both lethal and sublethal effects on organisms. The latter are receiving increased attention, as these may have harmful ecological outcomes. For example, recent explorations of heavy metals in freshwater habitats reveal that they can modify chemical communication between individuals, resulting in "info-disruption" that can impact ecological relationships within and between species. Info-disruption can affect animal behavior and social structure, which in turn can modify both intraspecies and interspecies interactions. In terrestrial habitats, info-disruption by metals is not well studied, but recent demonstrations of chemical signaling between plants via both roots and volatile organic molecules provide potential opportunities for info-disruption. Metals in terrestrial habitats also can form elemental plant defenses, in which they can defend a plant against natural enemies. For example, hyperaccumulation of metals by terrestrial plants has been shown to provide defensive benefits, although in almost all known cases the metals are not anthropogenic pollutants but are naturally present in soils inhabited by these plants. Info-disruption among microbes is another arena in which metal pollutants may have ecological effects, as recent discoveries regarding quorum sensing in bacteria provide an avenue for metals to affect interactions among bacteria or between bacteria and other organisms. Metal pollutants also may influence immune responses of organisms, and thus affect pathogen/host relationships. Immunomodulation (modification of immune system function) has been tied to some metal pollutants, although specific metals may boost or reduce immune system function depending on dose. Finally, the study of metal pollutants is complicated by their frequent occurrence as mixtures, either with other metals or with organic pollutants. Most studies of metal pollutants focus on single metals and therefore oversimplify complex field conditions. Study of pollutant impacts on chemical ecology also are difficult due to the necessity of studying effects at varying ecological scales: "dynamic scaling" of chemical ecology studies is rarely done completely. It is clear that much remains to be learned about how heavy metal pollution impacts organisms, and that exciting new research frontiers are available for experimental exploration.

Keywords Behavior · Elemental defense · Heavy metals · Immunomodulation · Information disruption · Infochemical effect · Pollution · Quorum sensing

Introduction

Humanity's ability to mine and use metals has played a major role in development of modern human society (Wilson 1996). Many metals have a wide range of uses, but these have come at a significant environmental price: some (generally called heavy metals) have serious negative environmental consequences, yet our dependence on them continues to result in large inputs into our environment (Han et al. 2002). Heavy metals are an important category of pollutants and as such have major detrimental impacts on both human health (Duruibe et al. 2007) and the health of terrestrial and aquatic communities and ecosystems (Sánchez 2008). A number of metals have been included in the term "heavy metal," but the term has not been used consistently in the literature (Sánchez 2008). The primary focus here will be on those commonly

R. S. Boyd (✉)
101 Life Sciences Building, Department of Biological Sciences, Auburn University,
Auburn, AL 36849-5407, USA
e-mail: boydrob@auburn.edu

studied as pollutants (Han et al. 2002), including Cd, Cu, Cr, Hg, Pb, Ni, and Zn.

Much research on heavy metal pollutants has focused on their direct negative effects on organisms. These direct effects stem from the general toxicity of heavy metals to a variety of biological processes. The negative effects often result in serious toxicological symptoms, including mortality. These effects are generally dose-dependent, but some heavy metals are required in relatively small amounts as micronutrients for many organisms, so that low doses can have positive direct effects (Lefcort et al. 2008). In addition to direct effects, pollutants may have indirect effects through their impact on the structure of food webs in communities (Fleeger et al. 2003). For example, Lefcort et al. (2002) reported that snails in a heavy metal-polluted lake were less sensitive to the metals than their internal parasites, so that parasite loads were markedly reduced in the polluted lake and this resulted in greater abundance of the snail. In another example, Stepanauskas et al. (2006) dosed freshwater microcosms with a range of Cd or Ni concentrations and found that bacterioplankton densities increased at very low metal concentrations. They explained this effect as a consequence of low metal doses suppressing growth of bacteriovore eukaryotes. There are many cases in which organisms differ in susceptibility to pollutants, and hence community structure is altered in polluted locations by both direct and indirect effects.

Recently, there has been recognition that serious sublethal direct effects of many pollutants (including heavy metals) can occur. These may have subtle impacts on species performance that in turn can lead to important indirect effects. "Info-disruption" is a recently defined category of sublethal direct effect (Lürling and Scheffer 2007) in which a pollutant interferes with the chemical communication networks that inform organisms about their biotic and abiotic environments. For example, a review by Lürling and Scheffer (2007) described the importance of the "smellscape" to organisms and how pollutants can alter their perception of it. Klaschka (2008) referred to info-disruption as the "infochemical effect" and provided an additional recent review of this burgeoning field. Klaschka (2008) points out that the ecological importance of info-disruption, in both laboratory and field studies, is greater than was previously believed. Both these reviews broadly illustrate how anthropogenic pollutants (including heavy metals) can affect organismal ecology and summarize recent advances in this area.

This mini-review is not intended to comprehensively cover the literature regarding all heavy metals and their ecological effects. Instead, I seek to extract from recent literature concepts regarding the biological effects of metal pollutants that might be of interest to chemical ecologists. My emphasis is in illustrating these concepts to demonstrate their potential ecological importance and as a stimulus for additional research. Because most of the research regarding info-disruption by pollutants has emerged from studies in freshwater aquatic systems (Lürling and Scheffer 2007; Klaschka 2008), those systems will be the starting place for this review. From there, I will move to terrestrial systems and examine how concepts being developed from aquatic systems might apply to relationships in terrestrial habitats. Then, I will summarize some of the recent excitement in microbiology regarding chemical communication (and potential social organization) in bacteria, thus illustrating the potential for heavy metals to impact these aspects of microbial biology and touching upon two other metal/microbial issues of great recent interest, antibiotic resistance and immunomodulation. Finally, I will review briefly some other issues that complicate our understanding of heavy metal pollutant effects on organisms and communities (including impacts of multiple pollutants, and how issues of scale add complexity to ecological studies), and conclude with a few considerations of future research directions.

Heavy Metals in Aquatic Communities

Heavy Metals and Predator/Prey Relationships Perhaps the most active research area regarding info-disruption by heavy metals in aquatic communities deals with the effects of metals on predator/prey interactions. The concern is that heavy metals can influence predator/prey interactions by degrading the ability of prey to respond to predators, ultimately resulting in decreased prey population sizes due to increased predator success (McPherson et al. 2004). Another reason for the interest in heavy metals and behavior in aquatic communities is that heavy metals may have behavioral effects at concentrations much less than at which they have lethal effects (Scott and Sloman 2004), suggesting that regulatory pollution limits based upon standard toxicological studies may be too high to prevent damage to aquatic communities through these sublethal behavioral effects. This realization has led to calls for increased integration of behavioral studies into ecotoxicological investigations (e.g., Clotfelter et al. 2004; Klaschka 2008).

With regard to predator/prey relations, a number of investigations have shown decreased ability of fish exposed to heavy metals to respond to skin extracts, which can serve as an alarm signal for prey species (Smith 1992; Kats and Dill 1998). Heavy metal pollutants for which these effects have been shown include Cu (Carreau and Pyle 2005; Pyle and Mirza 2007), Cd (Honda et al. 2008; Kusch et al. 2008), and Hg (Smith and Weiss 1997). Some studies have taken these investigations into the field to show that fish in metal-contaminated lakes respond differently from fish in

uncontaminated lakes. For example, McPherson et al. (2004) showed that prey fish in a Ni/Zn contaminated lake did not respond to skin extract of another prey species, whereas prey fish in an uncontaminated lake did. In other cases, fish from polluted and uncontaminated lakes have been brought into the laboratory for comparison of responses to skin extracts. Mirza et al. (2009) studied wild yellow perch (*Perca flavescens*) from a lake contaminated by a mixture of heavy metals (mainly Cu, Ni, and Zn), finding that the fish from the contaminated lake did not respond to a chemical alarm cue whereas those from an uncontaminated lake did.

Although the studies referred to above have focused upon ability of prey to detect and avoid predators, heavy metals may affect mechanisms other than escape behavior that are used by prey to avoid predation. For example, the bioluminescence ability of some marine organisms (or the presence of bioluminescent organisms as symbionts in hosts) is thought to be an anti-predator behavior (Buskey and Swift 1983; Jones and Nishiguchi 2004; Cronin 2005), and there is evidence that heavy metal pollution can affect bioluminescence ability. For example, Deheyn et al. (2000) collected individuals of an echinoderm, the brittle star *Amphipholis squamata*, along a heavy metal (Cd, Cu, Fe, Pb, Zn) gradient in a polluted bay in Spain. Those from the most polluted area had less intense and more slowly generated bioluminescence responses, and bioluminescence responses of individuals transferred from a less- to a more-polluted area became weaker and slower. The authors suggest that, since light production is a defense for some bioluminescent organisms, this defense would be less effective for this species in polluted areas. Bioluminescence of marine organisms also may play a role in mate attraction (Deheyn and Latz 2009) or attraction of prey (Cronin 2005), so that interference with bioluminescence by heavy metals may impact other organismal interactions.

While most studies show that info-disruption can result in lowered defensive ability and thus increased predation, info-disruption may act to enhance predator defense in some cases. I know of no case involving heavy metals in which prey defense against predation is enhanced by a metal pollutant, but Lürling (2006) reported that an organic pollutant (the surfactant FFD-6) affects a green alga (*Scenedesmus obliquus*) in a way that mimics effects of predator compounds that stimulate an anti-predator trait (formation of relatively large colonies of cells). Presumably, algal cells in the larger colonies formed in a polluted lake would be less susceptible to predation by zooplankton (Lass and Spaak 2003). Info-disruption also may benefit prey by negatively affecting predator search ability. For example, Smith and Weiss (1997) studied effects of Hg pollution on the behavior of mummichogs (*Fundulus heteroclitus*), a tidal creek fish that preys upon invertebrates but in turn is preyed upon by blue crabs (*Callinectes sapidus*). Fish from a polluted site attempted to capture prey less frequently than those from an unpolluted site, and the polluted site fish also were more likely to be captured by blue crabs. Although the effects of Hg were not shown to be due to info-disruption (rather than other sublethal effects), these results suggest that the net result of info-disruption will depend on the suite of species involved, their sensitivities to the pollutant, etc.

Another way by which a heavy metal can affect an organism negatively is for the metal to prevent that organism from detecting and avoiding areas that contain toxic heavy metal concentrations. Hansen et al. (1999a) found that low levels of Cu can damage olfaction in Chinook salmon (*Oncorhynchus tshawytscha)* and rainbow trout (*Oncorhynchus mykiss*), and fish actively avoided Cu-contaminated water. However, if fish were exposed to sublethal Cu levels that damaged their olfactory abilities, they were unable to detect waters containing lethally high Cu concentrations, thus suggesting that exposure to low levels of metal pollutants may result in mortality if fish are later exposed to greater concentrations.

Although I have emphasized studies of fish behavior in the previous sections, studies of invertebrates have shown heavy metal effects on their behavior as well. For example, Vuori (1994) concluded that sublethal concentrations of Cd changed behavior of caddisfly larvae. Larvae exposed to low (12 μg/L) Cd were less aggressive in intraspecific encounters, and behavior of both intruder and resident larvae that had been exposed to this low concentration of Cd differed from that of larvae not exposed to Cd. In a second example, Michels et al. (2000) showed that sublethal doses of Cd affected the phototactic behavior of the crustacean *Daphnia magna*. A recent review of chemically-induced predator defenses in plankton summarized a number of cases of behavioral predator defenses present in this invertebrate group (Lass and Spaak 2003), illustrating the potential for heavy metals to impact these and thus affect ecological relationships.

To this point I have discussed how heavy metals may influence predator/prey interactions via info-disruption, by affecting the ability of prey to detect predators or vice versa. Heavy metals may influence predator/prey interactions in ways other than through their effects on behavior, such as through effects on physical defenses. Physical defense (involving a change in morphology) is common in plankton (Lass and Spaak 2003), and a number of studies have reported morphological reactions of plankton species to the presence of predator kairomones. For example, *Daphnia* produce neckteeth that reduce the rate of predation

by *Chaoborus* midge larvae (Parejko 1991). Mirza and Pyle (2009) reported that low levels of Cu (10 μg/L) interfered with ability of *Daphnia* to respond to the *Chaoborus* midge kairomone that induces neckteeth formation. *Daphnia* exposed to Cu and kairomone had fewer and smaller neckteeth than those exposed to kairomone alone. These less well-defended offspring had lower survival when exposed to predators (*Chaoborus* midge larvae).

Heavy Metal Effects on Social Structure and Reproductive Behavior The effects of heavy metals on an animal species are likely complex, and one of the factors that can add to that complexity is the social structure of the organism(s) involved. Social structure is important in many organisms, especially vertebrates such as fish, and a recent review by Sloman (2007) of trace heavy metal effects on salmonid fish social hierarchies illustrates how sociality can influence pollutant effects on species. Her review shows that social status can change the physiology of an individual and, as a result, its susceptibility to pollutants. Furthermore, uptake may vary among heavy metals depending upon the physiological effects of a fish's social status. For example, she reported that dominant fish accumulated less Cu (Sloman et al. 2002; Sloman 2007) whereas studies of Cd showed that dominant fish accumulated more Cd (Sloman et al. 2003). In this case, the specific effect may depend on the mechanism by which the heavy metal is taken up by cells. Copper is taken up via Na transport pathways (Sloman et al. 2002), and subordinate fish have increased Na uptake due to increased social stress (Sloman et al. 2004). On the other hand, Cd crosses gills via Ca transport pathways (Sloman 2007). If dominant fish take up more Ca to support the higher growth rates that result from their social position, then Cd uptake may be greater than for subordinate fish (Sloman et al. 2003). In addition to an impact of hierarchical position on metal effects, heavy metal pollutants also can affect social hierarchy formation itself (Sloman 2007). For example, Sloman et al. (2003) showed that rainbow trout exposed to Cd formed social hierarchies more quickly. The reason for this more rapid hierarchy formation was unclear, as Cd both increased fish growth rate and also damaged olfactory sensing cells. Interestingly, waterborne Cu (Sloman et al. 2002) or Cd (Sloman et al. 2003) did not affect hierarchies that had been formed in fish populations prior to exposure to the heavy metals.

The route of exposure of a heavy metal may determine if social structure will affect metal concentration in a fish species (Sloman 2007). For example, if exposure is dietary rather than waterborne, then a fish's social status and a contaminated food item's perceived value may influence metal intake. This could occur if a dominant individual that monopolizes a prized but contaminated food source receives an increased dose, whereas a subordinate forced to eat less prized but less contaminated foods will have lesser exposure. This scenario suggests an interesting possibility for accelerated social turnover, where dominants are harmed by diet, become less aggressive and/or are less healthy, and are replaced by subordinates, which in turn become harmed, etc.

Reproductive behaviors are another arena in which heavy metals may exert negative effects on aquatic species. The above information regarding chemical cue detection and social hierarchies illustrates two ways in which metals may affect reproductive behaviors, since reproduction in aquatic animals often involves chemical cues (Paul and Ritson-Williams 2008) and may involve social hierarchies (Sloman 2007). However, heavy metals also may directly affect gamete interactions, as illustrated by a study of the effects of Cu on fertilization of eggs of an intertidal polychaete, *Galeolaria caespitosa* (Hollows et al. 2007). This worm is a broadcast spawner, so that the sperm are diluted rapidly over distance. Laboratory tests showed that Cu had stronger effects on fertilization at low sperm concentrations than at high ones. The authors also took their study into the field, finding that Cu also reduced fertilization success there. They concluded that eggs needed to be much closer to a sperm source in the presence of Cu for successful fertilization to occur in the field. Relatively sedentary aquatic species may require a minimum population density to be reproductively successful. This is an example of an Allee effect in population ecology, broadly defined as a positive relationship between individual fitness and population size or density (Stephens et al. 1999). Hollows et al. (2007) point out that their research on *Galeolaria caespitosa* shows that Cu pollution can magnify an Allee effect: the negative effect of Cu on fertilization success means that successful fertilization will occur at greater population densities than when Cu pollution is absent. Given that Allee effects are increasingly recognized as important in ecology and conservation biology (e.g., Levitan and McGovern 2005; Liu et al. 2008), potential effects of metal pollutants on reproductive processes deserve more investigation.

Mode of Action of Heavy Metals Reports of the effects of heavy metals on behavior have stimulated researchers to investigate the mechanisms by which those effects operate. Some studies of fish suggest that heavy metals directly damage sensing cells in the olfactory epithelium (e.g., Hansen et al. 1999b), but in some cases fish that return to unpolluted conditions recover their olfactory function (e.g., Beyers and Farmer 2001; Baldwin et al. 2003; Sandahl et al. 2006), depending on the degree and extent of exposure to the metals. More recent research has revealed cases of lasting damage from short-term embryonic exposure. For example,

Carreau and Pyle (2005) showed that short (5–7 days) embryonic exposure of fathead minnows (*Pimephales promelas*) to Cu caused decreased olfactory function several months after they were removed from Cu-contaminated conditions. A study by Blechinger et al. (2007), using embryos of zebrafish (*Danio rerio*) exposed for 3 hr to Cd, documented olfactory cell death, the effects of which decreased olfactory ability 4–6 weeks later.

Besides direct effects on sensing cells, there is an additional pathway by which heavy metal pollutants can interfere with fish senses. As summarized by Sloman (2007), certain metals (Cd, Hg, Ni, Mn) can be transported along nerves into more central portions of the nervous system and can exert disruptive effects there. Therefore, the examination of the olfactory epithelium for damage to detect olfactory impairment may not reveal functional failure. For example, Mirza et al. (2009) studied yellow perch from unpolluted and metal-contaminated lakes in Canada. They found that fish from the contaminated lakes had functional olfactory epithelium, but failed to respond to chemical alarm cues. They hypothesized that heavy metals of these chronically exposed fish may be accumulating in signal generating olfactory cells and interfering with signal processing rather than signal reception.

Heavy Metals as Elemental Defenses It also is possible that heavy metals have a direct chemical defense function. In general, studies of chemical defense in both marine (summarized in Pawlik 1993; Hay 1996) and terrestrial (summarized in Dearing et al. 2005; Schowalter 2006) environments have focused on the tremendous variety of organic chemicals (termed secondary chemicals) that are produced by organisms and that protect them from natural enemies. Elemental defense, a concept that has emerged mainly from studies of some terrestrial systems (see terrestrial system section below), suggests that certain elements present at high concentrations in an organism's tissues may protect it from natural enemies (Boyd 2004). In general, elemental defenses differ from secondary chemicals because they are not carbon-based structures synthesized via the metabolic pathways that generate secondary chemical defenses but instead are based upon other elements absorbed from the environment (Martens and Boyd 1994).

Elemental defense by heavy metals has been suggested in a few instances from studies in marine environments. For example, Capon et al. (1993) reported extremely high concentrations of Cd and Zn in an Antarctic marine sponge (*Tedania charcoti*) and demonstrated that those concentrations were capable of antibacterial effects. A recent survey of Antarctic sea spiders (Pycnogonida) found high levels of Cd, Cu, Ni, or Zn in some samples (Jöst and Zauke 2008): the authors suggested that the high Ni levels in some samples (up to 200 mg/kg on a dry mass basis) may have defensive effects, although no experimental investigation was done. In another example, Pawlik (1993) suggested that some tunicates that contain high levels of metals (notably V, but also Cr, Fe, Mg, Mn, Mo, and Ti) may be protected by those elements. In particular, feeding experiments with V (Stoecker 1980) showed that artificial food amended with V was less palatable to two species of representative generalist marine predators. Recently, Odate and Pawlik (2007) tested whether the high V concentration in a marine tunicate has a defensive effect. They were unable to confirm that V in the form present in the tunicate was defensively active, and suspected that low tissue pH was the primary chemical defense in the case they examined. Thus, unlike in terrestrial systems, there currently is little evidence that heavy metals (including metal pollutants) have defensive effects in marine systems. The topic, however, is not well explored.

Heavy Metals in Terrestrial Communities

Heavy Metals and Plant Behavior/Communication Whereas the literature from aquatic (particularly freshwater) communities suggests heavy metals may be important info-disruptors, similar effects have not been reported from terrestrial communities. This may be due to fundamental differences between aquatic and terrestrial habitats (Zimmer and Zimmer 2008): for example, heavy metals in above-ground terrestrial communities generally are not suspended in the medium (air) in high concentrations, whereas heavy metals in polluted aquatic communities usually are dissolved in the medium that bathes those organisms. On the other hand, effects of some heavy metals (e.g, Hg and Pb) on the nervous systems of terrestrial animals are well known, and these can include effects on behavior (Duruibe et al. 2007), illustrating a potential for heavy metal effects on terrestrial animal behavior.

The lack of information on heavy metal info-disruption in terrestrial communities might also stem from a lack of investigation. For example, the section above on effects of metals on aquatic animal behavior brings up the question of whether metals in soil might affect plant root behavior. Whether plants "behave" in a way that is analogous to animals is debatable (Trewavas 2009), but McNickle et al. (2009) have attempted to define common ground for studies of both animal and plant "behavior." In terms of plant root foraging, it has long been known that plant roots respond to heterogeneity in the soil environment (Hodge 2009), and research on the Zn hyperaccumulator plant *Thlaspi caerulescens* revealed existence of "zincophilic" foraging (Schwartz et al. 1999;

Haines 2002), the preferential proliferation of roots in high-Zn soil patches. Studies of plants that hyperaccumulate other metals (e.g., Liu et al. 2009) have documented similar cases of root proliferation in high-metal soil patches. The opposite situation, plant roots avoiding high-metal soil patches (perhaps in response to metal pollution of the soil), may exist for less metal tolerant species. Thus, soil heavy metal concentration may affect root growth patterns of plants and thus root behavior.

Direct response of plant roots to soil heavy metals may be analogous to direct responses of aquatic animals to waterborne heavy metals, but the more subtle info-disruption effects of heavy metals that are beginning to be identified in aquatic communities are difficult to relate to plants. Recent research, however, suggests plant roots may be more environmentally aware than previously thought. In particular, studies have suggested that roots can discriminate between self- and non-self roots (e.g., Falik et al. 2003; Gruntman and Novoplansky 2004). Furthermore, Dudley and File (2007) reported that roots of sea rocket (*Cakile edentula* var. *lacustris*) detect and respond to roots of conspecifics, and particularly suggested that they are capable of kin recognition. Debate has occurred regarding both the claim of kin selection by plants (Klemens 2008; Milla et al. 2009) and root mediated kin recognition (Hess and de Kroon 2007), but the field seems open for further experimental work (de Kroon 2007). Early work with desert plant roots suggested that *Larrea tridentata* and *Ambrosia dumosa* shrubs can detect roots of heterospecifics (Mahall and Callaway 1992, 1996) and the concept of chemical signaling between plant roots seems generally accepted (Hodge 2009).

Plant communication is not limited to the belowground realm. Communication between plants by volatile airborne chemicals has attracted interest since at least the 1970s, but the signals may be non-specific and susceptible to “eavesdropping” by neighboring plants (e.g., Karban et al. 2004). A recent report (Karban and Shiojiri 2009) showed that sagebrush (*Artemisia tridentata*) individuals discriminate between self-generated and non-self-generated signals. In this study, herbivore damage was significantly less when a clone of the target plant generated a volatile signal that induces herbivore chemical defense (compared to a volatile signal generated by a non-clone plant). Volatile chemical communication provides another pathway by which info-disruption may occur. The ability of a plant to detect volatile chemical signals may be changed due to exposure to heavy metals, most likely through impacts of metal-contaminated soil. Stress from exposure to the contaminated soil (Koricheva et al. 1998) may affect a plant’s physiology and thus the volatile chemical signaling system. Dry deposition of metal-containing dust is another avenue by which heavy metals can impact plants. Entry of metal-laden dust into stomata, or dissolution of metals from dust during rain or dew deposition, may allow metals to penetrate directly into tissues and ultimately cells (Greger 2004). How metal exposure may influence volatile chemical generation or detection remains to be explored.

The existence of chemical signaling between plants provides an opportunity for heavy metal pollutants to interfere with these signal mechanisms, perhaps in ways similar to the info-disruption documented in aquatic animal research. As in aquatic systems, signal disruption may occur in several ways: heavy metals may prevent the signal from reaching the receptor, interfere with the receptor’s reception, or interfere with signal transmission. To my knowledge there is no evidence of such info-disruption, but now that the question has been raised it can receive research attention.

Heavy metal pollutants, via other indirect pathways, also may influence plant interactions with natural enemies. As with other pollutants, heavy metal pollutants cause stress that changes plant characteristics, including secondary chemistry. Mithöfer et al. (2004) pointed out that heavy metals may stimulate production of oxylipins in plants: some of these, such as jasmonic acid, have well investigated roles as plant defense signals (e.g., Kessler et al. 2004). Koricheva et al. (1998) reviewed papers regarding the plant stress hypothesis: the concept that increased stress may cause changes in plant chemistry that favor natural enemy performance. They concluded that the hypothesis was supported in some cases but not in others, depending on many factors including the type of stress and insect feeding mode. It is clear from their review, however, that stress caused by heavy metal pollutants is another pathway by which plant ecological relationships with natural enemies can be impacted.

Heavy Metals as Elemental Defenses Heavy metal pollutants in terrestrial environments may directly affect plants by providing an elemental defense against plant natural enemies. The elemental defense hypothesis was originally applied to plants that accumulate extraordinary concentrations of elements, often heavy metals, in their tissues (Boyd and Martens 1992). These “hyperaccumulator” plants most often occur in habitats that contain naturally elevated levels of the element the plants accumulate. For example, many hyperaccumulators of Ni (Ni hyperaccumulators are the most numerous category of hyperaccumulators: Baker et al. 2000) occur on serpentine soils, and these often are naturally Ni-rich (Alexander et al. 2007). Tests of elemental defense (reviewed by Boyd 2007) have reported defensive effects (in some but not all cases) for a number of elements (As, Cd, Ni, Se, and Zn), most of which are heavy metals. These elements and others can be pollutants, leading to the question of whether a heavy metal

pollutant might form an elemental defense for a plant exposed to that pollutant.

I know of one study that may illustrate an elemental defensive effect by a pollutant. Scheirs et al. (2006) studied effects of Cd pollution on herbivory by a leaf-mining fly (*Chromatomyia milii*) on the grass *Holcus lanatus*. Plants were exposed to a range of relatively low Cd concentrations, and their suitability as hosts was determined by monitoring the performance of both adult flies and their offspring. Plant growth decreased in response to Cd treatments, and plant tissue Cd concentrations increased. Feeding and oviposition by adult flies, as well as performance of fly offspring, decreased as Cd concentration in plant tissues increased. Although this experiment showed plants exposed to Cd pollution were less suitable hosts, the authors were unable to determine if this was a direct effect of Cd or an indirect effect of plant host quality changes that occurred due to Cd exposure (in this case, for example, soluble sugar concentrations in plant tissues decreased with increasing Cd exposure). Thus, it cannot be concluded that the pollutant was directly responsible for the defensive effect, but this study does provide an example of a metal pollutant changing a plant-herbivore relationship in favor of the plant.

The concept of elemental defense is not limited to the producer trophic level. Elements (including heavy metals) found at relatively high concentrations in herbivores (or higher trophic levels) may have defensive benefits (Boyd and Martens 1998), but they have rarely been investigated. Nickel hyperaccumulator plants in serpentine ecosystems can be fed upon by specialized herbivores that themselves have high whole-body Ni levels (Boyd 2009). If high body Ni concentrations have defensive effects, these “high-Ni insects” (Boyd 2009), defined as those containing >500 µg Ni/g (on a whole-body, dry mass basis), are good choices for experimental tests of elemental defense against predators or pathogens. An initial investigation (Boyd and Wall 2001), that used the high-Ni mirid bug *Melanotrichus boydi,* found that mortality of crab spiders (*Misumena vatia*) was significantly greater when they were fed the high-Ni insect compared to those fed low-Ni prey. No defensive effect was found, however, for tests of two other predators (Boyd and Wall 2001) or for several pathogens (Boyd 2002), so that no general conclusion about elemental defense in this high-Ni herbivore could be reached. Elemental pollutants other than heavy metals also may have defensive effects in herbivores: Vickerman and Trumble (2003) showed that consumption of high-Se herbivore prey negatively affected the predacious bug *Podisus maculiventris*. Elemental defensive benefits likely will be positively correlated with heavy metal concentration. Biomagnification (sometimes used synonymously with bioconcentration) is generally defined as an increased element concentration in one trophic level relative to the previous one (Gray 2002). If biomagnification were to occur with a heavy metal, defensive metal effects would be more likely for organisms higher in a food web due to their higher body concentrations of that metal. Many studies have examined biomagnification, with mixed evidence that heavy metals biomagnify (e.g., Goodyear and McNeill 1999; Gray 2002; Burger 2008). Focus of elemental defense studies on systems that show biomagnification would be most likely to demonstrate elemental defense at higher trophic levels: yet even without biomagnification, differing abilities to bioaccumulate can result in defensive metal effects at higher trophic levels.

Heavy Metals and Pathogen/Microbial Communities

Heavy Metals as Pathogen/Microbial Info-disruptors Chemosensation is a vital way in which bacteria gain information about their environment. During the last 20 years, microbiologists have been excited to learn that chemical signaling among bacteria can be extensive and can lead to behaviors that suggest analogies to animal behaviors (West et al. 2007). For example, quorum sensing is the production of molecules by bacterial cells that indicate bacterial density and stimulate a population to act in a coordinated fashion (West et al. 2007; Williams et al. 2007). Discovery of this phenomenon stimulated much research into the mechanisms involved (Waters and Bassler 2005), the potential uses of these molecules in treating diseases (Ni et al. 2009), and even exploration of social phenomena (West et al. 2007) such as kin selection (Diggle et al. 2007) and cheating (Zhang et al. 2009) in bacteria. Although not all microbiologists are in agreement regarding the phenomenon’s behavioral analogies with eukaryotes (Turovskiy et al. 2007), it is interesting to consider how metal pollutants might impact the behavioral ecology of microbes.

Metals have some direct connections to quorum sensing. For example, Fe is an important and often limiting resource for bacteria, and they may produce chelating chemicals (siderophores) in order to sequester it from their environment (Challis 2005). These siderophores may have multiple functions, including acting as quorum sensors or antibiotics (Schertzer et al. 2009). Tolerance of some bacteria to heavy metals is increased greatly by formation of a bacterial biofilm, and biofilm formation can be controlled by quorum sensing molecules (e.g., Sarkar and Chakraborty 2008). At least one quorum sensing molecule contains a heavy metal (Zn) ion as part of its structure (Hilgers and Ludwig 2001).

Recognition of the importance of chemical signaling to microbes raises questions regarding the potential for heavy

metal pollutants to disrupt communication similar to those discussed for macrobiotic organisms in the preceding sections. Do heavy metal pollutants interfere with signal production, inactivate a signal, or interfere with signal reception? And, if they do, then what are the consequences for ecological relationships between organisms? Research on quorum sensing disruption appears to focus primarily on organic molecules: recent reviews (González and Keshavan 2006; Rasmussen and Givskov 2006; Ni et al. 2009) do not mention interference by heavy metals yet contain a plethora of examples involving organic compounds. The potential for pollutants to interfere with quorum sensing appears unevaluated, but there are tantalizing glimpses of how this could work. For example, the green alga *Chlamydomonas reinhardtii* produces organic chemicals that apparently mimic quorum sensing molecules produced by some bacteria (Teplitski et al. 2004). In another case, Manefield et al. (2002) report that a red alga (*Delisea pulchra*) produces organic chemicals (halogenated furanones) that interfere with quorum sensing of bacteria and may prevent algal biofilm formation on the surface of the algal thallus. There also are many examples in which chemicals produced by eukaryotes influence bacterial quorum sensing (e.g., González and Keshavan 2006; Ni et al. 2009), providing chemical interactions that might be influenced by heavy metal pollutants.

Heavy Metals and Antibiotic Resistance Another connection between heavy metals and disease incidence is concern that metal pollutants may act as co-selection agents for antibiotic resistance in bacteria. Co-selection occurs when selection for one trait simultaneously selects for a second trait: in this case, selection for metal resistance also selects for resistance to antibiotics (Baker-Austin et al. 2006). In fact, one explanation for the evolution of antibiotic resistance genes, some of which have had extensive evolutionary histories prior to widespread human uses of antibiotics, is their ability to function in heavy metal resistance (Aminov and Mackie 2007): essentially pre-adapting them to current human uses of antibiotics. Antibiotic resistance is a serious threat to human health, and how such resistance evolves and the role of the environment in this process is of increasing interest (Baquero et al. 2008). One reason for this interest is that antibiotics may degrade in the environment but metals do not, and heavy metal pollution continues to increase (Han et al. 2002). Thus, heavy metal pollution may help maintain antibiotic resistant bacterial strains even if input of antibiotics into the environment is reduced. For example, chloramphenicol has been banned in China since 1999 (Dang et al. 2008). A recent survey (Dang et al. 2008) of antibiotic-resistant bacteria found resistant bacteria remained in Jiaozhou Bay, and suggested that pollutants (including heavy metals) were providing a reinforcing selective pressure that maintained the antibiotic resistance of bacterial populations. This indicates that antibiotic resistance, once evolved, will be difficult to eradicate and thus will remain a serious human health concern.

Co-selection includes two mechanisms: co-resistance and cross-resistance. In co-resistance, a gene that confers heavy metal tolerance is present on the same genetic element as a gene for antibiotic resistance. Aminov and Mackie (2007) point out that antibiotic resistance genes may occur on plasmids large enough to include other genes, including heavy metal resistance genes. In such situations, a bacterium that possesses that plasmid would benefit from the physiological capability to resist both stresses. In cross-resistance, the same gene codes for resistance to both heavy metals and antibiotics. Baker-Austin et al. (2006) point out that cross-resistance is probably common because resistance to stresses induced by both heavy metals and antibiotics rely on similar cellular mechanisms. Co-selection also may occur because of a bacterial behavior (such as biofilm formation) that indirectly increases resistance to both heavy metals and antibiotics (Baker-Austin et al. 2006).

Evidence for co-resistance between heavy metal pollutants and antibiotics takes two forms. First, studies of metal polluted versus unpolluted areas show greater antibiotic resistance in bacteria from the polluted areas. For example, Stepanauskas et al. (2005) measured both heavy metal and antibiotic resistance of bacterial communities both before and after they were exposed to metal-polluted wastewater, finding significant increases in resistance to both stressors after metal exposure. In a study of freshwater microcosms, Stepanauskas et al. (2006) showed that bacteria from Cd- and Ni-treated microcosms were significantly more resistant to antibiotics than those from controls. A number of studies, in a variety of environments exposed to a variety of heavy metals, have shown similar results, suggesting that co-selection is widespread (Baker-Austin et al. 2006). Second, co-selection has been demonstrated experimentally by adding heavy metals to a system and measuring antibiotic resistance change. Berg et al. (2005) found that Cu-amended soils contained more Cu-resistant and antibiotic-resistant bacteria than unamended soils.

Heavy Metals as Immunomodulators The previous section regarding antibiotic resistance and heavy metals suggests a related issue that has been connected to metal pollutants: immunomodulation. Immunomodulation is a change in immune system function due to effects of a chemical (Lawrence and McCabe 2002), including pollutants such as heavy metals: it includes immunotoxicity (or immunodepression), a suppressing effect on the immune system, as well as immunostimulation (an accelerating effect). Either of these phenomena can negatively affect an organism's

health. As with other heavy metal effects, immune system effects can vary depending on metal dose (Lawrence and McCabe 2002). For example, low doses of some heavy metals, such as Cd, Hg, and Pb, can improve immune system function, whereas higher doses are suppressive (Cabassi 2007). Many studies have shown immunomodulation associated with sublethal metal exposure in the environment or in experimental laboratory settings (e.g., Lawrence and McCabe 2002; Dietert and Piepenbrink 2006; Ilbäck et al. 2008). Most studies have targeted vertebrates, but recent work has revealed negative effects of heavy metal pollutants on host defense response systems of marine invertebrates (e.g., Oweson and Hernroth 2009; Vijayavel et al. 2009) as well as terrestrial insects (e.g., Sorvari et al. 2007; van Ooik et al. 2008).

Although there are many reports of effects of heavy metals on immune system function, in general the ultimate mechanisms are not clear. One mode of action is for metals to affect directly the ability of the immune system to identify and respond to pathogen attack. In other words, the complicated chemical communication that occurs during generation of the immune response provides an opportunity for info-disruption by metals. In a simple example, phagocytic activities of cells, measured by their ability to engulf labeled particles, may decrease in the presence of some heavy metals (Fournier et al. 2000). Furthermore, Bishayi and Sengupta (2003) showed that chemotaxis was impaired in mouse splenic macrophages when animals were treated with As or Pb. It is not clear whether these effects stem from info-disruption or more direct effects of heavy metals on cellular health, but the potential parallel with the info-disruption issues being investigated by aquatic biologists is interesting.

Final Considerations

A complicating factor for studies of pollutants (including heavy metals) is that contamination by multiple pollutants often occurs (Fleeger et al. 2003). With regard to metals, these may be multiple metals or one or more metals mixed with one or more organic chemical pollutants. Despite the prevalence of mixtures, toxicological studies often focus on effects of single pollutants (Yang 1994) and so may not be realistic models for judging actual impacts. Generally, combinations of potentially toxic materials may act additively, synergistically, or antagonistically, and all of these effects have been observed in some cases for some heavy metals. For example, additive effects occur when one chemical acts independently of another. Jhee et al. (2006) explored the effectiveness of combinations of certain plant elemental defenses toward an herbivorous moth (*Plutella xylostella*) using an artificial diet amended with metals. When Zn was combined in a pairwise fashion with Cd, Ni, or Pb, toxic effects were additive, and the combination treatments caused greater mortality than single metals alone. Synergy occurs when chemicals interact in a way that increases their joint toxicity beyond that expected if their effects were additive. Jensen et al. (2006) reported an example of synergy of methylmercury and selenate in a study of effects of these chemicals on an insect detritivore (the fly *Megaselia scalaris*). Mixtures containing as little as the LC5 (Lethal Concentration 5%) of both chemicals resulted in 100% larval mortality. Finally, antagonism occurs when effects of one chemical can reduce a negative impact of another. For example, Sanchez-Dardon et al. (1999) studied effects of heavy metals on the immune system of rainbow trout. They found that individual doses of Cd, Hg, or Zn affected immune system function, but when Zn was combined with Cd or Hg no changes occurred. The positive effect of a small dose of a potentially toxic substance is termed a “hormetic effect” (Lefcort et al. 2008), and antagonism is one mechanism by which hormetic effects of a heavy metal pollutant can occur.

Another complicating factor for studies of pollutant impacts is that of scale. While the challenges of scale to ecological studies have been recognized for a long time, Zimmer and Zimmer (2008) point out that these are especially important in chemical ecology. In their recent review, Zimmer and Zimmer (2008) discuss dynamic scaling: the need to scale chemical ecology studies to the physical and chemical environments in which chemical ecology occurs. They point out that complete dynamic scaling is usually not done in laboratory or field studies and this can make it difficult to judge actual ecological effects. They also conclude that many chemical ecology studies have an organism-level emphasis, and point out that including population, community, and ecosystem scales is important to achieve a full understanding of ecological effects. These points certainly apply to studies of metals as info-disruptors, and illustrate the extensive and careful research needed to explore these aspects.

This mini-review has covered a number of ways in which anthropogenic heavy metal pollution may affect organisms and their interactions. It is interesting to consider how the human-generated increase in heavy metal concentrations in biological communities may be affecting evolution of species within them. It is clear that heavy metal pollution (or in fact, release of many pollutants) has provided an opportunity for some species to evolve tolerant populations. In the case of heavy metals, examples range from prokaryotes (e.g., Piotrowska-Seget et al. 2005) to eukaryotes (Janssens et al. 2009). In light of the concept of elemental defenses: is human-caused increase in heavy metal availability providing an opportunity for metal-based

defenses to become more frequent? There are two ways this may occur: 1) through changes in host traits (other than metal concentration) that occur as consequence of exposure to the heavy metal, 2) directly through increased heavy metal concentration serving as an elemental defense. The latter case (increased heavy metal concentration) occurs when organisms are exposed to heavy metal pollutants, and it depends on the balance of intake and excretion. The evolution of metal tolerance in organisms exposed to metal pollutants is a first step in formation of an elemental defense, allowing subsequent evolution of uptake and sequestration mechanisms that may result in concentrations of heavy metals adequate to deter natural enemies. It is difficult, however, to differentiate between direct and indirect effects of heavy metal pollutants. For example, in the section on terrestrial communities (above), the study of Scheirs et al. (2006) showed that Cd exposure of the grass (*Holcus lanatus*) negatively impacted an herbivore (the fly *Chromatomyia milii*). However, the authors were unable to determine if this was due to the direct effect of Cd on the fly, or an indirect effect of plant host quality changes that also changed due to Cd exposure.

Bioaccumulation of heavy metals may be another example by which metal pollutants can have defensive effects. In fact, in a sense this has been the case in some polluted locations where animals that contain dangerous levels of pollutants have been banned from human consumption. If this resulted in decreased human predation upon those animals, then a heavy metal pollutant would function as an elemental defense against humans. For example, in 2001 the U.S. government recommended that pregnant women limit intake of some fish (notably tuna) due to Hg contamination, and consumption by this target group decreased significantly (Oken et al. 2003). This apparently is not (at least not yet) an effective defense, as overfishing of tuna continues (e.g., MacKenzie et al. 2008), but it serves to illustrate a potential defensive consequence of heavy metal pollution.

Future Research Directions

Most of this mini-review has suggested future research directions, but I will close with some summary considerations. Research on direct toxic effects of heavy metals on organisms, a research area that has received much attention, needs further exploration. The discovery of important sublethal metal effects, however, provides a new area for research. The concept of info-disruption, now well established in freshwater systems, may be extended into other habitats and could uncover previously unrecognized heavy metal pollution impacts. For example, are there parallels between the effects of metals on immunomodulation in animals and effects of heavy metals on plant responses to pathogen attack? Poschenrieder et al. (2006) suggested there are, for example, pointing out that Cd treatment can induce production of plant signal molecules (e.g., jasmonic acid, ethylene) that mobilize plant defenses against natural enemies.

The concept of elemental defense is another research area in need of investigation. Examples from communities that are exposed to naturally elevated heavy metal levels, such as terrestrial serpentine communities (Boyd et al. 2009), suggest ways to explore similar questions in metal polluted situations. Finally, there are opportunities to explore the effects of heavy metal pollutants on ecological units larger than species. Communities and ecosystems are difficult to study due to their complexity, but a complete understanding of metal pollutant effects cannot be accomplished without such studies. Hopefully, a more complete understanding will enable us to limit harmful effects of anthropogenic heavy metal pollutants on Earth's biota.

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REVIEW ARTICLE

Direct and Indirect Effects of Invasive Plants on Soil Chemistry and Ecosystem Function

Jeffrey D. Weidenhamer · Ragan M. Callaway

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Abstract Invasive plants have a multitude of impacts on plant communities through their direct and indirect effects on soil chemistry and ecosystem function. For example, plants modify the soil environment through root exudates that affect soil structure, and mobilize and/or chelate nutrients. The long-term impact of litter and root exudates can modify soil nutrient pools, and there is evidence that invasive plant species may alter nutrient cycles differently from native species. The effects of plants on ecosystem biogeochemistry may be caused by differences in leaf tissue nutrient stoichiometry or secondary metabolites, although evidence for the importance of allelochemicals in driving these processes is lacking. Some invasive species may gain a competitive advantage through the release of compounds or combinations of compounds that are unique to the invaded community—the "novel weapons hypothesis." Invasive plants also can exert profound impact on plant communities indirectly through the herbicides used to control them. Glyphosate, the most widely used herbicide in the world, often is used to help control invasive weeds, and generally is considered to have minimal environmental impacts. Most studies show little to no effect of glyphosate and other herbicides on soil microbial communities. However, herbicide applications can reduce or promote rhizobium nodulation and mycorrhiza formation. Herbicide drift can affect the growth of non-target plants, and glyphosate and other herbicides can impact significantly the secondary chemistry of plants at sublethal doses. In summary, the literature indicates that invasive species can alter the biogeochemistry of ecosystems, that secondary metabolites released by invasive species may play important roles in soil chemistry as well as plant-plant and plant-microbe interactions, and that the herbicides used to control invasive species can impact plant chemistry and ecosystems in ways that have yet to be fully explored.

Keywords Invasive plants · Novel weapons hypothesis · Nutrient cycling · Allelopathy · Glyphosate

Introduction

Plants modify their environment in many ways that include the alteration of some of the most basic aspects of the physical and chemical substrates in which they grow. Through these effects, plants provide a crucial component of biogeochemical cycling, as they shape biogeochemical processes by producing "weathering agents", driving mineral cycles, and altering the hydrology of the soil (Kelly et al. 1998). Plants can affect soil structure (particle aggregation) by creating pores in soil, and plant growth promotes rapid wetting and drying cycles that cause shrinkage and strengthening of the soil (Angers and Caron 1998). This is amplified by root exudation of material that stabilizes the developing aggregations. Plants decrease the bulk density of soils through root channeling and litter deposition, thus making it easier for water and roots to penetrate (Tiedemann and Klemmedson 1986; Joffre and Rambal 1993; Callaway et al. 1991). However, the most thoroughly documented effects of plants on substrates involve resources, and in particular soil nutrients. Plants take up nutrients, but over long periods of time plant litter

J. D. Weidenhamer (✉)
Department of Chemistry, Ashland University,
Ashland, OH 44805, USA
e-mail: jweiden@ashland.edu

R. M. Callaway
Division of Biological Sciences, The University of Montana,
Missoula, MT 59812, USA

and root exudates modify and build soils so that the potential for storage and retention of soil nutrients increases, and concomitantly total pools of most nutrients also increase (e.g., Crocker and Major 1955).

There is a substantial literature base that indicates exotic invasive plant species may alter nutrient cycles in ways that differ from resident natives. Many invasive plant species have high specific leaf areas, growth rates, and leaf nutrient concentrations, relative to the natives at the same sites, and these traits have the potential to increase rates of decomposition and nutrient cycling (Allison and Vitousek 2004). Recently, Liao et al. (2008) conducted a meta-analysis and found that on average, invaders were correlated with much higher litter decomposition rates and increases in soil nitrogen mineralization and nitrification (also see Ashton et al. 2005; Feng et al. 2009). Ehrenfeld (2004) and Ehrenfeld et al. (2001) suggested that unusual traits of many invasives, relative to local natives, might affect the particular way in which they alter ecosystem properties. These traits include greater size, higher growth rates and photosynthetic rates, higher live tissue and litter nutrient concentrations, and highly effective nitrogen-fixing symbioses. Rout and Callaway (2009) hypothesized that if invasive species enhance net primary productivity and nitrogen cycling in invaded ranges but not in their native ranges, the inherent traits of plants may be unlikely to drive these processes, because then the same ecosystem effects should occur in the native ranges. An alternative possibility is that once they are successful, invasive plants may undergo rapid natural selection for key leaf traits related to nutrient cycling. For example, Feng et al. (2009) provided evidence that the invasive *Ageratina adenophora,* native to Mexico but an invader throughout the subtropics, appears to have evolved leaves that are easier to decompose in its invaded range. Regardless, understanding whether exotic invasives alter ecosystems differently from native species will help resolve important questions in ecology, evolution, and conservation.

Plants to some degree affect their substrates through processes that are general to all plant species, such as driving nutrient cycles and increasing acidity through inputs of CO_2 into substrate (Kelly et al. 1998). However, plants have the potential to affect substrates, and thus, important ecosystem processes in a more species-specific way through the vast array of different secondary biochemicals and combinations of biochemicals that they produce and release into their environments. Secondary biochemicals are loosely defined as organic compounds that not directly involved in primary metabolic processes such as photosynthesis, cell respiration, cell division or cell growth (Metlen et al. 2009). Not only are these compounds highly diverse, but specific combinations may be species-specific. Ugolini and Sletten (1991) have argued that organic acids produced by plants, in general, have crucial roles in weathering through soil acidification, but we know little about how specific secondary metabolites might affect ecosystem processes in other ways. However, it is worth considering the possibility that organic compounds produced by invasive species may affect their substrates in unique ways. It has been proposed that some exotic invaders may gain an advantage by possessing secondary compounds that are novel to the invaded community—the "novel weapons hypothesis". This is the idea that that some invaders possess allelopathic, defensive, or antimicrobial chemicals to which native organisms have not adapted (Callaway and Aschehoug 2000; Mallik and Pellissier 2000; Callaway and Ridenour 2004; Cappuccino and Arnason 2006; Callaway et al. 2008). If indeed some invasives become dominant because of the novelty of their chemical composition, and if the secondary metabolite composition of particular species can uniquely affect soil biogeochemistry, then the potential for invasives to have unusual effects on soil chemistry through the novel biochemistry of their leachates, litter, volatiles, or root exudates may be high.

Invasive plants also may indirectly alter soil chemistry through the synthetic herbicides used to control them. For example, the most widely used herbicide in the world, glyphosate, has long been considered an ideal herbicide because of its rapid breakdown in the environment, low toxicity to animal species, and lack of carcinogenic or teratogenic effects (Baylis 2000; Borrgaard and Gimsing 2008; Duke and Powles 2008). Glyphosate has disadvantages for the control of invasive plants because it is toxic to a broad spectrum of plant species, but it is a common tool in the management of invaders both in native plant communities (e.g., Spencer et al. 2008) and in agricultural settings where there is increasing use of transgenic crops modified to be glyphosate-resistant (Duke and Powles 2008). Herbicides can affect the growth of non-target plants in treated areas (Crone et al. 2009), or through drift to neighboring untreated areas. Sublethal herbicide applications can alter plant secondary chemistry significantly (Lydon and Duke 1988), with consequences that are little understood. Herbicides can influence plant communities indirectly by changing soil microbial communities, and by altering plant chemistry and plant associations with microorganisms, particularly mycorrhizal fungi and nitrogen-fixing bacteria. Here, we review and discuss the literature on how exotic invasive plants affect soil chemistry, ecosystem processes, and community structure, with a focus on the secondary biochemistry of the invaders and herbicides used to eliminate them.

Direct Effects of Invasive Plants—Litter and Exudates

Plants produce and release constitutive levels of unique secondary compounds and unique combinations of compounds through leaf leachates, leaf litter, leaf volatiles, and root exudates and volatiles (Langenheim 1994; Lin et al. 2007; Ens et al. 2009a; Metlen et al. 2009; Jamieson and Bowers 2009). The production and release of these compounds, however, can be induced by environmental conditions (Agrawal 1998; Ormeño et al. 2007; Soler et al. 2007; Metlen et al. 2009). Importantly, many of these compounds have the potential to alter the fundamental nature of the substrates in which plants grow. For example, low nutrient availability and toxic metals in soils can elicit the production and release of secondary biochemicals from roots that increase nutrient availability and chelate toxic metals in soil (Li et al. 2007). When grown in phosphorus-poor conditions, *Vicia faba* acidifies its rhizosphere with malate and citrate, substantially lowering the pH of growth media and increasing phosphorus availability. *Banksia grandis*, *Lupinus albus*, and *Caustis blakei* also have been shown to exude specific blends of carboxylates (e.g., malate and citrate), in response to specific soil chemistry, to make phosphorus available (Lambers et al. 2002; Misson et al. 2005; Playsted et al. 2006; Wang et al. 2007, 2008). In turn, these exudates affect the biogeochemistry of their environment and competitive dynamics in these plant communities. Similarly, Tharayil et al. (2009) found that the roots of *Centaurea diffusa* exude an allelochemical, 8-hydroxyquinoline, on a diurnal basis, and in response to iron deficiency. *Centaurea diffusa* utilizes this phytotoxin for its own acquisition of iron in alkaline soils. These effects however, appear to be ephemeral, and few studies have attempted to understand the potential long-term effects of either exuded or litter-based chemicals on soil characteristics.

Higher soil phosphorus often is correlated with invasion (but see Martin et al. 2009), yet it is not usually clear whether invaders prefer microsites with high soil phosphorus or if they cause increases in available soil phosphorus. For example, Herr et al. (2007) found that plots in Europe with *Solidago gigantea* had lower soil pH and increased labile phosphorus fractions than plots without the invader (but see Scharfy et al. 2009). Thorpe et al. (2006) found that phosphorus concentration in the invasive forb *Centaurea maculosa*, growing in natural field conditions, was more than twice that of three abundant native species, and that at very low levels of soil phosphorus availability, phosphorus uptake by *C. maculosa* was six times greater than that by the native forb, *Lupinus argenteus*. However, natural field soil phosphorus levels were elevated in sites with *C. maculosa* relative to where the weed had been eliminated with herbicide, thus providing somewhat stronger evidence that the invader was actually the cause of the elevated phosphorus concentrations in the soil. Higher phosphorus under *C. maculosa* is consistent with increases in soluble phosphorus found in the rhizospheres of plants that exude phosphatases (Grierson and Adams 2000) or chelating compounds (Grierson 1992; Stevenson and Cole 1999). Roots of *C. maculosa* appear to exude the polyphenol catechin (Blair et al. 2006; Pollock et al. 2009; Triebwasser et al. 2009; but see Stermitz et al. 2009), and high concentrations can be found periodically in soils (Perry et al. 2007). Although the exudation of catechin by *C. maculosa* is debatable, catechins also are produced by many other plant species. Much like 8-hydroxyquinoline, catechin is phytotoxic (Inderjit et al. 2008a, b; Pollock et al. 2009; Thorpe et al. 2009 but see Duke et al. 2009), and also can chelate metals. Tharayil et al. (2008) found that 8-hydroxyquinoline and catechin mobilize metal-nutrients in soils, thus increasing metal-nutrient acquisition by plants. Catechin is highly ephemeral in soils and solutions (Pollock et al. 2009; Triebwasser et al. 2009), and thus seems unlikely to have long-lasting direct ecosystem effects; however, the indirect chelating effects on metal-nutrient compounds may last longer.

DeLuca et al. (2006) measured the effects of catechin on phosphorus availability in calcareous alluvial soils (pH=8) collected near Missoula, Montana, and the effects of aluminum and 8-hydroxyquinoline on phosphorus availability in aluminum rich soils (pH=6) collected near the Dalles, Oregon. In the Montana soils, phosphorus likely is bound primarily to calcium as calcium phosphates, while in the Oregon soils, phosphorus likely is bound to aluminum. Both soils were amended with a small amount of rock phosphate. They found that application of catechin substantially increased phosphorus availability in calcareous soils and that 8-hydroxyquinoline increased phosphorus availability in acidic soils. This may be due to chelation of calcium by catechin and of aluminum by 8-hydroxyquinoline. Interestingly, catechin had no effect on phosphorus availability in acidic, aluminum-rich soils, and 8-hydroxyquinoline had no effect in calcareous soils (T.H. DeLuca, unpublished data). Furthermore, adding “biochar” (charcoal derived from natural fires) eliminated or reduced the effects of these allelochemicals on phosphorus availability. These results demonstrated the potential for different secondary metabolites exuded from roots to have system-specific effects. The addition of biochar to phenolic-rich soils in natural ecosystems also has been shown to strongly affect nitrogen transformations in temperate and boreal forests (DeLuca et al. 2002; Gundale and Deluca 2006).

The effects of root exudates may be altered by interactions of plants with other organisms. Hamilton and Frank (2001) conducted a ^{13}C pulse-chase and simulated

herbivory experiment on *Poa pratensis* in grasslands of Yellowstone National Park and measured carbon flow into the soil rhizosphere, microbial biomass, and the associated effects on soil nitrogen dynamics. They found that grazing promoted root exudation of carbon, which was then assimilated by soil microbes, although they did not determine the nature of the exudate. Herbivore-induced carbon flow from roots affected soil nutrient pools, plant uptake, leaf concentrations of nitrogen, and photosynthesis.

Rather than releasing chelators, plants may release enzymes involved in phosphorus acquisition. Caldwell (2005) found that soils under *Cytisus scoparius* (scotch broom), a species that has invaded widely in the Pacific Northwest, had much higher activities of two soil enzymes involved in phosphorus availability, phosphatase and β-glucosidase, but lower phosphorus:carbon ratios under *C. scoparius* than in prairie vegetation, perhaps due to higher phosphorus uptake by the invader. Similarly, Blank and Young (2004) found that three invasive species increased calcium, magnesium, and nitrogen mineralization in soil solution of a "high-resource" soil and also greater enzyme activity in both high- and low-resource soils.

In some cases, the release of biologically active compounds by plants has been documented, but the ecological effects are unknown. *Pteridium aquilinum* (bracken fern) and *Heracleum mantegazzianum* (giant hog weed) are both exotic invaders in parts of the world (Kollmann et al. 2009). *Pteridium* produces ptaquiloside, a norsesquiterpene glycoside, and *Heracleum* produces furanocoumarins, and these compounds can affect surface and ground water quality. Kollmann et al. (2009) report that ptaquiloside is transferred to soil from where it can leach into aquatic systems. They also report that ptaquiloside is carcinogenic and that very low concentrations can be dangerous to humans in drinking water. When cows eat ptaquiloside, the toxin can accumulate in milk (Alonso-Amelot et al. 1998).

Some of the most salient aspects of highly invasive plant species are their powerful competitive effects in non-native ranges relative to effects in their native ranges (Hierro et al. 2005). There is evidence that this biogeographic shift in competitive impact may be directly contributed to by novel allelopathic effects (Callaway and Aschehoug 2000; Mallik and Pellissier 2000; McKenney et al. 2007; Ens et al. 2009b; He et al. 2009). Competition between native species can be affected by the indirect effects of secondary metabolites exuded by roots and contained in litter (Meier and Bowman 2008); however, little is known about how the indirect effects may operate in invaded ecosystems.

As noted above, whether through leachates, litter, or exudates, invasive plant dominance often is correlated with changes in ecosystem processes in soils. Also, these changes frequently are measured as increases in available nutrients and nutrient pools (Liao et al. 2008). For example, Duda et al. (2003) found that as the exotic *Halogeton glomeratus* invades native *Krascheninnikovia lanata*-dominated vegetation, soil concentrations of nitrate, phosphorus, potassium, and sodium increased with the cover of the invader. Rodgers et al. (2008) found that soils in North American temperate deciduous forest invaded by the European forb *Alliaria petiolata* were consistently and significantly higher in nitrogen, phosphorus, calcium, and magnesium availability, and soil pH. In the laboratory, the release of volatile compounds from the leaves of *A. petiolata* did not significantly alter soil nitrogen availability, nor did the colonization of native soils by *A. petiolata* roots alter soil nutrient cycling. However, in a leaf litter decomposition experiment, the leaves of *A. petiolata* significantly increased the rate of decomposition of native tree species, thus indicating that fundamental changes in nutrient cycling were more important than inhibitory effects of *A. petiolata* secondary compounds on the activity of the microbes that decompose the native litter. Sanon et al. (2009) also found that microbial communities may have a role in the ecosystem-scale changes in soil biochemistry caused by invasive plants. They found that invasion by *Amaranthus viridis* increased concentrations of nitrogen, carbon, total phosphorus, and soluble phosphorus in the top 15 cm of soil. Total phosphorus was almost three times higher in invaded soils, whereas soluble phosphorus was approximately twice as high in invaded soils. These increases were correlated with increases in bacterial abundance and soil microbial activity. However, despite this increase in important soil resources, arbuscular mycorrhizae (AM) fungi, rhizobial development, and the growth of native *Acacia* species were severely reduced in soil invaded by *A. viridis*.

McGrath and Binkley (2009) measured the soil chemical changes induced by the invasive *Microstegium vimineum* (Japanese stiltgrass) in acidic, nutrient-poor temperate forests. In a greenhouse study, soil pH under *M. vimineum* was significantly higher than that of soil without the exotic, and in invaded forests they found higher pH, phosphorus, levels of several cations, and lower aluminum beneath dense *M. vimineum* than when *M. vimineum* was not present. Similarly, *Lantana camara* invasion in India is correlated with increases in soil available nitrogen, ammonification, nitrification rate, and nitrogen mineralization, which in turn is correlated with high nitrogen, low lignin, low lignin:nitrogen ratios, and low carbon:nitrogen ratios in *L. camara* litter (Sharma and Raghubanshi 2009). Blank (2008) found that invasion by *Bromus tectorum*, a Eurasian annual grass that has spread throughout western North America, increased the availability of manganese, nitrogen, phosphorus, copper, iron, calcium, and potassium. They suggested that *B. tectorum* affects the flow of nutrients

through the soil system with the potential to alter the vertical distribution of nutrients in the soil profile (also see Evans et al. 2001). Similar increases have been reported in invasions by *Lepidium latifolium* in the western United States (Renz and Blank 2004). Dassonville et al. (2007) hypothesized that a substantial increase in a wide range of nutrients occurred under the canopies of the invasive *Fallopia japonica* through “nutrient uplift” and redistribution.

Dassonville et al. (2008; see also Vanderhoeven et al. 2005) examined the effects of seven invasive plant species in Europe on nutrient pools in the topsoil and found that the intensity of the impact and whether or not pools increased or decreased depended on the site. Strong increases in nutrient concentrations with invasion occurred primarily in sites that were initially nutrient poor, but decreases typically occurred in soils that initially were nutrient rich. In the nutrient-poor tropical forest of the Seychelles, Kueffer et al. (2008) did not find strong differences among ecosystem effects of native and invasive trees. They compared leaf traits and understory soil characteristics of six native and six invasive tree species. Litter of invasive canopy and pioneer species had higher specific leaf areas, and litter decomposition was faster than for comparable native species; however, these traits did not differ between native and invasive understory species (also see Batten et al. 2005). However, whether exotics demonstrated strong invasive tendencies or not was not clear. Allison and Vitousek (2004) also explored the effects of invaders on ecosystem processes in nutrient-poor substrates on an island (Hawai’i). They measured leaf litter properties, decomposition rates, and nutrient dynamics for five common native species and six clearly identified aggressive invasive species. They found far higher litter decay rates for invaders.

Invaders also appear to alter the way that native litter is processed through nutrient cycles in some cases. Standish et al. (2004) found that native litter placed under mats of the invasive *Tradescantia fluminensis* in a remnant of New Zealand lowland podocarp–broadleaf forest decomposed at almost twice the rate of litter placed outside the mat. They also pointed out that the effect of *Tradescantia* on such cycles could be seen in the smaller forest floor mass where *Tradescantia* was abundant. Also, nitrate availability was higher in plots with *Tradescantia.*

Interestingly, the frequent increases in soil nutrient concentrations that are observed over time with many invaders has been proposed as a sort of feedback process in which elevated concentrations of nutrients, especially nitrogen, promote the greater success of invaders relative to natives. Prober and Lunt (2009) took an especially creative approach to this possibility by investigating whether restored stands of the native tussock grass, *Themeda australis*, in southeast Australia could reduce soil nitrate concentrations that appeared to be promoting a suite of exotic invaders, and thus might increase resistance to invasion. They found that successful establishment of *Themeda* plots treated by fire or by adding carbon supplements reduced soil nitrate to levels comparable with non-invaded, *Themeda*-dominated sites, and significantly reduced exotic cover compared to controls. When plots were not seeded with *Themeda*, soil nitrate concentrations increased after carbon treatments ended, and invasive abundance increased to levels of the control plots. They argued that “*Themeda* is a keystone species that regulates nitrate cycling, thereby imparting ecological resistance to invasion by nitrophilic annuals”.

In sum, the literature indicates that exotic invasive plants can have strong effects on the biogeochemistry of ecosystems and that these effects can be caused by differences in leaf tissue composition or secondary metabolites. However, effects of secondary metabolites on soil ecosystem processes appear to be less important than the stoichiometric ratios of nutrients and structural and non-structural carbon.

Indirect Effects of Invasive Plants—The Effects of Herbicides

While the primary use of herbicides is the control of weeds in agricultural lands, herbicide treatment increasingly is used to control invasive weeds in non-agricultural settings. The spectrum of compounds used as herbicides and growth regulators is far less than that of secondary metabolites in plants, but the large quantities of these compounds applied annually certainly have the potential to impact ecosystem processes. Globally, more than 800 million kg of herbicides and plant growth regulators were used in 2001. More than 200 million kg were used in the United States, including 40 million kg of glyphosate (US Environmental Protection Agency 2009).

The effects of herbicides on soil microorganisms and ecosystem processes have been studied in many different systems, and addressed in numerous reviews and texts (e.g., Grover and Cessna 1991). Our intent here is not to review this literature comprehensively, but rather to point out some of the ways in which herbicides may affect ecosystem processes and plant communities as they are applied to control invasive weeds. We have emphasized the effects of glyphosate (N-(phosphonomethy)glycine) because it is the most widely used herbicide in the US, but we also review selected studies with other herbicides to illustrate some of the potential indirect effects that these compounds can have.

The impact of glyphosate on soil microbial communities has been widely studied in both agricultural and non-agricultural communities, and most studies have shown

little to no lasting effects (Wardle and Parkinson 1991; Haney et al. 2000; Busse et al. 2001; Ratcliff et al. 2006; Weaver et al. 2007). Santos and Flores (1995) evaluated the effect of glyphosate on nitrogen fixation by free-living *Azotobacter chroococcum* and *A. vinelandii* and observed no effect at recommended applications rates of glyphosate. Busse et al. (2001) measured microbial biomass, respiration, and metabolic diversity in three ponderosa pine communities following 9–13 years of glyphosate applications. Soils had varying clay and aluminum and iron oxide contents, providing a range of capacities for glyphosate binding . Minimal effects on soil microbial communities were detected. Weaver et al. (2007) examined the impact of glyphosate on microbial communities in an agricultural soil. Profiles of microbial fatty acid methyl esters showed no effect of glyphosate application. Further laboratory experiments in which high doses of glyphosate were applied (up to three times the recommended rate) had a detectable impact on soil microorganisms for less than 1 week. They concluded that the effects of glyphosate on soil microbes were “small” and “transient.”

However, other studies have shown subtle effects with the potential to impact plant communities. Feng and Thompson (1990) measured rapid initial degradation of glyphosate in soils, but detected residues of the herbicide and aminomethyl phosphoric acid (AMPA), the primary metabolite of glyphosate, for up to 360 days after application. Araújo et al. (2003) found that in vitro application of glyphosate to two Brazilian soils resulted in increases of fungal and actinomycete populations, but very small decreases in the abundance of soil bacteria. Carbon dioxide release from soil was increased by glyphosate application, and the glyphosate metabolite AMPA (aminomethyl phosphonic acid) was found in soils treated with glyphosate, indicating that the herbicide was broken down by soil microorganisms. Microbial degradation of glyphosate is well-established, and organisms capable of degrading it have been characterized (Moore et al. 1983; Pipke and Amrhein 1988), suggesting at least the potential for glyphosate to alter soil microbial composition. Widenfalk et al. (2008) applied four pesticides including glyphosate to sediment in experimental microcosms. Glyphosate was found to cause small but significant shifts in bacterial community composition over 1 month, and the effects of glyphosate were greater than those of isoproturon, the other herbicide included in the study. Lupwayi et al. (2009) measured the impacts of applications of 2,4-D and glyphosate to glyphosate-resistant canola prior to seeding, and observed that the functional structure of the soil bacterial community was changed and functional diversity was reduced by both herbicides. Microbial biomass C increased when these herbicides were applied, and they concluded that these changes, while minor, could affect soil biological processes and food webs. Zabaloy et al. (2008) found minor impacts on microbial populations and activities from additions of 2,4-D, metsulfuron-methyl and glyphosate at ten times the recommended label rates. Effects of glyphosate exceeded those of the other two herbicides, and at high doses glyphosate could influence soil microbes as a phosphorus source. The authors cautioned that because less than 1% of soil microbes are cultivable, it is unclear whether all significant changes to soil microbial community structure can be detected. Powell et al. (2009) found no permanent negative effects of glyphosate-tolerant crops and herbicide regimes on soil microbiota and litter decomposition. However, in some treatments, glyphosate increased the fungus: bacteria biomass ratio, and there was some indication of reduced litter decomposition rates, although results were inconsistent. These authors concluded that further work was needed to determine whether there might be longer term effects on organic matter accumulation and nutrient cycling. Tejada (2009) characterized microbial biomass and several enzyme activities up to 180 days following laboratory applications of glyphosate and the herbicide diflufenican to two soils. Herbicides were applied singly and in combination. The glyphosate-diflufenican mixture was the most toxic, reducing microbial biomass, and decreasing the activities of important metabolic indicators including dehydrogenase, urease, β-glucosidase, phosphatase, and arylsulfatase. Toxicity was observed up to 180 days following application, and also was greater in a sandy loam soil than in clay soil. Because glyphosate is often used in combination with other herbicides that provide residual activity, these results imply that the impact of glyphosate on soil microorganisms and biochemistry may be greater than currently recognized.

Herbicides also can indirectly affect plant growth through effects on rhizobium nodulation and mycorrhizal formation, although these effects have not often been investigated. Eberbach and Douglas (1989) examined how the nodulation of subclover *Trifolium subterraneum* L. was affected in nutrient culture by nine herbicides (2, 4-D, amitrole, atrazine, chlorsulfuron, diclofop-methyl, diquat, glyphosate, paraquat, and trifluralin). Diquat, paraquat, glyphosate, and chlorsulfuron inhibited growth of *Rhizobium trifolii*, and amitrol, diclofop-methyl, and glyphosate decreased nodulation. Busse et al. (2004) reported no effect of the herbicides triclorpyr, imazapyr, and sulfometuron on ectomycorrhizal formation of Ponderosa pine, Douglas fir, and white fir. The herbicides were applied at rates up to double the recommended application rate. While seedling growth was inhibited by many of the treatments, ectomycorrhizal formation was unaffected. In contrast, Abd-Alla et al. (2000) reported that the herbicides bromoxynil and paraquat reduced

nodulation and colonization of roots of three legumes by arbuscular mycorrhizal fungi. Accumulation of nitrogen, phosphorus, and potassium in affected plants was reduced. The effects observed varied by species and compound. In an interesting early report, Schwab et al. (1982) indicated that at concentrations above 1 mg kg^{-1} simazine, the non-mycorrhizal species *Chenopodium quinona* was induced to form arbuscular mycorrhizas.

Herbicides and herbicide drift to adjacent land can have substantial effects on non-target plants. Picloram is used to control invasion by spotted knapweed (*Centaurea maculosa*). Crone et al. (2009) followed the effects of picloram on the native arrowleaf balsamroot, *Balsamorhiza sagittata*, and found that a single application of picloram reduced flowering and seed set of this native wildflower for 4 years. Marrs et al. (1991) conducted experiments in which eight native dicot species were grown in experimental microcosms with and without the perennial grass *Lolium perenne* and exposed to drift of the herbicide mecoprop. Effects ranged from reduction of growth to stimulation, depending on species, and flowering performance of some species also was affected. The results imply that herbicide drift could impact competitive balance and fecundity of species in communities adjacent to sprayed sites. Måren et al. (2008) explored the impact of the herbicide asulam on native heathland vegetation. Asulam is the most popular strategy for restoration of heathlands invaded by bracken (*Pteridium aquilinum*). In a seven-year field study, asulam treatment did eliminate bracken, but had both positive and negative effects on several minor heathland species. A group of "bracken-suppressed, yet herbicide-sensitive" species, was identified, and it was argued that abundance of these native species could be reduced because of herbicide use.

Gove et al. (2007) studied the effect of low doses of glyphosate on six woodland understory species. Glyphosate treatment increased mortality and reduced biomass and fecundity for all species. Species differed in their sensitivity to glyphosate, and differences in species distribution were measured to a distance of 4 m into woodland margins. Neumann et al. (2006) demonstrated that non-target transfer of glyphosate can occur not only through drift but also by release from treated plants into the rhizosphere. Glyphosate applications at 6% of the recommended rate reduced iron and manganese uptake in non-target sunflower plants, suggesting that glyphosate has the potential to alter micronutrient nutrition in non-target plants (Eker et al. 2006).

Two recent papers report stimulatory effects of sublethal doses of glyphosate, which imply that glyphosate drift may affect competitive outcomes of affected plants in ways that are difficult to predict. Velini et al. (2008) found that low doses of glyphosate can stimulate the growth of crops (corn and soybean), an herb (*Commelina benghalensis*), and two tree species (*Eucalyptus grandis and Pinus caribea)*. The doses required for stimulation varied with species and tissue type, with both root and/or shoot stimulation observed depending on species. Cedergreen (2008) treated barley with low doses of glyphosate and found that the growth stimulation observed lasted for 6 weeks in comparison to untreated controls, but did not persist until harvest. It was suggested that competitive abilities of plants may be affected by glyphosate stimulation, but this hypothesis remains untested.

A potentially important effect of glyphosate is the alteration of plant secondary chemistry that can be caused by sublethal applications. In a study of the effects of sixteen herbicides on soybean, Hoagland and Duke (1983) found that concentrations of hydroxyphenolics and anthocyanins were either increased or decreased, depending on the herbicide. Glyphosate inhibits the shikimic acid pathway, and thus accumulation of certain plant metabolites is characteristic of glyphosate exposure. Lydon and Duke (1988) reported increases in concentrations of gallic acid, 4-hydroxybenzoic acid, and protocatechuic acid in response to treatment of velvetleaf (*Abutilon theophrasti*) with glyphosate. Effects were dramatic, with protocatechuic acid concentrations increasing from 1.8 to 112 ng/mg dry weight. Four other species tested showed varying impacts of glyphosate on phenolic metabolism. Lydon and Duke (1988) hypothesized that the release of high concentrations of certain secondary metabolites caused by glyphosate exposure could have allelopathic impacts, but this has not been investigated. Adding weight to this hypothesis, Einhellig (1996) reported additive inhibition in greenhouse experiments for combinations of the allelochemical ferulic acid with low levels of atrazine, alachlor, and trifluralin. Despite this intriguing report, the interactive effects of herbicides and allelochemicals remain largely unknown. Disruption of phenolic metabolism by glyphosate also has been shown for nodulated soybean (Hernandez et al. 1999). Both glucosinolate and phenolic metabolism of rapeseed (*Brassica napus)* was affected by glyphosate, with concentrations of glucosinolates affected in complex ways—methionine-derived glucosinolates increased at glyphosate concentrations exceeding 5 μM, but decreased at 1 μM concentration (Peterson et al. 2007). Sublethal doses of other herbicides also can impact plant secondary chemistry. Alla and Younis (1995) treated corn and soybean seedlings with five different herbicides (trifluralin, fluometuron, atrazine, alachlor, and rimsulfuron) and found that the herbicides affected concentrations of hydroxyphenolic compounds and anthocyanins. They increased or decreased depending on the particular compound and species combination.

Future Directions

Understanding whether and how exotic invasive plants change ecosystems in ways fundamentally different from native species can contribute to our understanding of plant-soil microbe interactions, the role of secondary metabolites in ecology in general, and the potential for regional evolutionary trajectories in different parts of the Earth (Callaway et al. 2005). This also is an important issue to resolve for accurate evaluation of the biological and economic impact of invaders. We have learned a great deal about general ecosystems effects and some of the operating mechanisms. However, little is known about the substantial potential for long-term effects through exuded or litter-based secondary metabolites on soil characteristics. Recent work demonstrates that plants respond to environmental conditions by releasing particular allelochemicals that can play a role in chelation or solubilization of nutrients, but it is not known how this mechanism or other mechanisms differ between invaded communities and those in native ranges of the same species. Active chelation of scarce nutrients presumably may alter competitive relationships among species, but the extent and importance of these effects is not known. Little is known also about how secondary metabolites may indirectly impact competitive relationships through effects on soil microbial and other processes. The literature demonstrates that invasive plant dominance often is correlated with changes in nutrient status and ecosystem processes in soils, but the relative importance of secondary metabolites relative to mineral composition in driving these effects is not well understood and needs further investigation.

Numerous reports suggest the importance of the indirect impacts of invasive species through the herbicides used to control them. Herbicide applications can alter plant secondary chemistry significantly, and impact important symbioses with nitrogen-fixing bacteria and mycorrhizae, but the consequences of these effects are not yet understood. While several reports provide evidence for differential decomposition of invasive plant litter, the alterations in plant chemistry that have been demonstrated in response to glyphosate applications suggest that there could be a significant impact on how litter from these plants is processed. The potential interactive effects of herbicides and plant allelochemicals, on plant-plant interactions and on soil microbiology and chemistry, are largely unknown. The potential impact of combinations of herbicides on soil microorganisms and soil chemistry and interactions with allelochemicals is another area where further study is needed.

In summary, invasive plants have the potential to directly affect soil chemistry and ecosystem function through the direct effects of secondary metabolites in their tissues, leachates, and exudates; and indirectly through chemical attempts to control them. In this context, much is known about highly local effects (e.g. in pots, common gardens, or smallplots), but little is known about the scale at which these effects function. Thus, scaling up from small scale experiments to large scale field-based monitoring of these sorts of effects will be a necessary step for a complete understanding of the impact of invasive plants on soil chemistry.

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Iridoid Glycoside Variation in the Invasive Plant Dalmatian Toadflax, *Linaria dalmatica* (Plantaginaceae), and Sequestration by the Biological Control Agent, *Calophasia lunula*

Mary A. Jamieson · M. Deane Bowers

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Abstract Invasive plant species can have significant ecological and economic impacts. Although numerous hypotheses highlight the importance of the chemical defenses of invasive plant species, the chemical ecology of many invasive plants has not yet been investigated. In this study, we provide the first quantitative investigation of variation in iridoid glycoside concentrations of the invasive plant Dalmatian toadflax (*Linaria dalmatica*). We examined variation in chemical defenses at three levels: (1) variation within and among populations; (2) variation due to phenology and/or seasonal differences; and (3) variation among plant parts (leaves, flowers, and stems). Further, we examined two biological control agents introduced to control *L. dalmatica* for the ability to sequester iridoid glycosides from this invasive plant. Results indicate that *L. dalmatica* plants can contain high concentrations of iridoid glycosides (up to 17.4% dry weight of leaves; mean = 6.28±0.5 SE). We found significant variation in iridoid glycoside concentrations both within and among plant populations, over the course of the growing season, and among plant parts. We also found that one biological control agent, *Calophasia lunula* (Lepidoptera: Noctuidae), was capable of sequestering antirrhinoside, an iridoid glycoside found in *L. dalmatica*, at levels ranging from 2.7 to 7.5% dry weight. A second biological control agent, *Mecinus janthinus* (Coleoptera: Curculionidae), a stem-mining weevil, did not sequester iridoid glycosides. The demonstrated variation in *L. dalmatica* chemical defenses may have implications for understanding variation in the degree of invasiveness of different populations as well as variation in the efficacy of biological control efforts.

Keywords Iridoid glycosides · Antirrhinoside · Linarioside · *Linaria dalmatica* · Sequestration · *Calophasia lunula* · *Mecinus janthinus* · *Junonia coenia* · Chemical defenses · Invasive plant · Biological control · Lepidoptera · Noctuidae

M. A. Jamieson (✉)
Department of Ecology and Evolutionary Biology,
University of Colorado,
Boulder, CO 80309, USA
e-mail: mary.jamieson@colorado.edu

M. D. Bowers
University of Colorado Museum and Department of Ecology and Evolutionary Biology, University of Colorado,
Boulder, CO 80309, USA

Introduction

The invasion of non-native plant species has affected ecosystems throughout the world and represents a major threat to native biodiversity (Wilcove et al. 1998; Levine et al. 2003; Myers and Bazely 2003; Mitchell et al. 2006). Numerous hypotheses have been proposed to elucidate the mechanisms that underlie plant invasions. Many of these hypotheses, including the enemy release, evolution of increased competitive ability, novel weapons, and novel chemistry hypotheses, highlight the importance of plant defensive chemistry in the success and invasion dynamics of introduced species (Blossey and Notzold 1995; Keane and Crawley 2002; Callaway and Ridenour 2004; Cappuccino and Arnason 2006; Inderjit et al. 2006). Moreover, plant secondary compounds are known to play an important role in mediating species interactions, including plant-plant, plant-pathogen, and plant-herbivore interactions. Thus, research that examines the chemical ecology of invasive plants may contribute greatly to understanding factors that facilitate invasion success as well as factors that influence management and biological control efforts.

In this paper, we present the first quantitative investigation of the chemical defenses of Dalmatian toadflax, *Linaria dalmatica* (L.) P. Mill. (Plantaginaceae), which has been identified as both an ecologically and economically important invasive plant species (Duncan et al. 2004). This species contains iridoid glycosides (Handjieva et al. 1993; Ilieva et al. 1993; Franzyk et al. 1999), but its iridoid content has not been quantitatively examined. Iridoid glycosides are a group of cyclopentanoid monoterpene-derived compounds found in approximately 50 plant families (Bobbitt and Segebarth 1969; Jensen et al. 1975; El-Naggar and Beal 1980; Boros and Stermitz 1990). These compounds mediate plant interactions with both specialist and generalist herbivores, acting as deterrents and toxins for generalists and as attractants and feeding stimulants for certain specialists (review in Bowers 1991). Further, these compounds can be sequestered by some specialist insect herbivores and provide protection against their natural enemies (Bowers 1991; Rimpler 1991; Nishida 2002).

We quantified the two major iridoid glycosides, antirrhinoside and linarioside (Fig. 1), found in *L. dalmatica*, and examined three levels of variation in iridoid glycoside concentrations: (1) variation within and among populations; (2) variation due to phenology and/or seasonal differences; and (3) variation among plant parts (leaves, flowers, and stems). Additionally, we examined two introduced biological control agents, the toadflax defoliator, *Calophasia lunula* Hufnagel (Lepidoptera: Noctuidae) and the stem-mining weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), for their ability to sequester iridoids. We were interested especially in examining *C. lunula* because the bright coloration of larvae (white, black, and yellow) suggested aposematism and because a number of lepidopteran species are known to sequester iridoid glycosides that provide protection against natural enemies (Bowers 1991; Nishida 2002). For comparison, we also analyzed larvae of the common buckeye butterfly, *Junonia coenia* Hübner (Lepidoptera: Nymphalidae), for its ability to sequester iridoid glycosides from *L. dalmatica* because it is known to sequester other iridoid glycosides (Bowers and Collinge 1992) and will feed on *L. dalmatica* (pers. obs.).

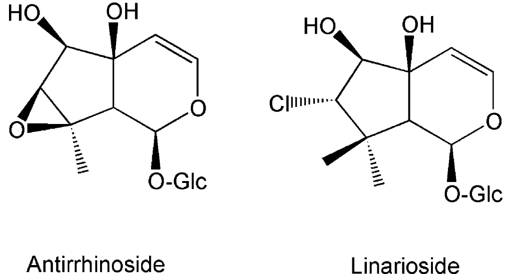


Fig. 1 Structures of the iridoid glycosides antirrhinoside and linarioside

Methods and Materials

Study System Linaria dalmatica is a perennial species native to Eurasia that escaped cultivation in North America during the early 1900's and since has become a land management concern throughout the western United States and Canada (Vujnovic and Wein 1997; Wilson et al. 2005). Synonyms of this species include *L. dalmatica* ssp. *dalmatica* and *L. genistifolia* ssp. *dalmatica* (Vujnovic and Wein 1997; Weber and Wittmann 2001; USDA 2009). *Linaria dalmatica* is designated as a noxious weed in 12 states throughout the western United States and has been reported to occur in at least 33 states (USDA 2009). Once established, *L. dalmatica* can be a strong competitor and may reduce the abundance of native grasses and forbs (Carpenter and Murray 1998).

Several iridoid glycosides have been identified in *L. dalmatica* and other *Linaria* species (Ilieva et al. 1992, 1993; Handjieva et al. 1993; Franzyk et al. 1999). *Linaria dalmatica* is reported to contain five iridoid glycosides: antirrhinoside, linarioside, isolinarioside, 5-O-glucosylantirrinoside, and 5-O-allosylantirrhinoside (Handjieva et al. 1993; Ilieva et al. 1993; Franzyk et al. 1999). To date, the iridoid content of *L. dalmatica* has not been investigated quantitatively; however, one study has examined other closely related *Linaria* species. Nikolova-Damyanova et al. (1994) used quantitative thin-layer chromatography (TLC) to estimate iridoid glycosides from the dried ground aerial plant tissue of *L. vulgaris*, *L. simplex*, *L. pelliseriana*, and *L. genistifolia*, and they found that antirrhinoside and linarioside represent the two major iridoids found in these species. In *L. genistifolia*, antirrhinoside represented approximately 60–70% of the total iridoid content and linarioside represented 10–20% (Nikolova-Damyanova et al. 1994).

Management of *L. dalmatica* by using chemical and mechanical control often is unsuccessful due to waxy leaves that protect the plant from herbicides as well as long taproots and the ability to reproduce vegetatively from root buds and fragments (Carpenter and Murray 1998; Wilson et al. 2005). Consequently, biological control may be the most effective management strategy for managing invasive populations of this species. Seven specialist herbivores of *L. dalmatica* have been introduced into North America, either intentionally as biological control agents or accidentally (Wilson et al. 2005). Iridoids are likely to play a role in mediating interactions between *L. damatica* and these specialist herbivores; however, little is known about the iridoid content and chemical ecology of this invasive plant.

Mecinus janthinus is a stem-mining weevil native to Eurasia, which was approved for release as a biocontrol agent in Canada and the US in the early 1990s (Wilson et

al. 2005). *Mecinus janthinus* larvae develop from early to mid-summer within the stems of *L. dalmatica*. Larvae pupate during late summer and overwinter in dead stems. Adults emerge the following spring and feed on the leaves and stems of the plant (Jeanneret and Schroeder 1992). Because *L. dalmatica* population growth appears to be limited by interspecific competition rather than seed availability, Grieshop and Nowierski (2002) suggest that biocontrol agents that reduce the competitive ability of plants by attacking stems and roots are most effective at reducing population densities. Furthermore, research by Peterson et al. (2005) demonstrated that *M. janthinus* larval injury had a significant deleterious effect on the primary physiology of *L. dalmatica*, whereas *Calophasia lunula* larval injury did not result in any significant physiological responses. Accordingly, *Mecinus janthinus* seems to be the most promising biological control agent for management of *L. dalmatica* populations.

Calophasia lunula is a defoliating lepidopteran, also native to Eurasia, which was introduced into North America in the early 1960's as a biocontrol agent for *L. dalmatica* and *L. vulgaris* (Wilson et al. 2005). Adult moths emerge in May, and caterpillars can be found feeding on *L. dalmatica* from late May through August. Depending on weather and the length of the growing season, *C. lunula* will complete one to three generations per year. This specialist herbivore is found throughout the range of *L. dalmatica* and its occurrence is widespread in the United States. Caterpillars can cause severe defoliation, but larval injury is likely only significant at the seedling stage, not at the adult stage of *L. dalmatica* (Wilson et al. 2005).

Junonia coenia is a lepidopteran native to North and Central America and is not a specialist on *L. dalmatica*, but it is a specialist on plants that contain iridoid glycosides (Bowers 1984). Common host plants of this species include native and cultivated snapdragons, *Antirrhinum majus* (Plantaginaceae) (Robinson et al. 2002). Moreover, larvae also will feed on *L. dalmatica* (pers. obs.). Snapdragons are known to contain antirrhinoside and other iridoid glycosides (Beninger et al. 2007). Although *J. coenia* larvae have been shown to sequester the iridoid glycosides aucubin and catalpol (Bowers and Collinge 1992), this species has not been examined for its ability to sequester antirrhinoside.

Sample Collection Plant samples were collected from three field sites in the northwestern region of Boulder County, Colorado, USA, in the spring and summer of 2006. Two field sites were located on Boulder County Parks and Open Space property: Rabbit Mountain (40° 14′ 13″ N; 105° 12′ 53″ W) and Hall Ranch (40º 12′ 42″ N; 105º 17′ 20″ W). The third site was located on private property in Lefthand Canyon (40° 7′ 14″ N; 105° 19′ 26″ W). Field sites ranged from 1675–2075 m in elevation and were characterized as dry and rocky foothills grassland habitat with vegetation dominated by mixed grasses, forbs, and shrubs. Plant samples were collected on three dates that spanned the major phenological stages of *L. dalmatica*: pre-flowering (late-May), peak flowering (mid-July), and during fruiting and seed set (mid-September). We collected plant samples from 10 patches at each field site (N=10 samples per date/site). Each sample was comprised of three ramets collected from a distinct patch. Because *L. dalmatica* reproduces vegetatively and from seed, it was not possible to determine whether ramets come from a single or multiple individuals without genetic analyses. Although *L. dalmatica* can hybridize with *L. vulgaris* in natural habitats (Ward et al. 2009), we are confident that our samples are pure *L. dalmatica* because *L. vulgaris* is not present at our study sites and the sampled plants in these populations clearly showed the morphology of *L. dalmatica*.

Mecinus janthinus adults (N=10) were collected from Lefthand Canyon in early June 2006. *Calophasia lunula* samples came from a lab culture started from eggs obtained from the Colorado Department of Agriculture Insectary (Palisade, CO). Larvae were reared in a growth chamber (model Percival 36-LLVL; interior volume = 0.84 m^3) with a 16:8 h L/D photoperiod and temperatures set to 25°C day and 20°C night. Larvae were fed *L. dalmatica* plants collected from a field population located behind the University of Colorado 30th St. Greenhouse, Boulder, CO during August 2006. Larvae were selected for sample preparation at three stages: newly molted 4th and 5th instars and midway through the 5th instar (larvae were starved for 12–18 h to empty gut contents). Male and female pupae, newly emerged adults, and eggs also were prepared for iridoid analyses. The sample size for each stage was N=10 individuals, except in the case of eggs, which were analyzed as 4 samples of 50–100 eggs. In addition to caterpillars, pupae, and eggs, we examined *C. lunula* hemolymph collected from 5th instar larvae (N=4) for the presence of iridoid glycosides. Hemolymph was collected with a 10 µl capillary tube from an incision made on a larval proleg. *Junonia coenia* larvae were obtained from a laboratory culture maintained at the University of Colorado, and were reared mid-August through mid-September 2006 from hatching until they molted to the 5th instar on either field collected *L. dalmatica* or garden collected *Antirrhinum majus* (N=10 individuals per host plant). For *J. coenia*, we analyzed iridoid glycoside concentrations for 5th instars only.

Sample Preparation Plant tissues were separated into leaves, stems, and flowers, oven-dried at 50°C to a constant mass, weighed to the nearest 0.01 g, ground into a fine powder, and then 25–30 mg of each plant sample was prepared for chemical analysis. Sample preparation

methods have been described previously (Gardner and Stermitz 1988; Bowers and Stamp 1993). Briefly, sample preparation involves extracting plant material in methanol, filtering out plant material from the methanol extract, evaporating extracts to dryness, and then partitioning the dried extract between water and ether to remove hydrophobic compounds.

Calophasia lunula and *J. coenia* samples were weighed fresh, killed by freezing at −40°C, ground and extracted fresh in methanol, and then prepared for iridoid analyses by using the same method as plant tissues. For *C. lunula* and *J. coenia* samples, a separate set of individuals at each life stage ($N=5$ per life stage) was weighed fresh, oven dried at 50°C to constant mass, reweighed, and data were used to calculate wet to dry weight conversion factors for the various life stages. *Calophasia lunula* eggs ($N=4$ composite samples) were weighed and extracted fresh (no conversion factors calculated). The 10 *M. janthinus* adults were combined into a single composite sample, weighed, and extracted fresh (no conversion factors calculated). These samples were then prepared for iridoid analysis as described above.

Chemical Analyses In this study, we compared two methods for examining iridoid glycosides: gas chromatography (GC) and high performance liquid chromatography (HPLC). Standards of antirrhinoside and linarioside were provided by Søren Jensen. Methods for analyzing iridoids using GC with phenyl-β-D-gluco-pyranoside (PBG) as an internal standard have been described previously (Gardner and Stermitz 1988; Bowers and Stamp 1993). For GC analysis, iridoid glycosides were derivatized to the corresponding trimethylsilyl derivatives by using Tri-sil Z™ (Pierce Chemical Company). GC analyses were performed on a Hewlett-Packard (HP) 5890A system (Agilent Technologies), and data were processed with HP ChemStation software (version A.03.34).

HPLC analyses were performed on a Hewlett-Packard 1090 system (Agilent Technologies) equipped with a diode array detector (DAD) and Apex (Jones Chromatography, U.K.) ODS 5 μm reverse phase C-18 column (250× 4.6 mm o.d.) protected with a guard column of the same material (7.5×4.6 mm). HPLC data were processed with HP ChemStation software (version A.10.02) that included a DAD spectral evaluation module. Our method for HPLC analysis was modified from a previous method described in Høgedal and Mølgaard (2000). Specifically, we used an isocratic method with a sample injection volume of 20 μl, mobile phase consisting of 3% acetonitrile in water (0.01% phosphoric acid added to bring pH to 4.0), flow rate of 1 ml/min, analysis time of 60 min, and spectrophotometric detection at 205 nm. Calibration curves were made using standard solutions with concentrations of 0.125–3.5 mg/ml. Standards included antirrhinoside, linarioside, and phenyl-β-D-gluco-pyranoside (PBG), which was the internal standard. The HPLC method was validated carefully and system suitability standards (<5% C.V for standard solution injections) were verified for each group of sample runs.

Statistical Analyses Iridoid glycosides were analyzed as proportions of dry weight (concentration). Data were arcsine square root transformed to meet assumptions of normality. Simple linear correlation analyses using Pearson correlation coefficients were performed to examine correspondence between iridoid glycosides measured with GC and HPLC methods and to determine if antirrhinoside and linarioside concentrations measured in plant tissues were correlated.

Because antirrhinoside and linarioside are potentially correlated, we used multivariate analysis of variance (MANOVA) to examine variation in iridoid glycoside concentrations due to (1) site and/or population differences, (2) date of sample collection and/or phenology, and (3) the part of the plant from which tissues were analyzed (flowers, leaves, or stems). We examined the main effects of date and plant part in separate analyses due to the unbalanced nature of our sampling design (i.e., iridoid glycoside concentrations from flowers and stems were measured only on one sampling date). The first MANOVA model, which examined iridoid glycoside concentrations of leaves only, included site, date, and a site by date interaction term. In the second MANOVA model, site, plant part, and a site by plant part interaction term were the main factors. MANOVAs were performed by using the general linear model (GLM) procedure in SYSTAT (version 11). When significant effects were detected by MANOVA, we followed up with univariate repeated-measures ANOVAs for each dependent variable (antirrhinoside and linarioside). Variation in iridoid glycoside concentrations (% dry weight) and total amount of iridoid glycosides (mg) of different life stages of *Calophasia lunula* were examined by ANOVA. For all univariate ANOVAs, Tukey's *post hoc* multiple comparisons tests were used to examine pairwise differences among groups when significant differences were detected. All statistical analyses were performed in SYSTAT (version 11).

Results

Comparison of GC and HPLC Methods Derivatized antirrhinoside and linarioside co-eluted when analyzed by GC, and the mean retention time for both compounds was 7.4 min. Derivatized PBG had a mean retention time of 4.1 min. With HPLC methods, separation of antirrhinoside

and linarioside peaks was achieved. Mean retention times for standard compounds were 20.1 min for antirrhinoside, 37.1 min for PBG, and 42.6 min for linarioside. Calibration curves indicated good linearity with standard solution concentrations between 0.125–3.5 mg/ml. The correlation coefficients for calibration curves were 0.995, 0.976, 0.995 for antirrhinoside, linarioside, and PBG, respectively. There was a highly significant positive relationship between GC and HPLC data (i.e., combined iridoid glycosides) (r=0.978; P<0.001; Fig. 2).

Iridoid Glycoside Variation in Linaria dalmatica Combined iridoid glycoside (antirrhinoside + linarioside) concentrations ranged from as high as 17.4% dry weight to as low as 0.2% dry weight of leaves (mean = 6.28±0.5 SE). We found antirrhinoside concentrations up to 16.5% (mean = 5.02±0.4 SE) dry weight of leaves and linarioside concentrations up to 6.7% (mean = 1.26±0.1 SE). The simple linear correlation analysis revealed a significant positive relationship between concentrations of antirrhinoside and linarioside (r=0.459; P<0.001; Fig. 3). On average, antirrhinoside represented 75.7% of the combined iridoid glycosides measured in leaves.

In our analysis of leaf tissue collected from plants on three sampling dates, we found significant variation in iridoid glycoside concentrations due to both site (*Wilks' λ*= 0.566, $F_{4,160}$=13.19, P<0.001) and date (*Wilks' λ*=0.378, $F_{4,160}$=25.07, P<0.001). There was no site by date interaction effect (*Wilks' λ*=0.958, $F_{8,160}$=0.43, P=0.900). Iridoid glycoside concentrations varied significantly both within and among populations (Fig. 4). Univariate ANOVAs revealed that antirrhinoside and linarioside showed similar patterns of variation among populations (site effect) and over the course of the growing season (date effect) (Table 1). Of the three populations, plants collected from Rabbit Mountain had the highest iridoid glycoside concentrations (Tukey's *post-hoc* pairwise comparisons: P<0.05 in both cases). In this one population, combined iridoid glycoside concentrations of leaf tissues ranged from 17.4 to 9.5% (mean = 12.97±0.7 SE) in May and from 14.2 to 0.5% (mean = 5.50±1.6 SE) in September, showing an average decline of 40% in the concentrations of these defense compounds over the course of the peak growing season. Across all field sites, iridoid glycoside concentrations were highest early in the growing season and declined over time (Fig. 4).

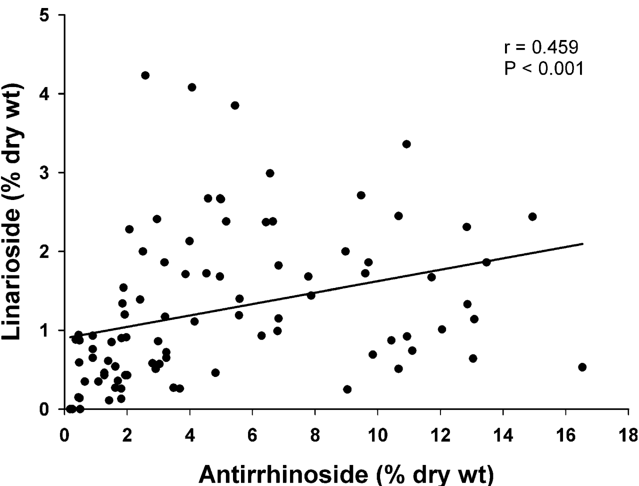


Fig. 3 Relationship between antirrhinoside and linarioside concentrations (% dry weight) measured in *Linaria dalmatica* leaves

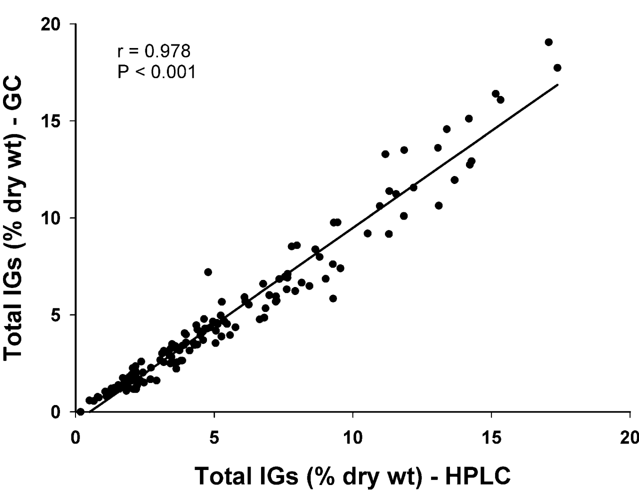


Fig. 2 Comparison of combined iridoid glycosides (antirrhinoside + linarioside) measured in *Linaria dalmatica* using GC and HPLC methods. Data are reported as % dry weight of leaf tissues. Antirrhinoside and linarioside co-eluted when analyzed by GC. Separation of compounds was acheived by HPLC

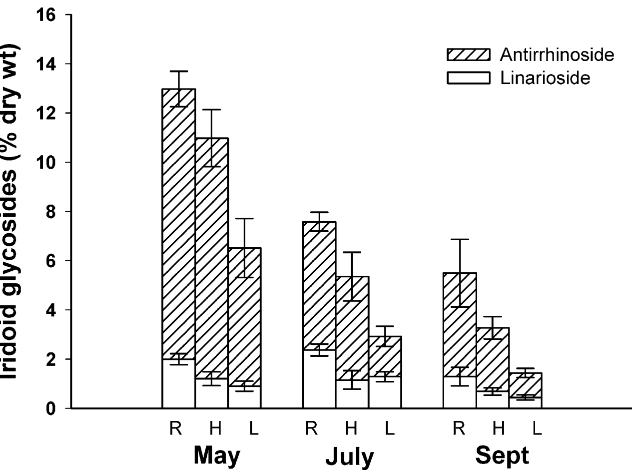


Fig. 4 Pattern of variation in iridoid glycoside concentrations of *Linaria dalmatica* leaves. Plant samples were collected in late-May, mid-July, and mid-September from three populations located in Boulder County, Colorado (USA): Rabbit Mountain (R), Hall Ranch (H), and Lefthand Canyon (L). Data reported are mean % dry weight ± SE (N=10 per site per date)

Table 1 ANOVA results summarizing the effects of site and date on iridoid glycoside concentrations of *Linaria dalmatica* leaves

Source	Antirrhinoside		Linarioside	
	F (df)	*P*	*F (df)*	*P*
Between Subjects				
Site[a]	18.18 (2)	**<0.001**	10.81 (2)	**<0.001**
Error	(27)		(27)	
Within Subjects				
Date[b]	52.33 (2)	**<0.001**	11.51 (2)	**<0.001**
Date x Site	0.17 (4)	0.953	0.67 (4)	0.615
Error	(54)		(54)	

[a] Field sites (Rabbit Mountain, Hall Ranch, Lefthand Canyon) were located in Boulder County, Colorado (USA)

[b] Samples were collected in May, July, and September of 2006 (N=10 per site per date)

Table 2 ANOVA results summarizing the effects of site and plant part on iridoid glycoside concentrations of *Linaria dalmatica* plants

Source	Antirrhinoside		Linarioside	
	F (df)	*P*	*F (df)*	*P*
Between Subjects				
Site[a]	15.10 (2)	**<0.001**	7.19 (2)	**0.003**
Error	(27)		(27)	
Within Subjects				
Plant part[b]	71.53 (2)	**<0.001**	26.77 (2)	**<0.001**
Plant part x Site	3.08 (4)	**0.024**	9.68 (4)	**<0.001**
Error	(54)		(54)	

[a] Field sites (Rabbit Mountain, Hall Ranch, Lefthand Canyon) were located in Boulder County, Colorado (USA)

[b] Tissues from leaves, flowers, and stems were analyzed for samples collected in July of 2006 (N=10 per site)

Results of the second MANOVA, which examined iridoid glycoside concentrations of flowers, leaves, and stems, indicated effects of site (*Wilks'* λ=0.506; $F_{4,160}$=16.25; P<0.001) and plant part (*Wilks'* λ=0.358; $F_{4,160}$=26.90; P<0.001) as well as a site by plant part interaction effect (*Wilks'* λ=0.673; $F_{8,160}$=4.38; P<0.001; Fig. 5). Again, univariate ANOVAs revealed that antirrhinoside and linarioside demonstrated similar response patterns, this time in response to the effects of site, plant part, and the interaction of these two factors (Table 2). Across field sites, combined iridoid glycoside concentrations of flowers (mean = 5.20±0.3 SE) and leaves (mean = 5.29±0.5 SE) were more than double the concentrations found in stems (mean = 2.07±0.2). At Hall Ranch and Lefthand Canyon, flowers had similar iridoid glycoside concentrations compared to leaves (Tukey's *post-hoc* pairwise comparisons: P>0.05 in all cases); however, at Rabbit Mountain, flower tissues contained slightly higher concentrations of iridoid glycosides compared to leaves (P<0.05). Site-specific differences in iridoid glycoside concentrations of flowers compared to leaves resulted in the observed plant part site by interaction effect (Table 2).

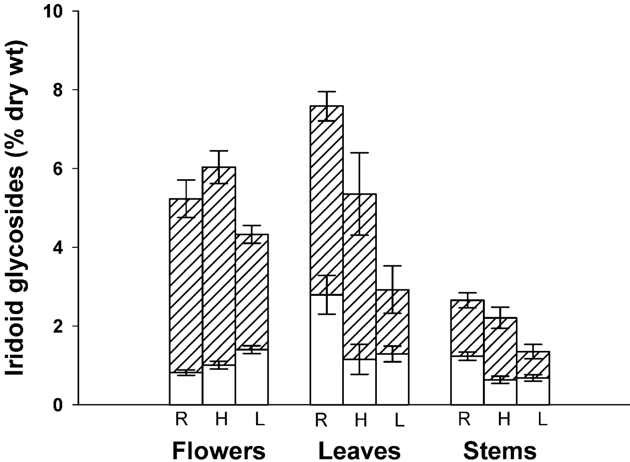
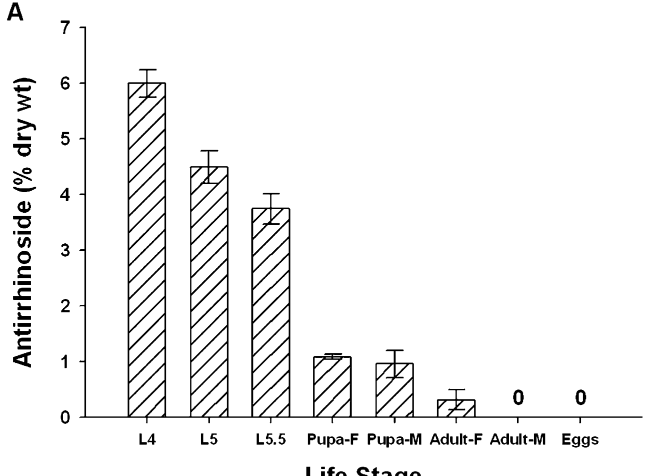


Fig. 5 Iridoid glycoside concentrations of flowers, leaves, and stems of *Linaria dalmatica*. Plant samples were collected in July 2006 from three populations located in Boulder County, Colorado (USA): Rabbit Mountain (R), Hall Ranch (H), and Lefthand Canyon (L). Data reported are mean % dry weight ± SE (N=10 per site)

Iridoid Glycoside Sequestration by Herbivores We found no evidence of iridoid sequestration by *M. janthinus*. However, results indicated that both *C. lunula* and *J. coenia* sequestered antirrhinoside from *L. dalmatica*. Antirrhinoside was found in larvae, pupae, and adult females of *C. lunula*, but was not found in eggs or adult males. In adult females, only three of the 10 samples contained antirrhinoside. Both the concentrations (% dry weight) and total amounts (mg) of antirrhinoside varied significantly among different life stages (P<0.001 in both cases; Fig. 6). The highest levels of antirrhinoside were found in larvae, with concentrations ranging from 2.7 to 7.5% dry weight (mean = 4.8±0.3 SE), and total amounts equal to 0.64± 0.07 mg (mean ± SE) in the three larval stages analyzed. Antirrinoside amounts in hemolymph ranged from 1.5 to 5.5 µg/µl, indicating that hemolymph is one important site of iridoid storage in *C. lunula* larvae. *Junonia coenia* also sequestered antirrhinoside. For 5th instars reared on *L. dalmatica*, we found antirrhinoside concentrations as high as 15.7% dry weight (mean = 12.1±0.5 SE), and total antirrhinoside amounts were 2.72±0.17 mg (mean ± SE). For 5th instar larvae reared on *A. majus*, antirrhinoside represented 9.6% (±0.5 SE) of the larval dry weight, and total amounts were 2.06±0.15 mg (mean ± SE). We found no evidence for sequestration of linarioside by either *C. lunula* or *J. coenia*, despite the presence of this iridoid glycoside in *L. dalmatica* host plants.



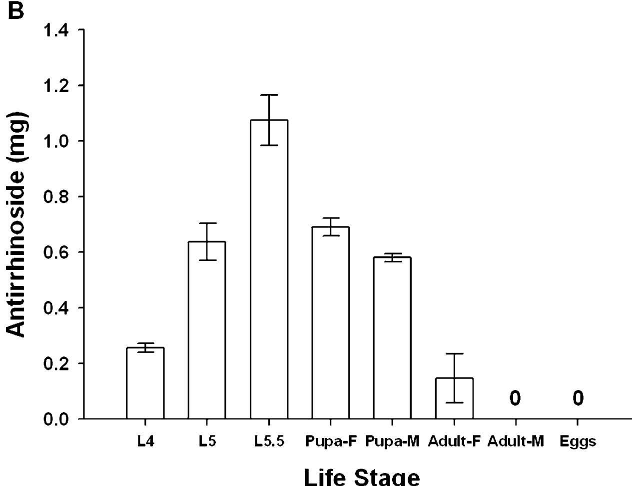
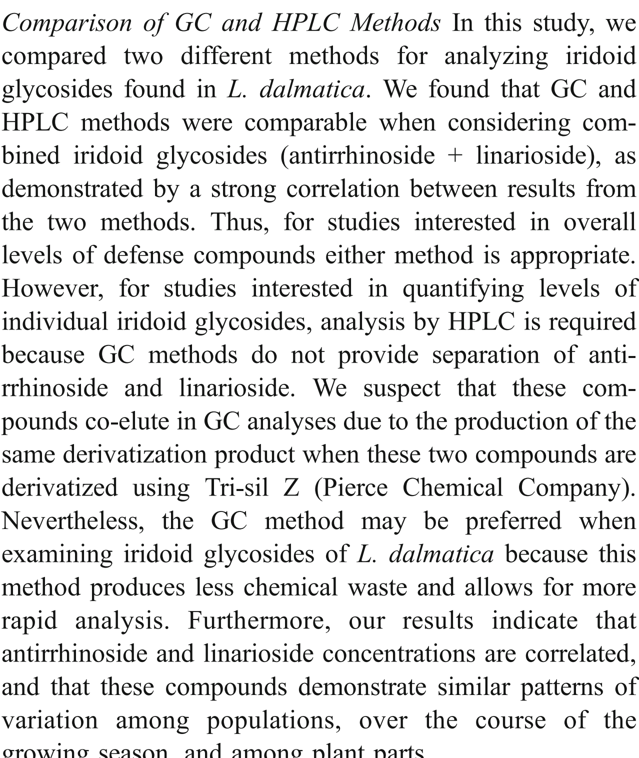


Fig. 6 Sequestration of antirrhinoside by *Calophasia lunula* (Noctuidae) at various life stages (**a**) concentration (% dry weight) (**b**) total amount (mg). N=10 individuals for each larval (L) stage, pupae, and adults. For eggs, N=4 samples each with 50–100 eggs

Discussion

Of the many plants introduced into novel habitats, few become established, and even fewer become invasive (Williamson and Fitter 1996). The novel chemistry and novel weapons hypotheses propose that some introduced plant species are successful invaders because natural enemies in the introduced range are not adapted to their unique chemical profiles (Callaway and Ridenour 2004; Cappuccino and Arnason 2006). Although many invasive plant species are thought to be phytochemically unique in their introduced range (Cappuccino and Arnason 2006), often little is known about the quantitative variation of defense compounds and the chemical ecology of plant-herbivore interactions for these species. Our research provides the first investigation of quantitative variation in the chemical defenses of the invasive plant *Linaria dalmatica.*


Comparison of GC and HPLC Methods In this study, we compared two different methods for analyzing iridoid glycosides found in *L. dalmatica*. We found that GC and HPLC methods were comparable when considering combined iridoid glycosides (antirrhinoside + linarioside), as demonstrated by a strong correlation between results from the two methods. Thus, for studies interested in overall levels of defense compounds either method is appropriate. However, for studies interested in quantifying levels of individual iridoid glycosides, analysis by HPLC is required because GC methods do not provide separation of antirrhinoside and linarioside. We suspect that these compounds co-elute in GC analyses due to the production of the same derivatization product when these two compounds are derivatized using Tri-sil Z (Pierce Chemical Company). Nevertheless, the GC method may be preferred when examining iridoid glycosides of *L. dalmatica* because this method produces less chemical waste and allows for more rapid analysis. Furthermore, our results indicate that antirrhinoside and linarioside concentrations are correlated, and that these compounds demonstrate similar patterns of variation among populations, over the course of the growing season, and among plant parts.

Iridoid Glycoside Variation in Linaria dalmatica We found that *L. dalmatica* contains high levels of iridoid glycosides, up to 17% dry weight with a mean of 6%. These results suggest that the species is well defended against generalist natural enemies, in particular naïve species. However, different generalist species can demonstrate varying responses to iridoid glycosides. For example, Beninger et al. (2008) found that gypsy moth larvae (*Lymantria dispar*: Lymantriidae) avoided leaves of cultivated snapdragon, *Antirrhinum majus* (Plantaginaceae), which contain antirrhinoside, and larval growth was reduced when fed a diet with 3.3% antirrhinoside concentrations. By contrast, cabbage looper (*Trichoplusia ni*: Noctuidae) larvae readily fed on *A. majus,* and larval growth actually increased on diets with antirrhinoside. The overall defensive nature of *L. dalmatica* iridoid glycosides against generalist herbivores requires further investigation.

Our study indicates that *L. dalmatica* plants are both spatially and temporally variable in levels of iridoid glycosides and that plant parts have varying concentrations of iridoid glycosides. Similar patterns of variation in iridoid glycoside concentrations have been documented for other plant species containing iridoid glycosides (e.g., Darrow and Bowers 1997). Although in contrast to *L. dalmatica*, Darrow and Bowers (1997) found that iridoid glycoside concentrations of *Plantago lanceolata* (Plantaginaceae) increased over the course of the growing season. Also, iridoid glycoside concentrations found in *P. lanceolata* were lower in general than those observed in *L. dalmatica*.

The results of our study suggest that there may be opportunities for generalist herbivores to avoid the deterrent or toxic properties of these compounds. For example, generalist herbivores, including both insects and grazing animals, may be able to utilize *L. dalmatica* as a resource in certain populations where plants produce lower levels of iridoid glycosides and at certain times of the year such as the later part of the growing season when levels are low. Also, stems are not well defended compared to leaves and flowers. Consequently, *L. dalmatica* may be especially susceptible to stem-mining or boring insects in the introduced range. It should be noted, however, that it is unknown whether iridoid glycosides found in *L. dalmatica* are inducible, as demonstrated in other plants that contain iridoids (e.g., Darrow and Bowers 1999; Fuchs and Bowers 2004).

Our research indicates that a number of factors, including genotype, ontogeny, phenology, and site differences may influence iridoid glycosides concentrations found in *L. dalmatica*. Genetic, ontogenetic, biotic, and abiotic environmental factors have all been shown to contribute to quantitative variation in iridoid glycosides in *Plantago* spp. (Bowers et al. 1992; Bowers and Stamp 1993; Adler et al. 1995; Darrow and Bowers 1999; Marak et al. 2002; Fuchs and Bowers 2004; Barton and Bowers 2006; Barton 2007; Wurst et al. 2008). Furthermore, such quantitative variation in these compounds has been shown to influence the degree to which individual plants are defended against generalist natural enemies, including pathogens and herbivores (e.g., Biere et al. 2004; Harvey et al. 2005).

In addition to influencing generalists, quantitative variation in iridoid glycosides of *L. dalmatica* also may affect specialists, including biological control agents used to manage *L. dalmatica* populations. Iridoid glycosides have been shown to act as feeding stimulants and oviposition cues, as well as to affect the performance of specialist insect herbivores (reviewed in Bowers 1991). In general, iridoid glycosides have been positively associated with both oviposition preference and performance of specialist insect herbivores (Bowers 1984; Pereyra and Bowers 1988; Bowers and Puttick 1989; Klockars et al. 1993; Nieminen et al. 2003; Harvey et al. 2005; Prudic et al. 2005; Saastamoinen et al. 2007; Reudler Talsma et al. 2008). Thus, iridoid glycosides may influence interactions between *L. dalmatica* and its biological control agents, potentially affecting host plant choice of ovipositing females as well as performance of offspring. Interestingly, we found that stems contain much lower levels of iridoid glycosides than flowers and leaves, which may have implications for biological control efforts, in particular for the use of *Mecinus janthinus*, which is a stem-miner. Gaining an understanding of the factors that contribute to variation in iridoid glycoside content of *L. dalmatica* may contribute to improving the management of this species.

Iridoid Glycoside Sequestration by Herbivores This study is the first to document sequestration of iridoid glycosides by *Calophasia lunula*. We found that *C. lunula* sequesters intermediate levels of antirrhinoside, on average about 5% of the caterpillar dry weight. At these concentrations, sequestered iridoid glycosides have been shown to be deterrent or toxic to a wide variety of predators, including birds, spiders, stink bugs, wasps, and ants (Bowers 1980; De la Fuente et al. 1994; Dyer and Bowers 1996; Strohmeyer et al. 1998; Theodoratus and Bowers 1999; Stamp 2001; Rayor and Munson 2002). We were surprised to find low levels of antirrhinoside in a few female adult moths; however, moths were frozen soon after emergence and may have retained some meconium, which can contain iridoid glycosides (Bowers and Puttick 1986; Bowers unpublished data). Variation in iridoid glycoside content of host plants has been shown to influence levels of iridoids found in sequestering species (e.g., Theodoratus and Bowers 1999; Prudic et al. 2005). Thus, factors that influence iridoid glycoside concentrations of *L. dalmatica* may also affect the degree to which *C. lunula* is defended against its natural enemies.

Compared to *Junonia coenia*, *C. lunula* is less efficient at sequestering antirrhinoside, as *J. coenia* larvae sequestered approximately twice as much antirrhinoside as *C. lunula* larvae. In general, *J. coenia* sequesters iridoid glycosides at high levels, with observed amounts up to 25% dry weight (Theodoratus and Bowers 1999). Larvae of three other lepidopteran species (*Meris paradoxa* Rindge (Geometridae), *Lepipolys perscripta* Gn (Noctuidae), and an undescribed species of *Lepipolys*) have been shown to sequester antirrhinoside at levels as high as 9.6 mg or 11.2% dry weight (Boros et al. 1991). Larvae of all three species feed on *Maurandya antirrhiniflora* (Plantaginaceae), which contains antirrhinoside in very high concentrations, up to 36% dry weight (Boros et al. 1991). Like *C. lunula*, these larvae are warningly colored; *M. paradoxa* is black, white, and yellow and the two *Lepipolys* species are gray with yellow, black, and white markings.

Plant defensive chemistry has been linked to the invasion success of numerous introduced species (Callaway and Aschehoug 2000; Callaway and Ridenour 2004; Cappuccino and Arnason 2006). Defense compounds are known to be of key importance in protecting plants from attack by generalist enemies and in host-plant selection by specialist enemies. However, as found in our study, plant defense compounds can demonstrate significant variation at multiple levels, including at the individual plant and population levels as well as over time. Such variation will ultimately influence the degree to which plants are

defended against generalist herbivores. Moreover, the influence of variation in plant chemical defenses on insect host plant selection and performance may contribute to variable success in biological control efforts. Thus, future studies that examine variation in the chemical defenses and chemical ecology of *L. dalmatica* may prove useful for understanding factors that influence the invasion success, impacts, and management of this species.

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REVIEW ARTICLE

Sex Pheromones and Their Impact on Pest Management

Peter Witzgall · Philipp Kirsch · Alan Cork

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Abstract The idea of using species-specific behavior-modifying chemicals for the management of noxious insects in agriculture, horticulture, forestry, stored products, and for insect vectors of diseases has been a driving ambition through five decades of pheromone research. Hundreds of pheromones and other semiochemicals have been discovered that are used to monitor the presence and abundance of insects and to protect plants and animals against insects. The estimated annual production of lures for monitoring and mass trapping is on the order of tens of millions, covering at least 10 million hectares. Insect populations are controlled by air permeation and attract-and-kill techniques on at least 1 million hectares. Here, we review the most important and widespread practical applications. Pheromones are increasingly efficient at low population densities, they do not adversely affect natural enemies, and they can, therefore, bring about a long-term reduction in insect populations that cannot be accomplished with conventional insecticides. A changing climate with higher growing season temperatures and altered rainfall patterns makes control of native and invasive insects an increasingly urgent challenge. Intensified insecticide use will not provide a solution, but pheromones and other semiochemicals instead can be implemented for sustainable area-wide management and will thus improve food security for a growing population. Given the scale of the challenges we face to mitigate the impacts of climate change, the time is right to intensify goal-oriented interdisciplinary research on semiochemicals, involving chemists, entomologists, and plant protection experts, in order to provide the urgently needed, and cost-effective technical solutions for sustainable insect management worldwide.

Keywords Sex pheromone · Attraction · Monitoring · Attracticide · Mating disruption · Insect control · Integrated pest management · Food security

P. Witzgall (✉)
SLU, Chemical Ecology Group,
23053 Alnarp, Sweden
e-mail: peter.witzgall@phero.net

P. Kirsch
APTIV,
Portland, OR 97202, USA

A. Cork
Natural Resources Institute, University of Greenwich,
Chatham Maritime, Kent ME4 4TB, UK

50 Years of Pheromone Research

Fifty years of curiosity driven pheromone research have yielded a profound understanding of sexual communication in insects. The discovery that minute amounts of species-specific chemical signals, encoded by discrete receptors on the antenna, instantaneously elicit a conspicuous upwind flight orientation behavior has been a source of inspiration for fundamental research on the insect olfactory system; research ranges from biosynthetic production of sex pheromones to peripheral perception by odorant receptor neurons, central processing of the olfactory input, and the resulting behavior (Jacquin-Joly and Merlin 2004; Jefferis et al. 2007; Xue et al. 2007; Cardé and Willis 2008; De Bruyne and Baker 2008).

The progress that has been made from the identification of the first sex pheromone in the silk moth by Butenandt and coworkers in 1959 to the identification of olfactory receptors in *Drosophila* (Clyne et al. 1999; Vosshall et al. 1999) and in the silk moth (Krieger et al. 2005) is spectacular. Pheromone communication, including the

generation of behavioral responses, is now being dissected at a molecular level (Benton et al. 2007; Dickson 2008). Our imagination is not sufficient to envision knowledge in insect olfaction and chemical ecology another 50 years from now, provided that researchers have the mandate and the resources to carry on this work.

Fundamental research derives, in part, its motivation and justification from the prospect of applying the acquired knowledge. A characteristic of insect chemical ecology is that the know-how can be transferred and used for the control of insects that are noxious to plants or animals. The interconnection between fundamental and applied research, the academic sector, chemical industries, agriculture, horticulture, and forestry, has been a driving force in fundamental and applied pheromone research. Many who began pheromone research in the sixties were influenced by the concept of integrated pest management (Stern et al. 1959) and Rachel Carson's (1962) plea for biorational pesticides. The use of synthetic pheromones for environmentally safe insect control was postulated soon after the discovery of silk moth pheromone (Butenandt et al. 1959; Wright 1964), well before pheromones of economically important insect pests were known.

Fifty years after bombykol, the database of insect pheromones and related attractants contains hundreds of chemicals (Arn et al. 1992; El-Sayed 2008). Pheromones are used as monitoring tools worldwide, and pheromone-based control applications cover large areas (Ridgway et al. 1990b; Howse et al. 1998; Baker and Heath 2004). Behavior-modifying chemicals are elegant tools for insect control, and the prospect of a wide range of future applications in agricultural and medical entomology continues to fuel research in insect olfactory physiology and chemical ecology (Van der Goes van Naters and Carlson 2006). The idea of replacing hazardous insecticides with environmentally benign and species-specific odorants is still a current research challenge, but the emphasis is shifting. The motivating force for green, sustainable insect control is no longer merely the health of the rural work force, the safety of agriculture and horticultural products, nor even the attempt to promote organic farming. The matter is more urgent than it was 50 years ago, and our concerns are the necessity of establishing sustainable insect control methods in times of increasing food insecurity.

Sustainable Insect Control and Food Security

Population growth, creating an increased demand for food, intensifies the pressure on our natural resources, and the adverse effects of climate change on agroecosystems further accentuates the magnitude of this challenge (Ehrlich et al. 1993). Foremost among the Millennium Development Goals endorsed by the United Nations is to eradicate extreme hunger and poverty and, more precisely, to halve between 1990 and 2015 the proportion of people who suffer from hunger. (www.un.org/millenniumgoals). Obviously, our endeavor to secure food for a growing population is closely related to another millennium goal, i.e., to reduce the loss of environmental resources and biodiversity. As we approach the deadline for the fulfillment of these goals, advances have begun to slow or even to reverse.

Environmental security and food security are closely interrelated and mutually dependent. Intensified pressure on ecosystems leads to depletion of resources that are vital for agriculture, including natural enemies of insect pests, pollinators, and carbon sequestration (Ehrlich et al. 1993; Thrupp 2000; van Mantgem et al. 2009). Moreover, future crops will grow under a different climate. The predicted associated effects of higher growing season temperatures and altered patterns of precipitation will have substantial impact on all forms of land use, from agriculture land and forests to aquatic environments. (Battisti and Naylor 2009; Schlenker and Roberts 2009).

Climate changes also will influence plant health and vigor, directly and indirectly through a modified reproductive performance of their associated herbivores. Climatic change will alter outbreak patterns and geographical ranges of insects, including those that vector diseases. The consequences are difficult to predict, especially in view of the complex interactions between crops, herbivores, and pathogens, but climate-related changes most likely will combine to reduce yields (Hunter 2001; Gregory et al. 2009). Forest insects have provided the first conspicuous examples of how insect outbreaks are intensified by global warming. Higher temperatures and drought are blamed for violent bark beetle attacks across Northern America that impact forest structure and in consequence carbon sequestration (Kurz et al. 2008; Van Mantgem et al. 2009).

Up to one third of worldwide food production is destroyed by insects, not including the damage done in storage. During decades of insecticide use, a permanent decrease in the abundance of targeted insect populations never has been achieved. Many of our top agricultural pests instead have been created by the use of pesticides that often have a stronger effect on natural antagonists than on the target species, and also because of widespread insecticide resistance (Pimentel et al. 1992; Elzen and Hardee 2003; Oerke 2006). This is particularly relevant in developing countries, where agricultural production must be increased to feed the population (Thrupp 2000; Pretty et al. 2003; Nwilene et al. 2008).

While crop protection against insects has long relied on insecticides, it is clear that they alone cannot provide a solution, not even by further intensification of their application. Shortcomings are particularly obvious in

regions with warm climates and long growing seasons. Recognition of pesticide limitations has, for example, led to the development of pheromone-based methods for control of the rice stem borer *Scirpophaga incertulas* in Bangladesh. Rice covers 70% of the land available for agriculture. Yield has increased by over 40% from 1996 to 2001, yet it does not match consumption. The annual insecticide application has increased from 7,000 t in 1997 to more than 16,000 t in the year 2000, some 90% of which is used in rice production. Even some agrochemical industries now have reached the view that a further increase is not feasible, and thus they support the development of mating disruption and mass trapping of rice stem borer in order to maintain a sustainable level of pesticide use (Cork et al. 2005b).

Chemical ecology produces the knowledge of non-toxic and species-specific pheromones and other semiochemicals that do not harm beneficial species and thus, the basis for efficient and sustainable insect management strategies. The paradox is that currently available know-how is not sufficiently exploited, and we may not be investing enough in research and development to provide breakthroughs quickly enough, especially in food-deficient countries in the developing world. Development aid to agriculture declined by almost 60% between 1980 and 2005, even though the total development aid bill increased over the same period (Fig. 1). In this review, one of our goals was to demonstrate that it is timely and meaningful to invest further in research on behavior-modifying chemicals for sustainable insect management.

Successes and Constraints of Pheromone-Based Methods

"There are many other reasons for using pheromones; one is that they are elegant" (Arn 1990). Three main elements account for the fascination of insect sex pheromones and their feasibility for insect management: 1) they are species-specific, 2) they are active in very small amounts, and 3) the vast majority are not known to be toxic to animals.

Pheromones are by definition species-specific, since the discrimination of conspecific and heterospecific pheromone signals is a key element in the evolution of specific mate recognition systems in many insects. Even synthetic, incomplete pheromone blends usually affect only the target, with the possible exception of taxonomically closely related species (Cardé and Haynes 2004).

Insects use extremely small amounts of pheromone for communication. For example, calling females of codling moth *Cydia pomonella* release pheromone at a rate of several ng/h. [In comparison, apple trees in orchards release one main volatile compound, (*E*,*E*)-α-farnesene, at an estimated rate of several g/ha/h (Witzgall et al. 2008)]. Pheromone trap lures used for detection and monitoring release typically ten to 100 times more than a calling female, and mating disruption dispensers used in orchards release up to 10,000 times more codlemone, which amounts to release rates of 10–100 mg/ha/h. The seasonal application rate of codlemone for mating disruption of codling moth in orchards is up to 100 g/ha. Worldwide annual production of codlemone is ca. 25.000 kg (Fig. 2), for codling moth control on ca. 210.00 ha.

Regulatory agencies in several countries consider it safe to use lepidopteran pheromones. This was corroborated by a recent evaluation by the California Environmental Protection Agency on the occasion of an area-wide eradication campaign against light brown apple moth (Ting 2009). Many pheromones have been registered for pest control, and there is no evidence of adverse effects on public health, non-target organisms, or the environment. Pheromones are applied in slow release formulations, thus resulting in low exposure; residues of lepidopteran pheromones in pheromone-treated food crops have not been detected (Tinsworth 1990).

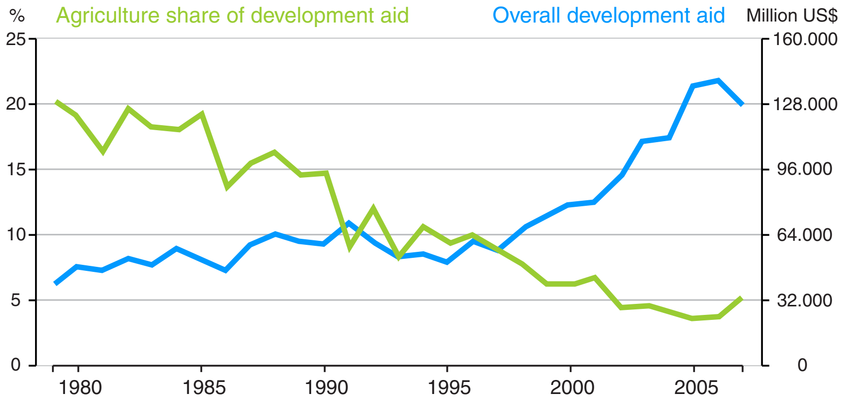


Fig. 1 Overall development aid (*right* scale, million US$), and the share dedicated to agriculture (*left* scale), 1979 to 2007 (Food and Agriculture Organization, FAO; World Summit on Food Security, Rome, November 2009)

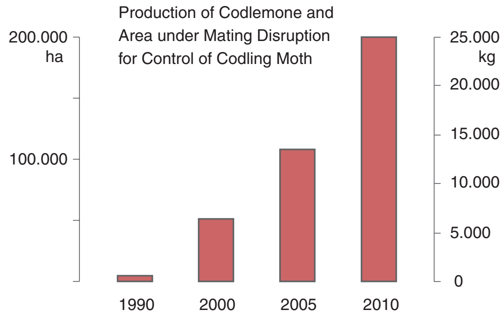


Fig. 2 Production of codling moth *Cydia pomonella* pheromone and area under mating disruption against codling moth worldwide (above; data courtesy of Shin-Etsu Chemical Co., Tokyo)

Insecticides vs. Pheromones

Insecticides do not achieve a long-term pest population decrease. In contrast, an observation shared by many working with pheromone-based control is that continuous long-term use *does* decrease population levels of target species (Fig. 3; Witzgall et al. 1999; Varner et al. 2001; Ioriatti et al. 2008; Weddle et al. 2009). This is attributable to a recovering fauna of beneficials, and to an increasing efficacy of pheromones at low population densities, when communication distance between sexes is increasing.

Insecticide overuse also induces outbreaks of secondary pests. Predatory and phytophagous mites provide the classic example of how the natural regulation of herbivores by their antagonists is disturbed by broad-spectrum pesticides (Agnello et al. 2003). Replacing insecticide with pheromone treatments in vineyards and orchards has rendered treatments against phytophagous mites superfluous, which compensates for the cost of the pheromone treatment (Louis et al. 1997; Waldner 1997; Jones et al. 2009). This emphasizes the contributing vital role of natural enemies for population control, and it corroborates that pheromone-based methods produce better results in the long run, due to recovery of the beneficial fauna.

Insects with hidden, protected lifestyles, including those with underground or woodboring larval habits, cannot easily be controlled with cover sprays of insecticides. Here, control with pheromones is advantageous, since it aims at the mobile adult life stage, and functions to prevent oviposition altogether. Examples of successful pheromone applications are provided in the sections below, and many of these concern insects that inflict severe damage and that are difficult or expensive to control with insecticides.

Slow Development of Pheromone-Based Pest Management

Despite their advantages, progress with practical implementation and commercial exploitation of pheromones has been slow. In Europe, pheromones have been used widely for almost two decades; against the grapevine moths *Lobesia botrana* and *Eupoecilia ambiguella* in Germany, Switzerland, and Northern Italy (Fig. 3; Arn and Louis 1996; Varner et al. 2001; Ioriatti et al. 2008) and against codling moth *Cydia pomonella* in Switzerland and Northern Italy (Mani et al. 1996; Waldner 1997). Although similar climatic, faunistic, and economic conditions exist in other

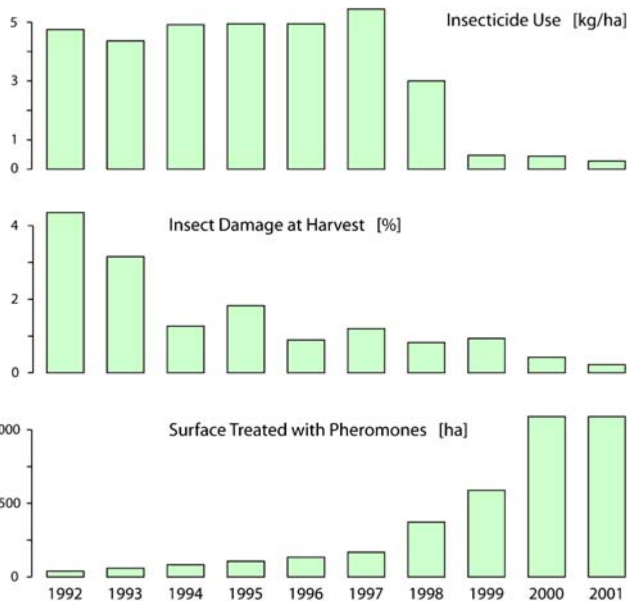


Fig. 3 Mating disruption in the Mezzocorona vineyards in Trento, Italy (Mauro Varner and Claudio Ioriatti, pers. comm.; photo by Mauro Varner)

European wine- and fruit-growing areas, pheromones have not been used much until very recently.

This suggests that motivation and determination among those involved in development and operation of pheromonal pest control methods is a key factor. A certain inertia in the pest control sector to adopt new technologies may be sustained by the lack of interest among research scientists in promoting and transferring existing knowledge. Ehrlich et al. (1993) convincingly argued that the historical separation of agriculture and pure biology at most universities has prevented the implementation of ecological principles in agriculture. To some extent, this may still be true today.

Another key to a more widespread use of pheromones is that the technologies currently in use must become more reliable. Continued goal-oriented research will lead to both more reliable and hence more widespread applications, but funding for applied research at academic institutions and extension services is being cut in many countries (Jones et al. 2009). Field implementation should become a focus of future pheromone research.

Few conventional chemical industries have invested in pheromones, and this lack of engagement has led to the belief that pheromone technology is not viable economically. More smaller companies that specialize in a particular product type, and have the flexibility and low overheads to make their investments in semiochemical products financially viable, are needed.

Practical pheromone applications depend on availability of efficient dispenser materials and on the economic synthesis of pheromone chemicals. The price of synthetic codlemone in the beginning of the nineties, for example, was far too elevated for commercial area-wide applications, but development of large-scale synthesis (Yamamoto and Ogawa 1989) made it possible to exploit it commercially. The annual production of codlemone, the main pheromone compound of codling moth, is on the order of 25 t (Fig. 2), and the price of codlemone is now well below 1,000 US$/kg.

Motives for Area-Wide Pheromone Use

The pheromone application concept goes beyond the conventional control paradigm of protecting plants against larval infestation with sprays of toxic compounds. Conditions for pheromone use are more favorable in area-wide programs, where the effect of immigrating, mated insect females becomes negligible. Successful use is frequently based on a joint effort that involves research scientists, extension entomologists, growers' associations, and also pheromone industries. Area-wide projects facilitate and provide support for this organizational effort (Ioriatti et al. 2008; Jones et al. 2009; Weddle et al. 2009).

The economic benefits of implementing sustainable techniques become evident at the landscape level. A close inspection of pheromone technologies that have been in place over several years shows that the price of pheromones vs. insecticides should not be confounded with economy of use. Farmers' efforts and successes in establishing sustainable production methods contribute to rural development (Ioriatti et al. 2008).

Safe insect control techniques not only improve product quality, but also contribute to the image of a region. Insecticide drift and run-off pollutes air and groundwater, and reduction of insecticide sprays alleviates growing conflicts between rural and urban areas. Orchards and vineyards, where pheromone-mediated mating disruption is widely used, are a part of cultural landscapes that produces, in addition to horticultural products, revenues by attracting tourists, enterprises, investors, and people who wish to inhabit this land.

Detection and Monitoring

The most widespread and successful applications of sex pheromones concern their use in detection and population monitoring. Captures in traps baited with synthetic pheromone lures accurately show whether a specific insect is present, and when its seasonal flight period starts. A simple and widespread strategy is to time insecticide sprays accordingly.

Population monitoring relates trap captures to the abundance of, or to the damage caused by an insect species. The magnitude of trap captures is used to determine thresholds, either for the timing of control procedures, or for making the decision whether or not remedial action is to be taken. One of the first widely used monitoring systems that include an action threshold based on trap captures was established for pea moth *Cydia nigricana* (Wall et al. 1987). Pheromone traps are sensitive enough to detect low-density populations, and are, therefore, effective for tracking invasive species in the establishment phase (El-Sayed et al. 2006; Liebhold and Tobin 2008).

Since pheromones lures are inexpensive and usually reliable, they facilitate the Integrated Pest Management (IPM) concept, which relies on frequent scouting of target species for planning of control measures and evaluating their efficacy. This is particularly so when more specific tools such as semiochemicals, microbials, or beneficials are used, rather than broad-spectrum insecticides.

Invariably, pheromone traps capture adults, and often only males, as is the case with lepidopteran pheromones. When trapping information is to be used in a predictive manner, such as in the damage done by the next generation of larvae, a good understanding of the biology of the pest and the effect of weather and crop stage on development is

needed. For pheromone-based monitoring it is further essential that a number of parameters, including the attractant, dispenser, trap design, and trap location are standardized and kept constant. The attractant and dispenser material must be under strict quality control, since release rates and chemical impurities, even in trace amounts, will strongly affect the attractiveness of a lure (Arn et al. 1997). Lure constancy, not overall attractiveness, is decisive.

One future goal is data capture. Insect monitoring can be facilitated by supplying farmers with additional information that includes current and historical seasonal records of trap catch, infestation rates, climate data, and possibly even the geographical distribution of the crop and target insect. This can be aided by the use of geographical information systems (GIS), for example, in area-wide control programs in forests (see below; Tobin et al. 2004, 2007).

Practical Use of Pheromone-Baited Traps

Hundreds of pheromone compounds have been identified, most of them in Lepidoptera, but also in other insect orders, particularly beetles and flies (Arn et al. 1992; El-Sayed 2008). Table 1 shows widely used pheromone lures. In several species, such as the tomato leafminer *Tuta absoluta* and in stored product insects, lures are used for both monitoring and mass trapping (see below). Lures are distributed by many companies worldwide, and there are no reliable data on the total number used, especially in emerging markets in Asia and South America. We estimate that at least 20 million pheromone lures are produced for monitoring or mass trapping every year. This includes all pest control sectors, horticulture, agriculture, stored products, forests, and also private use in households and gardens.

Gall midges (Diptera: Cecidomyiidae) are, because of their small size, difficult to see, and pheromones are cost-effective tools for tracking these tiny flies. Gall midge pheromone identifications require state-of-the-art analytical techniques, since they are produced in pico- to femtogram amounts. The chemical structures are carbon chains with one or two ester functionalities, and have been identified from several species including Hessian fly *Mayetolia destructor* (Andersson et al. 2009), swede midge *Contarinia nasturtii* (Hillbur et al. 2005), and raspberry cane midge *Resseliella theobaldi* (Hall et al. 2009). Swede midge traps have been deployed along the US-Canadian border to determine the geographical range of this invasive insect. A combination of a predictive model with pheromone traps accurately assesses and times control strategies (Hallett et al. 2009).

The tomato leafminer, *Tuta absoluta*, is an example of how the absence of an efficient conventional chemical control or other biological method encourages the use of an immature pheromone technology for insect control, merely because other methods are not available. *T. absoluta* is a multivoltine species that mines leaves and fruits of solanaceous plants. Effective chemical control is difficult to achieve (Pereyra and Sanchez 2006). Originally of neotropical distribution, *T. absoluta* recently has been introduced to Southern Europe and Northern Africa. This has fuelled the demand for monitoring lures, which are now employed in mass trapping campaigns in greenhouses. The main sex pheromone component is a triene, (E,Z,Z)-3,8,11-tetradecatrien-1-yl acetate (Svatos et al. 1996), but the lack of an economic synthesis currently precludes mating disruption tests.

Pheromone identifications in beetles are less advanced than in moths. Aggregation pheromones currently are used for mass trapping of weevils and scarabs (see below), and sex pheromones of several other families are under investigation. Larvae of click beetles are hard to control with pesticides due to their underground life habit. One main compound of click beetle pheromones is geranyl octanoate, and specific blends have been identified in several species. Pheromones have been used to survey species distributions and to monitor in the field, for example, *Agriotes* sp. in Europe and North America (Vernon and Toth 2007; Toth et al. 2008).

A number of pheromones have been elucidated in cerambycid beetles over the past decade. Even weak attraction to generic blends may be sufficient for monitoring distribution and phenology. However, some pheromones even attract females, and many of these species have long life cycles with short adult stages, which should favor the use of pheromones for control (Maier 2008; Ray et al. 2009; Rodstein et al. 2009). The coffee white stem borer *Xylotrechus quadripes* is a serious pest of coffee in South Asia. Male beetles have been shown to attract females, and (S)-2-hydroxy-3-decanone has been identified as the main attractive compound. Pheromone traps have been rapidly adopted for mass trapping (Table 1; Hall et al. 2006) reflecting exceptional grower interest in the absence of acceptable alternative control methods.

Mass Trapping and Annihilation

Control of insect populations with pheromones is achieved by two principle techniques, mating disruption and mass annihilation. Mating disruption (see below), causes disorientation and communication disruption between the sexes, and thus delays, reduces, or prevents fertilization of females. Mass annihilation, by mass trapping or attract-and-kill, relies on attraction of one or both sexes to a lure, in combination with a large-capacity trap or an insecticide-

Table 1 Use of sex pheromone lures for detection (D) and population monitoring (M), and for mass annihilation tactics, by mass trapping (MT) and attract-and-kill (AK)

Species	Purpose	Region	Lures/year
Horticulture			
Coleoptera			
Red palm weevil *Rynchophorus ferrugineus*	MT	Asia	1.175.000
American palm weevil *Rhynchophorus palmarum*	MT	Central and South America	25.000
Palm fruit stalk borer *Oryctes elegans*	MT	Asia	125.000
Banana weevil *Cosmopolites sordidus*	MT	Worldwide	120.000
Coffee white stem borer *Xylotrechus quadripes*	MT	India	40.000
Diptera			
Olive fruit fly *Bactrocera oleae*	MT, AK	EU	–[a]
Lepidoptera			
Grapevine moth *Lobesia botrana*	M	EU, Mediterranean countries, Chile, USA	–
Codling moth *Cydia pomonella*	M, AK	Worldwide	–
Oriental fruit moth *Grapholita molesta*	M, AK	Worldwide	–
Tomato leafminer *Tuta absoluta*	M, MT	South America, EU, North Africa	2.000.000
Brinjal fruit and shoot borer *Leucinodes orbonalis*	MT	India, Bangladesh	400.000
Fall armyworm *Spodoptera frugiperda*	MT	Central America	50.000
Agriculture			
Coleoptera			
Cotton boll weevil *Anthonomus grandis*	MT (AK)	North and South America	2.600.000
Click beetles *Agriotes spec.*	M	Europe	–
Lepidoptera			
Pink bollworm *Pectinophora gossypiella*	M, AK	North and South America, South Asia	–
Old World bollworm *Helicoverpa armigera*[b]	M, MT		830.000
Cotton leafworm *Spodoptera litura*[b]	M, MT		480.000
African armyworm *Spodoptera exempta*	D	East Africa	–
Spotted bollworm *Earias vittella*[b]	M, MT		280.000
Yellow rice stem borer *Scirpophaga incertulas*	M, MT	India	100.000
Southwestern Corn Borer *Diatraea grandiosella*	D	USA	–
Potato tuber moth *Phthorimaea operculella*	AK	South Africa	–
Forestry			
Coleoptera			
Spruce bark beetle *Ips typographus*	MT	Europe, China	800.000
Mountain pine beetle *Dendroctonus ponderosae*	MT	North America	–
Douglas-fir beetle *D. pseudotsugae*	MT	North America	–
Lepidoptera			
Gypsy moth *Lymantria dispar*	D	USA, EU	250.000
Spruce budworm, *Choristoneura fumiferana*	D	Canada, USA	–
Pine processionary moth, *Thaumetopoea pityocampa*	D, M	EU	–
Stored products			
Cigarette beetle *Lasioderma serricorne*	M, MT	Worldwide	2.500.000
Indian meal moth, *Plodia interpunctella*	M, MT	Worldwide	2.000.000
Households and gardens			
Japanese beetle *Popillia japonica*	MT	North America	–
Oriental beetle *Anomala orientalis*	MT	North America	–
House fly *Musca domestica*	MT	Worldwide	2.000.000
German cockroach, *Blattella germanica,* American cockroach, *Periplaneta americana*	MT	Worldwide	1.000.000

Examples of widely used lures. A distinction between monitoring and mass trapping is not always possible. The estimated number of lures used worldwide, based on turnover of leading companies in the US and Europe, exceeds 20 million lures

[a] No data available

[b] Data concern South Asia only

impregnated target. Unlike detection and monitoring, where only a small proportion of a population needs to be sampled, mass annihilation requires the use of the most attractive lure.

For attract-and-kill strategies, two different approaches are taken. Either, a semiochemical formulation, consisting of an attractant and inert carriers, is deployed as an additive tank-mix to insecticide products, or an attractant and insecticide are incorporated into a fully integrated matrix that can be applied as a stand-alone intervention. Each approach has specific merits. The additive approach has an advantage in that registration of semiochemicals is facilitated. When applying an integrated matrix product, blanket spray coverage of the crop is not necessary, so the amount of insecticide can be significantly reduced. However, specialized application technology is required.

With female-produced sex pheromones, only males are caught. Since male insects typically mate more than once, a high proportion of the male population must be removed to produce an effect. Male protandry, eclosion before females, will improve the effect of removing males. In addition, even a delay in mating, via a reduction in the number of available males, may also contribute to population control, as has been shown in mating disruption studies (Vickers 1997; Fraser and Trimble 2001; Jones et al. 2008; Stelinski and Gut 2009). These studies underscore the importance of integrating population biology and life history data into development of pheromone-based control applications. Population control is a dynamic and quantitative phenomenon, as illustrated by the importance of the Allee effects in the management of biological invasions (Liebhold and Tobin 2008).

Features of the biology and ecology of the target species that determine the efficacy of annihilation techniques include: the duration of the life cycle, the number of generations per season, the duration of the flight period, and the rate of population growth. Univoltine insects with short seasonal flight periods and a limited host range are easiest to control. Annihilation techniques become far more efficacious when using lures that attract females or both sexes, and when they include male-produced pheromones, aggregation pheromones, floral or plant volatiles that serve as ovipositional cues.

Mass trapping and attract-and-kill are cost-effective compared to mating disruption, since much smaller amounts of pheromones are needed. The insecticide component is environmentally rather safe, since small amounts are used and since crop contamination during application is much reduced. Nonetheless, the insecticide component is an obstacle to public acceptability of attract-and-kill methods. Fungal or viral insect pathogens might be used instead, but their slow mode of action and the short field-life of formulations have not been solved.

Practical Use of Mass-Trapping

Brinjal Fruit and Shoot Borer Eggplant is an important vegetable in South Asia, commercially produced by approximately 700,000 farmers on 570,000 ha in India alone. In Bangladesh, 40% of all vegetables produced are eggplants, providing farmers with a regular, year-round income. Severe yield losses are caused by the fruit and shoot borer *Leucinodes orbonalis*. Insecticides appear to be largely ineffective for control of *L. orbonalis*, because of protection offered by the fruit itself and because of insecticide resistance. A pheromone-based mass trapping strategy has been developed, from optimization of the pheromone blend and dose, trap design, and placement to field implementation (Cork et al. 2001, 2003, 2005a). Mass trapping, without the use of insecticides, has led to a 50% and higher increase in marketable fruit, which has been attributed to the combined effects of mass trapping and enhanced impact of natural enemies. Additionally, secondary pests, such as mites and whitefly, were reduced in the pheromone plots. The yield increase translates to earnings of $1,000 US$ per ha and yr for resource-poor families. Given estimated sales of pheromone lures reported in India and Bangladesh (Table 1), at least 15% of all farmers have now adopted the technology, and this is increasing year on year (A. Cork, unpublished).

Bark Beetles Bark beetles are of tremendous importance in coniferous forests worldwide, and the discovery of the aggregation pheromones of the most destructive European and North-American species was soon followed by area-wide mass trapping campaigns (McLean and Borden 1979; Bakke 1982). A more recent study corroborates that mass trapping is indeed a viable control strategy. In an isolated 2,000-ha forest reserve in China, traps for the double-spined spruce bark engraver *Ips duplicatus*, baited with a 2-component pheromone blend of ipsdienol and *E*-myrcenol, were employed at a rate of 1 trap/25 ha for 3 years. Yearly beetle captures between 0.5 and 1.7 million strongly reduced average tree mortality to 17% according to a 20-year record (Schlyter et al. 2003). This particular forest is isolated, but treatments on larger areas that use the same trap density should produce the same effect.

Palm Weevils Palm weevils are the most destructive pests of palm trees and cannot be efficiently controlled with insecticides. Adult weevils are not very susceptible to toxic compounds, and mining larvae are protected inside tree trunks. They provide an outstanding example of sustainable area-wide insect control by mass trapping, covering thousands of hectares of palm in all growing regions, particularly in Central America, the Middle East, and South Asia (Table 1). The beetles use aggregation pheromones for sexual communication prior to mating, attracting both

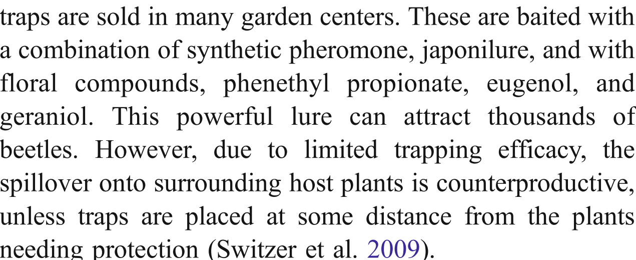
males and females. A facilitating factor is that overall population densities are lower than in smaller insects. Attractancy of the lures can be augmented by the use of plant and associated fermentation volatiles (Giblin-Davis et al. 1996; Oehlschlager et al. 2002).

Males of the American palm weevil, *Rhynchophorus palmarum*, release an aggregation pheromone, rhynchophorol: (4S)-2-methyl-(5E)-hepten-4-ol (Rochat et al. 1991; Oehlschlager et al. 1992). Captures with pure rhynchophorol increase considerably with the addition of plant material, leading to the development of an efficient control method based on mass trapping (Oehlschlager et al. 1993). Laboratory studies corroborate that aggregation is mediated by a male-produced pheromone and host-plant volatiles that include acetoin and ethyl acetate (Said et al. 2005). The American palm weevil is an important pest of several palm species in tropical America. Besides damaging the trees, it vectors a nematode that cause red ring disease. At less than one trap per 5 ha, even high palm weevil populations and nematode infestation rates have been reduced to very low infection levels, after only 1 year of trapping in combination with removal of infected palms (Oehlschlager et al. 2002).

The red palm weevil, *Rhynchophorus ferrugineus* originates in South-East Asia and is now widely distributed in Asia, Africa, and Oceania. It infests a range of tropical palms, including date, oil, and coconut palms. The larvae develop in the tree trunk, where they destroy the vascular system. Tens of thousands of date palm trees have been destroyed in the Middle East and North Africa since its appearance in the eighties because insecticide-based control is not sufficiently efficient (Soroker et al. 2005; Blumberg 2008). Mating in red palm weevil is mediated by an aggregation pheromone produced by the male weevil, composed of the main compound (4S,5S)-4-methyl-5-nonanol (ferrugineol) and 4-methyl-5-nonanone (Giblin-Davis et al. 1996; Perez et al. 1996). Traps loaded with ferrugineol, supplemented with ethyl acetate and plant volatiles, and a fermenting mixture of dates and sugarcane molasses, are placed at densities of up to 10 traps/ha for monitoring and mass trapping. Pheromone traps have played a significant role in the suppression of red palm weevil populations, for example in date palm plantations in Israel (Hallett et al. 1999; Soroker et al. 2005).

Banana Weevil The banana root borer *Cosmopolites sordidus* is a major pest of bananas throughout the world. The male-produced aggregation pheromone sordidin attracts both sexes, and mass trapping by using ground traps has the potential to replace inefficient insecticide treatments (Reddy et al. 2009).

Japanese Beetle The Japanese beetle is a devastating pest of urban landscape plants in the eastern United States and


traps are sold in many garden centers. These are baited with a combination of synthetic pheromone, japonilure, and with floral compounds, phenethyl propionate, eugenol, and geraniol. This powerful lure can attract thousands of beetles. However, due to limited trapping efficacy, the spillover onto surrounding host plants is counterproductive, unless traps are placed at some distance from the plants needing protection (Switzer et al. 2009).

Practical Use of Attract-and-Kill

Cotton Boll Weevil The cotton boll weevil *Anthonomus grandis* is a major pest of cotton in the Americas. Males produce an aggregation pheromone, grandlure (Tumlinson et al. 1969), that has been successfully incorporated into a pheromone-baited killing station known as "Boll Weevil Attract and Control Tubes". These tubes are produced in large numbers every year (Table 1). A density of 14 traps per ha achieves a strong reduction in weevil populations, at minimal crop damage. After successful control and eradication programs in the USA, bollweevil trapping is now also used in South America on at least 250,000 ha (Ridgway et al. 1990a; Smith 1998).

House Fly In addition to feeding attractants, house flies *Musca domestica* are attracted to pheromone, (Z)-9-tricosene (muscalure), which is widely used in combination with co-attractants in lure-and-kill approaches indoors and in livestock stables (Table 1; Butler et al. 2007; Geden et al. 2009).

Fruit Flies Male fruit flies (Diptera, Tephritidae) produce pheromones that attract females (Jang et al. 1994; Landolt and Averill 1999). However, these sex pheromones have not been a main research target, because of the efficacy of parapheromones and plant volatiles as attractants (methyl eugenol, trimedlure, cuelure, angelica seed oil, enriched ginger oil, raspberry ketone), and hydrolyzed protein baits (e.g., GF-120). These have been most widely used for monitoring and annihilation of several fruit flies, including Oriental fruit fly *Bacterocera dorsalis*, melon fly *Bacterocera cucurbitae*, and Mediterranean fruit fly, *Ceratitis capitata* for almost 50 years. Methyl eugenol, which is a male pheromone precursor, is a highly effective attractant and is used in IPM programs and for eradication of *Bactrocera* flies by male annihilation in the Pacific region, including Hawaii and California (Cunningham et al. 1990; Hee and Tan 2004; Vargas et al. 2008; El-Sayed et al. 2009).

A female-produced pheromone, a blend of (1,7)-dioxaspiro-[5,5,]-undane (olean), α-pinene, n-nonanal, and ethyl dodecanoate is exploited for control of the olive fly *Bactrocera oleae* by a lure-and-kill technology that incorpo-

rates the food attractant ammonium bicarbonate (Mazomenos and Haniotakis 1985; Broumas et al. 2002).

Orchard Tortricids A fully integrated attract-and-kill product, containing 0.16% pheromone and 6% permethrin, has provided control of codling moth at economic levels of less than 1% harvest infestation in apple orchards in Switzerland. Based on reductions in trap catch and the mating frequency of tethered moths, efficacy of the attract and kill droplets lasted 5–7 wk, requiring two seasonal applications. Subsequent experiments replaced permethrin with an alternative toxicant, the insect growth regulator, fenoxycarb, which has a sterilizing effect. Field tests showed that autosterilization, i.e., transfer of the insect growth regulator from a contaminated male to the female moth at mating, contributes to the control effect (Charmillot et al. 2000, 2002).

Studies of competition have shown that attracticide droplets are more attractive to male moths than calling females, and that the number of point sources is key to the ability of males to locate calling females (Krupke et al. 2002). Commercial applications require applications of 3,000 droplets per ha. At this rate, disruption of male orientation is likely to be a contributing mechanism. This has been substantiated with two further key orchard tortricids, Oriental fruit moth *Grapholita molesta* and lightbrown apple moth *Epiphyas postvittana* (Suckling and Brockerhoff 1999; Evenden and McLaughlin 2004).

Disorientation and Communication Disruption by Air Permeation

Insects rely on volatile sex pheromones to communicate for mating. Permeation of the crop with synthetic sex pheromone can disrupt chemical communication and thus prevent mating. Indeed, the mating disruption technique has become the most commonly utilized application of semiochemicals for population control (Baker and Heath 2004; Witzgall et al. 2008). Unlike with mass trapping, the natural pheromone is not required for mating disruption to be effective. Both attractive and non-attractive pheromone blends have been used, since off-blends can result in considerable cost savings (Bengtsson et al. 1994; Cork et al. 1996; Stelinski et al. 2008). Negative signals, including antiaggregation pheromones, have been combined with attractants into push-pull techniques (Borden 1997; Schlyter and Birgersson 1999; Cook et al. 2007).

The behavioral mechanisms by which mating disruption is achieved have been subject to investigation and discussion since Bartell (1982). If we understood the underlying mechanisms that cause or result in the behavioral modification that leads to disruption of mating, we will be better placed to understand why some applications are successful and others not (Cardé and Minks 1995; Sanders 1996, Miller et al. 2010). Attempts to interpret the behavioral response to air permeation treatments should, however, also build on field data on both the behavior of moths and molecules. Male moth behavior depends on a number of factors, including pheromone blend, release rate, and aerial concentration. Measurement of these factors and their contribution to efficacy will help to predict the outcome of mating disruption (Bengtsson et al. 1994).

Resistance to mating disruption is a remote risk in many species, because changes in female pheromone biosynthesis or male response are unlikely to lead to a new communication channel that is unaffected by synthetic pheromone treatments that do not precisely match the female-produced blend. However, resistance to treatments with a single pheromone component has occurred in the small tea tortrix *Adoxophyes honmai*. The efficacy of (Z)-11-tetradecenyl acetate, a ubiquitous leafroller pheromone component, decreased after 16 years. The composition of the pheromone blend produced by the females was unaltered, but the pheromone response was broader in resistant males. Efficacy of mating disruption returned to its former level, after changing to the natural 4-component pheromone blend (Mochizuki et al. 2002; Tabata et al. 2007a, b).

Commercial use of mating disruption became possible only after industrial scale synthesis became available at the end of the eighties. As a general guide, application rates of between 10 g and 100 g per ha per season are required to achieve communication disruption, thus resulting in aerial concentrations of at least 1 ng/m3 (Bengtsson et al. 1994; Cork et al. 2008).

A wide range of controlled release formulations has been developed for use in mating disruption. Early on, it had been assumed that a very high density of point sources was required to produce an effective fog of pheromone to disrupt male moths, and, therefore, formulations such as aqueous suspensions of micro-capsules and hollow fibers were developed. However, with the advent of hand-applied reservoir-type formulations, it was realized that fewer point sources that release higher quantities of pheromone could achieve the same result, by generating plumes with high concentrations of synthetic pheromone. Season-long field life is a main advantage of hand-applied dispensers. Renewed efforts to develop sprayable formulations is motivated by reduced application cost, either in combination with fungicides in orchards, or for applications on large areas (Leonhardt et al. 1990; Weatherston 1990; Trimble et al. 2003; Tcheslavskaia et al. 2005; Il'ichev et al. 2006).

A major flaw of current commercial pheromone dispensers is that pheromone release increases with ambient temperature. In apple orchards treated against codling moth,

ca. 90% of pheromone applied is released outside the diel flight period, mainly during daytime at peak ambient temperatures (Witzgall et al. 1999). In addition, dispensers must be applied early in season when population densities are still low and release rates decrease during the season, as population densities start to increase. These problems can be circumvented by using timed and metered pheromone sprayers that release constant and large amounts of pheromone only when the insects are active (Shorey and Gerber 1996; Mafra-Neto and Baker 1996; Fadamiro and Baker 2002). Such "puffers" are now increasingly used against navel orangeworm *Amyelois transitella* and codling moth.

Mating disruption is more efficacious over large areas. This is in part because large areas reduce the impact of gravid females that immigrate into treated plots, but also because homogenous air permeation is facilitated. Incomplete permeation with pheromone, especially along crop borders, is an obstacle. This has been confirmed by field EAG measurements of aerial pheromone concentrations (Milli et al. 1997). Border effects become negligible when large surfaces are treated. Indeed, dispenser spacing and overall pheromone application rate can be reduced as the treated area increases, resulting in considerable cost savings to farmers.

Adoption of mating disruption and reduction of insecticide leads to a decrease in the incidence of secondary pests due to conservation of natural enemies. In orchards and vineyards, mating disruption renders treatments against phytophagous mites superfluous since outbreaks are typically induced by the overuse of insecticides.

Other biological techniques rarely permit stand-alone containment of insects, and mating disruption, in addition to annihilation techniques, is often the only option when insecticides cannot be applied, as in organic crops, allotment gardens, or against insecticide-resistant insect populations (Suckling et al. 1990; Albert and Wolff 2000). From the nineties onwards, the area under mating disruption saw an almost exponential expansion into the first decade of this century (Fig. 2; Brunner et al. 2002; Ioriatti et al. 2008).

Mating Disruption in Vineyards

The history of mating disruption of grape moths in Europe, reviewed by Arn and Louis (1996) and Ioriatti et al. (2008), exemplifies the weight of interfacing research among extension people, growers, and pheromone industries for the development and implementation of this new technology.

The complete identification of the sex pheromones of the key European grape insects as a prerequisite for the development of mating disruption (Arn et al. 1986; Guerin et al. 1986; El-Sayed et al. 1999b) was the incentive for the development of techniques that still are widely used in chemical ecology research, such as the electroantennographic detector (Arn et al. 1975) and a wind tunnel bioassay with quantitative, controlled stimulus application (Rauscher et al. 1984; El-Sayed et al. 1999a, b).

A portable electroantennogram apparatus was designed for live measurements of ambient pheromone concentrations with an insect antenna, and for rapid optimization of pheromone dispenser placement (Sauer et al. 1992; Koch et al. 2009b). Field tents were used to determine the mating status of female moths and critical population densities, above which mating disruption is no longer effective (Feldhege et al. 1995). The latest methodological progress is appreciably simple but facilitates replicated field measurements of the behavioral effect of dispenser formulations or dispenser densities. Insects are released into portable 8.5 m^3 field cages that contain traps with live females and synthetic pheromone. The cages are placed in vineyards, into 20×20 m pheromone dispenser arrays that simulate full-scale vineyard treatments. The plot size is convenient for the investigation of experimental dispenser formulations (Koch et al. 2009a).

Experimental trials in vineyards were expanded to area-wide campaigns by involving plant protection entomologists, growers associations, and pheromone industries (Rauscher and Arn 1979; Neumann et al. 1993; Vogt et al. 1993). The coordination of mating disruption field campaigns is a complex task, indeed: 1,447 growers participated in Northern Italy in 1999 (Varner et al. 2001). By the end of the nineties, mating disruption had led to an area-wide reduction in population densities and it became widely accepted by growers in Germany, Switzerland, and Northern Italy (Fig. 3; Varner et al. 2001). A challenge in these European vineyards is now to develop novel strategies against new pests that are not affected by mating disruption, especially leafhoppers that vector plant diseases (Mazzoni et al. 2009).

Meanwhile, European grapevine moth *Lobesia botrana* has been found in Chile and Napa County, California. In Chile, 40,000 ha now are under mating disruption in an attempt to eradicate the newly established population (V. Veronelli, pers. comm.)

Mating Disruption in Orchards

Pheromone use in orchards concerns mainly the codling moth *Cydia pomonella*, Oriental fruit moth *Grapholita molesta* (Table 1), and lightbrown apple moth *Epiphyas postvittana*.

Lightbrown apple moth *Epiphyas postvittana* is native to Australia and New Zealand. It is a serious threat to agriculture, because of its polyphagous lifestyle on many fruit and ornamental crops and because it is a quarantine

pest in many countries. Suckling and Clearwater (1990) demonstrated that a 2-component blend provided better communication disruption than the main compound alone. Mating disruption then was conceived as a strategy to achieve, in combination with a reduced spray program, economically acceptable control in insecticide resistant populations in apple in New Zealand (Suckling et al. 1990; Suckling and Shaw 1995). More recently, efficient population control has been demonstrated in Australian citrus (Mo et al. 2006). Lightbrown apple moth now has been found in California. A mating disruption campaign uses ground-based sprays and hand-applied dispensers (Garvey 2008; Varela et al. 2008).

Codling moth *C. pomonella* exemplifies some main requirements for competitive pheromone use (Brunner et al. 2002; Witzgall et al. 2008). (1) The larvae damage the crop directly, and the economic damage threshold in apple, pear, and walnut is very low. (2) The hatching larvae are difficult to control with insecticides since they immediately bore into the fruit. A most efficient and widely used insecticide, azinphos-methyl, has been banned in many countries due to its acute neurotoxicity. Resistance problems have occurred with several other insecticides (Reyes et al. 2009). New insecticides, including neonicotinoids that provide more specific control than organophosphates are more costly and still harmful to beneficial arthropods (Beers et al. 2005; Brunner et al. 2005; Poletti et al. 2007). (3) Other, stand-alone biological control techniques are not available. (4) Overuse of insecticides is well-known to harm predatory mites and induce phytophagous mites (Waldner 1997; Epstein et al. 2000; Agnello et al. 2003). Miticide sprays are costly; avoiding them balances the cost of the pheromone treatment. (5) IPM was initiated in orchards and vineyards, and much emphasis has been placed on crop protection education. (6) Consumers are more wary of pesticide residues in fruit than in other food. (7) Sustainable pest control helps to reconcile conflicts between urban and adjacent rural areas, and corroborates the contribution of orchards to the aesthetic value of a region. The worldwide orchard area treated with codling moth mating disruption has now surpassed 200,000 ha, corresponding to, for example, almost half of the European orchard area (Table 2; Fig. 2).

Mating Disruption in Forests

Antipheromones and Bark Beetle Control Bark beetles, including mountain pine beetles *Dendroctonus* sp. can convert large regions of boreal and temperate forest from carbon sinks to carbon sources. It is, therefore, urgent to determine whether pheromones and other semiochemicals become effective in suppressing bark beetle outbreaks. Conifer-inhabiting bark beetles have evolved several olfactory mechanisms for finding, colonizing, and killing their hosts, and also for avoiding unsuitable, overcrowded host trees and resistant nonhost trees. The battery of semiochemicals, attractant and repellent, produced by beetles, host plants, and non-host plants is available for the design of innovative control technology (Borden 1997; Schlyter and Birgersson 1999; Zhang and Schlyter 2004; Seybold et al. 2006).

An alternative strategy to mass trapping with attractant pheromones (see above) are push-pull tactics. These combine aerial permeation of forest stands with anti-aggregation pheromone or repellent non-host volatiles with attractant pheromones. Recent tests confirm the potency of the anti-aggregation pheromones verbenone and methylcyclohexenone (MCH) in aerial forest treatments against mountain pine beetle *Dendroctonus ponderosae* and Douglas-fir beetle *D. pseudotsugae*, respectively (Gillette et al. 2009a, b). These compounds also have been combined with pheromone-based mass-trapping in a push-pull fashion, using hand-applied dispensers (Borden et al. 2006, 2007).

Gypsy Moth The largest application of mating disruption over many years is part of the area-wide management of gypsy moth *Lymantria dispar*, an invasive forest insect in the Eastern US. The "Slow the Spread" program has significantly reduced the spread of gypsy moth by detecting isolated populations in grids of pheromone-baited traps placed along the expanding population front. The detected populations are treated by using *Bacillus thuringiensis* or more frequently by mating disruption, by using aerial applications of plastic flakes (Sharov et al. 2002; Tcheslavskaia et al. 2005).

A prerequisite to the success of managing the spread or establishment of invasive insects is the availability of practical methods for detecting low-density populations. Much of the credit for the success of gypsy moth containment efforts is attributed to the availability of pheromone-baited traps that are inexpensive yet highly sensitive (Liebhold and Tobin 2008).

Area-Wide Programs

Natural insect populations are known to fluctuate in large-scale synchrony. Such spatio-temporal fluctuations are particularly conspicuous in unmanaged forest insects, which can defoliate entire regions (Peltonen et al. 2002). Population fluctuations have been largely neglected in horticultural and agricultural insects while conventional insecticides are the dominating management tactic. The knowledge of population changes on a regional scale is, however, vital for pheromone-based pest management programs (Kobro et al.

Table 2 Use of mating disruption and air permeation with pheromones and antipheromones

Species	Main crop	Region	Area (ha)
Mating disruption			
Gypsy moth *Lymantria dispar*	Forest	USA	230.000
Codling moth *Cydia pomonella* [a]	Apple, pear	Worldwide	210.000
Grapevine moth *Lobesia botrana*	Grape	EU, Chile	100.000
Oriental fruit moth *Grapholita molesta*	Peach, apple	Worldwide	50.000
Pink bollworm *Pectinophora gossypiella* [b]	Cotton	USA, Israel, South America, EU	50.000
Grapeberry moth *Eupoecilia ambiguella*	Grape	EU	45.000
Leafroller moths, Tortricidae	Apple, pear, peach, tea	USA, EU, Japan, Australia	25.000
Striped stem borer *Chilo suppressalis*	Rice	Spain	20.000
Other species	Fruit, vegetables		40.000
Total			770.000
Antipheromones			
Mountain pine beetle *Dendroctonus ponderosae*	Pine	USA, Canada	– [c]
Douglas-fir beetle *D. pseudotsugae*	Douglas-fir	USA, Canada	– [c]

[a] Annual estimated codlemone production is 25 tons (Fig. 2)

[b] Usage dropped significantly upon widespread adoption of transgenic cotton varieties

[c] No data available

2003). In addition, landscape ecology affects insect dynamics, and this should be taken into account for more efficient crop protection (Ricci et al. 2009).

Pheromone-based methods have been shown to produce reliable results especially in area-wide programs, and future applications should, therefore, be planned on a landscape level. Geographic information systems (GIS) make it possible to capture, organize and evaluate insect population data and to visualize spatial and temporal fluctuations on a regional scale. Geo-referenced insect monitoring data can, in addition be correlated with relevant parameters such as distribution of the crop and other vegetation, geography, climate, and insect control programs.

GISs are already in use, especially in forest insects, to document geographic variation, predict outbreaks, and to delimit invasive species (Tobin et al. 2004, 2007). The web adds yet another dimension to the analytical power of geographical information systems, as it provides worldwide connectivity. A web-based GIS permits one to quickly disseminate and share information, and it enables interactivity between end users, extension people, and researchers. A rigorous effort should be made to apply such techniques for landscape-level applications of semiochemical-based insect control.

Other Semiochemicals

Semiochemicals that attract insect females are tools for monitoring the occurrence and reproductive status of females. This is particularly important in species in which sexual communication relies on female-produced sex pheromones that attract only males. Moreover, some powerful annihilation strategies are based on female attractants (Table 3). Plant-derived chemicals also are known to improve attraction to pheromone lures (Giblin-Davis et al. 1996; Oehlschlager et al. 2002; Knight and Light 2005; Knight et al. 2005; Bengtsson et al. 2006; Schmidt-Büsser et al. 2009).

Even non-host volatiles may play a significant role in insect management, since some insects avoid volatiles of non-host plants. The know-how of negative plant volatile signals can be used to design push-pull techniques (Borden 1997; Zhang and Schlyter 2004; Cook et al. 2007).

Floral Compounds that Target Moths Floral scents play a key role in the coevolution of flowering plants and their pollinators (Raguso 2004; Bergström 2008). The bouquets of flowering plants, for example, Canada thistle and *Buddleja* butterfly bush, are strong attractants for Lepidoptera, Coleoptera, Diptera, and Hymenoptera (El-Sayed et al. 2008; Guedot et al. 2008). Synthetic floral attractants composed of phenylacetaldehyde and other volatiles, such as β-myrcene are known for several noctuid moths (Haynes et al. 1991; Heath et al. 1992; Landolt et al. 2006). Noctuids also respond to attractants that encode fermenting food sources, such as acetic acid and 3-methyl-1-butanol (Landolt and Alfaro 2001; Landolt et al. 2007).

A highly effective technique to control *Helicoverpa* spp. combines floral attractants and feeding stimulants with insecticide. The blend is sprayed on one out of 36 or 72 rows of a cotton field, thus minimizing the environmental

Table 3 Use of other semiochemicals in insect detection (D) and control by mass trapping (MT) and attract-and-kill (AK)

Species	Lure	Purpose	Region	Lures/year
Horticulture				
Mediterranean fruit fly *Ceratitis capitata*	Trimedlure	MT	Worldwide	3.000.000
Melon fly *Bactrocera cucurbitae*	Cue-lure	AK	South Asia, USA	300.000
Oriental fruit fly *Bactrocera dorsalis*	Methyl eugenol	AK	South Asia, USA	400.000
Agriculture				
Corn rootworm *Diabrotica spp.*[a]	Kairomone	AK	USA	40.000 ha
American bollworm *Helicoverpa armigera* [a]	Kairomone	AK	Australia	10.000 ha
Forestry				
Emerald ash borer *Agrilus planipennis*	Kairomone	D	USA	150.000
Medical entomology				
Sheep blowfly *Lucilia cuprina*	Kairomone	MT	Australia, South Africa	350.000
Tsetse fly, *Glossina pallidipes, G. morsitans morsitans*	Kairomone	AK	Southern Africa	–[b]
Bed bug, *Cimex lectularis*	Kairomone	D	USA, Canada, EU, Australia	50.000
Homes				
Social wasps	Food bait		USA, EU	–
Vinegar fly *Drosphila melanogaster*	Food bait		USA, EU	50.000

[a] Treated surface

[b] No data available

impact of the insecticide component. The attractant primarily targets female moths and removes them from the crop ecosystem prior to oviposition, showing that plant volatiles can be used to control insect populations (Del Socorro et al. 2003, 2010a, b; Gregg et al. 2010). The dispersal activity of insects, which is generally perceived as an obstacle for mating disruption and mass trapping, can be turned into an advantage when using female attractants.

This breakthrough development is based on a paradigm shift, since the attractant does not mimic a particular plant, it is instead a combination of compounds from several plants, producing a supernatural floral blend not found in nature (Del Socorro et al. 2003, 2010a, b; Gregg et al. 2010). Clearly, olfactory space would accommodate different floral attractants, since noctuid moths have been exposed to a changing guild of flowers during evolution. It is conceivable, given a more complete knowledge of olfactory receptor ligands, that such synthetic blends can be created also for other insect species.

Social Wasps Blends of acetic acid, with either butyl butyrate, heptyl butyrate, or isobutanol elicit food-finding behavior in a range of social wasps (Landolt et al. 2000). Lures for these wasps are widely distributed for the home and garden market (Table 3).

Corn Rootworms Diabroticine chrysomelid beetles evolved in the prairie ecosystem with larvae that feed on grass roots and adults that feed on vegetative parts of a broad range of plants, including maize. The western corn rootworm, *Diabrotica virgifera virgifera* is a species that is of particular concern in Europe, since it was introduced into the Balkans (Hummel 2003). Adult beetles are attracted to the volatiles of squash blossoms (Cucurbitaceae), and compulsive feeding and arrestment responses on cucurbit foliage are triggered by cucurbitacin secondary plant compounds (Metcalf et al. 1980). This behavior is exploited in the development of floral-baited traps for monitoring (Toth et al. 2007) and flowable and sprayable bait formulations that contain insecticides for area-wide management (Siegfried et al. 2004; French et al. 2007).

Emerald Ashborer The metallic and bright coloration of the elytra of jewel beetles (Buprestidae) points to the significance of visual cues in mate finding. However, host finding is likely mediated by olfactory cues, since many buprestids oviposit on stressed or dying trees. This has been demonstrated in the genus *Melanophila*, where females, which oviposit on wood of trees freshly killed by fire, can detect substances emitted in smoke from burning wood (Schütz et al. 1999).

The emerald ash borer is a rapidly spreading invasive species in the Eastern USA that kills ash trees. The cooperative emerald ash borer project includes a considerable trapping program (Table 3) for early detection in and around the Great Lakes district. Bark volatiles from green ash *Fraxinus pennsylvanica* contain a range of antennal active sesquiterpenes. Natural oil distillates, containing high concentrations of some of these ash volatiles, are currently used as a lure (Crook et al. 2008; Crook and Mastro 2010

this volume). Emerald ash borer trapping is another example of how even immature technology is adopted, due to the absence of other management tools.

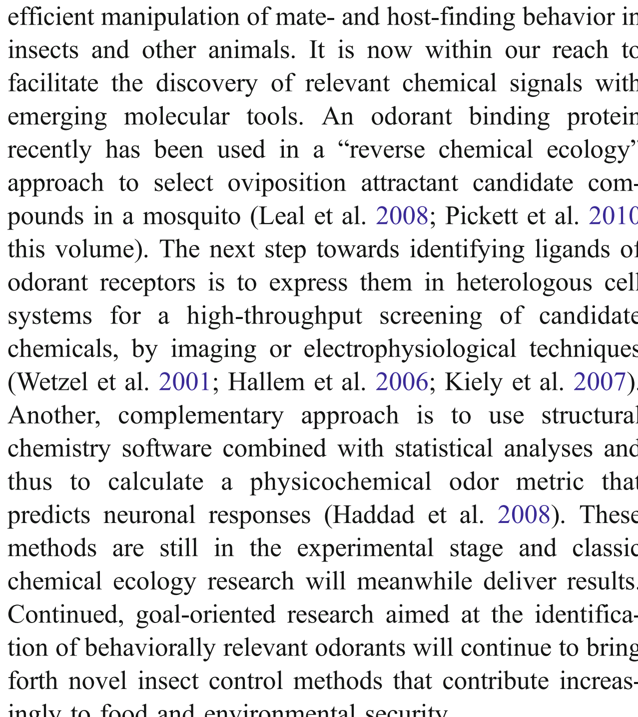
Tsetse Flies African trypanosomiasis is transmitted by tsetse flies *Glossina* spp., that are obligate haematophages. The prospect of eradicating tsetse over large areas has appeared to be a serious possibility because of the development of cost-effective semiochemical-based control technologies, notably odor-baited targets that mimic host odor. The search for further host volatiles is ongoing (Harraca et al. 2009). The best known attractant identified from cattle volatiles and buffalo urine comprises a blend of 3-n-propyl phenol, 1-octen-3-ol, and 4-methylphenol (p-cresol), and a separate dispenser containing methyl ethyl ketone or acetone, (Bursell et al. 1988; Vale et al. 1988). Importantly, the odor-baited targets are treated with a fast acting insecticide, typically deltamethrin, and provide visual cues, typically colored in panels of blue and black, that attract and elicit landing in tsetse flies (Torr et al. 1997). There has been a steady increase in the use of odor-baited targets for mass trapping in Southern Africa, with a concomitant insecticide treatment of cattle. In Zimbabwe, an area-wide vector management program reduced typanosomiasis in cattle from several thousand cases per year to two in 1995 (Lindh et al. 2009; Torr et al. 2010).

Sheep Blowflies Australian sheep blowfly *Lucilia cuprina* and related species are potentially controlled by a rather selective synthetic kairomone attractant (Table 3). Insecticidal control is problematic, because of the demand for residue-free wool and the resistance of blowflies to many insecticides. Fly population changes also are driven largely by climate, rather than by biotic factors, and are expected to increase under likely scenarios of climate change (Goulson et al. 2005).

Traditionally, blowfly traps have been baited with liver and sodium sulfide, but a synthetic kairomone, consisting of 2-mercaptoethanol, indole, butanoic acid, and a sodium sulfide solution is far more effective and selective for *L. cuprina* than the standard liver attractant. More importantly, the synthetic mix can be packaged in controlled-release dispensers to generate constant, prolonged release of the attractant. Field studies have confirmed that kairomone traps are a useful component of a blowfly control program (Ward and Farrell 2003; Urech et al. 2004, 2009).

Outlook

Future applications of pheromones and other semiochemicals depend on the availability of odorants that enable


efficient manipulation of mate- and host-finding behavior in insects and other animals. It is now within our reach to facilitate the discovery of relevant chemical signals with emerging molecular tools. An odorant binding protein recently has been used in a “reverse chemical ecology” approach to select oviposition attractant candidate compounds in a mosquito (Leal et al. 2008; Pickett et al. 2010 this volume). The next step towards identifying ligands of odorant receptors is to express them in heterologous cell systems for a high-throughput screening of candidate chemicals, by imaging or electrophysiological techniques (Wetzel et al. 2001; Hallem et al. 2006; Kiely et al. 2007). Another, complementary approach is to use structural chemistry software combined with statistical analyses and thus to calculate a physicochemical odor metric that predicts neuronal responses (Haddad et al. 2008). These methods are still in the experimental stage and classic chemical ecology research will meanwhile deliver results. Continued, goal-oriented research aimed at the identification of behaviorally relevant odorants will continue to bring forth novel insect control methods that contribute increasingly to food and environmental security.

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REVIEW ARTICLE

Chemical Ecology of the Emerald Ash Borer *Agrilus planipennis*

Damon J. Crook · Victor C. Mastro

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Abstract The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a serious invasive pest that has caused devastating mortality of ash trees (*Fraxinus sp*., Oleaceae) since it was first identified in North America in 2002. Shortly after its discovery, surveys were conducted, based on the visual inspection of trees. The shortcomings of visual surveys have led to a critical research need to find an efficient survey method for detecting *A. planipennis* infestations. Here, we present a review of research that has led to the development of effective trapping methods for *A. planipennis*. Studies on the insect's biology and behavior have led to the identification of several potential attractants as well as the design of a visually attractive trap. The ongoing challenge in developing an optimally efficient trapping methodology for *A. planipennis* will involve finding the best combination of variables, such as trap shape, trap color (or other visual properties), trap placement, lure components, as well as the ratios and release rates of those components.

Keywords *Agrilus planipennis* · Coleoptera · Buprestidae · Behavior · Sesquiterpenes · Green leaf volatiles · Contact pheromone

Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a serious invasive pest that has caused devastating mortality of ash trees (*Fraxinus sp*., Oleaceae) since it was first identified near Detroit Michigan, USA, and Windsor Ontario, Canada (Haack et al. 2002). It is not reported as a pest in its native range of China, Korea, Japan, Mongolia, Taiwan, or eastern Russia (Chinese Academy of Sciences, Institute of Zoology 1986; Yu 1992; Haack et al. 2002; Xu 2003; Gao et al. 2004; Wei et al. 2004). Despite intense suppression efforts by American and Canadian authorities, the beetle has increased its range rapidly, mainly due to the movement of logs, firewood, and nursery trees. More information on the introduction of *A. planipennis* into North America is provided in a thorough review by Poland and McCullough (2006). Since 2002 *A. planipennis* has been reported in Ohio and Maryland (2003), Indiana (2004), Illinois (2006), Pennsylvania and West Virginia (2007), and more recently in Virginia, Wisconsin, Missouri, Minnesota, Kentucky, and New York as well as Quebec, Canada (http://www.emeraldashborer.info). Since its introduction, *A. planipennis* has killed tens of millions of ash trees (*Fraxinus* spp.) in North America and Canada (http://www.emeraldashborer.info). North American ash species (in particular green ash *F. pennsylvanica* Marsh., white ash *F. Americana* L. and black ash *F. nigra* Marsh) are highly vulnerable to infestation by *A. planipennis*. When North American species have been planted in Asia (Liu et al. 2003), they have been shown to be highly susceptible to *A. planipennis* when compared to native asian ash species (mainly Chinese ash *F. chinensis* Roxb. and Manchurian ash, *F. mandshurica* Rupr.) (Eyles et al. 2007; Rebek et al. 2008). While Marshall et al. (2009b) reported that *A. planipennis* did not differentiate between different North American ash species in forest's, Anulewicz et al. (2008) found that *A. planipennis* preferred green over white ash, and white over blue ash in urban

D. J. Crook (✉) · V. C. Mastro
Otis Laboratory, USDA APHIS PPQ CPHST,
1398 West Truck Road,
Buzzards Bay, MA 02542-1329, USA
e-mail: Damon.J.Crook@aphis.usda.gov

environments. Laboratory studies also have found preferences among North American ash species (Pureswaran and Poland 2009a).

Studies of *Agrilus* species native to North America (Barter 1957, 1965; Cote and Allen 1980; McCullough and Katovich 2004; Cappaert et al. 2005a) reveal their biology and ecology are similar to those of *A. planipennis*. Larvae of *A. planipennis* feed in the phloem and cambial regions forming S-shaped galleries that disrupt nutrient and water flow, resulting in tree death, 2–3 years after first infestation (Liu et al. 2003). Larvae progress through four instars before overwintering as prepupae in the sapwood or outer bark. Pupation takes place in mid April before adults emerge in May and June through D-shaped exit holes (Bauer et al. 2004; McCullough and Katovich 2004). Adults emerge in May and June (in Michigan) with flight activity lasting until August. Adults feed on the margins of ash leaves throughout their lifetime and require a maturation period of ~1 week before females mate. Females continue to feed for another 5–7 days before they lay individual eggs (50–90) in bark crevices. Eggs hatch within 2 weeks (Bauer et al. 2004; Cappaert et al. 2005a). Adults continue to mate and feed for the remainder of their 3–6 week life span (Poland and McCullough 2006). A one-year life cycle is most common, but a two-year cycle does exist in China in the most northern reaches of its native range (Yu 1992; Marshall et al. 2009a). This 2 year larval development also has been reported in North America (Siegert et al. 2007; Tluczek et al. 2008; McCullough et al. 2009).

Agrilus planipennis—Mating Behavior and Pheromones

Although the genus *Agrilus* (Coleoptera: Buprestidae) includes thousands of species (Jendek 2000), it remains poorly understood, particularly with respect to chemical ecology and mating behavior. Once host trees are located, *Agrilus* beetles presumably find mates via one or more of the following signals: visual, auditory, vibration, tactile, or pheromonal (Fenton 1942; Carlson and Knight 1969; Gwynne and Rentz 1983; Dunn and Potter 1988).

Mating behavior of *A. planipennis* has been the subject of several studies. The primary mate-finding strategy appears to involve active visual searches by males for females (Lelito et al. 2007a, b, 2008; Rodriguez-Saona et al. 2007). Males will hover 0.3–1.0 m above conspecific beetles on ash leaves before rapidly and accurately descending onto them, and they respond similarly to naturally occurring or dead nonspecific's (Lelito et al. 2007b). Male mating behavior also has been tested using 'dummy' beetles that had been washed in dichloromethane (to remove any cuticular contact cues). Lelito et al. (2007b) found that males spent significantly less time attempting to copulate with pinned male or solvent washed female dummies than with unwashed females, indicating that a contact pheromone is utilized by males after the visual landing stage of mate location is completed. A detailed study on the distribution and structure of *A. planipennis* antennal sensilla showed that males had significantly more uniporous gustatory sensilla than females (Crook et al. 2008d), thus supporting the evidence that mate recognition by males involves female released contact cues. Sensory deprivation experiments in the laboratory using male—female pairs demonstrated that if male antennae were covered with paint they took significantly longer to find females and spent less time in copula compared to untreated males (Pureswaran and Poland 2009b). Blocking male eyes with paint did not appear to affect their mate finding capacity when compared to unpainted males, demonstrating that at short range (≤5 cm), olfactory cues were more important than visual ones.

Cuticular hydrocarbons recently were identified as contact pheromones for *A. planipennis* (Lelito et al. 2009; Silk et al. 2009). Lelito et al. (2009) identified 3-methyltricosane as being a potential contact pheromone. This long-chain hydrocarbon was present on the cuticle of mature females (10–12 days old) but found in only trace amounts on the cuticle of males or immature (3-day-old) females. In field assays, male *A. planipennis* spent significantly more time attempting to copulate with dead, pinned females coated with 3-methyltricosane (3 beetle equivalents) than with solvent-washed beetles, although males still spent the most time investigating pinned, dead, unwashed females. Lelito et al. (2009) suggested that the behavioral effect of 3-methyltricosane may be synergized by other compounds secreted onto the cuticular surface. Individual testing of the compound's two enantiomers may determine if chirality is important.

In another analysis of *A. planipennis* cuticular hydrocarbons, Silk et al. (2009) revealed nearly identical chemical profiles in males and females except for one compound, 9-methyl-pentacosane, which was exclusively present in 10–14 day old (sexually mature) females. Reapplication of synthetic 9-methyl-pentacosane to hexane rinsed female 'dummies' (at one female equivalent) restored almost all contact and male copulatory behavior in field bioassays. Time spent by males in contact with 9-methyl-pentacosane treated females and the time spent in attempted copulation was significantly greater than that observed with hexane washed females and not significantly different from unwashed female controls. 9-Methyl-pentacosane is, therefore, used as a contact pheromone component by male *A. planipennis*. This is interesting since 9-methyl-pentacosane also is a contact pheromone component of the cerambycid beetle *Xylotrechus colonus* F. (Ginzel et al. 2003).

Both 3-methyltricosane (Lelito et al. 2009) and 9-methyl-pentacosane (Silk et al. 2009) are more abundant or only present on the cuticle of 10–14 day old females, thus suggesting that their presence is associated with sexual maturation (or more specifically egg maturation). The relationship between insect sexual maturation and increased pheromone production is well documented with other insect species (Dillwith et al. 1983; Blomquist et al. 1987; Adams et al. 1995; Monnin et al. 1998; Schal et al. 2001; Howard and Blomquist 2005).

Olfactometer and behavioral data currently provide no evidence that *A. planipennis* uses long range pheromones (Otis et al. 2005; Rodriguez-Saona et al. 2006; Lelito et al. 2007b, 2008). A volatile, antennally active compound (for males and females), emitted primarily by female *A. planipennis*, was identified as (3*Z*)-dodecen-12-olide (3*Z*-lactone), but its biological activity has yet to be demonstrated (Bartelt et al. 2007; A. Cossé unpublished observations). Silk et al. (personal communication) synthesized both the 3*Z*-lactone and its stereoisomer (3*E*)-dodecen-12-olide (the 3*E*-lactone) and found that GC-EAD responses of *A. planipennis* were more consistent for the 3*E*-lactone. This is intriguing because the 3E-lactone has not yet been identified in male or female aeration samples. Despite the apparent lack of behavioral activity in preliminary field tests, the 3*E*-lactone (but not the 3*Z*-lactone) induced a significant orientation of males in preliminary olfactometer tests (P. Silk, personal communication). Female beetles were unresponsive in olfactometer tests. Further research is warranted but these results do indicate that (3*E*)-dodecen-12-olide (the 3*E*-lactone) could be a short range volatile pheromone for *A. planipennis.* This would support the observations of Pureswaran and Poland (2009b) who suggested that males can identify females at a short range (≤5 cm) before they come into contact with them.

This apparent lack of a useful pheromone for use in trap surveys has forced researchers to examine host volatile and visual stimuli in detail so that potential attractants can be identified and utilized.

Detection of *Agrilus planipennis*

The detection of *A. planipennis* at low population densities is the greatest challenge in being able to monitor and manage the beetle successfully (Marshall et al. 2009b). Without early detection, forest pests such as *A. planipennis* soon become well established, which in turn greatly limits the effectiveness of early control measures that would help limit the pest's movement to other areas (Liebhold et al. 1995; Marshall et al. 2009a, b). Shortly after its discovery in North America, *A. planipennis* surveys were conducted, based on the visual inspection of tree damage, as no traps, lures or other methods were available for operational use (McCullough et al. 2009). The detection of early *A. planipennis* infestations by using visual surveys proved exceedingly difficult for several reasons. Overt visible symptoms of *A. planipennis* infestation are bark cracks, woodpecker attacks, canopy dieback, and epicormic branching, but these are not clearly evident until ash trees are heavily attacked (Poland and McCullough 2006; McCullough et al. 2009). The D-shaped exit holes are visible earlier but are small and tend to occur in the upper canopy during the early stages of attack. The problems and inadequacies of visual surveys led to a research effort to develop a sensitive and efficient survey method for detecting newly established (and delimiting) infestations of *A. planipennis*.

Agrilus planipennis Attraction to Stressed Trees

Before *A. planipennis* was discovered in North America, very little was known about how buprestids locate potential mates and hosts. Buprestid mating appeared to be facilitated by host selection followed by visual, tactile, and possibly auditory cues, as opposed to involving a 'long distance' pheromone (Carlson and Knight 1969; Gwynne and Rentz 1983). Previous studies have shown that both the bronze birch borer (*A. anxius* Gory) and the two-lined chestnut borer (*A. bilineatus* Weber) colonize stressed trees rather than healthy or dead trees (Anderson 1944; Dunbar and Stephens 1976; Cote and Allen 1980; Haack and Benjamin 1982; Dunn 1985; Dunn et al. 1987; Katovich et al. 2001). To investigate stress-mediated attraction in *A. planipennis,* McCullough et al. (2009) compared the number of insects caught on sticky bands that were attached to healthy and stressed ash trees. Trees were stressed by herbicide injection or by mechanically girdling the tree (i.e., removing a 15-cm-wide band of outer bark and phloem, thereby disrupting nutrient translocation). They found a significantly higher number of *A. planipennis* adults on trees that had been stressed. As a result of these findings, girdled trees were used by survey agencies in Michigan, Ohio, and Indiana from 2004 to 2007 (McCullough et al. 2009) and they led to the detection of some previously unknown infestations. McCullough et al. (2009) suggested that girdled trees could also be used as 'trap trees' if they were destroyed after oviposition and before larvae completed development. One problem that limits the use of stressed trees in large-scale monitoring programs is that they must be felled and stripped of bark to determine presence or absence of larvae (Francese et al. 2008; Marshall et al. 2009a). This is labor intensive, destructive, expensive, and time consuming (Cappaert et al. 2005a). Also, suitable detection trees may not be available in areas

of interest (McCullough et al. 2009). Development of a practical trap and effective lure has, therefore, become a research priority for *A. planipennis* detection programs (Poland 2007).

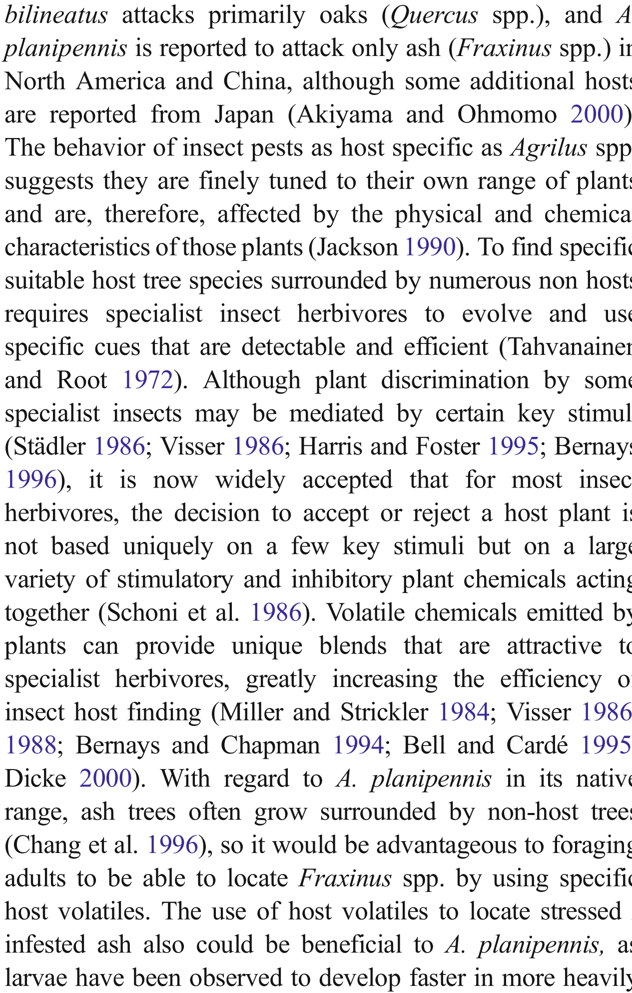
Traps—Design, Color and Placement Considerations

As pointed out by de Groot et al. (2008), an important component of any trap-based monitoring system is the trap itself. Recent studies based on color, visual cues, shape, and trap placement have led to good progress in developing an effective trap and trapping methods for *A. planipennis* (Francese et al. 2005, 2007, 2008; Otis et al. 2005; Crook et al. 2006, 2007, 2008a, b, c, 2009; Lelito et al. 2007a, b, 2008; McCullough et al. 2008; Marshall et al. 2009a, b). The trap currently used for *A. planipennis* survey is a prism-shaped sticky trap made from sheets of purple corrugated plastic (peak wavelengths of 430, 600, and 670 nm; trap described by Francese et al. 2008). This attraction to purple can be explained by recent electroretinogram (ERG) assays by Crook et al. (2009) who showed that while both male and female *A. planipennis* were sensitive to light in the blue (420–430 nm, 460 nm) range of the visible spectrum, females also were sensitive to light in the red (640–650, 670) range. Purple traps, accordingly, have typically caught more females than males (Francese et al. 2008; Crook et al. 2009).

Further ERG work by Crook et al. (2009) showed that *A. planipennis* are also sensitive to light in the green range (540–560 nm). In trapping assays, both dark and light green traps with a peak reflectance at 540 nm caught two to three times as many beetles as purple traps (either painted purple or made from purple plastic) when traps were hung at mid-canopy (~13 m). No significant differences were seen between purple and green traps at 1.5 m. The sex ratio on light and dark green traps, unlike purple, was heavily skewed toward males (Crook et al. 2009). Based on these results, Crook et al. (2009) suggested a green trap (placed at 13 m) for detecting incipient *A. planipennis* populations. Trap placement high in the tree canopy of tall trees, however, is logistically difficult and has not been widely adopted for large scale monitoring programs (Francese et al. 2008). When testing lures for *A. planipennis* for potential use in large scale monitoring programs, factors such as trap color and placement have to be considered, especially when interpreting sex ratio catch data on different treatments.

Host Volatiles and *Agrilus planipennis*

Agrilus buprestids typically have narrow host ranges; for example, *A. anxius* attacks only birch (*Betula* spp.), *A. bilineatus* attacks primarily oaks (*Quercus* spp.), and *A. planipennis* is reported to attack only ash (*Fraxinus* spp.) in North America and China, although some additional hosts are reported from Japan (Akiyama and Ohmomo 2000). The behavior of insect pests as host specific as *Agrilus* spp. suggests they are finely tuned to their own range of plants and are, therefore, affected by the physical and chemical characteristics of those plants (Jackson 1990). To find specific suitable host tree species surrounded by numerous non hosts requires specialist insect herbivores to evolve and use specific cues that are detectable and efficient (Tahvanainen and Root 1972). Although plant discrimination by some specialist insects may be mediated by certain key stimuli (Städler 1986; Visser 1986; Harris and Foster 1995; Bernays 1996), it is now widely accepted that for most insect herbivores, the decision to accept or reject a host plant is not based uniquely on a few key stimuli but on a large variety of stimulatory and inhibitory plant chemicals acting together (Schoni et al. 1986). Volatile chemicals emitted by plants can provide unique blends that are attractive to specialist herbivores, greatly increasing the efficiency of insect host finding (Miller and Strickler 1984; Visser 1986, 1988; Bernays and Chapman 1994; Bell and Cardé 1995; Dicke 2000). With regard to *A. planipennis* in its native range, ash trees often grow surrounded by non-host trees (Chang et al. 1996), so it would be advantageous to foraging adults to be able to locate *Fraxinus* spp. by using specific host volatiles. The use of host volatiles to locate stressed / infested ash also could be beneficial to *A. planipennis,* as larvae have been observed to develop faster in more heavily infested trees (Cappaert et al. 2005b).



Dunn (1985) reported that adult *A. bilineatus* were able to locate oaks within 24 hours of inducing a stress injury, suggesting that 'tree-stress' volatiles had an important role in tree colonization. This is supported by a further study that found that crude bark distillate of stressed oak trees, when used with sticky-banded trees and vane traps, caught significantly more *A. bilineatus* than did water controls (Dunn et al. 1986).

These findings on this related *Agrilus* species, along with the known attractancy of *A. planipennis* to girdled ash trees (Lyons et al. 2009; McCullough et al. 2009), suggested that the study of volatile attractants in stressed trees warranted further investigation.

Host Volatiles—Bark Sesquiterpenes

Crook et al. (2008b) collected headspace volatiles from the bark tissue at 0 and 24 h after manually girdling green ash (*F. pennsylvanica*) using methods similar to those of Zhang et al. (2000). Ratios of bark-emitted monoterpenes and sesquiterpenes were determined for both girdled and non-girdled

trees. Bark samples taken from girdled trees were seen to have elevated sesquiterpene levels. Gas chromatographic-electroantennographic detection (GC-EAD) revealed that six of the elevated compounds consistently elicited antennal responses in both male and female *A. planipennis*. Mated females gave larger and more consistent antennal responses than virgin females or males (D. J. Crook, unpublished observation), suggesting that mated females potentially used these volatile cues when searching for oviposition substrates.

Crook et al. (2008b) identified five of the antennally active compounds as α-cubebene, α-copaene, 7-epi-sesquithujene, *trans*-β-caryophyllene, and α-humulene (α-caryophyllene). The sixth antennally active compound was identified as eremophilene by Cossé et al. (2008). *Trans*-β-caryophyllene, α-humulene, and α-cubebene are systemic, as they have also been identified in green ash foliar volatiles that elicit antennal responses in *A. planipennis* (T. M. Poland, unpublished data).

These sesquiterpenes have been shown to be potentially important in several other plant-insect systems (Minyard et al. 1969; Pearce et al. 1975; Lanier et al. 1977; McKibben et al. 1977; Minks and Van Deventer 1978; Bejer 1979; Flint et al. 1979, 1981; Blight et al. 1980; Kamm and Buttery 1983; Millar et al. 1986; Vrkočová et al. 2000). Sesquiterpenes are notoriously difficult (and expensive) to isolate, identify, and synthesize (Gershenzon 1993), so natural sources were used for lure development and field testing. Crook et al. (2006, 2007, 2008a, b) found two sources of tree oils that contained high amounts of sesquiterpenes. The first, manuka oil, is a steam distillate from the New Zealand manuka tea tree *Leptospermum scoparium* J. R. and G. Forst (Myrtaceae). Manuka oil contains high amounts of four of the antennally active ash-bark sesquiterpenes: α-cubebene (35 g/liter), α-copaene (48 g/liter), *trans*-β-caryophyllene (24 g/liter), and ~3.4 g/liter of α-humulene. Eremophilene also is present in manuka oil in trace amounts (Cossé et al. 2008). Manuka oil lacks the sixth compound, 7-epi-sesquithujene (Crook et al. 2006, 2007, 2008a, b). The second oil, phoebe oil, is a steam distillate from Brazilian walnut, *Phoebe porosa* Mez. (Lauraceae), which grows in the Araucaria forests of Parana and Santa Catharina in Southern Brazil. This oil contains all six of the antennally active volatiles found in green ash in similar high quantities to manuka oil (Cossé et al. 2008).

Crook et al. (2008b) field tested manuka oil lures, using purple 'prism traps' (APHIS, USDA) set at 1.5 m, as they had previously been found to be visually attractive (described by Francese et al. 2008). Three daily release rates of manuka oil (5, 50, and 500 mg) were compared against unbaited controls. All three manuka oil treatments caught significantly more adult *A. planipennis* (both males and females) than unbaited traps, but there was no significant difference in insect catch among the three manuka oil release rates. Another field study that used purple traps placed at 1.5 m height, by Crook et al. (2008b), tested manuka oil (50 mg/day release rate), phoebe oil (50 mg/day release rate), manuka and phoebe oil (25 mg/day release rate each), and an unbaited trap. They found that all oil lure treatments caught significantly more adults than unbaited traps. Phoebe oil (84 mean trap catch) and manuka/phoebe oil (98 mean trap catch) baited traps caught significantly more insects than manuka oil (46 mean trap catch) traps and unbaited traps (32 mean trap catch). No significant differences were seen in trap catch between the two phoebe oil lure baited traps. Crook et al. (2008b) suggested that the increased catch on phoebe lure baited traps was due to the oil containing 7-epi-sesquithujene, which manuka oil lacked.

These findings showed that the antennally active 'ash' sesquiterpenes present in manuka oil and phoebe oil were attractive to *A. planipennis*. It appears that there is a change in the phloem chemistry of ash after girdling that causes a sudden increase in sesquiterpene levels. These levels are detectable and attractive to both male and female adult *A. planipennis*. Attractiveness of these oils to both sexes is not surprising, as both males and females respond to the sesquiterpenes found in girdled ash aerations, Manuka oil and phoebe oil when examined using GC-EAD techniques (Crook et al. 2008b).

Based on the consistent attractancy of manuka oil to *A. planipennis* in 2005–2007 field tests, the Emerald Ash Borer National Survey program (United States Department of Agriculture, Animal and Plant Health Inspection Service) implemented the use of purple prism traps baited with manuka oil for monitoring purposes in 2008 (Crook et al. 2009). Baited traps detected ten new infestations in 2008, including a new state record in Missouri (http://www.emeraldashborer.info). In 2009, the survey program used purple prism traps baited with a blend of manuka : phoebe oil (ratio 80:20, 50 mg/day nominal release rate). These traps detected 15 new infestations in counties within Kentucky, Pennsylvania, Ohio, Wisconsin, and West Virginia.

A recent study by Marshall et al. (2009b) quantified the capture rates and detection ability of manuka oil and phoebe oil baited traps (green and purple prism traps), along with girdled trap trees, in areas that had high and low population levels of *A. planipennis* (more typical of survey programs). In low beetle population sites, manuka oil baited purple traps, hung at 6 m, caught significantly more *A. planipennis* adults per day than girdled trap trees and green traps. Purple traps baited with phoebe oil or manuka oil had 70–88% detection rates (i.e., at least one adult caught) compared to 47% for girdled trap tree treatments. Adult catch on traps or trap trees was not compared to larval counts in trap trees (that had been peeled later, after the

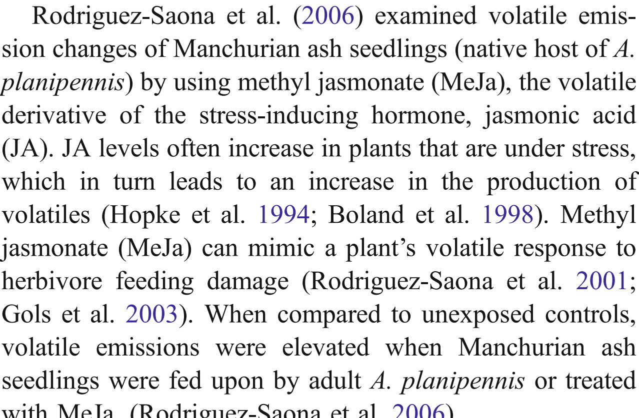
flight season). Marshall et al. (2009b) did mention that some sites with zero *A. planipennis* captured on traps had larvae in trap trees when examined later on. For detection surveys, it is recommended that girdled trees be peeled and examined for larvae because it increases their rate of detection.

Marshall et al. (2009b) also noted that there was a lack of significant differences with regard to traps associated with trees of different vigor ratings. This suggested for the first time that a baited trap may not be impacted by the ash tree from which it is hung. The lure and trap combination is, therefore, more important than the health and vigor of the tree in which it's hanging. Based on this study, baited purple traps (at 6 m) are currently, the most effective and practical method for detecting *A. planipennis* in newly established infestation areas.

There have been attempts to improve efficacy of the bark-oil distillate lures. One issue with the use of manuka and phoebe oil lures is that both oils are mixtures that include ~30 sesquiterpenes and triketones, some of which could potentially have negative effects on lure attractancy. The most recent research has concentrated on eremophilene and 7-epi-sesquithujene, which appear to be the most antennally active volatiles in aerations of both green ash (Crook et al. 2008b) and white ash aeration samples (Cossé et al. 2008). Unfortunately, neither of these biologically active sesquiterpenes are commercially available, and synthesis of 7-epi-sesquithujene is not practical. Eremophilene can, however, be obtained in fairly large, pure amounts through the chemical conversion of the natural ketone, eremophilone, which is commercially available in Buddha wood oil (*Eremophila mitchelli* Benth., *Myoporaceae*) as well as phoebe oil (Cossé et al. 2008). Recent field tests of lures that contain manuka oil and phoebe oil fractions (containing concentrated EAD active components) have not significantly improved trap catch when compared to standard phoebe oil based lures (D. J. Crook and A. Cossé unpublished results).

Host Volatiles—Ash Seedlings and Foliage

Green leaf volatiles (GLVs) are well-studied compounds released by deciduous trees. Their levels can become elevated due to stress from a variety of factors that can include physical damage and insect feeding (Ruther et al. 2000; Zhang and Schlyter 2004; Cossé et al. 2006; Rodriguez-Saona et al. 2006). GLVs can act as kairomones, pheromonal synergists, and host specific attractants for a number of coleopteran species (Dickens et al. 1990; Dickens 2000; Ruther et al. 2000, 2002; Reinecke et al. 2002, 2006; Ruther 2004; Cossé et al. 2006; Fernandez et al. 2007).



Rodriguez-Saona et al. (2006) examined volatile emission changes of Manchurian ash seedlings (native host of *A. planipennis*) by using methyl jasmonate (MeJa), the volatile derivative of the stress-inducing hormone, jasmonic acid (JA). JA levels often increase in plants that are under stress, which in turn leads to an increase in the production of volatiles (Hopke et al. 1994; Boland et al. 1998). Methyl jasmonate (MeJa) can mimic a plant's volatile response to herbivore feeding damage (Rodriguez-Saona et al. 2001; Gols et al. 2003). When compared to unexposed controls, volatile emissions were elevated when Manchurian ash seedlings were fed upon by adult *A. planipennis* or treated with MeJa. (Rodriguez-Saona et al. 2006).

Feeding by *A. planipennis* adults on Manchurian ash seedlings increased daytime volatile emissions by 6.8 times, which was similar to an increase of 6.3 times for MeJa treated seedlings. Olfactometer assays found that volatiles from beetle-damaged or MeJa-exposed ash were attractive to virgin female adult *A. planipennis*, suggesting that females were able to respond broadly to induced volatile emissions independent of the triggering factor. Mated female adults were not tested. Male *A. planipennis* did not respond significantly to insect damaged foliage or MeJa treated volatiles in olfactometer assays (Rodriguez-Saona et al. 2006).

Manchurian ash (considered a 'resistant' ash species that has co-evolved with *A. planipennis* in its native range) has been shown to release higher amounts of volatiles when compared to the highly susceptible North American green ash (Pureswaran and Poland 2009a). It is possible that low levels of volatile release indicate lower host resistance, and also that *A. planipennis* can detect that difference. In general, little is known about the underlying mechanisms that govern the behavior of *A. planipennis* with respect to ash tree resistance (Eyles et al. 2007; Rebek et al. 2008; Chen and Poland 2009).

Rodriguez-Saona et al. (2006) found at least 16 compounds from Manchurian ash that were antennally active to both male and female *A. planipennis*. GLVs elicited the strongest and most consistent antennal responses. Female *A. planipennis* showed a stronger electroantennogram (EAG) response to linalool than males. Male responses were greater to hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, 3-methyl-butylaldoxime, 2-methyl-butylaldoxime, and hexyl acetate. Of these, (*Z*)-3-hexenol elicited the largest EAG response overall.

In another study by de Groot et al. (2008), eight GLV's were detected in both white and green ash foliar aeration extracts. These were (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, hexanol, (*Z*)-3-hexenyl acetate, hexyl acetate, and hexanal. Of these, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate made up over 80% of the total GLV volatiles emitted by both ash species. GC-EAD test results

were similar to those of Rodriguez-Saona et al. (2006) in that the GLV's were antennally active to both males and females. Males produced larger EAD responses to all the alcohols and aldehydes than females. Females were more responsive than males to hexyl acetate. There was no difference in responses when both sexes were tested with (*Z*)-3-hexenyl acetate. de Groot et al. (2008) also showed that (*Z*)-3-hexenol was by far the most stimulatory volatile among the GLV's tested on males. Male responses to (*Z*)-3-hexenol were two-fold greater than to (*E*)-2-hexenal or (*E*)-2-hexenol at the same test concentration of 7 ng/μl. For females, (*Z*)-3-hexenol was no more stimulating than other GLV's tested.

Between 2004 and 2006, various lure combinations and dosages of these potential GLV's were tested by de Groot et al. (2008) in six field experiments. Males consistently responded to (*Z*)-3-hexenol in four experiments. In an experiment that tested (*Z*)-3-hexenol at two dosages (48 mg/day and 330 mg/day), males showed a positive response to the lower release rate; however, in two other non-dose studies, the 330 mg/day lure was an effective male attractant. Further field tests to establish the most attractive GLV release rate for *A. planipennis* were recommended (de Groot et al. 2008). GC-EAD results by de Groot et al. (2008) did however suggest that male *A. planipennis* were more sensitive to low concentrations of (*Z*)-3-hexenol (<1 ng) and the other GLV's than to higher ones. Hexanol and (*E*)-2-hexenol were not attractive to *A. planipennis* in field tests, and they did not enhance the attractiveness of (*Z*)-3-hexenol. The aldehydes hexanal, (*E*)-2-hexenal, and nonanal were not attractive to male or female *A. planipennis* in field tests despite being active in GC-EAD assays. The development of a GLV lure for *A. planipennis* obviously is a difficult, time consuming, and challenging task when there are numerous GC-EAD active GLV's to field test (at different release rates and in different blends). Of the GLV's tested to date, (*Z*)-3-hexenol, appears to be the most attractive to *A. planipennis*, particularly males, which correlates well to the strong antennal responses seen in the lab (Rodriguez-Saona et al. 2006; de Groot et al. 2008). de Groot et al. (2008) suggested (*Z*)-3-hexenol may serve as an important cue for finding females on foliage in the canopy of ash trees. Interestingly, (*Z*)-3-hexenol also is reported to be a kairomone for chafers (Genus *Melolontha*) in conjunction with a female pheromone (Ruther et al. 2000, 2002; Ruther 2004).

Several field studies have tested *A. planipennis* attraction to GLV based lures with manuka oil lures. McCullough et al. (2008) used a purple "double-decker trap" (a 10 foot tall PVC pipe with two purple prism traps attached at 1.8 and 3 m) to test various lure treatments. These traps were tested in full sunlight away from the edge of ash woodlots, which likely reduced competition from volatiles of nearby ash trees. A four-component leaf-blend lure (*Z*)-3-hexanol, (*E*)-2-hexanol, (*E*)-2-hexanal, and hexanal) developed by Poland et al. (2006) was tested with and without manuka oil lures. Their results showed that the traps baited with the GLV blend or the GLV blend + manuka oil lures caught significantly more *A. planipennis* than manuka oil baited traps. In addition, double-decker traps baited with the GLV blend + manuka oil were able to attract and intercept dispersing *A. planipennis* at least 300 m away from very lightly infested ash trees.

Crook et al. (2008b) tested the same four GLV components used by McCullough et al. (2008) with and without manuka oil lures (50 mg per day release) on individual purple traps at 1.5 m and 13 m heights, along the edges of infested ash stands. A GLV blend was released from individual bubble cap devices (daily release rates were (*Z*)-3-hexenol=3.8 mg, (*E*)-2-hexenol=3.8 mg, (*E*)-2-hexenal=13 mg, and hexanal=13 mg). Unbaited purple prism traps were tested along with three lure treatments (manuka oil + GLV blend, manuka oil alone, GLV blend alone). Overall, traps baited with manuka oil + GLV blend caught significantly more beetles than traps baited with the GLV blend or unbaited traps, but responses to the lures differed somewhat with trap height. When trap data were examined separately by trap height, at 13 m, manuka oil baited traps, with or without GLV lure, caught significantly more beetles than unbaited or GLV-baited traps, and the GLV lure did not measurably enhance effectiveness of the manuka lure. At 1.5 m, traps baited with manuka-oil or GLV caught significantly more *A. planipennis* than unbaited traps but were not significantly different when compared to each other; however, the manuka oil + GLV-baited traps caught significantly more *A. planipennis* than any other treatment. These results showed that the four-component GLV lure could enhance attraction to manuka oil, but its effectiveness appeared to be dependent on trap height. Effectiveness of the GLV lure at 13 m may have been compromised by volatiles from the surrounding ash foliage. Overall, traps caught significantly more beetles at 13 m than at 1.5 m, indicating that there is more *A. planipennis* activity higher in the ash canopy. Similarly, other studies have found that greater numbers of *A. planipennis*, as well as other buprestids, are caught in the canopy as opposed to near the ground (Francese et al. 2007, 2008; Wermelinger et al. 2007) along with observations on beetle behavior in the field (Lance et al. 2007; Lelito et al. 2007a, b; Rodriguez-Saona et al. 2007). Correlation between trap capture and the feeding, breeding, and dispersal habits of other beetle species is not uncommon (Atkinson et al. 1988; Safranyik et al. 2000).

Based on the most recent developments in lures (Crook et al. 2008b; de Groot et al. 2008), trap color (Crook et al. 2009), and trap placement (Francese et al. 2008), Grant et

al. (2009) evaluated (*Z*)-3-hexenol and manuka oil lures in two separate field trials. Phoebe oil based lures were not tested with (*Z*)-3-hexenol. Their first field trial tested lures on purple traps placed at 1.5 m above the ground; the second tested lures on light green traps hung on the lower canopy of small trees (approximately 3 m above the ground). The first test included two other GLV's, (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate, which had not been field tested previously (de Groot et al. 2008).

On purple traps hung at 1.5 m, (*Z*)-3-hexenol (7.6 mg/day) caught significantly more male beetles than females. Manuka-oil (50 mg/day) baited traps attracted equal numbers of males and females. The addition of (*Z*)-3-hexenal or (*Z*)-3-hexenyl acetate did not enhance purple trap catch in either binary or tertiary mixtures.

On green traps, 7.6-mg and 80-mg daily release rates of (*Z*)-3-hexenol caught significantly more males than females, and more males than the unbaited controls or manuka-oil baited traps. Female counts were similar for all treatments. A combination of the manuka oil and (*Z*)-3-hexenol did not enhance or reduce trap catch when compared to traps baited with just (*Z*)-3-hexenol.

Overall, (*Z*)-3-hexenol produced measurable increases in capture of male *A. planipennis* on sticky traps, and that effect was independent of trap height or color (Grant et al. 2009). Both low and high releases of (Z)-3-hexenol (7.6 and 80 mg) were equally attractive.

The studies by Crook et al. (2008b) and Grant et al. (2009) indicate that bark-released sesquiterpenes and GLV's act independently and affect males and females differently. This most recent study by Grant et al. (2009) supports findings by de Groot et al. (2008) that (*Z*)-3-hexenol does appear to have a role in canopy and/or mate finding by males. In comparison, sesquiterpene emissions from bark tissue may indicate the presence of a stressed ash tree and appear to attract both sexes. GC-EAD responses also suggest males are particularly sensitive to (Z)-3-hexenol (Rodriguez-Saona et al. 2006; de Groot et al. 2008), whereas mated females are more sensitive than males to sesquiterpenes (Crook et al. 2008b). Sesquiterpenes could have an important role for mated females when ovipositing on ash bark. More females than males are usually seen on the bark tissue of ash trees (Lelito et al. 2007b), including freshly girdled ash trees (D. J. Crook unpublished observations).

The one disadvantage of essential oils such as Manuka oil in monitoring programs (which require a standardized bait) is that the chemical composition can vary depending on the geographic region, cultivar, and growing conditions of the extracted plant material (Porter and Wilkins 1999). Despite this, manuka oil and phoebe oil are recommended as an efficient and cost effective attractant for the redbay ambrosia beetle, *Xyleborus glabratus* as they contain high levels of sesquiterpenes found in redbay wood, *Persea borbonia* L. Spreng (Hanula and Sullivan 2008). In comparison to essential oils, (Z)-3-hexenol and other GLV's, are single components that are easily synthesized, relatively inexpensive, and available commercially (Grant et al. 2009).

Final Comments

Despite its continued spread throughout North American ash resources, the known areas of *A. planipennis* infestation have been expanded due to the improvement of detection methods. Effective detection of adults (rather than larvae) now allows for the earlier planning and implementation of silvicultural management strategies by state, provincial and federal, regulatory, and natural resource agencies. This, working in conjunction with public outreach and education activities, will help to delimit and slow the spread of this devastating invasive pest in North America.

Effectiveness of monitoring and detection tools for *A. planipennis* will continue to improve as we learn more about the insect's behavior and biology. The ongoing challenge in developing an optimally efficient trapping methodology for *A. planipennis* will involve finding the best combination of variables such as trap shape, trap color (or other visual properties), trap placement, lure components, as well as the ratios and release rates of those components.

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REVIEW ARTICLE

Chemical Ecology of Animal and Human Pathogen Vectors in a Changing Global Climate

John A. Pickett · Michael A. Birkett · Sarah Y. Dewhirst · James G. Logan · Maurice O. Omolo · Baldwyn Torto · Julien Pelletier · Zainulabeuddin Syed · Walter S. Leal

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Abstract Infectious diseases affecting livestock and human health that involve vector-borne pathogens are a global problem, unrestricted by borders or boundaries, which may be exacerbated by changing global climate. Thus, the availability of effective tools for control of pathogen vectors is of the utmost importance. The aim of this article is to review, selectively, current knowledge of the chemical ecology of pathogen vectors that affect livestock and human health in the developed and developing world, based on key note lectures presented in a symposium on "The Chemical Ecology of Disease Vectors" at the 25th Annual ISCE meeting in Neuchatel, Switzerland. The focus is on the deployment of semiochemicals for monitoring and control strategies, and discusses briefly future directions that such research should proceed along, bearing in mind the environmental challenges associated with climate change that we will face during the 21st century.

Keywords Livestock · Human · Pathogen vector · Semiochemical · Climate change

J. A. Pickett (✉) · M. A. Birkett · S. Y. Dewhirst · J. G. Logan
Centre for Sustainable Pest and Disease Management,
Biological Chemistry Department, Rothamsted Research,
Harpenden, Hertfordshire AL5 2JQ, UK
e-mail: john.pickett@bbsrc.ac.uk

M. O. Omolo · B. Torto
Behavioural and Chemical Ecology Department,
International Centre of Insect Physiology and Ecology (icipe),
P.O. Box 30772-00100, Nairobi, Kenya

J. Pelletier · Z. Syed · W. S. Leal
Honorary Maeda-Duffey Laboratory, Department of Entomology,
University of California Davis,
Davis, CA 95616, USA

Introduction

Infectious diseases affecting livestock and human health that involve vector-borne pathogens are a global problem, unrestricted by borders or boundaries, which may be exacerbated by changing global climate. The global burden of disease through the impact of pathogens and other infectious organisms, including those transmitted by vectors, can be measured using a number of parameters. For farm animals, this can be measured directly as a reduction in the amount of available food (FAO 2005). This can contribute directly to food insecurity and poverty on a global scale. For human populations, the global burden of disease can be measured in disability adjusted life years (DALYs). Approximately 40% of all DALYs lost can be attributed specifically to vector-borne diseases, along with other major diseases including acute respiratory infections, HIV/AIDS, and TB (WHO 2000). In view of these startling figures, the availability of a range of tools for control of vectors of pathogens is of the utmost importance. The aim of this article is to review, selectively, current knowledge of the chemical ecology of vectors of pathogens that affect livestock and human health in developed and developing countries, based on key note lectures presented in a symposium on "The Chemical Ecology of Disease Vectors" at the 25th Annual ISCE meeting in Neuchatel, Switzerland, with a view to the deployment of semiochemicals for monitoring and control strategies, and to discuss briefly future directions that such research should proceed along, bearing in mind the environmental challenges associated with climate change that we will face during the 21st century.

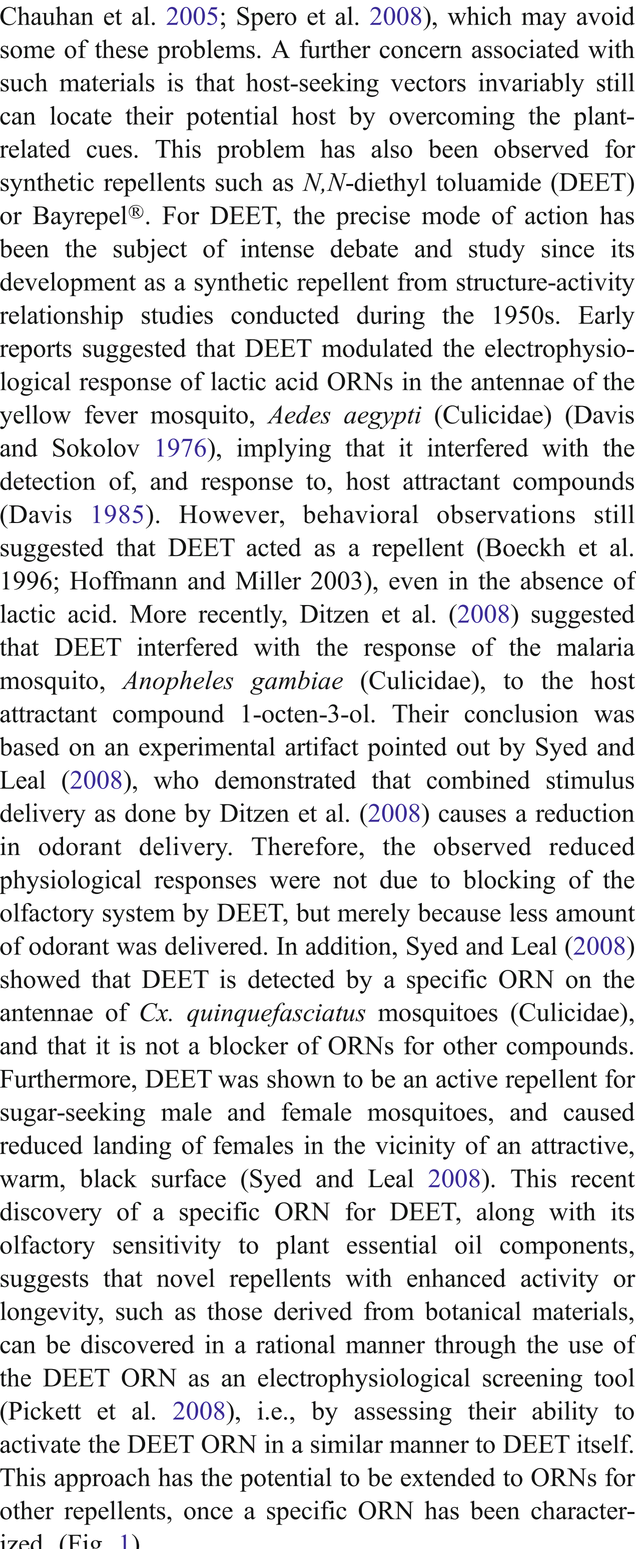
Compounds that act as broad-spectrum toxicants, i.e., insecticides, started to provide the main intervention against pathogen vectors during the latter half of the 20th century, and still do so. These include the synthetic pyrethroid

insecticides, which were largely invented during the 1960s and 1970s by Michael Elliott and his colleagues at Rothamsted (Elliott et al. 1973). However, due to a number of factors, such as the development of insecticide resistance, their perceived negative environmental impact upon human health and the environment, and the cost of maintaining registration of insecticides for pest control, such materials may no longer be acceptable for control of pathogen vectors. In spite of this, the insect nervous system still remains a major target for insect control. The emphasis now, however, relates to non-toxic mechanisms for interfering with pest behavior and development, e.g., host and mate-seeking behavior and oviposition site selection. These are mediated predominantly by the detection of small, lipophilic molecules (semiochemicals) that are detected by specialized olfactory receptor neurons (ORNs) located either on the antennae or on the maxillary palps (Pickett et al. 2009). The potential for manipulating the behavior of vectors of pathogens in host, mate, and oviposition site selection *via* olfactory detection of semiochemicals presents opportunities for their control, *via* the development of novel repellents and attractants, provided they can be deployed through an integrated management strategy, e.g., the push-pull strategy, which has been used highly successfully for pest management in some arable crop systems(Cook et al. 2007; Hassanali et al. 2008).

Hypotheses for Developing Novel Repellents for Pathogen Vectors

The ability to manipulate pathogen vector behavior can be exploited by developing repellents based on hypotheses that relate to evolution of repellency. These fall into three classes: (1) botanicals, (2) non-host species, and (3) host-derived repellents.

Botanicals In conveying a strong plant cue, botanicals interfere with host location by haematophagous (blood-sucking) and biting flies and other arthropods. Although botanically-derived materials such as citronella oil, *Eucalyptus* spp. oils containing *p*-menthane-3,8-diol and eucamalol, and gum resins from members of the Burseraceae have been investigated for use as repellents for human or livestock protection (Nishimura and Satoh 1999; Peterson and Coats 2001; Birkett et al. 2008), the active components are highly volatile, can be readily lost, and therefore lose efficacy at a rapid rate (Lindsay et al. 1996; Chou et al. 1997). Furthermore, these agents also can cause dermatitic problems, so repeated application is not a viable option. However, iridoid nepetalactones produced by *Nepeta* spp. plants (Lamiaceae), and structurally-related compounds, have been investigated as repellents (Bernier et al. 2005;



Chauhan et al. 2005; Spero et al. 2008), which may avoid some of these problems. A further concern associated with such materials is that host-seeking vectors invariably still can locate their potential host by overcoming the plant-related cues. This problem has also been observed for synthetic repellents such as *N,N*-diethyl toluamide (DEET) or Bayrepel®. For DEET, the precise mode of action has been the subject of intense debate and study since its development as a synthetic repellent from structure-activity relationship studies conducted during the 1950s. Early reports suggested that DEET modulated the electrophysiological response of lactic acid ORNs in the antennae of the yellow fever mosquito, *Aedes aegypti* (Culicidae) (Davis and Sokolov 1976), implying that it interfered with the detection of, and response to, host attractant compounds (Davis 1985). However, behavioral observations still suggested that DEET acted as a repellent (Boeckh et al. 1996; Hoffmann and Miller 2003), even in the absence of lactic acid. More recently, Ditzen et al. (2008) suggested that DEET interfered with the response of the malaria mosquito, *Anopheles gambiae* (Culicidae), to the host attractant compound 1-octen-3-ol. Their conclusion was based on an experimental artifact pointed out by Syed and Leal (2008), who demonstrated that combined stimulus delivery as done by Ditzen et al. (2008) causes a reduction in odorant delivery. Therefore, the observed reduced physiological responses were not due to blocking of the olfactory system by DEET, but merely because less amount of odorant was delivered. In addition, Syed and Leal (2008) showed that DEET is detected by a specific ORN on the antennae of *Cx. quinquefasciatus* mosquitoes (Culicidae), and that it is not a blocker of ORNs for other compounds. Furthermore, DEET was shown to be an active repellent for sugar-seeking male and female mosquitoes, and caused reduced landing of females in the vicinity of an attractive, warm, black surface (Syed and Leal 2008). This recent discovery of a specific ORN for DEET, along with its olfactory sensitivity to plant essential oil components, suggests that novel repellents with enhanced activity or longevity, such as those derived from botanical materials, can be discovered in a rational manner through the use of the DEET ORN as an electrophysiological screening tool (Pickett et al. 2008), i.e., by assessing their ability to activate the DEET ORN in a similar manner to DEET itself. This approach has the potential to be extended to ORNs for other repellents, once a specific ORN has been characterized. (Fig. 1).

Non-host Species The response of vectors to compounds from related non-host species is considered more adaptively valuable in their behavioral ecology than the response to botanically-derived repellents. Although there are plenty of anecdotal data to support this, scientific evidence is scarce.

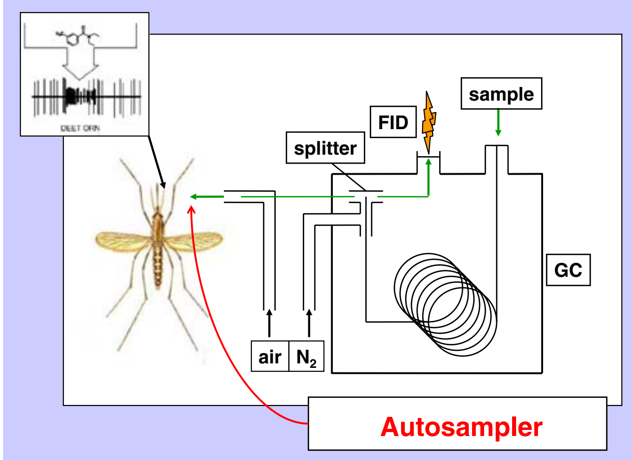


Fig. 1 Active compounds in samples from naturally repellent sources can be identified by gas chromatography (GC)-coupled electrophysiology. By replacing the GC with an autosampler and using a single olfactory neuron (e.g. one which responds to a known plant repellent and also responds to a synthetic repellent such as DEET or a repellent insecticide) compound libraries could be screened for new types of repellent

Evidence is provided, however, from the Morsitans group of tsetse flies, *Glossina* spp. (Glossinidae) that transmit trypanosomiasis (nagana) in cattle (Bovidae). Here, elegant chemical ecological studies have shown that the vertebrate non-host waterbuck, *Kobus defassa* (Bovidae), produces potent non-host repellents for this group of flies (see section below on Afro-tropical vectors). The hypothesis that underpins this work has allowed development of repellents in aquatic ecosystems, for example against the salmon louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae), a major arthropod pest that affects farmed and wild Atlantic salmon, *Salmo salar*, (Salmonidae) populations (Bailey et al. 2006; Mordue (Luntz) and Birkett 2009). It was shown that the non-host fish, the turbot, *Scophthalmus maximus* (Scophthalmidae), produces two compounds, 4-methylquinazoline and 2-aminoacetophenone, which when added to salmon (host)-conditioned seawater, interfere with *L. salmonis* host-seeking behavior (Bailey et al. 2006). Although unclear in this scenario, certainly for Dipterous flies, it is still possible for hosts to be detected in the presence of non-host species. For example, human beings are located easily by anthropophilic mosquitoes such as *An. gambiae*, even when surrounded by cattle (Costantini et al. 2001).

Host Derived Repellents Individuals within an animal population can be extremely unattractive, even to arthropods highly adapted to those species. Similar to the situation for related non-host species described above, there have been plenty of anecdotal accounts to support this hypothesis, but again only recently has evidence been provided on a scientific basis, specifically for Diptera. For dairy cattle, the loading of nuisance and pathogen-vectoring cattle flies (Muscidae) is uneven across a herd of same-breed individual heifers, *Bos taurus* (Jensen et al. 2004; Oyarzun et al. 2009), with the hypothesis being that differential fly-loads are mediated by volatile semiochemicals. Subsequent studies have shown that reduced fly-loads are due to the enhanced production and release of 6-methyl-5-hepten-2-one (MHO), and that addition of slow-release formulations that release this compound to “attractive” cattle could reduce fly loads (Birkett et al. 2004). The presence of “inappropriate” host signaling has been investigated in human beings. Field studies using odor-baited entry traps (OBETs) have shown that the addition of human-specific compounds (*E*)-, (*Z*)-3-methyl-2-hexenoic acid and 7-octenoic acid, major components of human axillary sweat, significantly reduce trap catches of *Anopheles gambiae* s.l. (Costantini et al. 2001). The differential attractiveness of human beings to mosquitoes (Culicidae) and biting midges (Ceratopogonidae) has been studied extensively (Logan et al. 2008, 2009). For the yellow fever mosquito, *Aedes aegypti*, and the Scottish biting midge, *Culicoides impunctatus*, differential attraction has been demonstrated and shown to be due to the presence of enhanced “inappropriate” host signaling, mainly *via* MHO, geranylacetone, octanal, nonanal, and decanal. The ability to reduce upwind flight activity of *Ae. aegypti* by using nanogram levels of compounds is indicative of how such signaling is adaptively valuable in the host-seeking ecology of this mosquito species (Logan et al. 2008, 2009).

Host-Derived Attractants and Attractant Pheromones

For the control of pathogen vectors, host-derived attractants and attractant pheromones can be exploited further by combined use of trapping with repellents as part of a push-pull strategy (Cook et al. 2007; Logan and Birkett 2007; Hassanali et al. 2008). Many pathogen vector species respond to attractants that are derived from host excretory products, such as urine, dung, exhaled breath, and skin secretions, including CO_2, lactic acid, ammonia, acetone, and fatty acids (Logan and Birkett 2007). Studies on the Morsitans group of tsetse flies, *Glossina* spp., (Glossinidae), have identified kairomones from cow odors (Hall et al. 1984) and urine (Owaga et al. 1988). These have been used in baited traps and targets for effective suppression of *Glossina* spp. populations (e.g., POCA; 3-n-Propylphenol: 1-Octen-3-ol: *p*-Cresol: Acetone) (Vale et al. 1988; Brightwell et al. 1991). However, such blends appear to be partially attractive, compared to natural host (cattle) odor, implying that other attractants need to be identified in order to provide full activity.

Pheromones, which although not always directly related to the vectoring component of the pathogen vector life-cycle, are highly species-specific, and, therefore, offer a potent means of vector control, through their deployment in trapping systems. Examples of pheromones for vectors are rare, but *Culex* spp. mosquitoes that are responsible for the transmission of West Nile Virus along with other viruses, and filarial nematodes, utilize an oviposition pheromone, (5*R*,6*S*)-6-acetoxy-5-hexadecanolide, to assess the suitability of an oviposition site (Laurence and Pickett 1982, 1985). This pheromone, which is released from the apical droplets of egg rafts, has been tested successfully in many field trials in several countries in afflicted regions (Otieno et al. 1988; Mboera et al. 2000a, b), and now is commercially available for trapping systems. The pheromone can be deployed effectively if used in conjunction with environmentally benign larvicides, such as the insect growth regulator pyriproxyfen or larvae-specific pathogens such as the fungus *Lagenidium giganteum* Couch (Pickett and Woodcock 1996). Use of the pheromone in such circumstances is tempered by three factors.

First, optimal activity requires use in conjunction with site-derived oviposition cues such as 3-methylindole (skatole), which is derived from stimuli such as decaying organic material or pit latrine water (Mordue (Luntz) et al. 1992; Blackwell et al. 1993; Mboera et al. 2000a), or other compounds such as trimethylamine and nonanal, which also are generated by decaying food material (Leal et al. 2008).

Second, *Culex* spp. mosquitoes habituate to local oviposition site cues. This was illustrated by the adaptation of mosquitoes based at the London School of Hygiene and Tropical Medicine to local, i.e., London, tap water (Pickett and Woodcock 1996). Thus, it appears that local oviposition cues need to be identified prior to their local application. While this may seem initially to be technically challenging, the development of new analytical techniques, e.g., stir bar sorptive extraction (SBSE), offers opportunities for rapid identification of further oviposition cues (Carson et al. 2009).

Third, the production of pheromone for their deployment, while being affordable in developed countries, represents a financial and technical challenge for local production. Thus, higher plants offer alternative, cheap, and renewable resources for production of pathogen vector pheromones in afflicted countries. These include production of the *Culex* spp. oviposition pheromone from the seed oil of *Kochia scoparia* (Chenopodiaceae) (Olagbemiro et al. 1999, 2004), and the sandfly, *Lutzomyia longipalpis*, pheromone, (*S*)-9-methylgermacrene-B, from the essential oil of *Geranium macrorrhizium* (Geraniaceae) (Hooper et al. 2006).

Host Shifts in Pathogen Vectors

Studies on the semiochemicals produced by human and birds hosts of *Culex* spp. mosquitoes revealed that one compound, nonanal, is a significant component of human, pigeon (*Columbia livia*), and chicken (*Gallus gallus*) odor (Syed and Leal 2009). Furthermore, this compound, along with others in host odor, is detected with extreme sensitivity by ORNs on the antennae of *Cx. quinquefasciatus*. Nonanal was detected by a large array of sensilla, and was by far the most potent stimulus. It has been suggested that *Cx. quinquefasciatus* can detect humans and birds, thereby facilitating host shifts, and thus, transmission of West Nile virus among human populations (Syed and Leal 2009). In field experiments, a combination of nonanal and CO_2 acts in a synergistic manner, leading to significantly higher catches of *Culex* mosquitoes in binary traps, compared to individual traps (Syed and Leal 2009).

Molecular Recognition Processes Underlying Pathogen Vector Behavior

The mechanisms that underlie olfactory reception of semiochemicals by insects currently are receiving much attention, with a view to providing new opportunities for pest control, including pathogen vectors. These are primarily targeted at two levels, odorant-binding proteins (OBPs) and olfactory receptors (ORs).

Odorant-binding proteins have been proposed as key agents involved in the movement of semiochemicals across the antennal sensillum lymph towards ORs (Pickett et al. 2009). Genes and cDNAs encoding the OBPs of a number of insect species have been cloned, including pathogen vectors, and recombinant OBPs that have been generated *via* suitable expression systems. The first OBP to be isolated from a mosquito was from *Cx. quinquefasciatus* (Ishida et al. 2002). Subsequent immunohistochemistry studies have shown that this OBP, termed CquiOBP1, is expressed in trichoid sensilla on the antennae that house an ORN sensitive to the oviposition pheromone, (5*R*,6*S*)-6-acetoxy-5-hexadecanolide (Leal et al. 2008). Furthermore, CquiOBP1 exists in monomeric and dimeric forms, with the monomer binding the oviposition pheromone in a pH-dependent manner, with a change in pH resulting in an apparent loss of binding due to changes in secondary structure (Leal et al. 2008). Interestingly, binding studies with enantiomers of the pheromone have shown that the non-naturally occurring antipode have higher affinity than the natural stereoisomer. Since the initial isolation of CquiOBP1, similar OBPs have been isolated from other *Culex* spp., including *Cx. tarsalis* (Ishida et al. 2003), *Cx. pipiens* and

Cx. molestus (Leal et al. 2008). cDNAs for putative OBPs have been isolated from *An. gambiae* (Biessmann et al. 2002).

The availability of genome sequences for mosquitoes has enabled the identification of genes that encode putative OBPs *via* genome and EST database searches. Their expression is detected in antennae by using quantitative real time PCR, and their production by using recombinant technology. These include genes for so-called "Plus-C" OBPs in *An. gambiae* (Zhou et al. 2004), genes for OBPs in anthrophilic *An. gambiae* s.s. and zoophilic *An. arabiensis* (Li et al. 2005), *Ae. aegypti*, and *An. gambiae* (Zhou et al. 2008), and for *Cx. quinquefasciatus* (Pelletier and Leal. 2009). Despite the discovery of putative OBPs for these pathogen vectors and other insects, the molecular recognition processes that involve OBP binding of semiochemicals are yet to be elucidated, Thus, experimental approaches are required that allow measurement of binding and specificity. To this end, several techniques have been developed to measure the non-covalent interaction between OBPs and semiochemicals. These include: (1) fluorescence binding assays, which have been used most recently to study the binding properties of OBPs from *Ae. aegypti* (Li et al. 2008) and the silkworm moth, *Bombyx mori* (Zhou et al. 2009), (2) cold-binding assays, which have been used to study the interaction between native and mutated pheromone-binding proteins (PBPs) from *B. mori*, and the pheromone bombykol (Leal et al. 2005), and (3) electrospray ionization mass spectrometry (ESI-MS), which was used to study the interaction between the *B. mori* pheromone-binding protein BmorPBP1 and bombykol (Oldham et al. 2000). Recently, high-throughput ESI-MS analysis of BmorPBP1 incubated with its' pheromone components (bombykol and bombykal) and analogues has been developed (Hooper et al. 2009). The availability of a high-throughput assay would allow *in vitro* screening of OBPs against compounds identified as pathogen vector semiochemicals, and would provide a means of searching libraries of compounds for new semiochemicals with enhanced activity. However, despite evidence that OBPs appear to be abundant in pathogen vectors and have the potential to be used in their control, functional evidence for the role of OBPs in mediating odor selectivity is still quite sparse, and *in vivo* evidence of their role is required. Studies on insect ORNs (see below) by Carlson and co-workers have apparently shown that ORNs are not matched to their cognate OBPs. This led to a suggestion that OBPs do not play a large role in specifying odor selectivity or sensitivity, at least in *Drosophila melanogaster*.

The identification and functional characterization of ORNs in pathogen vectors also offers new opportunities for control. The peripheral receptor system for *Cx quinquefasciatus*, i.e., maxillary palps and antennal ORNs, has been mapped, with all the sensilla on the palps being shown to house 3 ORNs and respond to CO_2 and plant/floral odors (Syed and Leal 2007). Antennal ORNs were shown in later studies to respond to a variety of chemicals (Syed and Leal 2009). High throughput electrophysiological assays have been used to identify volatile compounds that activate, inhibit, or block ORNs for *D. melanogaster*, with active compounds being tested for behavioral activity (Hallem and Carlson 2006; Kreher et al. 2008). Specialized ORNs that mediate the detection and avoidance of CO_2 have been identified in *D. melanogaster*, and recent studies by Turner and Ray (2009) have proposed that this phenomenon is due to inhibition of the CO_2 ORNs by volatile compounds present in food odor. The authors also report similar inhibition of CO_2 ORNs in *Culex* spp. mosquitoes, and highlight the important role that appropriate inhibitory odorants could have in disrupting CO_2-mediated host seeking behavior in pathogen vectors (Turner and Ray 2009). Similar to the OBP story, this approach potentially can be applied to search for new semiochemicals with superior activity. However, as peripheral responsiveness to stimuli must be integrated by the organism to mediate behavior, this suggests a potential limitation in developing such a strategy.

New Chemical Ecology Studies on Afro-Tropical Pathogen Vectors

The impact of pathogen vectors upon livestock and human health is greatest in sub-Saharan African countries. Chemical ecological research in these countries has been dominated by studies on the attraction of the Morsitans group of *Glossina* spp. tsetse flies, and *Anopheles* / *Culex* spp, mosquitoes to host odors and oviposition cues, as exemplified elsewhere in this review. Nevertheless, novel research is being undertaken that aims to identify: (1) semiochemicals from non-hosts that can be developed for livestock protection, (2) semiochemicals from hosts for the control of vectors of neglected diseases that can be deployed in the protection of livestock belonging to resource-poor farmers, e.g., nomadic communities, who are unable to access more advanced approaches to pathogen vector control, and (3) novel oviposition semiochemicals.

While host-derived attractants have been identified for the Morsitans group of *Glossina* spp. from host breath and urine (see above), more recent studies have focused on feeding preferences, including the existence of non-host allomones produced by the non-preferred host waterbuck, *K. defassa* (Gikonyo et al. 2000). Analysis of the odor composition of preferred (buffalo, *Syncerus caffer*, and ox, *Bos indicus*) and non-preferred (*K. defassa*) species, and electrophysiological studies, have shown that the odors of the two preferred hosts are comparable. They comprise medium-chain, saturated or unsaturated aldehydes and

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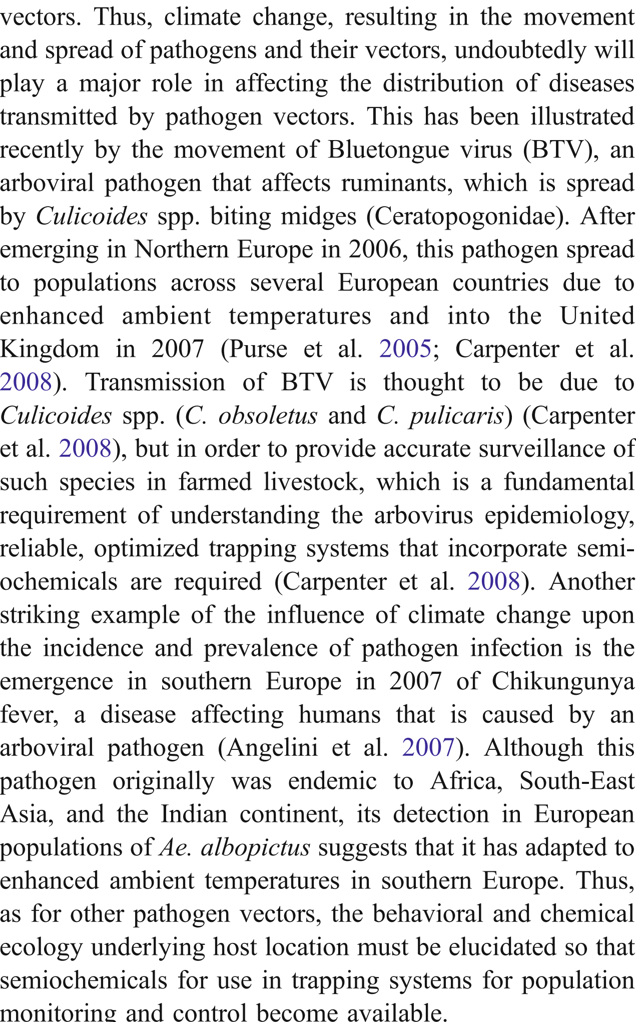
phenolic compounds, with the non-host odor containing fewer aldehydes but more phenolic components and a series of 2-ketones (C_8–C_{13}) and δ-octalactone, and moderate amounts of C_5–C_9 straight chain fatty acids. The electrophysiological responses of *Glossina* spp. show that 2-ketones and the lactone from the non-host odor are physiologically active (Gikonyo et al. 2002). Subsequent behavioral studies have shown that when presented with EAG-active components found specifically in the non-host odor, typical upwind flight behavior of flies is disrupted, with flies avoiding the non-host blend (Gikonyo et al. 2003). The behavioral responses of *Glossina* spp. to putative repellents based on guaiacol (2-methoxyphenol), a known mild repellent, also have been investigated, with the aim of identifying analogues with more potent repellent activity (Saini and Hassanali 2007). Of the compounds tested, the 4-methyl-substituted analogue (4-methylguaiacol) elicited stronger repellent effects, compared with guaiacol. Furthermore, the 4-methyl derivative reduced significantly trap catches of attractant-baited traps, and when applied to ox hosts, reduced the proportion of flies feeding on the host. Application of the repellent to approximately 75% of cattle herds has been shown to protect entire cattle herds (Saini and Hassanali 2007).

The Palpalis group of tsetse flies are responsible for the transmission of Human African Trypanosomiasis (HAT) across sub-Saharan Africa. In order to develop cost-effective control methods for *Glossina fuscipes* spp., the most important HAT vector, the responses of *G. fuscipes fuscipes* to host odors have been investigated in Kenya. Field trapping studies showed that odors from the monitor lizard, *Varanus niloticus*, significantly increased landing responses from *G. f. fuscipes* compared to ox and human odor, thereby suggesting that attractants in the lizard odor can be exploited in developing traps for controlling this major HAT vector (Omolo et al. 2009).

The oviposition behavior of gravid *An. gambiae* mosquitoes, in response to quality of oviposition site water and the density of larvae already at the site, has been investigated. The presence of larvae in distilled water deterred oviposition, but in natural breeding site water, a low density of larvae increased oviposition, whereas a higher density inhibited oviposition. These data suggest that a volatile pheromone is emitted by conspecific larvae, and that a non-olfactory cue deters oviposition at sites with high larval densities (Sumba et al. 2008).

Future Directions

For any vector-borne pathogens, the incidence and prevalence of pathogen infection in animals and human beings is dependent upon the distribution and abundance of their



vectors. Thus, climate change, resulting in the movement and spread of pathogens and their vectors, undoubtedly will play a major role in affecting the distribution of diseases transmitted by pathogen vectors. This has been illustrated recently by the movement of Bluetongue virus (BTV), an arboviral pathogen that affects ruminants, which is spread by *Culicoides* spp. biting midges (Ceratopogonidae). After emerging in Northern Europe in 2006, this pathogen spread to populations across several European countries due to enhanced ambient temperatures and into the United Kingdom in 2007 (Purse et al. 2005; Carpenter et al. 2008). Transmission of BTV is thought to be due to *Culicoides* spp. (*C. obsoletus* and *C. pulicaris*) (Carpenter et al. 2008), but in order to provide accurate surveillance of such species in farmed livestock, which is a fundamental requirement of understanding the arbovirus epidemiology, reliable, optimized trapping systems that incorporate semiochemicals are required (Carpenter et al. 2008). Another striking example of the influence of climate change upon the incidence and prevalence of pathogen infection is the emergence in southern Europe in 2007 of Chikungunya fever, a disease affecting humans that is caused by an arboviral pathogen (Angelini et al. 2007). Although this pathogen originally was endemic to Africa, South-East Asia, and the Indian continent, its detection in European populations of *Ae. albopictus* suggests that it has adapted to enhanced ambient temperatures in southern Europe. Thus, as for other pathogen vectors, the behavioral and chemical ecology underlying host location must be elucidated so that semiochemicals for use in trapping systems for population monitoring and control become available.

Pheromones (sex, aggregation, and oviposition), which although not always directly related to the pathogen transmission stage of the life cycle, also can be incorporated into optimized trapping systems. Although the underlying mechanisms associated with the chemical ecology of oviposition behavior have been studied extensively for *Culex* spp. mosquitoes, e.g., the oviposition pheromone and site-derived cues, such mechanisms need to be appropriately investigated for other vector species.

As stated above, host-derived attractants, and pheromones, can be used in trapping systems for vector population monitoring and control. However, their potential can be fully realized only when used in conjunction with repellents for livestock and human protection. The identification of host-derived repellents, as exemplified by the discovery of 6-methyl-5-hepten-2-one and geranylacetone from human beings (Logan et al. 2008), potentially will provide a new class of repellents that are highly active due to their ecological role in non-host avoidance. Deployment of such repellents that use slow-release formulations is technically challenging, but the availability of novel dispenser technologies will facilitate their commercial

development. A more sustainable approach is to enhance the production of repellents in hosts, by exploring their biosynthesis, and therefore, the genetic factors that are responsible for repellent production.

In addition to identifying new semiochemical tools for improved trapping systems, investigations into the olfactory processes that underlie the perception of semiochemicals also will potentially provide opportunities for improved detection of pathogen vectors, through the development of biosensors based either on whole insects, e.g., honeybee, *Apis mellifera*, olfaction (Pickett et al. 1998), or on over-expressed insect OBPs or ORs. Furthermore, the development of physical sensors such as portable, miniature mass spectrometers, for the detection of pathogen vector semiochemicals also provides a potential route to improved vector detection (Birkett and Pickett 2006).

In summary, in a changing global climate, the movement of vector-borne pathogens requires generation of additional scientific knowledge surrounding their chemical ecology. New and efficient tools for population monitoring and control will be needed that will both be acceptable to the general public, and affordable even to the poorest of communities in afflicted regions. This will be a grand challenge, on a global scale, for chemical ecologists in the 21st century.

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Trail Pheromone Disruption of Argentine Ant Trail Formation and Foraging

David Maxwell Suckling · Robert W. Peck · Lloyd D. Stringer · Kirsten Snook · Paul C. Banko

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Abstract Trail pheromone disruption of invasive ants is a novel tactic that builds on the development of pheromone-based pest management in other insects. Argentine ant trail pheromone, (*Z*)-9-hexadecenal, was formulated as a micro-encapsulated sprayable particle and applied against Argentine ant populations in 400 m^2 field plots in Hawai'i Volcanoes National Park. A widely dispersed point source strategy for trail pheromone disruption was used. Traffic rates of ants in bioassays of treated filter paper, protected from rainfall and sunlight, indicated the presence of behaviorally significant quantities of pheromone being released from the formulation for up to 59 days. The proportion of plots, under trade wind conditions (2–3 m s^{-1}), with visible trails was reduced for up to 14 days following treatment, and the number of foraging ants at randomly placed tuna-bait cards was similarly reduced. The success of these trail pheromone disruption trials in a natural ecosystem highlights the potential of this method for control of invasive ant species in this and other environments.

D. M. Suckling (✉) · L. D. Stringer
The New Zealand Institute for Plant and Food Research Limited,
PB 4704, Christchurch, New Zealand
e-mail: Max.Suckling@plantandfood.co.nz

R. W. Peck · K. Snook
Hawai'i Cooperative Studies Unit,
Pacific Aquaculture and Coastal Resources Center,
University of Hawai'i at Hilo,
Hawai'i National Park, HI 96718, USA

P. C. Banko
U.S. Geological Survey,
Pacific Island Ecosystems Research Center,
Hawai'i National Park, HI 96718, USA

Keywords Argentine ant · Trail pheromone · Disruption · Trail integrity · Invasive species

Introduction

The Argentine ant, *Linepithema humile* (Mayr), is a highly invasive species that has numerous negative impacts on native arthropods (Gillespie and Reimer 1993; Human and Gordon 1997; Liebherr and Krushelnycky 2007), including other ant species (Human and Gordon 1996; Holway 1999). When a species such as *L. humile* invades remote islands or other ecologically sensitive areas, native arthropod populations may be eliminated quickly causing problems for other organisms, such as plants, which may lose their native pollinators (Cole et al. 1992) and seed dispersers (Christian 2001). In human-modified landscapes, Argentine ants are both a nuisance (Vega and Rust 2001; Holway et al. 2002) and an agricultural pest, impacting the effectiveness of biological control agents in orchards (Moreno et al. 1987; Itioka and Inoue 1996). Poison baits have not proven entirely reliable for control of these pests (e.g., Krushelnycky and Reimer 1998; Hooper-Bui and Rust 2000; Klotz et al. 2000; Greenberg et al. 2006) and also pose risks to pets and wildlife. Thus, more effective and environmentally benign control tactics are needed for the management and control of these invasive ants.

Sensitive ecosystems require appropriate pest-control formulations to avoid non-target impacts at the landscape level. Pheromone-based pest management (e.g., El-Sayed 2009) offers both target specificity and low hazard. This approach could be used for managing invasive ants, such as Argentine ants, in urban or natural ecosystems. To date, exploration of the potential for using pheromones or other odorants for control of ant species has been limited

mainly to leaf cutting ants (Robinson and Cherrett 1978), the red imported fire ant (*Solenopsis invicta*), and the Argentine ant (Robinson and Cherrett 1978; Shorey et al. 1992).

Argentine ants use a trail pheromone, consisting of (*Z*)-9-hexadecenal (Z9-16:Ald) (Cavill et al. 1979), to orient to food and communicate the location of food to nest mates. The compound Z9-16:Ald also is a sex pheromone component of a number of moth species (El-Sayed 2009) and is readily available commercially. Wright (1964) first proposed the use of synthetic pheromones for mating disruption of moths, but until recently there has been no effort to expand this concept to flightless insects, such as foraging ants, through trail pheromone disruption.

Trail-following behaviors of Argentine ants have been well examined (Van Vorhis Key et al. 1981; Van Vorhis Key and Baker 1982). Van Vorhis Key and Baker (1982) recognized that it might be possible to modify trail following behavior by using synthetic compounds, although they did not elaborate on a possible method. The recent demonstration of close-range disruption of Argentine ant trails by using pheromone contained in “twist-tie” polyethylene tubing dispensers showed reduced ant traffic in small plots (Tatsuki et al. 2005; Tanaka et al. 2009). This type of polyethylene-tubing formulation has been used for mating disruption of moths, usually at a density up to one thousand discrete point sources per hectare in orchards and other crops (Suckling and Angerilli 1996; Suckling 2000). However, a discrete point-source approach may not give an effective concentration of trail pheromone to prevent trail following over large areas, except under very still conditions, because pheromone concentration from these dispensers is a function of application height (Suckling et al. 1999). Moreover, the boundary layer, which is the target for trail disruption of ants, is likely to receive the lowest concentration of pheromone.

In previous work, we quantified the integrity of Argentine ant trail-following behavior by video analysis of movement and track angles (Suckling et al. 2008). Trail integrity, defined as r^2 for the position of ants in a video frame, was devised as a measure of the ability of workers to follow a reasonably straight trail, before and after the application of a point source of synthetic trail pheromone. Well-formed trails were completely disrupted indoors by using a crude wax-based formulation. However, due to rapid pheromone release from the wax-coated quartz sand, only several hours of disruption was achieved in small (1 m^2 and 4 m^2) plots (Suckling et al. 2008).

In this study, we examined whether a more durable, sprayable formulation, containing Z9-16:Ald, could be used to disrupt trail-following behavior of Argentine ants in a hot and windy field setting. We used a widely dispersed point-source strategy within the boundary layer where the ants were actively foraging. A micro-encapsulated formulation was chosen for testing because a similar product is commercially available for mating disruption of moth species (Stelinski et al. 2007). The research followed two approaches: 1) laboratory bioassay and micro-plot testing of short-range disruption of trail-following behaviors, and 2) larger field trials to examine impact of disruption on trail formation and recruitment to baits.

Methods and Materials

Chemicals (*Z*)-9-Hexadecenal (94% isomeric purity) was obtained from Bedoukian Research (Danbury, CT, USA) and formulated into a micro-encapsulated sprayable product. The pheromone formulation was prepared by Suterra LLC (Bend, OR, USA), and was similar to that used for light brown apple moth in California (www.cdfa.ca.gov) and other moths (Stelinski et al. 2007), with ca 20% active ingredient as micro-encapsulated particles in water. This formulation had the advantage of being readily dispersible in the field with relatively even coverage of pheromone in the plots.

Field Site The field study took place at ca 1,200 m elevation in an exposed landscape with high Argentine ant populations, windy conditions, and occasional heavy rainfall, on friable cinder soil and rock. The area of Hawai‘i Volcanoes National Park called Broomsedge Burn (19°25′ 59.6″ N, 155°17′35.2″ W; Fig. 1) is mesic to dry ohia (*Metrosideros polymorpha*) woodland now dominated, after fires, by the invasive grasses broomsedge bluestem (*Andropogon virginicus*) and bush beardgrass (*Schizachyrium condenstum*). It is exposed to prevailing trade winds,

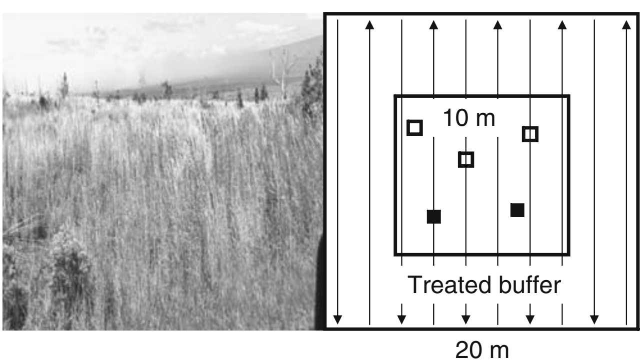

Fig. 1 Layout for trial testing the application of micro-encapsulated Argentine ant trail pheromone, (*Z*)-9-hexadecenal, in Moana Loa in Hawai‘i Volcanoes National Park (*left*), showing 400 m^2 plots treated by 10 swathes using a garden sprayer. Ant trailing and foraging at bait cards were made in the central square (5 m from the plot boundary). Locations were either fixed (*black squares*) at the same location, or randomly (*unfilled squares*) located within the assessment area on each sampling date

averaging 2–3 m s^{-1}, because of sparse canopy cover (Suckling et al. 2008). During the trial, daytime air temperatures of 15–29°C (mean 20.4°C) and ground temperatures of 13–30°C (mean 20.1°C) were experienced. Wind speed at 1 m above ground level ranged from 0.3 to 3.7 m s^{-1} (mean 1.5 m s^{-1}), while at ground level (1 cm) wind speed ranged from 0 to 2.4 m s^{-1} (mean 0.9 m s^{-1}).

Pre-treated Micro-plot Disruption We anticipated that forager numbers would be reduced under pheromone disruption conditions. A dose-response experiment was conducted over a square of paper on aluminum foil-covered boards (21.5×21.5 cm). The papers were treated with 0, 3.7, 37.5, 375, or 3,750 g a.i./ha of sprayable pheromone by using a low-pressure hand sprayer. The top rate was undiluted and only applied for calibration of deposition (7 replicates). Densities of particles on the board were determined with a stereomicroscope. Magnetic stirring of particles was needed because of the buoyancy (flotation) of the pheromone product. Twenty-eight pre-treated boards (5 m separation) were baited with approximately 1 ml of 1:1 macerated tuna (Bumblebee Foods, San Diego, CA, USA) and Karo Light Corn Syrup (ACH Food Companies, Memphis, TN, USA), centered on a plastic-coated card (3× 5 cm). Baits were protected from sunlight and heat with an inverted plastic plate (25 cm diam.) suspended 15 cm above the ground. Counts of ants on the cards were made one hour after bait placement.

Impact on Traffic Rates To test the longevity of the sprayable pheromone, a bioassay that measured the effect of the pheromone formulation on Argentine ant trail traffic was conducted on a 6-m section of ant trail on an external painted cinder block wall in Hawai‘i Volcanoes National Park. Pheromone was sprayed from a height of 1 m onto five 9 cm-diam. circular black craft paper discs (Pacon Corp., Appleton, WI, USA), for ca 0.5 s on 19 February 2008. Bioassays used treated half circles placed opposite each other, 2 cm either side of a vertical ant trail, at 0.5, 2, 4, 24, 48, and 72 h after treatment, then at approximately weekly intervals, for a total duration of 9 week. At each test time, a pre-treatment count of ant movement in a single direction along the trail was made for 1 min by using a bisected unsprayed disc placed along the trail as a control, followed by treated paper. For each observation (5 per date), two measurements of traffic rates were made, each 1 min apart, and pooled as pseudo-replicates (Hurlbert 1984) for analysis. It was expected that the short duration of the bioassay and the lack of complete disruption would enable 5 independent repeats at different locations along the trail but, to be certain, the data were analyzed for a downward trend in traffic. After the counts were completed, the half circles were removed from the trail and stored indoors at ambient temperature until the next test.

Small Field Plot Tests (400 m^2) To test whether treatment of larger areas would facilitate more sustained trail and/or foraging disruption, trials were conducted in 20×20 m plots, each with a 10×10 m central square (Fig. 1). Eight plots, 80 m apart from each other, were randomly designated as treatment (*N*=4) or as control (*N*=4). Trail following and foraging of ants was measured within the central square; the outer area acted as a buffer zone for any influence of wind. Pheromone was applied at a rate of 375 g a.i./ha by using a 3.8 liter garden sprayer (RL Flo-Master®, Root-Lowell Manufacturing Co., Lowell, MI, USA). The pheromone was divided into 10 equal portions so as to ensure uniform delivery of pheromone across the plot (Fig. 1). The sprayer was agitated continuously during application. Control plots were untreated.

Some 2.5 h prior to application of pheromone, five cards, baited with the 1:1 tuna-corn syrup mixture, were randomly placed within the 100 m^2 centers of treatment and control plots (Fig. 1), The number of ants on each card and the presence or absence of trails to each card were determined 0.02 d before pheromone treatment, and 0.02, 0.08, 0.16, 1, 2, 3, 8, 10, 14, 21, 28, and 35 d after treatment. Two of the 5 cards on each plot were randomly selected from those at which well-developed trails had been established (“fixed” locations). At later sampling dates, bait cards were placed at these fixed locations and at 3 new, randomly selected positions (Fig. 1). This differential placement of cards within plots was designed to challenge the ability of the ants to use established trails and to create new trails. Card placement simulated food resources that had either been previously identified by the ants or newly located on each sample date. The 2 fixed or 3 randomized cards from each plot were considered pseudo-replicates (Hurlbert 1984) and pooled for analysis.

The study was initiated on 29 January 2008, with two treatment and two control plots. The other 4 plots were started on 11 February 2008 because of poor weather.

Statistical Analysis Traffic rates and ant counts were log-transformed after initial inspection of the data indicated that this was warranted to stabilize the variance. Disruption in the bioassay and small plot test was calculated as = 100 – (count in the Treatment / count in the Control) * 100. Two-way ANOVA on log-transformed counts of ants was used to analyze the traffic rates for main effects (time and treatment) testing the longevity of the pheromone. Log-transformed counts of ants at baits in the micro-plot experiment were analyzed using two-way ANOVA. The experimental design for the small-plot test was a random-

ized complete block with two factors (treatment and bait card location) at two levels (control, treatment; fixed, random). A general linear model was conducted on log-transformed counts of ants at bait cards to stabilize the variance. The proportion of ant trails at the bait cards in the randomized and fixed plots was predicted using binary logistic regression (Quinn and Keough 2002). This predicted the probability of trail presence at the baits. The dependent-response variable was the presence or absence of ant trails to the cards, modeled using three explanatory factors: randomized/fixed cards, time, and treatment. The data were modeled against reference categories for each explanatory factor; reference categories, randomized = “fixed”, time = “before”, treatment = “no pheromone”. The predicted odds ratio, exp(β), indicated the odds of trail presence for the explanatory factors compared to their respective reference categories (see Stringer et al. 2007 for further explanation). For example, if the odds ratio, exp(β), is 20 for a point in time, then the odds of trail presence is 20 times greater than the reference time (before). A value of 1 would indicate that trail presence after application did not differ from that before. Only the main effects were modeled, as there were no significant interaction effects. Data were analyzed in GenStat (GenStat 2007) and SAS (2004).

Results

Pre-treated Micro-plot Disruption No evidence was found for short distance disruption in recruitment of ants to bait cards ($F_{3,24}$=2.47, P=0.09, mean ± standard deviation 89.6± 49.7 ants/card). There was no correlation between ant counts and the number of particles found on sample papers (r^2=0.15; range 0–6 particles per 38.5 cm^2).

Bioassay of Impact on Traffic Rates Two-way ANOVA revealed a significant effect for treatment ($F_{1,136}$=142.3, P<0.001) and time ($F_{16,136}$=3.41, P=0.001), with no significant interaction between the two factors. There was no impact of untreated cards on traffic rates (number of ants/min) over the course of the study, and no trend in traffic rate among repetitions (P>0.999, df=84). A significant downward trend in traffic rate with time since pheromone treatment was found (% reduction in traffic over time = −0.5083x + 72.92, where x = dose of pheromone treatment; r^2=0.63, P<0.01) (Fig. 2).

Small Field Plot Tests (400 m^2) Argentine ant trails were significantly disrupted shortly after pheromone application and up to 14 days later, measured by a reduction in the presence of visible trails in plots with both types of bait

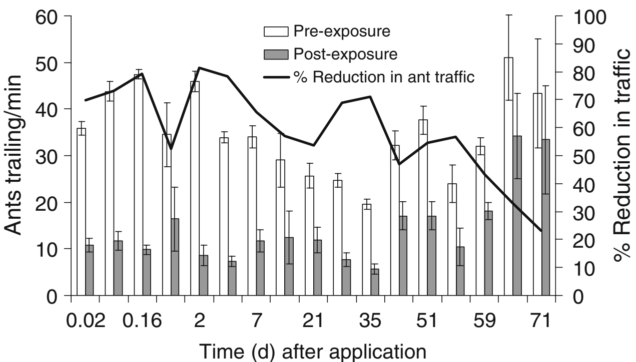

Fig. 2 Effect of application of micro-encapsulated synthetic trail pheromone, (*Z*)-9-hexadecenal, on Argentine ant trail-following over time; measured as mean (± SEM) traffic rates (*left axis*) and % reduction (compared to controls, pre-exposure) of ant counts, post-exposure (*right axis, line*)

locations and treatments (Fig. 3). Following pheromone application, there was a significant reduction in trail presence, with the odds ratio being 28 times less than for trail presence before pheromone application. The predicted odds of trail presence at randomized baits was eight times lower than at fixed bait positions, regardless of treatment (Table 1).

Randomizing the location of baited sites influenced ant counts at baits ($F_{(1,6)}$=19.25, P<0.005), with a reduction in worker number in the treated plots (Fig. 4). However, inspection of results on individual dates showed that the pheromone treatment disrupted ant trail formation on cards (as evidenced by the proportions of cards with trails) longer than it disrupted the counts of ants on cards (Figs. 3, 4, 5). Bait predation by introduced mongoose, *Herpestes javanicus*, occurred in several plots on Day 8. No error bars are shown on this day because of the resultant lack of degrees of freedom.

Discussion

We have demonstrated, using a micro-encapsulated trail pheromone formulation in a natural ecosystem, that significant disruption of trail following by Argentine ants is possible over at least a 14-day period. This is an improvement in longevity of disruption over the wax-based dispersible formulating originally tested, which disrupted visual trails and foraging at bait cards in 4-m^2 plots for only 24 h (Suckling et al. 2008). We also were able to demonstrate significant disruption of foraging by Argentine ant workers for bait cards.

Our bioassay that examined traffic rates proved to be a robust and reliable measure of trail disruption from a point source (Suckling et al. 2008; Tanaka et al. 2009). The

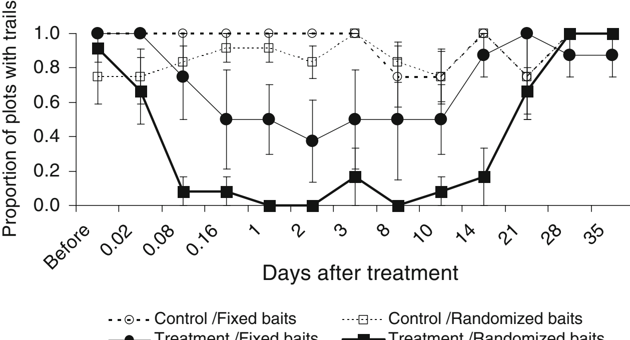

Fig. 3 Proportion of plots with visible Argentine ant trails before and after the 400-m^2 test areas were treated with micro-encapsulated synthetic trail pheromone or left as untreated control. Periodic 1 min observations for presence of trails were made, commencing 30 min after treatment, near baits placed repeatedly in the same location (fixed baits) or replaced at random positions (randomized baits). *Bars* indicate one standard error

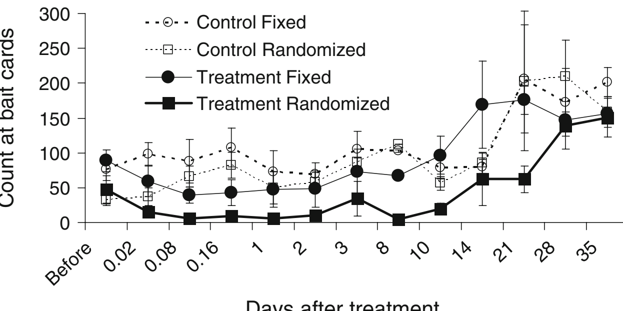

Fig. 4 Mean (±SEM) count of Argentine ants at bait cards before (control) and after synthetic trail pheromone treatment. Cards were placed in the field, repeatedly either at the same location (fixed) or randomly (randomized) within the central 10×10 m of the plot, 2 h before counts. *Bars* indicate one standard error

results showed that the treated bioassay cards, stored in the absence of sunlight or rainfall, maintained their efficacy at disrupting trail following for many weeks. Testing under less favorable conditions should be carried out to determine the robustness of the formulation in the field.

The micro-plot experiments offered a minimum walking distance of 6.3 cm from the edge of the sprayed area to the central bait card for disruption. That complete disruption was not achieved under these conditions, suggests that ant trails were formed near the treated boards, and that foraging ants regrouped onto trails in the vicinity of the bait. Argentine ants can orientate using cues other than trail pheromones to find food resources. For instance, they are known to use food odors (Wolf and Wehner 2000; Stanley et al. 2008), but not vision (Aron et al. 1993), to orient toward food sources. As trail following from the edge of the disrupted boards to the central food was unsuccessful, it appears likely that multiple individual discoveries of the food source were made, rather than a single discovery (or few discoveries) complemented by recruitment through trail pheromone.

The effect of disruption in the larger plots was less pronounced in the situation in which baits were replaced in the same locations (fixed), compared to when their positions were re-randomized. This suggests that ant nests may have learned the location of the fixed baits by associating different cues such as food olfactory cues (Wolf and Wehner 2000) or different pheromones (Dussutour et al. 2009) with the resource. It is not clear whether the responses of the ants to the fixed or randomized baits in this study are typical of ant responses to food supplies such as floral nectar or honeydew from homopterans. Finding of

Table 1 Binary logistic regression on the presence of Argentine ant trails modeled on explanatory factors of randomized/fixed bait cards, treatment, and time. Reference categories for explanatory factors: randomized/fixed bait cards = “fixed”, treatment = ‘no pheromone’ and time = ‘before’; exp(β) = predicted odds ratio

Factor	β	SE	Wald	*P*	exp(β)
Randomized	−2.054	0.340	−6.040	<.001	0.128
Treated	−3.323	0.363	−9.170	<.001	0.036
Time (days)					
0.021	−0.831	0.757	−1.100	0.272	0.436
0.08	−2.501	0.738	−3.390	<.001	0.082
0.16	−2.688	0.738	−3.640	<.001	0.068
1	−2.475	0.784	−3.160	0.002	0.084
2	−3.232	0.743	−4.350	<.001	0.039
3	−2.310	0.737	−3.130	0.002	0.099
8	−2.913	0.947	−3.080	0.002	0.054
10	−3.408	0.745	−4.570	<.001	0.033
14	−1.712	0.738	−2.320	0.020	0.181
21	0.244	0.868	0.280	0.778	1.277
28	1.610	1.160	1.390	0.164	5.025
35	1.610	1.160	1.390	0.164	5.025
Constant	6.295	0.784	8.03	<.001	542.0

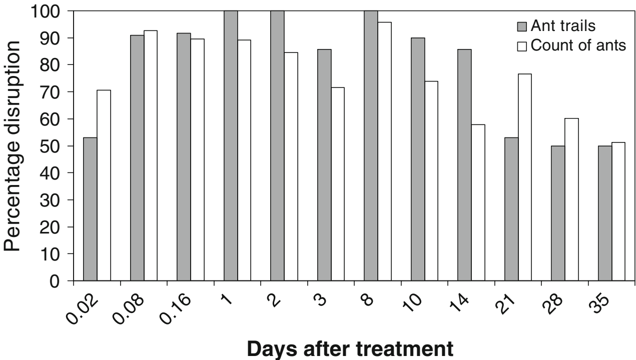

Fig. 5 Percentage trail pheromone disruption (relative to untreated controls) of Argentine ants in small plots (400 m^2) over time, as measured by reduction in presence of trails and ant counts at randomized bait cards

the randomized bait cards probably represents the opportunistic foraging efforts of the nest (i.e., the nest's ability to detect and use new food sources); reduced trail formation and forager numbers to such opportunistic food sources likely results in lesser utilization, and possibly an impact of nest productivity.

With our demonstration of the feasibility of long-duration disruption of Argentine ant trail following using sprayable pheromone, future research should concentrate on improving the efficacy of the method through studying the mechanisms involved both in trail following and disruption of the behavior. Additionally, disruption could be improved by the identification and utilization of other compounds involved in trail following (Cavill et al. 1979). We will continue examining the potential of disruption of pheromone trail integrity as a stand-alone pest management tactic against Argentine ants and as a tactic integrated with other control methods.

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Differences and Commonalities in Physical, Chemical and Mineralogical Properties of Zanzibari Geophagic Soils

Sera L. Young · M. Jeffrey Wilson · Stephen Hillier · Evelyne Delbos · Said M. Ali · Rebecca J. Stoltzfus

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Abstract The function of human geophagy has long been questioned. We sought to test hypotheses concerning its potential physiological effects through analysis of soils and patterns in geophagy behavior. Eleven samples of geophagic soils consumed by pregnant women on Pemba Island, Zanzibar, Tanzania, were characterized according to their color, texture, major element chemistry, trace element chemistry, bulk mineralogy, and clay mineralogy. An epidemiological study ($N=2367$) and ethnographic interviews ($N=57$) on Pemba yielded information about geophagic behaviors and socio-demographic and biological characteristics of those who consumed earth. The soils varied widely in color, ranging from light red to white through various shades of brown and yellow, and texture ranged from clay to sand. Major element chemistry of the soils also varied greatly; most were low in Fe and Ca. Trace elements, whether of biological or non-biological significance, were uniformly low when compared with normal ranges of mineral soils. The sole commonality among the samples is that all clay fractions were dominated by a kaolin mineral: kaolinite, halloysite, or a mixture of both. Geophagy behavior also varied greatly, with one major exception: a greater proportion of pregnant women (7.1%) and young children (4.5%) consumed earth than non-pregnant women (0.2%) or men (0%). The presence of kaolin mineral in all samples, its palliative and detoxifying properties, and the highest prevalence of geophagy among those most biologically vulnerable suggest that geophagy may be a protective behavior.

Keywords Pica · Geophagy · Nutrition · Detoxification · Africa · Pregnancy · Mineralogy · Health

S. L. Young (✉)
University of California Davis Medical Center,
Ticon II, 2516 Stockton Blvd.,
Sacramento, CA 95817, USA
e-mail: slyo@ucdavis.edu
e-mail: sera@younglucks.com

S. L. Young · R. J. Stoltzfus
Division of Nutritional Sciences, Cornell University,
Ithaca, NY 14853, USA

M. J. Wilson · S. Hillier · E. Delbos
Macaulay Land Use Research Institute, Craigiebuckler,
Aberdeen, Scotland AB15 8QH, UK

S. M. Ali
Public Health Laboratory-Ivo de Carneri, Chake Chake,
Pemba Island, Zanzibar, Tanzania

Introduction

Geophagy, the purposeful consumption of earth, has long been a source of fascination and puzzlement. Geophagy is a specific type of pica, which is defined as the craving and subsequent consumption of non-food substances. The motivation and consequences of this behavior remain unclear. Geophagy has been documented in many human cultures (Laufer 1930; Anell and Lagercrantz 1958) and throughout the animal kingdom (Jones and Hanson 1985; Kreulen 1985; Krishnamani and Mahaney 2000). It is a very old practice; the first written account of geophagy was by Hippocrates in the 4th century BCE (Hippocrates 1849), but archaeological evidence suggests it dates back to Homo habilis (Clark 2001).

There are three major groups of hypotheses concerning the physiological causes of pica: hunger, micronutrient

deficiency, and protection from toxins and pathogens (Young et al. 2008).

1. The hunger hypothesis posits that people consume non-food substances because they do not have anything else to eat (Laufer 1930).
2. The micronutrient deficiency hypothesis posits that people with micronutrient deficiencies eat non-food substances in an attempt to increase micronutrient intake of Fe (Hunter 1973), Zn (Smith and Halsted 1970), or Ca (Wiley and Katz 1998). Another version of this hypothesis is that a micronutrient deficiency causes disturbed taste sensitivities or malfunctioning of appetite-regulating brain enzymes that cause non-food substances to become appealing (von Bonsdorff 1977). In this scenario, pica is a consequence of micronutrient deficiency, but not an attempt to remedy it.
3. The protection hypothesis states that pica is motivated by an attempt to mitigate the harmful effects of plant chemicals or microbes (Johns 1986; Profet 1992). It is proposed that pica substances protect by either adsorbing pathogens and toxins within the gut lumen or by coating the surface of the intestinal endothelium, thereby rendering it less permeable to toxins and pathogens. According to this hypothesis, overt gastrointestinal distress, which can be the result of exposure to either toxins or pathogens (Simjee 2007), also trigger pica. Additionally, this hypothesis implies that pica substances would be ingested during periods of rapid growth, i.e., the times of greatest need for protection from toxins and microbes. Under this hypothesis, childhood and pregnancy, especially early pregnancy [which is the critical period of organogenesis (Moore and Persaud 1998)], are the periods when pica most likely would occur (Flaxman and Sherman 2000). Pregnant women, who are immunologically suppressed (Formby 1995; Fessler 2002), also may need protection from substances that would normally be harmless.

A limited number of studies have tested these hypotheses, fewer have studied the health status of those practicing pica, and rarer still are studies that have correctly examined the physical, chemical, and mineralogical nature of the soils consumed. In reviewing previous analytical work done on geophagic samples, there are a number of limitations that deserve special attention.

Most published chemical analyses of geophagic earths are not useful for testing the nutritional hypothesis because they are confined typically to the total elemental content, without consideration of the extent to which these elements are biologically available (Wilson 2003; Young et al. 2008). Human gut pH varies from pH 1–2 in the stomach to pH 7–8 in the small intestine, the site of the bulk of nutrient absorption. This fluctuation has major consequences for the bioavailability of elements, and must be considered when drawing conclusions about nutritional benefits.

Second, the amounts of the soils consumed need to be precisely specified; previous researchers have made calculations about intake based on amounts reported from another study, even if that study took place on a different continent, several decades in the past, or in a different age group (e.g., Hunter 1973; Smith et al. 1998).

Third, although it is critical to establish the mineralogy of geophagic samples, especially in relation to the protection hypothesis, previous studies have been vague in the characterization of their mineralogy. For example, halloysite is identified specifically as the main clay mineral in the soils consumed by humans (Aufreiter et al. 1997) and various primates (Mahaney et al. 1993, 1995a, b, 1997; Aufreiter et al. 2001), although Wilson (2003) concluded that the identification of the clay mineral in these papers was ambiguous. The distinction between different kaolin minerals could be important, as kaolinite and halloysite have different particle morphologies (flat platy vs. tubular or spheroidal), which could affect their dispersion/flocculation behavior (Itami and Fujitani 2005), as well as their viscosity and flow characteristics (Yuan and Murray 1997). These properties could be significant in affecting the ability of the clays to coat the gut wall, thereby acting as a barrier to harmful chemicals and microorganisms (Allen and Leonard 1985) and as a stimulant to mucus secretion (Leonard et al. 1994; Theodorou et al. 1994; Gonzalez et al. 2004). Additionally, it may be inferred from some studies of geophagic soils that animals and humans can distinguish halloysitic from kaolinitic soils (Wilson 2003), and knowledge of the exact mineralogy of the soil clays would provide useful information for testing this inference.

In this work, we attempted to increase our understanding of geophagy by a careful characterization of the physical, chemical, and mineralogical properties of 11 samples of consumed earth and then link it to epidemiological and ethnographic data on geophagy behavior. We performed these analyses to identify commonalities that may yield clues to the physiological motivation for the selection and consumption of these materials.

Methods and Materials

Study Site and Sample Collection Pemba was chosen as an appropriate research site because geophagy is a well-established practice there (Young and Ali 2005). Pemba is the second largest island in the Zanzibar archipelago located 50 km off the coast of Tanzania. The archipelago is part of the ancient Miocene Rufiji/Ruvu delta, and most

of Pemba is underlain by alluvial formations of Miocene age (Stockley 1942). Similar Miocene alluvial formations host the Pugu kaolinite deposits to the west of Dar es Salaam on the East African mainland. In general, the culture and ecology of Pemba are similar to the rest of coastal East Africa (Middleton 2004).

The geophagic earths for analysis were selected based on results from a large epidemiological study of maternal anaemia and pica in an obstetric population conducted between 2004 and 2006 (N=2367). Additionally, in-depth interviews about pica were conducted with a sample of 57 individuals who had professed their pica behavior. Participants in in-depth interviews were either pregnant participants in the larger study or resident in a study participant's household. Four respondents were male, and recounted their pica behaviors during childhood.

The 4 types of earth consumed in Pemba are known in Swahili as *udongo*, *mchanga*, *vitango pepeta* and *ufue* (Fig. 1). *Udongo* is a fine reddish-brown clayey earth that is found close to the surface or in termitaria, and is used in making structures like house walls. *Mchanga* is the sandiest of the earths eaten, and is collected close to the surface. It is often exposed during the construction of wells or latrines. *Vitango pepeta* (also called *vitango mlima*) consists of large soft light-colored chunks of earth, and is obtained from closer to the surface of the earth than *ufue*. *Ufue* is much whiter than *udongo* and is found by digging from 5 to 50 cm into the earth's surface. The terms *ufue* and *vitango pepeta* are frequently used interchangeably by consumers.

In the large epidemiological study, women were asked about their pica behavior in two questions: if they ate *udongo* and if they ate *vitango pepeta* or *ufue*. *Mchanga* was not inquired about because it was consumed far less frequently. The interviews with those who had engaged in pica lasted from 30–75 min, and covered the many pica materials consumed in Pemba, including the 4 earths. A number of other pica substances are consumed on Pemba, including uncooked rice, charcoal, ash, ice, chalk, and ground shell, but are beyond the scope of this paper (Young 2008).

During interviews, participants were asked how samples were identified, collected, stored, and prepared, as well as about the attractiveness of various qualities, e.g., color, texture, flavor. After the interview, a Pemban fieldworker and/or an author (SLY) accompanied participants to the source of the pica substance if they were still engaging in pica. The consumer then collected precise amounts of the materials they consumed, as well as a large amount for subsequent analysis. Of the 57 participants in in-depth interviews, 26 had eaten *udongo*, 10 had eaten *ufue*, 13 had eaten *vitango pepeta*, and 2 had eaten *mchanga*.

The samples chosen for laboratory analysis were selected to reflect the frequency of consumption reported of each type of earth in the interviews. In this study, five *udongo* samples (2 from house walls and 3 from soil exposures), two *ufue* samples (both soil exposures), three *vitango pepeta* samples (all soil exposures) and one *mchanga* sample (soil exposure) were characterized (Table 1). The study would have been strengthened by the analysis of undesirable samples, i.e., samples that were not considered suitable for consumption. This will be rectified in subsequent studies.

Sample analysis The color of the samples was established objectively by reference to a Munsell chart, which describes colors in order of their hue (actual color), value (degree of lightness), and chroma (strength of color). Soil pH measurements were made by an electrometric method that used glass-calomel electrodes on soil suspensions in a soil: water ratio of 1:2.5 following the procedure for agricultural soils outlined by Peech (1965). Particle size analysis was made by laser light scattering (diffraction) by using a Malvern Mastersizer 2000.

Determination of both the major and minor elements in the geophagic samples was done by X-ray fluorescence analysis (XRF). This is the method of choice for inorganic materials because the instrumentation is widely available and has become the standard method for the analysis of major and trace elements of rocks following the procedures developed by Norrish and Hutton (1969) and Leake et al. (1969). XRF analysis is performed on pressed-powder discs and involves no pre-treatment other than a simple crushing and milling procedure.

The mineralogy of the geophagic materials was determined by X-ray diffraction (XRD) methods. Bulk samples were micronized in water in a McCrone mill, and the resulting slurries spray dried as described by Hillier (1999). XRD patterns were obtained from the spray dried random

Fig. 1 The four geophagic earths on Pemba Island, Zanzibar. From left to right: *udongo*, *mchanga*, *vitango pepeta* and *ufue*

Table 1 Geophagic behavior on Pemba Island, Tanzania, based on 57 in-depth interviews

	Udongo (N = 26)[a]	*Ufue* (N = 10)	*Vitango pepeta* (N = 13)	*Mchanga* (N = 2)
Who collects material	Self (20); bought in store (1)	Self (6); other relatives (2)	Self (11); other relatives (2)	Self (2)
Frequency of collection	More than once daily (8); once daily (13); once weekly (2); once monthly (1)	Once daily (3); once weekly (4); once monthly (1)	Once weekly (10)	Once daily
Distance from home	1–30 min	10–120 min	5–60 min	From own home
Site	House wall (10); bought in store (1); Agricultural fields (4); “anywhere it’s found” (5)	Agricultural fields (4); digging “anywhere it’s found” (4)	Agricultural fields (3); hills (7)	Most sands are acceptable
Preparation	Sun-dried (4); brushed off (3); rinsed with water (1); ground (1); eaten as found (8)	Sun-dried (2); eaten as found (8)	Baked (2); sun-dried (5); brushed off (3)	Dried (1); filtered through cloth (1)
Prior to consumption	Nausea (5); excess salivation (2); no particular feeling, just a craving (7)	No particular feeling, just a craving (5)	___	___
Mean frequency of consumption per day (range)	2.75 (1–10)	2.5 (1–4)	4.3 (1–20)	2–3
Mean amount consumed per day in g (range)	71.4 (31.3–151.2)	62 (40.7–90.5)	53.3 (18.4 – 96.8)	180.2
Participants’ description of flavor	Sharp, sour (*ukali*) (5), appealing (10)	Sharp, sour (*ukali*) (7)	Sharp, sour (*ukali*) (12)	None
Participants’ description of smell	Very appealing (13)	Very appealing (2)	Similar to *udongo* (1)	
Reminds them of...	Smoke of woodfire (2); perfume (1); uncooked rice (2); cement (1); baobab candy (*ubuyu*) (1)	Raw rice frying (1); fruit juice (1); sour (*ukali*) candy (1)	Sour oranges (1), lime (2) lemon (2), unripe mango (2), partially broken raw rice (1), baobab candy (*ubuyu*) (1)	Fried flour
Reason for cessation of eating	No longer craved it after pregnancy (11)	No longer craved it after pregnancy (6); husband made her (2)	No longer craved it after childhood (3); pregnancy (6)	No longer craved it after pregnancy (2)
Other	Causes anaemia (10); good only if pregnant (10)	Causes anaemia (7); good only if pregnant (5); reduces nausea (1)	Causes anaemia (6); good only if pregnant (6); good any time (2); addictive (1); reduces nausea (2)	Good only if pregnant (1); never good (1)

[a] Not all participants discussed all facets of their geophagic behavior, so the information in each cell does not always sum to the number of respondents

powder samples by scanning from 2 to 75°2Θ, in 0.02 steps, counting for 2 seconds per step on a Siemens D5000, using Co Kα radiation, selected by a diffracted beam monochromator. Bulk mineralogical composition was determined by a full pattern fitting reference intensity ratio method as described in detail in Omotoso et al. (2006). For further characterization of the clay minerals, <2 micron clay fractions were separated from the bulk samples by sedimentation, and dried down onto glass slides. The resulting oriented preparations were scanned on the D5000 diffractometer, from 2–45°2Θ, with 0.02° steps, counting for 1 second per step, in the air-dried state, following solvation with ethylene glycol by a vapor pressure method and after heating to 300°C for 1 hour (Wilson 1987).

Identification of certain clay minerals requires the use of supplementary treatments. Distinguishing halloysite from kaolinite is done through the rapid formation of an intercalation complex with formamide (Churchman et al. 1984). Clay fractions sedimented onto glass slides were examined before and after treatment with formamide to determine the presence of halloysite. These samples were run on a Panalytical X-pert Pro diffractometer, using Ni-filtered Cu Kα radiation with an X-celerator position sensitive detector. Both the air-dried and the subsequently formamide-saturated clay samples were scanned from 2–45° 2Θ, the total scan taking approximately 7 minutes to complete. Formamide treated specimens were scanned 30 minutes after contact with formamide.

Coherent fragments of the materials were mounted onto standard SEM stubs using carbon paint as the adhesive coated with gold. The prepared samples were analyzed by using a Philips XL30 Field Emission Scanning Electron Microscope (FEG-SEM) operating at an accelerating voltage of 20 kV. Characterisation of the various minerals was aided by Energy Dispersive Spectroscopy (EDS), which provides an elemental composition of the material analyzed. The EDS detector is equipped with an ultra-thin window that allows detection of elements down to carbon.

Results

Geophagy in Pemba There was great variety in the collection and consumption of soils (Table 1). Most participants collected their own soils. There were many sites from which they could be obtained, including the walls of houses, agricultural fields, and hills. Some soils were prepared carefully by heating or cleaning, while others were eaten as found. Most consumers were not able to explain why they had cravings for earth, although a few mentioned nausea and excess salivation. The amounts consumed ranged from 18.4 to 180.2 g, and the frequency of daily consumption ranged from 1 to 20 times. Consumers frequently were enthusiastic about liking the smell and taste of the soils, but in non-specific ways. Many answers to inquiries about what they liked about it were variations of "It's just so good!". To better understand the smell and taste they experienced, we asked if there were items that the soil reminded them of.

One clear commonality in behavior is that most geophagy occurs during pregnancy. Of the 2,367 pregnant women interviewed, 169 (7.1%) stated that they had eaten earth in the course of their current pregnancy. Of children 2–5 years old, 4.5% engaged in geophagy, 0.2% of non-pregnant women engaged in geophagy, and no adult men or elderly men or women engaged in pica (Young 2008). No data are available on the behavior of 5–18 year olds. The marked relationship between geophagy and pregnancy was reinforced by participants' comments; many of them ceased craving earth after giving birth and thought that geophagy was "good" only if pregnant (Table 1).

Physical Characteristics Macroscopic Appearance Although the geophagic materials typically are described as "soils", a more accurate description for some would be "saprolites" in that they resemble decomposed and easily disaggregated rock, such as that often found at the base of soil profiles developed by *in situ* weathering (Table 2). Colors were highly variable, ranging from white to different shades of brown and red. Large textural differences also were apparent, with *udongo* and *mchanga* samples being sandy, whereas the *ufue* and *vitango pepeta* ones were more clay-rich. The latter appeared to consist of weathered shale or clay, while *udongo* and *mchanga* probably represented alluvial sandy material.

pH and Clay Content There was a surprisingly large range of pH values and proportion of clay and non-clay fractions (Table 3). Alkaline pH values characterized the *udongo* and *mchanga* samples, with one sample (835) yielding a pH of

Table 2 Color and macroscopic description of 11 geophagic samples from Pemba

Sample number	Name	Munsell color	Description
818	*Udongo*	Very pale brown (10YR 8/3)	Coarse blocky structure; sandy texture; contains fragments of carbonized wood; slakes immediately in water.
832	*Udongo* (house)	Light yellowish brown (2.5Y 6/3)	Sandy material containing white inclusions and organic matter; slakes immediately in water.
835	*Udongo* (house)	Dark grayish brown (10YR4/2)	Sandy material received in crushed disaggregated state; some white grains observed.
839	*Udongo*	Light brownish gray (2.5Y 6/2)	Blocky, slightly indurated sandy material; porous structure with some rootlets; slakes immediately in water.
849	*Udongo* (house)	Light red (2.5YR 6/6)	Coarse blocky decomposed material of silty texture; light and porous; slakes immediately in water.
833	*Ufue*	White (5Y 8/1)	Clayey sand; disperses easily but doesn't slake.
845	*Ufue*	White (7.5YR 8/1) with pinkish white (7.5YR 8/2) variegations	Soft decomposed clayey material; very coarse blocky; disperses easily but doesn't slake.
834	*Vitango pepeta*	Reddish yellow (7.5YR 7/6) with some red patches (10R 4/6), others pinkish white	Coarse blocky material, soft and decomposed; strongly variegated and patchy in color; clayey/silty texture.
812	*Vitango pepeta*	Pale whitish yellow (2.5Y 8/3) with yellowish (10YR 8/8) mottles	Coarse platy structure; weathered shale; clayey; disperses easily but doesn't slake immediately.
842	*Vitango pepeta*	White (7.5YR 8/1) with pink (7.5YR 8/2) patches on weathered surfaces	Soft decomposed clayey material; coarse crumbs (>10 mm) and medium blocks (10–20 mm); disperses easily but doesn't slake.
838	*Mchanga*	Pale yellowish (2.5YR 7/3)	Fine sandy material.

Table 3 pH and particle size analysis (%) data of 11 geophagic samples from Pemba

Sample number	Material	pH (H_2O)	<2 μm	2–20 μm	20–2000 μm
818	*Udongo* (field)	7.20	8.2	7.5	84.2
832	*Udongo* (house)	7.62	11.0	11.7	77.3
835	*Udongo* (house)	10.44	12.8	11.9	75.3
839	*Udongo* (field)	8.17	6.5	5.3	88.1
849	*Udongo* (field)	4.72	13.8	7.9	78.3
833	*Ufue*	5.02	4.8	15.6	79.6
845	*Ufue*	4.95	25.9	12.3	61.8
834	*Vitango pepeta*	4.94	22.9	25.2	51.9
812	*Vitango pepeta*	4.80	33.6	31.6	34.8
842	*Vitango pepeta*	4.54	17.6	23.0	59.4
838	*Mchanga*	8.45	0.7	1.4	97.9

10.4. This sample came from a house wall to which lime may have been added. In contrast, the *ufue* and *vitango pepeta* materials were distinctly acidic with pH values in the 4.54–5.02 range. Analysis of the clay contents confirmed the impression gained from the assessment of texture, with the *mchanga* sample containing <1% clay in contrast to *vitango pepeta* with 23–34% clay.

Chemical Characteristics. Major Elements Both *udongo* and *mchanga* samples were very siliceous; total silica contents ranged from 77 to 94% and with correspondingly low alumina and ferric oxide contents (Table 4). Values for alkalis and alkaline earths were variable. In contrast, the *ufue* and *vitango pepeta* samples were less siliceous and more aluminous. In the former, ferric oxide contents were comparable to those of *udongo*, and alkalis and alkaline earths showed a slightly smaller range of values. The *vitango pepeta* samples, however, were more iron-rich than any of the samples, with ferric oxide ranging from 1.56 to 8.06%.

Trace Elements Total trace element contents of the geophagic soils (Table 5) usually were lower than the range of values found for mineral soils with <5% organic matter derived from all types of parent materials, as assessed by Mitchell (1964). Not all trace elements analyzed are of biological significance from either a nutritional or toxicity point of view, but for the sake of completeness all results are shown. Trace elements of interest in the context of human nutrition, Co, Cu, I, and Zn, are all in the low or normal range when compared to the usual range in mineral soils. The same is true of trace elements such as As and Pb, which are associated with toxicity.

Table 4 Bulk chemical (major element) analyses in geophagic samples[1]

	Udongo					*Ufue*		*Vitango pepeta*			*Mchanga*
Sample number	818	832	835	839	849	833	845	834	812	842	838
SiO_2	90.97	89.07	81.45	93.49	77.02	71.58	78.61	61.61	52.08	54.05	94.48
TiO_2	0.45	0.37	0.29	0.39	0.63	0.15	0.24	0.92	0.60	0.38	0.21
Al_2O_3	7.58	7.75	7.92	5.04	16.13	19.78	17.78	23.20	32.30	34.58	3.12
Fe_2O_3[1]	0.75	0.88	0.74	0.73	2.72	0.84	0.46	8.06	5.74	1.56	0.57
MnO	0.01	0.01	0.04	0.02	0.01	0.00	0.01	0.01	0.01	0.01	0.01
MgO	0.14	0.18	0.47	0.17	0.25	0.20	0.17	0.70	1.16	0.74	0.13
CaO	0.18	0.77	2.97	0.14	0.04	0.17	0.06	0.11	0.04	0.04	1.79
K_2O	0.60	0.33	1.83	0.32	0.14	2.12	0.05	0.34	0.72	0.30	0.33
Na_2O	0.03	0.01	0.76	0.04	0.00	0.44	0.03	0.00	0.06	0.00	0.05
P_2O_5	0.01	0.04	0.09	0.02	0.00	0.01	0.00	0.02	0.02	0.00	0.02
Sum	100.7	99.42	96.56	100.4	96.94	95.31	97.41	94.96	92.73	91.65	100.7
LOI[2]	0	0.44	3.22	0	2.84	5.14	2.15	4.87	7.12	8.01	0

[1] All Fe expressed as ferric

[2] LOI—loss on ignition is calculated by difference from 100% oxides, not measured LOI

Table 5 Trace element contents (ppm) of 11 Pemban geophagic soils and usual content of trace elements in mineral soils

	Udongo					*Ufue*		*Vitango pepeta*			*Mchanga*	*Usual Mineral Soil Content*[a]
Sample number	818	832	835	839	849	833	845	834	812	842	838	
As	4	3	2	2	5	3	1	11	7	2	2	1–80
Ba	195	105	99	67	54	795	23	72	66	53	120	100–6000
Ce	15	17	7	16	23	13	8	61	27	19	17	–
Co	1	1	1	2	0	3	1	0	2	2	0	1–80
Cr	77	47	42	37	86	82	63	190	130	123	29	7–1000
Cu	<2	<2	<2	<2	<2	<2	<2	<2	8	<2	<2	3–100
Ga	6	7	7	5	17	19	17	26	22	26	3	10–100
I	5	9	5	7	20	3	8	10	3	9	4	2–8
La	8	7	6	7	6	9	8	34	13	9	5	–
Nb	10	10	9	10	13	7	9	16	12	9	8	–
Ni	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	7–800
Pb	12	25	9	10	11	20	7	22	16	13	15	3–120
Rb	10	6	24	6	5	40	1	12	22	8	5	12–900
Sr	32	32	123	16	14	115	9	21	15	12	43	70–1000
Th	4	3	2	3	4	2	3	9	5	3	2	–
U	0	0	1	1	1	1	0	2	1	1	0	–
V	15	16	18	14	50	31	20	120	111	72	8	12–800
Y	9	9	8	9	9	8	8	15	9	9	8	–
Zn	12	37	24	9	14	14	4	26	39	29	44	10–500
Zr	420	291	240	316	384	58	125	446	88	159	218	80–3000

[a] According to Mitchell (1964), for iodine according to Goldschmidt (1954)

Mineralogical Characteristics. Bulk Soil Analysis Mineral analyses of the bulk soils (Table 6) were reasonably consistent with the results of bulk chemical analysis (Table 4). The *udongo* and *mchanga* samples were quartz-rich with contents ranging from 69–92% and corresponding closely with SiO_2 values. In contrast, the clay-rich *vitango pepeta* samples contained smaller amounts of quartz (4–35%) and higher kaolin contents (58–95%) corresponding to their lower SiO_2 (52–62%) and higher $A_{12}O_3$ (23–35%) values. The *ufue* samples occupied a position intermediate between *udongo* and *vitango pepeta* with respect to quartz and kaolin contents. The main iron oxide mineral in the geophagic soils was goethite [FeO(OH)] rather than hematite (Fe_2O_3), with *vitango pepeta* samples being the most goethitic (8–11%) and correspondingly having the highest ferric oxide content (1.6–8.2% Fe_2O_3). There is a general correspondence between amounts of calcite and K-feldspar, particularly at the high end of the range, with CaO and K_2O values, respectively.

Clay Mineral Analyses Clay fractions of the geophagic soils were all dominated by kaolin-type minerals (Table 6). Representative XRD traces of the clay fractions from the various geophagic soils are shown in Fig. 2. In determining the relative kaolinite/halloysite contents of the soils, an initial assessment was made by comparing the intensities of the basal reflection at ~10 Å, due to the formation of the formamide/halloysite complex with the basal reflection at ~7Å, which represents the uncomplexed kaolinite mineral. From this, it was evident that kaolinite was dominant in 5

Table 6 Relative kaolinite/halloysite contents in the clay fractions of the geophagic soils as assessed from visual inspection of the XRD patterns before and after formamide treatment

Mineralogy	*Udongo*					*Ufue*		*Vitango pepeta*			*Mchanga*
	818	832	835	839	849	833	845	834	812	842	838
Kaolinite/ Halloysite	K dom.[a]	K≈ H	K dom.	K≈ H	K dom.	H dom.	K dom.	K dom.	H dom.	H dom.	H>K

[a] Dom. = dominant

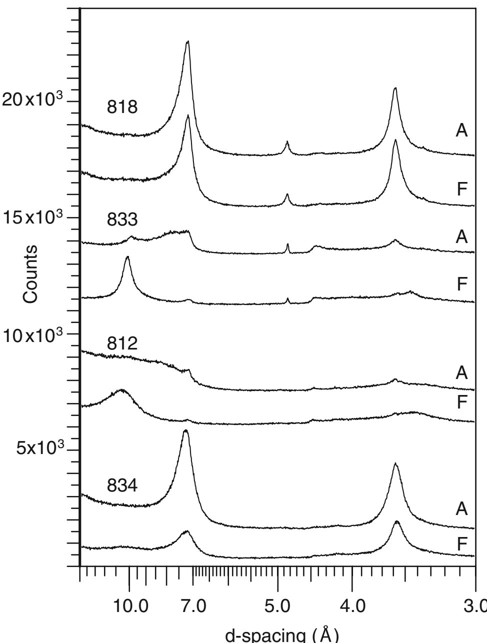


Fig. 2 XRD pattern of the air-dried (A) and formamide-treated (F) clay fractions. *Udongo* soil (sample 818) showing dominance of kaolinite (strong peak at 7Å) over halloysite (weak peak at 10Å) after formamide. A small amount of gibbsite is indicated (peak at 4.85Å). Two *Ufue* soil (sample 833) showing dominance of halloysite (strong peak at 10Å) over kaolinite (weak peak at 7Å) after formamide. A small amount of gibbsite is indicated (peak at 4.85Å)..*Vitango pepeta* soil (sample 812) showing dominance of halloysite (strong peak at 10Å) over kaolinite (weak peak at 7Å after formamide). Other features of these patterns suggest that this clay may be interstratified with smectite*, Vitango pepeta* soil (sample 834) showing dominance of kaolinite (strong peak at 7Å) over halloysite (weak peak at 10Å) after formamide

of the 11 samples, halloysite was dominant in 3 samples and sub-dominant in 1 sample (Table 6). In 2 samples, there were roughly equal amounts of both minerals. Both *ufue* and *vitango pepeta* samples could be either kaolinite dominant or halloysite dominant. *Udongo* tended to be kaolinite dominant whereas *mchanga* was more halloysitic. Quantification of the two minerals by a full pattern fitting method yielded results that were consistent with this initial assessment (Table 7), apart from sample 812 that had special features concerning mixed layering. Most clays also contained minor to trace amounts of gibbsite ($Al(OH)_3$), a mineral characteristic of highly weathered soils.

SEM Observations Geophagic materials examined by SEM included one sample of *udongo* (818), one of *ufue* (833), and two of *vitango pepeta* (812, 834) (Fig. 3). At low magnification, the *udongo* sample was found to consist of dense masses of aggregated clay arranged in a sub-parallel fashion that coated and bound together the majority sand-size particles (Fig. 3a). Clay coatings often have a globular appearance, but high magnification showed that they are composed primarily of individual, equant, flat, platy particles ~100–500 nm diameter (Fig. 3b). Occasional elongated particles also were observed. EDS analysis of the fine-grained particles revealed a dominance of Si and Al in roughly equal proportions, consistent with their identification as a kaolin mineral and with the XRD analysis of the clay fraction as being kaolinite-dominant.

The *ufue* sample also showed individual sand grains bound together by globular aggregates of clay. In some cases, the clay was observed to coat relatively fresh surfaces of feldspar (Fig. 3c). Higher magnification revealed that the coatings consisted of spherical micro-aggregates about 5 μm diameter and consisted of radially-arranged tube-like or lath-like particles that imparted a pin-cushion appearance to the micro-aggregate as a whole (Fig. 3d). Individual particles are 1–2 μm long and

Table 7 Full pattern fitting mineral analysis (wt. %) of bulk geophagic soils

Mineralogy	Udongo					Ufue		Vitango pepeta			Mchanga
	818	832	835	839	849	833	845	834	812	842	838
Quartz	88.2	85.8	76.0	90.2	68.6	47.9	70.0	35.2	3.5	18.4	91.8
Plagioclase	0.0	0.2	0.7	0.0	0.1	2.3	0.0	0.1	0.0	0.0	0.0
K-feldspar	2.4	1.0	1.4	0.6	0.5	11.6	0.0	1.7	0.0	0.0	1.4
Calcite	0.0	0.6	4.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5
Hematite	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0
Goethite	0.0	0.0	0.0	0.0	2.0	0.0	0.0	3.6	1.8	0.4	0.0
Gibbsite	0.0	0.6	0.0	0.0	0.5	0.4	0.0	0.4	0.0	0.4	0.0
Kaolin/smectite?	1.0	2.6	4.2	2.8	4.2	4.6	0.0	18.1	44.2	20.9	0.4
Kaolinite	5.0	4.4	10.1	3.0	14.3	8.6	23.4	25.1	21.3	21.4	1.2
Halloysite	3.3	4.9	3.2	3.3	9.8	24.7	6.6	14.3	29.1	38.5	1.7
Total-clay	9.3	11.9	17.5	9.1	28.3	37.9	30.0	57.5	94.6	80.8	3.3

Total-clay is sum of kaolin/smectite, kaolinite, and halloysite

Fig. 3 SEM images and EDS spectra of selected samples **a**: *Udongo* (818) showing aggregated clay coating and binding sand particles together. **b**: *Udongo* (818) showing platy morphology and small size of constituent particles. **c**: *Ufue* (833) showing clay layer with globular morphology coating relatively unweathered feldspar grain. **d**: *Ufue* (833) showing delicate tubular morphology of halloysite with a pure Al, Si, and O composition. **e**: *Vitango pepeta* (834) showing open sandy texture and binding network of clay. **f**: *Vitango pepeta* (834) showing crinkled mass of platy clay particles containing minor Mg in their composition. **g**: *Vitango pepeta* (812) showing aggregated clay texture. H: *Vitango pepeta* (812) showing crinkled platy morphology and curled edges of constituent clay particles, also containing minor Mg in their composition

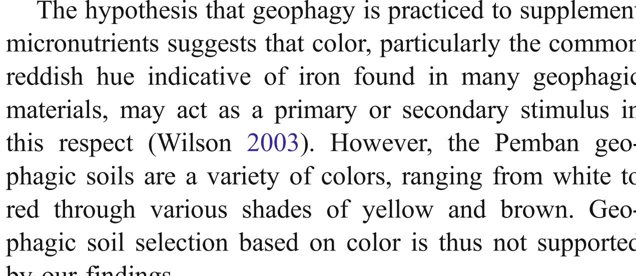
~0.2 μm diameter. The morphology of these particles is identical with that of tubular halloysite. Identification as halloysite is consistent with both the EDS analysis that showed Si and Al in equal proportions, and with the XRD analysis of the clay fraction.

The two *vitango pepeta* samples examined by SEM had somewhat different clay contents and, according to both clay fraction and bulk sample XRD analyses, also differed in the relative importance of kaolinite, halloysite, and mixed-layer kaolin. Sample 834 was kaolinite-dominant whereas sample 812 appeared to consist mainly of mixed-layer kaolin/smectite of halloysitic character with respect to the formamide test. Low magnification SEMs of 834 showed sandy material with abundant pore space held together by a meshwork of clay coatings over relatively fresh quartz and feldspar grains (Fig. 3e). The clay itself was composed of a crinkled mass of flat, platy particles, often with curled edges, about 1–2 μm diameter (Fig. 3f). There was abundant micro-pore space between the platy particles. The clay-rich nature of sample 812 is evident from low magnification SEMs, the whole consisting of crinkled flakes and aggregates arranged in sub-parallel fashion and with abundant void space (Fig. 3g). The constituent particles were mostly of an equant, platy shape, often bent or sinuous with curled edges, and ranged from ~0.5 to 2 μm diam (Fig. 3h). These crinkled, curled, and sinuous morphologies lend support to the identification by XRD of kaolin/smectite in the *vitango pepeta* samples. Furthermore, in both samples EDS analysis showed that the composition of the clay particles may be different from those described for *ufue* and *udongo* above in that they have a slightly higher Si:Al ratio and minor, but obvious, K, Mg, and Fe contents. Although minor in amount, the presence of Mg in the *vitango pepeta* clay particles is significant because it is consistent with the relatively high MgO content of the bulk *vitango pepeta* samples as determined by XRF (Table 4).

Discussion

In general, the geophagic soils we analyzed were highly variable in color and chemical composition, and had low concentrations of trace elements. The sole commonality was the presence of a kaolin mineral. Geophagy was practiced almost uniquely by pregnant women and young children.

No respondents described eating earth as a response to hunger or because other food was not available (Table 1). There was no significant difference in economic status or food intake between geophagists and non-geophagists (Young 2008). Therefore, hunger does not explain geophagy in this population.



The hypothesis that geophagy is practiced to supplement micronutrients suggests that color, particularly the common reddish hue indicative of iron found in many geophagic materials, may act as a primary or secondary stimulus in this respect (Wilson 2003). However, the Pemban geophagic soils are a variety of colors, ranging from white to red through various shades of yellow and brown. Geophagic soil selection based on color is thus not supported by our findings.

A second piece of evidence against the micronutrient deficiency hypothesis is the highly variable major chemical composition of the geophagic soils. *Ufue* and *vitango pepeta* soils are extremely low in Ca (<0.2% CaO), whereas the concentration in *udongo* soils is variable. Indeed, the correlation of CaO with calcite in *udongo* samples from house walls suggests that the CaO content is a result of lime addition during construction, rather than a property of the original soil. As for Fe, the *vitango pepeta* samples are notably rich in this element (1.6–8.1% Fe_2O_3), whereas all of the other soils, with one exception, are poor (<1% Fe_2O_3).

Furthermore, the concentrations of trace elements of biological significance in the soils are uniformly low when compared to the usual range found in all types of mineral soils. It is difficult to conceive that such a negative similarity could act as stimulus to the selection of soils to be consumed. Furthermore, micronutrient cravings have yet to be identified in humans (Johns and Duquette 1991).

Our research would be strengthened by determining the bioavailability of these trace elements. However, it is probable that none of them is bioavailable to any significant extent because of the highly weathered nature of the soils. Weathering tends to remove mobile or "available" nutrients leading to a highly leached soil where any nutrient elements are associated with resistant minerals not susceptible to decomposition. Furthermore, the dominant mineral form of Fe is goethite, which is the least soluble of any of the common iron oxide minerals (Schwertmann and Taylor 1989). It seems unlikely that geophagists are regularly obtaining any mineral nutrients from these soils.

It has been suggested that geophagic soils are selected because of their pleasant unctuous texture associated with richness in clay (Wilson 2003). Clayey soils are consistent with the protection hypothesis; a number of clays have been proven efficacious at quelling gastro-intestinal distress and detoxifying harmful pathogens and chemicals (Gonzalez et al. 2004; Leonard et al. 1994; Theodorou et al. 1994). However, our data show that the texture of these soils is extremely diverse ranging from sand, i.e., *udongo* and *mchanga* materials that consist predominantly of quartz, to clay i.e., *vitango*

pepeta largely made up of clay minerals. The *ufue* soils form an intermediate group between these two extremes. Selection of these geophagic soils simply on the basis of overall clay mineral content is not supported by the findings presented here, although it is notable that the four Swahili names are obviously correlated with total clay mineral content as determined by XRD.

Despite the many differences, the samples have one similarity: clay mineralogy. All clay fractions are dominated by a kaolin-type mineral, either kaolinite, halloysite, or a mixture of both. Additionally the *vitango pepeta* samples contain a significant amount of kaolin/smectite. Thus, it seems that the dominant type of kaolin mineral in the Zanzibar geophagic soils could be kaolinite, halloysite, or kaolin/smectite. This mineralogical similarity is consistent with the protection hypothesis, given that kaolin minerals have long been used in pharmaceutical formulations to both treat the causes and the symptoms of gastrointestinal distress (Vermeer and Ferrell 1985; Wakibara et al. 2001; Carretero 2002). The beneficial role of the kaolin minerals is based upon their ability to coat and adhere to the gastric and intestinal mucus membrane, thus protecting against toxins, bacteria, and viruses, and adsorbing excess water in the feces. (Allen and Leonard 1985; Leonard et al. 1994; Theodorou et al. 1994; Gonzalez et al. 2004).

It is plausible that the occurrence of kaolin-type minerals in all Zanzibari geophagic soil samples serves to protect the health of the consumer. The high prevalence of geophagy during pregnancy and early childhood, the times during which individuals experience the greatest biological vulnerability, lends support to this hypothesis. However, future analyses of Pemban soils must include mineralogical analysis of those not eaten, to ascertain that the clay mineralogy of all soils in Pemba is not dominated by kaolin.

No one has yet elucidated a mechanism by which humans can identify the presence of kaolin minerals in soils. One clue perhaps is the importance of smell in the selection of geophagic materials. The scent of earth, especially when wet, has been mentioned by many geophagists around the world (Hooper and Mann 1906; Forsyth and Benoit 1989; McIntyre 2000). Further study of human's capacity to identify and distinguish between odors of different clay minerals could be done easily, and may contribute further to our understanding of the selection and function of geophagy.

In summary, the geophagic soils of Pemba Island are diverse in color, texture, major element chemistry, and clay mineral content. The trace element contents of the soils, whether of biological or non-biological significance, are uniformly low, and the bioavailability of those that are present is dubious. The two commonalities observed among these soils is the dominance of kaolin in the clay fraction and the practice of geophagy by those who are biologically vulnerable. The palliative, protective and detoxifying properties of kaolin lend support to the hypothesis that geophagy is a protective behavior.

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Semiochemical-Mediated Oviposition Avoidance by Female House Flies, *Musca domestica*, on Animal Feces Colonized with Harmful Fungi

Kevin Lam · Michelle Tsang · Audrey Labrie · Regine Gries · Gerhard Gries

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Abstract House flies, *Musca domestica*, utilize ephemeral resources such as animal feces for oviposition and development of larval offspring, but they face competition with fungi that colonize the same resource. We predicted that house flies avoid oviposition on feces well-colonized with fungi, thereby reducing fungal competition for larval offspring. Working with fungal isolates from chicken feces, we have previously shown that prior establishment of *Phoma* spp., *Fusarium* spp., or *Rhizopus* spp. on feces significantly reduced oviposition by house flies. Here, we report that, in the headspace volatiles of these three fungal genera, five compounds (dimethyl trisulfide, an unknown, 2-phenylethanol, citronellal, norphytone) elicit responses from house fly antennae. In behavioral bioassays, dimethyl trisulfide and 2-phenylethanol significantly reduced oviposition by house flies. We conclude that fungus-derived volatiles serve as semiochemical cues that help house flies avoid resources colonized with fungal competitors for the development of larval offspring.

Keywords House flies · *Musca domestica* · *Phoma* spp. · *Rhizopus* spp. · Fungi · Animal feces · Resource competition · Oviposition · Semiochemicals · Dimethyl trisulfide · 2-Phenylethanol

Introduction

Microorganisms competing with insect larvae for ephemeral resources can adversely affect larval development, particularly when these microorganisms are first to colonize the resource. For example, microorganisms have been shown to curtail the number of burying beetle, *Nicrophorus quadripunctatus*, offspring that complete development (Wilson et al. 1984). Competition with fungal (Fuentes-Contreras et al. 1998; Askary and Brodeur 1999), viral (Hochberg 1991), or bacterial (Chilcutt and Tabashnik 1997) pathogens of host insects adversely affects even parasitoid larvae, which exploit living organisms. The outcome of this competition is dependent upon the relative timing of infection. Within pea aphids, *Acyrthosiphon pisum*, the presence of bacteria is so detrimental to larvae of the parasitoid *Aphidius ervi* that these bacteria persist as beneficial endosymbionts of the aphid (Oliver et al. 2003).

Competition with fungi is particularly costly for larvae of the house fly, *Musca domestica.* Females lay their eggs on fleeting organic resources such as manure (Keiding 1974; Fatchurochim et al. 1989). Larvae hatch within 24 h and develop through three instars in 5–7 days. After a 5-d pupal period, adult males and females eclose. When fungi were established on a fleeting resource 3 days before house flies oviposited, all of the resulting larvae perished (Zvereva 1987).

When the relative timing of resource colonization strongly affects the outcome of competition, harmful competitors often are recognized and avoided. Many parasites and parasitoids recognize host-marking pheromones and avoid oviposition on the same host (Li 2006; Stelinski et al. 2007). The mistletoe-feeding pierid butterflies *Delias argenthona* and *D. nigrina* have overlapping host ranges, but they oviposit more selectively on specific host species when heterospecific competitors are present (Orr 2008). Mosquitoes avoid larval habitats with intra- or interspecific competitors (Munga et al. 2006). Adult ladybirds avoid oviposition on plants with other aphid predators already present (Sarmento et al. 2007). Leaf beetles

K. Lam · M. Tsang · A. Labrie · R. Gries · G. Gries (✉)
Department of Biological Sciences, Simon Fraser University, Burnaby, BC V5A 1S6, Canada
e-mail: gries@sfu.ca

(Roeder et al. 2007) and Australian weevils (Rayamajhi et al. 2006) avoid plants already colonized with a rust fungus, and larvae and adults disperse rapidly when placed on fungus-infected host plants. Finally, the hymenopteran parasitoid *Aphidius ervi* avoids oviposition on plants where aphid hosts were killed by the entomopathogenic fungus *P. neoaphidis* (Baverstock 2005).

All the above examples are interactions between specific insects and microorganisms. House flies, in contrast, face diverse fungi as resource competitors, each with a different establishment rate and competitive effect on house fly larvae. To determine whether houseflies have adapted to avoid fungus-infested resources, we isolated fungal strains from different types of animal feces, and tested the hypotheses that house flies avoid oviposition on feces that is colonized with competitive fungi, and that the avoidance is due to fungus-derived semiochemicals.

Methods and Materials

Experimental Insects Adult house flies were kept in cages at 50–80% relative humidity (RH), 22–30°C and a 16L:8D photoperiod, and were provisioned with water, sugar cubes, and skim milk powder *ad libitum*. House fly eggs were collected on glass Petri dishes (50×10 mm) containing sterile skim milk agar (SMA; 15 ml water, 0.12 g nutrient broth powder, 0.24 g skim milk powder, 0.3 g agar) and reared to adult insects in artificial diet prepared from wheat bran (400 g), brewers yeast (15 g), molasses (15 ml), and water (700 ml), with a supplemental protein paste prepared from skim milk powder and water (Lam et al. 2007).

Isolation and Identification of Fungal Strains To isolate feces-colonizing fungi, feces pats from free-range organic chickens, sheep, horses, and wild barn swallows were collected at the Wind's Reach Farm, Langley, B.C., Canada. A sterile inoculating loop was inserted into the center of each pat and streaked across Petri plates (90×15 mm) containing feces agar (20 ml water, 2.5 g chicken feces that had been sterilized by stirring and autoclaving >3 times at 121°C for 45 min, plus 0.4 g agar). This procedure was to select for fungi that can grow on chicken feces nutrients alone. Mycelia from separate fungal colonies were isolated and incubated on Petri plates (90×15 mm) containing potato dextrose agar (PDA; 20 ml water, 0.48 g potato dextrose broth powder, 0.4 g agar) at 24°C and 85% RH. To identify the fungi that expressed behavioral activity in experiments 1–6, each strain was grown in a Sabouraud dextrose broth, and a Qiagen DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen®, Canada, Cat. No. 51304) according to the manufacturer's instructions. Sequencing of 18S rRNA was performed using Fun 1 and Fun 2 primers (unpublished; designed by Dr. Bryne, Animal Health Centre, Abbotsford, B.C., Canada). Fungi were identified based on colony morphology and by comparing 18S rRNA sequences to the Genbank database.

Hypothesis 1: House Flies Avoid Oviposition on Feces that is Colonized with Fungi In two-choice experiments 1–6 (N=10 each), we compared oviposition by house flies on sterilized feces (control) and sterilized feces mixed with one of six fungal isolates (treatment) as follows: *Phoma* sp., *Rhizopus* sp., *Fusarium* sp. (SFU-1), *Fusarium* sp. (SFU-2), Unknown 1, and unknown 2.

Chicken feces pats (0-24 h old) were blended and sterilized. An 8-g aliquot of treatment feces then was thoroughly mixed with the mycelia and spores (if present) of a 1-wk-old PDA culture of one fungal isolate. An 8-g aliquot of control feces was mixed without addition of fungi. Aliquots (8 g) of each feces mixture were packed tightly into separate sterile plastic Petri dishes (35×10 mm). To generate an attractive crevice for oviposition, a 5-mm diam well was poked into the center of each dish with a sterile metal spatula.

Control and treatment dishes then were incubated separately for 3 d in a sterile glass chamber (15 cm ID× 28 cm) through which filtered and humidified air was drawn at 0.5 l/min. Control and treatment dishes were placed in randomly assigned opposite corners of a mesh cage (30×30×45 cm) containing 20 male and 20 female house flies. When oviposition ceased after 3–6 h, the eggs on each dish were removed and weighed or counted (~15,000 eggs/g).

The weights/counts of eggs deposited on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited. This approach was chosen because of variation in the total amount of eggs deposited by the flies in each replicate. Two-tailed one-sample bootstrap tests on the means were conducted to determine whether the mean percentages of eggs deposited on treated feces was significantly different from 50%. For each bootstrap test, 1000 bootstrap samples were randomly generated with replacement, and each sample contained the same number of replicates as the original data set. The mean percentage of the original data set was then compared with the mean percentages of its bootstrap samples (SAS, ver. 9.1; SAS Institute, Cary, NC, U.S.A.) with α=0.05.

Hypothesis 2: Fungi Produce Semiochemicals that Inhibit Oviposition by Houseflies; Acquisition of Headspace Volatiles Ten Petri plates (90×15 mm) containing Potato Dextrose+ Feces agar (20 ml water, 0.48 g potato dextrose broth powder, 2.5 g sterilized chicken feces, 0.4 g agar) were

inoculated with mycelia of a fungal isolate that inhibited oviposition in experiments 1–6. Plates in staggered form were placed into a sterile glass chamber (15 cm ID×28 cm) through which filtered and humidified air (see next paragraph) was drawn at 1.0 l/min for 1 wk. Volatiles were adsorbed on 0.2 g of Porapak Q (50–80 mesh, Waters Associates Inc. Milford, MA, USA) inside a Pyrex glass tube (3.8×40 mm), and desorbed with 2 ml of pentane. Headspace volatiles of sterile potato dextrose+feces agar served as a control and were also adsorbed on Porapak Q.

To ensure that clean air entered the autoclaved aeration chamber and to prevent introduction of bacteria onto the fungal plates, air was filtered through 3.5 g of autoclaved (45 min at 121°C) and oven-dried (overnight at 110°C) Porapak Q that was tightly packed into a glass tube (140×8 mm ID). Each day, agar plates were visually monitored for bacterial and fungal contamination, and none was observed.

Analyses of Headspace Volatiles To identify volatiles that elicited responses from house fly antennae, Porapak Q extracts were concentrated and 1-µl aliquots analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al. 1975; Gries et al. 2002). Analyses employed a Hewlett Packard (HP) 5890A gas chromatograph equipped with a GC column (30 m×0.25 mm ID) coated with DB-5 (J&W Scientific, Folsom, CA, USA) and helium as a carrier gas (35 cm s^{-1}) with the following temperature program: 50°C (1 min), 20°C/min to 280°C. For GC-EAD recordings, an antenna was pulled from a live female house fly and suspended between two glass capillary electrodes (1.0×0.58×100 mm) (A-M Systems, Inc., Carlsborg, WA, USA) filled with saline solution (Staddon and Everton 1980). Mass spectra of antenna-stimulating components were obtained with a Saturn 2000 Ion Trap (Varian) fitted with a DB-5 column. Compounds were identified by comparing their retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature [dimethyl trisulfide (Swearingen et al. 2006), phenylacetaldehyde, norphytone (Pherobase 2010), citronellal, 2-phenylethanol (Adams 1989)], and with those of authentic samples.

Two-choice Experiments with Candidate Semiochemicals Experiments 7–11 (N=10–24 each) tested each of the candidate semiochemicals (dimethyl trisulfide, phenylacetaldehyde, 2-phenylethanol, cironellal, norphytone) to determine its effect on oviposition by house flies. Two glass Petri dishes (50×10 mm) containing sterile skim milk agar (15 ml water, 0.12 g nutrient broth powder, 0.3 g agar) served as oviposition sites. A thin strip of agar (width< 5 mm) was excised across the middle of the plate to provide a crevice conducive for oviposition. The candidate neat semiochemical was released from a 5-µl micro-capillary tube which was sealed with plasticine at one end and placed within the crevice such that the open end was near the center of the plate. An empty tube was prepared analogously and placed into the crevice of the control plate. In each replicate, the two plates were randomly assigned to opposite corners of a mesh cage (30×30×45 cm) that contained 20 male and 20 female house flies. Resulting egg counts/weights were analyzed as described above. In all replicates, release rates (µg/h) of candidate semiochemicals were determined volumetrically by measuring with a digital calliper the capillary tubes' length of released chemical, and by taking into account (*i*) the total length of the tube (40 mm), (*ii*) its total holding capacity (5 µl), (*iii*) the compound's density, and (*iv*) the duration (16 h) of the bioassay.

Results

Hypothesis 1: House Flies Avoid Oviposition on Feces Colonized with Fungi In experiments 1 and 2, sterilized feces inoculated with *Phoma* sp. or *Rhizopus* sp. received significantly fewer eggs from female house flies than sterilized feces with no added fungi (Fig. 1, P<0.001). In contrast, in experiments 3–5, the presence of *Fusarium* sp. (SFU-1 or SFU-2) or Unknown Fungal Isolate 1 had no effect on oviposition decisions (Fig. 1, P>0.05). In experiment 6, feces inoculated with Unknown Fungal Isolate 2 received significantly more eggs than feces devoid of fungi (Fig. 1, P<0.05).

Hypothesis 2: Fungi Produce Semiochemicals that Inhibit Oviposition by House Flies GC-EAD analyses of headspace volatile extracts of *Phoma* and *Rhizopus* fungi (which strongly inhibited oviposition by female house flies) and of *Fusarium* sp. (SFU-1) (which appeared to have some effect on oviposition), revealed six compounds that consistently elicited responses from house fly antennae (Fig. 2). By comparing their mass spectra and retention indices with those of authentic standards, antenna-stimulatory components 1, 2, 4, 5, and 6 were identified as dimethyl trisulfide, phenylacetaldehyde, 2-phenylethanol, citronellal, and norphytone, respectively. Component 3 remains unknown. Of these six components, only phenylacetaldehyde also was found in headspace volatiles of sterile feces agar (data not shown). Thus, phenylacetaldehyde was excluded form experiments 7–10 that tested the effect of each of the fungal volatiles dimethyl trisulfide, 2-phenylethanol, citronellal, or norphytone on oviposition decisions by house flies In experiments 7–10, dimethyl trisulfide and 2-phenylethanol strongly reduced oviposition by females (Fig. 3A, experiments 7–9), whereas neither citronellal nor norphytone had a behavior-modifying effect (Fig. 3A, experiment 9,10). These

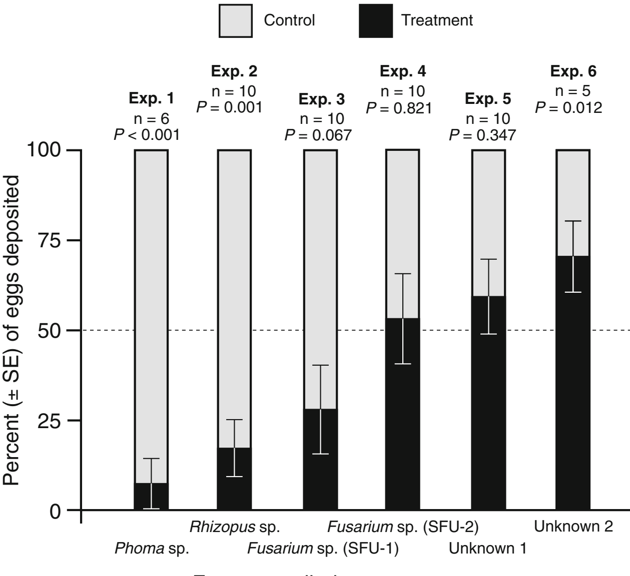


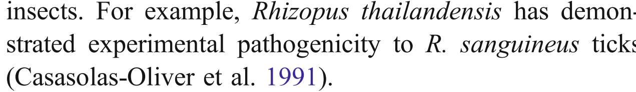
Fig. 1 Percent of house fly eggs oviposited on sterile chicken manure incubated for 3 days with (treatment) and without (control) experimental infestation with a fungal strain. Replicates in which no oviposition occurred were omitted

contrasting results could not be attributed to contrasting release rates of semiochemicals because citronellal and norphytone had greater release rates than the oviposition-inhibiting semiochemicals (Fig. 3B).

Discussion

Our data show that female house flies avoid oviposition on ephemeral resources that have been colonized by specific fungal resource competitors, likely due to detection of fungal volatiles.

The presence of particular genera of fungi such as *Phoma* and *Rhizopus*, rather than just the presence of any fungus, affected oviposition by house flies on feces. *Phoma* is a genus of common soil fungi that contains many plant pathogenic species that cause rots and blight (Gray et al. 2008; Davidson et al. 2009), but it also contains species affecting insects. *Phoma aspidioticola*, for example, is highly pathogenic to the scale insect *Aspidiotus destructor* but not to the plant host *Syzygium cumini* (Narendra and Rao 1974). *Rhizopus* is a genus of molds that includes fungi found in soil, decaying fruit and vegetables, old bread, and animal feces (El-Mougy et al. 2008; Rodriguez et al. 2008; Serrano-Garcia et al. 2008; Takahashi et al. 2008). However, some species are plant pathogens (Prom and Perumal 2008; Zhao et al. 2008), cause infections in humans and animals (Landlinger et al. 2008), or affect


insects. For example, *Rhizopus thailandensis* has demonstrated experimental pathogenicity to *R. sanguineus* ticks (Casasolas-Oliver et al. 1991).

By avoiding oviposition sites colonized with potentially pathogenic fungi, female house flies make choices that enhance their fitness. Larval diet inoculated with mycelia and/or spores of the oviposition-inhibiting fungi *Fusarium* spp., *Phoma* spp., and *Rhizopus* spp. had detrimental effects on the number of house fly larvae that completed development (Lam et al. 2009). This decrease in larval survival may not occur with other fungi [e.g., *Fusarium* spp. (SFU-1)] that had little or no impact on oviposition by female house flies. Alternatively, these fungi may simply lack the semiochemical cues that indicate the presence of (harmful) fungi.

Release rates of synthetic semiochemicals from glass capillary tubes in oviposition experiments 7–10 exceeded those from agar cultures of *Rhizopus* spp. by 100–500 times. This, however, seemed justified because levels of semiochemical emission from *Rhizopus* spp. are likely to have varied greatly during the 7-d aeration period, with peak emissions assumed to have coincided with a short (~1 d) period of rapid fungal growth (when nutrients were plentiful), and with much lower levels of emissions before and after that period.

Each fungal isolate with adverse effects on the development of house fly larvae (Lam et al. 2009) emitted dimethyl trisulfide and 2-phenylethanol, each of which strongly reduced oviposition by house flies on feces. Neither of these two semiochemicals has been reported to affect oviposition decisions by flies, but 2-phenylethanol is a bacteria-derived putative oviposition semiochemical for *Anopheles gambiae* mosquitoes (Lindh et al. 2008).

Dimethyl trisulfide and 2-phenylethanol are well known in diverse ecological contexts. For examples, 2-phenylethanol is released from the hair pencils of courting males in noctuid and pyralid moths (Kuwahara 1980; and references cited therein). As a plant semiochemical, 2-phenylethanol adds to the attractiveness of plants to their respective herbivores (e.g., Zilkowski et al. 1999), pollinators (e.g., Knudsen and Tollsten 1993), or predators in tritrophic systems (e.g., Zhu and Park 2005). Dimethyl trisulfide is a major constituent of the flower of the voodoo lily, *Sauramutum guttatum,* and of the stinking mushroom, *Phallus impudicus* (Borg-Karlson et al. 1994). It is also present in floral volatiles of bat-pollinated plants, and in the essential oils of garlic, *Allium sativum* (Harborne et al. 1999; Kim et al. 2004). It is a major aroma component of cooked brassicaceous vegetables (Maruyama 1970), an indicator of decaying meat (Brown 1982), and a semiochemical of the dead-horse arum, *Helicodiceros muscivorus,* a plant which fools flies into pollinating it by emitting a smell like a rotting carcass (Stensmyr et al. 2002). Dimethyl trisulfide is also a volatile odor constituent of

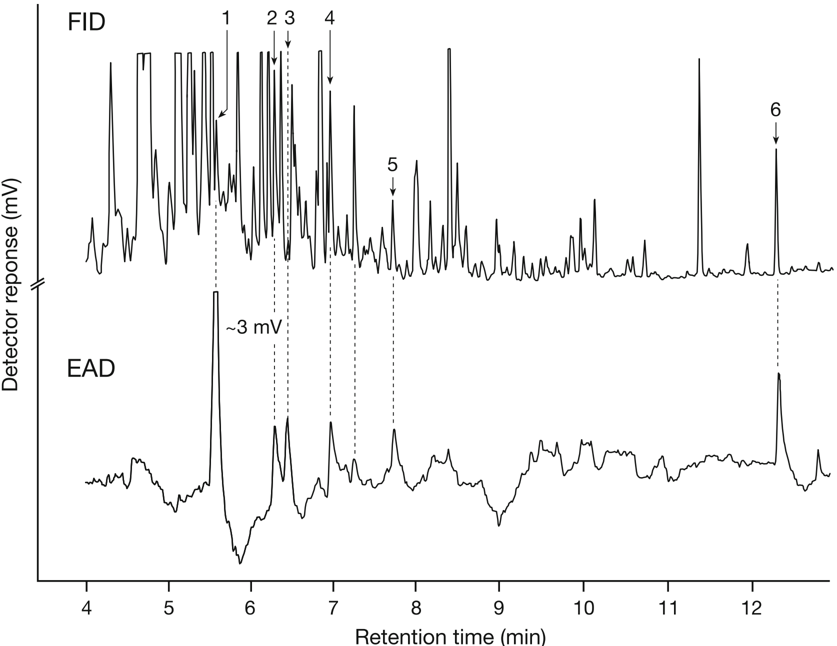


Fig. 2 Results of gas chromatographic-electroantennographic detection analysis of headspace volatiles captured from *Rhizopus* spp. growing on feces agar. No additional responses from house fly antennae were observed with the volatiles of the other two oviposition-inhibiting fungal strains (data not shown). Compounds that consistently (N=3) elicited antennal responses are marked with arrows and are labeled: (1) dimethyl trisulfide; (2) phenylacetaldehyde; (3) unknown; (4) 2-phenylethanol; (5) citronellal; and (6) norphytone. Phenlyacetaldehyde was found in headspace volatiles of *sterile* manure agar, and thus was omitted from experiments 7–10

various types of animal manure (Cai et al. 2007), and, as a single component, it is known to attract some calliphorid and one muscid fly (Nilssen et al. 1996).

Feces-emitted dimethyl trisulfide may, at least in part, be produced by fungi colonizing it. That some flies are attracted to dimethyl trisulfide (Nilssen et al. 1996) implies that it may serve as a foraging cue indicative of food or oviposition sites. Feces is rich in protein and could provide nutrients to adult flies even though they may not use it as a resource for larval offspring.

It would now be intriguing to determine whether volatile profiles of fungal isolates that are harmful or harmless to larval offspring of house flies differ, and how the differences might facilitate oviposition choices by female flies. It would

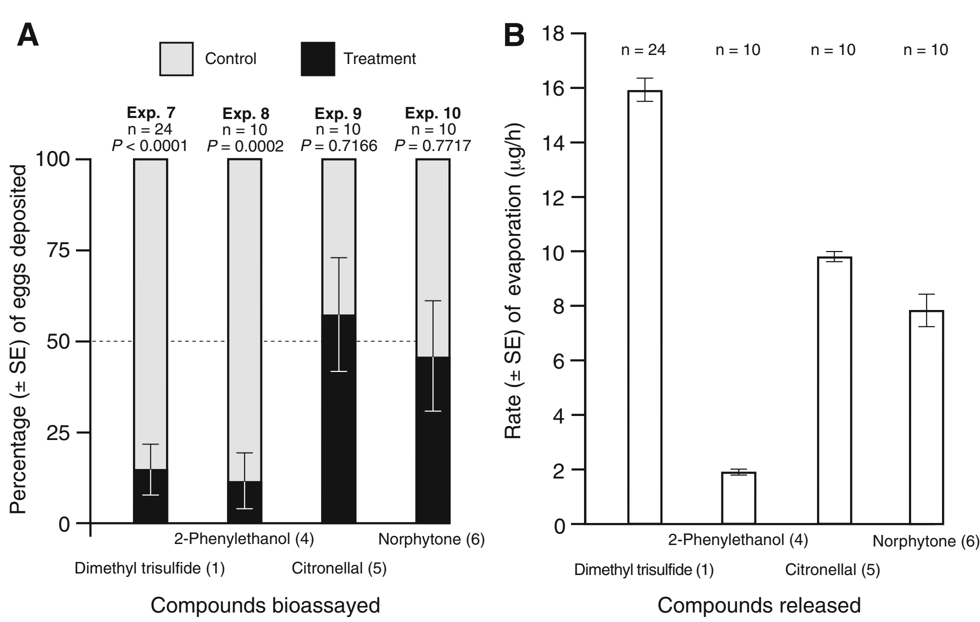


Fig. 3 **A** Percent of house fly eggs oviposited on artificial agar oviposition sites with (treatment) or without (control) a candidate semiochemical released from a micro-capillary tube at the centre of the oviposition site; **B** Rates of evaporation of each candidate semiochemical from micro-capillary tubes (5 µm ID×40 mm)

also be worth investigating whether the semiochemicals, or the fungi producing them, could be exploited as a tactic within programs for managing house flies in livestock production facilities.

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Sequestration of Glucosinolates and Iridoid Glucosides in Sawfly Species of the Genus *Athalia* and Their Role in Defense Against Ants

Sebastian E. W. Opitz · Søren R. Jensen · Caroline Müller

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Abstract In this study, the larval sequestration abilities and defense effectiveness of four sawfly species of the genus *Athalia* (Hymenoptera: Tenthredinidae) that feed as larvae either on members of the Brassicaceae or Plantaginaceae were investigated. Brassicaceae are characterized by glucosinolates (GLSs), whereas Plantaginaceae contain iridoid glucosides (IGs) as characteristic secondary compounds. *Athalia rosae* and *A. liberta* feed on members of the Brassicaceae. Larvae of *A. rosae* sequester aromatic and aliphatic GLSs of *Sinapis alba* in their hemolymph, as shown previously, but no indolic GLSs; *A. liberta* larvae with a narrower host range sequester aliphatic as well as indolic GLSs from their host plant *Alliaria petiolata*. Larvae of *A. circularis* and *A. cordata* are specialized on members of the Plantaginaceae. *Athalia circularis* utilizes mainly *Veronica beccabunga* as host plant, whereas *A. cordata* feeds additionally on *Plantago lanceolata*. Both sawfly species sequester the IGs aucubin and catalpol. In *V. beccabunga*, catalpol esters and carboxylated IGs also occur. The high catalpol concentrations in hemolymph of *A. circularis* can only be explained by a metabolization of catalpol esters and subsequent uptake of the resulting catalpol. The carboxylated IGs of the plant are excreted. The IG-sequestering sawfly species are able to accumulate much higher glucoside concentrations in their hemolymph than the GLS-sequestering species, and the concentration of IGs in hemolymph increases constantly during larval development. The defensive effectiveness of hemolymph that contains GLSs or IGs and of the respective glucosides was tested in feeding-bioassays against a potential predator, the ant *Myrmica rubra* (Hymenoptera: Formicidae). Hemolymph of IG-sequestering cryptic *A. cordata* larvae has a higher deterrence potential than hemolymph of the GLS-sequestering conspicuous *A. rosae* larvae. The results show that glucoside sequestration is widespread in the genus *Athalia,* but that the specific glucoside uptake can result in different defense effectiveness against a predator species.

Keywords Tenthredinidae · Hemolymph · Glucosinolates · Iridoid glucosides · Sequestration · Defense

S. E. W. Opitz · C. Müller (✉)
Department of Chemical Ecology, Bielefeld University,
Universitätsstr. 25,
33615 Bielefeld, Germany
e-mail: caroline.mueller@uni-bielefeld.de

S. R. Jensen
Department of Chemistry, Technical University of Denmark,
Kemitorvet - Build. 201,
2800 Lyngby, Denmark

Introduction

Most plant families contain secondary plant metabolites that are characteristic at the taxon level (Schoonhoven et al. 2006). These metabolites can act as a defense against generalist herbivores, however, some adapted specialized insect species are able not only to tolerate, but even to use these secondary metabolites for their own advantage. These species have evolved the ability to take up, accumulate, and store the plant-derived metabolites, a process referred to as sequestration (Duffey 1980). Sequestering insects often are conspicuously colored, which is interpreted as an alarm signal to predators (Rothschild 1973; Bowers 1993). Sequestration is a fairly widespread mechanism to deal with harmful plant metabolites and is commonly found in several phytophagous insect orders, mainly within the Coleoptera, Lepidoptera, and Heteroptera/Sternorryncha but also in Hymenoptera (for review see Opitz and Müller

2009). Within tenthredinids, sequestration of plant metabolites in the larval hemolymph has been described for several species (Bowers et al. 1993; Schaffner et al. 1994; Müller et al. 2001; Prieto et al. 2007) and seems to be related to the phenomenon of 'easy bleeding' (Boevé and Schaffner 2003). Thereby hemolymph is presented rapidly upon attack of biting predators through the easily disrupted integument of the larvae. Several potential predators are deterred by the hemolymph, whereas the sawfly larvae survive the attack (Boevé and Müller 2005).

Within the genus *Athalia* (Hymenoptera: Tenthredinidae), herbivorous larvae of *A. rosae* L. feed on various species of the Brassicaceae and are known to sequester glucosinolates (GLSs) up to a certain concentration level in the hemolymph (Müller et al. 2001; Müller 2009). Subsequently, GLSs are metabolized mainly in the larval body and to a minor extent excreted intact with the frass (Müller and Wittstock 2005). GLSs are amino acid derived ß-thioglucoside-*N*-hydroxysulfates with aromatic, aliphatic, or indolic side chains. They are characteristic for Brassicaceae and a few other plant families, and they differ species—specifically in their qualitative composition (Fahey et al. 2001). In *A. rosae* hemolymph, aromatic GLSs such as sinalbin (*p*-hydroxybenzyl GLS, **9**, Fig. 1) and glucotropaeolin (benzyl GLS) as well as aliphatic GLSs such as sinigrin (2-propenyl GLS) **10** have been found in high concentrations (Müller et al. 2001). In contrast, indolic GLSs such as glucobrassicin (indol-3-ylmethyl GLS) **11** are detectable only in traces in individuals fed on plants containing high indolic GLS concentrations (Müller 2009). The hemolymph of *A. rosae* has been shown to be deterrent against predatory insects, such as *Myrmica rubra* L. (Hymenoptera: Formicidae) and *Vespula vulgaris* L. (Hymenoptera: Vespidae). The defensive principle is, in part, based on the GLSs present in the hemolymph (Müller et al. 2002; Müller and Brakefield 2003). Sequestration of GLSs also is known for one bug and two aphid species (Aliabadi et al. 2002; Bridges et al. 2002).

In Europe, the genus *Athalia* comprises 17 species that are specialized on different plant families (Liston 1995). Several species, including *A. rosae* and *A. liberta* (Klug), feed on members of the Brassicaceae, whereas others, including *A. cordata* Lepeletier and *A. circularis* (Klug), feed on members of the chemically different Plantaginaceae, which are characterized by iridoid glucosides (IGs). These cyclopentanoid monoterpene-derived compounds are produced by the mevalonic acid pathway, and usually are *O*-linked to glucosides at C-1 (Jensen 1991; von Poser et al. 2000). Aucubin **1** and catalpol **2** are the most common IGs. Catalpol also can occur esterified with different acids forming catalpol esters. The 6-*O*-catalpol esters are limited to the genera *Veronica*, *Paederota*, *Picrorhiza* (Plantaginaceae), and some Bignoniaceae (Taskova et al. 2006; von Poser et al. 2000; Jensen et al. 2005). Sequestration of IGs has been found in species of different insect orders (for review see Opitz and Müller 2009), and renders some of

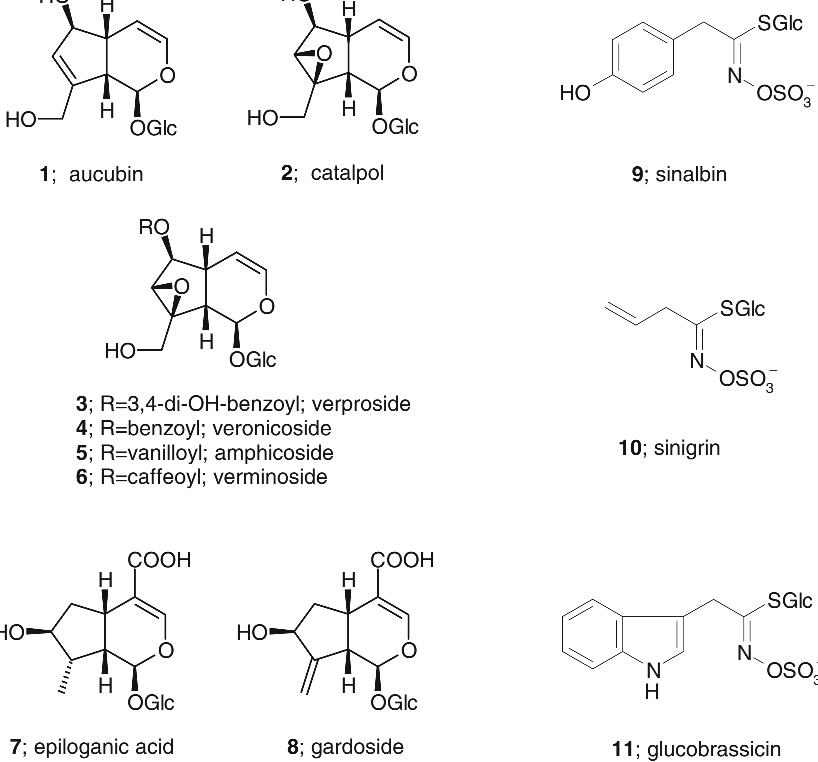

Fig. 1 Chemical structures of the main glucosides found in the investigated plant species. The IGs aucubin (1) and catalpol (2) occur in *Plantago lanceolata* and *Veronica beccabunga* (Plantaginaceae), whereas the latter also contains the carboxylated IGs epiloganic acid (7) and gardoside (8) as well as four catalpol esters (3 to 6). The diversity of GLSs in Brassicaceae is much higher and only one representative of each of the three main classes are shown; the aromatic sinalbin (p-hydoxybenzyl GLS, 9) as main component of *Sinapis alba*, the aliphatic sinigrin (2-propenyl GLS, 10) as main component of *Alliaria petiolata* and the indolic GLS glucobrassicin (indol-3-ylmethyl GLS, 11) occurring in both species

these species unpalatable to bird predators (Bowers 1980, 1992; Bowers and Farley 1990). The catalpol esters have not been found to be sequestered, and the corresponding esterifying acids are excreted with the frass in *Euphydryas anicia* (Doubleday and Hewitson) (Lepidoptera: Nymphalidae) (Gardner and Stermitz 1988).

In Brassicaceae, GLSs co-occur with myrosinases, plant thioglucosidases that hydrolyze the GLSs upon tissue damage into toxic, volatile isothiocyanates and other products via an unstable aglucone (Fahey et al. 2001). IGs can be hydrolyzed by insect β-glucosidases in the gut forming unstable aglyca with differing biological activity (Bowers 1991). Thus, sequestration of both glucoside types is possible only if these degradation processes are at least in part circumvented (Konno et al. 1999). Apart from *A. rosae* (Müller 2009), nothing is known about the sequestration abilities of other *Athalia* spp., nor about their defense effectiveness against predators. Therefore, this study aimed to investigate the sequestration ability of four *Athalia* species, *A. rosae*, *A. liberta*, *A. cordata*, and *A. circularis* that feed on two different glucoside-containing plant families (Brassicaceae and Plantaginaceae). Further, the defense capability of sequestered glucosides was examined against the ant *Myrmica rubra*. The glucoside concentrations were quantified and tracked over the entire larval development in IG-sequestering insects. Results are discussed with regard to the conspicuousness of the different species.

Methods and Materials

Plant and Insect Rearing White mustard (*Sinapis alba* L. cv. Silenda, Brassicaceae), garlic mustard [*Alliaria petiolata* (M. Bieb.) Cavara & Grande, Brassicaceae] and brooklime [*Veronica beccabunga* L., Plantaginaceae (earlier Scrophulariaceae)] were grown in a greenhouse at 20°C and a photoperiod of L16:D8. Ribwort plantain (*Plantago lanceolata* L., Plantaginaceae) was grown in a climate chamber (20°C, 70% r. h., L16:D8). Seeds of *S. alba* were obtained from Kiepenkerl (Norken, Germany), seeds of *P. lanceolata* from Rühlemann's (Horstedt, Germany), and seeds of *A. petiolata* and *V. beccabunga* from the Botanical Garden of Berlin, Germany. *Veronica beccabunga* was sown once, and subsequently was propagated by cuttings.

Adults of four *Athalia* species, *A. rosae*, *A. cordata*, *A. liberta*, and *A. circularis*, were collected in the surroundings of Würzburg (Bavaria, Germany). The Brassicaceae-feeder *A. rosae* was reared on flowering plants of *S. alba*, and *A. liberta* on 1st-year, non-flowering *A. petiolata* plants in the greenhouse. The Plantaginaceae-feeder *A. cordata* was reared on non-flowering *P. lanceolata*, and *A. circularis* on non-flowering plants of *V. beccabunga*. Adults were allowed to oviposit on potted plants of the respective species in cages (51×36×35 cm and 61×61×66 cm for *A. rosae*) kept in the greenhouse. Larvae were reared on these plants until pupation in the soil. Adults were provided with a mixture of honey and water (1:10). New plants were added to the cages when necessary. A colony of the ant *M. rubra* was transferred from the surrounding of Bielefeld University, Germany, and maintained in the laboratory (22°C, 70% r.h., and L16:D8).

Collection of Insect Samples and Plant Material Hemolymph samples were taken from *Athalia* spp. larvae (last feeding instar) that had fed on their respective host plants. Hemolymph was collected from living individuals (6 to 7 replicates per species) by piercing the integument carefully with a needle without damaging the gut. Exuded hemolymph was collected from every individual with glass capillaries in 1.5 ml Eppendorf tubes. Additionally 10 to 15 larvae were transferred in Petri dishes without food for 3 h, and their frass was collected (5 to 6 replicates), thereby avoiding any contamination with plant material. Moreover, six male adults of each species were taken shortly after emergence. In order to evaluate sequestration qualities and quantities, 3 to 7 replicates of mature leaf material of the four host plant species, *S. alba*, *A. petiolata*, *P. lanceolata*, and *V. beccabunga*, were sampled as well. All samples were weighed, frozen in liquid nitrogen, lyophilized, and analyzed for their glucoside contents either by HPLC-DAD or GC-MS (see below).

Analysis of Development-Dependent Sequestration of Iridoid Glucosides in A. circularis and A. cordata The time-course of GLS-sequestration throughout larval development is known for *A. rosae* (Müller and Wittstock 2005). To compare this pattern with that of IG-sequestering species, hemolymph samples of individual *A. cordata* larvae of mixed sex reared on *P. lanceolata* were analyzed 7, 9, and 11 d (N=4 to 6 per day) after larval hatching. It was not possible to collect hemolymph of younger (smaller) larvae or of pre-pupae (eonymphs), which occurred at the twelfth day. Similarly, male *A. circularis* larvae were reared on *V. beccabunga*, and their hemolymph was analyzed 8 (larval stage L3), 10 (L4), and 12 d (L4) after larval hatch (N=8 per day). To quantify the total IG amount in larvae throughout development, every other day *A. circularis* larvae of mixed sex (N=3 per day) were taken from larval hatch until reaching the eonymph stage after 14 d (males 5 instars, females 6 instars). The larvae were starved 1.5 to 1.8 h before sampling. All samples were weighed, frozen in liquid nitrogen, and processed as described below. A Kruskal-Wallis analysis of ranks followed by a multiple comparison test was used to compare the hemolymph

concentrations at different developmental times in *A. cordata* and *A. circularis*.

Analysis of Glucosinolate and Iridoid Glucoside Concentrations For analysis of GLSs, larval hemolymph, frass, adults, and leaf samples were extracted in 80% methanol with sinigrin (for samples related to *S. alba*) and sinalbin (for *A. petiolata*), respectively, as internal standards (both obtained from Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany). GLSs were converted to desulfo GLSs as described previously (Agerbirk et al. 2001). Analysis of desulfo GLSs was carried out on an HPLC (1200 series, Agilent, Santa Clara, CA, USA) with a Supelcosil LC-18 column (Supelco, Bellefonte, PA, USA) with a gradient (solvent A: water, solvent B: methanol) of 5–10% B within 10 min, 10–38% B within the next 14 min, 38–50% B in 2 min, and 50–60% B in 4 min, followed by a cleaning cycle. For identification, retention times and UV-spectra were compared to those of standards. GLSs sequestered by *A. rosae* had been identified earlier by LC-MS (Müller et al. 2001). For quantification, response factors were applied (aromatic GLS: 0.5, benzyl GLS 0.86, aliphatic GLS: 1, indolic GLS: 0.26).

For IG analysis, samples were extracted in 80% methanol with phenyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) as internal standard. After drying of samples in a rotation vacuum concentrator (RVC 2-18, Martin Christ, Osterode am Harz, Germany), they were re-dissolved in 500 µl water (TKA MicroPure, Niederelbert, Germany). An aliquot of 100 µl was transferred in GC-vials and evaporated to dryness under a nitrogen stream. For derivatization, 100 µl silylation agent (*N*-trimethylsilylimidazole:pyridine 1:4, Sylon TP Kit, Supelco, USA) were added, and samples were heated to 80°C for 30 min in a thermoblock (Reacti-Therm, Pierce, Rockford, IL, USA). GC-MS analyses were performed on a Focus GC-DSQ II (Thermo Electron S.p.A., Rodano, Italy), equipped with a VF-5 ms column (30 m×0.25 mm ID, 10 m guard column, Varian, Palo Alto, CA, USA) in electron impact ionization mode. The injection mode was splitless at a temperature of 225°C with a helium flowrate of 1.2 ml/min. Samples were analyzed with two different programs adapted from Gardner and Stermitz (1988). For the analysis of aucubin and catalpol only, temperature program A was applied, for the less volatile catalpol esters and carboxylated IGs, program B. Program A started at 100°C for 1 min, the first ramp of 70°C/min was heating to 200°C with 1 min hold, followed by a second ramp of 3°C/min to 250°C with a 1 min hold. The final temperature of 280°C was reached with a ramp of 5°C/min followed by a 5 min hold. Program B also started at 100°C for 1 min and heated with a 70°C/min ramp to 200°C with 1 min hold, but was followed by a 12°C/min ramp to 260°C with a 6 min hold. The third ramp of 20°C/min to the final temperature of 325°C ended with a hold of 20 min. IGs were identified by comparison of MS spectra and retention times to those of purified authentic standards. Aucubin **1** and catalpol **2** were obtained from Phytoplan (Diehm & Neuberger GmbH, Heidelberg, Germany), while the catalpol esters verproside **3**, veronicoside **4**, amphicoside **5**, and verminoside **6** and carboxylated IGs (epiloganic acid **7**, and gardoside **8**) were isolated from dry plant material of *V. beccabunga*. Plant material was extracted with ethanol, and the water-soluble fraction was purified by preparative chromatography using a Merck Lobar RP-18 column size B (Merck, Darmstadt, Germany). The initial eluent was H_2O followed by H_2O:MeOH mixtures (15:1 to 1:1) and finally by MeOH. The isolated compounds were identified by means of ^{1}H NMR spectroscopy (Jensen et al. 2005).

Deterrence Bioassay against Ants The response of ants towards larval hemolymph and towards purified glucosides was tested with *M. rubra* ant workers according to Müller et al. (2002). Single worker ants were placed in a glass Petri dish (4 cm diam) and offered a 4 µl test and a 4 µl control droplet (droplets 0.7 cm apart). For preparing the hemolymph solutions, 2 µl of freshly collected hemolymph of *A. rosae* or *A. cordata* larvae, respectively, were dissolved in 23 µl of 0.1 M sucrose solution (resulting dilution factor of 1:12.5). This was subsequently diluted further with 0.1 M sucrose solution. The test droplets of hemolymph-sucrose solution were tested for *A. rosae* in dilutions of 1:125 or 1:625 (Müller et al. 2002) and for *A. cordata* in dilutions of 1:312 and 1:1,562. The control droplets consisted of hemolymph-equivalent amounts of water diluted in 0.1 M sucrose solution, thus resulting in the same sucrose concentrations. Glucoside standards were tested in a concentration of 0.8 mM for sinalbin and 0.08 mM for catalpol. Number of visiting events and time spent drinking at the test or the control droplet were recorded for 14–15 ants for a period of 5 min.

To compare the defense effectiveness of GLS and IG sequestering larvae directly, the ant response was tested to larval hemolymph solutions of *A. rosae* and *A. cordata* offered as described above against each other in dilutions of 1:125 and 1:625, respectively. Three batches containing pooled hemolymph of three *A. rosae* larvae (reared on *S. alba*) were compared to three batches containing pooled hemolymph of three *A. cordata* larvae (reared on *P. lanceolata*). For each batch, 6 ants were recorded, in total 18 ants for each dilution. Differences in the drinking duration of ants on the two sucrose solution droplets were tested by Wilcoxon matched-pairs signed-rank tests. Hemolymph samples were divided before use to determine the GLS and IG concentrations of the respective samples by HPLC and GC-MS as described above.

Results

Sequestration Pattern in Athalia Species Sequestration abilities were studied in four *Athalia* species after rearing them on their primary host plants (Fig. 2). All species showed a similar glucoside (IGs or GLSs, respectively) sequestration pattern. Highest concentrations of glucosides were found in larval hemolymph, followed by adult sawflies, whereas the frass contained the lowest concentrations or only traces of glucosides. Regarding the sequestration efficiency, *A. cordata* and *A. circularis* that fed on Plantaginaceae were able to sequester much higher concentrations of glucosides in the hemolymph than the species feeding on members of Brassicaceae (100–250 µmol IG/g FW as compared to 10–40 µmol GLS/g FW; Fig. 2). Glucoside concentrations varied highly within each of the four plant species. However, glucoside concentrations of the larval hemolymph were not related to the concentrations present in the respective host plants across species.

Leaves of *A. petiolata* contained only low concentrations (below 1 µmol/g FW) mainly of sinigrin, whereas *S. alba* provided around 10 times higher concentrations of aromatic GLSs (sinalbin and glucotropaeolin). Comparably low concentrations of GLSs were found in larval hemolymph

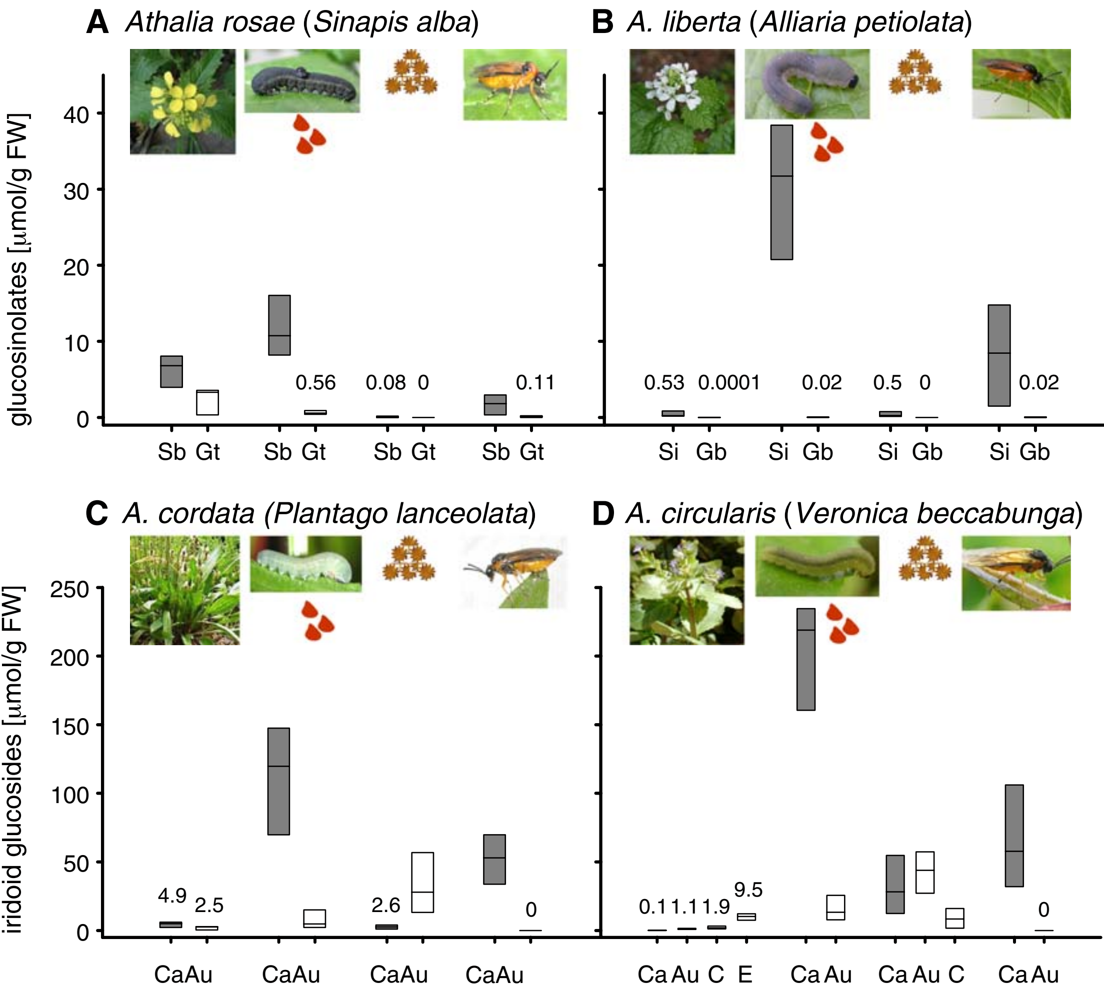

Fig. 2 Glucoside concentrations (median, 25th and 75th percentiles) of the prominent glucosides (*grey*: dominant sequestered glucoside) in the respective host plant species (*N*=3 to 7), larval hemolymph (*N*=6 to 7) and frass (*N*=5 to 6) of last feeding instar larvae and in adults (*N* =5 to 6) of four *Athalia* species: (**a**) *A. rosae* fed on *Sinapis alba*, (**b**) *A. liberta* fed on *Alliaria petiolata*, (**c**) *A. cordata* fed on *Plantago lanceolata*, and (**d**) *A. circularis* fed on *Veronica beccabunga*. For the Brassicaceae, only the main plant glucosides [for *S. alba*: Sb, sinalbin (*p*-hydroxybenzyl GLS), Gt, glucotropaeolin (benzyl GLS); for *A. petiolata*: Si, sinigrin (2-propenyl GLS), Gb, glucobrassicin (indol-3-ylmethyl GLS)] were taken into account. For the Plantaginaceae, the IGs aucubin (Au) and catalpol (Ca) found in *Plantago lanceolata* and *Veronica beccabunga* and the carboxylated IGs (C, sum of epiloganic acid, gardoside, arborescosidic acid and low concentrations of mussaenosidic acid) and catalpol esters (E, sum of verproside, veronicoside, amphicoside, and verminoside) of *V. beccabunga* were quantified. Small pictures of the host plant species, larval hemolymph (blood droplets), the frass and adults indicate the corresponding results and visualize the differences in the larval coloration as well as the similarities in coloration of the adults. Median values of very low concentrations are written above the respective boxes

as well as in adults of *A. rosae* (Fig. 2a). In contrast, *A. liberta* larvae concentrated sinigrin of their host plant *A. petiolata* 30-fold in their hemolymph. Moreover, indolic GLSs, e.g., glucobrassicin, always were detectable in the larval hemolymph and in adult sawflies of *A. liberta*, although indolic GLSs were present in the plant only in low concentrations (Fig. 2b). Only minute concentrations of GLSs were excreted with the frass in both *A. rosae* and *A. liberta*.

Within the Plantaginaceae, leaves of *P. lanceolata* contained the IGs aucubin and catalpol in substantial concentrations. Leaves of *V. beccabunga* contained only low concentrations of these IGs but high concentrations of various catalpol esters, i.e., verproside and veronicoside, small amounts of amphicoside and verminoside, and carboxylated IGs, e.g., epiloganic acid and gardoside (Fig. 2c, d). Larvae of *A. cordata* that fed on *P. lanceolata* sequestered 15-times higher concentrations of catalpol than aucubin in their hemolymph. The frass contained more aucubin than catalpol. Adult *A. cordata* contained catalpol only (Fig. 2c). The catalpol esters and carboxylated IGs of *V. beccabunga* were not sequestered by *A. circularis*; the larvae sequestered only aucubin and very high concentrations of catalpol (Fig. 2d). The host plant IGs were concentrated in the hemolymph up to 100-fold. Additionally, catalpol esters were not detected in the frass, but the carboxylated IGs and up to 50 µmol/g FW of aucubin and less catalpol were excreted. Furthermore, minor concentrations of 3,4-dihydroxy benzoic acid, the esterifying acid of verproside, also were detectable in the frass (data not shown). Adults of *A. circularis* contained only catalpol, as found for *A. cordata*.

Development-Dependent Sequestration of Iridoid Glucosides in A. cordata and A. circularis The development-dependent investigation of IG sequestration in both species revealed a cumulative uptake of IGs. The concentration of IGs (aucubin and catalpol) increased significantly over time in hemolymph of *A. cordata* larvae reared on *P. lanceolata* (Fig. 3a) and in hemolymph of *A. circularis* fed on *V. beccabunga* (Fig. 3b), respectively. In accordance, the total amount of IGs in whole larvae of *A. circularis* reared on *V. beccabunga* increased constantly during larval development (Fig. 3c). After 7 days, the total IG concentration in the hemolymph was around 30 µmol/g FW in *A. circularis* and reached concentrations up to 200 µmol/g FW after 11 days. In eonymphs, the total IG amount dropped to the level found in larvae around day 5. The eonymphs of *A. circularis* contained only catalpol (Fig. 3c) in a concentration range similar to that found in adults (about 50 µmol/g FW) (see above, Fig. 2d). Note that sequestration values of Figs. 2 and 3 can not be compared directly because the sequestration was investigated in larvae feeding on different host plant batches at different times.

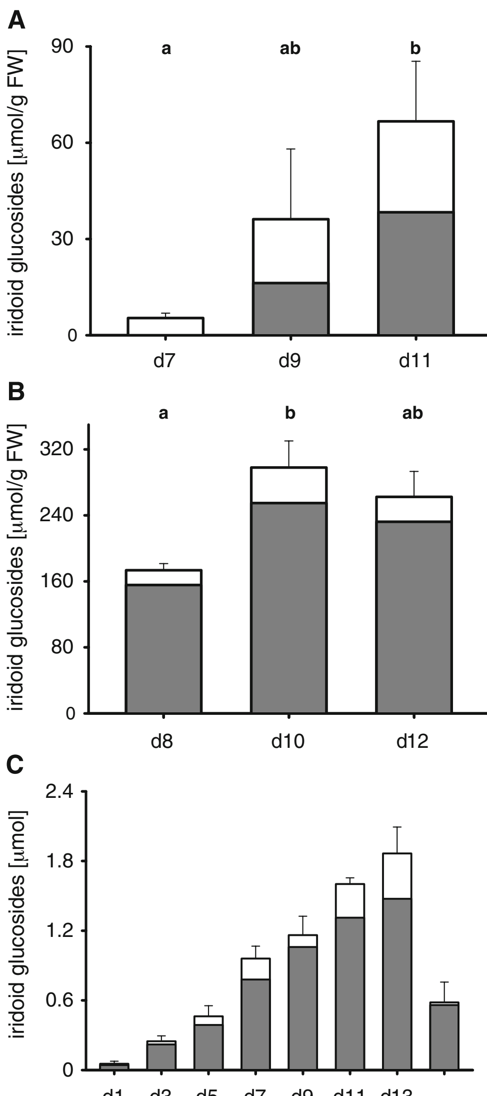

Fig. 3 Mean concentrations of IGs (+ s.e.) in (**a**) hemolymph of *Athalia cordata* larvae (N=4 to 6) at day 7, 9 and 11 after hatching and (**b**) hemolymph of *A. circularis* larvae (N=8) at day 8, 10 and 12 after hatching. (**c**) Mean total IG amounts (+ s.e.) in *A. circularis* larvae at different days after hatching and in eonypmhs (N=3 individuals per time point). *Stacked bars*: *grey*: catalpol, *white*: aucubin. Larval hemolymph concentrations were compared by Kruskal-Wallis analysis of ranks (*A. cordata*: $H_{(2,\ N=16)}=6.93$, $P=0.03$ for total IGs, *A. circularis*: $H_{(2,\ N=24)}=9.36$, $P=0.009$ for total IGs), followed by multiple comparisons tests; *lower case letters* denote significant differences ($P<0.05$); *FW* fresh weight, *Eo* eonymph

Defense Effectiveness of Hemolymph and the Sequestered Glucosides Against Ants Hemolymph of *A. rosae* (main glucoside sinalbin) and *A. cordata* (main glucoside catalpol) as well as sinalbin (GLS) and catalpol (IG) were tested for their deterrent activity against the generalist predator *M. rubra*. The response of individual workers differed highly, as indicated by the high standard errors. Sucrose solution embittered with either of the two hemolymph samples

evoked a clear deterring effect; the ants preferred to drink the pure sucrose solution. Different dilutions were tested to determine the deterrence limit of the two hemolymph samples. The hemolymph of *A. rosae* was still significantly deterrent in a dilution of 1:125, but not in a dilution of 1:625 (data adapted from Müller et al. 2002). The hemolymph of *A. cordata* was deterrent in a dilution of 1:312 and even in a dilution of 1:1,562 (Table 1). The glucoside concentrations in the tested hemolymph droplets were equal approximately to 0.1 mM sinalbin in the 1:125 dilution of *A. rosae* hemolymph and 0.09 mM catalpol in the 1:1,562 dilution of *A. cordata* hemolymph (calculated from empirical sequestration values). Similarly, sucrose droplets containing 0.8 mM sinalbin or 0.08 mM catalpol were less ingested than the control droplets, demonstrating that the glucosides of the sawfly hemolymphs were at least in part responsible for the deterrent effects on the ants. In addition, catalpol was deterrent even in a 10-fold lower concentration as compared to sinalbin (Table 1).

Finally, the deterrent effectiveness of hemolymph of *A. rosae* and of *A. cordata* was tested against each other. In a dilution of 1:125, ants preferred to drink sucrose solution with *A. rosae* hemolymph as compared to droplets with *A. cordata* hemolymph. One batch with a particularly high GLS concentration in *A. rosae* hemolymph (20 µmol/g FW) resulted in an overall low drinking duration on both droplets. Overall, the drinking period was comparably low when the hemolymph samples of *A. rosae* and *A. cordata* were tested against each other. In these cases, the ants showed a cleaning of mouth parts and antennae after feeding on the deterrent test dilutions offered in both concentrations. In a higher dilution, ants did not discriminate between hemolymph samples of either species.

Discussion

All investigated *Athalia* species are able to sequester glucosides from their host plant species of different plant families and able to concentrate them in their hemolymph regardless of the glucoside class. However, high quantitative differences became evident among the sequestration patterns of the four species. IGs were much higher concentrated in larval hemolymph of Plantaginaceae-feeders than GLSs in hemolymph of Brassicaceae-feeders. Larvae of *A. rosae*, for which GLS sequestration has been shown previously (Müller et al. 2001), had the lowest sequestration ability among the four *Athalia* species. Generally, they contained the lowest glucoside concentra-

Table 1 Drinking duration of individual *Myrmica rubra* ant workers in dual-choice assays

	N	Mean drinking duration (± SE) on test droplet [s][a]	Mean drinking duration (± SE) on control droplet [s][b]	*P*-value[c]
Athalia rosae hemolymph[d] Dilution 1:125[e]	14	10.6±6.0	85.8±16.1	0.004
A. rosae hemolymph[d] Dilution 1:625[e]	15	45.3±13.7	92.2±18.3	0.156
Sinalbin concentration 0.8 mM[e]	15	22.1±5.7	71.6±8.5	0.001
Athalia cordata hemolymph[f] Dilution 1:312	15	48.7±15.3	123.9±19.8	0.035
A. cordata hemolymph[f] Dilution 1:1,562	15	60.5±18.3	135.7±4.3	0.035
Catalpol concentration 0.08 mM	15	13.8±7.4	173.1±16.1	0.0007
	N	Mean drinking duration (± SE) on *A. cordata* hemolymph[f] samples [s]	Mean drinking duration (± SE) on *A. rosae* hemolymph[d] samples [s]	*P*-value
Dilution 1:125	18	3.2±1.5	35.4±6.9	0.002
Dilution 1:625	18	27.3±8.4	32.5±9.7	0.744

[a] Test droplet (4 µl) of crude *Athalia* spp. hemolymph or major glucosides, respectively, diluted in 0.1 M sucrose solution or with major glucosides, respectively; drinking duration within 5 min

[b] Control droplet (4 µl) consisted of the equivalent amount of water diluted in 0.1 M sucrose solution; drinking duration within 5 min

[c] Statistical comparison between drinking duration on both droplets by Wilcoxon matched-pairs signed-rank tests

[d] Hemolymph samples were gained from *A. rosae* larvae reared on *S. alba*

[e] Results integrated from Müller et al. (2002)

[f] Hemolymph samples were gained from *A. cordata* larvae reared on *P. lanceolata*

tions in larval hemolymph and in adults despite the highest glucoside concentrations available in the host plant *S. alba*. GLS uptake in *A. rosae* occurs only to a certain level, which is, however, also related to the GLS concentration of the host plants (Müller et al. 2003; Müller and Wittstock 2005; Müller and Sieling 2006). Moreover, GLSs are degraded rapidly and excreted after some hours as unknown metabolites in *A. rosae* (Müller and Wittstock 2005). This can explain the comparably low GLS concentrations in the hemolymph. Larvae of *A. rosae* are able to sequester aromatic GLSs from *S. alba* and aliphatic GLSs from other Brassicaceae, but almost no indolic GLSs (Müller et al. 2001; Müller 2009). In contrast, *A. liberta* stored three to ten times higher GLS concentration than its host plant *A. petiolata* in larval hemolymph and adults. Besides the dominant sinigrin, considerable concentrations of gluco-brassicin (indolic GLS) also were sequestered, although only low concentrations of this indolic GLS were present in the host plant. Another Brassicaceae feeder, *A. lugens* (Klug), also is able to sequester indolic GLSs from *B. rapa* (Opitz, personal observation), thus indicating different sequestration abilities among the *Athalia* spp. specialized on members of Brassicacae. It is unknown whether GLSs also are metabolized and degraded in *A. liberta*. Adults of the Japanese subspecies *A. rosae ruficornis* (Jakovlev) are known to feed pharmacophagously on e.g., *Ajuga decumbens* Thunb. (Lamiaceae) and to sequester clerodane diterpenoids from this plant species (Nishida et al. 2004). It needs to be studied whether the *Athalia* spp. investigated in the present study are also pharmacophagous on species of Lamiaceae or other plant families.

In *A. rosae* larvae, GLS concentrations varied in a fluctuating manner throughout larval development (see Müller and Wittstock 2005). In contrast, the IG concentration of the Plantaginaceae-feeders *A. cordata* and *A. circularis* increased in the hemolymph constantly throughout larval development. This resulted in very high concentrations of IGs in the last instar (Fig. 3). The IGs found in both *Athalia* species that fed on Plantaginaceae were always comprised of high amounts of catalpol, although other IGs were present in the host plants as well. Usually, *P. lanceolata* contains more aucubin than catalpol, with lower concentrations in older leaves (Bowers 1988, 1991). This relation is highly variable, however, and the mature leaves of *P. lanceolata* were found to contain five-times higher concentrations of catalpol than aucubin in the present study. These subsequently increased in the larvae of *A. cordata* to a ratio of 30:1 catalpol:aucubin (Fig. 2c). The presence of high concentrations of catalpol in the hemolymph of *A. cordata* as compared to aucubin could be due both to higher catalpol concentrations in the host plants and to a higher sequestration efficiency of catalpol. Two additional IGs, 8-epiloganic acid and gardoside, were found in *P. lanceolata* in low concentrations in another study (Willinger and Dobler 2001). None of these IGs was detected in the samples of *P. lanceolata* or in the hemolymph of *A. cordata* in this study. The high catalpol concentrations in *A. circularis* probably are acquired differently. Whereas *V. beccabunga* contains only low concentrations of catalpol, the main IG fraction in the genus *Veronica* is comprised of several catalpol esters and carboxylated IGs (Jensen et al. 2005). The four identified catalpol esters of *V. beccabunga*, verproside, veronicoside, amphicoside, and verminoside, were not detectable in larval hemolymph of *A. circularis* and were not excreted with the frass either. Most likely, the catalpol esters were hydrolyzed and the released catalpol was sequestered, thus resulting in high catalpol concentrations in the larval hemolymph of *A. circularis*. In accordance with this hypothesis, the esterifying acid of the dominant catalpol ester verproside, 3,4-dihydroxybenzoic acid, was detectable in the frass of some *A. cordata* samples (3 out of 7) after verproside had been fed in high concentrations to the larvae (data not shown). A similar way of coping with catalpol esters has been shown earlier for butterfly species of the genus *Euphydryas* (Gardner and Stermitz 1988). In both *A. cordata* and *A. circularis*, the higher amount of catalpol as compared to aucubin in larval hemolymph also may be due to an epoxidation of the precursor aucubin into catalpol. Unfortunately, *Athalia* spp. cannot be reared on artificial diet to test this hypothesis. Caterpillars of *Precis* (*Junonia*) *coenia* Hübner (Lepidoptera: Nymphalidae) did not convert aucubin to catalpol or *vice versa*, when they were fed on an artificial diet containing the respective IGs (Bowers 1992).

Only traces of GLSs were detectable in frass of the two Brassicaceae-feeders, which suggests an efficient transport system of the hydrophilic GLSs through the gut membrane and an almost complete degradation of GLSs before excretion in *A. rosae* and *A. liberta*. In contrast to the GLS-sequestering species, frass of the Plantaginaceae-feeding *Athalia* species contained considerably more IGs, with generally more aucubin than catalpol. This finding indicates a favored, but not complete uptake of catalpol in these species. Other IG sequestering insect species likewise preferably take up catalpol, but aucubin is sequestered as well (for review see Opitz and Müller 2009). In few insect species, other IGs are sequestered in low amounts, but no catalpol esters or carboxylated IGs (Stermitz et al. 1986; Nishida and Fukami 1989; Boros et al. 1991; Mead et al. 1993).

As found previously for *A. rosae* (Müller and Wittstock 2005), the glucoside amount in *A. circularis* dropped drastically from the last feeding instar to the eonymph (Fig. 3c). This indicates that glucosides are either metabolized to a large extent before pupation or eliminated with the molt to the eonymph. In adults of all four species,

glucoside concentrations were relatively low. Furthermore, only catalpol was present in adults of both Plantaginaceae-feeders. Either these species eliminated aucubin, metabolized it to catalpol before pupation, or the concentrations of aucubin were below the detection threshold. In the IG sequestering species *Poladryas minuta* Edwards (Lepidoptera: Nymphalidae), large amounts of catalpol are excreted with the meconium (L'Empereur and Stermitz 1990).

For tenthredinid sawflies, sequestration seems to be a common way to handle plant secondary metabolites (Boevé and Schaffner 2003), and is often related to easy bleeding in this taxon. All investigated *Athalia* species show the phenomenon of easy bleeding as larvae, by which they immediately after injury confront an attacking predator with the glucosides in their hemolymph. Within this genus, IG sequestration resulted in a higher defense potential than GLS sequestration against a potential predator, the ant *M. rubra* (Table 1). The hemolymph of *A. cordata* significantly deterred ants from drinking embittered sucrose solution in higher dilutions (1:1,562) than the hemolymph of *A. rosae* (1:125). When offered together in comparable concentrations to ant workers, the hemolymph of *A. cordata* was more deterrent than hemolymph of *A. rosae*. In accordance, catalpol still evoked a high deterrence when tested in a tenfold lower concentration than sinalbin. The drinking duration of ants generally was low on hemolymph solutions of both species, and they often showed cleaning behavior after contacting a hemolymph solution. In general, the glucoside concentration in the hemolymph of all species is sufficient to offer protection against this generalist predator. The superior defense of the IG sequestering species (high activity of catalpol as well as high IG concentration in hemolymph) is puzzling because of their rather cryptic lifestyle. Larvae of *A. cordata* as well as *A. circularis* are camouflaged, and their green–grayish coloration merges well with the plant coloration. Moreover, *A. circularis* larvae often hide at the basis of the plant or even in the soil especially during the first larval days (personal observation). In contrast, *A. rosae* larvae promote their unpalatability with a blackish, conspicuous coloration (Ohara et al. 1993), whereas *A. liberta* is grey and rather inconspicuous. Furthermore, *A. rosae* larvae move during larval development to the yellow flowers of *S. alba* (Bandeili and Müller 2010), where the contrast is even stronger as compared to larvae feeding on leaves. Of all investigated species, *A. rosae* is most visible to visually-hunting predators, although it is the species least effective in sequestration. Other selection pressures must be assumed for the evolution of this prominent coloration of *A. rosae*.

To our knowledge, nothing is known about the exact mechanism of GLS or IG sequestration. Although the polar, non-volatile GLSs and IGs differ in their structure, both are glucosidically-bound hydrophilic metabolites, whereby the chemistry of the glucose moiety might be sufficient to facilitate sequestration. A transporter may be needed for their uptake, as has been postulated for the uptake of the hydrophilic glucoside salicin in order to pass the gut membrane in larvae of *Chrysomela populi* L. (Coleoptera: Chrysomelidae) (Discher et al. 2009). In contrast, the rather lipophilic cardenolides do not seem to need a transporter in *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae); studies on this system postulated a passive diffusion across the gut membrane (Isman 1977; Scudder and Meredith 1982). The specificity of glucoside uptake in *Athalia* spp. needs to be investigated in more detail to determine whether the transport is dependent on the glucosidic nature of the compounds. This would include testing whether the *Athalia* species also are able to sequester respective glucosides from non host plant families. Such investigations might further allow one to postulate the ancestral and derived host plant family use in the genus *Athalia* and to reveal phylogenetic relationships of sawfly species on the basis of their differing sequestration abilities.

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Naphthoquinones and Anthraquinones from Scent Glands of a Dyspnoid Harvestman, *Paranemastoma quadripunctatum*

Günther Raspotnig · Verena Leutgeb · Miriam Schaider · Christian Komposch

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Abstract Extracts of *Paranemastoma quadripunctatum* (Opiliones, Dyspnoi, Nemastomatidae) contained seven components, all of which likely originated from the secretion of well-developed prosomal scent glands. The two main components (together accounting for more than 90% of the secretion) were identified as 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone. The minor components were 1,4-naphthalenediol, two methoxy-naphthoquinones (2-methoxy-1,4-naphthoquinone, and 2-methoxy-6-methyl-1,4-naphthoquinone) and two anthraquinones (2-methyl-9,10-anthraquinone and a dimethyl-9,10-anthraquinone). While some chemical data on scent gland secretions of the other suborders of Opiliones (Cyphophthalmi, palpatorean Eupnoi, and Laniatores) already exist, this is the first report on the scent gland chemistry in the Dyspnoi. Naphthoquinones are known scent gland exudates of Cyphophthalmi and certain Eupnoi, methoxy-naphthoquinones and anthraquinones are new for opilionid scent gland secretions.

Keywords Opiliones · Arachnida · Nemastomatidae · Chemical ecology · Exocrine secretions · Chemical defense

G. Raspotnig · V. Leutgeb · M. Schaider
Institute of Zoology, Karl-Franzens University,
Universitätsplatz 2,
8010 Graz, Austria

G. Raspotnig (✉)
Research Unit of Osteology and Analytical Mass Spectrometry,
Medical University, University Children's Hospital,
Auenbruggerplatz 30,
8036 Graz, Austria
e-mail: guenther.raspotnig@uni-graz.at

C. Komposch
Institute of Animal Ecology and Landscape Planning,
Bergmanngasse 22,
8010 Graz, Austria

Introduction

Large prosomal sac-like glands—so-called repugnatorial or scent glands—represent the main exocrine system in harvestmen, and they are a synapomorphic character of all Opiliones. These glands typically open to the body outside via one large orifice on the lateral margins of the carapace, either dorsal to coxae I (Palpatores), dorsal to coxae II (Laniatores), or atop tubercles ("ozophores") that protrude from the prosoma (Cyphophthalmi). Scent glands are primarily thought to serve chemical defense (e.g., Martens 1978) although, in certain groups, additional functions may have evolved (Holmberg 1986; Machado et al. 2002). Since the 1950s, the chemistry of scent gland secretions of various opilionids has been investigated sporadically, nevertheless leading to a considerable amount of chemical data (review in Gnaspini and Hara 2007): Specific sets of benzoquinones and phenols occur in gonyleptoid Laniatores (Grassatores) (e.g., Hara et al. 2005), while terpenes, esters, and nitrogen-containing compounds have been found in one representative of travunioid Laniatores (Insidiatores) (Ekpa et al. 1984). In the Palpatores, chemical knowledge on scent glands is biased: In Eupnoi (at least in some Sclerosomatidae), acyclic components such as ethyl ketones and their corresponding alcohols are common (Ekpa et al. 1985), while naphthoquinones were reported from Phalangiidae (Wiemer et al. 1978). Recently, both acyclic ketones and naphthoquinones were also detected in Cyphophthalmi (Raspotnig et al. 2005). By contrast, data on the chemical composition of scent gland exudates in the Dyspnoi are lacking.

Dyspnoid scent glands generally are more inconspicuous and cryptic than in other groups and show many aberrant features. Ozopores may be hidden or covered (instead of being exposed, as characteristic for defensive glands), and in the majority of Dyspnoi, noticeable emission of secretion from ozopores, as occurs in other Opiliones, does not occur at all. In some taxa, scent glands obviously produce and store solid secretion; this may be true for Ischyropsalididae (Juberthie et al. 1991) and Trogulidae (Schaider and Raspotnig 2009). In these cases, the emission of secretion is thought to be gaseous, by slow sublimation of solid glandular contents (Juberthie et al. 1991). In species of *Trogulus*, however, emission has never been detected, not even under heavy mechanical disturbance (Pabst 1953). Scent gland features in Dyspnoi have led to doubts concerning their defensive nature (Schaider and Raspotnig 2009), with the chemistry of secretion remaining a major open question. Since scent gland secretions and their chemical profiles, respectively, also seem to constitute phylogenetically useful information (e.g., Roach et al. 1980; Hara et al. 2005), and since the relationship of Palpatores and Laniatores is still uncertain (Shultz and Regier 2001; Giribet et al. 2002), the scent gland chemistry of Dyspnoi may help clarify phylogenetic questions.

We here present the first chemical analysis of a dyspnoid scent gland secretion using the model nemastomatid species, *Paranemastoma quadripunctatum*.

Methods and Materials

Specimens of *Paranemastoma quadripunctatum* (Perty 1833) (Opiliones, Dyspnoi, Nemastomatidae) were collected by hand in Styria and Carinthia (Austria). Extraction of scent gland secretions was performed from living specimens. In total, individual extracts from 18 adults of both sexes were prepared by two methods: 1) by dabbing scent gland secretion on filter paper pieces that were subsequently extracted in 100 µl of hexane for 10 min (8 extracts); 2) by whole body extraction of individuals in 150 µl of hexane or ethyl acetate for about 30 min (10 extracts). With respect to the latter method, in 8 of 10 cases, the solvent was heated to 60°C and sonicated to dissolve the components. Aliquots of extracts (2 µl) were subject to gas chromatographic-mass spectrometric (GC-MS) analysis, using a Trace GC2000 coupled to a Voyager MS (both from Thermo, Vienna, Austria). The GC was equipped with a ZB-5MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Germany). Injection was splitless with helium (at 1.5 ml min^{-1}) as carrier gas. The column temperature was programmed from 50°C (held for 1 min) to 200°C at 10°C min^{-1}, and then to 300°C at 15°C min^{-1}. The ion source of the MS and the transfer line were kept at 170°C and 310°C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Reference compounds for comparisons of GC-MS data were purchased from Sigma (Vienna, Austria) (1,4-naphthalenediol, 1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, 2-methyl-9,10-anthraquinone). For authentic 6-methyl-1,4-naphthoquinone, we used a natural source, namely the scent gland secretion of another opilionid, *Phalangium opilio*, in which 6-methyl-1,4-naphthoquinone was identified by both MS and NMR-studies (Wiemer et al. 1978).

Results

Chemical Identification of Extract Components Seven components (A–G) were detected in the extracts by GC (Fig. 1; Table 1). The two major compounds (A and C), together accounting for about 90% of the extract profile, were known previously from a study with the cyphophthalmid harvestman *Cyphophthalmus duricorius* (Raspotnig et al. 2005), and appeared to be 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone, respectively. For final identification, we compared both their MS patterns as well as GC retention times to those of the authentic standards, finding full correspondence. The remaining minor components were identified either: 1) by comparison of mass spectra and retention times to commercially available compounds (compounds B, D, and F) or 2) tentatively only, by interpretation and comparison of mass spectra to spectra from the NIST-library (compounds E and G) (Table 1).

The mass spectrum of compound B clearly was related to that of compound A (1,4-naphthoquinone), and was identified as 1,4-naphthalenediol. Compounds D and E also appeared to be homologous naphthoquinones, namely methoxy-naphthoquinones. Compound D was identified as 2-methoxy-1,4-naphthoquinone (M^+ at *m/z* 188). Compound E exhibited a comparable mass spectrum, but with fragment ions in the higher mass range, all shifted 14 mass units higher (M^+ was at *m/z* 202), indicating the presence of an extra methyl-group. The position of the additional methyl-group in compound E was not determined but, by analogy to component C (6-methyl-naphthoquinone), we tentatively identify this compound as 2-methoxy-6-methyl-1,4-naphthoquinone. Components F and G are anthraquinones, namely a 2-methyl-9,10-anthraquinone (M^+ at *m/z* 222; component F), and a homologous component that again differs only by the presence of an additional methyl-group (M^+ at *m/z* 236; compound G). The latter compound is probably a dimethyl- or an ethyl-anthraquinone.

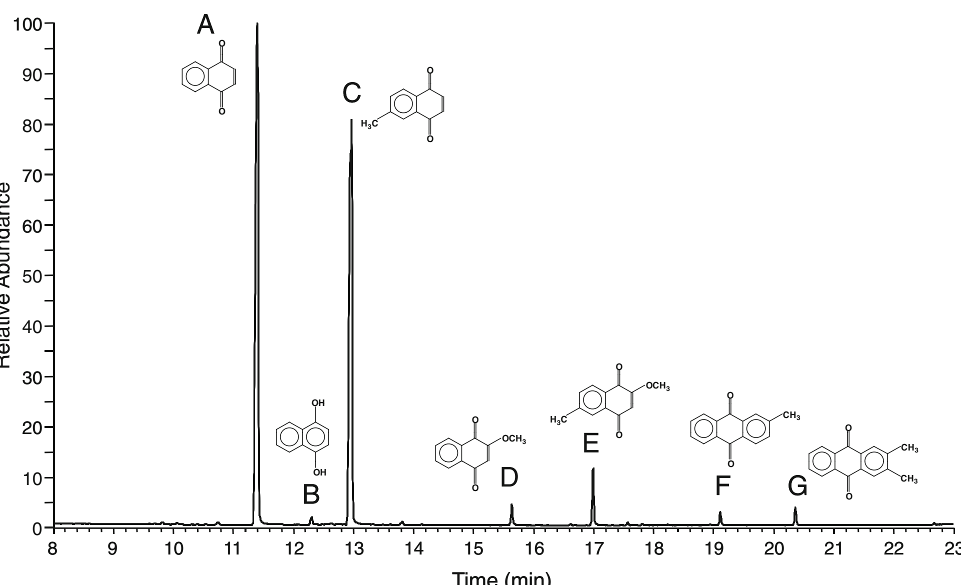

Fig. 1 Typical gas chromatographic profile of a droplet loaded with scent gland secretion dabbed onto filter paper from the mouth of a *Paranemastoma quadripunctatam* male. Components: Peak A (1,4-naphthoquinone), B (1,4-naphthalenediol), C (6-methyl-1,4-naphthoquinone), D (2-methoxy-1,4-naphthoquinone), E* (2-methoxy-6-methyl-1,4-naphthoquinone), F (2-methyl-9,10-anthraquinone), G* (2,3-dimethyl-9,10-anthraquinone). [Components E and G, marked with an asterisk were only tentatively identified (see text).]

Chromatographic Profiles and Secretory Glands The compounds found in the extracts are most likely from the scent glands that open to the body outside via one large ozopore on either side of the prosoma in the region of coxae I. In whole body-extracts obtained from short extraction times (30 min) at room temperature and hexane or ethyl acetate as a solvent, the chemical profile observed from filter-paper extraction was detected only in two out of 10 individuals. The remaining eight individuals (where no secretion was detected) were extracted again in hot hexane for 1 h, followed by ultrasonic treatment (10 min), and these extracts contained the same set of components as in filter-paper extracts, but in reduced amounts. In particular, the anthraquinones were found present only in trace amounts. In order to link unequivocally the extracted components with the scent glands, living specimens were squeezed gently with a forceps, which led to the emission of a droplet of clear enteric fluid from the mouth. Subsequently, and beginning from the lateral sides of the droplet, color was mixed into the enteric fluid, until the whole droplet became brownish. GC profiles of filter-paper extracts of this droplet clearly showed the full chemical profile of all seven components as described above. Such droplets were dabbed from eight individuals, and each exhibited a consistent chemical profile corresponding to the profiles obtained from whole body-extracted individuals (Fig. 1). No differences in secretion profiles were recognized between males and females. If clear droplets were examined (dabbed from

Table 1 Gas chromatographic—mass spectrometric identification of secretion components in *Paranemastoma quadripunctatum*

Peak	RT (min)	Rel. abund. (in % peak area)	Fragmentation pattern m/z (relative intensity)	Identified as
A	**11.39**	**49**	159 (12), 158 (100), 130 (70), 104 (75), 102 (78), 76 (68), 75 (23), 74 (21), 66 (11), 51 (16), 50 (35)	**1,4-naphthoquinone**
B	12.31	< 1	161 (9), 160 (88), 132 (15), 131 (19), 105 (27), 104 (100), 77 (13), 76 (41), 57 (18), 50 (21)	1,4-naphthalenediol
C	**12.97**	**43**	173 (15), 172 (100), 157 (13), 144 (45), 118 (61), 116 (58), 115 (63), 90 (28), 89 (43), 63 (19)	**6-methyl-1,4-naphthoquinone**
D	15.64	1	189 (12), 188 (100), 173 (37), 160 (36), 159 (41), 158 (45), 132 (11), 131 (19), 130 (30), 104 (20), 102 (81), 101 (17), 89 (89), 76 (42), 69 (23), 50 (26)	2-methoxy-1,4-naphthoquinone
E	17.00	4	203 (13) , 202 (100), 187 (34), 174 (26), 173 (48), 172 (33), 146 (6), 145 (14), 144 (17), 131 (15), 118 (13), 116 (54), 115 (40), 103 (76), 89 (29), 77 (27), 69 (13), 63 (17)	2-methoxy-6-methyl-1,4-naphthoquinone*
F	19.11	< 1	223 (16), 222 (100), 221 (14), 207 (17), 194 (44), 193 (13), 166 (33), 165 (92), 164 (17), 163 (14), 139 (11), 115 (6), 97 (9), 89 (7), 82 (29)	2-methyl-9,10-anthraquinone
G	20.36	1	237 (18), 236 (100), 235 (10), 221 (31), 208 (33), 207 (12), 193 (17), 180 (8), 179 (17), 178 (24), 165 (54), 152 (9), 139 (4), 104 (10), 89 (23), 76 (12)	dimethyl-9,10-anthraquinone*

Components in bold are main components of the secretion; components marked with * have been identified tentatively (on the basis of their mass spectra) only. Quantification of components is based on the relative abundance of peak areas (whole secretion=total of all peak areas = 100%).

the mouth before the colored secretion was mixed in), none of the profile-components was detected.

Discussion

As outlined in the introduction, problems in extracting dyspnoid secretions are due to: 1) their possibly solid nature and, 2) the general reluctance of Dyspnoi to release secretions. Thus, after several previous unsuccessful attempts to extract and analyze secretions of some Dyspnoi (e.g., species of *Trogulus* and *Anelasmocephalus*), we present here the first example of dyspnoid scent gland chemistry. Behavioral experiments with *P. quadripunctatum* could not easily evoke emission of secretion; in fact, observable discharge of secretion from ozopores (common in many other Opiliones) did not occur. In case of disturbance, however, individuals of *P. quadripunctatum* regurgitate a droplet from the mouth, which subsequently is mixed with scent gland secretion, indicated by a gradual color change of the droplet from clear to brownish. A similar situation is known for certain Laniatores where a regurgitated droplet runs from the mouth to the ozopores, mixing with scent gland secretion, thereby also gradually changing color (Eisner et al. 2004). In *P. quadripunctatum*, we might have observed the reversed emission sequence; secretion from scent glands may run towards the mouth region where it is dissolved in a regurgitated droplet of enteric fluid. Another possibility is that regurgitated enteric fluid runs first to the ozopores, partly dissolving (viscous or even solid) scent gland exudates. Subsequently, the scent gland secretion-enteric fluid mixture may run back to the mouth, finally mixing with more enteric fluid, thus creating an applicable defensive droplet. Since the chemistry indicates a viscous or partly solid exudate, the latter scenario may be the most probable. In either case, secretion or a secretion-fluid-mix reaches the regurgitated droplet at the mouth from both sides at the same time, as indicated by the change of color beginning at the lateral margins of the oral droplet. Additionally, the location of ozopores is consistent with this view; i.e., ozopores in *P. quadripunctatum* (dorsal to coxae I as in all Palpatores) are hidden beneath a dorso-lateral fold of the carapace, and are directed ventrally. Thus, secretion, or a secretion-enteric mixture, is discharged towards coxae I, and flows ventrally towards the mouth. The scent gland secretion-enteric fluid mixture in *P. quadripunctatum* may be applied to a potential predator with the chelicerae. As such, application of secretion may represent a special kind of "leg dabbing" as known from Cyphophthalmi and certain laniatoreans (Juberthie 1961; Eisner et al. 2004).

Furthermore, the anthraquinones found in the *P. quadripunctatum* exudate are highly unusual compounds for exocrine secretions of arthropods, since these are solid, poorly soluble compounds under normal ambient temperatures. Thus, dissolving or diluting scent gland exudates in enteric fluid may be necessary in order to release secretion quickly. The rapid solubility of the scent gland exudates of *P. quadripunctatum* in enteric fluid is, nevertheless, remarkable because attempts to extract these exudates from whole bodies by using different solvents at elevated temperature with sonication did not produce concentrated extracts. In the rare cases where we found large amounts of the scent gland components in whole-body extracts, we suppose that we extracted individuals at the very moment of "loading" droplets of enteric fluid with scent gland secretion. Large amounts of these components were detected in droplets of both sexes, excluding the participation of male cheliceral glands as a possible source of chemical material (these glands are present in male nemastomatids, see e.g., Martens and Schawaller 1977).

We have preliminary data that the use of enteric fluid to dissolve (viscous or even solid) scent gland exudates may be characteristic of Dyspnoi. Even in trogulids and ischyropsalidids, this mechanism of secretion release appears to be more likely than the hitherto proposed slow sublimation of solid scent gland contents (e.g., Juberthie et al. 1991). In any case, the *Paranemastoma* exudate may represent a "prestage" to these completely solid secretion boli in the groups mentioned above. From a chemosystematic point of view, anthraquinones and methoxy-naphthoquinones are new for opilionid scent gland secretions but are expected to occur in other Dyspnoi as well. By contrast, 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone (the two main components of the *Paranemastoma* exudate) may be widespread, and are previously known from scent glands of Cyphophthalmi (Raspotnig et al. 2005), and from glands of at least one representative of Eupnoi, *Phalangium opilio* (Wiemer et al. 1978).

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Isolation of a Pyrazine Alarm Pheromone Component from the Fire Ant, *Solenopsis invicta*

Robert K. Vander Meer · Catherine A. Preston · Man-Yeon Choi

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Abstract Alarm pheromones in social insects are an essential part of a complex of pheromone interactions that contribute to the maintenance of colony integrity and sociality. The alarm pheromones of ants were among the first examples of animal pheromones identified, primarily because of the large amount of chemical produced and the distinctive responses of ants to the pheromone. However, the alarm pheromone of the fire ant, *Solenopsis invicta*, eluded identification for over four decades. We identified 2-ethyl-3,6-dimethylpyrazine as an alarm pheromone component of *S. invicta*. Worker fire ants detect the pyrazine alarm pheromone at 30 pg/ml, which is comparable to alarm pheromone sensitivities reported for other ant species. The source of this alarm pheromone are the mandibular glands, which, in fire ants, are not well developed and contain only about 300 pg of the compound, much less than the microgram quantities of alarm pheromones reported for several other ant species. Female and male sexuals and workers produce the pyrazine, which suggests that it may be involved in fire ant mating flight initiation, as well as the typical worker alarm response. This is the first report of 2-ethyl-3,6-dimethylpyrazine from a *Solenopsis* species and the first example of this alkaloid functioning as an alarm pheromone.

R. K. Vander Meer (✉) · M.-Y. Choi
CMAVE, ARS, USDA,
1600 SW 23rd Drive,
Gainesville, FL 32608, USA
e-mail: bob.vandermeer@ars.usda.gov

C. A. Preston
USDA, APHIS, PPQ,
8100 NW 15th PL,
Gainesville, FL 32606, USA

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Introduction

In social insects, maintenance of colony cohesiveness, sociality, and defense depends on sophisticated pheromonal communication. Worker/worker chemical interactions include recruitment, colony immigration, alarm, and nestmate recognition. Male and female alate sexuals use pheromones to induce mating flights and mediate mating behaviors. Workers are attracted to queen-produced releaser pheromones that are related to queen grooming, feeding and care of eggs (Vander Meer and Alonso 1998). Queens also release primer pheromones that influence sexual competition, directly or through the workers (Vargo 1998; Vander Meer and Alonso 2002). Pheromone diversity in social insects is matched by the diversity of their exocrine glands. Sixty three different glands have been described for social insects; in ants (Formicidae), alone, there are at least 39 described exocrine glands (Billen and Morgan 1998).

Alarm pheromones are a major class of social insect releaser pheromone, which have no direct benefit to the recipient but serve to put other colony members in a high state of alertness. In response to alarm pheromone, a variety of alarm behaviors have been described, including rapid running, attraction to the source, colony dispersal, and aggressive postures (Vander Meer and Alonso 1998). The chemistry of ant alarm pheromones is diverse, but components typically have a molecular weight of 100–200 and have 5–10 carbons. The low molecular weight, and consequent high volatility, of alarm pheromones is necessary for a quick and highly transient information transfer. Alarm pheromones can be terpenoids, alcohols, aldehydes,

ketones, esters, nitrogen heterocycles, sulfur-containing compounds, or other types of molecules (Hölldobler and Wilson 1990; Billen and Morgan 1998).

The highly evolved fire ant, *Solenopsis invicta* Buren, uses a complex of pheromones, including a recruitment pheromone (Vander Meer 1986) and a queen-produced recognition pheromone (Glancey et al. 1984). Decades ago, Wilson (1962) defined alarm behavior in fire ants as the rapid, erratic movement of workers toward a disturbed worker, and suggested that the source was from the head and, secondarily, from Dufour's gland components. Interestingly, alarm pheromones also are likely involved in fire ant mating flights. Fire ant mating flights are characterized by the opening of the normally closed nest tumulus, and frenzied, alarm-like, activity by workers (sterile), and male and female sexual alates, prior to the alates taking flight (Markin et al. 1971; Obin and Vander Meer 1994). The alarm-like behavior of workers and alate sexuals is mediated by a releaser pheromone linked to the mandibular gland (Obin and Vander Meer 1994; Alonso and Vander Meer 1997).

Elucidation of the structure(s) of the alarm pheromone of *S. invicta* has been a perplexing problem since it was first described over four decades ago (Wilson 1962). We present here the isolation, identification, and source of an alarm pheromone component from workers, and female and male sexual fire ants.

Methods and Materials

Source of S. invicta Colonies All colonies were monogyne and queenright (functional queen, brood, and workers), and derived from newly mated queens or from monogyne colonies collected from the Gainesville, FL, USA area, at least one year previously. Colonies were maintained as previously described (Banks et al. 1981), fed crickets three times a week, and had access to a test tube containing water and a test tube containing 10% aqueous sucrose solution.

General Alarm Bioassay Ten to 15 colonies were used for each bioassay, unless otherwise specified. At least 2 h prior to beginning an experiment, approximately 100–200 workers and some brood from each colony were added to small plastic containers with the inside walls coated with Fluon® to prevent escape. Additionally, a piece of red cellophane was placed over one end of the container to simulate dark conditions and to encourage the ants to move their brood and settle into a quiescent group; the experiment was not started if the worker ants had not settled into quiescent groups. All treatments and controls were placed in 20-ml scintillation vials. Just prior to the time of evaluation, the vial cap was removed and 3 ml of headspace were quickly drawn into a 5-ml plastic syringe (Henke-Sass Wolf Co.). A single syringe was used for each control and treatment. One milliliter of headspace air then was discharged over a group of quiescent ants, at a rate for which clean air (blank) would not generate a response, and the response of the ants observed and recorded. The response was evaluated on a scale of 1 to 4, with 1 = no response, 2 = some antennation, 3 = one to three workers running out of the resting group, and 4 = >3 workers running erratically from the resting group. Each evaluation lasted no longer than the length of time required to exhaust 1 ml of control air without alarming the ants. The experiment was conducted blind, with one person preparing the headspace sample and a second applying an unknown sample and reporting the response. All ant bioassay units were tested with all of the controls and treatments. Bioassay units were evaluated in sequence, but the samples given to the evaluator were randomized. In this way, a test unit that was alarmed had enough time to recover prior to the next test. To avoid complicating intra-colony responses, the workers in the bioassay units did not receive headspace samples from their own colony in the treatment/control vials. If negative or positive controls did not elicit the appropriate response, the experiment was terminated.

The *McNemar* test for significance of changes was used to analyze the alarm bioassay data. For analysis, bioassay scores of 1–2 were considered no reaction and scores of 3–4 were counted as alarm. The number of worker groups that displayed an alarm reaction to the test sample, but not to the negative control, was compared to the number of workers that reacted to the negative control but not to the test sample.

Shaken vs. Unshaken Workers Quiescent fire ant workers have an alarm response to headspace volatiles from shaken workers, but not to unshaken workers or clean air (Vander Meer et al. 2002). Unless specified otherwise, shaken and unshaken workers (100–200 in 20-ml glass scintillation vials coated on the upper inside surface with Fluon®) served as positive and negative controls, respectively. Air drawn from an empty 20-ml vial served as another negative control.

Amount of Venom Alkaloids in Shaken Ant Vials We tested whether shaking caused fire ant workers to release venom alkaloids. A thin layer of Fluon® was painted on the upper quarter of the inside surface of 20-ml scintillation vials, and about 100 fire ants were placed in each vial and weighed. The vials were shaken vigorously for 3–5 sec, and the ants were removed. *n*-Pentacosane (200 ng in 20 µl hexane) was added as an internal standard to the vial, which was then rinsed × 3 with hexane (100 µl each). The rinses were combined and the samples concentrated, if necessary, with

a gentle stream of nitrogen. A Varian 3700 GC, equipped with a flame ionization detector (FID) and a DB-1 fused silica column (0.32i.d.×30 m, 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA), programmed from 150 to 285°C at 10°C min^{-1}, following a 2 min hold, was used for analysis. Injector and detector temperatures were 300°C. The data were analyzed with a Turbochome data analysis Workstation (Perkin-Elmer). Peak assignments were based on retention times of authentic *S. invicta* alkaloids, obtained by soaking workers in hexane overnight (see Ross et al. 1987). The total alkaloid quantity, relative to the internal standard, was determined This procedure was repeated five times.

Fire Ant Alarm Response to Venom Components Fire ant worker venom sacs were dissected (Vander Meer et al. 2002) and extracted in hexane at concentrations that approximated the amount found in vials from shaken ants (0.01 WE/10 µl hexane; WE = worker equivalent) and at 1 WE (per 10 µl hexane), used previously by Wilson (1962). Extracts (10 µl) were deposited on 1-cm square pieces of filter paper, the solvent allowed to evaporate, and the squares placed in 20-ml scintillation vials. The solvent control was prepared in the same way. Samples were evaluated in the alarm bioassay, as described above, along with shaken-ant positive controls.

Amount of Recruitment Pheromone in Shaken Ant Vials Shaken ants may release recruitment pheromone from their Dufour's glands, which could induce an alarm reaction in quiescent ants, as described by Wilson (1962). A sensitive recruitment orientation bioassay (modified from Barlin et al. 1976; Jouvenaz et al. 1978) was used to detect Dufour's gland products from shaken ants. This method utilizes a natural fire ant food trail that goes from the floor of the foraging area up a tongue depressor connected to two plastic platforms, the first platform used for the bioassay observation and the second containing food (crickets and 20% sucrose solution absorbed unto pieces of cotton). The base of each platform was coated with Fluon® to prevent ants from diverting their food trail down the sides of the platforms. A piece of paper (trail paper), the size of the platform, was placed on top of the first platform while the trail was developing. Pieces of paper (the same size as the platform) were marked with arcs on both sides, with both ends of the two arcs meeting. Ten microliters of treatment or control were applied to the two marked arcs of the paper (bioassay paper), and the solvent was allowed to evaporate. The bioassay paper was exchanged with the trail paper and the behavior of the ants observed. If at least one of the trailing ants followed the treatment trail from beginning to end, the test was scored positive; however, if the ants were confused at the two ends of the trail and no ants followed the test trail, the bioassay was scored negative. Due to the volatility of the recruitment pheromone, the bioassay was terminated after 2 min.

Three separate Dufour's gland extracts were prepared (Vander Meer et al. 2002) and diluted to a concentration of 0.1 WE/10 µl hexane. This concentration was subjected to bioassay and repeated dilution by a third until activity was lost. This allowed us to determine the approximate minimum Dufour's gland concentration needed for a positive bioassay. Extracts of shaken ant vials were prepared as described above, except no internal standard was added. Samples of these extracts were concentrated to 20 µl, with 10 µl used in the initial bioassay and the remaining 10 µl used for dilutions, if necessary. Responses of ants to these extracts were compared with responses of ants to the standard Dufour's gland dilutions, allowing determination of the approximate Dufour's gland concentration in WE.

Fire Ant Alarm Response to Recruitment Pheromone Dufour's glands were dissected as previously described (Vander Meer et al. 2002). Two concentrations were prepared: a) 1 WE/10 µl hexane, used by Wilson (1962) and b) 0.01 WE/10 µl hexane, the approximate amount released by shaken ants (see Results). Dufour's gland extract (10 µl) was deposited on a 1-cm square piece of filter paper, the solvent allowed to evaporate (ca. 20 sec), and the paper placed in a 20-ml scintillation vial. The solvent control was prepared in the same way. Samples were evaluated in the alarm bioassay as described above.

Solid Phase Micro Extraction (SPME) Collection Black (Carboxen/ Polydimethylsiloxane) SPME fibers (Supelco, Belefonte, PA, USA) were used to collect and desorb alarm pheromone volatiles for analysis. The fibers were thermally cleaned in the injection port of a GC for 2 h at 290°C or overnight at 280°C, prior to and after use. Approximately 1–1.2 g of worker ants were placed in a 20-ml glass scintillation vial, lined with Fluon® along the upper quarter to prevent escape. Each vial was sealed with a cap containing a small hole (large enough for insertion of the SPME needle) in the center. The ants were allowed to settle for approximately 1 h before headspace collection. For the unshaken ant treatment, a vial with worker ants was placed carefully under the SPME holder, and the fiber was lowered into the vial; volatiles were collected for 1 min. The SPME fiber then was retracted and the process repeated nine more times prior to desorption. For the shaken-ant treatment, a vial, containing worker ants, was shaken vigorously for 5 sec and then sampled by SPME, as described for unshaken ants.

Compounds absorbed onto the SPME fiber were analyzed initially on an Agilent 6890 GC, equipped with a FID (250°C), splitless injector (250°C), and a 30 m× 0.25 mm i.d. DB-23 capillary column. The column oven was

programmed from 40°C to 100°C at then 5°C min^{-1}, then to 250°C at 25°C min^{-1}. The SPME fiber was retracted 2.5 min. after insertion into the injector. SPME fibers also were analyzed by gas chromatography-mass spectrometry (GC-MS), using the same model GC and conditions, interfaced with an Agilent Mass Selective Detector 5973 (Palo Alto, CA) in the electron impact (EI) mode.

Identification of the Pyrazine Released by Shaken Fire Ant Workers The tentatively identified alarm pheromone component, 2-ethyl-3,5-dimethylpyrazine, was commercially available as a mixture with the 2-ethyl-3,6-dimethylpyrazine isomer (Aldrich Chemical Co, Milwaukee, WI, USA). Samples of these two isomers also were obtained (purified by preparative gas chromatography and 99% pure individually; Buttery and Ling 1997) from the laboratory of Dr R. M. Buttery. The identity of the natural alarm pheromone compound was established by co-injection and comparison of its retention time with those of the standards.

Synthetic chemicals were tested in the alarm bioassay at 1 ng/µl and 100 ng/µl in light mineral oil (Fisher Scientific, Fairlawn, NJ, USA). The oil did not elicit an alarm reaction and acted to slow the release of the volatile pyrazines. The negative controls for these tests were mineral oil, air, and unshaken ants. The positive control was the shaken ants. For all mineral oil bioassays, 1 ml of test material was placed in a 20-ml scintillation vial and tightly capped until use.

Quantification of Pyrazine Concentration in Headspace of Mineral Oil Standards Solutions of 100, 66, 33, 10, 6, and 3 pg/µl, in hexane, of 2-ethyl-3,6(3,5)-dimethylpyrazine were analyzed on the Agilent 6890 N-5973, in the Selective Ion Monitoring (SIM) mode, monitoring *m/z* 39, 42, 56, 81, 108, 135, and 136, in order to generate a standard curve. Since the standard consisted of two isomers, the ion abundances for each isomer were added to generate the standard curve. To determine synthetic pyrazine concentrations in the headspace of the mineral oil treatments used in the alarm bioassay, standards in light mineral oil, of concentration 100, 50, 25, 10, and 1 ng/µl, were prepared, and 1 ml of each was transferred to separate 20-ml scintillation vials. The headspace from each of these standards was used in the alarm bioassay, while another set of identically prepared standards was analyzed by GC-MS (sampling 100 µl of headspace) and used to determine pyrazine headspace concentration by comparison with the standard curve.

Qualitative Analysis of 2-Ethyl-3,6-Dimethylpyrazine in Mandibular Gland, Head and Thorax Quantities of the pyrazine in mandibular gland extracts from male and female alate sexuals and workers were determined. Female or male alate sexuals, or large workers, were collected in a scintillation vial with the upper quarter inner surface painted with Fluon®. The ants were allowed to become quiescent for at least 20 min and then frozen in a dry-ice acetone bath. An ant was placed on a watch glass, resting on ice under the microscope, with dorsal side up, and the top of the head punctured, and the cuticle layer was peeled away toward the front. The remaining (bottom) part of the head exoskeleton was held, and one of the mandibles was pulled gently. If the mandible disengaged from the associated musculature and there was no visible lumen, the sample was discarded. If the mandible was pulled out with some musculature and/or a visible lumen, it was transferred to a clean probe and placed in hexane in a vial insert. Twenty mandibles were accumulated per sample, and each sample was extracted in 50 µl hexane at 4°C overnight prior to analysis. An internal standard (IS), 2-methyl-3-ethyl pyrazine (100 pg), was added to the samples. Later, in order to shorten the time required for mandibular gland dissections, we dissected a minimum frontal part of the head that included mandibular glands, but excluded the postpharyngeal gland and antennae. Head and thorax samples (20 of each) of female and male alates, and workers also were collected and extracted as above.

Samples were analyzed by GC-MS using the DB-23 column, with helium as carrier gas. The GC oven was held at 40°C for 2 min, then increased to 150°C at 15°C min^{-1}, held for 5 min., then increased to 250°C at 10°C min^{-1}. Compounds were quantified by SIM, monitoring *m/z* 121 and 122 (for the IS) and 108, 135, and 136 (for 2-ethyl-3,6-dimethylpyrazine). Quantification of the alarm pheromone was achieved by comparing the total ion area of the pyrazine component to the total ion area of the IS. Analyses were carried out on workers (N=16), female alates (N=19), and male alates (N=10). The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) using Statview 5.0 software.

Results

Amount of Venom Alkaloids in Shaken Ant Vials The mean weight of ca. 100 worker ants was 73.3±4.4 mg (mean ± SE, N=5). The mean weight of venom alkaloids released by the ants onto the vial after being shaken was 211.0±48.6 ng per vial (mean ± SE, N=5). Each venom sac contains ca. 30 µg of total alkaloid (Vander Meer 1988); therefore, the shaken ants deposited only 0.007 poison sac equivalents onto the glass vial.

Amount of Recruitment Pheromone in Shaken Ant Vials The lowest detectable Dufour's Gland equivalent from standard Dufour's gland extracts in the bioassay was 0.0033±0.0013 WE (mean ± SE, N=3). This represents

approximately 1.3±0.8 pg of *Z,E*-α-farnesene per cm of trail, (mean ± SE, N=3), since a worker Dufour's gland contains approximately 4 ng of *Z,E*-α-farnesene (RVM, unpublished). Recruitment orientation bioassays with residues in the vials from shaken ants showed no activity in 2 of 3 replicates. The active replicate showed minimal activity (one ant completely followed the trail within the 2 min. time limit of the bioassay), and activity was lost with the next serial dilution. Therefore, shaken ants probably release extremely low levels of recruitment pheromone, between 0.0067 and 0.0033 WE of a Dufour's gland.

Fire Ant Alarm Response to Poison Sac and Dufour's Gland Components Headspace from 1 WE poison sac samples elicited a lower alarm response in fire ant workers than the shaken ant positive control (G=4.167, P=0.041, df=1, N=15), but elicited a significantly greater response than the air negative control (G=6.125, P=0.013, df=1, N=15) (Fig. 1). The alarm response from the lower poison sac concentration (0.01 WE) was not different from the air negative control (G=0.8, P=0.371, df=1, N=15) (Fig. 1). One WE of poison sac can generate an alarm response in fire ants; however, the small amount of poison sac contents released by shaken ants did not contribute significantly to the observed alarm response induced by the headspace above shaken ants (Fig. 1). Dufour's gland extracts at 1.0 WE gave an alarm response that was not different from the alarm response from shaken ants (G=1.333, P=0.248, df=1; N=14). When the concentration was reduced to 0.01 WE of a Dufour's gland, the alarm response was not different from that of the negative air control (G=0.000, P=1.000, df=1; N=14). The alarm response of the 1.0 WE Dufour's gland concentration was greater than the response of the 0.01 WE concentration (G=8.100, P=0.004, df=1; N=14). As shaken fire ant workers release less than 0.01 WE of Dufour's gland products, these results suggest that the alarm reaction from shaken ants is not due to the release of Dufour's gland products.

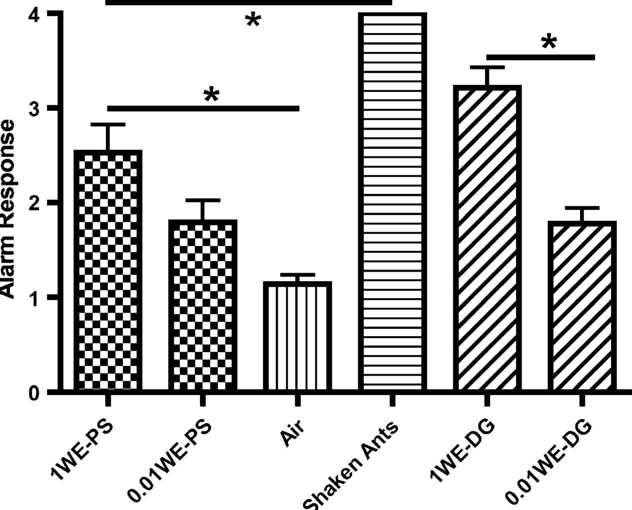

Fig. 1 Fire ant worker alarm responses to the headspace above, two poison sac extract concentrations (*WE* worker equivalent), two Dufour's gland (*DG*) extract concentrations, a positive control (shaken ants), and a negative control (air). The mean + SEM are shown (N=15). An * between two columns indicates a statistical difference (P< 0.05 *McNemar*'s Test)

Fig. 2 Structures of the two pyrazine isomers, 2-ethyl-3,6-dimethylpyrazine and 2,6-dimethyl-3-ethylpyrazine

Analysis of SPME Absorbed Volatiles Comparison of GC profiles from quiescent and shaken workers showed a difference of one major compound. The mass spectrum of this peak had a fragmentation pattern that matched 2-ethyl-3,5-dimethylpyrazine (2,6-dimethyl-3-ethylpyrazine; NIST 98 Mass Spectral Library, Rev.D.02.00, Palo Alto, CA, USA). Gas chromatographic analysis of the standards of this compound and the 2-ethyl-3,6-dimethylpyrazine isomer (Fig. 2), on a polar column, showed that the 2-ethyl-3,6-dimethylpyrazine eluted first (Buttery and Ling 1997). Our GC analysis showed that the natural compound co-eluted with this compound.

Alarm Pheromone Bioassay of 2-Ethyl-3,6-Dimethylpyrazine and 2-Ethyl-3,5-Dimethylpyrazine The alarm bioassay results for the commercial mixture of 2-ethyl-3,5(3,6)-dimethylpyrazine (ca. 60% 3,5-dimethyl isomer and 40% 3,6-dimethyl isomer) and the individual isomers are shown in Fig. 3. Responses to 1 ng/µl 2-ethyl-3,6-dimethylpyrazine were not significantly different (G=3.20, df=1, P=0.074, N=15) from the responses to the positive control of shaken ants, but were significantly greater (G=6.125, df=1, P=0.013, N=15) than those to the negative control of unshaken ants. In contrast, the alarm response to the 1 ng/µl synthetic mixture of 2-ethyl-3,5(3,6)-dimethylpyrazine was less (G=6.125, df=1, P=0.013, N=15) than that to the shaken ant positive control, but not different from that to the negative control of unshaken ants (G=3.2, df=1, P=0.074, N=15). By inference, the alarm response induced by the headspace above 1 ng/µl 2-ethyl-3,5-dimethylpyrazine was significantly less than that induced by the shaken ants, and not different from that by the unshaken ants. Responses to all 100 ng/µl treatment samples were not significantly different from the shaken ant positive control. In summary, 2-ethyl-3,6-dimethylpyrazine, had a lower active alarm response threshold than the 2-ethyl-3,5-dimethylpyrazine isomer and the mixture of the two isomers.

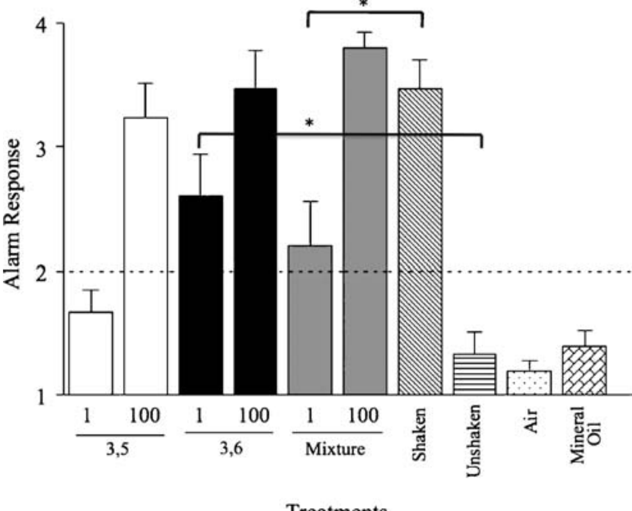

Fig. 3 Alarm responses of worker fire ants to two concentrations (in mineral oil) each of 2-ethyl-3,5-dimethylpyrazine (3,5), 2-ethyl-3,6-dimethylpyrazine (3,6), and a commercially available mixture of the two isomers (Mixture), negative controls (unshaken ants, air and mineral oil), and a positive (shaken ants) control. An * between two columns indicates a statistical difference ($P<0.05$, *McNemar*'s Test). An alarm response below the dashed line represents no alarm and alarm responses above the dashed line represents an alarm reaction

Alarm Response to Pyrazine Alarm Pheromone Concentration The fire ant alarm response to the headspace from a series of concentrations of the synthetic mixture of 2-ethyl-3,6(3,5)-dimethylpyrazine is shown in Fig. 4. The alarm response elicited from the headspace above the 1 ng/µl mixture was greater than that to the headspace above unshaken ants ($G=4.900$, $df=1$, $P=0.027$, $N=15$), but not different from that to headspace of the positive shaken ant control ($G=3.200$, $df=1$, $P=0.074$, $N=15$). By inference, all concentrations of the synthetic mixture of 2-ethyl-3,6(3,5)-dimethylpyrazine evaluated produced a greater alarm response than volatiles from the headspace above unshaken ants, with the exception of the lowest concentration, 100 pg/µl ($G=0.167$, $df=1$, $P=0.683$, $N=15$).

By using the standard curve ($y=1.3*10^6\times-628$; $R^2=0.9974$), derived from GC analysis of concentrations of the synthetic pyrazine mixture in hexane, the headspace concentrations of 100, 50, 25, 10, 1, and 0.1 ng/µl of the 2-ethyl-3,6(3,5)-dimethylpyrazine mixture in light mineral oil were determined as 0.5, 0.36, 0.24, 0.1, 0.03, and not detectable, pg/µl, respectively.

Pyrazine Alarm Pheromone in Adult Fire Ants Extracts of female and male alate and worker heads, and mandibles plus mandibular glands, all contained detectable amounts of 2-ethyl-3,6-dimethylpyrazine. In contrast, this compound was undetectable in thoracic extracts from these insects, demonstrating its specificity to the mandibular glands.

Workers contained significantly less 2-ethyl-3,6-dimethylpyrazine (316.6±42 pg/ant, $N=16$) than either female (746.9±80.8 pg/ant, $N=19$) or male (959.4±215.3 pg/ant, $N=10$) alates (Fisher PLSD, ANOVA, $P=0.002$ and $P<0.001$, respectively). The amounts of 2-ethyl-3,6-dimethylpyrazine in male and female alates were not significantly different (Fisher PLSD, ANOVA, $P=0.197$) (Fig. 5).

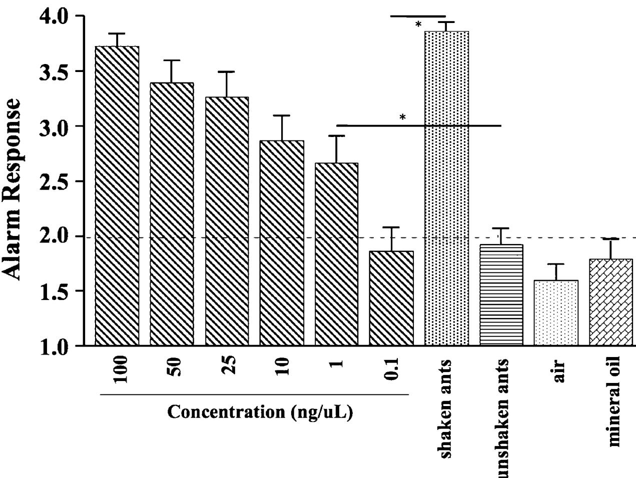

Fig. 4 Fire ant worker alarm responses to headspace above different concentrations of 2-ethyl-3,5(3,6)-pyrazine (in light mineral oil), negative controls (unshaken ants, air and mineral oil), and a positive (shaken ants) control. An * between two columns indicates a statistical difference ($P<0.05$, *McNemar*'s Test). An alarm response below the dashed line represents no alarm and alarm responses above the dashed line represents an alarm reaction

Discussion

Ant alarm pheromones are biosynthesized in a variety of exocrine glands and consist of chemicals of diverse

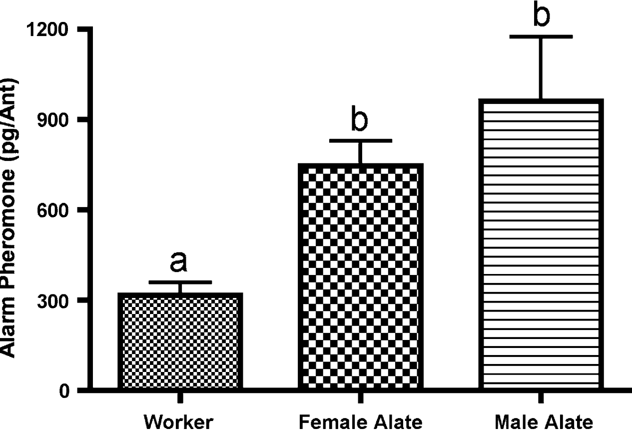

Fig. 5 The amount (mean + SEM) of 2-ethyl-3,6-dimethylpyrazine in fire ant workers (sterile females, $N=16$), and female ($N=19$) and male ($N=10$) sexual alates. Different letters above columns indicates means that are statistically different (*Fisher PLSD, ANOVA*, $P<0.05$)

structures (Vander Meer and Alonso 1998). Our identification of 2-ethyl-3,6-dimethylpyrazine as an alarm pheromone component of *S. invicta* increases the known diversity of ant alarm pheromones.

Pyrazines have been reported to have a variety of functions, including trailing (recruitment), defense, and alarm (El-Sayed 2009). The pyrazine reported here, 2-ethyl-3,6-dimethylpyrazine, has been reported primarily from the ant Subfamily Myrmicinae, which includes the genus *Solenopsis*, and also from the Ectatomminae, Dolichoderinae, and Ponerinae. In all cases, the function attributed to 2-ethyl-3,6-dimethylpyrazine is trailing (recruitment). While there are reports of 2-ethyl-3,6-dimethylpyrazine in mandibular glands of ants, no function has been attributed previously to this compound in ants (Cavill et al. 1984; Morgan et al. 1999). Another isomer, 2-ethyl-3,5-dimethylpyrazine, however, has been reported to be a part of the alarm pheromone of *Odontomachus brunneus* (Longhurst et al. 1978). This is the first report of 2-ethyl-3,6-dimethylpyrazine from a *Solenopsis* species, and is the first report of this alkaloid functioning as an ant alarm pheromone.

Although responses of fire ants to alarm pheromone previously have been documented (Wilson 1962), and the primary source of the pheromone known to be a cephalic gland, with attraction derived from a combination of the cephalic gland and Dufour's gland (Wilson 1962), the chemical identification of the fire ant alarm pheromone has eluded scientists. Our previous work had demonstrated that excited fire ant female and male sexual alates produce an alarm reaction in workers and that mandibular glands were the source of the alarm pheromone (Alonso and Vander Meer 1997). The ephemeral nature of fire ant alarm pheromones was demonstrated when headspace taken immediately after worker ants in a vial were electrically stimulated, elicited an alarm reaction. However, leaving the vial open for 2 min resulted in no alarm reaction (Vander Meer et al. 2002). This also partly explains the inability of some researchers to verify a cephalic source for fire ant alarm pheromones (Blum 1980).

Shaking a vial of worker ants revealed that both Dufour's gland and poison sac contents were deposited onto the vial, but at an amount less than one hundredth of a gland equivalent. This concentration did not elicit an alarm response from worker ants, although one Dufour's or poison gland equivalent did produce a significant alarm reaction. These results support the early alarm pheromone report by Wilson (1962), who used crushed worker body parts (one WE) to determine that the head, and probably the Dufour's gland, induced fire ant alarm responses. However, our results indicate that at physiologically relevant levels, neither Dufour's gland nor poison gland products are involved in eliciting the natural alarm response in fire ants, and that fire ant mandibular glands are the source of the alarm pheromone.

Because of the difficulty in obtaining behaviorally active extracts from worker mandibular glands, we used SPME to compare headspace above quiet and shaken workers to identify 2-ethyl-3,6-dimethylpyrazine as a unique component released from shaken fire ant workers. We then were able to detect and quantify this compound in the mandibular glands of workers, and male and female sexual alates. Our results showed that workers contain only about 300 pg of the pyrazine, which is consistent with the morphological description of fire ant mandibular glands as small and comprised of only a few secretory cells (Phillips and Vinson 1980; Billen 1990). The small quantity of alarm pheromone from fire ant workers is in sharp contrast to the microgram quantities (>3,000 fold more) of alarm pheromone found in other ant species, e.g., *Lasius alienus* Dufour's gland contains microgram quantities of alarm pheromone (Regnier and Wilson 1969). The high volatility of the fire ant pyrazine and the small quantity produced explains why isolation and identification of the alarm pheromone of *S. invicta* had proven difficult.

Fire ants respond to the pyrazine mixture at a concentration of 30 pg/cm^3, or 1.3×10^{11} molecules/cm^3, which is comparable to the alarm sensitivity of 10^{10}–10^{13} molecules/cm^3 predicted by a model (Regnier and Wilson 1968), and to alarm threshold measurements of $>10^{11}$ molecules/cm^3 for *Pogonomyrmex badius* (Wilson 1958). The detection threshold of 2-ethyl-3,6-dimethylpyrazine by humans is extremely low, roughly 3.8×10^{13} molecules/cm^3 (Buttery and Ling 1997). In comparison, the fire ant is about 300 times more sensitive to this compound.

Ants produce a wide variety of alkaloids (see El-Sayed 2009). Fire ants produce a variety of 2-methyl-6-alkyl or alkenylpiperidines (MacConnell et al. 1971) that are used by workers in a variety of defensive contexts (Obin and Vander Meer 1985) and for prey procurement. These alkaloids are produced by the poison gland and stored in large quantities in the venom sac (Vander Meer 1988) and have a variety of physiological functions e.g., insecticidal, antimicrobial, and herbicidal (Obin and Vander Meer 1985; Escoubas and Blum 1990). The patterns of piperidine alkaloid homologues have been shown to be species-specific and are useful taxonomic tools (Brand 1978; Vander Meer and Lofgren 1988). Our identification of a pyrazine alarm pheromone component, produced by mandibular glands of fire ants, adds to our knowledge of the variety of alkaloid biosynthesis in glands of ants.

Future research will focus on the ontogeny of 2-ethyl-3,6-dimethylpyrazine production in the fire ant, isolation and identification of additional fire ant alarm pheromone components, and the species specificity of 2-ethyl-3,6-dimethylpyrazine.

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Kairomonal Response of the Parasitoid, *Bracon hebetor* Say, to the Male-Produced Sex Pheromone of Its Host, the Greater Waxmoth, *Galleria mellonella* (L.)

Hany K. M. Dweck · Glenn P. Svensson · Eylem Akman Gündüz · Olle Anderbrant

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Abstract *Bracon hebetor* is a larval ectoparasitoid that utilizes several pests belonging to the family Pyralidae (Lepidoptera) as hosts. In the present study, we analyzed the kairomonal response of this wasp to the male-produced sex pheromone of a host, the greater wax moth *Galleria mellonella*, an economically important pest of honeybees, *Apis mellifera*. Coupled gas chromatography-electroantennographic detection (GC-EAD) revealed three compounds in headspace collections from male *G. mellonella* that elicited responses from *B. hebetor* antennae: decanal and the previously identified sex pheromone components, nonanal and undecanal. Y-tube olfactometer tests that used naïve, mated wasps showed that females, but not males, were highly attracted to (a) male *G. mellonella* headspace samples, (b) two synthetic blends of nonanal and undecanal (in ratios matching that found in male moth samples), and (c) the two aldehydes tested individually. Further, female wasps did not discriminate between a blend of aldehydes and male *G. mellonella* headspace. In dose-response trials that used octanal, nonanal, decanal, and undecanal, no difference in EAG responses of the two sexes was observed, except for undecanal at the second highest dose, for which female antennae showed significantly larger responses than did male antennae. When the two binary blends were tested at different doses, female wasps were significantly attracted to the two highest doses (1 µg and 10 µg), but not to the lowest dose (100 ng). Our results show that females of this economically important parasitoid utilize the male-produced sex pheromone of a host as an indirect cue to guide them to potential oviposition sites.

Keywords Host-parasitoid interaction · Semiochemical · Attraction · Behavioral experiment · Electrophysiology · GC-EAD · Hymenoptera · Braconidae · Lepidoptera · Pyralidae

H. K. M. Dweck
Entomology Department, Faculty of Science,
Cairo University,
12613 Giza, Egypt

G. P. Svensson · O. Anderbrant (✉)
Department of Ecology, Lund University,
Sölvegatan 37,
223 62 Lund, Sweden
e-mail: olle.anderbrant@ekol.lu.se

E. A. Gündüz
Department of Biology, Faculty of Science and Arts,
Ondokuz Mayıs University,
Kurupelit,
55139 Samsun, Turkey

Introduction

The greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), is an economically important pest of the honeybee, *Apis mellifera* (L.). Larval stages of the moth feed on wax, pollen, and honey, thus causing heavy economic damage as they burrow through the comb. One of the main biological control agents of *G. mellonella* is *Bracon hebetor* Say (Hymenoptera: Braconidae), a cosmopolitan, gregarious larval ectoparasitoid of many pests belonging to the family Pyralidae (Lepidoptera), including the Mediterranean flour moth, *Ephestia kuehniella* (Zeller), the Indian meal moth, *Plodia interpunctella* (Hübner), and the almond moth, *Cadra cautella* (Walker) (Benson 1973; Taylor 1988; Brower and Press 1990; Parra et al. 1996; Gündüz and Gülel 2004). The broad host range, high reproductive rate,

and short generation time of *B. hebetor* makes it an excellent candidate for biological control of many pyralid pests, including *G. mellonella*.

During host foraging, hymenopteran parasitoids commonly use semiochemicals emitted by the host, sources indirectly associated with the presence of the host (e.g., microorganisms), or the host habitat (Madden 1968; Vinson 1986; Godfray 1994; Rutledge 1996; Colazza et al. 2004; Laumann et al. 2009). Successful parasitism depends on appropriate habitat identification (i.e., long-range host location or host-habitat location), followed by short-range host location that includes host finding, host acceptance, host suitability assessment, and host regulation (Vinson et al. 1998). Parasitoids may use different semiochemicals emitted from different sources at various stages of the host selection process. In general, host habitat cues are most important at long distances, while cues from the host itself are more important at short distances (Weseloh 1981).

Sex pheromones released by host species have been shown to serve as kairomones in host location by a number of egg parasitoids, e.g., *Trichogramma* spp., *Telenomus* spp., *Chrysonotomyia ruforum* (Krausse), *Dipriocampe diprioni* (Ferrière), and *Ooencyrtus pityocampae* (Mercet) (Powell 1999; Bruni et al. 2000; Hilker et al. 2000; Reddy et al. 2002; Fiaboe et al. 2003), and by several larval/pupal parasitoids, e.g., *Apanteles ruficrus* (Haliday), *Microplitis rufiventris* (Kok), and *Eupelmus vuilleti* (CRW) (Powell 1999). Sex pheromones of aphids also are utilized as kairomones by several parasitoids, including *Diaeretiella rapae* (McIntosh), *Praon* spp., and *Aphidius* spp. (Powell 1999; Birkett and Pickett 2003; Powell and Pickett 2003). The parasitoids *Aphytis* spp., *Aphytis chilensis* (Howard), and *Encarsia perniciosa* (Tower), which attack the California red scale, *Aonidiella aurantii* (Maskell), the Oleander scale, *Aspidiotus nerii* (Bouché), and the San José scale, *Diaspidiotus* (=*Quadraspidiotus*) *perniciosus* (Comstock), respectively, also respond to host sex pheromone (Dunkelblum 1999; Powell 1999). All these interactions involve female-produced sex pheromones of the host; few studies have reported attraction of a parasitoid to an identified male-produced sex pheromone of the host (but see Borges et al. 1999; Silva et al. 2006).

Previously, it was not known whether the sex pheromone of *G. mellonella* is exploited by *B. hebetor* during the location of host larvae. Males of *G. mellonella* produce a sex pheromone in specific glands located on their forewings (Barth 1937; Roller et al. 1968), which has been identified as a mixture of nonanal and undecanal (Leyrer and Monroe 1973; Finn and Payne 1977; Romel et al. 1992). In this study, we evaluated the electrophysiological and behavioral responses of naïve, mated females and males of *B. hebetor* to the sex pheromone of male *G. mellonella*.

Methods and Materials

Insect Origin and Rearing The stock culture of *G. mellonella* originated from infested honeycomb stored in a warehouse in Samsun, Turkey, in 1997, and it has been maintained since on the same diet in the Laboratory of Animal Physiology at Ondokuz Mayıs University, Turkey. For the experiments, adult moths were collected from the stock culture and held in 1-liter glass jars, along with honeycomb for oviposition. Last instars were transferred to containers with fan-folded pieces of stiff paper and kept in an environmental chamber at 23°C, 60% r.h. and 16:8 L:D photoperiod. Individuals were separated by sex during the pupal stage.

Bracon hebetor was collected from a stock culture of *G. mellonella* in 1997 and has since been maintained on *G. mellonella* infesting honeycomb. Individuals used in the electrophysiological experiments, and the behavioral experiments with the 7:3 nonanal:undecanal blend (see results), were reared on *C. cautella*, whereas individuals used in the remaining behavioral experiments were reared on *G. mellonella* or *E. kuehniella*, from stock cultures in our laboratories. One *G. mellonella* last instar, or 1–3 *E. kuehniella*/*C. cautella* last instars, was introduced into a glass vial containing a honey-saturated cotton pad. One pair (female and male) of *B. hebetor* (<24-h-old) was introduced into the vial and allowed to attack the host for 48 h. Mated females and males were obtained by placing newly emerged female parasitoids with males in vials for 72 h. All parasitoids used in bioassays were 3 to 5-d-old. The test insects were reared in a controlled environment chamber at 25°C, about 60% r.h., with a 14:10 L:D photoperiod.

Preparation of Odor Stimuli Headspace collection was performed using small Teflon filters (3 mm i.d.), filled with adsorbent and connected to a battery-driven air pump (GroTech, Gothenburg, Sweden) via an aquarium hose. Initially, collection was performed on 10 male *G. mellonella* for 7 h, after the moths had been kept in the rearing jar for several days. In this experiment, 30 mg of SuperQ (Alltech, PA, USA, catalogue #2735) were used as adsorbent. Additional, 2–4 h collections (N=4) were later performed on 1–2 males placed in 50-ml glass jars. In these collections, 40 mg of Tenax GR 60/80 (Alltech, PA, USA, catalogue #4937) were used as adsorbent. The opening of the collection chamber was covered with part of an oven bag (Toppits®, Melitta Scandinavia AB, Klippan, Sweden). The flow rate of the pump was set to 200 ml min^{-1}, and odor collections were performed at the beginning of the scotophase at 25°C, 60% r.h., and 100 Lux red light. Collections from empty jars served as controls to identify possible contaminants.

After collection, filters were eluted with 200 µl of hexane with methyl stearate (10 ng/µl) added as an internal

standard. Prior to analysis by coupled gas chromatography and mass spectrometry (GC-MS), samples were concentrated to 50 µl with a stream of N_2. Synthetic aldehydes (nonanal, decanal, undecanal, of purity 95–99%) were purchased from Aldrich Chemical Ltd. (Dorset, England) or PolyScience Corp. (Niles, IL, USA). Male *G. mellonella* samples were diluted to match the concentrations of synthetic nonanal and undecanal in binary blends used in behavioral experiments.

Chemical Analysis Male *G. mellonella* headspace samples were analyzed with a Hewlett-Packard 5890 gas chromatograph, equipped with a non-polar HP-1 column (30 m×0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA), linked to an HP 5972 mass spectrometer. Helium was used as carrier gas at a velocity of 40 cm sec^{-1}, and injector temperature was 220°C. Oven temperature was programmed from 50°C (held for 2 min.) to 250°C at 10°C min^{-1}. Aldehydes in headspace samples were identified by using mass spectral libraries and by comparison of retention times and mass spectra with those of synthetic standards.

Electrophysiology Coupled gas chromatography-electroantennographic detection (GC-EAD) was used to identify physiologically active compounds in headspace samples of male *G. mellonella*. The head of a *B. hebetor* wasp was cut and mounted to a PRG-2 EAG (10× gain) probe (Syntech, Kirchzarten, Germany), with the head placed on one electrode and the tips of both antennae placed on the other electrode with conductive gel (Blågel, Cefar, Malmö, Sweden). Charcoal-filtered and humidified air, at 0.5 m sec^{-1}, passed over the antennae via a glass tube (0.6 cm i.d.), located 10 mm from the preparation. Headspace samples, or a blend of synthetic nonanal, decanal, and undecanal (10 ng/µl of each compound) were injected into a Hewlett-Packard 5890 Series II plus gas chromatograph, equipped with the non-polar HP-1 column. The effluent from the column was split, 1:1, between the flame ionization detector (FID) and a heated transfer line (230°C) that led to the antennal preparation. Hydrogen, at 40 cm sec^{-1}, was used as carrier gas. Oven temperature was programmed from 50°C (held for 2 min.) to 250°C at 10°C min^{-1}. A wasp preparation was used only for a single GC-EAD run.

In addition to GC-EAD, electroantennogram (EAG) dose-response tests were performed on female and male *B. hebetor* antennae by using octanal [found in male *G. mellonella* from Russia (Lebedeva et al. 2002)], nonanal, decanal, and undecanal, in hexane, as stimuli. A Pasteur pipette, containing 10 µl of the test stimulus on a strip of filter paper, was inserted through a hole in the glass tube, 10 cm before the outlet. The pipette was linked to an air-control system (Syntech, Kirchzarten, Germany), which generated 0.5 sec puffs through the pipette into the air stream of the glass tube. The amount of aldehyde loaded into a pipette ranged from 10 ng to 10 µg. Pipettes loaded with 10 µl of hexane served as controls. A dose-response trial for a specific aldehyde always started with the lowest dose and finished with the highest dose, and was always followed by a control stimulus. Each antennal preparation was stimulated with all four aldehydes at all four doses, but the order of aldehydes tested was randomized among antennal preparations.

Data obtained from GC-EAD and EAG experiments were analyzed with Autospike Version 3.3 (Syntech, Kirchzarten, Germany). In dose-response experiments, the EAG amplitude for each dose of a test compound was divided by the amplitude of the control stimulus associated with that compound, and the calculated ratios used in the statistical analyses. For each compound and dose, responses of female and male antennae were compared by using unpaired *t*-tests (SPSS 11.5).

Behavioral Experiments A Y-tube glass olfactometer was used to test the attraction of naïve, mated female and male *B. hebetor* to headspace samples, as well as to the two sex pheromone components of male *G. mellonella* (nonanal and undecanal) that showed EAD/EAG responses for both sexes of wasps (see Results). All bioassays were conducted during the photophase, between 0900 and 1700 h. The bioassay room was maintained at 28±1°C, with r.h. between 50% and 70%.

The olfactometer consisted of a central tube (15 cm long, 1 cm i.d.) and two lateral arms (5 cm long, 1 cm i.d.), which were separately connected to an extending glass tube (10 cm long, 1 cm i.d.). Humidified and purified air, at 150 ml min^{-1}, was passed into the extending glass tube through a Teflon connection. Illumination was provided by hanging an office lamp (20 W) vertically, 50 cm above the olfactometer. In each experiment, we used strips of filter paper (1 x 1 cm) loaded with either 10 µl of the test stimulus or hexane (control) as lures.

Based on the analysis of headspace samples (see Results), the following test stimuli (Table 1) were used: male *G. mellonella* headspace sample (stimulus #1), two synthetic nonanal:undecanal blends at a total dose and ratios matching those found in different headspace sampling methods (#3 and #6), and nonanal (#8) and undecanal (#9) at doses matching those found in headspace samples. In addition, to test the dose-response relationship of *B. hebetor* to the host sex pheromone components, the two binary blends were tested at three concentrations each (#2–4, and #5–7).

For each bioassay, a single parasitoid was introduced individually into the central arm of the Y-tube. We recorded

Table 1 Odor stimuli used in behavioural assays with *Bracon hebetor*

Stimulus	Amount applied onto filter paper (µg)	
	Nonanal	Undecanal
1. Male *Galleria mellonella* headspace sample	≈0.4	≈0.6
2. Two-component blend[a] (7:3) low	0.07	0.03
3. Two-component blend (7:3) medium	0.7	0.3
4. Two-component blend (7:3) high	7.0	3.0
5. Two-component blend (2:3) low	0.04	0.06
6. Two-component blend (2:3) medium	0.4	0.6
7. Two-component blend (2:3) high	4.0	6.0
8. Nonanal	0.7	–
9. Undecanal	–	0.3

[a] Ratio in parentheses refers to nonanal:undecanal

the initial choice of each wasp that walked into one of the arms (choice chambers) and remained there for at least 30 sec. If a wasp did not make a choice within 8 min. after being released into the olfactometer, it was considered as a non-responder and excluded from the analysis. After five wasps had been tested, the olfactometer arms were rotated 180°, to randomize any positional effects, and the filter strips were changed. After ten wasps had been bioassayed, the olfactometer was exchanged for a clean one. Olfactometer data were analyzed by chi-square tests (SPSS 11.5).

Results

Chemical Analyses All headspace samples of *G. mellonella* males contained large amounts of nonanal and undecanal, small amounts of the corresponding primary alcohols and fatty acids, as well as intermediate amounts of decanal and 6,10,14-trimethylpentadecan-2-one. The nonanal:undecanal ratio in the sample from male moths in a rearing jar was approximately 7:3, whereas the average ratio (±s.d.) in four samples from male moths in empty glass jars was 0.58±0.04. Based on these data, headspace samples with a nonanal:undecanal ratio of ≈2:3, and two synthetic blends with a nonanal:undecanal ratio of 7:3 or 2:3, were tested in behavioral experiments (see below). Decanal was probably a contaminant, as this compound was found in similar amounts in male headspace samples and control samples.

Electrophysiology Three compounds in male *G. mellonella* headspace samples consistently elicited responses from female wasp antennae: nonanal, decanal, and undecanal (Fig. 1). The GC-EAD activity of these compounds was confirmed by using a synthetic mixture of the three aldehydes (Fig. 1). In the dose-response trials using the three aldehydes and octanal, no difference in EAG responses was observed between sexes, except to undecanal at the second highest dose (1 µg), to which female antennae showed significantly higher responses than male antennae ($P<0.01$; Fig. 2).

Behavioral Experiments In the Y-tube bioassay, naïve mated *B. hebetor* males did not respond to male headspace ($\chi^2=0.36$; $P>0.05$; Fig. 3), or to the mixture of nonanal and

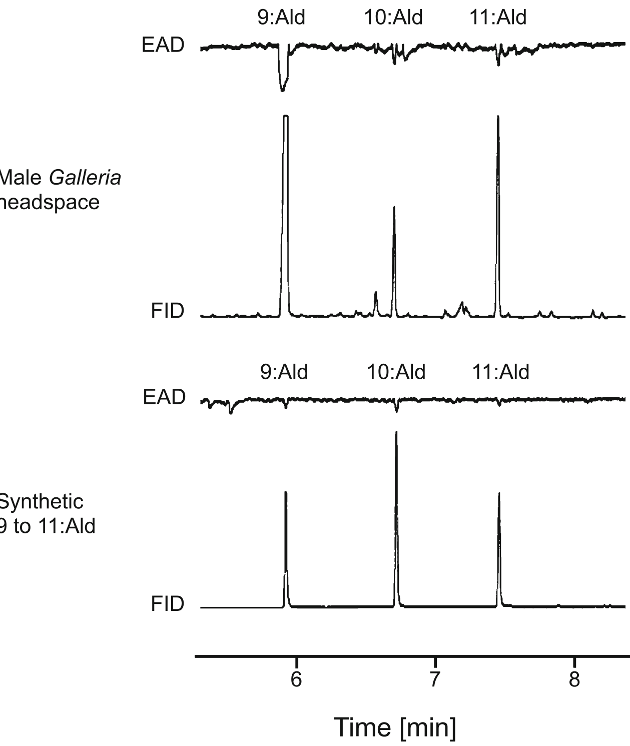

Fig. 1 Gas chromatography-electroantennogram detection (GC-EAD) responses of antennae of female *Bracon hebetor* to nonanal (9:Ald), decanal (10:Ald), and undecanal (11:Ald) in male *Galleria mellonella* headspace sample (*upper graph*) and synthetic reference compounds (*lower graph*). *FID* flame ionization detection

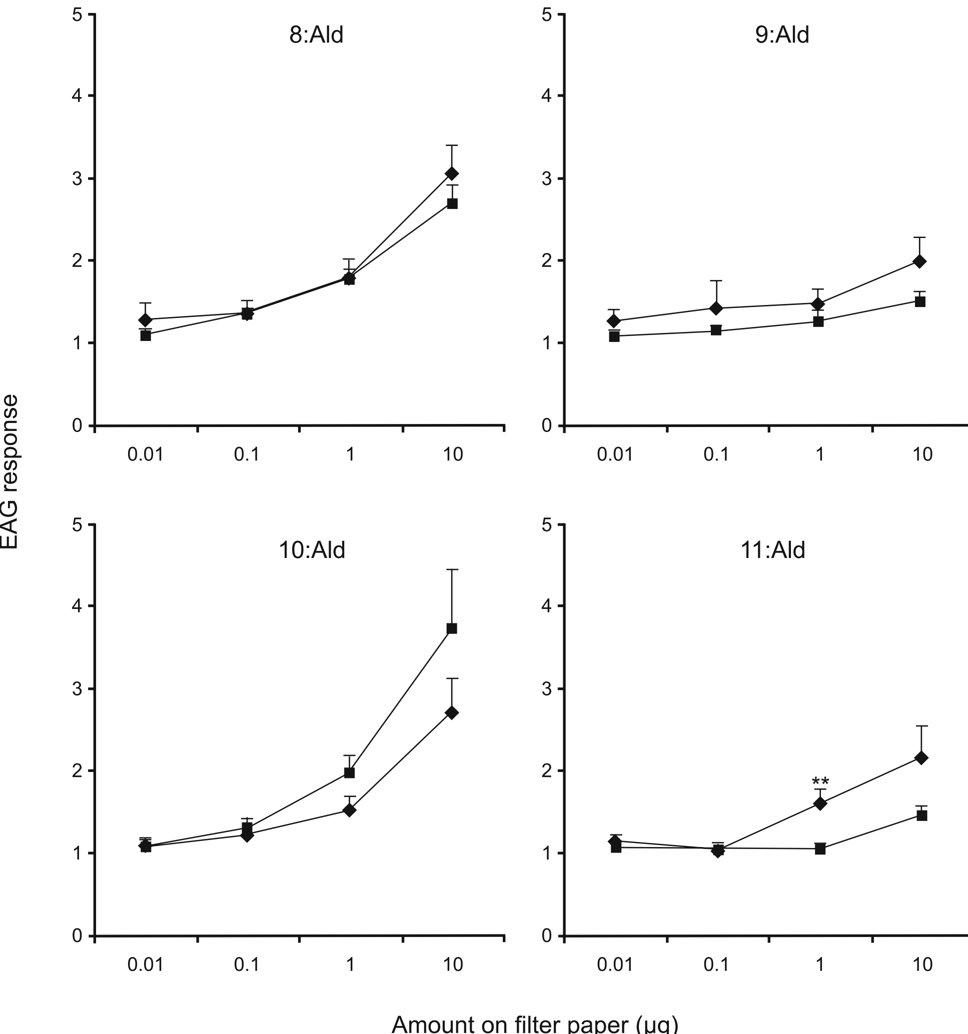

Fig. 2 Electroantennogram (EAG) responses of female (♦ N=14) and male (■ N=15) antennae of *Bracon hebetor* upon stimulation with various doses of octanal (8:Ald), nonanal (9:Ald), decanal (10:Ald), and undecanal (11:Ald). EAG responses were calculated as the ratio of the stimulation amplitude (test compound) to the control amplitude (air). *Error bars* show the standard error of the mean. The *asterisks* indicate a significant difference in response between sexes at a specific dose (unpaired *t*-test: ** $P<0.01$)

undecanal at the same dose (1 µg) as the headspace sample (7:3 ratio: χ^2=2.6; $P>0.05$; 2:3 ratio: χ^2=0.40; $P>0.05$; Fig. 3). In contrast, naïve, mated *B. hebetor* females were attracted significantly to the headspace sample (χ^2=14.4; $P<0.001$; Fig. 3), as well as to both synthetic binary blends at 1 µg (7:3 ratio: χ^2=4.8; $P<0.05$; 2:3 ratio: χ^2=9.0; $P<0.01$). When the headspace sample was tested against the binary blend at a nonanal:undecanal ratio of 2:3, at the same total amount of compounds (1 µg), no difference in attraction of female wasps was observed (χ^2=1.0; $P>0.05$, Fig. 3). Interestingly, when tested individually, both nonanal at 700 ng (χ^2=9.0; $P<0.01$) and undecanal at 300 ng (χ^2=4.8; $P<0.05$) were attractive to female wasps (Fig. 4). Dose-response trials that used the two binary blends showed that female wasps were attracted to the highest dose (10 µg; 7:3 ratio: χ^2=6.5; $P<0.05$; 2:3 ratio: χ^2=9; $P<0.01$), but not to the lowest dose (100 ng; 7:3 ratio: χ^2=0.13; $P>0.05$; 2:3 ratio: χ^2=0.04; $P>0.05$), of these mixtures (Fig. 4). In general, there were few non-responders ("No choice" in Figs. 3 and 4).

Discussion

Semiochemicals play an important role in mediating the interactions between parasitoids and their hosts. In some cases, these chemical cues are not directly associated with the attacked host stage, but help the parasitoids through restricting the search area. This is the case in several systems in which parasitoids utilize the host's sex pheromone to search an area where host mating is in progress and where oviposition has probably taken place or is soon to occur (Vet and Dicke 1992; Godfray 1994). In the present study, we demonstrated that an economically important parasitoid, *B. hebetor*, uses the male-produced sex pheromone components of its host, *G. mellonella*, as a kairomone for locating potential oviposition sites.

Electrophysiological analyses revealed that antennae of *B. hebetor* responded selectively to three compounds in male *G. mellonella* headspace samples: the two previously identified pheromone components, nonanal and undecanal (Barth 1937; Roller et al. 1968), as well as decanal. Other

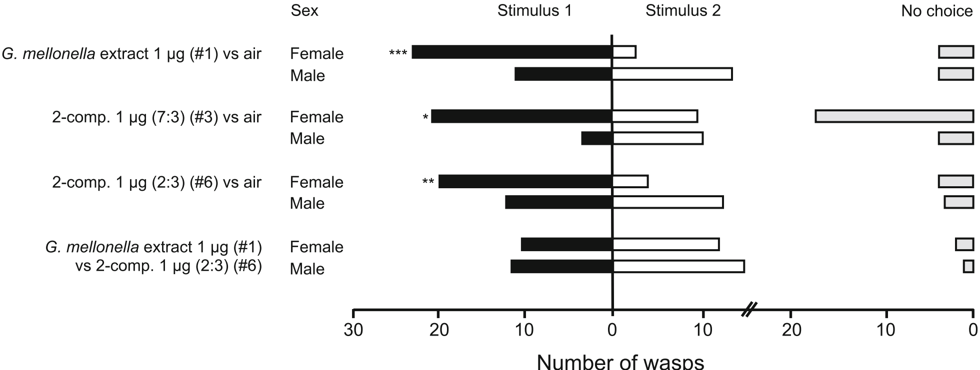

Fig. 3 Responses of naïve, mated females and males of *Bracon hebetor* to headspace sample of male *Galleria mellonella*, and to 7:3 and 2:3 mixtures of nonanal:undecanal (2-comp), in Y-tube olfactometer experiments. The total amount of aldehydes applied to the filter paper is shown (*chi-square* test: * $P<0.05$, ** $P<0.01$, *** $P<0.001$). The numbers of wasps that did not walk into one of the arms of the olfactometer and remain there for at least 30 sec are shown as "No choice"

compounds found in headspace samples in this study and in previous studies [1-nonanol, 1-undecanol, nonanoic acid, undecanoic acid (Romel et al. 1992), and 6,10,14-trimethylpentadecan-2-one (Lebedeva et al. 2002)] did not elicit antennal responses. Naïve mated female wasps were attracted significantly to two different blends of nonanal and undecanal in Y-tube tests. Further, female wasps did not discriminate between the synthetic binary blend and the headspace sample of male *G. mellonella*. This attraction to the compounds apparently was independent of the host species on which the test individuals were reared upon as larvae.

Few studies have reported attraction of female parasitoids to male-produced volatiles of the host. The egg parasitoid *Trissolcus basalis* (Wollaston) is attracted to volatiles released from virgin males of its host, *Nezara viridula* (L.) (Colazza et al. 1999). The same pattern has been observed for the congener *T. brochymenae* (Ashmead) that attacks *Murgantia histrionica* (Hahn) (Conti et al. 2003). However, the chemicals that mediate parasitoid attraction in these interactions were not identified. The only studies we know that show parasitoid attraction to an identified male sex pheromone of the host are by Borges et al. (1999) and Silva et al. (2006). They demonstrated that *Telenomus podisi* (Ash.) was attracted to methyl 2,6,10-trimethyltridecanoate, the male-produced pheromone of *Euschistus heros* (Fabr.) (Borges et al. 1998).

We used two different blends of aldehydes in the behavioral tests, based on the contrasting ratios obtained from the two different collection procedures (i.e., 10 males kept in the rearing jar for several days before collection was performed, or 1–2 males kept in a glass jar only during the time of odor collection). The nonanal:undecanal ratio of 7:3, obtained by the former method, was the same as that reported by Leyrer and Monroe (1973), who collected headspace from 50 males over 1 or 2 days. This ratio probably reflects the faster accumulation of the more volatile nonanal, relative to the less volatile undecanal, over longer collection periods. In contrast, the ratio of 2:3 obtained in the latter experiments is similar to that reported by Romel et al. (1992), who collected headspace for just a few hours. Regardless, female wasps were attracted similarly to both ratios in Y-tube experiments.

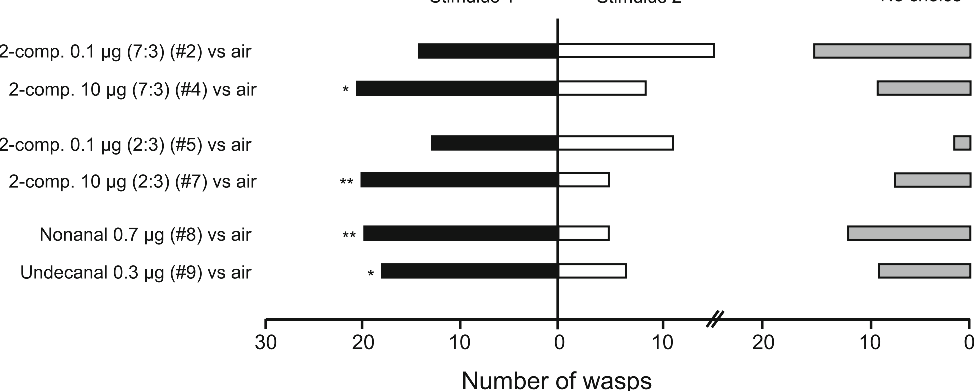

Fig. 4 Responses of naïve, mated females of *Bracon hebetor* to nonanal, undecanal, and 7:3 and 2:3 ratios of nonanal:undecanal (2-comp), in Y-tube olfactometer experiments. The total amount of aldehydes applied onto the filter paper is shown (*chi-square* test: * $P<0.05$, ** $P<0.01$). The numbers of wasps that did not walk into one of the arms of the olfactometer and remain there for at least 30 sec are shown as "No choice"

Another indication of a flexible response by female *B. hebetor* to the *G. mellonella* sex pheromone was its attraction to both aldehydes, individually, in contrast to many other parasitoid-host interactions for which mixtures of host sex pheromone components are needed to elicit parasitoid attraction. Another example of parasitoid attraction to individual component(s) of host sex pheromone is *Trichogramma chilonis* (Ishii). This parasitoid utilizes several moth species as hosts, including *Plutella xylostella* (L.) and *Ostrinia furnacalis* (Guenée). Female *T. chilonis* are attracted to (*Z*)-11-hexadecenyl acetate, a sex pheromone component of *P. xylostella* (Reddy et al. 2002), as well as to (*E*)-12-tetradecenyl acetate, a sex pheromone component of *O. furnacalis* (Boo and Yang 2000). Other natural enemies of *P. xylostella*, such as *Cotesia plutellae* (Kurdjumov) and *Chrysoperla carnea* (Stephens), respond only to more complete blends of the *P. xylostella* pheromone and not to individual components (Reddy et al. 2002).

The kairomonal response of *B. hebetor* females to the *G. mellonella* sex pheromone appears to be innate, as the wasps had no previous experience with odors of that host. Glinwood et al. (1999) demonstrated that naïve (i.e., received no experience of aphids or sex pheromone prior to testing) *Aphidius ervi* (Haliday) responded to aphid sex pheromone in olfactometer bioassays, providing further evidence that the response is innate. Innate responses to species-specific odor cues, such as adult sex pheromones, were reported by Mbata et al. (2004) for the generalist parasitoid *Pteromalus cerealellae* (Ashmead). This species is known to utilize insect hosts from two orders and four families (Brower 1991). Steidle et al. (2001, 2003), reported that a related generalist parasitoid, *Lariophagus distinguendus* (F.), also responded innately to the pheromone of one of its hosts.

Despite the sexual dimorphism in behavioral responses to host odors, no major difference in antennal sensitivity was observed between female and male *B. hebetor*, suggesting that the response difference was not a result of sexual dimorphism in the peripheral olfactory system (see Dweck and Gadallah 2008). Instead, our data imply that the behavioral differences arose from different processing by female and male central nervous systems of similar sensory input. Similar findings have been shown for the pteromalid bark beetle parasitoids, *Roptrocerus xylophagorum* (Ratzeburg) and *Rhopalicus tutela* (Walker) (Pettersson et al. 2000; Pettersson 2001).

Our study is not the first to demonstrate the use of semiochemicals for host location by *B. hebetor*. Parra et al. (1996) suggested that the species is attracted to grain moth infestations by volatile chemicals emanating from *E. kuehniella* frass. In addition, female *B. hebetor* are attracted to frass extracts from several host species (Dweck, Gündüz, Svensson, Anderbrant, unpubl. data). This use of olfactory cues by *B. hebetor* for location of hosts may be able to be exploited in pest control programs that utilize this parasitoid.

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Plants on Constant Alert: Elevated Levels of Jasmonic Acid and Jasmonate-Induced Transcripts in Caterpillar-Resistant Maize

Renuka Shivaji · Alberto Camas · Arunkanth Ankala · Jurgen Engelberth · James H. Tumlinson · W. Paul Williams · Jeff R. Wilkinson · Dawn Sywassink Luthe

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Abstract This study was conducted to determine if constitutive levels of jasmonic acid (JA) and other octadecanoid compounds were elevated prior to herbivory in a maize genotype with documented resistance to fall armyworm (*Spodoptera frugiperda*) and other lepidopteran pests. The resistant inbred Mp708 had approximately 3-fold higher levels of jasmonic acid (JA) prior to herbivore feeding than the susceptible inbred Tx601. Constitutive levels of *cis*-12-oxo-phytodienoic acid (OPDA) also were higher in Mp708 than Tx601. In addition, the constitutive expression of JA-inducible genes, including those in the JA biosynthetic pathway, was higher in Mp708 than Tx601. In response to herbivory, Mp708 generated comparatively higher levels of hydrogen peroxide, and had a greater abundance of NADPH oxidase transcripts before and after caterpillar feeding. Before herbivore feeding, low levels of transcripts encoding the maize insect resistance cysteine protease (Mir1-CP) and the Mir1-CP protein were detected consistently. Thus, Mp708 appears to have a portion of its defense pathway primed, which results in constitutive defenses and the ability to mount a stronger defense when caterpillars attack. Although the molecular mechanisms that regulate the constitutive accumulation of JA in Mp708 are unknown, it might account for its enhanced resistance to lepidopteran pests. This genotype could be valuable in studying the signaling pathways that maize uses to response to insect herbivores.

Keywords Maize · Induced defenses · Jasmonic acid · Defense gene expression · Herbivory · Plant-herbivore interactions · Monocot · *Spodoptera frugiperda* · Fall armyworm

R. Shivaji · A. Camas · A. Ankala · J. R. Wilkinson
Department of Biochemistry and Molecular Biology,
Mississippi State University,
Mississippi State, MS 39762, USA

J. Engelberth
Department of Biology, University of Texas San Antonio,
San Antonio, TX 78249, USA

J. H. Tumlinson
Department of Entomology and Center for Chemical Ecology,
The Pennsylvania State University,
University Park, PA 16802, USA

W. P. Williams
USDA-ARS Corn Host Plant Resistance Research Laboratory,
Mississippi State, MS 39762, USA

D. S. Luthe (✉)
Department of Crop and Soil Sciences,
The Pennsylvania State University,
University Park, PA 16802, USA
e-mail: dsl14@psu.edu

A. Camas
Cinvestav-Unidad Irapuato, Dpto. Ingenieria Genetica,
Km. 6.9 Libramiento Norte, Carr Irapuato-Leon,
Gto 36821, Mexico
e-mail: jcamas@ira.cinvestav.mx

Introduction

Numerous arthropod pests attack maize, an important crop grown throughout the world. Some of the most devastating of these insects are larvae in the order Lepidoptera, and they include various corn borers (*Ostrinia nubialis*, *Diatraea grandiosella*, *Sesamia nonagrioides*), the corn earworm (*Helicoverpa zea*), and fall armyworm (*Spodoptera frugiperda*). Although transgenic technology has vastly improved control of these pests, it is not always publicly

acceptable. Consequently, the development of host plant resistance to insect herbivores, especially against lepidopteran larvae, is an alternative and/or supplement to transgenic technology and chemical control methods. Over a number of years, the maize inbred lines Mp704 and Mp708 that are resistant to fall armyworm and southwestern corn borer (*Diatraea grandiosella*) have been developed using classical plant breeding (Williams et al. 1990b). These maize inbreds exhibit a remarkable resistance to a number of lepidopteran pests (Davis et al. 1988) in both field and laboratory tests, and they are often designated multiple-borer-resistant genotypes. The germplasm used to develop these lines originated in Antigua, where it was probably selected for its ability to yield well under high insect pressure. The phenotype exhibited by these lines is a reduction in larval weight by approximately 50%. Larvae recovered from resistance plants grown in the field are significantly smaller than are those reared in laboratory bioassays on fresh or freeze-dried material (Williams et al. 1990a). Not only are larvae reared on whorl tissue from resistant plants smaller, their development is delayed and they pupate later. This results in smaller larvae that are susceptible to predators and parasitoids in the field for a longer period of time.

A number of studies using Mp704 and Mp708 have been conducted to elucidate the mechanism of resistance, which is a multigene trait regulated by approximately 12 QTL (Brooks et al. 2005, 2007). The plants do not contain significant levels of secondary compounds like DIMBOA (Hedin et al. 1984), but there are differences in protein and hemicellulose levels that are associated with resistance (Williams et al. 1998). Other studies have shown that larval feeding results in the accumulation of a cysteine protease, Mir1-CP, at the wound site in the yellow-green region of the maize whorl (Pechan et al. 2000). Transgenic plant material expressing Mir1-CP inhibited fall armyworm larval growth by approximately 80% (Pechan et al. 2000). The LC_{50} for purified recombinant Mir1-CP ranged from 0.6 to 8.0 ppm depending on the lepidopteran species (Mohan et al. 2008). Both in vitro and in vivo studies indicated that Mir1-CP perforates the insects' peritrophic matrix (PM), which is an acellular structure that surrounds the food bolus and protects the midgut microvilli from chemical and physical damage (Pechan et al. 2002; Mohan et al. 2006). Physiological studies have shown that larvae feeding on whorl tissue from resistant plants are less able to utilize nutrients from ingested and digested food (Chang et al. 2000), which could be attributed to disruption of the PM that prevents nutrient recycling and accounts for the reduction in larval growth (Pechan et al. 2002).

Because resistance in these lines is a multigene trait, it would be useful to determine what other characteristics contribute to the resistance phenotype. Since chemical signals play a vital role in protecting plants from insect herbivores, they might account for some of the resistance found in Mp704 and Mp708. Some chemicals attract insect predators, others prime the plant for subsequent herbivory, while others trigger a signaling cascade within the plant that results in the accumulation of a plethora of defensive molecules (Walling 2000; Kessler and Baldwin 2002; Frost et al. 2008; Zhu-Salzman et al. 2008). These signals include jasmonic acid (JA), its conjugates, and other molecules in the octadecanoid pathway that signal plant responses to both abiotic and biotic stresses and regulate plant developmental pathways (see recent reviews by Wasternack 2007; Balbi and Devoto 2008; Browse and Howe 2008; Howe and Jander 2008; Kazan and Manners 2008). However, one of the crucial functions of JA is to transmit the signals that enable the plant to mount defenses against insect herbivores (Wasternack 2007; Browse and Howe 2008; Howe and Jander 2008). As would be expected for a signaling compound, JA biosynthesis is tightly regulated, and its concentration in plants prior to insect infestation generally is low (Hildebrand et al. 2000). However, JA levels dramatically increase in response to herbivory resulting in the increased expression of enzymes in the JA biosynthesis pathway and other defensive molecules. Although the JA signaling pathway has been studied extensively in dicot plant species, far less is known about it in monocots (Kazan and Manners 2008). Little is known about the role of JA and other octadecanoids in maize response to herbivory, especially at the midwhorl growth stage approximately five weeks after planting, when plants are particularly vulnerable to lepidopteran feeding in the field. The role of JA in positively regulating the accumulation of Mir1-CP in Mp708 recently has been demonstrated (Ankala et al. 2009). JA-treatment increased the expression of Mir1-CP and its transcripts, whereas blocking the octadecanoid pathway with ibuprofen or salicylic acid (SA) prevented its accumulation and resulted in plants more susceptible to herbivory (Ankala et al. 2009). Although the accumulation of Mir1-CP in Mp708 in response to caterpillar feeding or JA application is rapid and abundant, it has been observed consistently that low amounts of the protein and its transcripts are present prior to feeding (Pechan et al. 2000; Ankala et al. 2009). In addition, maize whorls collected in the field prior to herbivory and immediately lyophilized also retard caterpillar growth by 50% (Williams et al. 1990a). These observations suggested that parts of herbivore defense pathways are constitutively expressed in the resistant genotypes and that these plants may be "primed" to respond to herbivore attack. Priming has been defined as "the physiological state that enables plants to activate better or stronger defense responses" (Beckers and Conrath 2007). Generally plants are primed by external factors such as volatile organic compounds (Engelberth et al. 2004; Ton et al. 2006; Frost et al. 2008), but in this case it appears that the

insect resistant genotype Mp708 may be "naturally or genetically primed". We propose that Mp708 has constitutively elevated JA levels that cause the expression of certain defense genes prior to herbivory and that allows the plants to mount a rapid and strong response to subsequent herbivore feeding. In this study, we measured the levels of JA and *cis*-12-oxo-phytodienoic acid (OPDA) in the resistant and susceptible inbreds prior to and during herbivory. We also examined the expression of key genes that lead to the production of defensive proteins in maize, as shown in our model of the herbivore-defense signaling pathway, to determine if their transcript levels were elevated prior to herbivore attack (Fig. 1). These included genes in the JA-biosynthetic and signal transduction pathways and several genes that encode proteins involved in direct defenses.

Methods and Materials

Plant Materials and Fall Armyworm Feeding Two inbred lines of maize (*Zea mays*) Mp708 and Tx601 that are resistant and susceptible to fall armyworm feeding, respectively, were used (Williams et al. 1990b). Mp708 was selected from a cross between the resistant inbred Mp704 and Tx601 (Williams et al. 1990b). In numerous studies over 15 years, we have not detected the expression of Mir1-CP or its transcript in Tx601. However, Tx601 does have a defense response that includes the induction of defense gene expression in response to fall armyworm feeding (see Fig. 4). Plants were grown under greenhouse conditions at the Plant Science Research Center, Mississippi Agricultural and Forestry Experiment Station (Mississippi State University, USA). Fall armyworm larvae were reared on artificial diet at the Corn Host Plant Research Unit Insect-Rearing Laboratory of the USDA-Agricultural Research Service. Larvae that were 5-7-d post hatch (*ca*. 3rd instar) were used and seven larvae were placed in the whorl of 4–5-wk-old corn plants. This experiment was repeated three times with five plants for each time point and genotype. Tissue surrounding the feeding area in the yellow-green region of the whorl was collected at 0 time (no feeding) or after 1.5 or 24 h of feeding. Collected samples were frozen immediately in liquid nitrogen and stored at −70°C until use.

Quantification of Jasmonates and Salicylate Approximately, 100 mg of each sample tissue (frozen in liquid

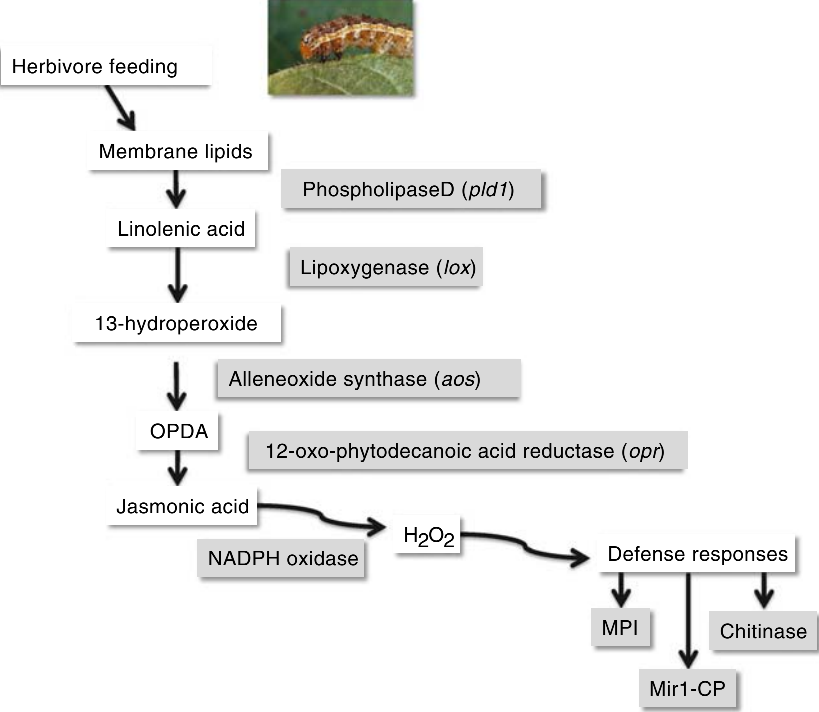

Fig. 1 A generalized diagram of the steps that occur in response to caterpillar feeding and that lead to the production of plant proteins involved in direct defenses. The enzyme phospholipase D releases linolenic acid from membranes. Linolenic acid is converted to jasmonic acid (JA) through a series of reactions. Key enzymes catalyzing some of the reactions are lipoxygenase (LOX), allene oxide synthase (AOS), and 12-oxo-phytodecanoic acid reductase (OPR). NADPH oxidase forms superoxide that is converted to the signaling molecule H_2O_2, which in turn triggers the production of maize protease inhibitor (MPI), maize insect resistance 1 cysteine protease (Mir1-CP), and chitinase. Transcript levels for the genes encoding the enzymes in the gray boxes and the three defense proteins were measured in this study

N_2) were used for the extraction and quantification of JA, OPDA, and SA as described previously (Engelberth et al. 2003). After extraction, the samples were analyzed by Chemical Ionization (CI)-GC/MS (Engelberth et al. 2003; Schmelz et al. 2003). Because appropriate internal standards are not commercially available, OPDA was quantified by comparison with an external standard curve as described previously (Schmelz et al. 2003).

RNA Extraction and Reverse Transcription Total RNA was isolated from the frozen leaf samples ground in liquid nitrogen using the Rneasy mini-kit (Qiagen) as per manufactures protocol. RNA quality was verified electrophoretically on 1% agarose gels followed by ethidium bromide staining. RNA samples were quantified spectrophotometrically by absorbance at 260 nm. Synthesis of cDNA and RT-PCR was carried out using the ThermoScript RT-PCR System (Invitrogen). cDNA synthesis was carried out with Oligo $(dT)_{20}$ primers using 500 ng of each RNA sample. RT-PCR conditions were optimized for each primer in a gradient thermocycler (Master Cycler, Gradient, Eppendorf) with regard to annealing temperatures. RT-PCR products were analyzed on a 1% agarose gel, and the optimized annealing temperatures were used for quantitative real-time PCR (qRT-PCR) experiments.

Quantitative RT-PCR qRT-PCR was carried out in a Roche Light Cycler 2.0 instrument using 0.5 μM each of forward and reverse primers (HPLC purified) (Table 1), 2 μl of Fast Start DNA Master SYBR Green I (Roche Diagnostics), and 1 μl cDNA (equivalent to 25 ng of mRNA). A no-template negative control was run with each set of samples to check for primer dimers or contaminants in the reagents. The following Light Cycler protocol was used. Pre-incubation (95°C for 10 min); amplification and quantification (95° for 10 sec, annealing temperature for specific primer for 10 sec, 72° for 5 sec) repeated 45 cycles: melting curve (65°–95° with 0.1°C/sec heating rate). A trial run was carried out for each primer set with all samples, and the one showing the highest expression (lowest CP) was used for construction of a standard curve using serial dilutions. Primer efficiency was determined for each primer from the standard curve (Pfaffl 2001). The relative expression for each target gene was calculated using the mathematical model proposed by Pfaffl (Pfaffl 2001). Ubiquitin was used as the reference gene. Specificity of RT products was confirmed by agarose gel electrophoresis of the product as well as evaluation of the melting curve.

Statistical Analysis Jasmonate and salicylate quantification data were obtained from three sets of experimental repeats

Table 1 Genes selected for qRT-PCR, accession number, primer sequence, resulting product size and annealing temperature

Gene	Accession number	Primer sequence (5’——3’)	Product size (bp)	Annealing temp (°C)
Phospholipase D	D73410	GTGAGGTAAAGCAGGAGGGAGAAT GGCGCGGGTGTCGGGGAAGT	503	60
ZmLOX1	AF271894	GAACGGGGAAACGCAAACAATCTA GCCGGGCAGCGACGAGTA	450	60
Allene oxide synthase	AY488135	CCAGGTGAGGAAGGGCGAGATGCT GTGAAGGTGGGGCCGAGGGTGAGA	484	60
ZmOPR7	AY921644	CGGCTGTTCATCGCTAATCCCGA CAATCGCGGCATTACCCAGATGT	248	65
ZmOPR1	AY921638	ATAGCGGCTTGCTCTACTGC CATCCAGCTCGAACCTCCTA	244	60
ZmOPR2	AY921639	ACCGCTCCATCTTCTACACGCAAG CCACAATGGCCAATAATGAATG	161	60
ZmOPR6	AY921643	AGCAGGCTTTGATGGAGTGGA TTGGCAAAACGCATCGGAAGG	516	65
PRm3 (chitinase)	AY107125	CGCCGCAGTGCCCCTACCC TCTCCCGATGATCCGCTCTTATATTA	477	60
ZmLOX3	AF149803	TCACGAGCCAGATCCAGACCA ATTCGATTCACCAGCCCACACG	474	60
Mir1	AF019145	CTACTGGATCGTGAAGAACTCGTG CTCCCTCCTAAGCAAGAACCATC	211	64
NADPH oxidase	CK849936	ACCAGCGCGTCGGAGTGTTC TGGCATTTCGATCATTAGTTCTTC	310	60
MPI	X78988	ATGAGCTCCACGGAGTGC TCAGCCGATGTGGGGCGTC	222	60
Ubiquitin	U29162	TGATAATGTGAAGGCCAAGATCCAG GGTCTGGGGGAATCCCCTCCTTGTC	277	60

where each experiment had four to five biological repeats for each time point and each genotype. qRT-PCR data are from three biological repeats for each genotype and time point. Statistical analysis was carried out with a mixed model analysis of variance using the MIXED procedure of SAS (SAS Institute, Cary NC, USA).

Immunoblot Analysis Immunoblot analysis was conducted from whorl samples as previously described (Pechan et al. 2000).

Detection of H_2O_2 in Leaves Presence of H_2O_2 was detected by using 3,3- diaminobenzidine (DAB) as a substrate (Orozco-Cardenas and Ryan 1999) The DAB solution (1 mg/ml) was prepared by stirring DAB in PBS buffer of pH 3.2 for 5 h and adjusting the final pH to 5.6 with KOH.

Detection of H_2O_2 in Wounded Leaves The plants were cut at the soil level, and the cut stems were placed separately in these solutions for 12 h: (a) DAB solution; (b) DAB solution containing 100 μM diphenylene iodonium (DPI), an inhibitor of NADPH oxidase (Frahry and Schopfer 1,998); or (c) DAB solution containing 10 mM ascorbic acid, an antioxidant. The whorl region was crushed with a hemostat five times. This resulted in severe wounding to the tissue, but the plants remained structurally intact. The damaged whorl region was collected 10 min after wounding. The leaf segments were boiled in ethanol for 10 min to decolorize the leaves allowing clear visualization of the reddish brown color formed by the polymerization of DAB in the presence of H_2O_2. As control, unwounded leaves were stained in DAB solution and boiled in ethanol.

Detection in Fall Armyworm Infested Tissues The outer leaves of 5-day old plants were gently peeled off to expose the yellow-green region of the whorl region. Five-d-old fall armyworm larvae that had been starved for 6 h were placed in the whorl region and were allowed to feed for 3 or 24 h. The feeding site then was dipped in a Petri dish containing 15 ml of DAB solution for 3 min while the leaf was still attached to the plant. This was done to ensure that the peroxide formed was due to larval feeding and not to wounding caused by cutting the leaf. A separate replicate was performed with DAB solution containing 10 mM ascorbic acid. The stained area was cut from the plant and was boiled in ethanol for 10 min.

Results and Discussion

JA and OPDA Levels in Mp708 and Tx601 Three time points were selected for the determination of JA levels in Mp708 and Tx601. Since the primary objective of this study was to determine if JA levels in Mp708 were higher than those in Tx601 prior to herbivore feeding, samples were collected at the control time point (0 h). The 1.5 h time point was used because this is the minimum amount of time needed to detect the accumulation of Mir1-CP protein in response to fall armyworm feeding. The 24 h time point was selected because prior investigations indicated that Mir1-CP transcripts (*mir1*) were present prior to herbivory and did not increase during the first 24 h of feeding, which suggested that endogenous JA levels might maintain elevated transcript levels during this time. When both *cis*- and *trans*-JA levels were determined in whorls of the resistant and susceptible inbreds prior to herbivory, the results indicated that the resistant line Mp708 had *ca.* 3 to 4-fold higher levels of *cis*-JA and *trans*-JA than Tx601 (Fig. 2a, b). In addition to higher JA levels, OPDA, an intermediate in the JA biosynthetic pathway, was *ca.* 4-fold higher in Mp708 than Tx601 (Fig. 2c, d). In Mp708 and Tx601, *cis*-JA levels increased in response to fall armyworm feeding from 0 to 24 h and its levels were almost equal in both genotypes after 24 h of feeding (Fig. 2b). In Mp708, there was a larger increase in *cis*-OPDA as well as *trans*-OPDA in response to herbivory at 24 h (Fig. 2c, d). The susceptible genotype Tx601 had a "typical" response to herbivory marked by low constitutive levels of *cis*-JA and *cis*-OPDA prior to herbivory followed by an increase in both after larval feeding (Fig. 2b, d). Tx601 did not show an increase in *trans*-OPDA in response to larval feeding. Hence, it appears that herbivore defense in Mp708 could employ both JA and OPDA. OPDA can trigger the JA signal transduction pathway, and both compounds are needed for complete activation of the wound response (Böttcher and Pollman 2009). In addition OPDA can activate the expression of a unique set of defense genes not regulated by JA (Böther and Pollman, 2009).

In addition to OPDA and *cis*-JA, the level of the phytohormone salicylic acid (SA), which is also involved in plant defense signaling, was determined at 0 and 1.5 h after infestation (Fig. 2e). While JA plays a major role in defense against feeding by chewing insects, SA plays a central role in defense against pathogens (Ryals et al. 1996). Although there is evidence for both positive and negative interactions between the JA and SA pathways, the primary mode of interaction appears to be mutual antagonism. Reduction in SA levels has been shown to increase the JA response and vice versa. The significantly reduced level of SA in Mp708 relative to Tx601 at these two time points supports the mutual antagonism that has been observed in other plants. Unfortunately, SA levels were not determined at subsequent time points and we do not know if this trend continues at 24 h.

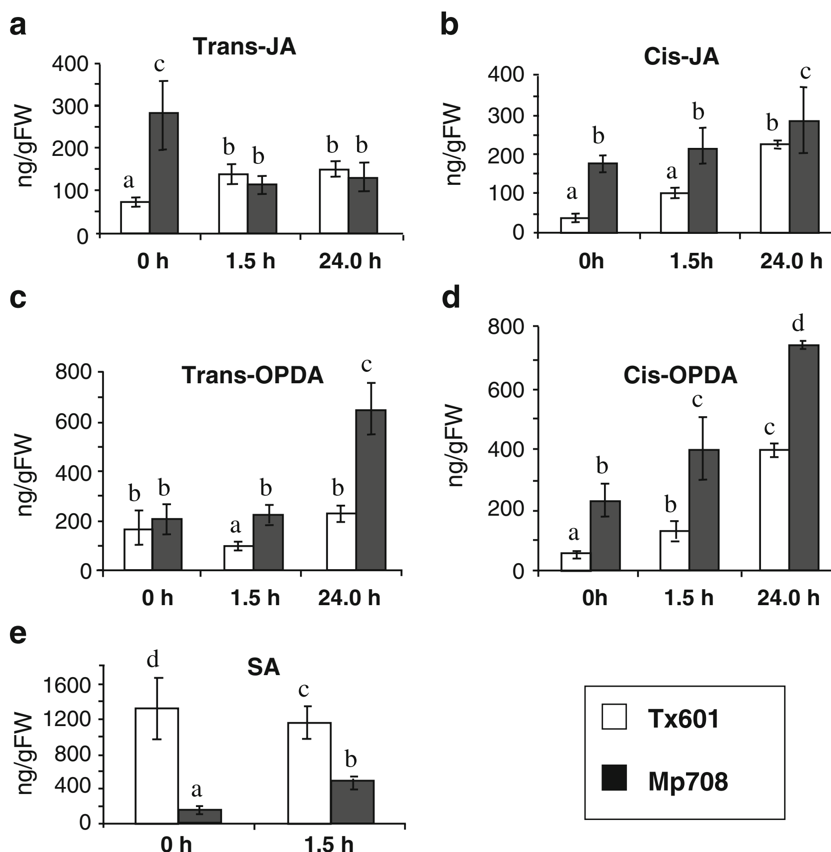

Fig. 2 Quantification of jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA) in maize whorls. Larvae were allowed to feed on 5-wk-old plants of Mp708 and Tx601 for 1.5 and 24 h. Leaf tissue surrounding the feeding site was harvested, whereas tissue in the same position of the whorl of uninfested plants was collected as control. *Trans*-JA (**a**), *cis*-JA (**b**), *trans*-OPDA (**c**), *cis*-OPDA (**d**), and SA (**e**) were measured with CI-GC/MS. There were five samples per treatment, and the experiment was repeated three times. The lines on each bar represent standard error

Transcript Levels of JA Biosynthetic Pathway Genes Correlate with Higher Constitutive Levels of JA in Mp708 To determine if the constitutively elevated JA levels were affecting the expression of genes involved in herbivore defense response, the transcript abundance of key genes in the JA signaling, biosynthesis and response pathways was examined. Figure 1 shows a schematic diagram of the proposed JA signaling pathway leading to plant defense responses. Upon caterpillar feeding, phospholipase D (PLD) releases linolenic acid from the chloroplast membrane (Wang et al. 2000). Linolenic acid then is converted to JA in a series of reactions that occur in the chloroplast and peroxisome. Although there are many steps in this pathway, we measured the abundance of transcripts that encode a few key enzymes in the pathway, lipoxygenase (LOX), allene oxide synthase (AOS), and 12-oxo-phytodecanoic acid reductase (OPR). The JA signaling pathway ultimately activates NAPDH oxidase leading to the production of H_2O_2, which in turn, triggers the plant to accumulate proteins involved in direct defenses. We measured the transcript levels of NADPH oxidase and three proteins involved in direct defense, Mir1-CP, chitinase and maize protease inhibitor (MPI).

PLD catalyzes the first step in the signaling pathway. The transcript abundance for PLD was significantly higher in Mp708 at both 0 and 24 h (Fig. 3a, $P<0.001$). The abundance of transcripts for a lipoxygenase gene, *ZmLOX1,* was determined because this gene is induced by wounding and MeJA (Kim et al. 2003). In addition, the enzyme encoded by *ZmLOX1* produces a 13-LOX product that could be the precursor for JA (Kim et al. 2003). Message levels for *ZmLOX1* were higher in Mp708 at both time points (Fig. 3b, $P<0.001$). In fact, transcript levels of *ZmLOX1* were > 100-fold higher in Mp708 than in Tx601 after 24 h of insect feeding. The second *LOX* gene studied was *ZmLOX3*, which was selected because it is involved in maize defense against root nematodes (Gao et al. 2008). The expression of *ZmLOX3 also* was higher in both control and herbivore fed plants (Fig. 3c, $P<0.001$). In addition transcripts for *AOS* , which encodes an enzyme in the JA biosynthesis pathway, were higher in Mp708 than in Tx601 at both 0 and 24 h (Fig. 3d, $P<0.001$). These results indicate that in Mp708 key transcript levels in the herbivore-induced signaling pathway are constitutively elevated when compared to Tx601. Furthermore, the levels of these transcripts are elevated more in Mp708 than Tx601 following 24 h of herbivory, thus suggesting that priming is occurring in Mp708, but not Tx601.

The next enzyme involved in JA biosynthesis is 12-OPDA reductase (OPR). Several OPR genes have been identified in maize including *ZmOPR7,* which has been predicted to encode one of the specific maize OPRs

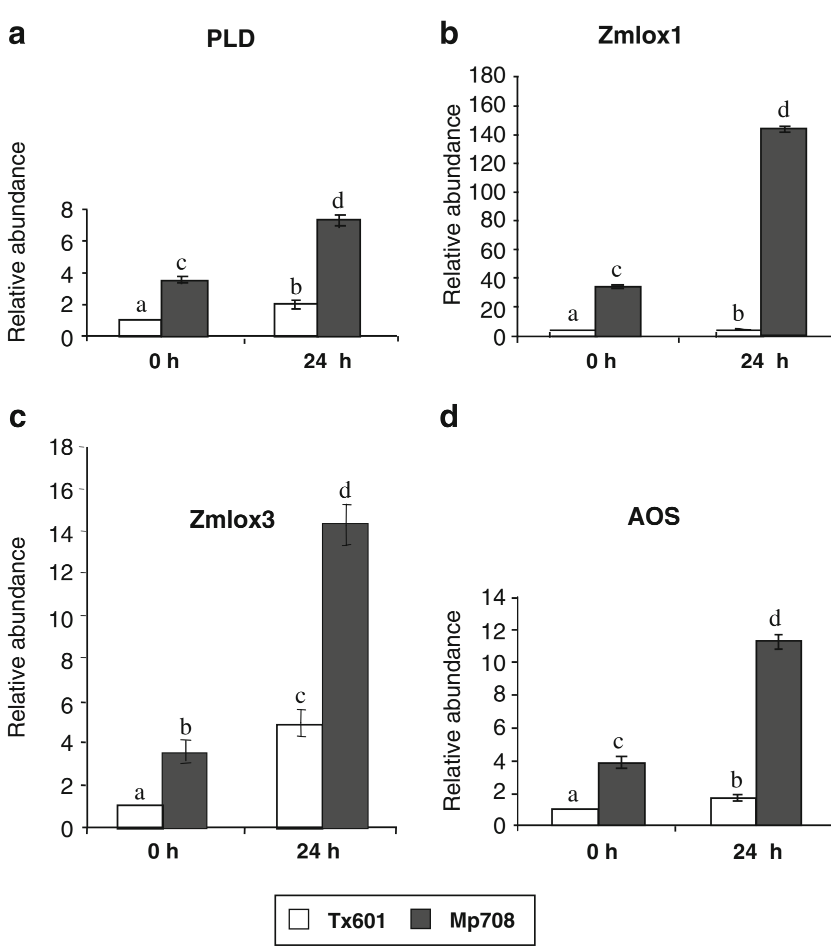

Fig. 3 Relative abundance of transcripts for enzymes in the JA signaling and biosynthetic pathway. RNA was extracted from the whorl region of 5-wk-old Mp708 and Tx601 plants infested with 3rd instar fall armyworm larvae for 24 h and from uninfested plants (0 h). cDNA was synthesized from these samples using oligo-dT primers. qRT-PCR was performed to determine the relative transcript abundance of *PLD* (**a**), *ZmLOX1* (**b**), *ZmLOX3* (**c**), and *AOS* (**d**). The relative abundance was calculated using the Pfaffl model with ubiquitin as reference gene. There were three biological and three technical replicates for each treatment. The lines on each bar represent standard error

involved in JA biosynthesis (Zhang et al. 2005). Consistent with the higher levels of *cis*- JA and *cis*-OPDA seen in control Mp708 plants, transcript levels for *OPR7* were higher in Mp708 than in Tx601 prior to herbivory (Fig. 4a, $P<0.001$). Larval feeding increased *OPR7* transcript levels in Tx601 ($P<0.001$), whereas *OPR7* transcript levels decreased in response to herbivory in Mp708 ($P<0.001$).

Expression of Other OPR Genes A significant increase in both *ZmOPR1* and *ZmOPR2* transcript levels was observed in response to larval feeding in Mp708 but not in Tx601 (Fig. 4b, c, $P<0.001$). These OPRs are similar to the *Arabidopsis OPR1* and *OPR2*, which do not participate in the JA biosynthetic pathway (Schaller 2001). *OPR1* appears to be activated by mechanical wounding (Biesgen and Weiler 1999). In maize, *ZmOPR2* expression increases in response to treatment with green leafy volatiles, mechanical wounding, or the regurgitant elicitor from beet armyworm (*Spodoptera exigua*) larvae (Engelberth et al. 2007). In Mp708, the large increase in OPDA without the commensurate increase in JA correlates with the increased abundance of transcript for *OPRs* that are not involved in JA biosynthesis. Although it remains to be tested, this suggests that OPDA might regulate an alternative herbivore defense pathway in Mp708. Both genotypes responded to feeding by reducing the expression of *OPR6* transcripts Fig. 3d, $P<0.001$), which is cytoplasmic and wound-inducible (Farag et al. 2005). Treatment of maize leaves with green leafy volatiles as well as crude regurgitant from beet armyworm larvae also failed to induce increase in *OPR6* transcripts (Engelberth et al. 2007).

Induction of Other Defensive Proteins In many plants, wound-induced synthesis of JA leads to activation of genes that encode proteinase inhibitors and other defensive proteins (Farmer and Ryan 1992). Therefore, transcript levels for maize protease inhibitor (*mpi*) (Cordero et al. 1994), a chitinase (*PRm3*) (Rakwal et al. 2004), and *mir1* (Pechan et al. 2000) were examined. *mpi*, which is induced by wounding and exogenous JA, was expressed at low levels in the controls of both genotypes, and fall armyworm feeding resulted in increased transcript levels in both genotypes, but the increase was larger in Mp708 (Fig. 5a, $P<0.001$). If the induction of *mpi* is dependent solely on JA, high constitutive expression in Mp708 would be expected. However, this was not the case, and this suggests that the induction of *mpi* in both Mp708 and Tx601

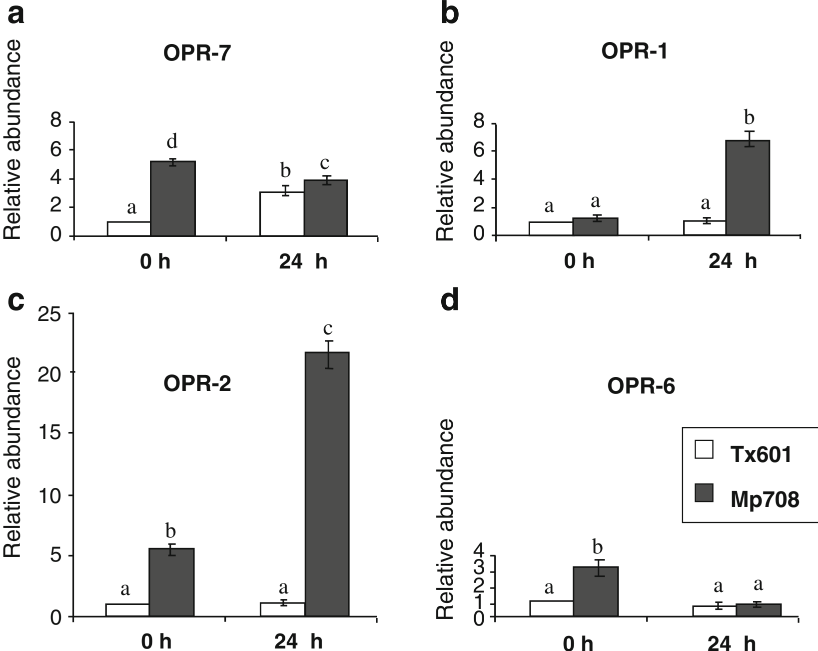

Fig. 4 Relative abundance of transcripts for several *OPR* genes. RNA was extracted and cDNA was synthesized as described in Fig. 3. qRT-PCR was performed to determine relative abundance for transcripts of *ZmOPR7* (**a**), *ZmOPR1* (**b**), *ZmOPR2* (**c**), and *ZmOPR6* (**d**). The relative abundance was calculated using the Pfaffl model with ubiquitin as reference gene. There were three biological and three technical replicates for each treatment. The lines on each bar represent standard error

requires another signal in addition to JA. It has been shown that blocking ethylene perception or synthesis decreases the accumulation of Mir1-CP and increases the susceptibility to herbivory in both Mp708 and Tx601 (Harfouche et al. 2006; Ankala et al. 2009). Thus, it is likely that ethylene is also required for MPI accumulation.

Although chitinases have been classified as antifungal proteins (Rakwal et al. 2004), they have been shown to be effective against aphid herbivory in sugarcane (Falco et al. 2001) and wheat (van der Westhuizen et al. 1998). In addition, chitinases that are induced by herbivory may attack the lepidopteran peritrophic matrix (PM), which is rich in chitin. Transcript levels of *PRm3*, which encodes a maize chitinase, were higher in Mp708 than Tx601 prior to herbivory (Fig. 5b, $P<0.001$), and although message abundance increased in response to herbivory in both genotypes, the level in Mp708 was much larger than in Tx601 (Fig. 5b, $P<0.001$). These results suggest that high chitinase expression in Mp708 could contribute to the PM perforation that is observed when fall armyworm feeds on this maize inbred (Pechan et al. 2002).

Mir1 Expression in Response to Fall Armyworm Feeding Low basal levels of Mir1-CP protein and its transcripts have been detected consistently in control plants prior to herbivore feeding (Fig. 6a, Pechan et al. 2000, Ankala et al. 2009). Upon larvae feeding, Mir1-CP begins to accumulate in the whorl within 1 h (Pechan et al. 2000). Despite this rapid accumulation, there is no commensurate increase in *mir1* transcripts at 30 min or 3 h (Fig. 6b). In fact, *mir1* transcripts appeared to decrease between 30 min and 24 h as shown by RT-PCR and real-time PCR (Fig. 6b, c). The paradox of rapid Mir1-CP accumulation in the absence of increased transcript levels during the first 24 h of herbivory could be due to post-transcriptional regulation of expression. However, a more likely explanation is that Mir1-CP is transported to the feeding site from the maize roots (Lopéz et al. 2007). This is supported by data indicating that 1, Mir1-CP accumulates in the roots in response to foliar herbivory, 2, it is found in the xylem and phloem of Mp708, and 3, root removal limits its accumulation in the whorl in response to herbivory (Lopéz et al. 2007). This suggests that there is communication between the above and below parts of maize in response to herbivore attack, which is consistent with the results of Erb et al. (2009).

Mp708 Appears to Generate More H_2O_2 and Accumulate More NADPH Oxidase Transcripts than Tx601 H_2O_2 has been shown to be a secondary messenger in JA-mediated defense signaling that acts downstream from JA (Orozco-Cardenas et al. 2001). Because JA and OPDA levels were elevated in Mp708 prior to herbivory, we speculated that there also might be increased production of H_2O_2. We visualized H_2O_2 by DAB staining and found that Mp708

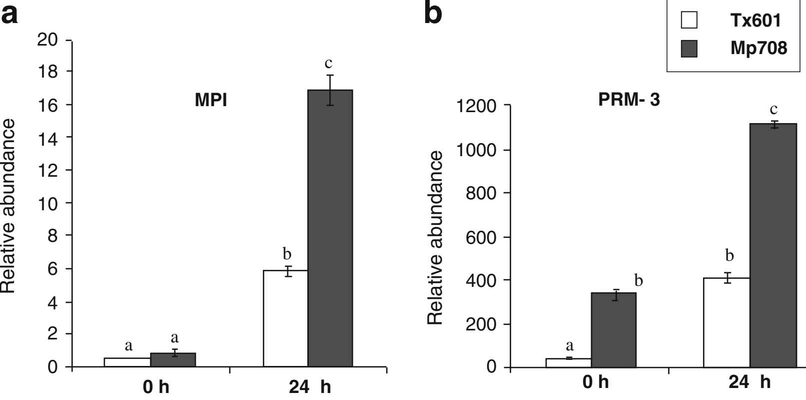

Fig. 5 Relative abundance of transcripts for *MPI* and *PRm3* (chitinase). RNA was extracted and cDNA was synthesized as described in Fig. 3. qRT-PCR was performed to determine the relative transcript abundance for *MPI* (**a**), and *PRm3* (**b**), was calculated using the Pfaffl model with ubiquitin as reference gene. There were three biological and three technical replicates for each treatment. The lines on each bar represent standard error

generated more H_2O_2 compared to Tx601 in response to wounding (Fig. 7a) or insect feeding (Fig. 7b). The release of H_2O_2 in response to wounding occurred within 10 min (Fig. 7a), but its production in response to feeding was slower and barely detectable in either genotype after 3 h (Fig. 7b). However, after 24 h of herbivory, there was intense red coloration surrounding the damaged areas of Mp708, whereas there was little coloration in Tx601 (Fig. 7b), which also suggests that Mp708 is able to mount a stronger defense after herbivory than Tx601.

A membrane-bound NADPH oxidase catalyzes the production of wound-induced superoxide (O_2^-), which then

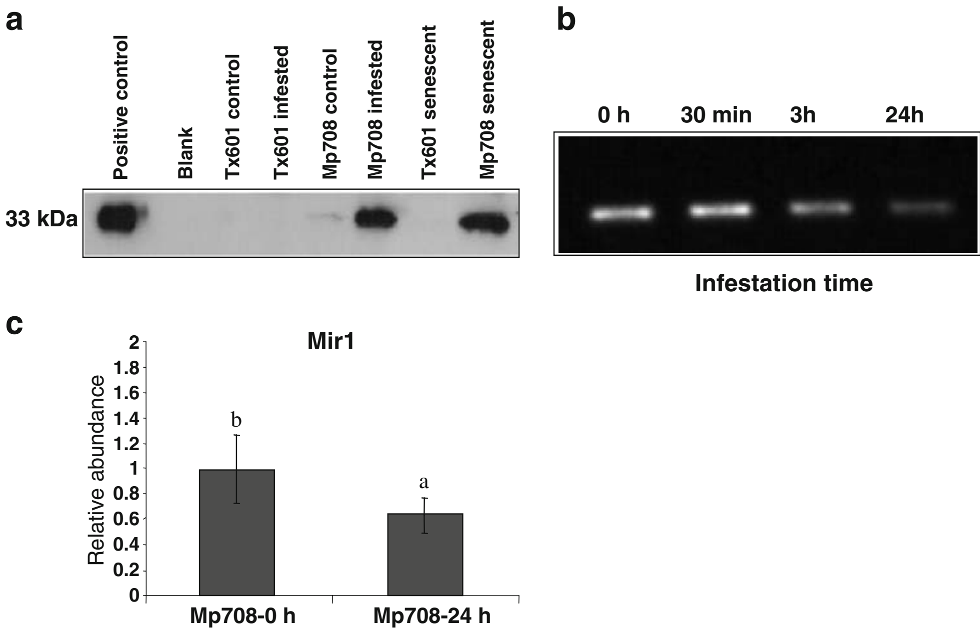

Fig. 6 *Mir1* expression and Mir1-CP accumulation in Mp708. **a** Immunoblot analysis of Mir1-CP expression in Mp708 and Tx601 plants. Total protein was extracted from samples collected near the larval feeding site or from senescing leaves. Controls were collected from the whorls of uninfested plants. The positive control is a protein extract from callus generated from Mp708 embryos. **b** RT-PCR of products generated with primers for *mir1* using RNA extracted from whorls of Mp708 around the feeding site after 0 h, 30 min, 3 h and 24 h feeding by 3rd instar fall armyworm larvae. **c** Relative abundance of transcripts for *mir1*. RNA was extracted and cDNA synthesized as described in Fig. 3. qRT-PCR was preformed to determine the abundance of *mir1* and ubiquitin transcripts. Relative abundance was calculated as the ratio between *mir1* and ubiquitin expression. The relative abundance was calculated using the Pfaffl model with ubiquitin as reference gene. There were three biological and three technical replicates for each treatment. The lines on each bar represent standard error

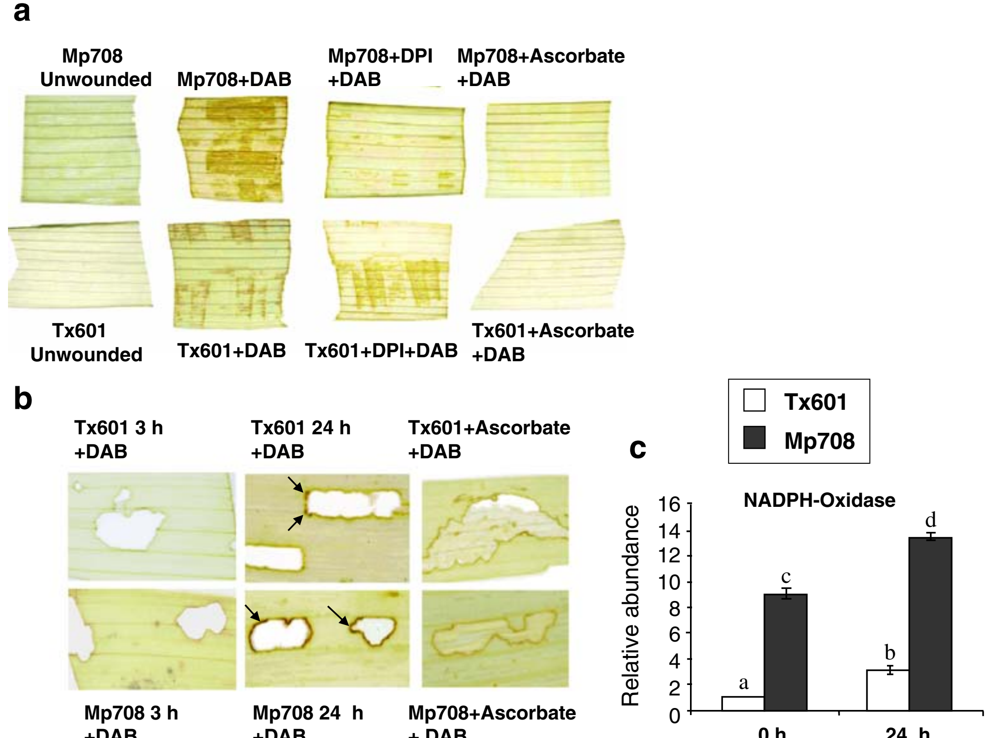

Fig. 7 H_2O_2 generation in wounded (**a**) and fall armyworm infested whorls (**b**). For the wounding experiment, plants were treated with DAB, DPI, and ascorbate as described in Materials and Methods, and the whorl region was crushed with a hemostat. For feeding experiments, 3rd instar fall armyworm larvae were placed on the yellow-green whorl region of 4-week old plants and caged to restrict the feeding area. Larvae were removed at specified times, and feeding areas were stained with DAB or DAB containing ascorbic acid. **c** NADPH oxidase transcript levels were measured by qRT-PCR conducted with cDNA synthesized as described in Fig. 3. The relative abundance was calculated using the Pfaffl model with ubiquitin as reference gene. Arrows indicate the reddish brown coloring in the wounded and fed areas. There were three biological and three technical replicates for each treatment. The lines on each bar represent standard error

is dismutated to H_2O_2 (Doke et al. 1996). When diphenyleneiodonium sulfate (DPI) was used as an inhibitor of NADPH oxidase (Frahry and Schopfer 1998), there was no detectable release of H_2O_2 in Mp708 in response to wounding (Fig. 7a), while its generation in Tx601 remained unchanged (Fig. 7a). These results suggest that H_2O_2 generation in these two inbreds could occur via two different pathways with NADPH oxidase functioning in Mp708 and other enzymes such as cell wall peroxidases functioning in Tx601. Absence of reddish brown coloration in the presence of the free radical scavenger ascorbic acid confirms that the coloration was due to H_2O_2 reacting with DAB. Prior to herbivory, NADPH oxidase transcript levels were higher in Mp708 than in Tx601, and they increased slightly in response to larval feeding (Fig. 7c). In Tx601, the level of NADPH oxidase transcripts increased due to feeding, but the overall expression levels were lower than those of Mp708 ($P<0.001$).

The maize inbred Mp708 was selected for its resistance to caterpillar feeding in the field. We propose that a portion of its resistance stems from expression of genes in the JA-induced defense pathway prior to herbivore attack and a response to subsequent larval feeding with even higher levels of defense gene transcripts than the susceptible inbred Tx601. These findings are consistent with the idea that Mp708 is genetically primed to withstand herbivore attack. Currently the cause of the JA over-production in Mp708 is unknown. Although there are a number of mutations in *Arabidopsis* that impair JA synthesis, conjugation to isoleucine, and perception (Wasternack 2007; Balbi and Devoto 2008; Browse and Howe 2008; Howe and Jander 2008; Kazan and Manners 2008), there are fewer known mutations that cause JA to be over-produced or to accumulate in the absence of wounding or herbivory (Cano-Delgado et al. 2000, 2003; Ellis and Turner 2001; Hilpert et al. 2001; Ellis et al. 2002; Jensen et al. 2002; Ko et al. 2006; Bonaventure et al. 2007a, b). The *fou2* mutant of *Arabidopsis* has a lesion in the Two Pore Channel 1 gene that encodes a Ca^+ permeant non-selective cation channel that produces more JA than wild type both constitutively and upon wounding. This mutant also has increased ability to catalyze the synthesis of LOX metabolites, and the

expression of a suite of JA and defense genes is up-regulated (Bonaventure et al. 2007a, b). Several other mutations resulting in constitutive JA production and constitutive defense appear to be involved in some aspect of cell wall deposition (Ellis et al. 2002; Cano-Delgado et al. 2003; Ko et al. 2006). Mutations that affect cellulose synthesis appear to alter the cell wall structure and result in continuous JA production (Ellis et al. 2002). Interestingly, there are differences in cell wall composition between Mp708 and Tx601. The resistant plants had more cellulose and hemicellulose than susceptible plants (Williams et al. 1998). The hemicellulose from resistant lines contained more neutral sugars and higher cross-linking than that from susceptible lines (Hedin et al. 1984). Although there could be a number of alternative scenarios, we speculate that changes in cell wall composition might alter its structure, which could result in the increased constitutive levels of JA and defense gene expression that we observed in Mp708.

In this study, we compared the defense responses of two maize inbreds that have different levels of resistance to caterpillar feeding. Mp708 is unique because it has a high level of resistance to herbivory, hence, we cannot extrapolate these results to all maize inbred lines. Nevertheless, the results of this study show that constitutive *cis*-JA and *cis*- OPDA levels are elevated in the caterpillar-resistant inbred Mp708, and the transcripts of many genes in the octadecanoid defense pathway are more abundant in resistant plants both prior to and after herbivory. Furthermore, Mp708 appears to rapidly generate higher levels of peroxide than the susceptible maize line. Hence, the remarkable ability of Mp708 to resist caterpillar feeding is probably because the entire herbivore defense signaling pathway, from PLD to H_2O_2, is primed prior to herbivory, suggesting that these plants are on constant alert and poised to mount a rapid and strong defense to caterpillar attack.

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Herbivore- and Elicitor-Induced Resistance in Rice to the Rice Water Weevil (*Lissorhoptrus oryzophilus* Kuschel) in the Laboratory and Field

Jason C. Hamm · Michael J. Stout · Rita M. Riggio

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Abstract Feeding by herbivores can change plants in ways that make them more resistant to subsequent herbivory. Such induced responses are better-studied in a number of model dicots than in rice and other cereals. In a series of greenhouse and field experiments, we assessed the effects of prior herbivory by the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) and of exogenous applications of jasmonic acid (JA) on the resistance of rice plants to the rice water weevil, *Lissorhoptrus oryzophilus* (Kuschel), the major pest of rice in the United States. Prior feeding by *S. frugiperda* and treatment of plants with exogenous JA resulted in increases in the resistance of plants to the weevil. Increases in resistance were manifested as reduced numbers of eggs and first-instars associated with armyworm-injured or JA-treated plants relative to control plants. In field experiments, there was a transient but significant reduction in the number of immature *L. oryzophilus* on JA-treated plants relative to untreated plants. To our knowledge, this is the first example of direct induced resistance in rice demonstrated in small-plot field experiments. We discuss the potential for the use of elicitor induced resistance in rice.

Keywords Jasmonic acid · Induced resistance · Elicitors · *Oryza sativa* · *Lissorhoptrus oryzophilus* · *Spodoptera frugiperda*

J. C. Hamm · M. J. Stout (✉) · R. M. Riggio
Department of Entomology, Louisiana State University Agricultural Center,
Baton Rouge, LA 70803, USA
e-mail: mstout@agcenter.lsu.edu

Introduction

Feeding by arthropod herbivores often causes changes in the expression of resistance-related genes and traits in plants, and these changes often make the plants less suitable for subsequent herbivores. This phenomenon has been termed induced resistance (Karban and Baldwin 1997). Induced resistance to herbivory can be broadly classified as direct or indirect. Direct induced resistance refers to changes that have direct negative effects on herbivore behavior, growth, or physiology. These effects can be manifested in a multitude of ways, such as reduced feeding, oviposition, fecundity, and survival of herbivores on previously damaged plants (Walling 2000). Indirect induced resistance refers to changes that attract or retain natural enemies of herbivorous arthropods (Dicke et al. 2003). Direct and indirect induced resistance has been reported in a wide variety of plants. However, such responses are less studied in rice and other economically important monocots (Kogel and Langen 2005; Karban and Chen 2007).

Jasmonic acid (JA) is a plant hormone that mediates the expression of both direct and indirect defenses against herbivory (Thaler et al. 2002; Wasternack 2007; Browse and Howe 2008). Jasmonic acid accumulates rapidly in plant tissues near the site of herbivore attack (Korth and Thompson 2006). Increases in endogenous JA lead, through a series of intermediary steps, to changes in the expression of resistance-related genes and metabolites, and to enhanced resistance to herbivory (Korth and Thompson 2006; Bruinsma and Dicke 2008). Consistent with its role as an endogenous signal, treating plants with exogenous JA often simulates the changes induced by natural herbivory (Farmer and Ryan 1990; Kessler and Baldwin 2002; Browse and Howe 2008).

The broad-spectrum nature of induced resistance in many plants (Stout and Bostock 1999) and the ability to stimulate induced resistance by applying exogenous elicitors like JA has raised the possibility of using induced resistance in agriculture. One way it could be used in agriculture is to use elicitors like JA to stimulate resistance at appropriate times in the life cycles of the pest and crop (Stout et al. 2002). Most tests of this idea have been conducted in greenhouses, and few studies have shown significant reductions in the preference, feeding, and survival of pests in field settings (Thaler et al. 1996; Black et al. 2003).

Rice, which serves as a staple food for a large portion of the world's population, has been relatively neglected as a model for the study of induced resistance to herbivorous arthropods (Karban and Chen 2007). What research that has been done has primarily focused on indirect induced resistance and/or induced responses to feeding by sucking insects (Bentur and Kalode 1996; Seino et al. 1996; Matsumura and Suzuki 2003; Xu et al. 2002, 2003; Kanno et al. 2005; Lou et al. 2005; Satoh et al. 2005; Senthil-Nathan et al. 2009). Recently, however, Stout et al. (2009) demonstrated direct induced resistance in rice to a chewing insect, the fall armyworm (*Spodoptera frugiperda* J.E. Smith), following earlier feeding by the same insect. Resistance to the fall armyworm also was induced by exogenous JA. The fall armyworm defoliates a wide assortment of plants, including rice, but rarely achieves pest status in rice in the United States.

The experiments described herein extend the prior work on direct induced resistance in rice by studying the effects of prior fall armyworm herbivory and exogenous JA on the resistance of rice to its major early-season insect pest in the United States, the rice water weevil, *Lissorhoptrus oryzophilus* Kuschel. Adult rice water weevils move from overwintering sites to rice fields in early spring and feed on rice leaves, resulting in longitudinal feeding scars that run parallel to leaf veins. Oviposition is triggered by flooding of rice fields, which typically occurs within five weeks of planting (Everett and Trahan 1967; Stout et al. 2002). Upon hatching, neonate larvae migrate down the plant to roots, where they feed and pass through four instars in 21–27 days (Zou et al. 2004). Feeding by adults generally does not result in economic injury, but root pruning by larvae can severely reduce both the growth and yield of rice (Smith 1983; Zou et al. 2004). In Louisiana, yield losses typically exceed 5% and can approach 25% or more (Stout et al. 2000). The objectives of this study were to determine if *S. frugiperda* herbivory and exogenous application of jasmonic acid induce resistance to *L. oryzophilus* in both greenhouse and small plot field experiments.

Methods and Materials

Plant and Insect Culture A total of four experiments were conducted in a greenhouse on the campus of Louisiana State University, Baton Rouge. For each experiment, seeds were planted in a soil mix (2:1:1, soil: peat moss: sand) in 11.4 cm^2 square pots, and plants were maintained in greenhouse conditions under ambient lighting at approximately 29°C–33°C. Approximately 1.2 g of 19:6:12 controlled release fertilizer (Osmocote, Scotts Miracle-Gro, Marysville, OH, USA) was added to soil at planting. Plants were grown, and experiments were conducted in large wooden basins lined with heavy black plastic that allowed plants to be flooded. Plants were thinned to a density of three plants per pot five to 7 d after planting.

Adult rice water weevils used in these experiments were collected from rice fields at the LSU AgCenter's Rice Research Station in Crowley, Acadia Parish, Louisiana, 1 d prior to use in experiments. Weevils were maintained from collection until use in plastic containers with water and rice leaves. In order to ensure equal ratios of males and females, weevils were captured in-copula and placed in small plastic cups just prior to use.

Fall armyworm larvae used to damage plants were obtained from a colony maintained year-round on artificial diet in the laboratory. The colony originated from larvae collected in bermudagrass pastures near Baton Rouge in 1997. Insects collected from pastures or rice fields are added annually to the colony to maintain genetic variability and vigor.

Two separate experiments were conducted to investigate whether feeding by *S. frugiperda* larvae induces resistance to *L. oryzophilus*. In the first, the rice cultivar 'Rosemont' was used; in the second, the cultivar 'Jackson' was used. These varieties were used because prior studies had shown them to be responsive to fall armyworm feeding and JA treatment (Stout et al. 2009). Rice seedlings were grown to the early three-leaf stage as described above. Pots then were randomly assigned to two treatment groups, 'control' and 'damaged'. Plants in the latter group were damaged by confining one 4th to 5th instar *S. frugiperda* larvae per plant in cages constructed of clear plastic cylinders (8.5 cm diam, 23 cm height) with one end inserted into the soil and the top end covered with a mesh-screen lid. Cylinder cages had two mesh-lined holes to allow for air circulation. Larvae were allowed to feed for 4–6 h, and on average, consumed between 20% and 30% of total leaf area, typically damaging portions of every leaf. Cages with no larvae were placed over plants assigned to the control group. Cages and larvae were removed from plants after 4–6 h of feeding, and plants were maintained in the greenhouse for later evaluation of resistance to *L. oryzophilus*.

Evaluations of resistance to *L. oryzophilus* were conducted 13–15 d after injury by *S. frugiperda*. By this time a new leaf or, in some cases, two new leaves had emerged on both damaged and control plants. Four pots of each treatment were placed into infestation cages, which were constructed of cylindrical wire frames (46 cm diam, 61 cm tall) covered with a mesh fabric screening. A total of 5 cages were used in each experiment. Weevils were then placed in cages at a density of one male:female pair per plant (24 pairs per cage). Basins were flooded to a depth of approximately 24 cm, and weevils were allowed to feed, mate, and oviposit on plants in cages for 4 d. Plants then were removed from cages and any weevils found on plants were removed.

Densities of eggs and 1st instars on or associated with plants were used to estimate levels of weevil infestation. Procedures for estimating egg and larval densities were adapted from Heinrichs et al. (1985) and Stout and Riggio (2002). Estimating egg densities provides information regarding oviposition preference, and estimating larval densities provides further information on oviposition preference and possibly on survival of eggs and early instars. Densities of eggs were determined by removing one plant from each pot. Soil was removed carefully from the roots, and plants were then labeled and placed in 75% ethanol until bleached. The numbers of eggs on plants were determined by carefully examining plants under a dissecting microscope (Meiji Techno Co. Ltd, Tokyo, Japan).

The densities of 1st instars emerging from plants were determined by removing two plants from each pot, carefully washing soil from the roots, and suspending individual plants in test tubes containing distilled water. Test tubes were labeled, arranged in a rack, and placed in a growth chamber (28°C,14:10 h L:D). Weevils infesting plants treated in this manner hatch from eggs, emerge from leaf sheaths, and settle on the bottom of test tubes (Heinrichs et al. 1985). First instars were removed by shaking roots free of larvae and then pouring water from test tubes into a Petri dish for counting. Plants were placed back into their respective test tubes immediately after counting and replenished with distilled water. Larvae were counted daily until no larvae were found for three consecutive days.

Two additional experiments were conducted to investigate JA induced resistance, each using the two rice cultivars 'Jasmine' and 'Rosemont.' Elicitor treatments were prepared by dissolving 21 mg (1 mM) or 105 mg (5 mM) jasmonic acid (Sigma-Aldrich, St. Louis, MO, USA) in 1 ml of 95% ethanol and adding the ethanol solution to 100 ml distilled water. Three weeks after planting, 6 pots of each cultivar were assigned to treatment groups and were sprayed until run-off with 100 ml of 1 mM or 5 mM JA solutions or with 100 ml of water containing 1 ml of ethanol (control) using a hand held aerosol sprayer. Each plant received approximately 5.5 ml of elicitor or control solutions. The following day, one pot of each treatment/cultivar combination was placed in each of 6 infestation cages, resulting in 6 pots per cage, and plants were infested with weevils as described earlier. Estimation of larvae and egg densities were carried out as previously described, except that egg densities were not determined in the second experiment.

Analysis of Data Data for each of the four experiments described above were analyzed separately. For each experiment, counts of eggs and 1st instar larvae were taken using separate plants, thus providing independent measures of plant resistance. Data from *S. frugiperda* experiments were analyzed as a randomized complete block design using a mixed-model analysis of variance (ANOVA) (PROC MIXED) in SAS (SAS Institute 2007), with damage treatment (damaged or control) as a fixed effect and cage as a random effect. Data from JA experiments were analyzed as a 2×3 factorial using PROC MIXED, with infestation cage as a random effect and cultivar ('Rosemont' and 'Jackson') and JA concentration (0, 1, and 5 mM) as fixed effects. Means were separated using least significant differences (LSD) test.

Two experiments were conducted during the 2008 growing season at the Louisiana State University Agricultural Center Rice Research Station, Crowley, Acadia Parish, Louisiana. In both experiments, rice was planted by placing seeds into soil using a plywood template measuring1.5× 1.5 m, with 3 cm diam holes spaced 7.5 cm apart and arranged in five rows and five columns. Two seeds were inserted into each hole at a depth of approximately 2 cm. The area defined by the template was considered a plot, and plots were spaced 1 m apart in a completely randomized design. In the first experiment, seeds of the cultivar 'Jackson' were hand planted on 8 April with 6 plots (replicates) of each of 3 treatments. Treatments consisted of exogenous applications of 1 mM and 5 mM solutions of JA and an untreated control; JA and control solutions were prepared as described in greenhouse experiments. On 29 April, 21 d after planting, plots were fertilized at a rate of 68 kg per acre of nitrogen as urea, and plants were subsequently sprayed with JA until runoff. A permanent flood was established 1 wk after JA treatment.

A second experiment using the rice cultivar 'Rosemont' was planted on 10 June using the same templates as above with 1.8 m spacing between plots. The experimental design was a completely randomized design with two treatments—control and JA (5 mM)—with 15 replicates for each treatment. On 10 July, 30 d after planting, plots were fertilized at a rate of 68 kg per acre of nitrogen as urea, and plants were subsequently sprayed with JA until runoff and permanent flood was established the following day.

In the first field experiment, plants were sampled for eggs 6 and 12 d after establishment of permanent flood (13 and 19 d after JA treatment). In the second experiment, plants were sampled for eggs 2 d after permanent flood was established (3 d after JA treatment). For egg sampling, two plants from each plot were removed, and soil was washed from roots. Plants were labeled and stored in 75% ethanol until bleached. The numbers of eggs on plants were determined by examining the leaf sheaths under a dissecting microscope (Meiji Techno Co. Ltd, Tokyo, Japan).

Densities of *L. oryzophilus* larvae and pupae in plots were determined by using a soil-root core sampler with a diam of 9.2 cm and a depth of 7.6 cm. Core samples were taken 19 and 27 after permanent flood in the first experiment, and 14 and 19 d after permanent flood in the second experiment. For each sampling date, 1 to 4 core samples were taken from each plot. Core samples were processed by placing them in a sieve bucket (40-mesh screen) and washing soil from roots. Buckets then were placed into plastic basins containing salt water, which facilitated larval and pupal counts as they floated to the water surface (N'Guessan et al. 1994).

Prior to analysis, the number of immature *L. oryzophilus* observed in each core sample was converted to number of larvae per plant by dividing the total number of immature larvae by the number of plants in each core sample. Generally, one or two plants were contained in each core sample. Data were analyzed as completely randomized design experiments by one-way ANOVA using PROC MIXED with treatment (JA or control) as a fixed effect. Means were separated using least significant difference (LSD) test.

Results

Characterization of Induced Resistance Following Fall Armyworm Herbivory In the first experiment, significantly fewer first instars emerged from 'Rosemont' rice plants previously damaged by *S. frugiperda* than from undamaged plants (Fig. 1; $F_{1,33}$=5.30, P=0.028). Numbers of eggs per plant did not differ between control and damaged plants ($F_{1,33}$=1.66, P=0.21) in this experiment. In the second experiment, densities of both eggs ($F_{1,33}$=8.78, P=0.006) and first instars ($F_{1,33}$=15.02, P<0.001) of *L. oryzophilus* were significantly lower in cultivar 'Jackson' that were previously injured by *S. frugiperda* than in undamaged plants (Fig. 2).

Characterization of Induced Resistance Following JA Applications In the first experiment with exogenous JA, densities of eggs were lower in plants treated with 1mM and 5mM JA than in control plants of both varieties (Fig. 3; $F_{2,50}$=12.39, P<0.001). In addition, there were fewer eggs per plant on 'Rosemont' than on 'Jasmine' ($F_{1,50}$=5.89, P=0.02), but the interaction between cultivar and treatment was not significant ($F_{2,50}$=0.39, P=0.68), indicating that the effect of JA did not differ between cultivars. The high rate of exogenous JA also reduced the number of first

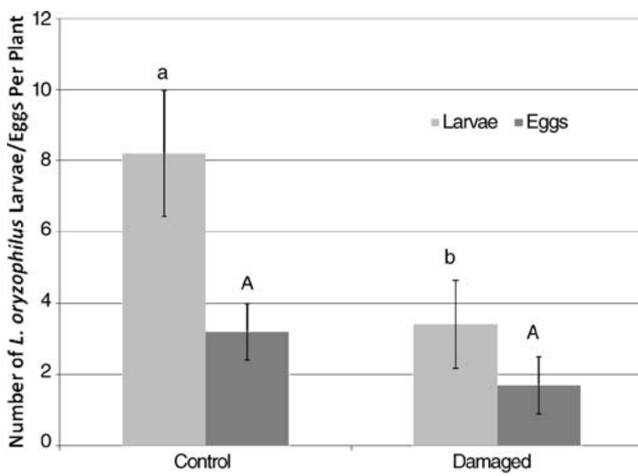

Fig. 1 Mean number of *Lissorhoptrus oryzophilus* larvae and eggs per plant (± se) in initial experiment using *Spodoptera frugiperda* damaged and undamaged plants of rice cultivar Rosemont. Plants were damaged by allowing one 4th–5th instar *S. frugiperda* to feed on each plant for 4–6 h. *Bars* accompanied by different *upper case letters* (eggs) or *lower case letters* (larvae) indicate that means differ significantly (P≤0.05)

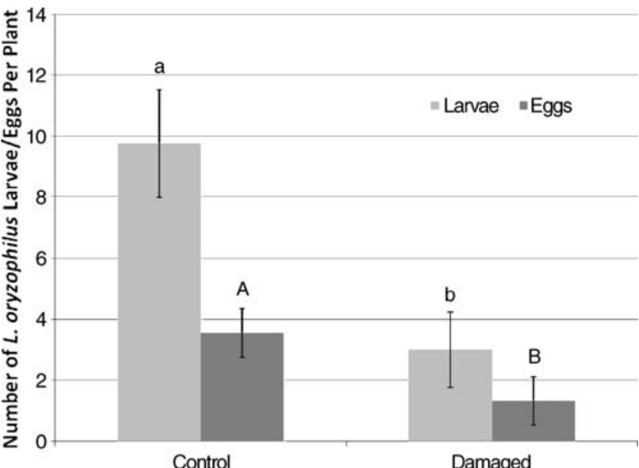

Fig. 2 Mean number of *Lissorhoptrus oryzophilus* larvae and eggs per plant (± se) in second greenhouse experiment using *Spodoptera frugiperda* damaged and undamaged plants of rice variety 'Jackson.' Plants were damaged by allowing one 4th–5th instar *S. frugiperda* to feed on each plant for 4–6 h and assays for weevil resistance were conducted 15 d later. *Bars* accompanied by different *upper case letters* (eggs) or *lower case letters* (larvae) indicate that means differ significantly (P≤0.05)

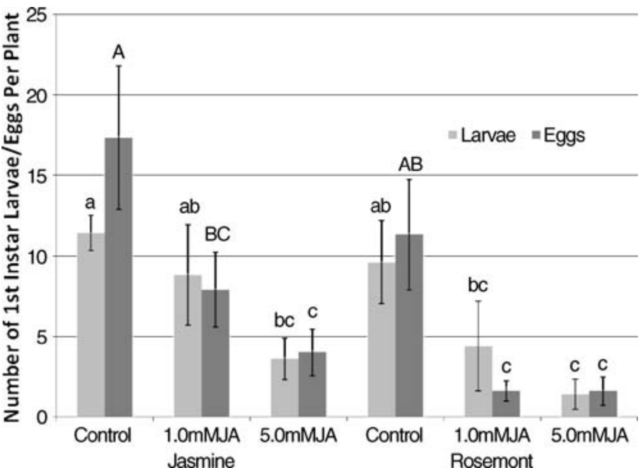

Fig. 3 Mean number of *Lissorhoptrus oryzophilus* eggs and larvae per plant (± SE) in greenhouse two-way experiment using two different varieties and three different concentrations of exogenous JA applications. *Bars* accompanied by different *lower case* (eggs) or *upper case* (larvae) letters indicate that means differ significantly ($P \leq 0.05$)

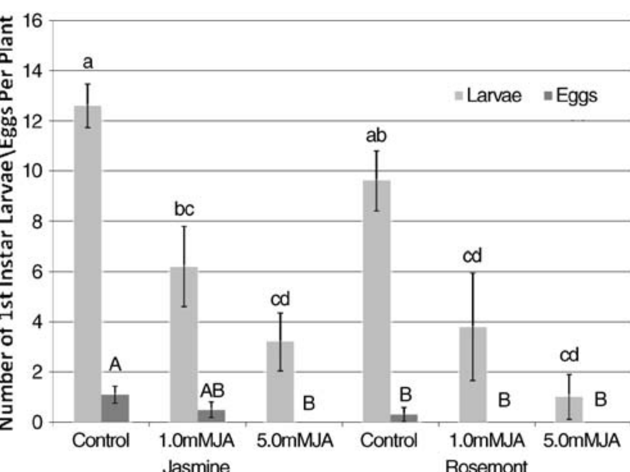

Fig. 4 Mean number of *Lissorhoptrus oryzophilus* larvae (±SE) per plant in second greenhouse experiment using two different varieties and three different concentrations of exogenous JA applications. *Bars* accompanied by different *letters* indicate that means differ significantly ($P \leq 0.05$). The effect of variety is also significant (P=0.038)

instars relative to the 1mM and untreated plants (Fig. 3; $F_{2,20}$=6.82, P=0.006). No cultivar effect on first instars was observed ($F_{1,20}$=2.51, P=0.13), and the interaction between cultivar and treatment was not significant ($F_{2,20}$=0.21, P=0.81).

In the second JA experiment, densities of larvae in both the 1mM and 5mM JA treatments were reduced compared to untreated plants (Fig. 4; $F_{2,20}$=21.57, $P<$ 0.001). A varietal difference in larval densities was also observed ($F_{1,20}$=4.92, P=0.038), but the interaction between cultivar and treatment was not significant ($F_{2,20}$=0.04, P=0.96).

Field Experiments In the first field experiment (cultivar 'Jackson'), numbers of eggs per plant did not differ among treatments at 6 and 12 days after flood (Table 1; $F_{2,15}$=0.53, P=0.60 and $F_{2,15}$=0.15, P=0.86 at 6 and 12 d after JA treatment, respectively). Numbers of *L. oryzophilus* larvae per plant in the first core sampling were reduced by 52% (1 mM) and 62% (5 mM) in the JA treated plants compared to control (Table 1; $F_{2,15}$=3.90, P=0.04). In the second core sampling, numbers of larvae per plant were not significantly different among treatments ($F_{2,14}$=0.71, P=0.51).

In the second field- experiment (cultivar 'Rosemont') JA treatment did not significantly reduce egg densities. Numbers of *L. oryzophilus* larvae per plant in the first core sampling were lower in the JA-treated plots than in control plots (Table 2; $F_{1,20}$=4.23, P=0.05). The second core sampling showed no treatment effect on the number of larvae per plant ($F_{1,18}$=0.36, P=0.55).

Discussion

While there is a growing body of literature pertaining to induced responses to insects in rice (Karban and Chen 2007), most of this research has involved piercing-sucking insect pests or indirect resistance (Xu et al. 2002; Matsumura and Suzuki 2003; Zhou et al. 2003; Lou et al. 2005). Rice thus remains relatively under-utilized for the study of direct induced resistance to chewing insects. This is a critical lack of knowledge, as many of the most important pests of rice in the U.S. and globally are chewing insects. Recently, Stout et al. (2009) showed that feeding by *S. frugiperda* and exogenous JA induced a long-lasting systemic resistance to subsequent feeding by *S. frugiperda.* The results of this prior study led us to hypothesize that *S. frugiperda* herbivory and exogenous JA would induce resistance to *L. oryzophilus*, the most important insect pest of rice in the United States (Smith 1983; Way 1990). The goal of this study was to provide further information on the nature and importance of direct induced resistance in rice and its possible use in pest management.

Our results demonstrate that feeding by *S. frugiperda* induces resistance to an unrelated insect species (*L. oryzophilus*), which is consistent with the broad spectrum nature of induced resistance in many systems (Stout and Bostock 1999). Female *L. oryzophilus* laid 37–53% fewer eggs on plants damaged by fall armyworm 13–15 days earlier than they did on undamaged plants. Additionally, 30–40% fewer *L. oryzophilus* first instars were recovered from previously damaged plants than from undamaged plants. This reduction in densities of *L. oryzophilus* eggs and first instars on armyworm-damaged plants is likely due

Table 1 Mean number of *Lissorhoptrus. oryzophilus* eggs and larvae per plant (± SE) in field samples using rice cultivar 'Jackson.' Egg samples were taken 13 and 19 d after exogenous JA applications. Core samples were taken 19 and 27 d after permanent flood was established. Means within the same column followed by different letters indicate a significant difference ($P<0.05$)

Treatment	First egg sample Eggs/Plant	Second egg sample Eggs/Plant	First core sample Larvae/Plant	Second core sample Larvae/Plant
Control	0.83±0.74a	2.17±1.48a	4.84±1.32a	7.4±1.7a
1.0 mM JA	0.17±0.16a	1.82±0.8a	2.31±0.50b	4.76±0.75a
5.0 mM JA	0.67±0.33a	4.10±1.8a	1.92±0.50b	6.32±1.1a

to an induced response rather than a reduction in oviposition sites for weevils because *L. oryzophilus* females oviposit inside leaf sheaths and not in leaf blades (Stout and Riggio 2002), the primary tissue removed by armyworm feeding. Also, plants had put on between 1 and 2 new leaves between the time of armyworm feeding and weevil infestations, and weevils prefer the sheaths of younger leaves for oviposition (Stout et al. 2002).

Exogenous JA also stimulated resistance to rice water weevils. Plants treated with 1 mM or 5 mM exogenous JA received 54 to 66% fewer eggs on 'Jasmine' and 'Rosemont', respectively, than untreated plants. Moreover, exogenous JA reduced the number of *L. oryzophilus* larvae per plant by 23% (1 mM) to 69% (5 mM) in 'Jasmine' and 54% (1 mM) to 85% (5 mM) in 'Rosemont'. There also was a significant effect of cultivar in one of the JA experiments (more eggs and larvae were found on 'Jasmine' than on 'Rosemont'), but the effect of JA on egg and larval mortality was stronger than the effect of cultivar. Our results are consistent with research in many dicot species that has found the activation of the JA pathway can provide generalized protection against an assortment of herbivorous insects (Inbar et al. 1988; Thaler 1999; Omer et al. 2000, 2001).

Our demonstration of direct induced resistance in rice contributes to a growing body of literature that documents responses induced by chewing insects in rice. Recently, Yuan et al. (2008) identified genes that underlie enhanced volatile emission from rice plants damaged by *Spodoptera frugiperda* (F.) herbivory. They also demonstrated that induced volatiles from *S. frugiperda* herbivory were highly attractive to female *Cotesia marginiventris* (Cresson) parasitoids. Xu et al. (2002) demonstrated increased volatile emission following *S. litura* herbivory compared with *Nilaparvata lugens* (Stål) damaged, mechanically damaged, and undamaged rice plants. Moreover, *S. litura* females avoided plants infested with *N. lugens* in a dual-choice flight tunnel bioassay.

The ability of JA to induce resistance to *L. oryzophilus* in greenhouse experiments led us to hypothesize that exogenous JA would induce resistance in field grown plants. Although adult rice water weevils can be found in rice fields before flooding, oviposition and larval infestations largely commence after flooding (Everett and Trahan 1967; Stout et al. 2002). Densities of eggs in rice fields in Louisiana generally are highest 1 to 3 weeks after flooding, while peak larval densities usually occur two to three weeks later (Stout et al. 2000; Zou et al. 2004). Because our greenhouse experiments had shown that JA-induced resistance reduces oviposition, applications of JA were made 7 days and 1 day prior to flooding in the first and second field experiments, respectively. Although densities of rice water weevil eggs were not significantly reduced in JA treated plants compared with untreated plants in either experiment, consistent trends in the data suggest that JA-treatment made plants less attractive for oviposition (see Tables 1 and 2). Most probably, sampling of eggs was not extensive enough (both in terms of number of plants sampled and frequency of sampling) to detect transient JA-induced differences in egg densities. This interpretation is particularly likely in light of the results of the core sampling, which in both experiments were consistent with the hypothesis that JA treatment induces a transient increase in rice resistance to rice water weevils. In both experiments, initial core samplings, which were conducted 15 to 28 days after flooding, revealed significant

Table 2 Mean number of *Lissorhoptrus. oryzophilus* eggs and larvae per plant (± SE) in field samples using cultivar 'Rosemont.' Egg samples were taken 3 d after exogenous JA applications. Core samples were taken 14 and 19 d after permanent flood was established. Means within the same column followed by different letters indicate a significant difference ($P<0.05$)

Treatment	First egg sample Eggs/Plant	First core sample Larvae/Plant	Second core sample Larvae/Plant
Control	2.18±0.7a	2.88±0.68a	8.12±2.56a
5.0 mM JA	0.88±0.43a	1.46±0.34b	6.54±1.33a

and substantial (up to 60%) reductions in densities of larvae and pupae in JA treated plants compared to untreated plants. However, weevil densities in the second core sampling were not reduced in JA treated plants compared to untreated plants in either experiment.

There are few studies that have examined the use of JA on economically important crops in field settings (but see Thaler 1999; Black et al. 2003), and, to our knowledge, the results reported here are the first to describe direct, JA-induced resistance in rice to herbivores in field-based experiments. The use of chemical elicitors, such as JA, holds potential as a tool for use in agriculture (Stout et al. 2002). Negative effects associated with a reliance on conventional insecticides, such as the development of insecticide resistance, environmental contamination, and threats to human safety, can be mitigated by the use and exploitation of elicitor-induced host plant resistance. Elicitors can stimulate broad-spectrum resistance to a variety of pests; in rice, for example, this study and prior studies (Mei et al. 2006; Senthil-Nathan 2009; Stout et al. 2009) have shown that exogenous JA induces resistance against a variety of important insect and disease pests. In addition, the use of elicitors allows producers to manage both the intensity and timing of induction. However, the relative high cost of elicitors compared to insecticides, coupled with the transient nature of elicitor-induced resistance, may serve as limiting factors in the widespread adoption and use of elicitors in agriculture.

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RAPID COMMUNICATION

Elucidation of a Diurnal Pattern of Catechin Exudation by *Centaurea stoebe*

Nishanth Tharayil · Daniella J. Triebwasser

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Abstract The exudation of secondary metabolites at phytotoxic concentrations has been proposed as a mechanism of invasion for some exotic plant species. Catechin is a natural flavanoid implicated in the potential allelopathic interactions of *Centaurea stoebe*. However, recent studies have shown that catechin is highly unstable and not likely to accumulate in growing medium at phytotoxic concentrations. All previous studies that investigated the allelopathic potential of catechin assumed a continuous exudation of this compound by *C. stoebe*. Contrary to this, but similar to many other plant secondary metabolites, we hypothesized that catechin exudation may exhibit a pulsed pattern that could facilitate its transient accumulation. Further, we aimed at optimizing a more sensitive detection technique. We tested the hypothesis by quantifying the diurnal pattern of catechin release by *C. stoebe* in a hydroponic system. Using sample processing, based on a solid phase extraction technique, and more sensitive fluorescent detection parameters, we were able to quantify catechin in the picomolar range from the growing medium. Catechin exudation exhibited a possible diurnal rhythm with respect to light intensity, with the highest concentration at 6 h after exposure to sunlight. Catechin also was found to undergo a degradation reaction resulting in a transient abundance of pyrocatechol in our system.

Keywords *Centaurea stoebe* · *Centaurea maculosa* · Spotted knapweed · Allelopathy · Catechin · Fluorescence spectroscopy · Allelochemical

Introduction

Plants produce and exude a diverse array of secondary metabolites, under various environmental conditions. These exuded compounds can modify species competition in favor of donor plants by providing uncontested access to the limited resources (Rice 1984), by increasing chemical nutrient foraging efficiency (Tharayil et al. 2009), by preventing intraspecific competition through autotoxicity (Ervin and Wetzel 2000), and by facilitating root navigation in soil (Falik et al. 2005). *Centaurea stoebe*, spotted knapweed, is a noxious and economically destructive exotic weed that has invaded 2.9 Mha in North America (DiTomaso 2000). Roots of *C. stoebe* appear to exude the polyphenol catechin (Blair et al. 2005), but early reports of exudation have not been reproducible (Stermitz et al. 2009). Catechin has been reported at very low concentrations in the rhizosphere soil of *C. stoebe* (Blair et al. 2005, 2006) but high concentrations may occur periodically (Perry et al. 2007).

Exudation of metabolites by plant roots and their concentration in growth medium is a dynamic process, as the exudates are degraded, transformed, or resorbed. Hence, the time of sampling and the sensitivity of sampling procedures are major determinants in understanding the magnitude of root exudates by plants. In a previous study, we demonstrated that the exudation of 8-hydroxyquinoline by the related species *C. diffusa* follows a diurnal pattern of light intensity (Tharayil et al. 2009). Many plant metabolites are subjected to similar diurnal patterns of light

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N. Tharayil (✉) · D. J. Triebwasser
Entomology Soils & Plant Sciences, Clemson University,
Clemson, SC, USA
e-mail: ntharay@clemson.edu

intensity (Urbanczyk-Wochniak et al. 2005), which can be linked to the diurnal regulation of photosynthetic carbon metabolism (Geiger and Servaites 1994). Methods used in previous studies that investigated the exudation of catechin seemed to assume a continuous exudation of this compound by *C. stoebe* (Blair et al. 2005). In contrast, in the present study we hypothesized that catechin could exhibit a pulsatile exudation pattern. Further, we used a more sensitive detection technique for catechin.

Methods and Materials

Plant Material Seeds of *Centaurea stoebe* were collected from Missoula, MT, USA during 2004. The root exudates were quantified in hydroponic cultures. Although hydroponic cultures oversimplify the complex interactions that exudates undergo in soil matrices, they were superior for the objectives of this study due to the less complex matrix influence (thus providing a realistic measure of root exudates) and also due to the convenience of repeated sampling at relatively short intervals. *Centaurea stoebe* were grown in a system similar to that used for growing *C. diffusa* as described in Tharayil et al. (2009) [Supplementary material]. Plants were grown for 10 wk in hydroponics to obtain enough root biomass for catechin quantification. At the day of harvest, after twice thoroughly washing the roots, plants were transferred to the appropriate half-strength Hoaglands solution (2 l, only distal half of roots immersed). We monitored the catechin exudation pattern in relation to the diurnal variation in sunlight intensity by harvesting root exudates of nutrient-sufficient plants (batches of 12) 2, 5, 7, 10, 14, and 24 h after nutrient solution replacement (nutrient solution changed 7:00 am [time zero], light intensity crossed 10 μmol S^{-1} m^{-2} at 8:00 am).

Catechin Analysis The fluorescent spectra of an aqueous solution of catechin were studied with a PerkinElmer Fluorescence Spectrometer (LS-45) with an excitation-emission scan range from 200–800 nm and at a scan speed of 5 nm S^{-1}. Raman scatter lines were removed by subtracting a background (DI-H_2O) from the sample.

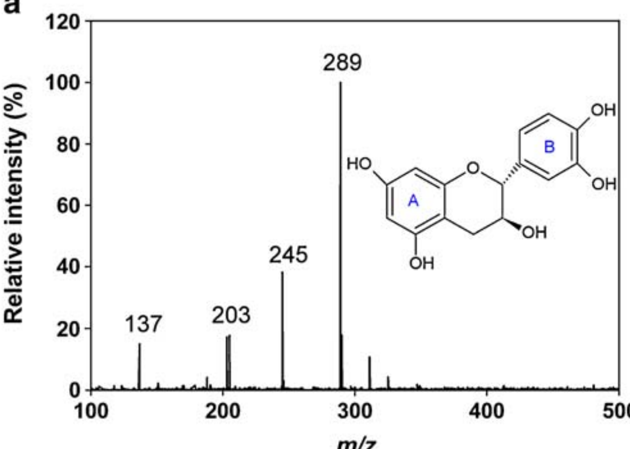
Analysis of Exudates Exudate solutions were filtered through a 1.8-μm glass fiber filter (metal-free) followed by a 0.45-μm cellulose acetate membrane filter. Samples were concentrated by solid-phase extraction using Strata-X 33-μm polymeric reversed phase cartridges (200 mg/3 ml; Phenomenex, Torrance, CA, USA); samples were loaded on a preconditioned column (4 ml min^{-1}), thereafter the cartridges were washed with 25% (v/v) MeOH, and the retained catechin was eluted with a 1.5 ml mixture of MeOH and MeCN (20:60%; v/v). The elutent was concentrated further under nitrogen to 0.5 ml before analysis. Preliminary studies with spiked (5 picomolar; 4 l) sample matrix gave 90% recovery of catechin. All samples were analyzed with a Shimadzu quaternary pump HPLC system equipped with an autosampler, inline degasser, UV–visible diode array detector (DAD) and fluorescence detector. Separations were performed on a Gemini C_{18} column (5 μm $110A^0$; 250 mm×4.6 mm I.D.; Phenomenex, Torrance, CA, USA). Absorbance of samples was monitored at 280 nm with the DAD and at excitation-emission wavelength of 205–316 nm and 280–316 with the fluorescent detector. The photo multiplier tube gain of fluorescence detector was adjusted to 4. The mobile phase consisted of MeCN : acetic acid: water (15 : 0.5 : 84.5, v/v). Standards were prepared in solid-phase elution solvent to avoid interference by the sample matrix during quantification of the analyte. Sample peaks were identified by comparing their retention time and spectra with those of authentic standards. Peaks of interests were collected and further identified qualitatively by using a liquid chromatograph (Waters ACQUITY UPLC™ system, Waters Corp., MA, USA) coupled with electrospray ionization quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer (Micromass MS Technologies, Manchester, UK, ion source- 3,000 V, detector 1,200 V, Lockmass: NaFormate, scan range from 100 to 800 *m/z*), operating in negative ion mode.

Statistics Data were analyzed using one-way ANOVA, and significance is reported at the 95% confidence interval. All statistical tests were performed with SAS 9.1 (SAS Institute, Cary, NC)

Results and Discussion

The presence of catechin and catechol in the growing medium was positively determined based on mass spectra of fractions of interest. Catechin was identified based on following the fragmentation pattern in negative ion mode (Fig. 1a): 289 $[M-H]^-$, 245 subsequent loss of –CH2–CHOH–, 203 subsequent loss of C_2H_2O, 137 results from A-ring fragment of catechin (retro-Diels-Alder cleavage of heterocyclic flavanoid ring, Stöggl et al. 2004; Tharayil et al. 2008). Catechol was identified based on *m/z* 109 which corresponds to $[M-H]^-$.

Considering the minute and transient exudation of most plant secondary metabolites, the analytical techniques adopted for their qualitative and quantitative determination should be robust in the extraction, pre-concentration, and cleanup steps. Automated methods for purification and concentration of catechin from tea leaves using in-tube



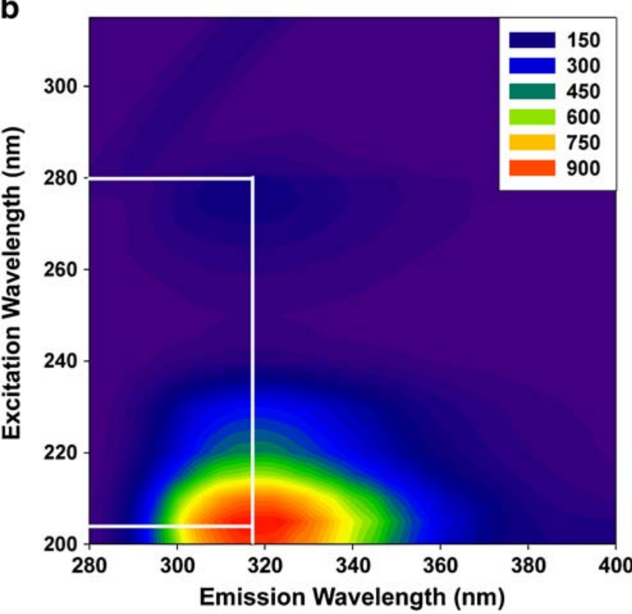
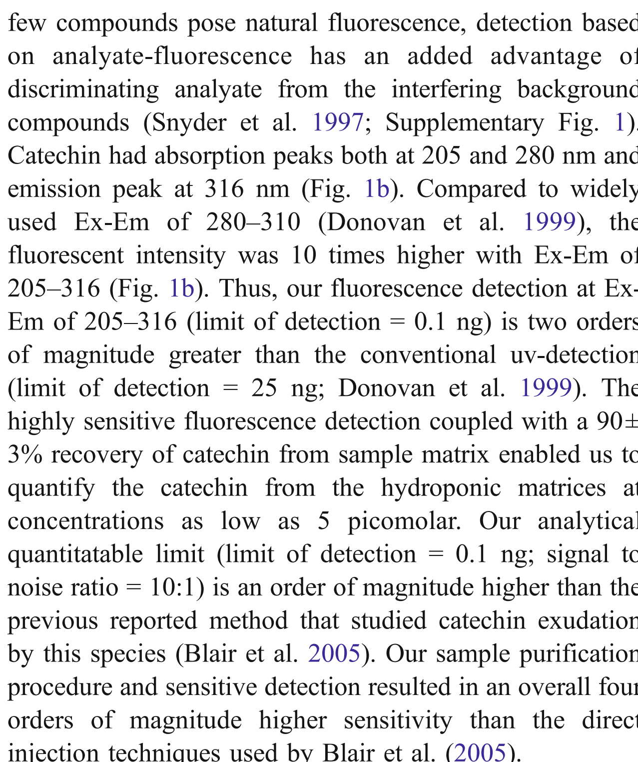


Fig. 1 Spectra of catechin. **a** LC-ESI-MS/MS of catechin and its molecular structure Refer to result section for spectral interpretation. **b** Background-corrected fluorescent spectrum of catechin showing higher fluorescence emission (at 316 nm) when excited with 205 nm compared to 280 nm. The contours represent the relative fluorescence intensity

solid phase microextraction (SPME) have been previously published (Wu et al. 2000). Our method, although more cumbersome than the automated method, results in superior extraction efficacy (90%) compared to 10.1 % using SPME (Wu et al. 2000). Additionally, in-tube SPME cannot be employed when sample size is large, which is typical of any soil-less root exudate studies. Mass-spectrometer detection that uses a gas-chromatograph, although more sensitive (Donovan et al. 1999), requires careful derivatization with *N,O*-bis(trimethylsilyl)-trifluroacetamide (BSTFA), which can be cumbersome.

Compared to uv-dectection, fluorescence detection generally is three orders of magnitude more sensitive. Since


few compounds pose natural fluorescence, detection based on analyate-fluorescence has an added advantage of discriminating analyate from the interfering background compounds (Snyder et al. 1997; Supplementary Fig. 1). Catechin had absorption peaks both at 205 and 280 nm and emission peak at 316 nm (Fig. 1b). Compared to widely used Ex-Em of 280–310 (Donovan et al. 1999), the fluorescent intensity was 10 times higher with Ex-Em of 205–316 (Fig. 1b). Thus, our fluorescence detection at Ex-Em of 205–316 (limit of detection = 0.1 ng) is two orders of magnitude greater than the conventional uv-detection (limit of detection = 25 ng; Donovan et al. 1999). The highly sensitive fluorescence detection coupled with a 90± 3% recovery of catechin from sample matrix enabled us to quantify the catechin from the hydroponic matrices at concentrations as low as 5 picomolar. Our analytical quantitatable limit (limit of detection = 0.1 ng; signal to noise ratio = 10:1) is an order of magnitude higher than the previous reported method that studied catechin exudation by this species (Blair et al. 2005). Our sample purification procedure and sensitive detection resulted in an overall four orders of magnitude higher sensitivity than the direct injection techniques used by Blair et al. (2005).

Centaurea stoebe produced actively growing roots in our system (root : shoot ratio of 8:1; fresh weight; Supplementary Fig. 2). Previous studies have expressed catechin exudation based on its concentration in culture media (Blair

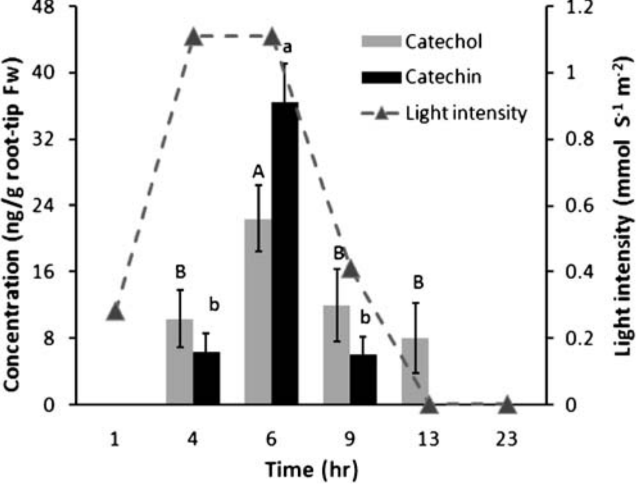


Fig. 2 Appearance of catechin and catechol in hydroponic medium as a function of diurnal light intensity. *Bars* represent mean value ± SD (*N*=4). X-axis represent the time in hours of six sample harvest after the light intensity crossed 10 µmol S^{-1} m^{-2} (8:00 am). Since only the distal half of the roots were immersed in sampling solution, catechin production is normalized based on fresh weight of root tips. Catechin concentration at 1, 13, and 23 h and catechol concentration at 1 and 23 h were below our detection limit. *Triangles* represent the mean sunlight intensities at respective sampling periods. *Bars* with the same letter are not significantly different (*P*<0.05, *N*=4; Duncan's New Multiple Range Test)

et al. 2005). Since the amount of compounds secreted would be directly proportional to the active root-biomass, and since only the distal half of the roots were immersed in sampling medium, the exudation of catechin was normalized to root-tip-fresh-weight collected at the end of the experiment. This overcame the dilution effect due to the amount of media. Root-tip biomass on an average consisted of 30–38% of total root fresh weight.

Centaurea stoebe exhibited a diurnal pattern in catechin exudation, and exudation reached a maximum at 6 h duration of light exposure (7 h after time zero, Fig. 2, $P < 0.05$, $N=4$). The exhibited diurnal pattern could be explained by the fact that flavan-3-ols (catechins) are derived from flavanones, and flavonoid biosynthesis takes place in the presence of light (Seigler 1995).

Catechin was found to undergo a degradation reaction that resulted in a transient abundance of catechol in our system (Fig. 2). Further, both catechin and catechol were unstable in the medium, and the concentration dropped below the detection limit overnight (Fig. 2). To test whether the catechol in the media is a degradation product of catechin, catechin was added to 24 h sample-containers after removing the plants, and the catechin concentrations and catechol were monitored. In a subset of containers, sucrose was added as a source of carbon (200 μg ml^{-1}). Disappearance of the added catechin and a corresponding but transient appearance of catechol identified the source of catechol as catechin. Although the experiments were not conducted in axenic conditions, the absence of catechin in the above plant-less cultures suggests that the source of catechin would be *C. stoebe* rather than microbes.

Catechol is more persistent in the hydroponic media (up to 13 h) compared to catechin (Fig. 2), which further suggests that catechol is a degradation product of catechin. Previous studies in soil media have demonstrated a similar breakdown of exogenously applied catechin to catechol with an accompanying increase in nutrient-mobility attributed to the chelation properties of catechol (Tharayil et al. 2008). Catechin production in our system was below the detection limit on cloudy days (>0.1 mmols S^{-1} m^{-2}). The exudation measured in our system is an order of magnitude lower than the previous conservative estimates by Blair et al. (2005). Apart from the high variability in catechin production by individual Centaurea plants (Blair et al. 2005), another explanation for low concentrations in our system could be possible microbial degradation. Considering the lower catechin phytotoxicity in soil-less medium observed in previous studies (Blair et al. 2005; Tharayil et al. 2008), the catechin concentration in our system would be below that required for phytotoxic concentration.

Pulsed release could increase the bioavailability of a compound by temporarily overwhelming the biological and chemical degradative capacity of soil (Tharayil et al. 2009). Additionally, catechin has been shown to be more persistent in a sandy loam soil when present along with phenolic acids that are released during litter decomposition of *C. stoebe* (Tharayil et al. 2008). Hence, how a pulsed release of catechin could interact with other soil edaphic and biotic factors needs further investigation. Since most earlier studies assumed continuous exudation, our results showing the diurnal pattern could provide some explanation for the previously reported highly variable catechin concentration in natural soils and in growing media. This affirms the importance of the sensitivity of sampling techniques and the time of sampling for studying plant secondary metabolites exuded through roots.

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RAPID COMMUNICATION

Reduced Photosynthetic Activity is Directly Correlated with 2-(3*H*)-benzoxazolinone Accumulation in Lettuce Leaves

Adela M. Sánchez-Moreiras · Alberto Oliveros-Bastidas · Manuel J. Reigosa

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Abstract 2-(3*H*)-Benzoxazolinone (BOA) is a secondary plant metabolite previously found to inhibit plant growth and development. The phytotoxic activity of BOA has been extensively demonstrated over the last years. However, the relation of BOA phytotoxicity with BOA accumulation in plant leaves has not been thoroughly investigated. In this work, BOA phytotoxicity on photosynthesis (ΦPSII and *P*n) of lettuce (*Lactuca sativa* L. cv. Great Lakes) was studied, and these results were correlated with BOA quantities in the leaves. BOA-treated plants showed reduced photosynthesis rate 6 h after the beginning of the treatment, and the efficiency of photosystem II started to be affected 10 h after treatment. These results were correlated with an increasing concentration of BOA in leaves that starts 6 h after treatment and shows a maximum at 96 h.

Keywords BOA action · HPLC · Photosynthetic activity · Phytotoxicity

Introduction

2-(3*H*)-Benzoxazolinone (BOA) is a stable decomposition product derived from the highly reactive and toxic but unstable plant secondary metabolite 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, especially found in cereals. This benzoxazolinone is a potent allelochemical in rye and other plant species, and it has been shown previously to have phytotoxic activity (Baerson et al. 2005). This classical Gramineae compound has been associated with dose-dependent germination and growth inhibition and more in-depth effects on energy metabolism in higher plants. Recent studies demonstrated BOA-reduced seedling length, which was associated with detailed morphological characteristics and impacts on cell division in the radicles (Sánchez-Moreiras and Reigosa 2008). Furthermore, a model of action related to oxidative damage has been proposed for BOA on adult plant metabolism (Sánchez-Moreiras et al. 2005; Batish et al. 2006). These studies concluded that BOA induces the generation of oxidative stress with high levels of lipid peroxidation and concomitant cell damage.

These previous studies demonstrate clear effects of BOA treatment. However, the relation between allelochemicals effects and the presence of the allelochemicals in the target organ has not been documented often. The ability of the cells to metabolize, accumulate, and extrude the natural compounds increases the probability that the toxicity measured after allelochemical application is due to some derivative compound and not necessarily to the 'original' allelochemical (Macías et al. 2006). The different strategies for avoiding allelochemical damage (extracellular excretion, vacuolar sequestration, vesicle transport, accumulation of the metabolite in a non-toxic form) usually are present in the plants as resistance mechanisms. Several studies on the detoxification of BOA in plant cells have concluded that, once into the cell, the cyclic hydroxamic acid can be metabolized by oxidation or hydroxylation and fast glycosylation to obtain a detoxification product storable in the vacuole (Friebe et al. 1998; Hofman et al. 2006). The intermediate toxic compounds generated during these

A. M. Sánchez-Moreiras (✉) · M. J. Reigosa
Facultade de Bioloxía,
Departamento de Bioloxía Vexetal e Ciencia do Solo,
Universidade de Vigo,
Campus As Lagoas, Marcosende,
36310 Vigo, Spain
e-mail: adela@uvigo.es

A. Oliveros-Bastidas
Facultad de Ciencias, Departamento de Química Orgánica,
Universidad de Cádiz,
C/ República Saharaui s/n 11510 Puerto Real,
Cádiz, Spain

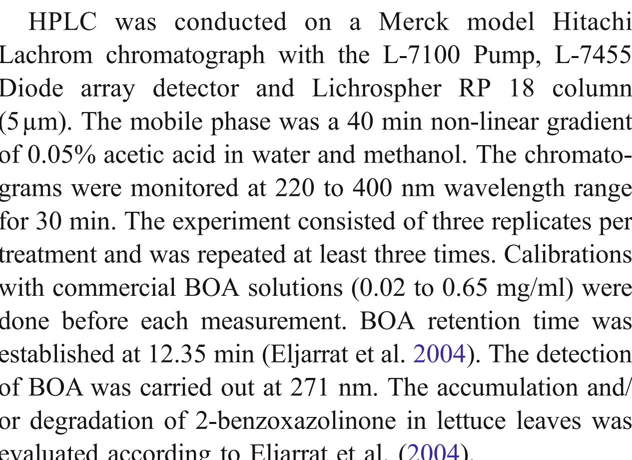
detoxification reactions (i.e., BOA-6-OH) could interfere and distort the measurement of BOA effects (Sicker et al. 2001; Schulz et al. 2006). Most of these studies, however, were done on roots of treated plants, and little information is available about the amount of BOA that reaches the leaves, the stability of this benzoxazolinone in the cells, and the correlation of BOA and phytotoxicity. Chiapusio et al. (2004) measured the translocation and accumulation of radio-labelled BOA in radish seeds and seedlings. They concluded that cotyledons were the sink organ for this allelochemical. However, these results were obtained in BOA-germinating seedlings, which makes it difficult to predict at which precise moment BOA reaches the leaf and to what extent this presence can be correlated with the observed phytotoxicity. Although BOA shows stronger effects on germinating seedlings, its impact on adult plant development also is important due to its continuous presence in the field when the BOA-containing crops are used as cover crops, mulch, smother crops, green manure, or grown in rotational sequences (Dhima et al. 2006). The ecological meaning of BOA action is based on its small but constant effect, which can be highly effective in plant to plant interactions.

Therefore, our goal in this study was to measure the presence of BOA in lettuce leaves after treatment and to correlate these results with the effects on photosynthesis. HPLC was used to measure BOA in leaf extracts, and phytotoxicity was assessed by photosynthesis and fluorescence measurements.

Methods and Materials

BOA solutions (2-benzoxazolinone; Aldrich Chem.Co. 15,705-8) were prepared with distilled water (pH 6.0) at 600 µM and 1 mM concentrations. Seeds of *Lactuca sativa* L. cv. Great Lakes (California) were placed in a thermostatically controlled chamber (16/8 h photoperiod at 150 µmol m^{-2} s^{-1} and 18/8 ºC and germinated in plastic trays with perlite, and 500 ml nutrient solution were added every other day. One week after germination, seedlings were treated with three different treatments. BOA concentrations were based on the dose-response curve for BOA (Sánchez-Moreiras et al. 2008): control, 600 µM BOA (first BOA concentration with significant effects), and 1 mM BOA (I_{50}). All solutions were prepared in 1:1 Hoagland's solution with 0.1% EtOH as solvent.

About 1 g fresh weight leaf tissue was harvested (3 samples per treatment) at 0, 2, 6, 12, 24, 48, 72, and 96 h after BOA exposure for quantification of BOA in leaves by high performance liquid chromatography (HPLC). For HPLC analyses, fresh lettuce leaves were cut into small pieces and immediately treated with ultrasounds at 50 ºC in a solution of 2% HOAc-MeOH for 10 min. The extract was filtered and dissolved to 5 ml.



HPLC was conducted on a Merck model Hitachi Lachrom chromatograph with the L-7100 Pump, L-7455 Diode array detector and Lichrospher RP 18 column (5 µm). The mobile phase was a 40 min non-linear gradient of 0.05% acetic acid in water and methanol. The chromatograms were monitored at 220 to 400 nm wavelength range for 30 min. The experiment consisted of three replicates per treatment and was repeated at least three times. Calibrations with commercial BOA solutions (0.02 to 0.65 mg/ml) were done before each measurement. BOA retention time was established at 12.35 min (Eljarrat et al. 2004). The detection of BOA was carried out at 271 nm. The accumulation and/or degradation of 2-benzoxazolinone in lettuce leaves was evaluated according to Eljarrat et al. (2004).

Photosynthesis rate (*P*n) was measured in the whole plant with an infrared gas analyser LI-6400 (Li-Cor model 6400, Lincoln, NE, USA). Photosynthesis values (µmol m^{-2} s^{-1}) were recorded in triplicate at 800 µmol m^{-2} s^{-1} quantum flux density (provided by a 6400-02B led light source), leaf temperature close to 20ºC, and a CO_2 flow value of 400 µmol s^{-1}. Immediately after gas-exchange measurements, the influence of BOA (1 mM) on the functional state of the photosynthetic apparatus and the ability of the plant to respond to this situation was investigated by the *in vivo* chlorophyll fluorescence of whole plants with a fluorescence monitoring system from Hansatech Ltd. Plants were exposed to ambient light for short time when the measurements were done during night period (see Figs. 1 and 2). Negative control (water) was used in order to ensure the correct development of the experiment (supplemental data). Data were subjected to analysis of variance, with a multiple range test (LSD) for subsequent pairwise comparisons. Data homocedasticity was tested with Levene's statistics.

Results and Discussion

The photosynthetic efficiency was monitored over a 96 h treatment period in order to get a clear picture of the evolution of BOA effects on lettuce leaves (as a model system). Figure 1 shows the monitored *P*n (photosynthetic rate) in plants treated with BOA and untreated controls. Photosynthetic activity clearly was enhanced briefly after treatment.

Figure 2 shows the monitored ΦPSII (chlorophyll *a* fluorescence) and *P*n (photosynthetic rate) in the plants after 1 mM BOA exposure. BOA started to affect photosynthetic rate about 6 h after treatment (day period), and reached the lowest rates 8 h after treatment. The *P*n transiently increased between 8 and 10 h, and then decreased during the night period. *P*n recovered after 16 to 18 h, and was relatively high 36 h after treatment. Probably the plant was coping with the stress produced by BOA application by compensating (most likely by heat

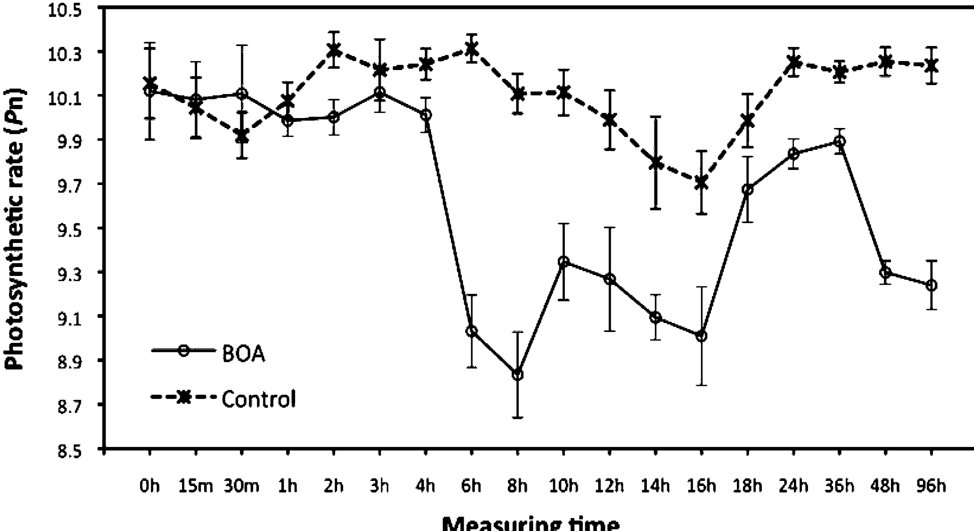


Fig. 1 Photosynthetic rate of control and BOA (1mM)-treated plants over 96 h treatment period (N=3 for each data point; means±SE are given)

dissipation) the excess energy. However, this was a short time of recovery, and the decline in *P*n became even more drastic after the second day of treatment. *P*n decreased between 36 and 48 h and remained low for the remainder of the experiment. At this time (48 h), no visible symptoms of BOA phytotoxicity were detected in the leaves. Finally, plants showed very low *P*n and Fv/Fm values 96 h after treatment, the last measurement time, when BOA-treated plants showed smaller size and more senescent leaves than control plants.

Pulse-modulated fluorescence was recorded for the same replicates per treatment used for gas-exchange measurements (Fig. 2). The photochemical yield of Photosystem II (PSII) was established by measuring the quantum yield of

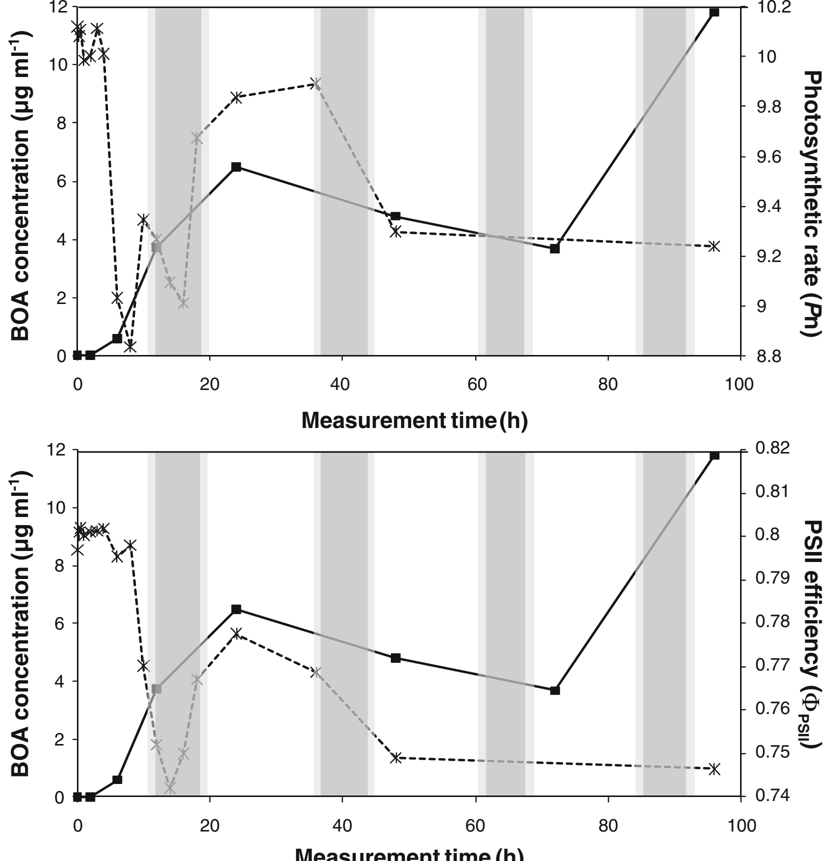


Fig. 2 BOA concentration and photosynthetic activity in lettuce leaves over 96 h treatment. Continuous line represents BOA concentration in the leaves (μg ml^{-1}) and dotted line represents photosynthetic activity (*P*n and Φ_{PSII}). Dark areas represent night periods and day-night transition periods

Table 1 Correlations between photosynthetic parameters and BOA quantities in lettuce leaves after BOA treatment (1mM)

	Correlations	ΦPSII	*P*n	[BOA]
ΦPSII	Pearson Correlation	1	0.546([a])	−0.773([a])
	Sig. (2-tailed)	–	0.000	0,000
*P*n	Pearson Correlation	0.546([a])	1	−0.464([b])
	Sig. (2-tailed)	0.000	–	0.013

[a] Correlation is significant at the 0.01 level (2-tailed)

[b] Correlation is significant at the 0.05 level (2-tailed)

Photosystem II photochemistry (Φ_{PSII}). The quantum yield of electron flow through PSII *in vivo*, i.e., the overall efficiency of PSII reaction centers in the light (Φ_{PSII}), measures the proportion of absorbed energy that is used in photochemistry (the reached efficiency). The inhibition of Φ_{PSII} values insinuates a weak efficiency of PSII reaction centers and indicates an alteration of the rate of linear electron transport, suggesting that the proportion of photons absorbed by PSII and used for photosynthesis is being reduced (Hall and Rao 1999). The temporal profile for ΦPSII was similar to that of *P*n but it started later. Decrease of PSII efficiency started 10 h after treatment and stayed statistically low for the overall period. Moreover, we detected a similar tendency for recovery at 36 h for ΦPSII and *P*n, although the recovery observed in ΦPSII was a trend only with statistically different values with respect to the control.

To quantify BOA concentration in lettuce leaves, HPLC measurements were conducted in plants treated with 600 µM and 1 mM BOA concentrations and in untreated plants as a negative control. Figure 1 shows BOA content in lettuce leaves at eight different exposure times after 1 mM BOA treatment. BOA accumulation in leaves slowly increased to a first peak of concentration 24 h after the treatment. BOA concentration in the leaves was highest 96 h after BOA exposure. Values obtained for 600 µM were similar to those found for 1 mM BOA treatment but in a lower range of values (data not shown).

When considering *P*n, ΦPSII, and HPLC measurements, we see a clear correlation between presence of BOA in the leaves and decreased photosynthetic activity (Table 1). The first time point at which BOA was detected in the leaves (6 h) correlates perfectly with the first time at which photosynthesis was strongly affected (6 h). When photosynthesis is low, BOA concentration in the leaves is high. In this way, Pearson's coefficient gave statistically significant correlations for these three measurements. BOA accumulation showed significantly inverse correlation with ΦPSII and *P*n non-stressed values (−0.773 and −0.464, respectively) while, as expected, ΦPSII showed a highly significant correlation with *P*n values. No new peaks were detected in the chromatograms over this time period although previous studies have shown the ability of lettuce to enzymatically detoxify BOA (Schulz et al. 2006). We conclude that the phytotoxicity observed in lettuce leaves at this time was due to the activity of BOA and not to some derivative or other degradation compounds. Nevertheless, the time course data demonstrated that photosynthetic rate (*P*n) dropped as BOA was first detected in the leaves, which also suggests that the signal or damage that reduced *P*n occurred in the roots and moved up to the leaves along with BOA. Altogether, the effect observed on lettuce after BOA treatment is directly correlated with addition and presence of BOA in the plants.

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Foliar Mono- and Sesquiterpene Contents in Relation to Leaf Economic Spectrum in Native and Alien Species in Oahu (Hawai'i)

Jordi Sardans · Joan Llusià · Ülo Niinemets · Sue Owen · Josep Peñuelas

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Abstract Capacity for terpene production may confer advantage in protection against abiotic stresses such as heat and drought, and also against herbivore and pathogen attack. Plant invasive success has been intense in the Hawaiian islands, but little is known about terpene content in native and alien plant species on these islands. We conducted a screening of leaf terpene concentrations in 35 native and 38 alien dominant plant species on Oahu island. Ten (29%) of the 35 native species and 15 (39%) of the 38 alien species contained terpenes in the leaves. This is the first report of terpene content for the ten native species, and for 10 of the 15 alien species. A total of 156 different terpenes (54 monoterpenes and 102 sesquiterpenes) were detected. Terpene content had no phylogenetic significance among the studied species. Alien species contained significantly more terpenes in leaves (average ± SE=1965±367 μg g^{-1}) than native species (830±227 μg g^{-1}). Alien species showed significantly higher photosynthetic capacity, N content, and lower Leaf Mass Area (LMA) than native species, and showed higher total terpene leaf content per N and P leaf content. Alien species, thus, did not follow the expected pattern of "excess carbon" in comparison with native species. Instead, patterns were consistent with the "nutrient driven synthesis" hypothesis. Comparing alien and native species, the results also support the modified Evolution of Increased Competitive Ability (EICA) hypothesis that suggests that alien success may be favored by a defense system based on an increase in concentrations of less costly defenses (terpenes) against generalist herbivores.

J. Sardans (✉) · J. Llusià · J. Peñuelas
Global Ecology Unit CSIC-CEAB-CREAF, Facultat de Ciencies, Edifici C, Universitat Autònoma de Barcelona,
08913 Bellaterra, Spain
e-mail: j.sardans@creaf.uab.cat

S. Owen
Centre for Ecology and Hydrology Edinburgh,
Bush Estate, Penicuik,
EH26 0QB Scotland, Great Britain

Ü. Niinemets
Estonian University of Life Sciences,
Institute of Agricultural and Environmental Sciences,
Kreutzwaldi 1,
51014 Tartu, Estonia

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Introduction

Plant invasion is an important component of current global change (Mooney and Hobbs 2000). Chemical factors such as terpenes can be involved in the competition between alien and native plant species. For example, Barney et al. (2005) stated that the terpene production capacity of *Artemisia vulgaris* can be a key factor in its establishment and proliferation in introduced habitats by phytotoxic effects on native species.

Many studies have investigated the physiological and ecological significance of terpenes in plants. Protection, defense, and infochemical function have been highlighted as roles of terpenes (Llusià and Peñuelas 2001; Wheeler et al. 2002; Peñuelas and Llusià 2003, 2004). Examples of these roles are photoprotection (Peñuelas and Munné-Bosch 2005), thermotolerance (Sharkey and Singsaas 1995; Peñuelas and Llusià 2001, 2002; Copolovici et al. 2005; Peñuelas et al. 2005), protection against drought stress

(Llusià and Peñuelas 1998; Kainulainen et al. 1991), and non-specific antioxidative capacity, whereby terpenes protect photosynthetic membranes against peroxidation and reactive oxygen species such as singlet oxygen (Loreto and Velikova 2001; Peñuelas and Llusià 2002; Loreto et al. 2004; Munné-Bosch et al. 2004; Llusià et al. 2005).

Although relative performance often depends on growth conditions, invaders are more likely to have higher leaf area and lower tissue construction costs that increase productivity, and also greater phenotypic plasticity that is advantageous in disturbed environments (Daehler 2005). Foliar traits such as higher photosynthetic capacity per dry mass (A_{mass}) and lower leaf construction costs associated with a lower leaf mass per area (LMA) partly explain the success of alien plant species (Baruch and Goldstein 1999; Funk and Vitousek 2007), since they may contribute to faster growth rates for invaders and confer a competitive advantage over native species (Reich et al. 1997; Peñuelas et al. 2010). Similarly, invasive plant species in Hawai'i have been found to have higher foliar N and P concentrations than native species (Peñuelas et al. 2010).

Changes in nutrient availability can affect terpene production (Son et al. 1998; Kainulainen et al. 2000; Lee et al. 2005). Greater terpene production in plants with higher nutrient concentration and photosynthetic rates can be expected from the "nutrient-driven synthesis" hypothesis that predicts a larger enzyme production with greater cellular N and P availability. Higher nutrient availability usually is expected to translate into higher carbon fixation and activity of the enzymes involved in isoprenoid production (Harley et al. 1994; Litvak et al. 1996). In contrast, a lower production of terpenes as carbon based secondary compounds under higher nutrient availabilities can be expected from the "carbon based secondary compounds" (CBSC) hypthesis and the source-sink "carbon-nutrient balance" or "excess carbon" (CNB) hypothesis (Loomis 1932; Bryant et al. 1983; Herms and Mattson 1992; Peñuelas and Estiarte 1998). These hypotheses assert that plants allocate carbon to secondary metabolism only after growth requirements are met, and that growth is constrained more by nutrients than by photosynthesis. According to these theories, the excess carbohydrates that accumulate in nutrient-limited plants when photosynthesis outpaces growth are diverted to the production of carbon-based secondary compounds (e.g., terpenes and phenolics).

Phenotypic plasticity has been an important mechanism that enables alien plants to colonize exotic habitats, and recent studies indicate that alien plants also can evolve quickly (Maron et al. 2004). Some invasive trees and herbs have proved to be able to evolve in periods from 1 to 3 hundred years or less, reaching a faster growth capacity, and changing their chemical defense strategies (Rogers and Siemann 2004; Siemann et al. 2006). The main cause of this increase in fitness has been proposed as the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Nötzold 1995). It predicts that introduced species, which lose contact with their natural specialist herbivores, may evolve, thus decreasing their investment in anti-herbivore chemical defenses. This way, resources no longer needed for defense can be reallocated to other functions that provide a selective advantage in the novel habitat. Recent modifications in the development of increased competitive ability (EICA) hypothesis propose that since invasive genotypes still may experience attack by local generalist herbivores (Müller-Schärer et al. 2004), selection may favor a reduction in the expression of metabolically expensive chemical defenses effective against specialist herbivores, and increase the concentrations of less costly qualitative defenses, such as terpenes, that may be more toxic to generalist herbivores (Joshi and Vrieling 2005; Stastny et al. 2005). In this context, Johnson et al. (2007) have observed that when North American native populations of *Solidago gigantea* grow under the same environment conditions as alien European population of the same species, the native plants have lower monoterpene and diterpene contents than invasive plants. This suggests that terpene content might be related to alien success.

The Hawaiian archipelago is the most isolated terrestrial region on Earth (Vitousek and Walker 1989), and is especially vulnerable to invasions by non indigenous species (Harrington and Ewel 1997). Alien plants in Hawai'i have strong impact on native Hawaiian ecosystems and their highly endemic flora (Mack and D'Antonio 2003; Hughes and Uowolo 2006). In these Islands, around 861 flowering plant species (47% of total Hawaiian angiosperm flora) are naturalized alien species (Wagner et al. 1999). As a result, approximately 25% of the Hawaiian native flora, 90% of which is endemic, has been listed as threatened or endangered. In fact, all tropical island ecosystems appear to be especially vulnerable to invasive species, and some experiments suggest that the high resource availability and the poor ability of native species to capture these resources, contributes to the vulnerability of island communities to the establishment and spread of alien species (Allison and Vitousek 2004).

There are published reports of terpene contents in species that are aliens in Hawai'i, but these studies have been conducted in other parts of the world (Ogunkoya et al. 1972; Schapoval et al. 1998; Kikuzaki et al. 2000; Wheeler et al. 2002; Chiang and Kuo 2002; Pino et al. 2005; Randrianalijaona, et al. 2005; Fernández and Torres 2006; Pachanawan et al. 2008). Generally, apart from scarce reports (Komai and Tang 1989) little is known about terpene content in Hawaiian native and alien flora.

In this study, we conducted a screening of leaf terpene content in 35 native and 38 alien dominant Hawaiian plant

species. Our aims were to: (i) estimate terpene content and composition of native and alien species that are dominant in Oahu, (ii) compare the mono- and sesquiterpene content of the 35 native plants with the content of 38 alien plants, (iii) compare the relationships of terpene content with the leaf traits defining "leaf economics spectrum" (Wright et al. 2004), such as photosynthetic rates (A_{mass}), leaf mass area (LMA), and C, N, P, and K leaf concentrations among native and alien species, and (iv) test the "nutrient driven synthesis", "excess carbon", and "modified EICA" hypotheses for terpene content in native and alien species.

Methods and Materials

Field Sites The study was conducted in May 2007 on Oahu, the third largest of the Hawaiian Islands. As typical of larger Hawaiian Islands, the climate is characterized by steep rainfall gradients over short distances (Müller-Dombois and Fosberg 1998). Lowlands at the leeward side have a pronounced dry summer season, while precipitation is distributed almost uniformly in lowland and mountain rain forests. Due to the oceanic tropical climate, interannual temperature oscillations are small with winters having on average 2–3°C cooler temperatures than summers. As large differences in the composition of native and alien vegetation occur in response to rainfall gradients, four sites with distinct precipitation regimes were selected for plant sampling in the leeward lowlands of Oahu, and at the leeward side of Koolau mountains (Table 1 and see detailed decription of the sampling sites, their climate and the studied species in Peñuelas et al. 2010).

The four key soil types found across the sites rank according to the state of soil weathering as *oxisols*>*ultisols*>*mollisols*>*inceptisols* (Uehara and Ikawa 2000; Deenik and McClellan 2007). *Mollisols* exhibit the highest fertility, while more leached *oxisols* and *ultisols* with lower pH are among the soils with lowest fertility (Uehara and Ikawa 2000; Deenik and McClellan 2007). *Inceptisols*, the youngest soils, typically show weak profile development, and, depending on genesis, exhibit tremendous variability in fertility (Deenik and McClellan 2007). The Tantalus series *inceptisols* are of moderate to high fertility, while the *inceptisols* in rocky soils and mountainous land are of low fertility. Thus, in our study, the broad soil classes rank according to fertility as *mollisols* > *inceptisols* (Tantalus) > *oxisols* ≅ *ultisols* > *inceptisols* (mountainous soils).

Study Species Altogether, 73 dominant,(35 native, and 38 alien) species were studied at four sites (Peñuelas et al. 2010). All native species sampled were evergreen, but in dry sites (St. Louis Heights, Hahaione Valley), four alien species were drought-deciduous (*Desmodium incanum*, *Falcataria moluccana*, *Senna surattensis*, *Tabebuia rosea*), and two were semi-deciduous (*Haematoxylum campechianum*, *Leucaena leucocephala*). The deciduous and semi-deciduous aliens were legumes, except for *Tabebuia rosea* (*Bignoniaceae*). Of the 73 studied species, 36 were trees, 29 shrubs, 3 woody vines to shrubs, 3 woody vines, one herb to subshrub, and one parastitic mistletoe (*Korthalsella complanata*). The distribution of species among the key life form classes was similar among native and alien species (14 alien and 15 native shrubs, 21 alien and 15 native trees; Peñuelas et al. 2010).

Each species was sampled in triplicate, with twigs or small branches taken from 3 individuals for each species. Samples were cut with a sharp knife and cut again immediately with the excised stem under water, to prevent ingress of air to the xylem vessels and subsequent stress. In the lab, the excised stems were enclosed loosely in plastic bags to prevent water loss by transpiration prior to terpene extraction. Leaf extractions were conducted during the next 12 h. Species coordinates and sampling altitude were noted in each site by using GPS, and this information was used to link species locations to specific soil types and to derive location-specific climatic data. Long-term average monthly and annual precipitation, precipitation of the three driest months and annual precipitation, and average, maximum

Table 1 Description of the study sites

Site	Coordinates	Average ± SD[a] altitude (m)	Average ± SD precipitation (mm)		Average ± SD annual temperature (°C)				
			Annual	Three driest months	Minimum	Maximum	n	N	A
St. Louis Heights	21°18′N, 157°48′W	171±65	1430±210	197±45	18.7±0.5	26.9±0.5	12	0	12
Hahaione Valley	21°19′N, 157°43′W	390±140	1268±22	157±7	17.1±0.6	25.7±0.5	14	2	12
Tantalus	21°N, 20′157°49′W	441±24	3670±440	705±41	16.2±0.6	24.1±0.6	22	11	11
Wiliwilinui	21°19′N, 157°45′W	660±120	2100±150	413±60	15.2±0.9	23.8±0.8	25	22	3

N number of species, *N* native species, *A* alien species

[a] averages are based on the number of species sampled and species-specific locations. In statistical analyses, exact species-specific environmental data were used

and minimum temperatures were estimated from high resolution climatic grids by using the database developed and continuously updated by Giambelluca and associates (Giambelluca et al. 1986; Cao et al. 2007). ARCGIS 9.1 was used to extrapolate between the isohyets (10 m square cells in the grid with appropriate elevation model), as applied previously in Hawaiian ecosystems (Porder et al. 2005; Dunbar-Co et al. 2009).

Species were classified according to site preference as dry, dry-mesic, mesic, dry-wet, mesic-wet, and wet forest species. Species invasiveness was scored by using a four-level scale as 0 (native species), 1 (low invasiveness), 2 (moderate-high), and 3 (very high). These simplified scores were based on the Australia/New Zealand weed risk assessment (WRA) system (Pheloung et al. 1999) modified to Hawai'i and other Pacific Islands (Daehler et al. 2004). For Hawaiian Islands, these scores are reported in Pacific Island Ecosystems at the Risk (PIER) project online database, maintained by U.S. Forest Service's Institute of Pacific Islands Forestry (http://www.hear.org/pier/), and on recent updates on species invasive potential in Oahu (Daehler and Baker 2006). The weed risk assessment is based on up to 49 questions about species biology. For 9 species that have not been scored in these assessments, weed risk assessment scores were derived based on the risk questionnaire (http://www.hear.org/pier/). As the risk assessment provides information of possible species invasiveness, but not on whether the species actually becomes invasive in the specific new habitat, finally, a simplified 3-level scale (1–3) was used to group aliens with varying invasive potential and known invasiveness throughout Oahu. (Daehler et al. 2004; Daehler and Baker 2006).

Leaf Terpene Extraction and Analysis Leaf samples were crushed in liquid nitrogen with a Teflon pestle in a Teflon tube until a homogeneous powder was obtained. Between 2–4 ml of pentane (depending on the leaf type) were added before the pulp defrosted. The Teflon tubes were maintained airtight at 25°C during 24 h for full extraction. After this, a sample of each extract was put into a 300 μl glass vial. Samples were injected automatically into the GC-MS following a split of 0.5:80, thus allowing only 0.625% of the injected sample to enter the column. The column was an HP-5 crosslinked 5% PH Me Silicone (Supelco Inc.). Solvent delay was 3 min. The initial temperature of 40°C was increased immediately with a ramp of 30°C min^{-1} to 60°C. The second ramp was 10°C min^{-1} to 150°C, which was maintained for 3 min. The third ramp was 70°C min^{-1} to 250°C, which was maintained for 5 min. Carrier gas was helium at 0.7 ml min^{-1}. The mass detector was used with an electron impact of 70 eV. Identification of monoterpenes was conducted by GC-MS and comparison with authentic standards from Fluka (Buchs, Switzerland), literature spectra, and GCD Chemstation G1074A HP. Calibration with common terpenes α-pinene, 3-carene, β-pinene, β-myrcene, *p*-cymene, limonene, sabinene (monoterpene), and α-humulene (sesquiterpene) standards was carried out once every 5 analyses. Standards were purchased from Sigma Aldrich (Gilingham, Dorset, UK). Terpene calibration curves (N=4 different terpene concentrations) were always highly significant (r^2>0.99 for the relationships between signal and terpene concentrations). The most abundant terpenes had similar sensitivity (differences were less than 5%). Quantification of the peaks was conducted according to the amount of ion 93 in the compound and by using the calibration of the most similar mono- or sesquiterpene standard depending on the compound investigated. The total GC run time was 23 min. All sampling and analytical procedures were applied in the same way for native and alien species.

Statistical Analyses The program Phylomatic (Webb and Donoghue 2005) was used to build a phylogenetic tree of the species studied (Fig. 1) as explained in Peñuelas et al. (2010). The stadistical significance of the genetic differences among different species in explaining the variability of the studied variables was calculated by employing Matlab 7.6.0 with the PHYSIG module developed by Blomberg et al. (2003).

Altitude was significantly correlated with all the main climate variables of each respective site (total annual precipitation, the precipitation of the three driest months, mean annual temperature, annual mean of the daily minimum temperatures, annual mean of the daily maximum temperatures, annual mean of monthly difference between the maximum and minimum temperature and annual mean of the coldest monthly temperature) (data not shown).

To analyze the effects of all studied characteristics on foliage terpene contents, we conducted a general linear model (GLM) with site (4 different sample sites), species origin (native and alien), and soil type (5 different soil types) as independent categorical variables, altitude as independent continuous variable, and in the case of variables with phylogenetic fingerprinting, phylogenetic distances also were included as continuous independent factors. We introduced the factor site in the GLM design to extract the variability due to site. Since the origin of the studied species (native vs. alien) showed a significant phylogenetic signal (k=0.309, P=0.022) mainly due to the high abundance of alien species of the Orders *Rosales*, *Lamiales*, and *Laurales*, we conducted all the statistical analyses that had the origin of species as the independent factor both by an ordinary GLM without phylogenetic distance matrix and also with phylogenetic distance matrix even when the dependent variable had no significant phylogenetic signal. Thereafter, the model with a lower

Fig. 1 Phylogenetic tree of the studied woody plant species obtained from PHYLOMATIC programme (Webb and Donoghue 2005). The scale depicts millions of years. The field sampling site is depicted between brackets to the right of each species. (SLH = St. Louis Heights. HV = Hahaione Valley. T = Tantalus. WR = Williwillinui Ridge). The origin (N = Native. A = Alien) is also depicted

Akaike information criterion (AIC) was selected. To conduct these analyses we used Matlab 7.6.0 with REGRESSIONV2 module (Lavin et al. 2008). We also conducted a PCA analysis with leaf economics spectrum variables (nutrients, A_{mass} and LMA) and the different sets of species as cases. Then, we looked at the correlation between the factor scores that characterized the functional spectrum and total leaf terpene contents by using Statistica 6.0 software package (StatSoft, Inc. Tule, OK, USA).

We employed the same rationale to analyze potential differences between native and alien species in terpene contents vis-à-vis leaf chemical, physiological, and anatomical traits. To analyze what variables are differently correlated between alien and native species, we conducted discriminant analysis among leaf total terpene contents and leaf traits by using Matlab 7.6.0 with REGRESSIONV2 module (Lavin et al. 2008) and Statistica 6.0 software (StatSoft, Inc. Tule, OK, USA). To analyze the effects of leaf economics spectrum on terpene emisssion and whether there were different relationships between native and alien species, we also conducted a PCA analysis with the species as cases and leaf economics spectrum (nutrients, amass, and LMA) as variables and calculated the factor scores for each species. Thereafter, we conducted a correlation between the factor scores that characterized the functional spectrum and the total terpene contents.

To compare possible differences in the proportion of species that produced and accumulated terpenes between the alien and native species set, we conducted a *Chi-square* test by using Statistica 6.0 software package (StatSoft, Inc. Tule, OK, USA).

Results

Foliage Terpene Concentration. Alien vs. Native Species Twenty-five (10 natives and 15 alien) of the 73 studied species contained at least one terpene (concentration above the detection limit of 0.6 ng g^{-1} in their leaves; Tables 2 and 3). No terpenes were detected in 48 of the 73 studied species (Appendix 1). Total terpene and total monoterpene concentrations were higher in the entire set of alien species than in the entire set of native species (P=0.02 and P=0.04, respectively) (Table 4, Fig. 2). In alien species, total terpene concentration was 1965±367 μg g^{-1} (4524±1225 μg g^{-1} when considering only storing species) and in native species it was 830±227 μg g^{-1} (2905±650 μg g^{-1} when considering only storing species). Total sesquiterpenes, cyclic monoterpenes, cyclic sesquiterpenes, and aromatic monoterpenes were also higher in alien than in native species but the differences were not significant (Table 4, Fig. 2). The greater average total terpene content in alien than in native species was due mainly to higher leaf monoterpenes in moderately-high invasive species and to higher sesquiterpenes in highly invasive species than in native species (Fig. 2). Neither the sampling site nor soil type had any significant effect on total terpene content (Table 4). The proportion of species that accumulated terpenes, although higher in alien species, was not significantly different between native (29%) and alien species (39%) (χ^2=0.97, P=0.42).

Several mono- and sesquiterpene compounds were found in 10 native species (*Cheirodendron trigynum*, *Melicope clusiifolia*, *Melicope peduncularis*, *Metrosideros macropus*, *Metrosideros polymorpha*, *Metrosideros rugosa*, *Metrosideros tremuloides*, *Myrsine lessertiana*, *Myrsine sandwicensis*, *Syzygium sandwicensis*), which had not been described previously as terpene accumulators to our knowledge. *Syzygium sandwicensis* accumulated 3 monoterpenes (camphene, E-β-ocimene, 3,7 dimethyl-octa-1,3,7-triene) and 11 sesquiterpenes (Tables 2 and 3) in its leaves. In the species of the genus *Metrosideros*, three monoterpenes (sabinene, 1,3,6-octatriene, 3,7-dimethyl E-β-ocimene) and 18 sesquiterpenes (Tables 2 and 3) were detected. In the two species of the genus *Myrsine*, 2 monoterpenes (limonene, myrcene) and 33 sesquiterpenes (Tables 2 and 3) were observed. In *Cheirodendron trigynum*, 11 monoterpenes and 11 sesquiterpenes were found (Tables 2 and 3). Finally, in the two *Melicope* species, 24 monoterpenes and 29 sesquiterpenes were found (Tables 2 and 3).

Among the 15 alien species that accumulated terpenes in their leaves, three species *Heliocarpus americanus* (1 monoterpene, 7 sesquiterpenes, Tables 2 and 3), *Schinus terebinthifolius* (7 monoterpenes, 9 sesquiterpenes), and *Persea americana* (7 monoterpenes, 3 sesquiterpenes) had not been reported previously as terpene accumulators.

Phylogenetic influence on the values of the variables was present only in 6 of the 156 different detected terpenes: β-cubebene, β-maaliene, epi-bicyclo-sesqui-phellandrene, γ-elemene, β-ocimene and l-β-pinene. Total terpene contents did not show a phylogenetic effect (data not shown).

Relationships of Leaf Terpenes to A_{mass}, LMA and Nutrient Leaf Concentrations and Climate No significant relationships of total terpenes with climatic characteristics and A_{mass} were observed in the corresponding GLM analysis either in native or alien species (data not shown). Discriminant analysis of the total terpenes (TT) and leaf traits: LMA (Wilk's Lambda = 0.58 and P=0.002), leaf N concentration (Wilk's Lambda = 0.65 and P=0.008), leaf K concentration (Wilk's Lambda = 0.82 and P=0.1) and A_{mass} (Wilk's Lambda = 0.67 and P=0.019), separated native and alien species (Fig. 3), showing that the set of native species had greater LMA and lower leaf economic traits and leaf terpenes than the set of alien species. Relationships of total

terpene contents with the main leaf economic and structural traits did not differ between native and alien species (Fig. 3). There was no significant correlation between total leaf terpenes and the PC1 scores of each species obtained in the PCA analysis of leaf economic traits (LMA, Amass, N, K) within native plants (R=0.17, P=0.64) nor within alien plants (R=0.090, P=0.77) (Fig. 3).

Discussion

Terpenes in Hawaiian Plants This study provides novel information about terpene contents in Hawaiian native plant species. It also contributes to advance our understanding of terpene content in several alien species that have worldwide distributions (Sharma et al. 1999; Olajide et al. 1999; Ghisalberti 2000; Kikuzaki et al. 2000; Wheeler et al. 2002; Pino et al. 2005; Zhao et al. 2009).

None of the 10 native species that accumulated terpenes has been reported previously as terpene-containing, at least to our best knowledge. At the genus level, only some species of the genus *Syzygium* had been reported as terpene accumulators (Chang et al. 1999). Among the 15 alien terpene-containing species, six had been previously identified as mono and sesquiterpene-containing species (*Psidium guajava*, Olajide et al. 1999; *Lantana camara*, Sharma et al. 1999; Ghisalberti 2000; *Pimenta dioica*, Kikuzaki et al. 2000; *Melaleuca quinquenervia*, Wheeler et al. 2002; *Mangifera indica*, Pino et al. 2005; *Ageratina adenophora*, Zhao et al. 2009). In these cases, the terpenes reported previously and those found in the present research generally were the same, but with some differences. Some terpenes found in *Lantana camara*, such as α-gurjunene or zingiberene, had not been reported previously in this species. Similarly, in *Melaleuca quinquenervia*, we found 7 terpenes (viridiflorol, 1–8 cineole, α-terpineol, α-pinene, β-pinene, γ-terpinene, α-terpinene) of the 10 already previously reported in this species (Wheeler et al. 2002), but in addition we also found 8 more monoterpenes and 22 sesquiterpenes (Tables 2–3). It is possible that the sampling technique may have induced some stress, which might have resulted in the production of these extra compounds. Five of the 17 alien species that accumulated several mono- and sesquiterpenes, *Cinnamomum burmannii, Eucalyptus robusta*, *Psidium cattleianum*, *Rubus rosifolius*, and *Syzygium cumini* (Tables 2–3) had not yet been reported as terpene-accumulator species, although members of the same genus have been described as terpene accumulators (Chang et al. 1999; Olajide et al. 1999; Moore et al. 2004; Chao et al. 2005; Yang et al. 2005; Malowicki et al. 2008). In another species, *Pluchea carolinensis*, known to have medical properties, terpene content has been suggested, but had not been described (Fernández and Torres 2006). In this study, 1 monoterpene and 3 sesquiterpenes were detected in this species. Finally, three alien species *Heliocarpus americanus*, *Schinus terebinthifolius* and *Persea americana* had not been reported previously as terpene accumulators. We did not detect a phylogenetic effect in the total terpene content among the set of studied species, nor in the content of the individual terpenes.

Higher Terpene Content in Alien Species and No Relationship to Leaf Economics In regard to the number of species that accumulate terpenes, although the difference was not significant, the proportion of species that accumulated terpenes in leaves was slightly higher in alien (39%) than in native (29%) species. In regard to the absolute terpene leaf accumulation, alien species accumulated greater amounts in leaves, and also had greater N and P leaf concentrations than native species, and their ratio of terpene contents to the concentrations of these two elements were higher than in native species. Thus, the differences between alien and native species were proportionally greater in leaf terpene accumulation than in N and P leaf content. Collectively, these data suggest a greater investment in terpene production with respect to nutrient absorption and carbon fixation in alien compared with native species. Thus, alien species in Hawai'i may have more productive leaves and invest more of their leaf primary production in terpene accumulation than native species.

The discriminant analyses based on the relationships between total terpene accumulation and LMA and nutrient content significantly separated alien from native plants, thus reflecting that alien species have greater total terpene contents, N and K concentrations, and A_{mass} and lower LMA than native species. When comparing native with alien species, a significantly higher leaf nutrient and terpene content are observed in alien species. This segregation suggests that these species may be using different resources and might also have greater capacities to capture and use nutrients. Despite this, however, there were no significant relationships between leaf terpene accumulation and leaf economic spectrum in either native or alien species.

"Nutrient Driving", "Excess Carbon" and "EICA Related" Hypotheses The comparison of alien and native species did not support the "excess carbon" hypotheses (Loomis 1932; Bryant et al. 1983; Herms and Mattson 1992; Peñuelas and Estiarte 1998). Alien species had higher N leaf contents but did not have lower terpene concentrations. Decreases of terpene production have been reported when leaf nutrient concentrations increase (Son et al. 1998). Kainulainen et al. (1996) observed that N fertilization had no effect on monoterpene concentrations in growing needles of *Pinus sylvestris*, but in mature needles, N fertilization significantly decreased concentrations of some individual and total monoterpenes. There also are reports of no relationships at

Table 2 Foliar contents of aromatic, cyclic, and non-cyclic monoterpenes (μg g^{-1}) detected in the studied native and alien species in Oahu (Hawai'i). The species lacking detectable amounts of aromatic and cyclic monoterpenes are shown in Appendix 1. A = alien; N = native

Species	Origin													
Ageratina adenophora	A	*p*-Cymene 340 ± 60	α-Pinene 27.3 ± 11.2	Camphene 707 + 105	β-Pinene 14 ± 12	Δ^3-Carene 18.8 ± 5.9	Limonene 91 ± 12	Thymoquinone 331 ± 105	Bornyl acetate 1060 ± 172	α-Terpinene 485 ± 93	Endo-borneol 30.6 ± 3.7	E-α-Ocimene 20 ± 7	E-β-Ocimene 11 ± 2	Linalool 21 ± 6
Cheirodendron trigynum	N	α-Thujene 6.3 ± 3.1	α-Phellandrene 130 ± 70	α-Pinene 3500 ± 1100	Sabinene 66 + 28	Camphene 30 ± 13	β-Pinene 673 ± 533	β-Terpinene 165 ± 67	α-Terpinolene 13 ± 5	α-Campholene aldehyde 36 ± 20	α-Terpinene 5 ± 2	Myrcene 163 ± 66		
Cinnamomum burmannii	A	*p*-Cymene 220 ± 100	Camphene 67 ± 21	β-Pinene 910 ± 260	Δ^3-Carene 117.0 + 3.0	Thymoquinone 45 ± 9	E-α-Ocimene 49.1 ± 17	1,8-Cineole 261 ± 72	γ-Terpinene 3.7 ± 2.6	α-Terpinolene 8 ± 2	Terpinen-4-ol 6 ± 2	α-Terpineol 79 ± 22	Terpinolene 133 ± 39	Myrcene 72 ± 24
		E-α-Ocimene 3.6 ± 1.7	L-3,7-dimethyl-1,3,7-Octatriene 21 ± 7	n. id. monoterpene 10 ± 5										
Eucalyptus robusta	A	*p*-Cymene 77 ± 11	α-Pinene 330 ± 150	α-Fenchene 22 ± 5.67	Camphene 14 ± 11.8	Phellandrene 79 ± 24	Limonene 33 ± 10	β-Phellandrene 5.7 ± 1.4	α-Campholene aldehyde 6 ± 1	E-Pinocarveol 278 ± 36	3-Cyclohexen-1-ol. 4-methyl-1-(1-methylethyl)- 7 ± 2	*p*-Mentha-1.5.8-triene 13 ± 2	α-Terpineol 41 ± 17	Z-*p*-Mentha-1(7),8-dien-2-ol 6 ± 6
		Bornyl formate 15 ± 2	Endo-fenchol 35 ± 7	Endo-borneol 74 ± 16	E-α-Ocimene 6 ± 1	E-Linalool Oxide 5.1 ± 1.6								
Heliocarpus americanus	A	Camphene 0.30 ± 0.22												
Lantana camara	A	α-Thujene 4.1 ± 0.4	α-Phellandrene 16 ± 3	α-Pinene 105 ± 10.7	Sabinene 90 ± 9	Camphene 43 ± 5.53	Δ^3-Carene 71.5 ± 7.8	Limonene 66 ± 14	l-Camphor 61 ± 9	α-Terpineol 27 ± 4	α-Terpinene 4.7 ± 1.9	Terpinolene 14 ± 4	E-α-Ocimene 18 ± 4	Linalool 31 ± 7
Mangifera indica	A	α-Pinene 10.3 ± 0.7	f-Phellandrene 5 ± 0	β-Phellandrene 30 ± 1	E-α-Ocimene 1.3 ± 0.5	E-β-Ocimene 3.4 ± 1.3								
Melaleuca quinquenervia	A	α-Thujene 6.1 ± 1.5	α-Pinene 746 ± 155	Camphene 12 ± 3.85	β-Pinene 188 ± 22	α-Terpinene 79 ± 15	1,8-Cineole 5963 ± 605	γ-Terpinene 85 ± 17	α-Terpinolene 9 ± 4	Isopulegol 37 ± 1	3-Cyclohexen-1-ol. 4-methyl-1-(1-methylethyl)- 15 ± 2	α-Terpineol 2155 ± 413	- exo-2-Hydroxycineole 17 ± 3	Myrcene 32 ± 22
		E-α-Ocimene 11 ± 8	Linalool 82 ± 25											
Melicope clusiifolia	N	*p*-Cymene 9 ± 5	α-Pinene 3661 ± 123	Camphene 15 ± 8.43	β-Pinene 57 ± 33	Limonene 31 ± 15	Z Linalool oxide 33 ± 19	α-Campholene aldehyde 98 ± 57	E-Pinocarveol 18 ± 11	E-Verbenol 52 ± 30	Myrtenol 7 ± 3	Verbenone 14 ± 8	Bornyl acetate 7 ± 3	Endo-borneol 6 ± 1
		Myrcene 22 ± 4	E-β-Ocimene 4.8 ± 0.5	Linalool oxide 56 ± 26	Linalool 18 ± 8	Geranyl acetate 27 ± 15								
Melicope peduncularis	N	α-Pinene 13 ± 6	(1R)-E-Isolimonene 13 ± 2	Limonene 149 ± 113	β-Terpinene 39 ± 17	*p*-Mentha-1.5.8-triene 6 ± 3	E-Carvyl acetate 8 ± 6	E-α-Ocimene 1.6 ± 0.2	p-Mentha-E-2,8-dien-1-ol 9 ± 7					

Metrosideros macropus	N	Sabinene 13 ± 5												
Metrosideros polymorpha	N	E-α-Ocimene 19 + 5	E-β-Ocimene 56 ± 17											
Myrsine sandwicensis	N	Limonene 1 ± 1	Myrcene 1.8 ± 1.0											
Persea americana	A	α-Phellandrene 6.4 ± 0.2	α-Pinene 113 ± 51	Sabinene 278 ± 27	β-Pinene 137 ± 76	1,8-Cineole 77 ± 9	E Sabinene hydrate 2.3 ± 0.2	Myrcene 11 ± 3						
Pimenta dioica	A	*p*-Cymene 0.13 ± 0.11	Eugenol 4177 ± 1950	α-Thujene 9.9 ± 8.1	α-Phellandrene 36 ± 3	α-Pinene 26 ± 15	β-Pinene 1.2 ± 1.0	Δ^3-Carene 3.13 ± 2.6	1,8-Cineole 133 ± 77	γ-Terpinene 15 ± 12	α-Terpinolene 64 ± 52	Terpinen-4-ol 0.17 ± 0.14	α-Terpineol 6 ± 5	Terpinolene 30 ± 25
		Myrcene 9 ± 8												
Pluchea carolinensis	A	α-Pinene 4 ± 68												
Psidium cattleianum	A	α-Pinene 26 ± 21												
Schinus terebinthifolius	A	α-Thujene 75 ± 9	α-Phellandrene 730 ± 73	α-Pinene 1115 ± 141	Sabinene 406 ± 49	β-Thujene 2544 ± 286	α-Terpinene 84 ± 21	Myrcene 53 ± 4t	E-β-Ocimene 17 ± 2					
Syzygium cumini	A	α-Phellandrene 2.6 ± 0.3	α-Pinene 110 ± 20	α-Fenchene 1.6 ± 0.65	β-Pinene 65 ± 36	l-Phellandrene 27 ± 3	Limonene 167 ± 17	α-Terpinolene 5.0 ± 1.9	α-Terpineol 6 ± 2	α-Terpinene 107 ± 10	Terpinolene 0.2 ± 0.1	Myrcene 17 + 2	E-α-Ocimene 1.5 ± 0.5	
Syzygium sandwicensis	N	Camphene 12 ± 1	E-β-Ocimene 523 ± 120	L-3,7-dimethyl-1,3,7-Octatriene 68 ± 6										

Table 3 Foliar cyclic sesquiterpene contents (μg g^{-1}) in native and alien species in Oahu (Hawai'i). Species without detectable sesquiterpene pools are listed in Appendix 1. A = alien; N = native

Species	Origin														
Ageratina adenophora	A	Sabinyl acetate 67.9 ± 7.0	E-α-Bergamotene 10 ± 1	β-Caryophyllene 501 ± 91	β-Funebrene 130 ± 21	α-Bergamotene 410 ± 60	α-Longipinene 14.3 ± 6.4	Germacrene-D 448 ± 366	α-Zingiberene 78.4 ± 14.2	Bicyclo-germacrene 330 ± 56	β-Bisabolene 612 ± 118	β-Sesqui phellandrene 284 ± 57	Cis-α-Bisabolene 176 ± 49		
		Cadina-1,4-diene 15 ± 6	γ-Curcumene 1793 ± 232	α-Bisabolol 1484 ± 216	E-Caryophyllene 17 ± 3	E-β-Farnesene 473 ± 105	σ-Nerolidol 71 ± 9	Nerolidol 124 ± 36	1(5),6-Guaiadiene 2385 ± 341	Calarene 119 ± 22					
Cheirodendron trigynum	N	α-Copaene 10 ± 2	β-Caryophyllene 7 ± 2	α-Humulene 187 ± 146	Germacrene-D 491 ± 220	α-Zingiberene 230 ± 168	Eremophilene 116 ± 54	β-Sesqui phellandrene 39 ± 21	Germacrene B 84 ± 24	Cycloisolongifol-5-OL (sesqui) 4.6 ± 0.6	Dehydro-aromadendrene 7 ± 2	(3E,5E,8Z)-3,7,11-Trimethyl-1,3,5,8,10-dodecapentanene 3.8 ± 1.2			
Cinnamomum burmannii	A	β-Caryophyllene 431 ± 112	Germacrene-D 526 ± 123	Bicyclo-germacrene - 626 ± 114	E–α-Bisabolene 63 ± 16	γ–Elemene 36 ± 11	Spathulenol 143 ± 68	Caryophyllene oxide 367 ± 17	Guaiol 152 ± 49	Isospathulenol 8 ± 2	α-Eudesmol 18 ± 6				
Eucalyptus robusta	A	Alloaroma-dendrene 13 ± 5	Globulol 1.6 ± 0.3												
Heliocarpus americanus	A	α-Cubebene 6 ± 1	β-Elemene 3.2 ± 2.0	Cis-α-Bisabolene 0.6 ± 0.5	β-Selinene 36 ± 7	α-Selinene 31 ± 6	Caryophyllene oxide 25 ± 3	γ-Gurjunene 21 ± 4							
Lantana camara	A	α-Copaene 11 ± 3	β-Caryophyllene 773 ± 106	α-Humulene 645 ± 79	Germacrene-D 644 ± 107	α-Zingiberene 43 ± 5	β-Cubebene 43 ± 7	Bicyclo-germacrene 680 ± 105	Germacrene A 86 ± 17	α-Gurjunene 115 ±17	E–α-Bisabolene 5 ± 2				
Mangifera indica	A	β-Caryophyllene 24 ± 1	α-Humulene 12.9 ± 0.3	β-Cubeben 7 ± 0.3	α-Gurjunene 18 ± 1	Germacrene B 140 ± 21	γ-Curcumene 26 ± 14								
Melaleuca quinquenervia	A	α-Copaene 49 ± 6	α-Gurjunene 57 ± 4	β-Caryophyllene 276 ± 9	Alloaromadendrene 43 ± 19	Aromadendrene 224 ± 24	α-Humulene 56 ± 0.3	Germacrene-D 2.1 ± 0.9	β-Selinene 138 ± 35	Dehydro aromadendrene 38 ± 0.5	α-Selinene 152 ± 25	α-Amorphene 139 ± 22	Δ-Cadinene 120 ± 15		
		Globulol 70 ± 12	Veridiflorol 3811 ± 714	Ledol 634 ± 141	α-Copaene 89 ± 12	γ-Eudesmol 99 ± 22	α-Eudesmol 336 ± 68	β-Eudesmol 101 ± 64	α-Amorphene 43 ± 3	Palustrol 92 ± 14	Caryophyllene oxide 197 ± 35				
Melicope clusiifolia	N	Z-Caryophyllene 52 ± 1	Aromadendrene 492 ± 230	α-Humulene 1442 ± 50	Aristolene 26± 13	β-Selinene 89 ± 20	γ-Cadinene 221 ± 113	Δ-Cadinene 376 ± 199	Epiglobulol 47 ± 27	β-Caryophyllene 1316 ± 77	Spathul enol 48 ± 1	Caryophyllene oxide 792 ± 234	Isoledene 28 ± 9		
		β-Eudesmol 52 ± 19	Globulol 188 ± 58	Calarene 18 ± 1	α-Eudesmol 145 ± 21										
Melicope peduncularis	N	α-Copaene 16 ± 7	β-Guaiene 9 ± 6	α-Humulene 142 ± 25	Eremophilene 10 ± 3	β-Caryophyllene 10 ± 4	Germacrene B 28 ± 8	Spathulenol 52 ± 27	Caryophyl lene oxide 70 ± 43	Guaiol 1082 ± 209	E-Caryoph yllene 6 ± 5	γ-Eudesmol 173 ± 37	Calarene 135 ± 25		
Metrosideros macropus	N	α-Humulene 42 ± 14	E-Caryophyllene 450 ± 167	α-Eudesmol 319 ± 31	β-Eudesmol 252 ± 60	Bulnesol 186 + 78									
Metrosideros polymorpha	N	α-Copaene 63 ± 51	β-Caryophyllene 1022 ± 33	β-Elemene 26 ± 5	α-Humulene 145 ± 53	2-Isopropyl-5-Bicyclo[4.4.0]Dec-1-en 26 ± 11	γ-Selinene 41 ± 7	Germacrene-D 25 ± 20	β-Selinene 665 ± 156	α-Selinene 783 ± 181	α-Gurjunene 8 ± 4	γ-Cadinene 10.2 ± 5.7	Δ-Cadinene 128 ± 70	α-Muurolene 2.3 ± 1.9	Caryophyllene oxide 9 ± 3
		Epi-Bicyclosesqui phellandrene 13± 9	γ-Gurjunene 27 ± 15	Junipene 3.3 ± 1.7											
Metrosideros rugosa	N	α-Cubebene 107 ± 50	α-Ylangene 33 ± 14	α-Copaene 324 ± 144	β-Caryophyllene 349 ± 140	β-Cubebene 379 ± 159	Aromadendrene 24 ± 10	α-Humulene 115 ± 46	2-Isopropyl-5-Bicyclo[4.4.0]Dec-1-en 139 ± 58	Germacrene-D 681 ± 317	α-Amorphene 127 ± 61	α-Gurjunene 76 ± 33	Eremophilene 142 + 90	γ-Cadinene 369 ± 159	
		α-Muurolene 50 ± 22	Germacrene B 433 ± 224	Ledol - 27 + 12	Δ-Selinene 108 ± 10	T-Muurolol 61 ± 49	(3S, 4R, 5S,6R,7S)-Aristol-9-en-3-ol 8 ± 3	α-Cadinol 166 ± 71	Cadina-1,4-diene 135 ± 65	γ-Gurjunene 6 ± 4	Δ-Cadinene 663 ± 303				

Metrosideros tremuloides	N	α-Humulene 4.9 ± 2.4	Germacrene-D 192 ± 119	Bicyclo-germacrene 9 ± 16	E-Caryophyllene 15 ± 7											
Myrsine lessertiana	N	α-Copaene 48 ± 16	(3Z)-Cembrene A 35 ± 12	β-Caryophyllene 1091 ± 593	α-Bergamotene 8 ± 2	Alloaromadendrene 1.1 ± 0.1	Aromadendrene 12 ± 3	α-Humulene 294 ± 15	2-Isopropyl-5-Bicyclo[4.4.0]Dec-1-en 8 ± 2	γ-Selinene 199 ± 46	Aristolene 20 ± 6	β-Selinene 686 ± 214	β-Cubebene 244 ± 169	α-Selinene 825 ± 266		
		γ-Muurolene 84 ± 22	Δ-Cadinene 163 ± 47	Spathulenol 83 ± 38	Caryophyllene oxide 87 ± 33	T-Muurolol 15 ± 5	γ-Cadinene 40 ± 9									
Myrsine sandwicensis	N	α-Cubebene 77 ± 19	α-Ylangene 34 ± 11	α-Copaene 298 ± 54	β-Caryophyllene 669 ± 160	Alloaromadendrene 11 ± 5	α-Humulene 108 ± 20	Germacrene-D 570 ± 147	β-Cubebene 41 ± 10	Bicyclo-germacrene 379 ± 166	α-Amorphene 388 ± 159	α-Gurjunene 9 ± 3	Δ-Cadinene 547 ± 158	α-Muurolene 3.6 ± 2.1	Spathulenol 11 ± 3	
		Caryophyllene oxide 19 ± 2	Isoledene 11 ± 2	α-Cadinol 87 ± 20	Calarene 51 ± 8	Cadina-1,4-diene 30 ± 4	γ-Gurjunene 2.9 ± 0.4	T-Muurolol 98 ± 52	Junipene 210 ± 114							
Persea americana	A	Germacrene-D 122 ± 27	β-Cubebene 58 ± 9	Δ-Cadinene 27 ± 12												
Pimenta dioica	A	α-Ylangene 1.6 ± 0.6	β-Caryophyllene 399 ± 163	β-Elemene 4.9 ± 3.9	Aromadendrene 24 ± 19	α-Humulene 93 ± 39	Δ-Cadinene 47 ± 19	Caryophyllene oxide 31 ± 13	Calarene 9 ± 4	γ-Gurjunene 3.6 + 1.5	T-Muurolol 4.2 ± 2.5					
Pluchea carolinensis	A	γ-Gurjunene 77 ± 55	α-Selinene 41 ± 30	α-Gurjunene 19 ± 9												
Psidium cattleianum	A	α-Cubebene 30 ± 10	α-Ylangene 43 ± 3	α-Copaene 385 ± 37	β-Maaliene 23 ± 3	β-Caryophyllene 3132 ± 331	Alloaromadendrene 39 ± 32	Aromadendrene 55.8 ± 7.5	α-Humulene 651 ± 47	Epizonare 153 ± 15	Germacrene-D 57 ± 8	β-Selinene 122 ± 15	α-Selinene 160 ± 23	α-Amorphene 106 ± 8		
		γ-Cadinene 165 ± 33	Δ-Cadinene 355 ± 29	E-γ-Bisabolene 79 ± 7	α-Muurolene 57 ± 5	Germacrene B 353 ± 99	Caryophyllene oxide 73 ± 7	Veridiflorol 37 ± 6	Valencene 197 ± 39	6,10,11,11-Tetramethyl-tricyclo[5-3-0-1(2,3)undec-1(7)ene 48 ± 4	E-β-Farnesene 27 ± 8	Calarene 21 ± 3	γ-Curcumene 116 ± 36	γ-Gurjunene 41 ± 7	α-Bisabolol 43 ± 18	Juniper camphor 67 ± 6
Psidium guajava	A	α-Ylangene 18 ± 7	α-Copaene 21 ± 17	β-Maaliene 37 ± 12	β-Caryophyllene 454 ± 129	Alloaromadendrene 4.7 ± 3.0	Aromadendrene 132 ± 50	α-Humulene 48 ± 15	Germacrene-D 12 ± 4	β-Selinene 46 ± 27	α-Selinene 110 ± 51	α-Amorphene 330 ± 104	γ-Cadinene 124 ± 55	Δ-Cadinene 242 ± 121	E-γ-Bisabolene 211 ± 85	
		α-Muurolene 36 ± 11	Caryophyllene oxide 34 ± 5	Veridiflorol 0.5 ± 0.4	Valencene 0.6 ± 0.5	6,10,11,11-Tetramethyl-tricyclo[5-3-0-1(2,3)undec-1(7)ene 28 ± 10	Calarene 16 ± 5	γ-Curcumene 20 ± 3	α-Bisabolol 135 ± 49	E-β-Farnesene 36 ± 16	Germacrene B 12 ± 6					
Rubus rosifolius	A	α-Cubebene 50 ± 23	α-Ylangene 69 ± 31	α-Copaene 164 ± 74	Germacrene-D 29 ± 13	β-Cubebene 78 ± 34	α-Amorphene 839 ± 381	γ-Cadinene 28 ± 13	Δ-Cadinene 194 ± 86	β-Copaen-4 α-ol 90.4 ± 39.3	6,10,11,11-Tetramethyl-tricyclo[5-3-0-1(2,3)undec-1(7)ene 499 ± 218	15-Copaenol 46 ± 21	T-Muurolol 255 ± 109	caryophylla-3,8(13)-dien-5β-ol 9 ± 5		
Schinus terebinthifolius	A	α-Copaene 32 ± 3	β-Caryophyllene 231 ± 77	β-Elemene 92 ± 15	α-Humulene 38 ± 9	Germacrene-D 4710 ± 823	Bicyclo-germacrene 2095 ± 521	Δ-Elemene 202 ± 43	Germacrene B 302 ± 72	E-Caryophyllene 30 ± 13						
Syzygium cumini	A	α-Copaene 6 ± 2	β-Elemene 89 ± 28	α-Guaiene 56 ± 9	α-Humulene 196 ± 23	Germacrene-D 226 ± 23	Aristolene 46± 3	Germacrene A 5700 ± 533	γ-Cadinene 41.9 ± 3.0	Δ-Elemene 56 ± 12	α-Muurolene 41 ± 7	Germacrene B 67 ± 6	Valencene 0.2 ± 0.2	6,10,11,11-Tetramethyl-tricyclo[5-3-0-1(2,3)undec-1(7)ene 139 ± 16	Isoledene 272 ± 53	
		Calarene 32 ± 4	Globulol 42 ± 18	E-Caryophyllene 423 ± 53												
Syzygium sandwicensis	N	α-Cubebene 14 ± 1	α-Copaene 0.30 ± 0.01	Aromadendrene 26.5 + 0.2	α-Humulene 21 + 0.4	β-Cubebene 21 ± 0.3	α-Gurjunene 16 ± 0.3	Δ-Guaiene 2.8 ± 0.4	Ledol 15 ± 0.3	Isoledene 23 ± 1	Cadina-1,4-diene 12 ± 2	E-Caryophyllene 31 ± 1				

Table 4 Mean values (SE) of the concentrations of the most abundant terpenes and their ratios to key nutrient contents and photosynthetic capacity per dry mass (A_{mass}) in relation to sampling site, species origin (native or alien) and soil type. *P*-values indicate the results of general linear models. OLS-Ordinary least squares regression (see "Methods")

Trait	Model	Site[1]					Origin			Soil[2]					
		Ta	Wi	HV	SLH	*P-value*	Native	Alien	*P-value*	Inc	Oxi	Ult	Inc_T	Moll	*P-value*
β-Caryophyllene μg g^{-1}	OLS	45.5 (30.6)	84.6 (51.3)	351 (201)	61.4 (42.0)	0.60	89.7 (46.0)	164 (86)	0.72	0 (0)	221 (48)	47.8 (32.1)	69.0 (69.0)	49.9 (49.9)	0.94
α-Humulene μg g^{-1}	OLS	0.85 (0.65)	102 (58)	95.5 (54.8)	135 (9)	0.82	71.4 (41.9)	45.8 (24.1)	0.67	39.6 (32.0)	110 (48)	0.65 (0.65)	15.2 (13.6)	11.7 (11.7)	0.86
Caryophyllene oxide μg g^{-1}	OLS	1.7 (1.7)	38.7 (31.7)	8.8 (5.0)	20.7 (17.8)	0.46	27.9 (22.7)	10.5 (5.6)	0.96	0 (0)	21.7 (22.7)	1.8 (1.8)	49.2 (49.2)	39 (39)	0.58
Myrcene μg g^{-1}	OLS	3.94 (3.42)	8.1 (6.5)	3.3 (3.3)	3.8 (2.0)	0.51	5.3 (4.7)	5.1 (2.4)	0.49	2.8 (2.8)	6.9 (4.9)	4.1 (3.6)	8.1 (8.1)	1.2 (1.2)	0.78
p-Cymene μg g^{-1}	OLS	26.6 (18.7)	0.36 (0.36)	0 (0)	7.0 (7.0)	0.53	0.3 (0.3)	16.7 (10.6)	0.063	0 (0)	0.26 (0.26)	28.0 (19.7)	19.2 (19.2)	0.02 (0.02)	0.78
α-Phellandrene μg g^{-1}	OLS	43.6 (43.6)	5.3 (5.2)	46.6 (45.5)	3.3 (3.3)	0.94	3.7 (3.7)	44.8 (30.2)	0.18	0.4 (0.4)	25.0 (21.9)	45.8 (45.8)	0 (0)	4.5 (4.5)	0.97
α-Pinene μg g^{-1}	OLS	12.8 (7.6)	291 (198)	83.1 (69.2)	101 (71)	0.41	164 (142)	74.0 (35.4)	0.97	18.3 (18.3)	242 (144)	13.4 (7.9)	270 (177)	3.2 (3.2)	0.54
Camphene μg g^{-1}	OLS	33.7 (33.7)	2.3 (1.4)	2.7 (2.7)	2.4 (1.6)	0.72	1.6 (1.0)	20.4 (18.6)	0.18	0 (0)	2.9 (1.5)	35.4 (35.4)	6.5 (3.8)	0 (0)	0.96
β-Pinene μg g^{-1}	OLS	7.2 (6.5)	31.8 (26.9)	0 (0)	17.2 (17.1)	0.36	20.8 (11.2)	10.7 (6.2)	0.80	10.8 (10.8)	20.8 (19.2)	7.1 (6.8)	46.9 (46.9)	0.15 (0.15)	0.63
Total monoterpene μg g^{-1}	OLS	247 (161)	291 (197)	360 (313)	1360 (904)	0.14	**195** (101)	**693** (304)	**0.044**	87.1 (84.1)	358 (197)	269 (169)	2614 (2287)	558 (558)	0.24
Total sesquiterpene μg g^{-1}	OLS	545 (454)	1150 (391)	1433 (628)	676 (313)	0.75	635 (202)	1272 (297)	0.08	1321 (1226)	1250 (346)	560 (478)	1748 (1664)	84.8 (84.8)	0.56
Total terpene μg g^{-1}	OLS	792 (660)	1141 (465)	1793 (875)	2034 (1488)	0.42	**830** (227)	**1965** (367)	**0.039**	1408 (1310)	1607 (470)	819 (640)	4370 (3942)	(642 (642)	0.12
Total monoterpene/N μg Terp mg^{-1} N	OLS	**10** (5.8)	**18.7** (13)	**17.4** (15.4)	**118** (83)	**0.047**	**12.8** (9.3)	**47.4** (13.3)	**0.01**	**4.5** (43)	**20.6** (11.5)	**10.3** (6.0)	**243** (216)	**41** (41)	**0.029**
Total monoterpene/P μg Terp mg^{-1} P	OLS	**180** (120)	**328** (219)	**223** (201)	**3275** (2347)	**0.042**	222 (158)	1153 (700)	0.22	**93.1** (87.6)	**321** (179)	**188** (107)	**6527** (160)	**1239** (1239)	**0.0034**
Total sesquiterpene/N μg Terp mg^{-1} N	OLS	19.3 (14.6)	81.6 (28.0)	80.6 (34.0)	62.3 (57.3)	0.46	52.8 (19.5)	67.7 (23.8)	0.40	71.2 (61.7)	82.9 (23.1)	19.4 (15.3)	163.5 (156.9)	622 (6.2)	0.14
Total sesquiterpene/P μg Terp mg^{-1} P	OLS	293 (199)	1637 (545)	1449 (505)	1759 (1613)	0.14	961 (361)	1346 (540)	0.36	1469 (1263)	1443 (404)	298 (210)	4509 (4440)	188 (188)	0.22

Total terpene/N μg Terp mg^{-1} N	OLS	29 (20)	100 (32)	98 (45)	180 (138)	0.13	**64** (19)	**115** (29)	**0.029**	75.7 (65.9)	104 (28.5)	29.6 (20.9)	406 (372)	47.1 (47.1)	0.1
Total terpene/P μg Terp mg^{-1} P	OLS	472 (289)	1966 (603)	1372 (633)	5032 (3913)	0.053	1183 (409)	2499 (1180)	0.19	**1562** (1352)	**1763** (470)	**486** (302)	**11030** (10590)	**1427** (1427)	**0.032**
Total monoterpene/A_{mass} [3]	OLS	2189 (1300)	4426 (2930)	5539 (5233)	20343 (15569)	0.063	3505 (2376)	8986 (4742)	0.63	**631** (557)	**5589** (3062)	**2345** (1386)	**40686** (29246)	0 (0)	**0.0019**
Total sesquiterpene/A_{mass} [3]	OLS	3341 (2068)	27170 (13888)	16171 (8866)	11127 (11053)	0.61	21841 (11357)	11718 (4779)	0.95	10768 (8137)	25777 (11265)	3412 (2201)	22840 (21841)	0 (0)	0.69
Total terpene/A_{mass} [3]	OLS	5529 (3218)	31596 (14277)	21709 (13581)	31456 (26305)	0.35	25345 (11719)	20700 (9031)	0.84	11399 (8684)	31366 (12199)	5757 (3448)	63497 (50450)	0 (0)	0.19

[1] Ta - Tantalus, Wi - Wiliwilinui, HV – Hahaione Valley, SLH – Saint Louis Heights (Table 1)

[2] Inc –Inceptisols (mountainous soils), Oxi - Oxisols, Ult - Ultisols, Inc_T – Inceptisols (Tantalus), Moll - Mollisols *Significant differences (P< 0.05) are highlighted in bold*

[3] A_{mass} = μmols CO_2 g^{-1} soil s^{-1}

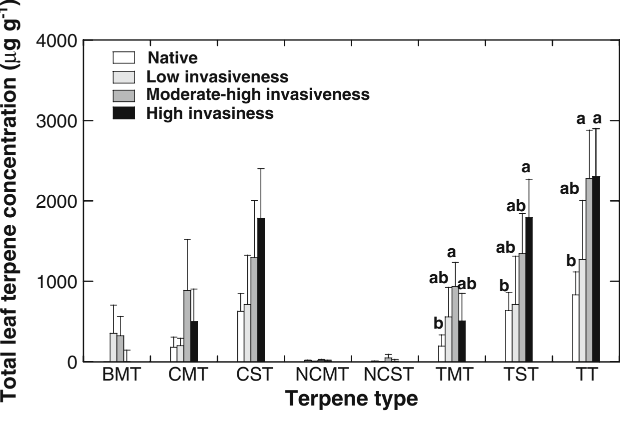

Fig. 2 Foliar benzenic monoterpene (BMT), cyclic monoterpene (CMT), cyclic sesquiterpene (CST), non-cyclic monoterpene (NCMT), non-cyclic-sesquiterpene (NCST), total monoterpene (TMT), total sesquiterpene (TST), and total terpene (TT) concentrations (μg g^{-1}) in native and alien species grouped according to their invasiveness index. Different letters indicate statistically significant differences at P<0.05 among species groups with differing invasiveness index

all between soil nutrients and terpene contents (Heyworth et al. 1998). Our results support the "nutrient driving synthesis hypothesis", which expects higher nutrient availability to translate into higher carbon fixation and activity of the enzymes involved in isoprenoid production (Harley et al. 1994; Litvak et al. 1996). Other studies have reported a significant and positive relationship between leaf terpene content and N availability in *Pinus halepensis* (Kainulainen et al. 2000), and NPK fertilization has shown to increase terpene contents in *Chrysanthemum boreale* (Lee et al. 2005). On the other hand, our results also support the modified EICA related hypothesis that suggests that alien success may be favored by an increase in the concentrations of less costly defenses such as terpenes that may be more toxic to generalist herbivores (Joshi and Vrieling 2005; Stastny et al. 2005), as has been observed in some previous studies (Johnson et al. 2007).

Terpene Content and Success of Aliens The results suggest that alien success may be related to higher levels of leaf terpene content that can have protective effects in response to environmental stress and/or prevent the attack of generalist herbivores and pathogens. The possible role of terpenes as cause of alien success due to herbivorism protection, however, should be taken with caution because the few studies that have examined herbivory pressure in Hawai'i are inconclusive. For example, a study by DeWalt et al. (2004) found little fungal and insect damage on one invader; however, Joe and Daehler (2008) found significant slug damage on several rare native species. Terpenes may produce advantages by other mechanisms, e.g., overall higher terpene production and accumulation in alien species could be involved in allelopathic or protective mechanisms

Fig. 3 Discriminant analysis using Leaf total terpenes (TT) vs. Photosynthetic capacity (A_{mass}), TT vs. Leaf mass per area (LMA), TT vs. leaf nitrogen concentration (N), TT vs. Leaf potassium concentration, TT and TT vs. PC1 first principal component factor scores of 'leaf economics spectrum' (covariation among leaf traits; LMA, A-mass, N, K) as independent continuous variables, and species origin: native (in black - bold) and alien (in red – light gray) as the dependent categorical factor. Only terpene containing species are considered in the discriminant analyses shown here. Similar results were found for the whole set of species studied including both terpene containing and non-containing species. (Ag = *Ageratina adenophara*, Cb = *Cinnamonium burmanii*, Ct = *Cheirodendrum trigynum*, Er = *Eucapyptus robusta*, He = *Heliocarpus americanus*, Lc = *Lantana camara*, Ma = *Metrosideros macropus*, Mc = *Melicope clusiifolia*, Md = *Myrsine lessertiana*, Me = *Metrosideros polimorpha*, Mi = *Magnifera indica*, Mp = *Melicope peduncularis*, Mq = *Melicope quinquenervia*, Mr = *Metrosideros rugosa*, Ms = *Myrsine sandwicensis*, Mt = *Metrosideros tremuloides*, Pc = *Pluchea carolinensis*, Pd = *Pimenta dioica*, Pe = *Persea Americana*, Pg = *Psidium guajava*, Pt = *Psidium cattleionum*, Ru = *Rubus rosifolius*, Sc = *Syzygium cumini*, St = *Schinnus terebinthifolius*, Sy = *Syzygium sandwisensis*)

that would confer competitive advantage with respect to native species, compensating for their greater nutritive value and palatability that results from their higher nutrient contents and lower LMA. Additionally, terpenes have other functions that can confer competitive advantage to aliens. For example, they have infochemical and communication roles (Peñuelas et al. 1995; Wheeler et al. 2002; Peñuelas and Llusià 2003, 2004), and confer photoprotection (Peñuelas and Munné-Bosch 2005) and thermotolerance (Sharkey and Singsaas 1995; Peñuelas and Llusià 2001 and 2002; Peñuelas et al. 2005). They also may confer protection against drought stress (Kainulainen et al. 1991; Llusià and Peñuelas 1998), and may act as general antioxidants, protecting vital membranes against peroxidation and reactive oxygen species such as singlet oxygen (Loreto and Velikova 2001; Peñuelas and Llusià 2002; Loreto et al. 2004; Munné-Bosch et al. 2004; Llusià et al. 2005).

In summary, total terpene contents were greater in alien than in native species. The frequency of leaf terpene-containing species, however, was not significantly greater in alien than in native species. The results also suggest that

the percentage of species that contain terpenes in leaves in Oahu is probably comparable with other floras. Alien species presented higher terpene contents, and also greater N and K leaf concentrations, and A_{mass}, but lower LMA than native species. These differences between alien and native species did not support the "exces carbon" and the "traditional EICA" hypothesis but were in accordance with the "nutrient driven synthesis" and with the "modified EICA related" hypotheses. The results suggest a possible different time-stage of adaptation to a novel Oahu habitat between native (old-alien-invaders) and recent alien plants. The different patterns in production and content of terpenes in native and alien species merit further investigation, given that plant invasive success is an emerging global phenomenon.

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Appendix 1. Species with not detected foliar terpene contents in this study

Acacia confusa, *Acacia koa*, *Senna surattensis*, *Clermontia oblongifolia*, *Desmodium incanum*, *Falcataria moluccana*, *Haematoxylum campechianum*, *Casuarina equisetifolia*, *Ficus macrophylla*, *Ficus microcarpa*, *Trema orientalis*, *Antidesma platyphyllum*, *Bischofia javanica*, *Ochna thomasiana*, *Passiflora suberosa*, *Elaeocarpus bifidus*, *Hibiscus arnottianus*, *Sida fallax*, *Wikstroemia oahuensis*, *Scaevola gaudichaudiana*, *Ilex anomala*, *Ilex paraguariensis*, *Alyxia stellata*, *Labordia tinifolia*, *Bobea elatior*, *Coffea arabica*, *Coprosma longifolia*, *Hedyotis acuminata*, *Hedyotis fosbergii*, *Hedyotis terminalis*, *Buddleja asiatica*, *Citharexylum caudatum*, *Clerodendrum macrostegium*, *Stachytarpheta cayennensis*, *Jasminum fluminense*, *Tabebuia rosea*, *Cestrum nocturnum*, *Carmona retusa*, *Ardisia elliptica*, *Diospyros sandwicensis*, *Pouteria sandwicensis*, *Vaccinium calycinum*, *Broussaisia arguta*, *Korthalsella complanata*, *Santalum freycinetianum*, *Pisonia umbellifera*, *Freycinetia arborea*, *Smilax melastomifolia*.

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Inactivation of Baculovirus by Isoflavonoids on Chickpea (*Cicer arietinum*) Leaf Surfaces Reduces the Efficacy of Nucleopolyhedrovirus Against *Helicoverpa armigera*

Philip C. Stevenson · Reju F. D'Cunha · David Grzywacz

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Abstract Biological pesticides based on nucleopolyhedroviruses (NPVs) can provide an effective and environmentally benign alternative to synthetic chemicals. On some crops, however, the efficacy and persistence of NPVs is known to be reduced by plant specific factors. The present study investigated the efficacy of *Helicoverpa armigera* NPV (*Hear*NPV) for control of *H. armigera* larvae, and showed that chickpea reduced the infectivity of virus occlusion bodies (OBs) exposed to the leaf surface of chickpea for at least 1 h. The degree of inactivation was greater on chickpea than that previously reported on cotton, and the mode of action is different from that of cotton. The effect was observed for larvae that consumed OBs on chickpea leaves, but it also occurred when OBs were removed after exposure to plants and inoculated onto artificial diet, indicating that inhibition was leaf surface-related and permanent. Despite their profuse exudation from trichomes on chickpea leaves and their low pH, organic acids—primarily oxalic and malic acid—caused no inhibition. When *Hear*NPV was incubated with biochanin A and sissotrin, however, two minor constituents of chickpea leaf extracts, OB activity was reduced significantly. These two isoflavonoids increased in concentration by up to 3 times within 1 h of spraying the virus suspension onto the plants and also when spraying only the carrier, indicating induction was in response to spraying and not a specific response to the *Hear*NPV. Although inactivation by the isoflavonoids did not account completely for the level of effect recorded on whole plants, this work constitutes evidence for a novel mechanism of NPV inactivation in legumes. Expanding the use of biological pesticides on legume crops will be dependent upon the development of suitable formulations for OBs to overcome plant secondary chemical effects.

Keywords Baculovirus · Biopesticide · Nucleopolyhedrovirus · *Helicoverpa armigera* · Chickpea · Induced resistance · Plant leaf chemistry · Isoflavonoid

Chickpea isoflavonoids inhibit *Hear*NPV

P. C. Stevenson · R. F. D'Cunha · D. Grzywacz (✉)
Natural Resources Institute, University of Greenwich,
Chatham, Kent ME4 4TB, UK
e-mail: D.Grzywacz@gre.ac.uk

P. C. Stevenson
Royal Botanic Gardens,
Kew, Surrey TW9 3AB, UK

Introduction

Helicoverpa armigera (Hubn.) is a major crop pest in Asia, Africa, and Australasia attacking a wide range of important crops including cotton, maize, tomato, peppers, chilies, and legumes such as chickpea and pigeonpea (King 1994; Gowda 2005). Its status as arguably the world's most important agricultural pest can be attributed to its wide geographical and host range coupled with its ability to develop high levels of resistance to chemical insecticides (Armes et al. 1992b; Kranthi et al. 2002). The baculovirus biopesticide *Helicoverpa armigera* nucleopolyhedrovirus (*Hear*NPV) is an ecologically benign alternative to chemical insecticides that is effective and can overcome problems of chemical insecticide resistance (Moscardi 1999; Grzywacz et al. 2005). *Hear*NPV is now commercially produced in Australia, Thailand, India, and China for control of *H. armigera* (Singhal 2004; Buerger et al. 2007, Sun and Peng 2007). However, the utility of baculoviruses for insect pest management is compromised by the fact that

some host plants adversely influence the severity of viral disease in insects and so reduce pest control efficacy (Felton and Duffey 1990; Duffey et al. 1995; Hoover et al. 1998a; Cory and Hoover 2006). It has for some time been recognized that *Heliothis zea* NPV, a closely related baculovirus, performed poorly on some crops such as cotton (Young and Yearian 1974; Forschler et al. 1992), a phenomenon linked to the direct action of glandular secretions in reducing the persistence of occlusion bodies (OBs) the infective stage of the virus (Young and Yearian 1977; Ellerman and Entwistle 1985). OBs are a protective crystalline protein matrix in which virions are embedded during transmission and in hostile environments (Hunter-Fujita et al. 1998). The maintenance of OB integrity is crucial to viral persistence outside the host and for initiating infections in new host insects. Host plant effects on biological pesticides are not restricted to baculoviruses, as plants such as cotton have been shown to reduce the efficacy of other biopesticides, especially *Bacillus thuringiensis* (Kushner and Harvey 1962; Johnson 1982; Ali et al. 2004). Inhibition of NPV infections on cotton also has been attributed to high peroxidase activity and subsequent free radical generation, which was associated with an increase in the sloughing off of midgut cells that are the point of entry for the NPV virions, thereby reducing virus-induced mortality (Hoover et al. 1998a, b, 2000). While the use of *Hear*NPV has been shown to be effective on chickpea (Jayaraj et al. 1987; Rabindra et al. 1992; Cherry et al. 2000), field trials have indicated OB persistence and activity to be much lower on chickpea leaf surfaces than on other crops such as tomato (Rabindra et al. 1994), suggestive of some degree of adverse interaction on chickpea. Chickpea produces copious glandular secretions rich in organic acids, and the leaf surface can subsequently have a low pH (<3) (Rembold and Weigner 1990; Stevenson and Aslam 2006). This could make it a challenging host plant for biopesticide use, as earlier work on *Lymantria dispar* NPV has shown that larvae can be less susceptible to OBs when inoculated on highly acidic (pH 3.8–4.6) oak foliage rather than other less acidic aspen foliage (Keating and Yendol 1987) an effect associated with low pH and high levels of organic acids (Keating et al. 1989).

The present study was undertaken to investigate the efficacy of *Hear*NPV on chickpea in comparison with tomato, a known favorable host (Forschler et al. 1992; Farrar and Ridgway 2000), and cotton, a host plant known to impair OB infectivity. We hoped to better understand what plant factors affect virus efficacy with a view to developing better recommendations for the efficacy of NPV-based insecticides on legume crops and to assist in the development of a suitable formulation for OBs for use on crops such as chickpea.

Methods and Materials

Virus The virus strain (NRI#0210) was provided by Professor R.J. Rabindra of Tamil Nadu Agricultural University, India, and stored at −80°C. This strain is typical in activity of strains of *Hear*NPV used in biopesticide products in India, having a mean LC_{50} of 2.78×10^3 OB ml^{-1} for neonate larvae. This is similar to that reported by others including Somasekar et al. (1993) and that had been used previously in field trials on chickpea in India (Cherry et al. 2000). It was multiplied up in third instars of *H. armigera*, then harvested and purified by using a standard NPV purification protocol (Hunter-Fujita et al. 1998). The virus was enumerated by using a standard Neubauer haemocytometer and phase contrast microscope at X400 magnification (Wigley 1980). The identity of the source and progeny of the virus was checked by using a standard DNA restriction analysis protocol for NPVs with EcoR1 (Hunter-Fujita et al. 1998).

Insects Insects for the bioassays were derived from a culture of *H. armigera* provided by the NERC Centre for Ecology and Hydrology at Oxford that had been maintained there for a number of years. The insects were reared at 26±2°C with a relative humidity of 50±5% and a 14:10 hL:D regime. Larvae were reared in groups in 250 ml plastic pots on an artificial wheatgerm casein diet until the second instar, and then individually in 30 ml plastic pots on wheatgerm diet using a method previously described (Armes et al. 1992a).

Plants Plants used were cotton (*Gossypium hirsutum*,) variety Ankur 651 (Ankur Seeds Ltd. Nagpur, India), chickpea (*Cicer arietinum*) variety ICC 11322 provided by ICRISAT, Hyderabad, India, and tomato (*Lycopersicon esculentum*) 'Moneymaker' variety. All were grown in plastic pots on John Innes no. 2 potting compost at 28±2°C in a glasshouse with a 14:10 hL:D cycle and a relative humidity of 60%. Plants were 5 wk old. The surface area of leaves was measured with a Quantimet 520-image analyzer (Leica Microsystems Cambridge Ltd., UK). Thus, the concentration of different compounds in a sample could be equated to an area of leaf surface to ensure that insects were presented with naturally occurring concentrations during feeding bioassays. These data together with the chemical analysis were used to calculate chemical concentration of leaf extracts in terms of unit area so that surface contamination bioassays could be calibrated to match concentrations found on leaf surfaces.

Viral Bioassays To assess OB activity, both leaf dip and surface contamination neonate larval bioassays were used under standard larval rearing conditions, 26°C with

a 14/10 hL:D cycle. In the leaf dip assays, a standard methodology was used (Evans and Shapiro 1997). The *Hear*NPV stock suspensions were prepared as fivefold dilution series in 50 ml of 0.02% Triton X-100 immediately prior to use in bioassays. Leaves were cut from the plant at the stem and dipped in the *Hear*NPV dilutions. Control leaves were dipped in 0.02% Triton X-100 only. After dipping, the stem of the treated leaves was mounted in molten agar in 250 ml round plastic containers, either one cotton leaf, two tomato leaves, or six compound chickpea leaves were used per container; fifty neonate larvae less than 18 h old were used for each treatment with 25 being placed in each container. Larvae were allowed to feed on the leaves for 24 h, after which they were transferred to 25 ml individual pots and reared individually on clean artificial diet, the mortality was recorded after 5 and 7 d. To ascertain OB activity separately from leaf surfaces OB treatments the mass surface contamination bioassay was employed (McKinley 1985; Jones 2000). Again, fivefold series dilutions of OBs in distilled water were prepared and then dispensed as 75 µl aliquots onto the surface of artificial diet in 30 ml plastic pots, spread evenly by tilting and left to dry. Two larvae were added to each pot, reared for 7 d under standard conditions, and mortality was counted on d 5 and 7. Fifty larvae were used for each treatment replicate. All assays were replicated 5–7 times with each assay including a control and a stock solution positive control. The results were subjected to probit analysis (Finney 1971) in SPSS. Comparisons of LC_{50} were performed on log transformed data, to equalize variances, using ANOVA procedure in SIGMASTAT software. Treatment means were compared by using the LSD test. In some bioassays where means differed by several orders of magnitude, transforming the data did not normalize variances, so the non-parametric Kruskal-Wallis test with Tukey multiple comparison procedure was adopted.

Effect of Exposure of HearNPV to Cotton, Tomato and Chickpea Leaf Surfaces To study plant surface chemistry and its effect on *Hear*NPV, OBs suspended in distilled water were applied to the leaf surfaces on whole plants at a concentration of 3×10^{7} OB ml^{-1} in 0.02% triton by using a hydraulic hand sprayer. They were applied at a rate sufficient to evenly wet the leaves. Plants used in experiments were, after application of OB, maintained in the laboratory at 26°C under the 14/10 hL:D cycle, and the virus was then left on the leaves for 1 or 24 h after which OBs were recovered by using a standard washing technique in water containing 0.1% sodium dodecycl sulphate for 1 h (Jones 1988). The samples and the OBs were concentrated by centrifugation at 2500 g at 5°C for 30 min (Hunter-Fujita et al. 1998). The supernatant was discarded, and the OBs were re-suspended in distilled water, then stored at −20°C prior to counting and bioassay. This procedure was found to have no significant effect on the LC_{50} of virus, and recovery of OBs from leaf surfaces was ascertained to be >95%; similar to that reported by other workers using this technique (McKinley 1985; Jones 1988).

Analysis of Organic Acids in Methanol Extract of Chickpea Leaf Surface by GC-MS The surfaces of 50 leaves were extracted in 300 ml methanol and analyzed by GC-MS. Purification of the organic acid fraction was carried out according to Stumpf and Burris (1979). The residue was resuspended in pyridine (50 µl) (Sigma-Aldrich) with a glutaric acid internal standard (1 mg ml^{-1}) (Sigma-Aldrich). Ten min before injection, 25 µl of *N, O*-bis (tri-methylsilyl)-acetamide (Supelco) were added; the vial was shaken and left to stand at room temperature for 5 min before injection. GC-MS was carried out on a Hewlett Packard HP6890 GC linked to an ion detector (HP 5973 Mass Selective Detector) operated in Electron Ionization (EI) mode. A fused silica capillary column (30 m×0.25 mm i.d., coating 0.25um) coated with non-polar HP-5MS (5% Phenyl Methyl Siloxane, Agilent 1909 IS-433) was used with a split/splitless injector with helium as carrier gas (0.5 kg cm^{-2}). The oven temperature was held at 60°C for 2 min and then raised to 250°C at 6°C per min. Compounds were identified by comparing EI-MS and GC retention indices with synthetic standards under the same operating conditions. A set of organic acid standards as reported to occur on chickpea leaf surfaces (Rembold and Weigner 1990) was prepared in sterile distilled water, derivatized and analyzed as described above.

Effect of Organic Acids Present on the Chickpea Leaf Surface on the Infectivity of OBs against H. armigera Neonates Organic acids (Sigma Aldrich, USA) were mixed at the concentration present on leaf surface as determined above, in 10 ml of sterile distilled water. A sample of *Hear*NPV (1×10^{10}OB) was added to the organic acid solution and then left in a rotator at 30 rpm for 1 h. OBs were recovered by centrifuging at 2500 g for 30 min, then re-suspended in 5 ml of distilled water, and then were counted. Serially diluted suspensions of OBs in distilled water were bioassayed alongside a control OB suspension not exposed to the organic acids.

HPLC Analysis of Chickpea Leaf Extracts after Spraying with OB Suspension To determine the effect of *Hear*NPV OBs on chickpea leaf chemistry, a suspension of 3×10^{7} OB ml^{-1} in 0.02% Triton was sprayed onto to the leaf surfaces of whole plants with a hydraulic hand sprayer sufficient to evenly wet the leaves. Control plants were sprayed with 0.02% Triton. Leaves were excised within 5 min or after 1, 4, or 24 h after spraying, and the surface

was extracted in methanol for 40 s. Extracts were filtered (Whatman No. 1), and evaporated to dryness under reduced pressure. Dried extracts were redissolved in 1 ml of 100% HPLC grade methanol for analysis. Aliquots (10 ul) were injected onto a reverse-phase column (Spherisorb 5ODS analytical column, 4.6 mm i.d. x 250 mm) and eluted at 1 ml min^{-1} using the gradient 90% A: 10% B at t=0 min to 50% A: 50% B at t=20 min to 20% A: 80% B at t=25 min to 100% B at t=30 min and 90% A: 10% B at t=37 min (A is 2% acetic acid and B is 2% acetic acid in acetonitrile).

Isolation of Leaf Surface Compounds and Their Effect on Activity of HearNPV OBs against H. armigera Larvae Compounds **1** and **2** were isolated by repetitive HPLC as described above, and fractions were collected manually at approximately 22 and 29 min. Combined fractions were evaporated under reduced pressure and weighed. LC-MS was carried out on a Thermo-Finnigan LC/MS/MS system consisting of a 'Surveyor' autosampling LC system interfaced to a LCQ Classic quadrupole ion trap mass spectrometer. Chromatographic separation was performed on a 150 mm×4.6 mm i.d. (5 μm particle size) Phenomenex Luna C18 column using a linear mobile phase gradient of 1 ml min^{-1} flow rate with water (A): MeOH (B): 5% Acetic Acid in MeOH (C). Initial conditions were 80% A, 0% B, and 20% C changing to 0% A, 80% B, and 20% C at t=20 min and maintained at these conditions to t=25 min. Injection volume was 10 μl, and data analysis was performed using Xcalibur 1.2 software. The ion trap MS was fitted with an Atmospheric Pressure Chemical Ionization (APCI) source operated under standard conditions; i.e., vaporizer temperature 450°C, needle current 5 mA, heated capillary temperature 150°C, sheath and auxiliary nitrogen gas pressure 80 and 20 psi, and the source voltages tuned for the optimal transmission of protonated rutin. The ion trap was set to monitor ions from m/z 125–1200 with collision energy of 45%. Authentic samples of genistein, daidzein, pratensein, biochanin A, and formononetin (Aldrich-Sigma) were co-chromatographed with methanol leaf extracts of chickpea leaf surface that had been sprayed with *Hear*NPV (suspended in 0.02% Triton X-100) and indicated that **2** was biochanin A. Compound **1** had a similar UV spectrum to **2** but eluted earlier (22 min) indicating a more polar nature and suggesting a glycoside. An aliquot of **1** that had been isolated from the leaf extracts as described above was analyzed by LC-MS and recorded a molecular ion signal in positive mode $[M+H]^+$ at m/e=447 indicating the molecular weight of 446 and a molecular formula $C_{22}H_{22}O_{10}$. Comparison of the mass spectrum with the library confirmed the structured to be biochanin A 7-*O*-glucoside (sissotrin) with good match in the lower range (m/e=100–300) of the spectrum. For example, the signal observed at $[M+H]^+$ m/e=285 indicated loss of a glucose moiety $[M\text{-}162 + H]^+$ and corresponded to biochanin A with a base peak at m/e=270 correlating to the loss of glucose and a methyl from the methoxy at C-4′ and a further fragment at m/e= 253 correlating to $[M\text{-}162\text{-}OCH_3]^+$ with the loss of the methoxy group. Subsequent co-chromatography using an authentic standard of sissotrin from natural products collection at Royal Botanic Gardens, Kew, confirmed this identification.

1 **2**

Compounds **1** and **2** were used subsequently in bioassays to evaluate their effects on *Hear*NPV.

The surface area of the leaves was measured as described above. A 200 μl aliquot of sissotrin (25 μg ml^{-1}) in methanol containing the equivalent sissotrin from 1250 mm^2 of chickpea leaf surface and equal to the surface area of artificial diet in a 30 ml container was placed onto the diet surface and allowed to evaporate. The control diets were treated with 200 μl methanol. *Hear*NPV concentrations on a five-fold dilution scale were prepared in distilled water. A control dose containing only distilled water also was prepared. An aliquot of each virus concentration was dispensed in a volume of 75 μl onto the surface of the diet and allowed to dry, after which 10 neonate larvae were released into each of the five pots. Larvae were allowed to feed for 24 h and then were transferred to clean artificial

diet pots at a rate of two per pot and reared under standard conditions. Mortality was recorded after 7 d. The experiment was replicated three times.

Effect of Biochanin A on the Efficacy of HearNPV against H. armigera Larvae Biochanin A (Sigma Aldrich, USA) was diluted to 500, 250, 100, and 10 ppm in distilled water, and was also tested against *Hear*NPV. A 200 µl aliquot of biochanin A at 500, 250, 100, or 10 ppm was spread over the surface of artificial diet. Control pots were treated with same amount of biochanin A. Bioassays were carried out as described above for sissotrin with 50 larvae treatment^{-1}, and the experiment again was replicated three times.

Results

Effect of Cotton, Tomato and Chickpea Plants on HearNPV against H. armigera Larvae using a Leaf Dip Bioassay Method The leaf dip bioassay showed that exposure of *Hear*NPV on chickpea leaf could impair *Hear*NPV activity. The LC_{50} values (Fig. 1) for the different plants were different (F=14.6, df=2,20, P=<0.001), and the LC_{50} for *Hear*NPV on chickpea of 3.96×10^4 OB ml-1 was significantly higher than that on tomato (2.65×10^3 OB ml^{-1}) and cotton (9.36×10^3 OB ml^{-1}). The result on tomato was not different from the mean LC_{50} of this virus strain obtained on artificial diet, which was 2.78×10^3 OB ml^{-1}. The bioassays of *Hear*NPV OBs exposed to tomato, cotton, and chickpea leaf surfaces also showed highly significant differences after 1 h (H=10.851, df=3, P=0.017) and 24 h (H=11.033, df=3, P=0.012) (Fig. 2); OBs on chickpea were markedly less infectious than OBs on tomato or cotton, which did not differ significantly from the LC_{50} of unexposed control OBs. Thus, exposure of OBs to the surface of chickpea for 1 and 24 h resulted in inactivation even after OBs were removed from the leaf surface. The LC_{50} values of *Hear*NPV OBs exposed to chickpea for 1 and 24 h did not differ significantly, indicating that the observed inactivation reaches its maximum effect within 1 h and exposure beyond that does not further affect OB infectivity.

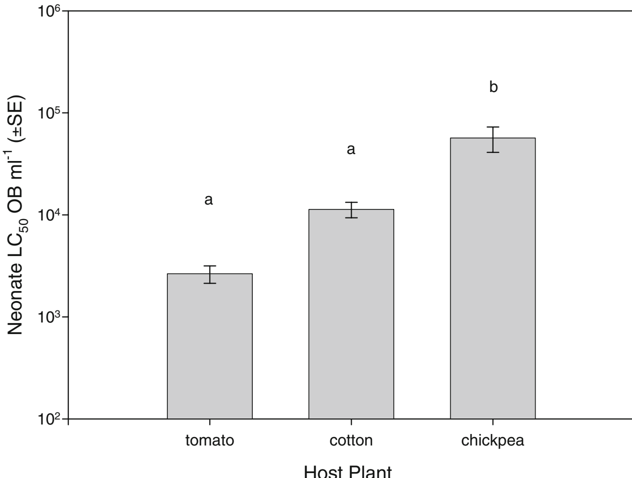
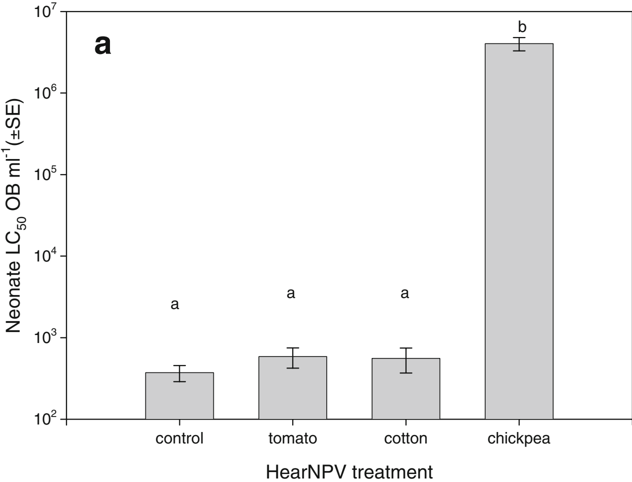


Fig. 1 Comparison of median lethal concentrations ($LC_{50}\pm$SE,) of *Hear*NPV against *Helicoverpa armigera* neonates bioassayed on leaves of cotton, tomato and chickpea with the leaf dip bioassay. Bars with different letters are statistically significant at $P<0.05$ (see detail in text)



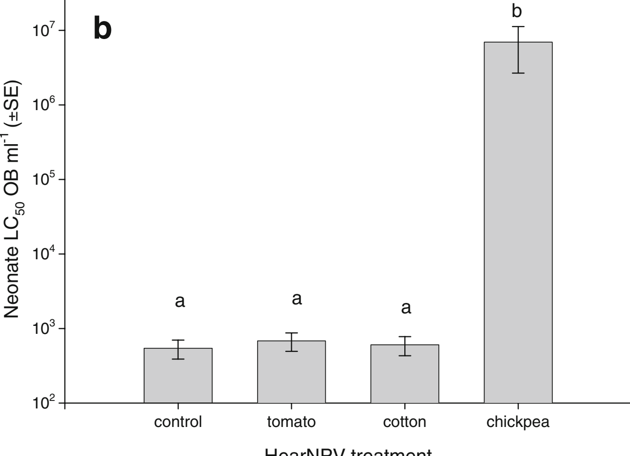


Fig. 2 Comparison of median lethal concentrations ($LC_{50}\pm$SE) of untreated *Hear*NPV and *Hear*NPV OB exposed to cotton, tomato and chickpea leaf surfaces for 1 hr (**a**) and 24 hr (**b**) bioassayed against *Helicoverpa armigera* neonates on artificial diet. Bars with different letters are statistically significant at $P<0.05$ (see detail in text)

Analysis of Organic Acids in Methanol Extract of Chickpea Leaf Surface by GC-MS The leaf surfaces of chickpea extracted with 100% methanol contained oxalic, malonic, malic, citramalic, and citric acid (Fig. 3). The compounds with retention times 13.47–13.48, and 16.01 min were silane impurities, while those at 24.80–24.81 min were sugars. Glucose-6-phosphate, oxalacetate, succinic, and

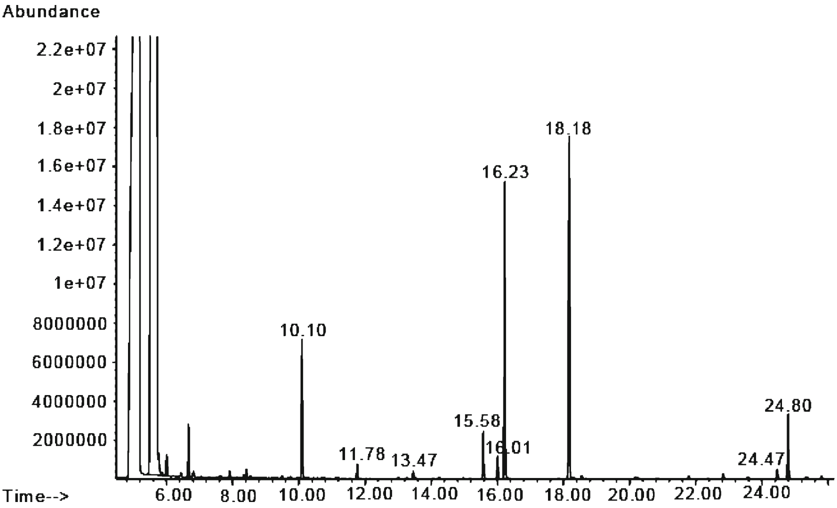


Fig. 3 Total ion gas chromatogram of chickpea leaf surface extract in methanol

Retention time (min)	Compound
10.10	Oxalic acid
11.78	Malonic acid
13.47	Silane impurities
15.58	Citramalic acid
16.01	Silane impurities
16.23	Glutaric acid
18.18	Malic acid
24.47	Citric acid
24.80	Sugars

fumaric acids were not found in any of the solvent extracts despite having been identified earlier by Rembold et al. (1980).

Effect of Organic Acids Present on the Chickpea Leaf Surface on the Efficacy of HearNPV against H. armigera Neonates The mean LC_{50} values of *Hear*NPV exposed to organic acids and for untreated *Hear*NPV by using a surface contamination bioassay system to neonates of *H. armigera* were 8.05×10^2 OB ml^{-1} and 6.16×10^2 OB ml^{-1}, respectively, and were not significantly different (t=0.484, P=0.762).

HPLC Analysis of Chickpea Leaf Surfaces after Spraying with NPV Chickpea plants were sprayed with *Hear*NPV in a 0.02% Triton X-100 suspension (to optimize spreading) and surface extracted in methanol within 5 min and after 1, 4, and 24 h. After 1 h, there was a more than four-fold increase in the concentration of **1** to 22 μg cm^{-2} compared with unsprayed leaf surfaces (5 μg cm^{-2}) in which the presence of **1** is constitutive. After 2, 4, and 24 h, the concentration of **1** was similar to pre spray quantities and remained there up to 24 h. Analysis of control plants that were sprayed with 0.02% Triton also showed only higher levels of **1** after 1 h, indicating that the process of spraying in the absence of virus was itself sufficient to induce the production of this compound and that it was not induced by the presence of the *Hear*NPV.

Effect of Sissotrin on the Efficacy of HearNPV against H. armigera Larvae The mean LC_{50} after exposure of *Hear*NPV to sissotrin for 1 h at a concentration equivalent to that found on the leaf surface after spraying was 1.23×10^4 OB ml^{-1}, and this was significantly higher than untreated *Hear*NPV at 2.30×10^3 OB ml^{-1} (F=44.24, df=1,4, P=0.003). However, this increase in LC_{50} for sissotrin treated *Hear*NPV is small compared to the LC_{50} values when *Hear*NPV OBs were exposed to chickpea plant surface for 1 h, thus suggesting that sissotrin does reduce the efficacy of *Hear*NPV but does not account for all the inhibition observed when *Hear*NPV was applied to the leaf.

The mean LC_{50}s of *Hear*NPV after exposure to different concentrations of biochanin A are shown in Fig. 4. There was a difference (F=4.16, df=4, 10, P=0.031) between the treatments, and it was shown by using least significant difference tests that mean LC_{50} values for *Hear*NPV exposed to biochanin A were not significantly different from each other but were significantly greater than the untreated sample. This indicates that biochanin A was effective even at concentrations as low as 10 ppm. As with

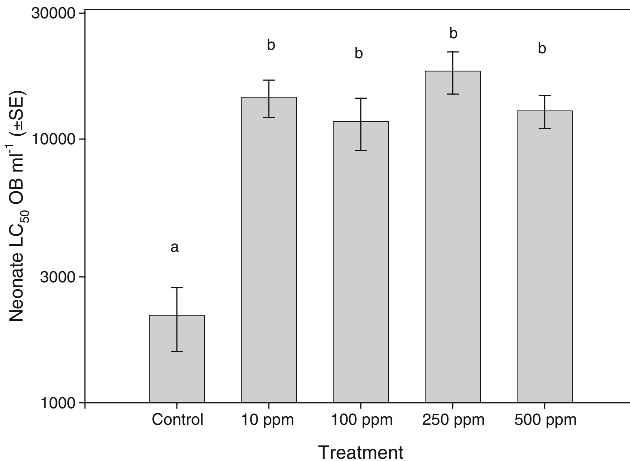


Fig. 4 Median lethal concentration (LC_{50}±SE) for *Hear*NPV exposed for 1 hr to different concentrations of biochanin A as determined by diet surface contamination bioassay on *Helicoverpa armigera* neonates. Bars with different letters are statistically significant at $P<0.05$ (see detail in text)

sissotrin, however, the effect of biochanin A does not explain fully the 5-fold increase in LC_{50} seen in *Hear*NPV after exposure on chickpea plants, suggesting that other factors must be involved.

Discussion

This study showed that the efficacy of *Hear*NPV OBs was inhibited considerably more on chickpea than on cotton, and that the effect was caused, at least in part, by surface isoflavonoids and not by organic acids. This was surprising since chickpea leaf surfaces have pH of <3 due the presence of organic acids (Rembold and Weigner 1990), and there is a well known association between low pH with NPV inactivation (Ignoffo and Garcia 1966). This study also has demonstrated that the inactivation of OBs on leaves is caused by their direct interaction with surface chemicals since OBs that had been exposed to the leaf surface were still inactive once removed. Thus, this differs from the mechanism of peroxidase inactivation reported previously for cotton (Hoover et al. 1998a, b,). The present work does not support an earlier proposition that the reduced efficacy of *Hear*NPV on chickpea could be related to a slower feeding rate of *H. armigera* on chickpea, thereby reducing the rate of OB ingestion (Rabindra et al. 1992). Sissotrin accumulated on the leaf surface at least for a short period of time after plants were sprayed with the OB suspension in 0.02% Triton or even with the 0.02% Triton control. This indicates that the process of spraying was sufficient to induce the production of these compounds, and was not induced by the presence of the *Hear*NPV. Thus, the induction of these compounds is not a response specific to the application of *Hear*NPV, but rather a response to either wetting or the presence of surfactant. The increased secretion of biologically active antimicrobial compounds by chickpea in response to wetting would be biologically explicable, as chickpea is subject to damaging fungal diseases such as Botrytis grey mould during periods of heavy dew or precipitation (Pande et al. 2005).

Plant chemicals previously have been shown to inhibit OB dissolution by binding irreversibly to OB structural proteins (Schultz and Keating 1991), a mechanism that is enhanced at least for orthodihydroxy moieties in the presence of peroxidases and polyphenoloxidases, particularly in damaged plant tissues (Felton and Duffey 1990). The present data do not shed light on the mechanism by which isoflavonoids impair NPV infectivity. Further work to understand this would be useful since the inactivation mechanism reported here may impact on other biological pesticides such as Bt or entomopathogenic fungi, given that chickpea isoflavonoids are toxic to numerous organisms including viruses, bacteria, fungi, and insects (Stevenson et al. 1997; Stevenson and Haware 1999; Simmonds and Stevenson 2001; Ito et al. 2003; Getti et al. 2006; Aslam et al. 2009). The identification of a new group of compounds that affect OBs, however, adds to the existing literature on this topic. The importance of the finding is highlighted by the LC_{50}s of OBs exposed on leaf surfaces being 3–5 orders of magnitude greater than that reported in cotton in both the present and earlier studies (Young and Yearian 1974; Forschler et al. 1992). It is not known if this mechanism is present or is as profound in all chickpea varieties. However, selective breeding for disease resistance (Pande et al. 2005) may have resulted in varieties with more biologically active compounds, and may explain the high OB inactivation reported here.

This study showed that *Hear*NPV OBs were inactivated when consumed on cotton leaf material, but showed no sign of inactivation when bioassayed on diets after exposure on and then removal from cotton; a result that concurs with those of Hoover et al. (1998a, b). However, there was no evidence of the OB inactivation by ionic cotton gland secretions reported previously (Ellerman and Entwistle 1985) on Ankur 651, the cotton variety tested here. This again may be explained by varietal differences in the chemistry of Ankur 651 and the Deltapine varieties studied earlier. Some Indian cottons are reportedly more detrimental to OB infectivity than chickpea (Rabindra et al. 1994).

While sissotrin and biochanin A have a significant inactivating action, the magnitude of inactivation by these compounds did not fully account for the effects observed on leaf surface assays. Therefore, other chemicals are likely to contribute to this inactivation and further work will be required to identify these.

In considering the results reported here, it may be surprising that *Hear*NPV is effective as a biopesticide on chickpea (Jayaraj et al. 1987; Rabindra et al. 1989; Cherry et al. 2000; Ahmed and Chandel 2004). However, on some crops, 90% of *H. armigera* larvae killed by *Hear*NPV sprayed onto plants acquire the infection within 1 h of application (D Murray, pers. comm.). The interaction of *Hear*NPV with chickpea also may be influenced by the variety of chickpea. Cowgill and Bhagwat (1996) for example reported a field trial in which *Hear*NPV was more effective at killing *H. armigera* when applied to the *H. armigera* susceptible genotype (ICCC 37) of chickpea than on a *H. armigera* resistant genotype (ICC 506). This may have been due to differences in their chemistry since the production of isoflavonoids in chickpeas is known to vary among cultivars at least in association with resistance to plant pathogens such as *Botrytis* and *Fusarium* (Stevenson et al. 1997).

Additives, including milk powder, casein, molasses, and Robin blue dye are reported to improve *Hear*NPV performance on chickpea (Rabindra et al. 1989). Although it has been assumed that they improved UV stability (Rabindra and Jayaraj 1988), given the present findings, it is possible that some additives also may contribute to improving OB efficacy by inhibiting chemical inactivation of OBs or by encouraging feeding and rapid viral acquisition before the OB inactivation processes have taken effect.

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Flavonoids Have Differential Effects on Glucose Absorption in Rats (*Rattus norvegicus*) and American Robins (*Turdis migratorius*)

Michele M. Skopec · Adam K. Green · William H. Karasov

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Abstract Mounting evidence suggests that small birds rely largely on non-mediated intestinal absorption of glucose through the paracellular pathway, while non-flying mammals rely on mediated absorption across the enterocyte membranes by using glucose transporters SGLT-1 and GLUT-2. Relying on non-mediated transport of glucose may decrease its absorption rate at low glucose concentrations but may release small birds from the effects of glucose transport inhibitors. We evaluated transport by using flavonoids known to inhibit glucose transport in vitro. Quercetin, isoquercetrin, and phloridzin were tested in rats (*Rattus norvegicus*) and robins (*Turdis migratirius*), and naringenin, naringenin-7-glucoside, genistein, epigallocatechin gallate (EGCG), and phloretin were used only in rats. By using a pharmacokinetic approach that involves serial blood collection and area under the curve calculations, we determined the bioavailability of 3-0-methyl D-glucose, the non-metabolized analogue of D-glucose. Six of the eight flavonoids tested in rats significantly decreased the absorption of 3-0-methyl D-glucose, while none of the flavonoids tested in robins significantly decreased the bioavailability of 3-0-methyl D-glucose. We conclude that flavonoids effectively decrease glucose absorption in rats, which rely on mediated absorption of glucose, but that flavonoids do not have an effect in robins, which rely on non-mediated absorption of glucose.

Keywords Flavonoids · Glucose absorption · Pharmacokinetics

M. M. Skopec (✉)
Department of Zoology, Weber State University,
Ogden, UT, USA
e-mail: MicheleSkopec@weber.edu

A. K. Green
Department of Biology Sciences, Santa Barbara City College,
Santa Barbara, CA, USA

W. H. Karasov
Department of Forest and Wildlife Ecology,
University of Wisconsin,
Madison, WI, USA

Introduction

Glucose is absorbed in the small intestine via the transcellular and paracellular pathways. Transcellular absorption is mediated by membrane-bound transporter proteins such as the sodium dependent glucose transporter (SGLT-1), found on the apical membrane of enterocytes, that actively absorbs glucose from the gut lumen. GLUT-2, a glucose transporter found on the basolateral membrane of enterocytes facilitates the diffusion of glucose out of the enterocytes and into the blood. Paracellular absorption involves movement of glucose through the tight junctions (TJs) of adjoining cells by diffusion or by solvent drag (Pappenheimer and Reiss 1987). This non-mediated route is quantitatively important in some species. For example, paracellular absorption accounts for the majority of glucose absorption in at least two avian species (Chang and Karavsov 2004; McWhorter et al. 2009). Paracellular absorption accounts for significantly more glucose absorption in small birds (<400 g) than in similar-sized non-flying mammals (Lavin and Karasov 2008). Enhanced paracellular absorption may compensate for the relatively small intestinal size in birds compared to nonflying mammals (Caviedes-Vidal et al. 2007).

The reliance on mediated absorption of glucose either by SGLT-1 or GLUT-2 ensures efficient and complete absorption from the intestine, but it requires energy either to drive

active transport or to produce and insert the transporter into the enterocyte membrane. Furthermore, reliance on mediated absorption of glucose makes mammals susceptible to transport inhibitors. Non-mediated transport, such as the paracellular pathway, may be less costly energetically than mediated absorption, but it may also be less efficient when glucose levels in the intestine are low. However, the use of non-mediated absorption of glucose may free small birds from susceptibility to transport inhibitors.

Flavonoids are widely distributed in higher land plants, and have numerous biological effects, including inhibition of glucose transporters. Phloretin and phloridzin are chalcones that inhibit GLUT-2 and SGLT-1, respectively. Numerous in vitro studies have demonstrated that tea catechins isoflavones, flavonols, and flavanones inhibit glucose transport (Song et al. 2002; Johnston et al. 2005; Kwon et al. 2007; Araujo et al. 2008). These in vitro experiments demonstrate only what might occur in the whole animal. For example, inhibition may not occur in vivo if flavonoids are greatly diluted by intestinal secretions or if lengthy intestinal residence time compensates for reduced membrane absorption rate. In vivo studies are needed to show whether flavonoids that inhibit glucose transport in vitro will also inhibit transport in vivo.

We tested whether flavonoids inhibit glucose absorption in vivo in laboratory rats (*Rattus norvegicus*) and robins (*Turdis migratorius*). We hypothesized that mammals would be more susceptible to the potential inhibitory effect of flavonoids than birds. We tested eight flavonoids from five different subclasses as inhibitors of glucose absorption in vivo in rats and three flavonoids from two different subclasses in robins. We employed a pharmacokinetic approach by using a non-metabolized, radiolabeled analogue of D-glucose, 3-0-methyl D-glucose (3OMD-glucose), to determine if flavonoids decrease glucose absorption from the intestine. 3OMD-glucose is used commonly along with other glucagogues to study glucose absorption in vivo (Uhing and Kimura 1995; Debru et al. 2001). By comparing the plasma concentration of 3OMD-glucose after oral dosing to the plasma concentration after injection, we calculated the bioavailability and elimination rate of orally administered 3OMD-glucose with or without flavonoids present. By using standard pharmacokinetic analysis, we determined the inhibitory potential of some common flavonoids in vivo in rats and robins.

Methods and Materials

Animals and Housing Male Sprague Dawley rats (Harlan) weighing 300–350 g were housed individually in shoebox cages. The room in which the rats were housed was maintained at an average ambient temperature of 22°C and an average relative humidity of 30%. Rats were fed rodent blocks (Teklad) ad libitum. There is evidence of diurnal rythmicity of SGLT-1 function (Tavakkolizadeh et al. 2001) so rats were placed on a reverse light cycle of 12D:12L to allow experiments to occur during their active period.

Adult American robins (*Turbidus migratorius*) with an average body mass of 69.2±1.4 g were captured with mist nets at the University of Wisconsin - Madison campus during 2003 and 2004. Robins were housed individually in wire cages (0.75 m×0.5 m×0.5 m) at an average ambient temperature of 22°C and an average relative humidity of 30%. The robins were fed a banana mash diet (Denslow et al. 1987) and water ad libitum. All procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the Research Animal Resource Center Animal Care Committee at the University of Wisconsin - Madison.

Experimental Design Rats were assigned randomly to 4 groups of 4 rats per group. Each group received 4 randomly assigned treatments, consisting of an injection treatment and 3 oral treatments, at least 1 wk apart. The oral treatments included a control treatment plus two flavonoid treatments. Eight flavonoids: quercetin, naringenin, genistein, epigallocatechin gallate (EGCG), phloridzin, phloretin, naringenin-7-glucoside, and isoquercetrin were tested (Fig. 1). Naringenin-7-glucoside and isoquercetrin were from Indofine, while all other flavonoids tested were from Sigma-Aldrich.

A total of 8 robins were assigned randomly to 5 treatments, consisting of an injection treatment and 4 oral treatments at least 1 wk apart. The oral treatments included a control plus three flavonoid treatments. The three flavonoids tested were phloridzin, quercetin, and isoquercetrin. Unlike the omnivorous rat, insectivorous/frugivorous robins are unlikely to have any ecologically relevant exposure to the flavonoids naringenin, naringenin-7-glucoside, genistein, EGCG, and phloretin that are found in citrus, soy, green tea, and apples Therefore, we tested only the flavonoids that were ecologically most relevant in the robin, quercetin and isoquercetrin, and phloridzin as a positive control because it is a potent inhibitor of SGLT-1.

Solution Composition The glucose analog 3-0-methyl [^{14}C (U)] D-glucose with a specific activity of 250 mCi/mmol was obtained from American Radiolabeled Chemicals. The injection treatment solution consisted of 5 μCi of ^{14}C 3OMD-glucose in 200 μl (rats) or 100 μl (robins) of isotonic saline. The oral control treatment solutions contained 5 μCi of ^{14}C 3OMD-glucose in 2 ml (rats) or 750 μl (robins) of 10 mM aqueous glucose containing 2% (*v*/*v*) dimethyl sulfoxide (DMSO, Sigma-Aldrich). For the

Fig. 1 Structures of flavonoids tested for inhibition of glucose absorption

flavonoid treatment solutions, the flavonoid was dissolved in DMSO and then diluted into the control oral treatment solution to yield 10 mM flavonoid and 2% (*v*/*v*) DMSO.

Experimental Procedure Food was withheld from the rats and robins for 15 h prior to experiments to allow their stomachs and small intestines to empty. The rats and robins were either gavaged with a blunt end feeding needle for the oral treatments or they were given an intramuscular (IM) injection in the caudal thigh muscle (rats) or the pectoralis muscle (robins) for the injection treatment. Dosing syringes were weighed immediately before and after dosing on an analytical balance to determine the exact mass of the treatment solution for each individual. Blood samples were taken from rats at 0, 10, 20, 30, 45, 60, 90, 150, and 240 min post administration (injection or oral). Blood (70–100 μl) was collected from rats with a 26 gauge, 0.5 in needle (Fisher Scientific) inserted into the tail vein of the rat, and a heparinized capillary tube was used to collect blood from the luer lock of the needle. Blood samples were taken from robins at 0, 5, 10, 15, 20, 45, 90, and 150 min post administration. Blood (~40 μl) was collected from the robins with a 26 gauge, 0.5 in needle inserted into the brachial vein of the robins, and heparinized capillary tubes were used to collect the blood from the luer lock of the needle. The capillary tubes were centrifuged at 4,000 rpm to separate the plasma. Plasma was transferred into pre-weighed 7 ml scintillation vials (Fisher Scientific), and the vials were reweighed to determine the plasma mass. Samples were counted on a Wallac WinSpectral 1414 liquid scintillation counter after adding 5 ml of scintillation fluid to each sample (Ecolume, ICN).

Pharmacokinetic Analysis The radioactivity in each plasma sample at time *t* was normalized to the mass of the sample (C_t, dpm/g plasma) and plotted against sampling time. The area under this curve (AUC_t) represents the amount of radiolabeled probe that has been absorbed from time zero up to time *t*, whereas AUC_{total} denotes the total amount of probe absorbed from time zero to infinity (∞).

Following typical procedures in pharmacokinetics (Gibaldi and Perrier 1982), the area from $t = 0$ to $t = x$ min (when the final blood sample was taken) was calculated by using the trapezoidal rule. The area from $t = x$ min to $t = \infty$ was calculated as:

$$AUC^{x\rightarrow\infty} = C_t(\text{at}\, t = x)/K_{el} \quad (1)$$

where K_{el} is the elimination rate constant, which can be determined for each animal in each experiment as the slope of the terminal portion of its plot of log (plasma ^{14}C concentration) vs.time (Riviere, 1999). K_{el} was determined typically from the last three log transformed values (Lavin et al. 2008; McWhorter et al. 2009). The total $AUC^{0\rightarrow\infty}$ was obtained by summing the two areas. Bioavailability (B) for each probe was estimated as the ratio of the area for oral gavage experiments to the area for injection experiments ($AUC_{injection}$) normalized to the dose given to the animal:

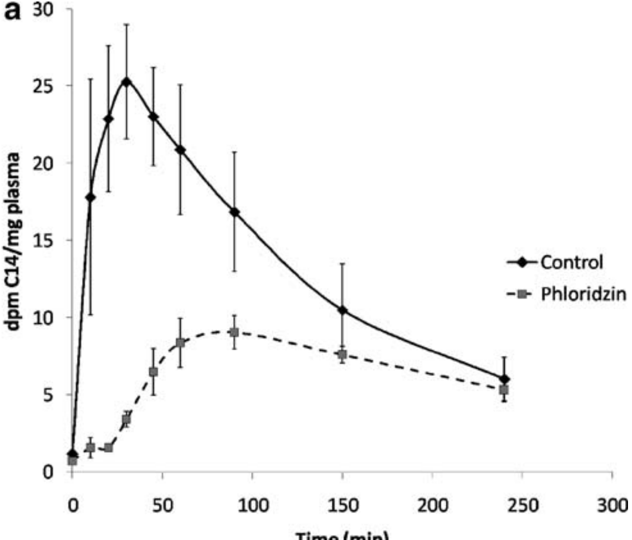
$$\text{Bioavailability}(B) = (AUC_{oral}/\text{dose}_{oral})/(AUC_{injection}/\text{dose}_{injection}) \quad (2)$$

This method of calculating B is favored because it makes no major assumptions about compartments or kinetics of absorption or elimination (Welling 1986). The dose of ^{14}C 3OMD-glucose was determined by liquid scintillation counting of 5 µl aliquots of the treatment solutions that were weighed and corrected to the mass of material actually administered.

Statistical Analysis Bioavailability data were normalized by arcsine square root transformations. Bioavailability and elimination rate were analyzed within species by repeated measure analysis of variance (ANOVA) with treatment as a factor using SYSTAT (Wilkinson and Coward 2000). *Post-hoc* pairwise comparisons using pooled variances were used to determine which flavonoid treatments significantly affected bioavailability or elimination rate compared to control within species. For between species comparisons, Bonferroni corrected two-tailed t-tests were used to determine if bioavailability and elimination rates differed. Data are represented as mean ± standard deviation (SD) unless otherwise stated. A P-value of 0.05 or less was used to establish significance.

Results

Bioavailability Rats and robins showed qualitatively similar uptake kinetics of ^{14}C 3OMD-glucose in the absence of flavonoids (Fig. 2). The bioavailability of ^{14}C 3OMD-glucose in rats was decreased ($P<0.05$) by six of the eight flavonoids tested (Table 1). The phloridzin treatment decreased absorption 38%, whereas bioavailability in the



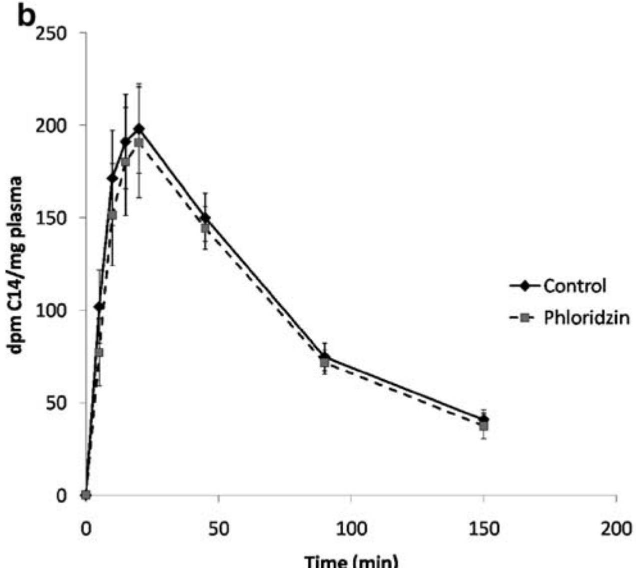


Fig. 2 **a** Absorption of orally administered ^{14}C 3OMD-glucose in laboratory rats. The presence of phloridzin in the oral treatment solution extended the absorption phase of ^{14}C 3OMD-glucose. Data represented are the means ± SE of the plasma ^{14}C at each time point (N=4). When rats were orally dosed with the control treatment (5 µCi ^{14}C 3OMD-glucose, 10 mM D-glucose) the plot is characterized by an early rapid increase in plasma ^{14}C that peaks around 45 minutes. When rats were orally dosed with the phloridzin treatment (10 mM phloridzin, 5 µCi ^{14}C 3oMD-glucose, and 10 mM D-glucose) the plot is instead characterized by an early slower increase in the plasma ^{14}C that does not peak until after 60 minutes. **b** Absorption of orally administered ^{14}C 3OMD-glucose in robins. The presence of phloridzin (10 mM) in the oral treatment solution (5 µCi ^{14}C 3OMD-glucose, 10 mM D-glucose) did not affect the absorption of ^{14}C 3OMD-glucose in robins. Data represented are the means ± SE of the plasma ^{14}C at each time point (N=8)

presence of quercetin was not significantly different from its respective control. The bioavailability of ^{14}C 3OMD-glucose in robins was not significantly affected by quercetin, isoquercetin, or phloridzin ($F_{3,21}$=0.447, P=

Table 1 The bioavailability of ^{14}C 3-O-methyl d-glucose was decreased in the presence of flavonoids in rats

Treatment	Bioavailability (%) mean ± SD	Repeated measure ANOVA using arcsine sqrt transformed FA data		Pairwise comparisons post-hoc using pooled variance	
		F	*P*-value	*F*	*P*-value
Group 1 (*n*=4)		10.263	0.012		
Control	97.0±3.9				
Quercetin	93.9±4.4			1.715	0.282
Naringenin[a]	83.5±9.6			12.321	0.039
Group 2 (*n*=4)		9.519	0.014		
Control	97.3±5.5				
Genistein	80.7±8.3			6.374	0.086
EGCG[a]	77.5±5.1			44.97	0.007
Group 3 (*n*=4)		12.298	0.008		
Control	90.4±15.5				
Phloridzin[a]	55.8±7.4			12.658	0.038
Phloretin[a]	79.9±11.9			12.593	0.038
Group 4 (*n*=4)		18.715	0.003		
Control	96.4±5.2				
Naringenin-7-glucoside[a]	83.5±6.8			41.352	0.008
Isoquercetrin[a]	86.0±7.7			12.889	0.037

[a] Significantly decreased the bioavailability of 3OMD-glucose $P<0.05$

0.722), in contrast to in rats in which both isoquercetin and phloridzin reduced absorption (Figs. 2 and 3).

The bioavailability of ^{14}C 3OMD-glucose was higher in rats than in robins in the control treatments ($P=0.004$; Fig. 3). The overall mean bioavailability for the control treatments in rats ($N=16$) was 95.3±8.4% while in robins ($N=8$) it was 75.1±9.3%.

Elimination Rate We also tested for treatment effects on elimination rate because SGLT-1 is responsible for reabsorption of glucose in kidney tubules. If the flavonoids reach the kidney, they might decrease the rate of glucose reabsorbtion and, thus, increase the rate of elimination. None of the flavonoids tested significantly altered the elimination rate of ^{14}C 3OMD-glucose in robins (Table 2,

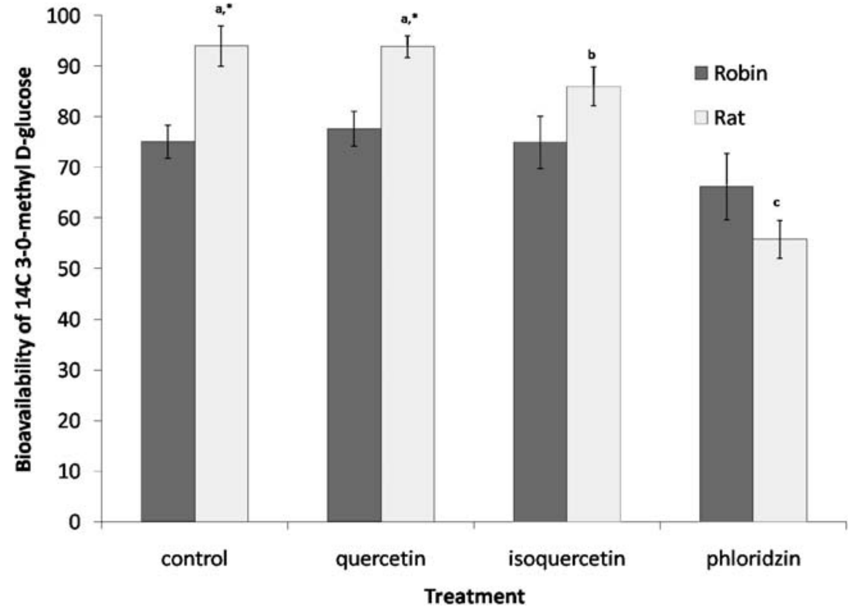


Fig. 3 Bioavailability of orally administered ^{14}C 3OMD-glucose in rats and robins. Data represented are the means ± SE of 5 μCi ^{14}C 3OMD-glucose bioavailability in the presence of 10 mM of the flavonoids and 10 mM D-glucose. Robin $N=8$ and rat $N=4$. Bars with * denotes means that are significantly different between species ($P<0.05$). Bars with different letters (a, b, c, d, and e) denote means significantly different within a species ($P<0.05$)

Table 2 Elimination rate of ^{14}C 3-O-methyl d-glucose in rats and robins

Treatment	Apparent Elimination Rate Constant[a] (dpmmg^{-1}min^{-1})	
	Rats	Robins
Injection	0.007±0.001 (*n*=16)	0.012±0.002[b] (*n*=8)
Control	0.008±0.001 (*n*=16)	0.013±0.002[b] (*n*=8)
Quercetin	0.009±0.001 (*n*=4)	0.013±0.004 (*n*=8)
Isoquercetrin	0.008±0.001 (*n*=4)	0.012±0.003 (*n*=8)
Phloridzin	0.005±0.001[c] (*n*=4)	0.013±0.003[b] (*n*=8)
Phloretin	0.008±0.001 (*n*=4)	
Naringenin	0.01±0.001 (*n*=4)	
Naringenin-7-glucoside	0.008±0.001 (*n*=4)	
Genistein	0.008±0.001 (*n*=4)	
EGCG	0.01±0.004 (*n*=4)	

[a] Rate constants are expressed as dpm of ^{14}C per mg plasma per min, and are presented as mean ± SD

[b] Significantly different from rat ($P<0.05$)

[c] Significantly different from all other rat treatments $P<0.05$)

$F_{4,28}=1.612$, $P=0.199$), and only phloridzin appeared to have a significant effect in rats (Table 2). Apparently, phloridzin slowed the absorption rate of ^{14}C 3OM D-glucose to such an extent that absorption was still occurring at the last three blood sampling time points (Fig. 2a; notice the 3rd to last plasma sample was in the peak). Therefore, the apparent elimination rate constant estimated for the phloridzin treatment that used the last three time points was likely influenced by absorption, and probably underestimated the true elimination rate constant.

Robins had higher apparent elimination rates of ^{14}C 3OMD-glucose than rats in all treatments (Table 2, Fig. 4). The overall mean apparent elimination rate constant for the injection treatments in rats ($N=16$) was 0.007±0.0001 dpm-mg^{-1}-min^{-1} while in robins ($N=8$) it was 0.012±0.002 dpm-mg^{-1}-min^{-1}.

Discussion

We found that flavonoids inhibited in vivo glucose absorption in rats but not in robins. We discuss the importance of testing the inhibitory activity of flavonoids in vivo, and possible ecological/evolutionary ramifications of differential effects of flavonoids on glucose absorption in rats vs. robins.

While numerous in vitro studies have determined that many flavonoids inhibit glucose transporters, only a handful

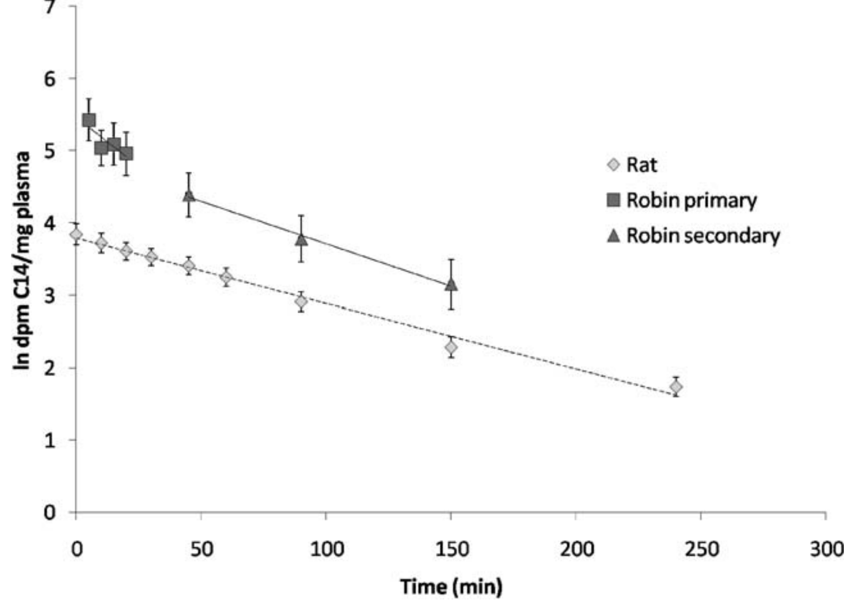


Fig. 4 Elimination rates of ^{14}C in rats and robins when injected intramuscularly with 5 µCi ^{14}C 3OMD-glucose. Data represented are the means ± SE of the plasma ^{14}C at each time point. In the robins the elimination of ^{14}C 3OMD-glucose is bi-exponential so only the last three time points 45, 90 and 150 minutes, which are labeled as robin secondary, were used from both the injection as well as oral treatments to calculate the elimination rate. Robins had a significantly faster elimination rate than the rats ($P<0.05$)

have investigated their in vivo effects. For example, in vivo studies have been performed to investigate the effect on blood glucose levels of apple juice (Johnston et al. 2002), quercetin (Song et al. 2002), and various herbal extracts that historically are used as treatments for diabetes (Shi et al. 1998; Ahmad et al. 2000; Yoshikawa and Matsuda 2000; Aybar et al. 2001; Ndong et al. 2007). In some of these studies, blood glucose concentrations were determined after dosing with extracts or flavonoids, while in others glucose tolerance tests were used to determine if the extracts dampened the spike in blood glucose following oral glucose loading. Neither method distinguishes between pre- and post-absorptive effects of the test chemicals.

By employing standard pharmacokinetic techniques, we demonstrated that six of eight flavonoids significantly decreased bioavailability of ^{14}C 3OMD-glucose in rats (Table 1). Differences in bioavailability may be due to changes in absorption or changes in rate of elimination (Riviere 1999). One flavonoid, phloridzin, decreased the apparent elimination rate (Table 2), but we believe this was an artifact. Apparently phloridzin decreased the absorption rate to such an extent that absorption was still occurring during the time points used to determine the elimination rate, thus leading to underestimation of the elimination rate constant (Fig. 2a). Crespy et al. (2001) estimated the bioavailability of phloridzin in rats to be approximately 10%, with no intact phloridzin found in the plasma, only unconjugated and conjugated phloretin. It is, therefore, not likely that phloridzin affected the elimination rate through systemic effects. Given the artifacts in our measurement, inhibition of glucose absorption by phloridzin may actually be larger than the 35% estimated in this study, and in fact phloridzin inhibited glucose uptake by around 66% when samples were taken over a longer time interval (Skopec 2003).

Robins and other small birds rely on non-mediated absorption of glucose (Chang and Karavsov 2004; McWhorter et al. 2009). Small birds have significantly less surface area in their small intestines than similarly sized non-flying mammals (Lavin et al. 2008). Less surface area may physically constrain the number of glucose transporters. Therefore, birds may need to rely on the paracellular pathway in order to absorb glucose. While non-mediated absorption may be a spatially and energtically efficient way to absorb glucose, it is not as efficient as mediated absorption for low levels of glucose. In our study, with low glucose concentrations in the treatment solutions, the 3OMD-glucose bioavailability was 20% lower in robins than in rats (Fig. 3). In house sparrows (*Passer domesticus*), which are similar in size to robins and, which also primarily utilize non-mediated absorption of glucose, bioavailability of 3OMD-glucose is 26% lower under low glucose concentrations (no D-glucose in the gavage solution) than with saturating levels of glucose (200 mM D-glucose in the gavage solution) (Chang and Karavsov 2004).

Another difference between the rats and robins was that robins eliminated 3OMD-glucose more rapidly than the rats (Table 2, Fig. 4). The robins were significantly smaller than the rats (70 g compared to 300–350 g), and elimination rates are typically slower in larger animals (Riviere 1999).

There may be several ecological/evolutionary ramifications of differential effects of flavonoids on glucose absorption in small birds vs. non-flying mammals. For mammals or large birds with well developed hindguts, inhibition of glucose absorption from the small intestine by dietary flavonoids may have little to no effect. In the presence of the inhibitor, glucose would be efficiently fermented in the hindgut, and much of the energy from the fermentation would be passed onto the animal. However, inhibition of glucose absorption by flavonoids might have negative consequences for frugivores. Frugivorous birds such as Yellow-rumped warblers, Cedar waxwings, and American robins have limited hindgut fermentative capacity and subsist for at least part of the year almost exclusively on fruit (Afik et al. 1997; Martinez Del Rio et al. 1992). In these birds, glucose that is not absorbed in the small intestine due to inhibition by flavonoids present in fruit would be lost in feces, significantly decreasing energy acquisition.

Non-mediated absorption of glucose from the intestine in small birds may have two benefits: It may allow decreased surface area and volume of the small intestine that may lead to lighter guts necessary for flight (Lavin et al. 2008). In addition, non-mediated glucose absorbtion may make small frugivorous birds tolerant to the inhibitory effects of the flavonoids present in their food.

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J Chem Ecol (2010) 36:244
DOI 10.1007/s10886-010-9759-5

ERRATUM

Erratum to: Sex Pheromone of the Pine False Webworm *Acantholyda erythrocephala*

Joseph K. Staples · Robert J. Bartelt · Allard A. Cossé · Douglas W. Whitman

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Erratum to: J Chem Ecol (2009) 35:1448–1460
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The presentation of Fig. 7 in the original version of this article contained a mistake. It is now corrected here.

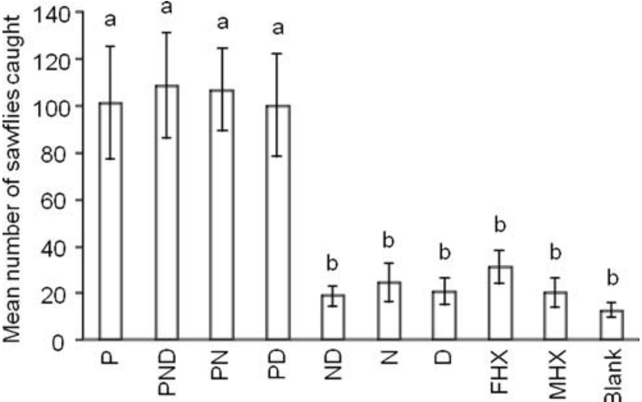

Fig. 7 Illinois field experiment 2002. Results show the mean number of male *Acantholyda erythrocephala* collected per treatment from April 17 through April 20. Error bars=standard error of untransformed data. Different letters indicate significant differences at $P<0.05$ (ANOVA followed by Tukey HSD test). Treatments include P=(*Z*)-6,14-pentdecadienal (50 μg), N=nonanal (1 mg), D=decanal (1 mg), ND=nonanal and decanal (1 mg each), FHX=female whole body washes (in hexane), MHX=male whole body washes (in hexane), and blank traps

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J. K. Staples (✉)
Department of Environmental Science,
University of Southern Maine,
37 College Avenue,
Gorham, ME 04038, USA
e-mail: jstaples@usm.maine.edu

R. J. Bartelt · A. A. Cossé
Crop Bioprotection Research Unit, USDA,
ARS National Center for Agricultural Utilization Research,
Peoria, IL 61604, USA

D. W. Whitman
School of Biological Sciences, Illinois State University,
Box 4120, Normal, IL 61790, USA

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RAPID COMMUNICATION

Knockdown of a Mosquito Odorant-binding Protein Involved in the Sensitive Detection of Oviposition Attractants

Julien Pelletier · Aline Guidolin · Zainulabeuddin Syed · Anthony J. Cornel · Walter S. Leal

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Abstract Odorant-binding proteins (OBPs) were discovered almost three decades ago, but there is still considerable debate regarding their role(s) in insect olfaction, particularly due to our inability to knockdown OBPs and demonstrate their direct phenotypic effects. By using RNA interference (RNAi), we reduced transcription of a major OBP gene, *CquiOBP1*, in the antennae of the Southern house mosquito, *Culex quinquefasciatus*. Previously, we had demonstrated that the mosquito oviposition pheromone (MOP) binds to CquiOBP1, which is expressed in MOP-sensitive sensilla. Antennae of RNAi-treated mosquitoes showed significantly lower electrophysiological responses to known mosquito oviposition attractants than the antennae of water-injected, control mosquitoes. While electroantennogram (EAG) responses to MOP, skatole, and indole were reduced in the knockdowns, there was no significant difference in the EAG responses from RNAi-treated and water-injected mosquito antennae to nonanal at all doses tested. These data suggest that CquiOBP1 is involved in the reception of some oviposition attractants, and that high levels of OBPs expression are essential for the sensitivity of the insect's olfactory system.

Keywords RNA interference · *Culex quinquefasciatus* antennae · CquiOBP1 · EAG · Oviposition attractants · MOP · Skatole · Indole

J. Pelletier · A. Guidolin · Z. Syed · A. J. Cornel · W. S. Leal (✉)
Department of Entomology, University of California-Davis,
1 Shields Ave,
Davis, CA 95616, USA
e-mail: wsleal@ucdavis.edu

Introduction

Odorant binding proteins (OBPs) were identified almost three decades ago (Vogt and Riddiford 1981), but their roles in insect olfaction are still a matter of considerable debate. That OBPs are involved in odorant reception was disputed after odorant receptors (ORs) were demonstrated to respond to semiochemicals when expressed in heterologous systems These expression systems, however, have limitations in addressing the role(s) of OBPs in olfaction. The heterologous expression system that uses *Drosophila* empty neurons (Dobritsa et al. 2003) includes surrogate OBPs, i.e., OBPs expressed in the ab3 sensilla, whereas in non-insect cell systems[1] (Forstner et al. 2009) odorants are solubilized with organic solvent or with the addition of recombinant OBPs. Thus, ultimately the role(s) of OBPs in insect olfaction must be addressed by examining insects with reduced levels (knockdowns) or devoid of a test OBP (knockouts). In *Drosophila*, analysis of a mutant defective for expression of an OBP revealed that DmelOBP76a (aka LUSH) is required for the activation of pheromone sensitive neurons by (*E*)-11-vaccenyl acetate and associated behavior (Xu et al. 2005), but other insect species are not amenable to this type of genetic manipulation. Previously, we employed the empty neuron system of *Drosophila* to express the pheromone receptor from the silkworm moth, *Bombyx mori*, BmorOR1 alone or co-expressed with a pheromone-binding protein, BmorPBP1 (Syed et al. 2006). Despite the low levels of BmorPBP1 expression in this heterologous system, we demonstrated clearly that PBPs enhance the sensitivity of the insect olfactory system (Syed

[1] We apologize for not being able to cite all the relevant literature due to reference limitations of a rapid communication.

et al. 2006). Recently, it was shown that addition of a recombinant PBP to a heterologous system that expresses a pheromone receptor from *Antheraea polyphemus* increases both sensitivity and selectivity (Forstner et al. 2009).

Given that our previous attempts to knockdown PBP expression in the silkworm moth were unsuccessful (Leal and Ishida, unpublished data), we explored knocking down OBP expression in mosquitoes. We then focused on CquiOBP1, which is highly expressed in the antennae of the Southern house mosquito *Culex pipiens quinquefasciatus* (=*Cx. quinquefasciatus*) (Ishida et al. 2002). Recently, CquiOBP1 was shown to bind a mosquito oviposition pheromone (MOP) (Laurence and Pickett 1982) in a pH dependent manner and to be expressed in antennal sensilla sensitive to this pheromone (Leal et al. 2008). In the present study, we used CquiOBP1 as a target in RNA interference (RNAi) experiments to examine its function in the reception of oviposition attractants. Mosquitoes injected with double strand RNA (dsRNA) showed reduced levels of *CquiOBP1* transcripts as well as reduced antennal responses to MOP, skatole, and indole when compared to water-injected controls. Interestingly, antennal response to nonanal, a major host cue detected with extremely high sensitivity by *Cx. quinquefasciatus* antennae (Syed and Leal 2009), was not significantly affected. These findings suggest that CquiOBP1 is involved in the detection of multiple oviposition attractants and plays a key role in the sensitivity of the mosquito olfactory system.

Methods and Materials

CquiOBP1 RNA Interference Full-length CquiOBP1 dsRNA was synthesized by in vitro transcription from purified PCR product that contained T7 promoter sequences in inverted orientations and purified by using RNeasy MinElute Cleanup Kit (Qiagen). Approximately 100 nl (350 ng) of dsRNA were injected through the intersegmental thorax membranes into 1-to-48 h-old *Cx. quinquefasciatus* female mosquitoes with a microINJECTOR™ System MINJ-1 (Tritech Research, Los Angeles, CA, USA). dsRNA-injected, water-injected, and non-injected mosquitoes were generated. Individual female heads were dissected in liquid nitrogen 4 d post-injection, RNA from each head was extracted with RNeasy Mini Kit (Qiagen), and individual cDNAs were synthesized from 0.1 μg of RNA using 100u SuperScript® II reverse transcriptase (Invitrogen). Real-time quantitative PCR (qPCR) was carried out by using EXPRESS SYBR® GreenER™ qPCR Super-Mix Universal (Invitrogen) in a final volume of 20 μl. Reactions were run with a standard cycling program, 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, on an AB7300 real-time PCR system (Applied Biosystems). Determination of transcripts abundance was based on two independent replicates for each sample. CquiOBP1 expression was normalized to the expression levels of an endogenous control, the ribosomal protein that encodes gene S7 (CquiRpS7). Relative quantification analysis based on the comparative C_t method ($\Delta\Delta C_t$) was performed using AB7300 system SDS software (Applied Biosystems). Non-injected mosquitoes were used for calibration purposes. Non quantitative PCR was carried out from the same cDNAs by using 2u GoTaq® DNA polymerase (Promega) in a final volume of 25 μl. CquiRpL8 amplification was used as a control of cDNA integrity.

Electrophysiological Recordings An excised head of an adult *Cx. quinquefasciatus* female was mounted on a Syntech EAG platform equipped with micromanipulator-12 and a high-impedance AC/DC preamplifier (Syntech, Germany). Chloridized silver wires in drawn-out glass capillaries filled with 0.1% KCl and 0.5% polyvinylpyrrolidone (PVP) were used for reference and recording electrodes. The recording electrode accommodated the two antennae of the excised head after the tips of the antennae were clipped to provide a better contact. Preparation was bathed in a high humidity air stream flowing at 20 ml/s to which a stimulus pulse of 2 ml/s was added for 500 ms. Any change in antennal deflection induced by the stimuli or control puffs was recoded for 10 s. Indole and 3-methyl indole (skatole) were purchased from Acros (USA) and were 95% pure; nonanal (99%) was from Sigma-Aldrich; racemic 6-acetoxy-5-hexadecanolide (MOP) was a gift from Bedoukian Research Incorporated, USA. Chemicals were dissolved in dichloromethane (DCM), wt/vol, to make a stock solution of 10 μg/μl and decadic dilutions were made. An aliquot (10 μl) of a stimulus was loaded onto a filter paper strip, the solvent was evaporated for 30 s, and the strip was placed in a 5 ml polypropylene syringe from which various volumes were dispensed. Solvent alone served as control. Data presented are from a pool of mosquitoes injected and tested in three different batches on different days. In each session, EAG responses of at least three of RNAi-treated and water-injected mosquitoes were recorded.

Results and Discussion

We employed a combination of RT-PCR and real-time quantitative PCR (qPCR) to examine mRNA levels of *CquiOBP1* in heads of RNAi (dsRNA-injected) and control (water-injected, non-injected) mosquitoes using *CquiRpS7* as a control gene. RT-PCR analysis showed a clear reduction of *CquiOBP1* transcript levels in dsRNA-injected mosquitoes, as compared to water-injected and non-injected mosquitoes (Fig. 1a). We then examined by

electroantennogram (EAG) the responses of sham-and RNAi-treated female mosquitoes to oviposition attractants. Silencing the *CquiOBP1* gene clearly affected antennal responses to MOP and indole, a putative oviposition attractant (Millar et al. 1992) (Fig. 1b), but the response to nonanal was not significantly affected. Next, we quantified the reduction of transcripts by qPCR (Fig. 1c), which confirmed the trend observed by a semi-quantitative method (Fig. 1a). dsRNA-injected mosquitoes displayed reduction of *CquiOBP1* transcript levels (average 59.9%) when compared to both water-injected (sham-treated) mosquitoes (average 97.3%) and non-injected controls (normalized to 100%). dsRNA-Injected individuals displayed significant reduction of *CquiOBP1* transcripts (47% to 65%) (Fig. 1c). Furthermore, water-injected and non-injected mosquitoes displayed almost equivalent levels of *CquiOBP1* transcripts, thus demonstrating that RNAi treatment is responsible for the observed reduction of *CquiOBP1* mRNA levels (Fig. 1c). This partial silencing of *CquiOBP1* shown by qPCR analysis demonstrates the feasibility of significantly reducing even highly expressed olfactory genes like OBPs by using the RNAi approach.

Fig. 1 PCR and EAG data. **a** RT-PCR analysis indicating that *CquiOBP1* transcripts were reduced in RNAi-treated females (RNA1 & RNA2) when compared to the transcript levels in water-injected (Water) and non-injected (NON) females. CquiRpL8, control gene. **b** EAG traces recorded from antennae of water- and RNAi-treated female mosquitoes challenged with MOP (100 µg), indole (10 µg), and nonanal (10 µg). Bars on the top of traces indicate the duration of the 500 ms stimulus. **c** Relative expression of *CquiOBP1* by qPCR using EXPRESS SYBR® Green ER™. RNAi-treated, water-injected, and non-injected mosquitoes (each $N=5$). **d**, **e**, **f** Dose-response EAG curves for skatole, indole, and nonanal, respectively ($N \geq 10$). The scale for skatole (**d**) and indole (**e**) graphics is the same, but the high sensitivity of nonanal (**f**) required a different scale

Correlation with EAG data (Fig. 1b) also suggests that ~50% transcripts reduction is enough to generate reduced responses to several semiochemicals.

Finally, we compared the responses of sham- and RNAi-treated female mosquitoes to various doses of these oviposition-related compounds. EAG responses of RNAi-treated females to MOP were below the detection limit, but the dose required to generate consistent EAG signals with water-treated or untreated mosquitoes was high (100 µg). In contrast, reduction of *CquiOBP1* transcripts led to a significantly reduced response to skatole (N=10, P<0.05) at all doses tested (Fig. 1d). Likewise, EAG responses to indole by RNAi-treated females were significantly lower than the responses recorded from water-treated female mosquitoes at all doses tested (Fig. 1e). Lastly, we observed an apparent trend towards smaller EAG responses to nonanal by RNAi-treated compared water-treated female mosquitoes, but the differences were not significant (Fig. 1f).

The simplest explanation for these findings is that OBPs play an important role for the sensitivity of the insect's olfactory system. Although we were not able to completely silence *CquiOBP1*, probably because of the high level of transcription, the partial knockdown clearly affected antennal response to physiologically relevant compounds. Previously, we demonstrated by in vitro assays that CquiOPB1 binds MOP in a pH-dependent manner, and we showed its expression in antennal sensilla sensitive to this oviposition attractant (Leal et al. 2008). These RNAi experiments are the first evidence in vivo that CquiOBP1 is involved in the reception of *Culex* mosquito oviposition attractants. Although it is tempting to speculate that CquiOBP1 is selective because responses to nonanal were not significantly different in sham- and RNAi-treated mosquitoes (Fig. 1f), the level of transcript reduction achieved by our RNAi treatments may not be high enough to affect EAG responses of semiochemicals such as nonanal for which the olfactory system responds with remarkable sensitivity (Syed and Leal 2009). By contrast, the reduced levels of *CquiOBP1* transcripts affected the responses of compounds with higher thresholds, thus allowing us to conclude that CquiOBP1 is indeed involved in the detection of oviposition attractants, and that high levels of OBPs expression are essential for the sensitivity of the insect's olfactory system.

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Note added in proof: Since this manuscript was accepted for publication, the authors became aware of a report by Biessmann et al. describing that RNAi is effective in knocking down accumulation of OBP1 transcripts in the antenna of *Anopheles gambiae* mosquitoes. Their results will be reported in the following paper in PLoS ONE: Harald Biessmann, Evi Andronopoulou, Max R. Biessmann, Vassilis Dourisb, Spiros D. Dimitratos, Elias Eliopoulos, Patrick M. Guerin, Kostas Iatrou, Robin W. Justice, Thomas Kröber, Osvaldo Marinotti, Panagiota Tsitoura, Daniel F. Woods, Marika F. Walter. The *Anopheles gambiae* Odorant Binding Protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. IN PRESS.

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Interaction Between Visual and Olfactory Cues During Host Finding in the Tomato Fruit Fly *Neoceratitis cyanescens*

Thierry Brévault · Serge Quilici

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Abstract Herbivorous insects searching for a host plant need to integrate a sequence of multimodal sensory inputs. We conducted a series of no-choice experiments in a laboratory wind tunnel to examine the behavioral response of the specialist fruit fly, *Neoceratitis cyanescens* (Diptera: Tephritidae), to host visual and olfactory stimuli presented singly or in combination (e.g., colored fruit model with or without host fruit odor). We also studied the influence of wind flow, age, and sex on the response of flies. In two-choice experiments, we evaluated the ability of mature females to discriminate between two fruit models emitting host vs. non-host fruit odor or clean air. *Neoceratitis cyanescens* mature females can use independently or interactively olfactory and visual stimuli to locate their host, whereas immature females and males respond primarily to host fruit odor. In the absence of wind, mature females mainly use visual information to locate the host fruit. In wind, host fruit odor significantly increases the probability and speed of locating the host fruit. In a two-choice situation between two bright orange spheres, flies accurately detected the sphere emitting host fruit odor vs. non-host fruit odor or odorless air. Nevertheless, they preferred to land on the bright orange sphere when the sphere emitting host fruit odor was blue. Furthermore, when odor source and fruit model were spatially decoupled (90 or 180°), >50% flies that landed on the fruit model initially performed an oriented flight toward the odor source, then turned back to the fruit model while in flight or after one landing, thus suggesting visual information to be the ultimate indicator of host fruit.

Keywords Host location · Multimodal cues · Host fruit odor · Color · Flight tunnel

T. Brévault (✉)
CIRAD, UPR 102,
34398 Montpellier, France
e-mail: brevault@cirad.fr

S. Quilici
CIRAD, UMR PVBMT,
97410 Saint-Pierre, La Réunion, France

Introduction

Finding and selecting suitable targets for egg laying, mating, feeding, or resting, is a key issue for reproductive success in insects (Schoonhoven et al. 2005). In phytophagous insects, orientation, including searching and finding host plants, involves the integration of a sequence of multimodal sensory inputs (Bernays and Chapman 1994; Bell et al. 1995; Cardé and Willis 2008). These inputs are received through peripheral receptors such as antenna and compound eyes, and then processed in the brain. Finch and Collier (2000) developed the hypothesis that once phytophagous insects have located the habitat by use of plant volatile chemical information, visual stimuli then govern the next phase of host plant location.

Frugivorous fruit flies (Diptera: Tephritidae) have evolved mechanisms that use plant volatiles and spectral properties during the host finding process (Roitberg 1985). In the specialist apple maggot fly, *Rhagoletis pomonella* (Walsh), fruit-seeking females are attracted at long distance by a blend of volatile esters from ripening fruit (Fein et al. 1982; Zhang et al. 1999), whereas vision is predominant at close range, within the host habitat (Prokopy and Owens 1983; Averill et al. 1988; Aluja and Prokopy 1992; Green et al. 1994). However, olfactory cues can interact in short-range searching for host fruit in the tree canopy when fruit are less apparent or scarce (Prokopy and Roitberg 1984;

Aluja and Prokopy 1993). Recent studies indicate that specific volatile blends, including non-host fruit volatiles, are used by apple, hawthorn, and flowering dogwood-infesting *Rhagoletis* flies to discriminate host fruit (Nojima et al. 2003a,b; Linn et al. 2003, 2004, 2005; Forbes and Feder 2006). The relative importance and the sequence by which these stimuli mediate host location at short distance, and how flies adapt their searching behavior in response to information, are not well known. Furthermore, the decision to engage in searching a plant is based not only on the perception of suitable information but also on the insect's physiological status (for a review, see Barton Browne et al. 1990). For various species, responses to visual and olfactory stimuli from host plants have been shown to be influenced by age and reproductive status. In *R. pomonella*, the age and sexual maturity of females significantly affect the probability and time to discover fruit models (Duan and Prokopy 1994).

As an oligophagous species specialized on solanaceous plants, the tomato fruit fly *Neoceratitis cyanescens* (Bezzi) (Diptera: Tephritidae) is a relevant model system to study the role of visual and olfactory cues and their interaction in guiding females to the host plant at short range. Classified in the subfamily Dacinae and the tribe Ceratitidini (Hancock and White 1997), this species is widespread throughout the Indian Ocean region, including Madagascar, Mauritius, Reunion, and Mayotte islands (OEPP/EPPO 1997). Females usually lay their eggs in fruit, causing serious damage to vegetable crops, particularly field-grown tomato (*Lycopersicum esculentum* Mill.), whereas wild plants such as bugweed (*Solanum mauritianum* Scop.) serve as natural reservoirs. Previous studies showed that flower and fruit volatiles assist gravid females (but also immature females and males) in finding the host plant (Brévault and Quilici 2010). At short range, i.e., within the host plant habitat, gravid females (but neither immature females nor males) are strongly attracted to fruit-mimicking bright orange spheres (Brévault and Quilici 1999, 2007a,b).

Because olfactory and visual cues were presented separately in these experimental settings, it is not clear how they interact during the fruit finding process at short range. Furthermore, many signals in nature are complex and multimodal, consisting of components from two or more sensory modalities, and the question of why organisms use multimodal signals remains poorly studied (Kulahci et al. 2008). We tested the following hypotheses: (a) visual stimuli are sufficient to elicit orientation response to host fruit; (b) the integration of more specific olfactory cues can improve the host finding efficiency in terms of speed and accuracy, especially when visual cues are inadequate or poor (obstructed or weakly attractive), or when visual cues are similar to those of non host plants; (c) conversely, since turbulence is likely to affect olfactory information, flies may rely on visual stimuli to locate host fruit when olfactory cues are inadequate or poor.

Using a laboratory wind tunnel, we studied the behavioral response of individually tested flies to an array of visual and olfactory cues presented singly or in combination (e.g., fruit model with or without host fruit odor). We also investigated the effect of wind flow, age (sexual maturity), and sex of flies on the response to a combination of visual and olfactory stimuli. In two-choice experiments, we evaluated the ability of gravid females to discriminate between two fruit models that emit host or non-host fruit odor, or clean air. The behavioral traits of *N. cyanescens* are discussed in the light of adaptive strategy and pest management.

Methods and Materials

Insects

Larvae of *Neoceratitis cyanescens* were collected from field-grown tomatoes in the western part of Reunion Island. Flies of both sexes emerged in 30×30×30 cm screen Plexiglas cages under controlled conditions (25±1°C, 70± 10% RH, and a LD 12:12 h photocycle). They had free access to granulated sugar, enzymatic yeast hydrolysate (ICN Biomedicals, Costa Mesa, CA, USA), and water until the time of testing. Behavioral experiments were conducted on laboratory-reared adult flies (generations 2 to 6) (Etienne 1973). The responses of 2-d-old immature females, 8-d-old mature females, and 6-d-old mature males were recorded. Based on Brévault and Quilici (2000a), 8-d-old females were supposed to have a high oviposition drive, as opposed to 2-d-old immature females. The flies used in all experiments were naive to fruit and plant odors and fruit visual cues, i.e., they were not allowed any contact with host-plant material and had no ovipositional experience prior to testing.

Laboratory Wind Tunnel

A semi-cylindrical plastic tunnel was used to assess the behavioral response of flies to olfactory cues (Fig. 1). A fan placed at the downwind end of the tunnel produced an adjustable wind flow of 0–60 cm sec^{-1}, measured with a telescopic 830 anemometer (TSI Inc., St. Paul, MN, USA). Clean air was pumped from above the building roof, and once passed through the tunnel was pushed outside. Two fabric filters placed in the upwind and downwind sections of the tunnel provided a laminar air flow. Grey adhesive bands were placed randomly on the tunnel floor to provide visual flow for the flying insects (Cardé 1984). Two sliding windows allowed the introduction and removal of insects from the tunnel. A neon ramp placed 50 cm above the

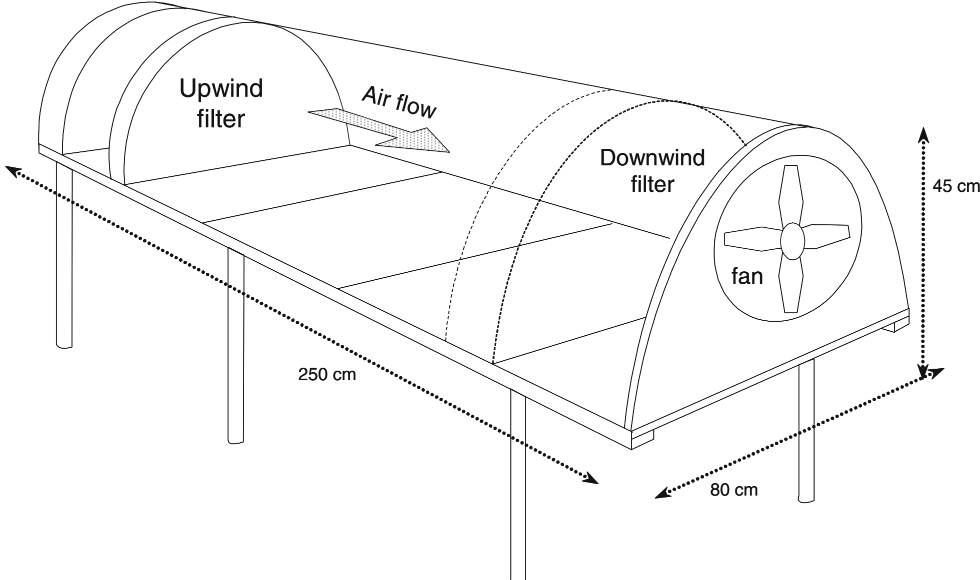

Fig. 1 Laboratory wind tunnel used to investigate the response of fruit flies to visual and olfactory cues

tunnel provided an illumination of 400 lux, and temperature was maintained at 25±1°C.

The behavioral response of flies to the combination of visual and olfactory stimuli was tested in no-choice or two-choice experiments. In both types of experiments, the odor sources were placed outside the tunnel in a sealed plastic bag (PET) with an inlet and outlet for air (Fig. 2). The inlet was connected via a silicon pipe to a vacuum pump that injected air at a flow of 1.0 l min^{-1}, whereas the outlet was connected to a sphere (3.7 cm diam) pierced with small holes (1 mm diam) and placed in the flight tunnel at 2 cm height (Fig. 2). As a result, odor was swept from the bag to the flight tunnel. Based on previous studies, air speed in the tunnel was fixed at 25 cm s^{-1} (Brévault and Quilici 2010).

Experimental Trials

In the first no-choice experiment (Exp. 1), the odor source was composed of unripe berries of bugweed (100 g), host fruit known to be highly attractive for *N. cyanescens* flies (Brévault and Quilici 2010), or an empty bag as control (clean air). Odor was conveyed to a colored sphere at location ‘0’ in the tunnel (100 cm from the released fly, Fig. 2), or no sphere as control. The color of the sphere was alternatively blue, yellow, or bright orange, respectively representing weak, moderate, and strong host fruit stimuli for mature females (Brévault and Quilici 2007a). In Exp. 2, flies were offered only a bright orange sphere at location ‘0’, with or without host fruit odor, in wind (30 cm sec^{-1}),

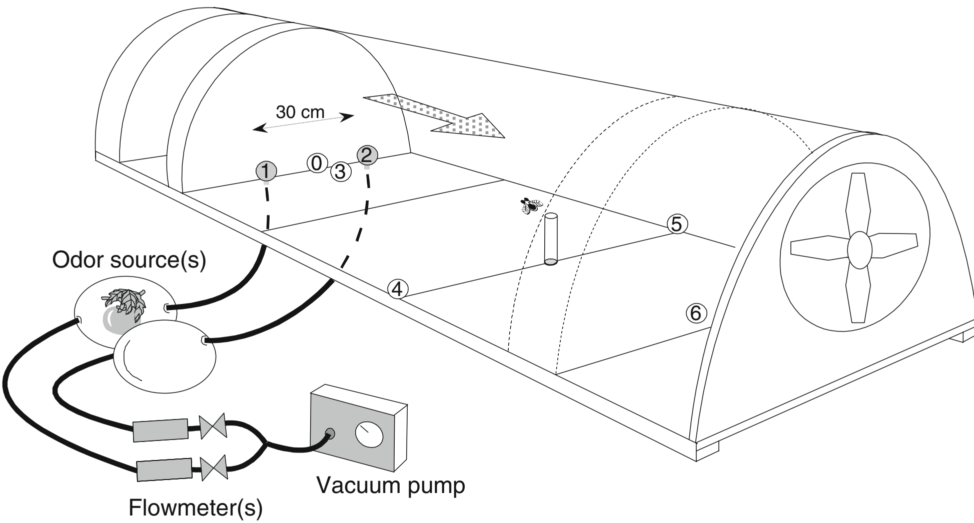

Fig. 2 Experimental trials designed for assessing behavioral response of flies to a combination of visual and olfactory stimuli. The odor source is placed in a sealed plastic bag and headspace is swept to the tunnel at locations 0–6. The release vial containing one individual fly is placed gently into the wind tunnel. Observations begin when the fly climbs onto the top of the release vial. Behavioral sequences and location of insects are time recorded using ‘The observer version 3.0’ software

or in still air (0 cm sec^{-1}). In Exp. 3, flies were offered only a bright orange sphere at location '0' (0°, 100 cm from the released fly, Fig. 2), on one side at location '4' or '5' (90°, 40 cm from the released fly, Fig. 2), or in the downwind end of the tunnel at location '6' (180°, 50 cm from the released fly, Fig. 2), with host fruit odor emitted at location '0' (Fig. 2).

In the two-choice experiments, a pair of spheres was presented in wind flow (25 cm sec^{-1}) to individually tested 8-d-old mature females. In Exp. 4, two bright orange spheres were placed at location '1' and '2' (30 cm between spheres, 100 cm from the released fly, Fig. 2). One odor source was composed of unripe berries of bugweed (100 g), and the other was composed of either unripe host fruit (tomato, *L. esculentum*), unripe non-host fruit (zucchini, *Cucurbita pepo* L., orange, *Citrus sinensis* L.), or clean air (empty bag as control). Bugweed odor vs. clean air also was tested in the absence of wind flow. In Exp. 5, a yellow or blue sphere emitting host fruit odor replaced the bright orange sphere emitting host fruit odor. In Exp. 6, a bright orange or yellow sphere without odor was placed at location '3', 10 cm in front of the bright orange sphere emitting host fruit odor at location '0' (Fig. 2).

Behavioral Observations

In the no-choice experiments, individual flies were gently transferred into a small glass vial (5×1.5 cm) that was deposited downwind at 100 cm of location '0' (Fig. 2). Observations started as soon as the fly climbed onto the top of the vial, and lasted 2 min, or less when the fly reached the sphere or the upwind section of the tunnel in the absence of any sphere. Behavioral sequences, locomotion, and location of flies were time recorded with 'The Observer version 3.0' software (Noldus Information Technology, Wageningen, The Netherlands). Only typical postures were analyzed, i.e., scan (motion around the tip of the release vial), orientation toward odor source ('arrestment'), lag time (time before leaving the vial), upwind flight (in the opposite direction of the wind flow), direct flight and landing on the sphere. In the two-choice experiments, observations also lasted 2 min or ended sooner when flies landed on a sphere. Behavioral observations lasted 1 min more after landing, to assess egg-laying behavior (Exp. 4) or landing on the other sphere (Exp. 6).

A minimum of 30 flies per treatment were tested. Flies were tested only once, individually, in series of 5 individuals per randomized treatment. Between treatments, the wind tunnel was ventilated with clean air for 5 min to prevent odor contamination. Observations were conducted in the afternoon between 2:30–5:30 pm when females usually seek for host fruits to lay their eggs (Brévault and Quilici 2000b).

Data Analysis

Quantitative data were analyzed using non parametric *Kruskal-Wallis* one-way *ANOVA*, because assumption of normality was not met. Behavioral frequencies were compared by χ^2-*tests* and Marascuilo procedure using XLSTAT (Fahmy 2006). To determine whether there was any interaction between olfactory and visual stimuli, a χ^2-*test* was used for a two-way contingency table (combinations of absence and presence of odor and visual stimuli).

Results

Do Visual and Olfactory Cues Interact during the Host Finding Process?

Orientation toward the odor source was more frequent in flies exposed to host fruit odor than to clean air (χ^2_7=99.4 for 8-d-old females; χ^2_3=83.6 for 2-d-old females; χ^2_3= 90.2 for 6-d-old males; P<0.001), and negatively associated to scan behavior (r_6=0.95, P<0.001) and lag time (r_6= 0.92, P=0.001) on the release vial (Fig. 3a, Table 1). Flies

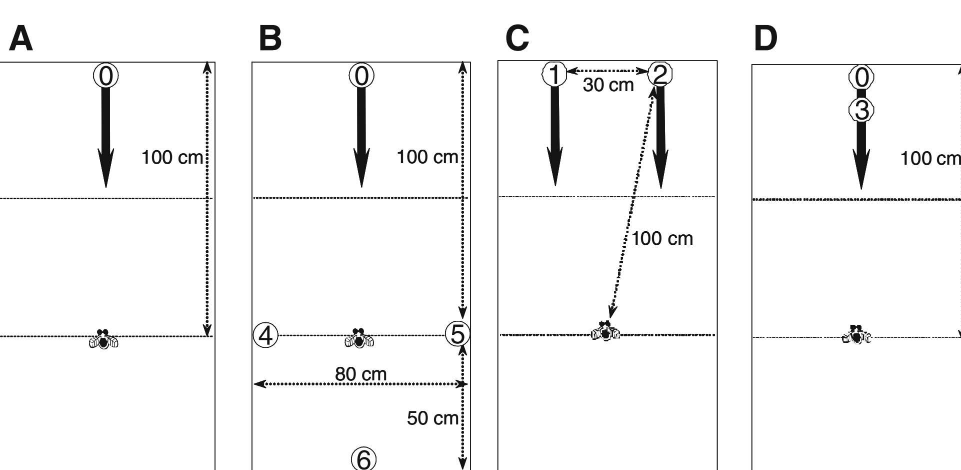

Fig. 3 Experimental setup used for the assays documented in Table 1 and 2 (**a**), Table 3 (**b**), Table 4 (**c**), and Table 5 (**d**)

Table 1 Behavioral response of individually tested *Neoceratitis cyanescens* flies to a combination of visual (bright orange, yellow, blue sphere, or control) and olfactory (host fruit odor or control) cues in a laboratory wind tunnel

Sphere	Odor source	N	From the release vial				In the tunnel	
			Scan (%)	Orientation to. Odor (%)	Lag time ±SEM* (sec)	Upwind flight (%)	Upwind section (%)	Landing on sphere (%)
8-d-old females								
–	Control	40	70.0 *a*	37.5 *c*	54±6 *a*	0.0 *c*	0.5 *e*	
–	Host fruit	40	30.0 *b*	87.5 *a*	17±4 *b*	77.5 *a*	65.0 *ab*	
Blue	Control	40	70.0 *a*	25.0 *c*	46±6 *a*	12.5 *bc*	12.5 *de*	0.0 *d*
Blue	Host fruit	40	17.5 *b*	82.5 *ab*	11±3 *b*	87.5 *a*	72.5 *ab*	10.0 *cd*
Yellow	Control	40	50.0 *ab*	45.0 *bc*	45±6 *a*	32.5 *b*	27.5 *cd*	5.0 *cd*
Yellow	Host fruit	40	12.5 *b*	80.0 *a*	14±4 *b*	85.0 *a*	77.5 *ab*	27.5 *bc*
Orange	Control	40	70.0 *a*	15.0 *c*	46±6 *a*	32.5 *b*	37.5 *cd*	37.5 *b*
Orange	Host fruit	40	17.5 *b*	92.5 *a*	15±4 *b*	87.5 *a*	85.0 *a*	75.0 *a*
	χ^2_7, K_7 *(P)*		76 (<0.001)	110 (<0.001)	112 (<0.001)	149 (<0.001)	112 (<0.001)	84 (<0.001)
2-d-old females								
–	Control	40	52.5 *a*	0.0 *b*	93±7 *a*	0.0 *b*	0.0 *b*	
–	Host fruit	40	17.5 *b*	65.0 *a*	39±7 *b*	62.5 *a*	50.0 *a*	
Orange	Control	40	40.0 *ab*	5.0 *b*	103±5 *a*	5.0 *b*	5.0 *b*	2.5 *b*
Orange	Host fruit	40	17.5 *b*	80.0 *a*	31±7 *b*	67.5 *a*	60.0 *a*	32.5 *a*
	χ^2_3, K_3 *(P)*		17 (0.001)	86 (<0.001)	67 (<0.001)	70 (<0.001)	55 (<0.001)	13 (<0.001)
6-d-old males								
–	Control	30	80.0 *a*	3.3 *b*	82±8 *a*	0.0 *b*	10.0 *b*	
–	Host fruit	30	3.3 *b*	93.3 *a*	14±5 *b*	73.3 *a*	60.0 *a*	
Orange	Control	30	56.7 *a*	6.7 *b*	92±8 *a*	16.7 *b*	16.7 *b*	6.7 *b*
Orange	Host fruit	30	10.0 *b*	90.0 *a*	22±6 *b*	80.0 *a*	70.0 *a*	43.3 *a*
	χ^2_3, K_3 *(P)*		52 (<0.001)	90 (<0.001)	57 (<0.001)	59 (<0.001)	35 (<0.001)	11 (0.001)

The sphere and odor source were placed at location '0', 100 cm distance from the released fly (Fig. 3a).

SEM standard error of mean. *N* number of tested flies

Data of the same experiment in the same column followed by different letters are significantly different according to *χ2-test* or *Kruskal-Wallis ANOVA**. The source of host fruit odor was composed of 100 g unripe berries from bugweed (*Solanum mauritianum*).

that oriented toward the odor source did not scan their environment and left the release vial earlier. Conversely, the presence of a sphere with clean air, regardless of its color, did not affect significantly those parameters (χ^2_{1-3}=0.1–6.1, P=0.107–0.702). Host fruit odor *per se* significantly triggered an upwind flight regardless of the age or sex of flies (Table 1). Accordingly, host fruit odor had a significant effect on the percentage of flies that reached the upwind section of the flight tunnel (χ^2_7=97.0 for 8-d-old females; χ^2_3=53.8 for 2-d-old females; χ^2_3=33.6 for 6-d-old males; P<0.001).

In the absence of host fruit odor, the percentage of 8-d-old females that reached the upwind section was higher in the presence of a bright orange or a yellow sphere than in the absence of sphere (χ^2_7=12.5, P=0.006). Furthermore, there was a significant additive interaction between olfactory and visual stimuli for the percentage of flies that engaged in an upwind flight and that reached the upwind section (χ^2_7=10.1, P=0.002; χ^2_7=7.6, P=0.006, respectively). However, the presence of a sphere did not affect the percentage of 2-d-old females and 6-d-old males that reached the upwind section (χ^2_3=1.1, P=0.295 for 2-d-old females; χ^2_3=0.9, P=0.350 for 6-d-old males). Only 2.5% of 2-d-old females and 6.7% of 6-d-old males landed on the bright orange sphere in the absence of host fruit odor, compared to, respectively, 32.5 and 43.3% when fruit odor was combined. The bright orange sphere *per se* significantly triggered landing of 8-d-old females on the sphere (37.5%) compared to the yellow (5.0%) or the blue sphere (0.0%), and this percentage increased synergistically when host fruit odor was combined with the bright orange sphere (75%), as compared to the yellow (27.5%) or blue (10.0%) sphere (χ^2_7=3.9, P=0.048; χ^2_7=4.8, P=0.028, respectively). In addition, flies reached the bright orange

sphere earlier in the presence (15±4 sec) than in the absence (49±10 sec) of host fruit odor.

In the absence of wind flow or host fruit odor, most 8-d-old females scanned their environment (Fig. 3a, Table 2). Conversely, in both wind flow and host fruit odor, most flies oriented themselves towards the odor source so that lag time on the release vial was significantly reduced (Table 2). In addition, there was a significant synergistic interaction between olfactory and wind stimuli for the percentage of flies that scanned their environment or orientated toward the source, and the mean time before leaving the release vial (χ^2=12.4, P< 0.001; χ^2_3=8.4, P<0.001; χ^2_3=5.2, P<0.001, respectively). A significant synergism was found between olfactory and wind stimuli for the percentage of flies that landed on the sphere (χ^2_3=13.9, P<0.001). Host fruit odor significantly increased the probability of locating the bright orange sphere in wind but not in still air. In the absence of host fruit odor, the percentage of flies that landed on the sphere in still air (45.0%) was higher than that in wind (27.5%). Time to reach the sphere was significantly reduced in the presence of host fruit odor and wind flow.

Within the 2 min assigned to behavioral observations, the percentage of flies that landed on the orange sphere was higher when the sphere was placed in the odor plume at 0° (90.6%) or outside the plume at 90° (78.1%) than in the plume at 180° (40.6%) (Fig. 3b, Table 3). At 0°, 86.2% of flies that landed on the sphere performed a direct flight. At 90°, 40.0% of flies that landed on the sphere performed a direct flight, whereas 52.0% turned back while in flight toward the odor source. At 180°, 46.2% of flies that landed on the sphere performed a direct flight, whereas 23.1% turned back while in flight toward the odor source, and 30.8% with at least one landing transition. The mean time to reach the sphere was significantly lower at 0° than at 90° and 180° (Table 3).

Does Host Fruit Odor Mediate Discrimination between Fruit Models?

In the absence of wind flow, 8-d-old females did not land preferentially on the bright orange sphere emitting host fruit odor (62.5%), but 66.6% of flies that first landed on the sphere emitting clean air left the sphere within 1 min (Fig. 3c, Table 4). Conversely, 80.0% of flies that landed on the sphere emitting host fruit odor drew their ovipositor. In wind (25 cm sec^{-1}), flies preferentially landed on the sphere emitting host fruit odor rather than on the sphere emitting clean air or non host fruits such as zucchini or orange odor (77.8, 83.3, and 80.6%, respectively), but did not show any preference when the second sphere emitted host fruit odor from unripe tomatoes (Table 4). Most flies extended their ovipositor regardless of the nature of the fruit (57.1–71.4%), and few left the odor-emitting sphere (0.0–14.3%).

Flies significantly preferred to land on the bright orange sphere when presented together with a yellow or a blue sphere (Fig. 4). However, they did not discriminate between the bright orange and the yellow sphere when the yellow sphere emitted host fruit odor, but continued to prefer the bright orange sphere to the blue one emitting host fruit odor. When a bright orange sphere emitting clean air was placed 10 cm in front of a bright orange sphere emitting host fruit odor, flies preferentially landed on the odorless sphere (83.3%) but this stop constituted a transitional step, as 90% of females switched to the sphere emitting the fruit odor within 1 min. When a yellow sphere was placed in front of the bright orange sphere emitting host fruit odor, 70% of flies flew around the yellow sphere and

Table 2 Effect of wind on the behavioral response of individually tested *Neoceratitis cyanescens* 8-d-old mature females to a bright orange sphere combined with host fruit odor, in a laboratory wind tunnel

Wind flow (cm sec^{-1})	Odor source	N	From the release vial						In the tunnel			
			Scan (%)		Orientation to. odor (%)		Lag time ±SEM* (sec)		Landing on sphere (%)		Mean time to reach sphere (sec)	
0	Control	40	75.0	*a*	2.5	*b*	25±4	*a*	45.0	*b*	44±9	*a*
	Host fruit	40	47.5	*a*	7.5	*b*	17±5	*b*	65.0	*ab*	50±7	*a*
30	Control	40	72.5	*a*	12.5	*b*	36±6	*a*	27.5	*c*	49±11	*a*
	Host fruit	40	2.5	*b*	97.5	*a*	8±3	*c*	85.0	*a*	15±6	*b*
	χ^2_3, K_3 (P)		54 (<0.001)		117 (<0.001)		53 (<0.001)		30 (<0.001)		36 (<0.001)	

The bright orange sphere and the odor source were placed at location '0', 100 cm distance from the released fly (Fig. 3a).

SEM standard error of mean. *N* number of tested flies.

Data in the same column followed by different letters are significantly different according to *χ2-test* or *Kruskal-Wallis ANOVA**. The source of host fruit odor was composed of 100 g unripe berries from bugweed (*Solanum mauritianum*).

Table 3 Behavioral response of individually tested *Neoceratitis cyanescens* 8-d-old mature females to spatially decoupled visual (bright orange sphere) and olfactory (host fruit odor) stimuli in a laboratory wind tunnel

In the tunnel

Sphere location	*N*	Landing on sphere (%)		Direct flight (%)		Upwind flight and turn back (%)		At least one landing transition (%)		Mean time to reach sphere ±SEM* (sec)	
0 (0°)	32	90.6	*a*	89.6	*a*	0.0	*b*	10.3	*a*	13±3	*b*
4 or 5 (90°)	32	78.1	*a*	40.0	*b*	52.0	*a*	8.0	*a*	14±3	*b*
6 (180°)	32	40.6	*b*	46.2	*b*	23.1	*ab*	30.8	*b*	20±2	*a*
χ^2_2, K_3 *(P)*		25 (<0.001)		16 (0.001)		20 (<0.001)		25 (<0.001)		13 (0.001)	

The bright orange sphere was placed at 100, 40, or 50 cm distance from the released fly, according to the location 0, 4–5, or 6 of the sphere. The odor source was placed at location '0' (Fig. 3b). SEM : standard error of mean. N: number of tested flies. Data in the same column followed by different letters are significantly different according to *χ2-test* or *Kruskal-Wallis ANOVA**. The source of host fruit odor was composed of 100 g unripe berries from bugweed (*Solanum mauritianum*).

landed on the bright orange sphere as a first choice (Fig. 3d, Table 5).

Discussion

Our results demonstrate that: 1) *N. cyanescens* gravid females can use independently or interactively olfactory and visual cues to locate host fruit; 2) olfactory information when carried by wind improves searching efficiency at short range, both in terms of speed and accuracy, but visual cues are predominant in the decision of landing; and 3) immature females and males respond primarily to olfactory cues. Consistent with previous reports on *N. cyanescens* responses to fruit models (Brévault and Quilici 2007a, b) and host fruit odor (Brévault and Quilici 2010), we observed that both bright orange spheres and host fruit odor in wind independently elicited positive responses of fruit-seeking females. The bright orange sphere *per se* significantly triggered landing of 8-d-old females (37.5%) compared to the yellow sphere (5.0%) or the blue sphere (0.0%), and this percentage increased synergistically when

Table 4 Behavioral response of individually tested *Neoceratitis cyanescens* 8-d-old mature females to olfactory cues released by two bright orange spheres in 2-choice tests

Wind speed (cm sec^{-1})	Odor source	N	Landing on sphere (%)	Drawing ovipositor on the sphere (%)	Leaving sphere within 30 s (%)
0	Bugweed	40	62.5	80.0	4.0
	Control		37.5	6.7	66.7
	χ^2_1 *(P)*		1.3 (0.260)	20.2 (<0.001)	18.9 (<0.001)
25	Bugweed	36	77.8	85.7	0.0
	Control		22.2	0.0	50.0
	χ^2_1 *(P)*		6.0 (0.014)	20.6 (<0.001)	15.8 (<0.001)
25	bugweed	30	53.3	87.5	6.3
	tomato		46.7	71.4	14.3
	χ^2_1 *(P)*		0.1 (0.796)	1.2 (0.272)	0.5 (0.464)
25	bugweed	30	83.3	88.0	0.0
	zucchini		16.7	80.0	0.0
	χ^2_1 *(P)*		7.5 (0.006)	0.2 (0.631)	N/A
25	bugweed	36	80.6	86.2	0.0
	orange		19.4	57.1	14.3
	χ^2_1 *(P)*		7.4 (0.006)	3.0 (0.081)	4.3 (0.039)

N number of tested flies

The two bright orange spheres and the odor sources were placed at location '1' and '2', 100 cm distance from the released fly (Fig. 3c). A *χ2-test* was performed to compare the distribution of landings of flies with a theoretical distribution (50–50%). The other frequencies were analyzed by using *χ2-tests*. The source of fruit odor was composed of 100 g unripe fruit from host plants (bugweed, *Solanum mauritianum*; tomato, *Lycopersicon esculentum*) or non host plants (zucchini, *Cucurbita pepo*; orange, *Citrus sinensis*).

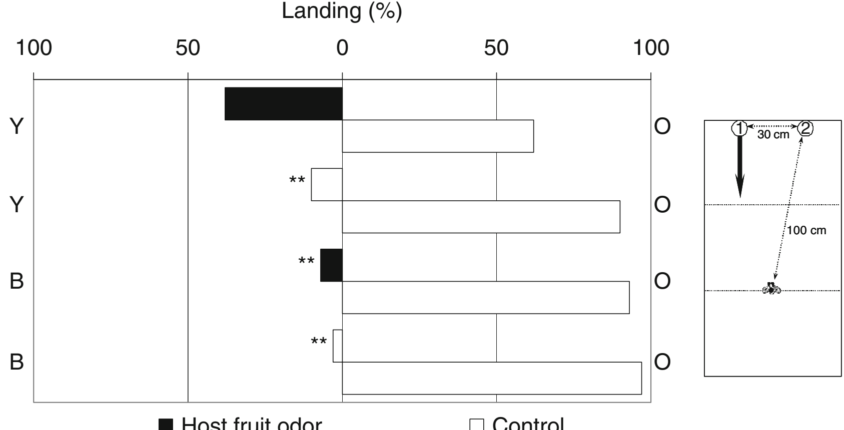

Fig. 4 Behavioral response of individually tested *Neoceratitis cyanescens* 8-d-old mature females to a combination of visual (B-blue, Y-yellow, or O-bright orange spheres) and olfactory (host fruit odor) cues in 2-choice tests. *N*=30 flies. The two spheres and the odor source are placed at location '1' or '2', 100 cm distance from the released fly. A χ*2-test* is performed to compare the distribution of landings of flies with a theoretical distribution (50–50%). The source of fruit odor is composed of 100 g unripe fruit from bugweed (*Solanum mauritianum*)

host fruit odor was combined with the bright orange sphere (75%).

In the specialist fruit fly *R. pomonella*, host fruit odor also interplays in stimulating visual search activity during host finding by females when fruit are less apparent or scarce (Aluja and Prokopy 1993). Szentesi et al. (1996) observed that olfactory stimulation favored landing of adults of the grasshopper, *Melanoplus sanguinipes* (F.) (Orthoptera, Acrididae) on visual models, with more directional searching flight and less time. Similar additive interactions between visual and olfactory stimuli in host location have been reported for the mountain pine beetle *Dendroctonus ponderosae* Hopkins (Campbell and Borden 2006) and the pine weevil, *Hylobius abietis* L. (Coleoptera, Scolytidae) in finding an undamaged conifer seedling (Björklund et al. 2005). In the leafhopper *Dalbulus maidis* (De Long and Wolcott) (Homoptera, Cicadellidae), plant volatiles act as a synergist by increasing the number of contacts made by insects with green light (Todd et al. 1990). Searching activity of parasitoids such as *Microplitis croceipes* (Cresson) (Wackers and Lewis 1993), *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera, Braconidae) (Jang et al. 2000), and *Apocephalus paraponerae* Borgmeier (Diptera, Phoridae) (Morehead and Feener 2000) increase when olfactory cues are combined with visual cues. In *Manduca sexta L.* (Lepidoptera, Sphingidae), a nectarivorous nocturnal hawkmoth attracted by a range of stimuli including floral volatiles and visual display, Goyret et al. (2007) showed that transient olfactory stimulation before visually guided approach (temporal decoupling) enhanced responsiveness to an odorless visual target. Under natural conditions, the integration of bi- or multi-modal information may be viewed as adaptive, as it improves the searching efficiency within resource patches of variable and complex environments.

In the absence of wind to carry the olfactory information, *N. cyanescens* mature females relied on visual information to locate the bright orange sphere. In a two-choice situation between two bright orange spheres, flies were able to detect

Table 5 Behavioral response of individually tested *Neoceratitis cyanescens* 8-d old mature females to a combination of visual (yellow or bright orange spheres) and olfactory (host fruit odor) cues in 2-choice tests

Color of sphere	Sphere location	Odor source	*N*	Landing site as first choice (%)	Landing site 1 min later (%)
Bright orange	3	control	30	83.3	10.0
Bright orange	0	Host fruit		16.7	90.0
		χ^2_1 *(P)*		7.5 (0.006)	11.4 (0.001)
Yellow	3	control	30	30.0	0.0
Bright orange	0	Host fruit		70.0	100.0
		χ^2_1 *(P)*		2.5 (0.114)	20.0 (<0.001)

N number of tested flies

The two spheres were placed at location '0' or '3', respectively 100 and 90 cm distance from the released fly. The odor source was placed at location '0' (Fig. 3d). A χ*2-test* was performed to compare the distribution of landings of flies with a theoretical distribution (50–50%). The source of fruit odor was composed of 100 g unripe fruit from bugweed (*Solanum mauritianum*).

the sphere emitting the host odor only in the wind, suggesting that host fruit odor can assist flies to locate the adequate fruit when visual stimuli are similar to those of non host plants. However, flies did not discriminate between an odorless bright orange sphere and a yellow sphere (poor signal) with host fruit odor, but they preferred to land on the odorless bright orange sphere when presented with a blue sphere with host fruit odor. This result indicates a dominance of visual cues in the decision of landing.

Furthermore, flies preferentially landed on the odorless bright orange sphere (83.3%) placed 10 cm in front of that emitting host fruit odor, although this stop constituted a transitional step. When the odorless orange sphere was replaced by a yellow sphere, flies landed preferentially on the bright orange sphere. When odor source and fruit model were spatially decoupled, most flies engaged in an oriented flight toward the odor source. However, some were able to turn back while in flight or after landing to orient themselves toward the fruit model, thus suggesting visual information to be the ultimate indicator of host fruit. In a comparable dual-choice experiment, *M. sexta* moths showed a strong bias for the visual display over the odor plume, suggesting the former to be the ultimate indicator of a nectar source (Goyret et al. 2007).

Efficiency of host finding by insects entails the use of informative, accurate and easy to assess multimodal cues (Fawcett and Johnstone 2003). Flies initially may use specific volatiles emitted by host fruit (including flower blends) and carried by wind to locate an appropriate habitat, by tracking wind-borne odor known as odor-guided optomotor anemotaxis (Schoonhoven et al. 2005; Cardé and Willis 2008) and 'aim-then-shoot' tactics (Brévault and Quilici 2010). Olfactory cues emitted by the host foliage then could be an indicator of an appropriate habitat, leading flies to search for a visual image to detect individual fruit at a short distance (Brévault and Quilici 2007b).

However, integration of olfactory signals enhances searching efficiency, both in terms of speed and accuracy. Synergism between olfactory and visual cues supports the more general hypothesis that foragers may benefit by integrating multimodal information of the plants they encounter in nature (Campbell and Borden 2006). Flies can be guided by the odor plume when fruit are scarce or obstructed by dense canopy, whereas visual orientation will enable them to stay on course when it loses the odor trail. The response of immature females and males to olfactory stimuli could favor, respectively, long range dispersal towards potential host plants, or the search for a sexual partner.

The positive response of flies to olfactory plant stimuli in the wind tunnel (quantity and reliability of odor release) may not reflect the natural situation that flies experience at a short distance to a host plant. Furthermore, complete absence of turbulence is rare under natural conditions where volatiles are carried away from the source with the prevailing direction of air flow and will be dispersed as pockets of odor. Moreover, host plants may be scattered between non-host plants (Schoonhoven et al. 2005). In this study, we observed the behavioral response of naive host-seeking flies that had no previous contact with the host plant. Experience may enhance decision-making or induce changes in preference due to enhanced egg maturation resulting from contact with hosts (Papaj 2000) or through behavioral changes such as sensitization (Bell 1990; Schoonhoven et al. 2005). For example, *Bactrocera dorsalis* (Hendel) females previously exposed to kumquats locate more easily this fruit than females exposed to various other hosts (Prokopy et al. 1990). Similarly, learning influences the visual ability of *R. pomonella* females to detect host fruit (Prokopy et al. 1994). Experience with the host then may modify the ranking and specificity of host location stimuli, so that a more specific search image and perhaps olfactory cues are used subsequently (Rausher 1978; Vet et al. 1990, 1995; Wackers and Lewis 1993; Landolt and Molina 1996).

As opposed to most tephritids of economic significance on Reunion Island, no effective lure or bait is known for *N. cyanescens*. Knowledge of the interaction between visual and olfactory stimuli involved in host location could be useful for developing trapping systems (Katsoyannos 1989). In commercial orchards, red sticky spheres mimicking apples were improved by the addition of specific volatile compounds for monitoring *R. pomonella* females seeking host fruit (Zhang et al. 1999). Our next step will be to identify and formulate the minimal blend of volatile compounds that causes attraction of *N. cyanescens* mature females.

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Aphid Feeding Activates Expression of a Transcriptome of Oxylipin-based Defense Signals in Wheat Involved in Resistance to Herbivory

Charles Michael Smith · Xuming Liu · Liang J. Wang · Xiang Liu · Ming-Shun Chen · Sharon Starkey · Jianfa Bai

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Abstract Damage by the Russian wheat aphid (RWA), *Diuraphis noxia*, significantly reduces wheat and barley yields worldwide. In compatible interactions, virulent RWA populations flourish and susceptible plants suffer extensive leaf chlorophyll loss. In incompatible interactions, RWA reproduction and population growth are significantly reduced and RWA-related chlorophyll loss in resistant plants is minor. The objectives of this study were to develop an understanding of the molecular and phytochemical bases of RWA resistance in plants containing the *Dnx* resistance gene. Microarray, real-time polymerase chain reaction, and phytohormone assays were conducted to identify transcriptome components unique to RWA-infested *Dnx* plants and susceptible (*Dn0*) plants, and to identify and characterize putative genes involved in *Dnx* plant defense responses. We found that RWA-infested *Dnx* plants upregulated >180 genes related to reactive oxygen species, signaling, pathogen defense, and arthropod allelochemical and physical defense. The expression of several of these genes in RWA-infested *Dnx* plants increased significantly from 6- to 24-h post infestation (hpi), but their expression in *Dn0* plants, when present, was delayed until 48- to 96 hpi. Concentrations of 16- and 18-carbon fatty acids, *trans*-methyl-12-oxophytodienoic acid, and abscisic acid were significantly greater in *Dnx* foliage than in *Dn0* foliage after RWA infestation, suggesting that *Dnx* RWA defense and resistance genes may be regulated via the oxylipin pathway. These findings provide a foundation for the elucidation of the molecular basis for compatible- and incompatible plant-aphid interactions.

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C. M. Smith (✉) · X. Liu · S. Starkey
Department of Entomology, Kansas State University,
Manhattan, KS 66506, USA
e-mail: cmsmith@ksu.edu

L. J. Wang
Division of Biology, Clemson University,
Clemson, SC 29631, USA

X. Liu
Department of Plant Biology, North Carolina State University,
Raleigh, NC 27695, USA

M.-S. Chen
USDA/ARS Plant Science Laboratory,
Manhattan, KS 66506, USA

J. Bai
Department of Diagnostic Medicine/Pathobiology,
College of Veterinary Medicine, Kansas State University,
Manhattan, KS 66506, USA

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Introduction

Aphids (Order Homoptera) are major arthropod pests of agriculture worldwide, damaging crops by removing photoassimilates and vectoring numerous devastating plant viruses. The limited tissue damage caused during aphid feeding and the prolonged interactions of aphid mouthparts with plant cells make plant responses to phloem-feeding aphids distinct from those of chewing insects (Walling 2000). Many species of aphids are resistant to insecticides (Devonshire and Field 1991), and some have developed virulence to plant aphid

resistance genes. Environmental concerns linked to insecticide use have led to the development and cultivation of many aphid-resistant crop varieties during the past century (Panda and Khush 1995; Smith 2005).

The Russian wheat aphid (RWA), *Diuraphis noxia*, is a serious pest of wheat, *Triticum aestivum*, and barley, *Hordeum vulgare*, and with the exception of Australia, the aphid has been introduced globally since the early 1900s (Quisenberry and Peairs 1998). In compatible interactions with RWA, susceptible wheat plants react to the injection of RWA saliva by rolling the leaves longitudinally around the main leaf vein to form a tubular refuge that protects aphids from predators. As a result, RWA populations flourish and plants suffer extensive leaf chlorophyll and carotenoid loss (Burd and Elliott 1996; Heng-Moss et al. 2003). These reductions also are manifested as significant reductions in photosynthetic efficiency that results in weakened plants with substantially lower grain yields (Smith et al. 1991). In incompatible interactions that involve plants containing the RWA-resistance genes, RWA reproduction and population growth are significantly reduced, and chlorophyll loss is minor.

Heritable resistance to pest insects has been widely documented in many cereal, forage, fruit, and vegetable crop plants (Smith 1989) and single resistance (R) genes inherited as dominant traits control resistance in both monocots and dicots (reviewed in Smith 2005). Resistance may be categorized as antibiosis (an adverse effect on insect biology—including mortality), antixenosis (an adverse effect on insect behavior), or tolerance (the ability of a plant to withstand insect damage). Many insect-resistant plants, including RWA-resistant barley and wheat, contain multi-category resistance (reviewed in Berzonsky et al. 2003; Smith 2005).

Ten RWA (*Dn*) resistance genes from cereal crops have been identified (Smith 2004; Liu et al. 2005) and are being deployed in the U.S. (Quick et al. 1996) and South Africa (Prinsloo 2000). However, RWA virulence occurs in Africa (Malinga et al. 2007; Tolmay et al. 2007), Asia (Dolatti et al. 2005), Europe (Basky 2003), North America (Burd et al. 2006), and South America (Smith et al. 2004).

An understanding of the molecular bases of plant-aphid interactions is progressing, albeit slowly, based on the identification of plant genes that control aphid resistance. The NBS-LRR gene *Mi-1.2* from *Lycopersicon peruvianum* controls resistance to the potato aphid, *Macrosiphum euphorbiae* (Thomas), and to three species of the root knot nematode, *Meloidogyne* spp. (Kaloshian et al. 1997; Vos et al. 1998). No monocot insect R genes have been cloned, but the transcript levels of NBS-LRR genes are affected in plants infested by aphids (Lacock et al. 2003; Klingler et al. 2005; Botha et al. 2006; Park et al. 2006). Aphid feeding activates plant defense signals similar to those involved in response to pathogen infection (Tjallingii and Hogen-Esch 1993; Walling 2000; Kaloshian 2004), but the origin of aphid elicitors of these signals is poorly understood (Urbanska et al. 1998; Miles 1999; Forslund et al. 2000).

The recognition of aphid probing and sustained feeding results in transmission of defense response signal cascades that involve jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and gibberellic acid (GA) (Smith and Boyko 2006). At the onset of aphid-plant interactions, aphid feeding results in the altered activation of peroxidases, intercellular chitinases, and β-1,3-glucanases involved in the release of plant cell wall oligosaccharides (Smith and Boyko 2006). Reactive oxygen species (ROS) elicitors that respond to aphid feeding may also upregulate the activity of these enzymes. Peroxidase (PER), glutathione transferase (GST), catalase (CAT), nitrate reductase, and quinone oxidoreductase genes are upregulated in aphid-infested plants (Martinez de Ilarduya et al. 2004; Zhu-Salzman et al. 2004; Divol et al. 2005; Boyko et al. 2006; Park et al. 2006; Couldridge et al. 2007).

Interactions between aphids and their host plants elicit the activation of both the JA and SA defense response pathways and the related upregulation of genes controlled by each of these plant hormones. Examples of these interactions include greenbug feeding on sorghum (Zhu-Salzman et al. 2004), the peach potato aphid (PPA), *Myzus persicae*, feeding on *Arabidopsis thaliana* and potato, *Solanum tuberosum* (Fidantsef et al. 1999; Moran and Thompson 2001; Moran et al. 2002), and potato aphid feeding on potato and tomato (Fidantsef et al. 1999; Martinez de Ilarduya et al. 2004). Results of experiments with RWA feeding on wheat, and the tobacco aphid, *Myzus nicotianae*, feeding on wild tobacco, *Nicotiana attenuate*, indicate the involvement of JA signaling but only marginal activity of SA signals in these interactions (Voelckel et al. 2004; Boyko et al. 2006).

Ethylene production increases significantly after aphid feeding on the foliage of aphid-resistant barley or wheat plants compared with susceptible plants (Miller et al. 1994; Argandona et al. 2001; Boyko et al. 2006), and sequences that code for proteins involved in ET production are over-expressed in aphid-infested *Arabidopsis* and celery, *Apium graveolens*, plants (Moran et al. 2002; Divol et al. 2005). Jasmosinc acid and ET are synergistic in the defense responses of *Arabidopsis* resistance to the peach potato aphid (Dong et al. 2004) and in the induction of defense responses in squash, *Cucurbita moschata*, foliage to feeding by the silver leaf whitefly, *Bemisia argentifolii*, (van de Ven et al. 2000). WRKY proteins (with the conserved amino acid sequence WRKYGQK) modulate JA-SA interactions in *Arabidopsis* pathogen response (Li et al. 2004) and *WRKY* upregulation in tobacco plants infested by the tobacco aphid suggests that JA-SA interactions also play a role in plant defense responses to aphids (Voelckel et al. 2004). Sequences putatively involved in ABA and GA biosynthesis are

upregulated in aphid-infested foliage of celery, sorghum, and wheat (Zhu-Salzman et al. 2004; Divol et al. 2005; Boyko et al. 2006; Park et al. 2006).

The objectives of this study were to develop an understanding of the molecular and phytochemical bases of RWA resistance in wheat plants that contain the *Dnx* RWA resistance gene. Microarray, real-time PCR, and phytohormone assays were used to identify transcriptome components and phytohormones that were differentially regulated in RWA-infested *Dnx* plants and susceptible (*Dn0*) plants. Previous research (Dong et al. 2004; Voelckel et al. 2004; Boyko et al. 2006; Park et al. 2006), led us to hypothesize that unique components of the *Dnx* transcriptome in the ROS, oxylipin, ABA, and terpenoid pathways are activated by RWA feeding, and that these proteins may function to mediate the expression of the *Dnx* plant phenotype.

Methods and Materials

Plant Genotypes and Aphids A wheat landrace from Afghanistan (USDA Plant Introduction 220127) (Harvey and Martin 1990) containing the RWA resistance gene *Dnx* (Liu et al. 2001) was crossed to the RWA-susceptible wheat genotype 'Sando's Selection 4040' (*Dn0*), originally developed at Oklahoma State University. F_2-derived F_3 family plants originating from this cross were bulked into groups of 10 resistant plants and 10 susceptible plants according to their reaction to RWA biotype 1 (RWA1) infestation (Liu et al. 2001). Bulk lines were advanced to the F_4 generation. Seeds of resistant and susceptible F_4 lines were planted in 12 cm diam plastic pots containing Jiffy® potting mix and grown in the greenhouse during July 2005 at 30°C day: 22°C night, and a photoperiod of 14:10 (L:D). Supplemental lighting was provided from sodium halide lamps. Experiments were conducted in the greenhouse under the same conditions. Seed stocks are currently maintained by the Plant Resistance Laboratory, Department of Entomology, Kansas State University (KSU), Manhattan, KS, USA. RWA biotype 1 (RWA1) used in all experiments originated from a culture established with aphids collected from Hays, KS, in 2004, courtesy of Dr. J. P. Michaud, KSU Dept. of Entomology, Hays, KS. The culture was maintained on RWA-susceptible 'Jagger' wheat plants at the environmental conditions described above for plant growth.

In total, 140 plants with the *Dnx* gene and 110 plants lacking the gene (*Dn0*) were assessed across five different experiments. These included plant phenotyping experiments to classify plant and aphid responses to each other (20 plants of each genotype), a microarray experiment to assess wheat EST expression (90 *Dnx* plants, 60 *Dn0* plants), northern blot verification of microarray results (9 plants of each genotype), real-time PCR verification of microarray results (9 plants of each genotype), and phytohormone experiments (12 plants of each genotype).

Phenotype Evaluations The previously reported resistance of *Dnx* and susceptibility of *Dn0* plants to RWA1 (Liu et al. 2001; Boyko et al. 2006) was confirmed in experiments to measure phenotypic damage expression, tolerance resistance, and antibiosis resistance with protocols of Boyko et al. (2006). Pre-germinated *Dnx* and *Dn0* F_4 seeds were planted in pots and allowed to grow to the two-leaf stage. Plants were paired for height and growth (*Dnx* with *Dnx*, *Dn0* with *Dn0*), and one plant of each pair was infested with five RWA1 late stage nymphs. The remaining plant of each pair was not infested and used as a control. All pots were covered with individual nylon-mesh cages, and 10 pairs (replicates) of plants were arranged in a randomized complete block design. When the infested *Dn0* plants showed complete leaf rolling and 95% chlorosis of the youngest leaf (~21 d), cages were removed and leaf chlorosis, leaf rolling and leaf folding damage sustained from RWA1 feeding was rated on a 0–3 scale for each symptom; where 0 = no damage, 1 = <50% symptoms, 2 = >50% symptoms and 3 = 100% symptoms/ dead plant) (Smith et al. 1991).

Tolerance resistance, the ability of a plant to withstand arthropod damage and yield significantly greater dry mass than a susceptible plant under similar conditions of infestation (Smith 2005), was measured by calculating the proportional plant dry weight change (DWT) of *Dnx* and *Dn0* plants as $DWT = [(WC - WT)/WC] \times 100$, where WC was the dry weight of the uninfested control plant, and WT was the dry weight of the infested plant (Reese et al. 1994). The shoots from uninfested and infested plants were cut at the soil surface, placed in aluminum foil pouches, dried in an oven at 75°C for 72 h, and tissue weights were determined.

Antibiosis resistance, in which the plant adversely affects the growth and development of the arthropod, was determined by counting the numbers of RWA1 on the infested plants in each of the pairs of plants in the tolerance experiment. Aphids produced on infested plants of each genotype were removed with a camel's-hair brush, collected on wax paper, funneled into vials of 95% EtOH, and counted. A tolerance index (TI) was calculated by using the equation: plant DWT/number of aphids produced on the infested plants (Reese et al. 1994). The plant TI was determined to compensate for the confounding effect of differing numbers of RWA1 on infested plants. Genotypes with TI values significantly lower than those of the susceptible control plants were considered tolerant. Data for plant damage, plant DWT, plant TI, and RWA1 population development were subjected to ANOVA by using the SAS GLM procedure (SAS 2001). Where significant, treatment means were separated by using the LSD at α=0.05.

RNA Extraction For the microarray hybridization experiments, RNA was extracted from leaves of *Dnx* and *Dn0* plants at the two-leaf stage of growth when the 3rd leaf was beginning to unfurl. Plants were grown in the greenhouse as mentioned previously and were not under drought stress. There were three replications of *Dnx* infested plants, three replications of *Dnx* uninfested plants, two replications of *Dn0* infested plants, and two replications of *Dn0* uninfested control plants. Each replicate of each treatment contained 15 plants. Each of the 15 treatment plants was infested with approximately 50 RWA1 late stage nymphs and adults. In each treatment replicate, leaves of all plants were harvested 24 h after infestation, pooled, quick frozen in a freezer at −80°C, and ground in liquid N_2. Total RNA was extracted from pooled leaves with TRI Reagent™ following the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH, USA). RNA samples were purified with a RNease Kit following the manufacturer's instruction from QIAGEN Inc. (Valencia, CA, USA). RNA concentrations were measured by using a NanoDrop-1000 Spectrophotometer from NanoDrop Technologies (Wilmington, DE, USA). RNA quality was checked with an Agilent 2100 Bioanalyzer following the Reagent Kit guide from Agilent Technologies (Foster City, CA, USA).

Microarray Hybridization and Data Analysis The Affymetrix GeneChip® Wheat Genome Arrays (Santa Clara, CA, USA) contain 61,127 probe sets representing 55,052 transcripts for all 42 chromosomes in the wheat genome on a single array. The array includes ESTs from *T. monococcum, T. turgidum*, and *Ae. tauschii,* and GenBank® full-length mRNAs from all species through May 18, 2004. Labeling and hybridization of arrays were performed according to the standard protocol provided by Affymetrix. (www.affymetrix.com/support/technical/manual/expression_manual.affx). After hybridization and washing, arrays were scanned with an Affymetrix GeneChip Scanner 3000. Hybridization quality was verified by scaling factor, overall hybridization rate, and signal strength of several bacterial spike controls. The spike controls were hybridized with labeled targets in different concentrations resulting in particular ratios between different spikes.

Image acquisition, image settings, and raw data generation were accomplished with Affymetrix GeneChip® Operating Software (GCOS). After alignment of the image settings for each chip, raw data were extracted and marked as "present" (well above the background), "marginal," or "absent" (similar to, or below the background) under default settings. The overall target signal intensity of each chip was scaled to 500 and then loaded into GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA) for further analysis. The data were pre-normalized to the 50th percentile per chip and then normalized based on per-gene normalization with the median method, from which the signal of each gene in a particular sample was divided by the median signal of the same gene in all samples.

Normalization per gene and per chip of the log2 values was performed to allow the comparison of the independent replicates performed in the experiment. Normalization was performed for all measurements by using the flags ("present," "marginal," or "absent") assigned by Affymetrix treatment of the arrays. However, only those transcripts that were declared present or marginal in at least 4 of the 6 microarrays hybridized with RWA-resistant *Dnx* probes and 3 of the 4 arrays hybridized with RWA-susceptible *Dn0* probes were taken into account. This procedure facilitated the elimination of transcripts with very low signals in both treatments (declared "absent").

The resulting genes that exhibited significant changes in expression in comparisons between treated and control *Dnx* or *Dn0* plants were selected by applying a *t*-test (one-way ANOVA Welch *t*-test, $P>0.05$ with "Benjamini and Hochberg False Discovery Rate" for multiple comparisons $\alpha=0.05$). A cutoff value of a 2-fold change, commonly used for microarray analysis, was used to discriminate the expression of genes that were differentially altered in response to RWA feeding. Fold change values were calculated between treatment and control samples based on the normalized average measurement of the signal intensity. The number of sequences examined was further narrowed after eliminating those with raw expression values of <600 units.

Comparisons of changes in differential up- or downregulation of gene expression between RWA-infested *Dnx* and *Dn0* plants were discriminated by using the CEDA (Comparative EST Data Analysis) virtual subtraction procedure developed by Wang and Zhang (2004). Genes expressed in uninfested control plants were removed from those in infested plants with the formula: [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)] for *Dnx* plants, and [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)] for *Dn0* plants. The CEDA output provided a list of candidate up- or downregulated genes, uniquely expressed in *Dnx* infested plants or *Dn0* infested plants, and sorted by the statistical significance of change in gene expression.

The nucleotide sequences identified after probing and hybridization of arrays were clustered by using the CAP3 software tool with default settings (Huang and Madan 1999). From 61,290 array probe sequences, a non-redundant dataset containing 7,511 contigs and 42,863 singletons was obtained that allowed the comparison of the expression of different probes mapped to a single contig. To understand gene function, non-redundant sequences were searched against the UniProt reference database (Bairoch et al. 2005) with the BLASTX program downloaded from the NCBI site (ftp://ftp.ncbi.nih.gov/blast/). Queried sequences were annotated by using the best hit in UniProt with an E value threshold of 1^{e-5}. Functional annotation included text

description as well as gene ontology terms of each matched reference sequence (Camon et al. 2003), to understand the biological processes involving genes of interest. Unique known transcripts in *Dnx* and *Dn0* plants were compared for similarity to known genes, and these genes were classified by function.

Northern Blot Analyses To validate the expression of ESTs in the microarray hybridzation experiment, a separate northern blot experiment was conducted with total RNA isolated from fresh leaf tissue of RWA-infested resistant (containing the *Dnx* gene) and susceptible (containing the *Dn0* gene) wheat plants infested with 20 RWA biotype 1 late stage nymphs per plant. Tissues were collected at 6-, 12-, 24-, 72-, and 120 h post-infestation, and RNA was extracted with TRI reagent™ (Molecular Research Center). Each treatment consisted of RNA pooled from two biological replicates, and each replicate contained leaf tissue from 3 plants, for a total of 6 plants. Three separate uninfested *Dnx* plants and 3 separate uninfested *Dn0* plants served as non-infested controls. Tissues were collected from plants at each time point. Five µg of total RNA from each treatment were subjected to electrophoresis in denaturing 1.5% agarose gels containing formaldehyde and transferred onto a GeneScreen membrane (Perkin-Elmer Life Science Inc., Boston, MA, USA). Gels were stained with ethidium bromide, and rRNA levels were compared to control for equal loading. Membranes were baked at 80°C for 2 h to fix the RNA and then hybridized separately to individual probes. Primers for PCR amplification were generated at the KSU Core Biotechnology Facility by using selected sequences from the Affymetrix GeneChip® Wheat Genome Array EST files (Table 1). Probes were derived by PCR amplification of plant template cDNAs and labeled with ^{32}P-dCTP by using the random labeling kit from Stratagene (La Jolla, CA, USA). Prehybridization (4 h) and hybridization (overnight) were carried out at 42°C in hybridization buffer (Chen et al. 2004). Membranes then were washed at medium stringency [2x SSC at room temperature for 30 min, 2x SSC, 1% sodium dodecyl sulphate (SDS) at 65°C for 30 min, and 0.1x SSC, 0.1% SDS at room temperature for 30 min]. Blots were placed against X-ray film (Kodak) and hybridization patterns were visualized by autoradiography.

Real-time Quantitative PCR Analyses Real-time PCR was used to confirm the expression of six stress response or cellular metabolism genes in leaf tissues from both *Dnx* and *Dn0* plants. These genes had been shown previously to be highly downregulated in the transcriptome of *Dnx* plants at 24 hpi. Genes included Q9P3N1 (hypothetical stress response protein), Q5ZD81 (calmodulin-like protein), Q6Z1A3 (putative NAC1 stress response protein), Q6Z1A3 (putative cytochrome P450), Q7XN01 (transcription protein), and Q6I5G9 (mitochondrial ATP synthase). Primers were designed based on Affymetrix™ Gene Chip EST sequences and GeneBank by using the software package Beacon Designer. Primers sequences are shown in Table 1.Wheat actin (AB181991) was used as an internal control. Total RNA was isolated as described for northern blotting. After purification with TURBO™ DNA-free (Ambion, Austin, TX), 2 µg total RNA were reverse transcribed into cDNA by using a SuperScript III First-Strand cDNA Synthesis System (Invitrogen) following the manufacture's protocols.

The change in expression of these genes was determined at 6-, 12-, 24-, 72-, 96-, and 120 hpi in tissues from *Dnx* and *Dn0* plants infested with 20 RWA biotype 1 late stage nymphs per plant. For each post-infestation—genotype treatment, RNA was collected from two biological replicates, each consisting of leaves pooled from 3 plants. Three separate uninfested *Dnx* plants and 3 separate uninfested *Dn0* plants served as non-infested controls. Real-time PCR was performed with iQ™ SYBR Green Supermix (BIO-RAD) by using the flowing amplification protocol: 5 min denaturation at 95°C, 40 cycles of 30 s at 95°C, 20 s at 53°C, and 45 s at 72°C. This was followed by product melt to confirm a single PCR product. Gene downregulation fold change was calculated as: $2^{\Delta\Delta Ct}[\Delta\Delta Ct = (Ct_{GOI}In - Ct_{GOI}Un) - (Ct_{HKG}In - Ct_{HKG}Un)]$, where GOI = gene of interest, In = infested sample, Un = uninfested sample, HKG = wheat-actin gene. Data for mean downregulation of each gene were subjected to ANOVA by using the SAS GLM procedure (SAS 2001). Where significant, treatment means were separated by using the LSD at α=0.05.

Phytohormone Analyses Individual two leaf stage plants containing *Dnx* or *Dn0* were grown in 10 cm diam. plastic pots filled with Pro-MixBx® potting mix, in the greenhouse [24°C day: 20°C night, photoperiod of 14:10 (L:D)]. Six *Dnx* or 6 *Dn0* plants were each infested for 12-, 24-, 48-, or 96 h with 20 to 30 RWA1 late stage nymphs and adults per plant. Six uninfested *Dnx* or *Dn0* plants collected at 24 h post-infestation served as uninfested controls for all infested *Dnx* or *Dn0* treatments. At the end of each infestation period, all above ground foliage from plants in each treatment were harvested, placed a in freezer at −80°C, ground to powder in liquid N_2, weighed, and analyzed.

Plant powders were extracted in 300 µl cold 1-propanol : H_2O : HCl (2:1:0.005), centrifuged for 1 min, the organic layer transferred to a glass tube, and diluted with 20 µl of 2 M trimethylsilyldiazomethane:methylene chloride (1:4). Each extract then was combined with 20 µl of (12:88 acetic acid:hexane) : methylene chloride (1:4) and allowed to stand overnight to quench methylation. Samples were placed in SuperQ columns, where phytohormones were

Table 1 Oligonucleotide primers used for isolation and expression analysis of wheat proteins involved in defense responses to Russian wheat aphid biotype 1

Purpose/Primer name	Sequence (5′—3′)
Northern Blot Analyses	
Q9XEN7 β-1,3-glucanase	
5′	CTCTTCAACCCGGACAAATC
3′	TGAAGAATTTGGGCGTTTTC
Q8W427 Chitinase III	
5′	CGACAACCTGGACTGCTACA
3′	ATGGATCGCACCATTATTCG
Q01482 *WIR1A* Membrane protein	
5′	CTCCTGCAGATCGCTCTCTT
3′	CCGGTGGTCTACATCCGTAA
Q43212 Peroxidase precursor	
5′	AACACTGTCCGGAACTTTGC
3′	TGTCGTGCTGGCTAGTATGC
Q8S702 Glutathione S-transferase	
5′	CCTCAGGGACTGCTCTAACG
3′	GTCCAACGATCCGAAGTTGT
Q8H8H7 Flavanone 3-hydroxylase	
5′	TACCGCAGCTACACCTACGA
3′	TGAGTAATGCTGCGTCGTG
Q9AVM3 Cytochrome P450	
5′	CATCATTGACATGTCCTGAAAA
3′	GGGCTTGCAGTAAGCAAAAA
Q5BQ31 Serine/threonine kinase	
5′	AAAAGGCACATAGCGTCCAT
3′	AGTGGTGGAGACCAGGTTTG
P27357 Thaumatin-like protein PWIR2 precursor	
5′	GCAGCACCCAGGACTTCTAC
3′	TGCGACGTATAGAGGCTTCA
P29114 *LOX1* Lipoxygenase 1	
5′	GATCGAGAGCAAGGTGGTG
3′	TCAGATGGAGATGCTGTTGG
Q5NTH3 Shikimate kinase 2	
5′	ATCCATACACAGCGGCTTTC
3′	GTAGGGCCTCGACAGCAATA
P12940 Bowman-Birk trypsin inhibitor	
5′	GACCCATCCCTCAACGTCT
3′	ACACCTGCTGGCGTATTCAT
Q6Z676 *phi-1* ABA dehydration signaling	
5′	CACCTGTTCGACCTTGGTGT
3′	GAAAGCCAGTGCAGCAATTT
P93671 *XET* Xyloglucan endotransglycosylase	
5′	GTGGGTGCAGAGCAACTACA
3′	GGCGTAAATGCCAAAGAAGA
Q43210 *PAL* Phenylalanine ammonia lyase	
5′	ACCAGGGTAAGCACATCGAC
3′	ATCTTTGGCAATGGCCTCTA
Expression Analysis (qRT PCR)	
Q9P3N1 Stress response protein	
5′	CTTCACATCTAACGGGCATC
3′	ATGGAGGTGCTTGAGACG
Q5ZD81 Calmodulin-like protein	
5′	AGGGAAGGGAAAGGATAAAGTG
3′	CGACCTACAGACAGTACGC
Q6Z1A3 NAC1 Stress response protein	
5′	GGAGGTTACATTACATTTGGAGAG
3′	TGGAGTAGCATTGGACTATTGG
Q6YXE1 Cytochrome P450	
5′	TCATGGAGAAGAACAAGCAG
3′	GAGGCGGGTGTAGAAGAG
Q7XN01 Transcription protein	
5′	GCCATTGCGGAGTCACAAG
3′	TGGTTCGTCCTTCACTATGC
Q6I5G9 Mitochondrial ATP synthase	
5′	TCATGGAGAAGAACAAGCAG
3′	GAGGCGGGTGTAGAAGAG
TA1868 Wheat actin	
5′	GAGTCGGTGAAGGTTGTTTAC
3′	CTTAGGCAGCGTTTGGAATAC

removed by vapor phase extraction and eluted from columns with 100–200 µl of methylene chloride into GC vials. The residual solvent from each SuperQ column was removed and added to each sample vial with a N_2 stream. Due to sampling errors from damage to tissues or sample loss during vapor phase extraction, the number of replications analyzed for each phytohormone treatment varied from N=3 to N=6.

Samples were subjected to quantitative analyses by using an HP 6890 gas chromotograph and HP 5973 mass spectrometer in the KSU Lipidomics Research Center. Total ion counts were acquired and processed by using Agilent Chemstation software, AMDIS (www.amdis.net), and MET-IDEA (Broeckling et al. 2006; http://www.noble.org/plantbio/ms/MET-IDEA/index.html). Plant tissue data were normalized by fresh weight, and sample ion counts were expressed as per cent ng/g standard. Spectral data were subjected to log e transformation and analyzed by using SAS PROC GLM with unequal variance (SAS 2001). The procedures used for tissue preparation, extraction, sample acquisition, and GC-MS analyses are detailed in Schmelz et al. (2004).

Samples were evaluated for content of the following phytohormones and fatty acids: methyl abscisic acid (ABA); methyl benzoate (BA); methyl *trans*-cinnamate (CA); methyl salicylate (SA); *cis*- and *trans* methyl jasmonate (JA); *cis*- and *trans*-methyl-12-oxophytodienoic

acid (12-OPDA); the saturated 16:0 and 18:0 fatty acids, and the 16:1, 16:3, 18:1, 18:2, and 18:3 unsaturated fatty acids. Internal standards were as follows: ABA – (*S*)-5-(1-hydroxy-2, 6, 6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-(2Z,4E)-pentadienoic acid; BA—(ring-$^{13}C_6$) benzoic acid methyl ester; CA – (E)-3-phenyl-2-propenoic acid methyl ester; SA – 2-hydroxybenzoic acid methyl ester; and JA – 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid. The CA internal standard was used to calculate the amount of each methyl fatty acid, OPDA-Me trans, and OPDA-Me cis. In several previous experiments, the relative instrument response of CA/methyl 19:0 fatty acid was 0.137 ng/ng.

Results

Phenotype Evaluations F_4 plants containing the RWA1 resistance gene *Dnx* displayed limited leaf rolling symptoms (1.8±0.62, mean ± SE) and leaf chlorosis symptoms (1.8±0.13, mean ± SE), compared to F_4 plants lacking *Dnx* (*Dn0*) (mean ± SE leaf rolling=2.9±0.12, mean ± SE chlorosis=3.0±0.34). (Both symptoms rated on a 0–3 scale; 0=no damage, 3=100% symptoms).

Both antibiosis and tolerance function in RWA1 resistance imparted by other *Dn* genes (Hein 1992; Smith et al. 2004; Voothuluru et al. 2006), and in tolerance experiments infested *Dnx* plants sustained significantly ($P<0.009$) smaller changes in proportional plant dry weight (DWT) compared with *Dn0* plants (Fig. 1b). However, when RWA1 population sizes on *Dnx* and *Dn0* plants were used to compute a tolerance index (TI) to compensate for different population sizes, the observed differences in plant DWT were not significant (data not shown). Nevertheless, leaves of *Dnx* plants exhibited significantly less leaf rolling and leaf chlorosis compared to *Dn0* plants, thus demonstrating their ability to tolerate RWA1 feeding and chloroplast destruction.

Dnx and Dn0 Plant Transcriptomes There were distinct and dramatic quantitative differences in the unannotated transcriptomes of RWA1-infested plants and uninfested plants containing *Dnx* and *Dn0*. Infested *Dnx* plants upregulated 1,137 genes and downregulated 171 genes that were expressed at significantly ($P\leq0.05$) greater levels compared to uninfested control plants (Table 2). In contrast, infested *Dn0* plants significantly upregulated only 201 genes and downregulated 16 genes.

The CEDA virtual subtraction procedure (Wang and Zhang 2004) was applied to the data in Table 2 to identify genes uniquely expressed in *Dnx* infested plants or *Dn0* infested plants. This was accomplished by using the formulae [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)] for *Dnx* plants, and [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)] for *Dn0* plants. CEDA subtraction yielded 551 upregulated genes and 43 downregulated genes in infested *Dnx* plants (≥2-fold expression differences, $P\leq0.05$). Of these genes, 401 of the upregulated transcripts and 27 of the downregulated transcripts were of unknown function. After ontology and grouping, 161genes unique to RWA1-infested *Dnx* plants were significantly upregulated and only 17 genes were significantly downregulated (Table 3). After virtual subtraction of the *Dn0* transcriptome, followed by ontology and grouping, 38 genes were found to be significantly upregulated and 14

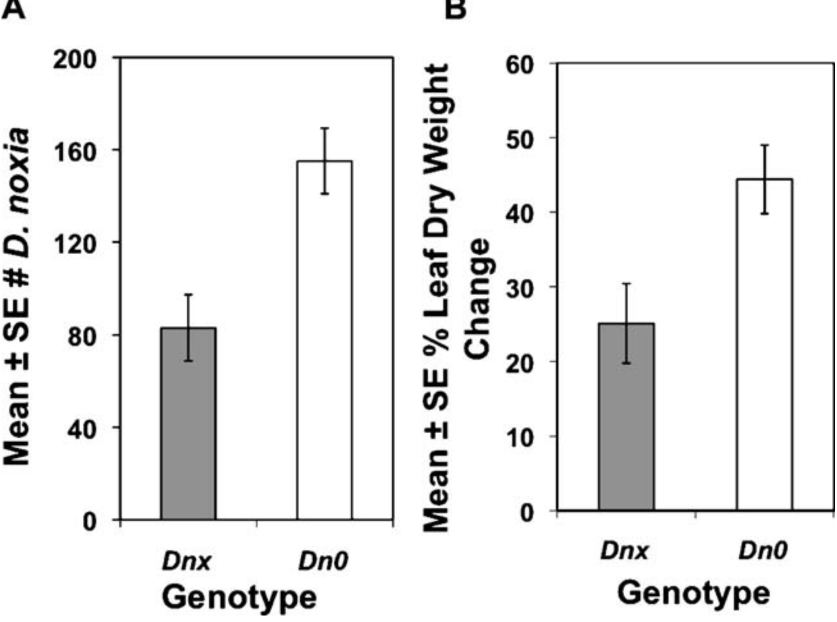

Fig. 1 Phenotypic responses of Russian wheat aphid biotype 1 (RWA1) and RWA1-resistant (*Dnx*) and susceptible (*Dn0*) plants at 21 d post infestation: (**a**) Mean ± S.E. RWA population development (differences significant at $P=0.001$); (**b**) Mean ± S.E. percent proportional leaf dry weight changes as determined by DWT = [(dry wt. of uninfested control plant−dry wt. of infested plant)/dry wt. of uninfested control] ×100 (differences significant at $P=0.009$); $N=10$

Table 2 Numbers of genes exhibiting significant changes in expression[a] in comparisons between infested[b] and uninfested plants of wheat genotypes resistant (*Dnx*) and susceptible (*Dn0*) to Russian wheat aphid biotype 1

Genotype/treatment comparison	Number of upregulated genes	Number of downregulated genes
Dnx infested versus *Dnx* uninfested control	1,137	171
Dn0 infested versus *Dn0* uninfested control	201	16

[a] $P \leq 0.05$, minimum 2-fold change

[b] 24 h post infestation

genes were found to be significantly downregulated (Table 3). The putative functions of affected transcripts in both genotypes were related to plant cell wall disruption; the initiation of defense responses; ROS production; ABA-, ET-, JA-, and SA signaling; phytopathogen defense responses; and arthropod allelochemical and physical defenses (Table 3). We found differences in the *Dnx* and *Dn0* transcriptomes after annotation (Table 3), compared to before annotation (Table 2), as well as before and after application of the CEDA virtual subtraction procedure.

Plant Cell Wall Disruption Numerous transcripts with putative functions in the cell wall were 2- to 6-fold upregulated in infested *Dnx* plants (Supplemental Data Table 1). These transcripts include cereal β-1,3-glucanase (*GLG*), chitinase (*Chia)*, protein kinase, and WIR1 (*WIR1*) membrane proteins based on sequence similarity. Among them, *GLG* and *Chia* were completely absent in infested *Dn0* plants. On the other hand, infested *Dn0* plants expressed two membrane proteins (Supplemental Data Table 2) that were not expressed in infested *Dnx* plants.

On northern blots, *GLG*, *Chia-3*, and *WIR1B* exhibited a similar expression pattern (Fig. 2a). They were expressed very little in uninfested *Dnx* plants, upregulated slightly at 6 hpi, and strongly at 12 hpi in infested *Dnx* plants. The elevated transcript levels in infested *Dnx* plants gradually decreased after 24 hpi. These three genes were either not upregulated or much less upregulated in infested *Dn0* plants until 72 or 120 hpi, when a significant elevation of the level of these transcripts was observed.

ROS Production Several genes related to *ROS* metabolism, including *GST*, Ca^{++} and Fe^{++} binding proteins, *CYP450*, oxidoreductases, and peroxidases, were 2- to 8-fold upregulated in infested *Dnx* plants responding to RWA1

Table 3 Numbers of annotated genes in different functional classes expressed[a] in wheat plant leaves containing the *Dnx* resistance gene or *Dn0* susceptibility gene after phloem feeding by Russian wheat aphid biotype 1[b] as determined by the Comparative EST Data Analysis virtual subtraction procedure[c]

Gene functional class	Number of upregulated genes		Number of downregulated genes	
	Dnx	*Dn0*	*Dnx*	*Dn0*
β-glucanases	7	0	0	0
Chitinases	5	0	0	0
Membrane proteins	7	2	4	1
Protein kinases	12	0	1	1
GST, Ca^{++}, Fe^{++} binding	10	7	4	2
Oxidoreductase/Hydroxylase	20	4	3	1
SA signaling	16	3	0	1
JA signaling	8	5	0	0
ABA signaling	4	0	0	0
ET signaling	2	0	0	0
AUX signaling	0	4	0	0
Arthropod allelochemical defense	18	4	0	0
Pathogen defense	15	1	0	0
Arthropod structural defense	37	1	0	2
Metabolism	0	7	5	6
Total number of genes	161	38	17	14

[a] $P \leq 0.05$, minimum 2-fold change

[b] 24 h post infestation

[c] *Dnx* plants = [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)];
Dn0 plants = [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)]

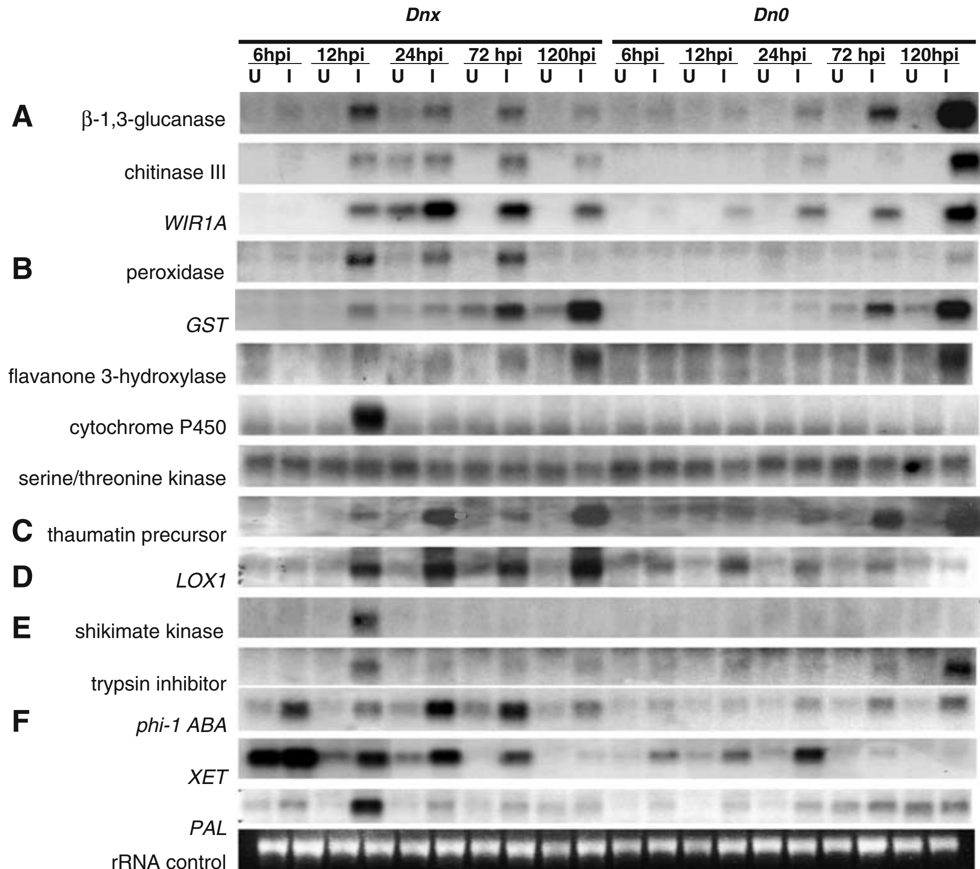

Fig. 2 Temporal upregulation of wheat transcripts from Russian wheat aphid biotype 1 (RWA1)—infested (I) and control uninfested (U) plants containing the RWA1 resistant (*Dnx*) or susceptible (*Dn0*) genes. (**a**) Membrane protein genes—Q9XEN7 *β*-1,3-glucanase, Q8W427 chitinase III, Q01482 *WIR1A* membrane protein; (**b**) ROS response genes—Q43212 peroxidase precursor, Q8S702 glutathione S-transferase, Q8H8H7 flavanone 3-hydroxylase, Q9AVM3 cytochrome P450, Q5BQ31 serine/threonine kinase; (**c**) P27357 thaumatin-like protein PWIR2 precursor (SA metabolism); (**d**) P29114 *LOX1* lipoxygenase 1 (JA precursor); and (**e**) Aphid anti-digestion/toxin genes—Q5NTH3 shikimate kinase 2, P12940 Bowman-Birk trypsin inhibitor; F. Dehydration response genes—Q6Z676 *phi-1* ABA dehydration signaling, P93671 *XET* xyloglucan endotransglycosylase, Q43210 *PAL* phenylalanine ammonia lyase. Northern blot analysis of 5 µg of total RNA from the leaves of *Dnx* or *Dn0* plants at 6-, 12-, 24-, 72- and 120 h post infestation (hpi) with RWA biotype 1 adults. Filters were hybridized with probes derived from cDNA clones of Affymetrix EST sequences encoding each gene shown on left. Gels were stained with ethidium bromide and rRNA levels compared to control for equal loading (shown is representative loading on a single gel). Each lane represents RNA pooled from two biological replicates, each consisting of leaves from three plants

feeding (Table 3, Supplemental Data Table 1), but their expression was approximately one-half that level in *Dn0* plants (Table 3, Supplemental Data Table 2). On northern blots, the levels of these transcripts were strongly upregulated in infested *Dnx* plants at least at one post infestation time point (Fig. 2b). The levels of these transcripts were either unaffected or less elevated in infested *Dn0* plants.

Defense Signaling Results of microarray analyses produced transcripts putatively associated with the SA signaling pathway. These included Pathogenesis-Related-1 (*PR1*), *PR4*, *PR5*, and *WRKY* (Yalpani et al. 1991; Dong et al. 2003), which were 2- to 7-fold upregulated in infested *Dnx* plants (Supplemental Data Table 1). In contrast, transcripts associated with the JA signaling pathway including *FAD3C*, *DAD1 LOX1*, *ACS1*, *12-OPR,* OPDA hydrolases, 12-OPDA ABC transporters (Dhondt et al. 2000; Ishiguro et al. 2001; Liechti and Farmer 2002; Theodoulou et al. 2005) were 2- to 4-fold upregulated in infested *Dnx* plants (Supplemental Data Table 1). Transcripts associated with ABA and ET signaling pathways increased from 2- to 4-fold in infested *Dnx* plants (Supplemental Data Table 1). These included EIN-3-like proteins, AP2 domain-containing transcription factors, C2 GRAM domain-containing proteins, and putative phi-1 proteins (Zhu 2002; Zhang et al. 2004) (Supplemental Data Table 1). None of these genes were upregulated in infested *Dn0* plants (Supplemental Data Table 2).

Pest/Pathogen Defenses Dnx-based resistance to RWA is manifested as reduced aphid population growth (Boyko et al. 2006; Khan et al. 2009; Lazzari et al. 2009), which may be a result of suppressed aphid feeding, an inhibition of aphid

digestion or a combination of both. Plant secondary metabolites suppress insect feeding, and several transcripts associated with secondary metabolite production including cycloartenol synthase, monoterpene synthase, and shikimate kinase were upregulated 2–4 fold in RWA1-infested *Dnx* plants (Supplemental Data Table 1). On northern blots, the transcript coding for shikimate kinase was expressed in infested *Dnx* leaves at 12 hpi but was absent in *Dn0* plants at all post-infestation time points (Fig. 2e). Further, the transcript encoding a UDP-glucose glucosyltransferase, an enzyme that transfers glucose UDP-glucose to terpenes (Xiong et al. 2001, Scharrenberg et al. 2003), was upregulated 2- to 4-fold (Supplemental Data Table 1). Various enzyme inhibitors limit digestion in the insect gut (Gatehouse and Boulter 1983). A transcript encoding a Bowman-Birk trypsin inhibitor was upregulated at 12 hpi in *Dnx* infested plants, but this transcript was not significantly affected by RWA1 feeding in *Dn0* plants until a very late time point (120 hpi) (Fig. 2e). Interestingly, several phytoalexin and pathogen resistance genes underwent 2- to 4-fold upregulation in infested *Dnx* plant foliage, including *ACRE, HHT, SMM:HSM, and SNAP* (Supplemental Data Table 1), but these transcripts were not expressed in *Dn0* infested plants (Supplemental Data Table 2).

Downregulated Transcripts Transcripts downregulated in plants of each genotype possessed various functions (Table 3, Supplemental Data Tables 3 and 4) and several representative downregulated transcripts were chosen for real-time PCR to confirm microarray results (Fig. 3, Supplemental Data Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14.). There were no significant differences in downregulation of the stress response protein in either *Dnx* or *Dn0* tissues at 6-, 12-, 24-, or 72 hpi (Fig. 3a). A calmodulin-like gene displayed similar patterns, and in both *Dnx* and *Dn0* infested tissues, downregulation was significantly greater at 24- and 120 hpi than at 6- or 12 hpi (Fig. 3b). A transcript encoding a NAC domain pathogen response protein (Collinge and Boller 2001) was strongly downregulated in infested *Dnx* plants (Fig. 3c), and was significantly greater in *Dnx* tissues at 72- and 120 hpi than at 6-, 12-, or 24 hpi. There were no differences in expression between any post-infestation times in *Dn0* tissues. Expression of *CYP450* CA619079 was relatively low in *Dnx* and *Dn0* tissues (Fig. 3d), but was significantly greater in *Dnx* infested tissues at 24- and 120 hpi compared to 6- and 12 hpi. There were no differences in expression between *Dnx* infested tissues collected at 72 hpi and any other post-infestation time (Fig. 3d). In *Dn0* infested tissues, there were no differences in the expression of CYP450 at any post-infestation time. Downregulation of a transcription protein and a mitochondrial ATP synthase in *Dnx* plants was significantly greater in *Dnx* tissues at 120 hpi than at 6 hpi (Fig. 3e, f). There were no significant temporal differences in downregulation of either protein in *Dn0* tissues.

Phytohormone Analyses The concentration of the 16:1 fatty acid in infested *Dnx* and *Dn0* plants increased significantly ($P<0.001$) at 12-, 24-, and 48 hpi, but returned to the control level at 96 hpi (Table 4). The concentration of 16:3 fatty acid increased dramatically in infested *Dnx* and *Dn0* plants at 48 hpi, where it was significantly greater in *Dnx* plants than *Dn0* plants. There were comparatively fewer significant differences between treatments for the 18:1 and 18:3 fatty acids. The 18:1 fatty acid content of both *Dnx* and *Dn0* infested tissues was significantly ($P<0.001$) greater than uninfested controls at 96 hpi. The 18:3 fatty acid content of *Dnx* infested plants was significantly ($P<0.001$) greater than the uninfested control at 12 hpi, and in *Dn0* plants, significantly ($P<0.001$) greater than the control at 12- and 24 hpi. This trend was similar for the 18:2 fatty acid, but the only significant increase in *Dnx* tissues over *Dn0* tissues occurred at 12 hpi (data not shown). There were no significant differences between two genotypes at any time point for 16:0 or 18:0 fatty acids (data not shown).

The *cis*- and *trans*-Me-OPDA content differed significantly ($P<0.001$) between post infestation times in leaves of *Dnx* and *Dn0* plants, and between the genotypes at each post infestation time (Table 5). In *Dnx* plants, *cis*-Me-OPDA content increased dramatically to 2.3% at 24 hpi, and was significantly ($P<0.001$) greater than the uninfested control or any other *Dnx* post infestation time. In contrast, *cis*-Me-OPDA content in *Dn0* plants was reduced at each hpi time compared to that produced in uninfested plants, and reductions were significant ($P<0.001$) at 12- and 24 hpi (Table 5). With the exception of the 24 hpi treatment, *cis*-Me-OPDA content was significantly greater in *Dn0* plants (including uninfested controls) than the corresponding *Dnx* plant treatments. Expression of *trans*-Me-OPDA was more pronounced than *cis*-Me-OPDA. In *Dnx* plants, *trans*-Me-OPDA content was significantly greater at 12-, 24-, and 48 hpi than in uninfested controls, and decreased to the level of the 0 h uninfested control at 96 hpi. As with *cis*-Me-OPDA, production of *trans*-Me-OPDA in uninfested *Dn0* plants was significantly greater than in uninfested *Dnx* plants (Table 5). However, the *trans*-Me-OPDA content of *Dn0* infested plants at all hpi intervals was significantly less than untreated *Dn0* control plants and significantly lower than that of each corresponding *Dnx* plant hpi time. In contrast to Me-OPDA, there were no significant trends in differences in *cis*- or *trans*-methyl jasmonate (meJA) content at different time points or between *Dnx* and *Dn0* plants (data not shown).

Abscisic acid (ABA) content in *Dnx* plant foliage was significantly ($P<0.001$) greater at 12- and 48 hpi than in uninfested plants (Table 6) and was significantly greater in

Fig. 3 Temporal downregulation (mean ± S.E fold change) of wheat transcripts from plants containing the RWA resistant (*Dnx*) or susceptible (*Dn0*) gene at 6-, 12-, 24-, 72- and 120 h post infestation with RWA biotype 1 adults. (**a**) Q9P3N1 hypothetical stress response protein; (**b**) Q5ZD81 calmodulin-like protein; (**c**) Q6Z1A3 NAC1 stress response protein; (**d**) Q6YXE1 cytochrome P450 (**e**) Q7XN01 transcription protein; and (**f**) Q6I5G9 mitochondrial ATP synthase. Each treatment mean represents two biological replicates, each consisting of leaves pooled from three *Dnx* or *Dn0* plants

Dnx plants than in *Dn0* plants at 48 hpi. ABA content in the foliage of *Dn0* plants was elevated at 12- and 24 hpi, but these amounts were no different from those of the uninfested control. The leaf content of benzoate, a salicylic acid intermediate, increased significantly ($P<0.001$) in *Dnx* foliage at 12-, 24-, and 48 hpi compared to the 0 h control, and a similar trend was absent in *Dn0* plants (Table 6). In addition, the benzoate content of infested *Dnx* plants was significantly

Table 4 Mass (% ng/g standard) of unsaturated 16:1 (7-hexadecenoic), 16:3 (7,10, 13-hexadecatrienoic), 18:1 (9-octadecenoic), and 18:3 (9,12,15-octadecatrienoic) fatty acids in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*) wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation (hpi), and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	Dnx	*Dn0*	*Dnx*	*Dn0*
	16:1 fatty acid		16:3 fatty acid	
0	0.52±0.03 ab[a]	0.66±0.07 bc	0.04±0.04 a	0.10±0.12 b
12	1.72±0.09 e	1.65±0.05 e	0.08±0.03 b	0.08±0.04 b
24	1.02±0.04 d	1.16±0.06 de	0.14±0.05 b	0.13±0.07 b
48	0.79±0.05 cd	0.36±0.02 a	2.08±0.14 d	0.47±0.23 c
96	0.51±0.08 ab	0.89±0.05 cd	0.03±0.03 a	0.12±0.04 b
	18:1 fatty acid		18:3 fatty acid	
0	1.81±0.11 ab[a]	2.00±0.34 ab	2.28±0.20 a	4.45±2.6 a
12	2.43±0.22abcd	1.88±0.36 ab	14.43±3.11c	11.25±3.01bc
24	2.10±0.13 abc	2.30±0.20abcd	5.08±1.08 a	12.44±3.14bc
48	2.63±0.30 bcd	1.63±0.12 a	7.26±1.47 ab	2.12±0.65 a
96	2.86±0.45 cd	3.06±0.42 d	2.59±0.61 a	2.40±0.42 a

[a] For each fatty acid, means within a column followed by the same letter are not significantly different at $P<0.001$

greater than in *Dn0* plants at one time point (48 hpi). However, there were no differences in methyl salicylate leaf content between any of the *Dnx* or *Dn0* post infestation treatments (data not shown). The amounts of the related SA intermediate *trans*-cinnamate also increased over time in both *Dnx* and *Dn0* plants, but the quantities were no different from those of uninfested control plants (data not shown).

Discussion

Plant resistance to insects is a complex process, often involving numerous plant biochemical pathways. We hypothesized that the products of unique defense proteins in the *Dnx* plant transcriptome are activated by RWA1 feeding probes, resulting in expression of the *Dnx* plant phenotype. Results from microarray hybridizations, northern blot and real-time PCR assays, and vapor phase extraction of foliar phytohormones support this hypothesis. The results that we obtained identify unique differences in the molecular responses of resistant *Dnx* plants to RWA attack in comparison with those of susceptible *Dn0* plants, and suggest that 16 carbon membrane fatty acids; oxylipins, ABA, and ET defense response signals; and downstream defense proteins are likely components for *Dnx*-mediated resistance to RWA. Most transcripts potentially involved in resistance were upregulated more rapidly and at a greater magnitude than in susceptible plants, suggesting a more rapid and stronger induction of a *Dnx*-mediated gene network as a likely mechanism for *Dnx* resistance. This mechanism has been observed with several plant resistance responses to various pathogens (van Loon et al. 2006).

Table 5 Mass (% ng/g standard) of *cis*- and *trans*-methyl-12-oxo-phytodienoic acid (12-OPDA) in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*)wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation, and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	cis Me-OPDA		*trans* Me-OPDA	
	Dnx	*Dn0*	*Dnx*	*Dn0*
0	0.21±0.13 ab[a]	1.76±0.04 c	0.08±0.08 a	1.15±0.33 b
12	0.14±0.14 a	0.25±0.07 b	0.90±0.01 b	0.05±0.07 a
24	2.29±0.03 c	0.22±0.16 ab	2.01±0.14 b	0.04±0.09 a
48	0.24±0.06 b	1.28±0.05 c	2.24±0.28 b	0.06±0.10 a
96	0.28±0.11 b	1.52±0.04 c	0.04±0.06 a	0.04±0.08 a

[a] For each OPDA isomer, means within a column followed by the same letter are not significantly different at $P<0.001$

Table 6 Mass (% ng/g standard) of methyl abscisic acid (Me-ABA) and methyl benzoate (Me-BA) in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*) wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation, and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	Me-abscisic acid		Me-benzoate	
	Dnx	*Dn0*	*Dnx*	*Dn0*
0	1.00±0.05 bc[a]	1.79±0.10 cde	0.01±0.04 ab	0.02±0.09 acb
12	2.34±0.05 de	1.98±0.04 de	0.43±0.22 e	0.80±0.31 de
24	1.43±0.05 cd	2.97±0.06 e	0.03±0.09 cd	0.03±0.14 cd
48	2.10±0.07 de	0.54±0.09 a	0.08±0.23 cd	0.02±0.14 ab
96	0.56±0.07 ab	0.61±0.18 a	0.01±0.08 a	0.01±0.11 a

[a] For Me-abscisic acid or Me-benzoate, means within a column followed by the same letter are not significantly different at $P<0.001$

Several results suggest the SA pathway to be less important in mediating *Dnx* resistance to RWA than the JA pathway. Fewer SA-related transcripts were upregulated in *Dn0* plants (3) versus *Dnx* plants (16), and none of those upregulated in *Dnx* plants was expressed in *Dn0* plants. Over five post-infestation time points, there were no differences between tissues of *Dnx* and *Dn0* infested plants in the content of the *trans*-cinnamate or SA. Content of the SA intermediate benzoate in *Dnx* plants was significantly greater than in *Dn0* plants at only 48 hpi.

The levels of expression of the majority of transcripts were similar between *Dnx* and *Dn0* plants. However, approximately 30% of the transcripts contained in the microarray were upregulated in *Dnx* plants, and most of them are plant defense-related genes (Table 3). In comparison, only 19% of the transcripts were upregulated in *Dn0* plants, and the proportion of these upregulated transcripts associated with plant defense was much lower. The transcripts upregulated in susceptible plants, therefore, may be related to general plant stress responses. Most, if not all ABA, ET, and JA signaling genes upregulated in *Dnx* plants were absent in *Dn0* plants. On the other hand, approximately 9% of the upregulated *Dn0* transcripts were related to auxin (AUX) signaling and these were absent in *Dnx* plants (Table 3). The expression of AUX-related transcripts may result in *Dn0* plants increasing their ROS production, as demonstrated by Boyko et al. (2006) and Kawano (2003). An additional 27% of the upregulated *Dn0* transcripts included metabolism genes (Table 3), which were not upregulated in *Dnx* plants. The downregulation of metabolic transcripts in *Dnx* plants at 120 hpi (Fig. 3) may decrease some plant metabolic activities that can enable the production of *Dnx*-related defense compounds. A related downregulation of stress response transcripts in *Dnx* plants could be due to a reduced need for these transcripts, in favor of downstream plant defenses.

At the onset of insect feeding, components of the cell wall membrane such as *GLG* and *Chita*, which were highly expressed in infested *Dnx* plants, participate in insect defense responses in plants (Kempema et al. 2007; Park et al. 2007; see review of Smith and Boyko 2006). The increased upregulation of defense response transcripts related to ROS metabolism in *Dnx* leaves (Fig. 2b) is similar to that induced by other plant feeding insects (Schmidt et al. 2005; Couldridge et al. 2007). The expression of *GLG*, *Chita*, and ROS-related transcripts in *Dnx* infested tissues suggests their involvement in *Dnx* defense responses to RWA feeding probes.

Further, the putative role of WIR1 membrane proteins in *Dnx* resistance is strengthened by the results of Gaupels et al. (2008), who identified a WIR1A-like protein in barley phloem sap, obtained from stylets of actively feeding *Rhopalosiphum padi* aphids. Our results were similar to those of Zhu-Salzman et al. (2004) and Park et al. (2006), who demonstrated expression of CYP450 monooxygenase (MO) in aphid-resistant sorghum plants. The precise role of the CYP450MOs in *Dnx*-infested plants is difficult to determine, because these compounds function in the synthesis of JA, SA, chemical defenses, and the detoxification of exogenous chemicals such as RWA salivary components.

LOX, a gene whose transcripts are associated with the JA signaling pathway, is strongly induced by foliar feeding of numerous insects (Sardesai et al. 2005; and see Smith and Boyko 2006 review), but plant JA-induced defense responses may be antagonized by those induced by SA (Spoel et al. 2003; Koornneef et al. 2008). For example, silver leaf whitefly herbivory suppresses *LOX2* and *FAD* expression and elevates *PR1* and *PR5* expression (Kempema et al. 2007). However, the upregulation of JA- and SA-related transcripts in *Dnx*-infested plants was similar to that noted by Salzman et al. (2005), in sorghum response to mechanical wounding and by Li et al. (2008) in soybean plant response to aphid feeding. We observed induction of homologs of genes composing much of the JA and SA pathways but expression of SA transcripts was ~ 2x greater than expression of JA synthesis transcripts (Supplemental Data Table 1). Nevertheless, although SA transcripts were more highly expressed in micro-

arrays than JA transcripts, results of northern blot and phytohormone assays indicated that SA does not contribute to *Dnx* resistance (Fig. 2c, Table 6). Liu et al. (2007) reported similar results in Hessian fly resistant wheat plants.

Aphid feeding stimulates *WRKY* expression (Voelckel et al. 2004; Park et al. 2006; Li et al. 2008) (Supplemental Data Table 1), yet *WRKY* transcription factors suppress JA (Kalde et al. 2003; Li et al. 2004; Mao et al. 2007), indicating that *WRKY* expression in *Dnx* tissues may partially explain the comparatively reduced expression of JA-signaling genes. Wound-induced JA production is regulated by the supply of substrate available to allene oxide synthase (Turner et al. 2002). Thus, the minimal foliar damage and related chloroplast loss sustained by *Dnx* plants in our experiments (Fig. 1b), may have limited the release of fatty acids from chloroplast lipids for JA metabolism.

Oxylipin analyses suggest that *LOX*, *trans*-OPDA, and the 18- and 16 carbon OPDA fatty acid precursors function in *Dnx* resistance (Fig. 2d, Tables 4 and 5). The direct role of OPDA in insect defense responses (Stintzi et al. 2001) and the production of OPDA-specific response gene homologs by mechanical wounding (Taki et al. 2005) support these results. Expression of oxylipin metabolites in *Dnx* plants appears to stimulate production of downstream feeding inhibitors and toxins (Fig. 2e), several of which have been reported as resistance factors (Moraes et al. 2000; Miller et al. 2005; Lou and Baldwin 2006; Smith and Boyko 2006; Liu et al. 2007). RWA1 phloem ingestion decreases markedly on *Dnx* plants within 8 hpi (Lazzari et al. 2009), further supporting the possibility that *Dnx* feeding inhibitors and/or toxins contribute to the significant ($P<0.001$) antibiotic effects exhibited in the reduced RWA1 population development shown in Fig. 1a. These antibiotic effects shown by RWA1 substantiate similar results of Khan et al. (2009) with plants containing *Dnx* in different genetic backgrounds. The ~50% RWA1 population reduction on *Dnx* plants in Fig. 1a is similar to that observed by Boyko et al. (2006) (57%, 66%) and Khan et al. (2009) (34%).

The lack of leaf wilting and interveinal collapse in RWA1 incompatible interactions indicates that *Dnx* resistance also may result from reduced tissue and water loss. For example, *PAL* and *CAD5*, which mediate lignin synthesis, were highly expressed in infested *Dnx* tissues (Fig. 2f), and both have been implicated in insect resistance (Ciepiela 1989; Kempema et al. 2007; Liu et al. 2007). Elevated expression of latex-abundant-, fiber (CA609522), and sorbitol transporter (BT009301) proteins (Supplemental Data Table 1) provide additional evidence of physical components of *Dnx* resistance and an additional explanation of the low tissue dry weight changes occurring in RWA-infested *Dnx* plants (Fig. 1b).

ABA- and ET- dehydration responses were uniquely upregulated in *Dnx* plants during RWA feeding (Fig. 2f, Table 6), as reported by Salzman et al. (2005) and Park et al. (2006). ABA- and ET over-expression in response aphid-infested plants (Moran et al. 2002; Divol et al. 2005; Boyko et al. 2006) and the upregulation of the ET signaling genes in *Dnx* plants (Supplemental Data Table 1) strongly suggests their role in *Dnx* resistance. Related dehydration response transcripts were highly expressed, and one - *XET*—was differentially expressed in *Dnx* plants (6–72 hpi) and *Dn0* plants (12–24 hpi) (Fig. 2f), also demonstrating their contribution to *Dnx* resistance.

Our results indicating the upregulation of homologs of the pathogen resistance genes *ACRE, AP, Bet, HHT*, and *SNAP* in RWA1-infested *Dnx* tissues is the first report of their expression in response to insect feeding. Validation of the role(s) of these genes and other candidate genes in *Dnx* resistance awaits functional confirmation experiments, likely involving gene silencing. In the interim, more than 400 *Dnx* expressed transcripts are presently of unknown function. Elucidation of their function will provide additional information about putative genes and their expression patterns involved in wheat plant responses to RWA herbivory.

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Resistance in the Plant, *Barbarea vulgaris*, and Counter-Adaptations in Flea Beetles Mediated by Saponins

Jens K. Nielsen · Tsuneatsu Nagao · Hikaru Okabe · Tetsuro Shinoda

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Abstract Three saponins and two sapogenins had differential effects on food consumption in five near-isogenic flea beetle lines, which differ in their ability to utilize a novel host plant, *Barbarea vulgaris* (Brassicaceae). The ability to live on this plant is controlled by major, dominant R-genes in the flea beetle, *Phyllotreta nemorum* (Coleoptera: Chrysomelidae: Alticinae). A susceptible genotype (rr) is unable to live on the plant, whereas resistant genotypes (RR and Rr) can utilize the novel host plant. Among compounds isolated from *B. vulgaris*, hederagenin cellobioside (hederagenin-3-O-(4-O-β-D-glucopyranosyl)-β-D-glucopyranoside) inhibited feeding, whereas the effect of oleanolic acid cellobioside was much weaker. The aglycones (sapogenins) were inactive. Although hederagenin cellobioside was active against all flea beetle lines, its effect on food consumption was much stronger on the susceptible genotype (rr) compared to the resistant genotype (Rr). Susceptible and resistant flea beetle genotypes were equally sensitive to a non-host saponin, α-hederin (hederagenin-3-O-(2-O-α-L-rhamnopyranosyl)-α-L-arabinopyranoside). These results suggest that R-alleles in flea beetles might be specific adaptations to defensive saponins in *B. vulgaris*. A possible mechanism of action of the R-alleles might be to encode for an enzyme (e.g. a glucosidase), which is able to cleave glycosidic bonds in hederagenin cellobioside, but not in α-hederin. The potential role of saponins as defensive compounds in *B. vulgaris* and as targets for counter-adaptations in flea beetles and other insects is discussed.

Keywords *Barbarea vulgaris* · Brassicaceae · Flea beetle · *Phyllotreta nemorum* · Chrysomelidae · Alticinae · Host plant resistance · Host shift · Deterrents · Triterpenoids · Saponins · Hederagenin cellobioside

Tsuneatsu Nagao deceased: Sept 24, 2002.

J. K. Nielsen (✉)
Department of Basic Sciences and Environment,
Faculty of Life Sciences, University of Copenhagen,
Thorvaldsensvej 40,
DK 1871 Frederiksberg C, Denmark
e-mail: jeni@life.ku.dk

T. Nagao · H. Okabe
Faculty of Pharmaceutical Science, Fukuoka University,
8-19-1, Nanakuma,
Jonan-ku, Fukuoka, Japan

T. Shinoda
National Institute of Agrobiological Sciences,
Ohwashi 1-2,
Tsukuba, Ibaraki 305-8634, Japan

Introduction

Green plants produce a large variety of secondary compounds that have important roles in the interaction between the plant and its environment (Hartmann 1996). Many of these compounds are toxic to or deter feeding in herbivorous insects, and plants that contain these compounds may have a selective advantage in habitats where herbivorous insects are abundant. Insects, on the other hand, may evolve counter-adaptations to particular secondary compounds, i.e., they may be able to detoxify them or tolerate them in their tissues without harmful effects (Schoonhoven et al. 2005).

The idea of a stepwise "coevolutionary arm's race" between plants and insects was first formulated by Ehrlich and Raven (1964) and later these ideas were expanded upon by Futuyma and Keese (1992) and Thompson (1994, 2005). This theory can explain the apparently paradoxical observations that: 1) green plants produce large amounts of chemicals with no obvious functions for plant growth and

reproduction in the absence of enemies (Hartmann 1996), and 2) that the host range of many herbivorous insects is restricted by the occurrence of secondary plant compounds, which may have positive as well as negative effects on insect behavior and performance (Schoonhoven et al. 2005). Coevolution can explain broad patterns of distribution of plant defenses and host use in herbivorous insects, but many processes still are not well understood, and there are only few examples, where reciprocal selection in natural populations has been rigorously proven (Thompson 2005; Gomulkiewicz et al. 2007).

The interaction between the plant *Barbarea vulgaris* ssp. *arcuata* (Opiz.) Simkovics (Brassicaceae) (hereafter called *Barbarea*) and the flea beetle, *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae: Alticinae) seems to be particularly well suited for studies of processes involved in coevolutionary interactions between two trophic levels, since both the plant and the insect are polymorphic. The pubescent P-type of *Barbarea* is susceptible to all known genotypes of the flea beetle, whereas the glabrous G-type is resistant to the most common flea beetle genotypes found in Denmark (Nielsen 1997a, b; Agerbirk et al. 2003b). The inheritance of resistance to flea beetles in *Barbarea* still is not fully understood, although levels of resistance in hybrids (F1) between the G- and the P-type are intermediate between those of the parents, whereas the full range from complete susceptibility to complete resistance is expressed in a segregating F2 population (Kuzina et al. 2009).

The flea beetle, *P. nemorum*, is an oligophagous species, which utilizes a few species from the crucifer family (Brassicaceae) as natural host plants. Although the G-type of *Barbarea* is resistant to most flea beetle genotypes, it is a major host plant for the species in some localities (Nielsen and de Jong 2005; de Jong et al. 2009). Local flea beetle populations that live on the G-type of *Barbarea* have high frequencies of particular resistance genes (named R-genes), whereas frequencies of these genes in populations that live on other host plants are lower (Nielsen and de Jong 2005). The R-genes, which may be sex-linked or autosomal, are dominant, since the genotypes RR and Rr develop equally well on the G-type of *Barbarea*, whereas rr does not grow at all on this plant type (Nielsen 1997a, b; de Jong et al. 2000). In order to study the effect of individual R-genes, a number of near-isogenic lines were developed by repeated back-crossing of the genotypes RR and Rr with rr. Detailed investigations of one of these lines demonstrated that its particular R-gene should be considered as an adaptation to defenses in *Barbarea*, since it conferred ability to live on this particular species but not to any other crucifer (Nielsen 1999).

Members of the crucifer family are characterized chemically by their content of glucosinolates. Being present in all members of the Brassicaceae, these compounds may be considered as a first generation of defensive compounds in the family (Feeny 1977; Nielsen 1978; Renwick 2002). Glucosinolates, however, do not protect the plants against a number of specialist insects that have evolved means to detoxify them (Ratzka et al. 2002; Wittstock et al. 2004) and indeed use them as host specific cues (Renwick 2002). As a putative response to renewed selection pressures imposed by herbivorous insects, a number of crucifers have evolved a second generation of defensive compounds, e.g. cucurbitacins in *Iberis* spp. and cardenolides in *Cheiranthus* and *Erysimum* species. These compounds are feeding deterrents for a number of flea beetles (Nielsen et al. 1977; Nielsen 1978), and in *Pieris* spp. they serve as feeding deterrents for caterpillars and as oviposition deterrents for adult females (Huang et al. 1993; Sachdev-Gupta et al. 1993a, b; Huang and Renwick 1994). These compounds can explain the differential utilization of *Iberis*, *Cheiranthus*, and *Erysimum* species by several herbivorous insects (Nielsen 1978; Renwick 2002).

The relationship between chemical content and suitability to insects in the genus *Barbarea* is less well understood. The P- and the G-type of *Barbarea* contain different optical isomers of 2-hydroxy-2-phenylethylglucosinolate (glucobarbarin and glucosibarin), but no correlation (neither quantitative nor qualitative) between glucosinolate content and resistance to flea beetles has been documented (Agerbirk et al. 2001, 2003b). More recently, resistance to flea beetles has been correlated with the content of a number of triterpenoid saponins in a segregating F2 population from a cross between the G- and the P-type (Kuzina et al. 2009). Two triterpenoid saponins previously have been isolated and identified from *B. vulgaris* (Shinoda et al. 2002; Agerbirk et al. 2003a). The major compound in the G-type is hederagenin-3-O-(4-O-β-D-glucopyranosyl)-β-D-glucopyranoside (hederagenin cellobioside), whereas oleanolic acid-3-O-(4-O-β-D-glucopyranosyl)-β-D-glucopyranoside (oleanolic acid cellobioside) is found in lower concentrations (Kuzina et al. 2009). These compounds are absent in the P-type, but occur in variable concentrations in the G-type and in F2 plants from a cross between the G- and the P-type (Kuzina et al. 2009). They are both potent feeding deterrents against caterpillars of the diamond back moth, *Plutella xylostella* (Shinoda et al. 2002; unpublished results), while their effects on flea beetles have yet to be evaluated.

The purpose of the present work was to determine whether resistance to flea beetles in *Barbarea* might be mediated by saponins. Saponins and their aglycones (sapogenins) have been recognized as defensive compounds in plants against several herbivorous insects and pathogenic fungi. In many cases, the biological effects were closely linked to the carbohydrate moiety. Removal of individual sugars might increase as well as decrease the biological activity (Bowyer et al. 1995; Osbourn 1996; Adel et al. 2000). In the present study, feeding deterrent activity of the two known triterpenoid

saponins from *B. vulgaris* were compared with the effects of the aglycones, hederagenin and oleanolic acid, and of a hederagenin glycoside (α-hederin) with a different carbohydrate moiety (Fig. 1). Furthermore, we investigated whether R-genes in flea beetles might be considered as adaptations to saponin mediated defenses in *Barbarea*.

The study addressed the following questions: 1) do saponins reduce feeding in *P. nemorum*? 2) are the aglycones more or less active than the parent glycosides? 3) Is there any relation between biological activity and the structure of the aglycone or the carbohydrate moiety of saponins? and 4) do near-isogenic flea beetles that are either susceptible or resistant to defenses in *B. vulgaris* show differential responses to saponins?

Methods and Materials

Plants Radish plants were grown in a growth chamber at 20±2°C and a L18:D6 photoperiod as described previously (Nielsen 1999).

Insects Feeding responses of five near-isogenic flea beetle lines were compared. A susceptible line from Taastrup (ST) originated from beetles collected on radish in Taastrup (Nielsen 1999). Beetles from this line (rr genotype) are unable to survive on *Barbarea*. Imagines of the ST-line were kept in groups of 15–25 beetles that were allowed to mate randomly. Larvae as well as adults were reared on radish leaves as described previously (Nielsen 1999).

Feeding responses of the susceptible ST-line were compared with those of four near-isogenic resistant lines that express R-genes from different Danish and Swiss populations. All the resistant lines are able to survive on the G-type of *Barbarea*. They are heterozygous (Rr genotype), and the lines were maintained by repeated backcrossing with the ST-line. When one heterozygous male (Rr) was mated with an ST-female (rr), 50% of their offspring was Rr. This genotype was selected by rearing the offspring on the G-type of *Barbarea*, since Rr genotypes survived on this plant, whereas rr did not. At least ten generations of backcrosses had been made with the lines used in the present study.

Four different resistant lines were maintained: 1) YE contained a Y-linked R-gene from Ejby, which only occurs in males and is inherited from fathers to sons (Nielsen 1997b, 1999); 2) the AE line contained an autosomal gene from Ejby (Nielsen 1997b); 3) the AK-line contained an autosomal gene from Kværkeby (de Jong et al. 2000); and 4) the DE-line contained a gene from Delemont (Switzerland) which was inherited autosomally under laboratory conditions (Nielsen, unpublished results). The founders of the YE, AE, and AK lines were collected in populations that use the G-type of *Barbarea* as a natural host plant, whereas the founders of the DE line were collected on *Sinapis arvensis* (Nielsen, unpublished results). Feeding responses of females from the AE, AK, and DE lines were compared with those of males

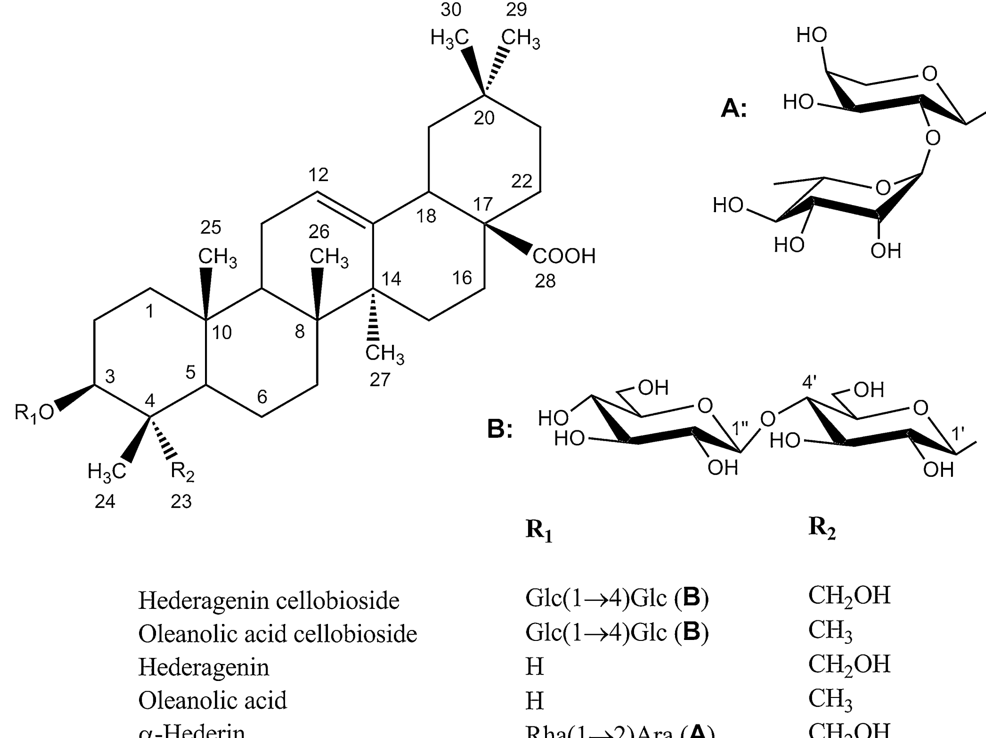

Fig. 1 Saponins and sapogenins evaluated as feeding deterrents against the flea beetle, *Phyllotreta nemorum*

from the YE line and females and males from the ST line. Beetles used in the bioassays were 3–7 d-old and had been feeding on radish leaf discs until the bioassays were initiated.

Chemicals Hederagenin cellobioside was isolated from *B. vulgaris* as described previously (Shinoda et al. 2002). Oleanolic acid cellobioside was isolated, and the structure was determined as follows: Fresh leaves (2.8 kg) of *B. vulgaris* var. *variegata* (Chiltern Seeds, England) were extracted with MeOH (14 l) and the aqueous MeOH ext. was partitioned with $CHCl_3$. The $CHCl_3$ layer was evaporated off and chromatographed on silica gel ($CHCl_3$ -> 20% MeOH/$CHCl_3$) to separate 12 fractions. The compound (96 mg) was purified as a white powder from Fr. 7 (1.3 g; 10% MeOH/$CHCl_3$ eluate) by the chromatography of silica gel ($CHCl_3$:MeOH:H_2O=150:30:2), reversed phase silica gel (YMC-GEL ODS-120A, 85% MeOH), and prep. HPLC (Shiseido Capcellpak C18, 82.5% MeOH). FAB-MS was obtained by using a JEOL HX-110 mass spectrometer, ^{1}H and ^{13}C NMR spectra were recorded on a JEOL A-500 spectrometer, and optical rotation was measured on a JASCO DIP-360 polarimeter.

Spectral data: A white powder, $[\alpha]_D^{26}$+18.2° (MeOH; *c*. 1.2). Positive ion FAB-MS (glycerol matrix) *m/z* 803.4551 $([M + Na])^+$, $C_{42}H_{68}NaO_{13}$ requires 803.4558. Negative ion FAB-MS (glycerol matrix) *m/z* 779 ($[M–H]^-$), 617 ($[M–glc-H]^-$), 455 ($[M–2glc-H]^-$). ^{1}H NMR (500 MHz, C_5D_5N): 0.84 (3H, *s*, H-25), 0.96 (3H, *s*, H-29), 1.00 (6H, *s*, H-24 and H-26), 1.02 (3H, *s*, H-30), 1.31 (6H, *s*, H-23 and H-27), 3.34 (1H, *dd*, *J*=4, 12 Hz, H-3), 3.95 (2H, *m*, H-5′ and H-5″), 4.01 (1H, *dd*, *J*=8, 8 Hz, H-2′), 4.09 (1H, *dd*, *J*=8, 8 Hz, H-2″), 4.18 (2H, *dd*-like, H-3″ and H-4″), 4.26 (1H, *dd*, *J*=8 Hz, H-3′), 4.27 (1H, *dd*, *J*=6, 12 Hz, H-6″a), 4.30 (1H, *dd*, *J*=8, 8 Hz, H-4′), 4.49 (1H, *dd*, *J*=3, 12 Hz, H-6″b), 4.50 (1H, *dd*, *J*=2, 12 Hz, H-6′a), 4.55 (1H, *dd*, *J*=4, 12 Hz, H-6′b), 4.86 (1H, *d*, *J*=8 Hz, H-1′), 5.18 (1H, *d*, *J*=8 Hz, H-1″), 5.48 (1H, *dd*, *J*=3, 3 Hz, H-12). ^{13}C NMR (125 MHz, C_5D_5N): 15.4 (C-25), 16.9 (C-24), 17.3 (C-26), 18.4 (C-6), 23.6 (C-16), 23.7 (C-11), 23.7 (C-30), 26.1 (C-27), 26.4 (C-2), 28.2 (C-23), 28.3 (C-15), 30.9 (C-20), 33.1 (C-7), 33.2 (C-22), 33.2 (C-29), 24.2 (C-21), 37.0 (C-10), 38.7 (C-1), 39.4 (C-4), 39.7 (C-8), 42.0 (C-18), 42.1 (C-14), 46.5 (C-19), 46.6 (C-17), 48.0 (C-9), 55.8 (C-5), 62.4 (C-6′), 62.4 (C-6″), 71.5 (C-4″), 74.8 (C-2″), 75.2 (C-2′), 76.2 (C-5′), 76.9 (C-3′), 78.2 (C-3″), 78.4 (C-5″), 81.6 (C-4′), 88.9 (C-3), 104.9 (C-1″), 106.4 (C-1′), 122.5 (C-12), 144.8 (C-13), 180.1 (C-28).

The high resolution FAB-MS analysis established the molecular formula, $C_{42}H_{68}O_{13}$. This molecular formula and the NMR spectral data suggested that the compound was an oleanolic acid 3-*O*-glycoside having two hexosyl groups as its sugar moiety (Nagao et al. 1991). The identification of the sugar moiety by using acid hydrolysis and methanolysis was performed as described previously (Shinoda et al. 2002). The linkages between the aglycon and sugar moieties were determined from correlations in the HMBC spectrum between H-3 (3.34) and C-1′ (C-1 of inner glucosyl group; 106.4), H-1′ (4.86) and C-3 (88.9), H-4′ (4.30) and C-1″ (C-1 of terminal glucosyl group; 104.9), and between H-1″ (5.18) and C-4′ (81.6). The spectral data are nearly identical to those published previously by Agerbirk et al. (2003a) that used slightly different conditions, and they confirm that the isolated compound is oleanolic acid-3-O-(4-O-β-D-glucopyranosyl)-β-D-glucopyranoside (oleanolic acid cellobioside).

α-Hederin was purchased from Extrasynthese, France, hederagenin from Carl Roth GmbH, Germany, and oleanolic acid from ICN Biomedical, USA.

Bioassays Saponins and sapogenins were dissolved in 75% ethanol. Radish leaf discs (11 mm diam) were treated on both sides with 15 μl of these solutions by using an automatic pipette. Control leaf discs were treated with solvent only. When the solvent had evaporated, the leaf discs were transferred to plastic containers (*V*=185 ml) with gypsum-charcoal bottom layers that supplied high humidity. Two leaf discs that had received the same treatment were transferred to each container (non-choice situation). In each container, one beetle was exposed to two leaf discs for 24 h at 24±2°C and a L18:D6 photoperiod. The leaf area consumed was measured under a stereo microscope.

In order to avoid biases due to day-to-day variations in availability of beetles from different lines and in radish leaf quality, the following measures were taken: 1) bioassays were made only on days when at least two different lines were available, and 2) consumption rates of individual beetles on radish leaf discs treated with saponins or sapogenins were adjusted with consumption rates on control leaf discs tested on the same day (see statistics).

Statistical Analysis Consumption by one beetle on two leaf discs for 24 h was treated as one replicate (C_i). From each replicate, a relative consumption rate (RCR) was calculated as 100×C_i / average consumption on control leaf discs tested on the same day. Confidence limits (95%) around RCR were calculated as $t_{0.975}$ × SE. Test and control treatments are significantly different when the 95% confidence interval around RCR does not include 100, which is the theoretical RCR if consumption rates of test and control leaf discs are equal. Analysis of variance (ANOVA) was performed with the GLM procedure in SAS on square root transformed values of RCR in order to obtain homogeneity of variances.

Results

Relative consumption rates (RCR) on treated radish leaf discs depended on compound, concentration and flea beetle

genotype (Tables 1, 2). Analysis of variance (ANOVA) on all data and on various subsets of them provided further information on the sources of variation. In the analysis of all data, a significant effect of flea beetle line was found (Table 2). This effect disappeared when data from resistant genotypes (Rr; lines: YE, AE, AK, and DE) and susceptible genotypes (rr; ST females and males) were analyzed separately (Table 2). This means that the variation observed among flea beetle lines in the overall ANOVA could be explained as a difference between on the one hand the susceptible ST line, and on the other hand all the resistant lines, whereas there were no significant differences among the resistant lines depending on the origin of the R-gene.

The two saponins isolated from *Barbarea vulgaris*, hederagenin cellobioside and oleanolic acid cellobioside had different effects on RCR in flea beetles. High concentrations of hederagenin cellobioside (2.0 mM and 0.5 mM) had significant, negative effects on RCR in all lines, since confidence intervals around RCR never included 100 (the theoretical value when consumption rates on treated and control leaf discs are equal) (Table 1). The effect of hederagenin cellobioside was much stronger on the susceptible ST-line compared to the resistant lines (see below). The effect of oleanolic acid cellobioside was weaker and in many cases not significant (Table 1). The aglycones (sapogenins) were inactive (Table 1). These

Table 1 Relative consumption rates (RCR) in near-isogenic lines of the flea beetle, *Phyllotreta nemorum,* on radish leaf discs treated with different concentrations of saponins or sapogenins. Deterrent effects of the saponins and sapogenins are significant, when RCR + c.l. < 100, since 100 is the theoretical value of RCR, when there is no difference between treated and control leaf discs

Line	Gender	RCR ± c.l.[a] 2.0mM	*N*[b]	RCR ± c.l.[a] 0.5mM	*N*[b]	RCR ± c.l.[a] 0.125mM	*N*[b]
Hederagenin-cellobioside:							
ST	Male	6.1±2.83	7	26.8±6.33	9	101.6±38.48	8
ST	Female	8.6±3.61	10	19.0±6.73	10	84.1±26.45	10
YE	Male	33.6±18.89	11	53.9±15.07	12	101.7±28.49	14
AE	Female	21.8±9.45	5	48.6±16.50	10	103.8±38.12	6
AK	Female	24.2±13.73	5	50.1±17.17	8	106.0±119.6	3
DE	Female	59.8±25.98	7	72.0±22.38	10	98.1±25.83	7
Hederagenin							
ST	Male	94.1±52.88	7	84.7±36.56	7		
ST	Female	59.2±40.82	11	76.1±33.87	11		
YE	Male	57.2±46.26	11	109.0±26.52	10		
AE	Female	95.0±48.59	10	109.8±45.28	8		
AK	Female	118.7±79.15	5	92.7±61.74	5		
DE	Female	80.9±53.23	7	86.6±46.00	7		
Oleanolic acid cellobioside							
ST	Male	43.5±21.66	6	66.8±23.10	8	88.2±13.75	8
ST	Female	68.9±36.88	6	76.4±22.59	8	91.8±26.34	8
YE	Male	66.5±16.22	8	79.6±15.28	9	94.0±37.15	6
AE	Female	85.3±20.17	6	93.2±30.61	9	111.6±38.31	6
AK	Female	76.6±15.84	6	92.9±36.83	8	74.5±29.17	6
DE	Female	71.8±43.10	5	78.4±59.71	6	100.4±11.36	5
Oleanolic acid							
ST	Male	87.7±25.18	10	48.2±33.15	7		
ST	Female	84.3±15.80	10	73.1±35.09	8		
YE	Male	76.1±29.44	9	82.9±18.99	8		
AE	Female	89.4±44.78	8	95.9±20.14	12		
AK	Female	102.2±26.14	9	87.3±33.96	7		
DE	Female	80.3±26.48	10	87.9±18.55	9		
α-Hederin							
ST	Male	4.3±1.48	12	18.9±5.89	14	87.4±24.81	8
ST	Female	5.8±2.46	12	18.8±5.52	12	101.8±13.69	8
YE	Male	7.2±1.62	10	30.7±10.54	9	87.4±18.88	6
AE	Female	7.9±5.74	8	33.4±6.41	6	57.7±137.9	2
AK	Female	6.8±2.01	13	33.2±10.45	14	96.8±12.50	12
DE	Female	7.4±3.35	14	21.3±5.73	12	93.5±16.42	12

[a] 95% confidence limits ($t_{0.975}$ * standard error of the mean)

[b] *N* = Number of beetles tested

Table 2 Statistical analysis (ANOVA) on effects of saponins and sapogenins on relative consumption rates (RCR) in near-isogenic lines (data from Table 1). The analysis was performed initially on all data, and subsequently on various subsets of them in order to illustrate the main sources of variation

Subset of data	Effect	*F*	*df*	*P*
All data	Compound	94.75	4	<0.001
	Concentration	152.09	2	<0.001
	Flea beetle line	8.68	4	<0.001
Resistant genotype (Rr) (YE, AE, AK, and DE)	Compound	61.87	4	<0.001
	Concentration	88.51	2	<0.001
	Flea beetle line	1.24	3	ns
Susceptible genotype (rr) (ST; females and males)	Compound	36.22	4	<0.001
	Concentration	68.27	2	<0.001
	Gender	0.09	1	ns
Responses of resistant lines (Rr) to hederagenin glycosides[a]	Compound	68.38	1	<0.001
	Concentration	190.46	2	<0.001
	Flea beetle line	1.35	3	ns
Responses of ST-line (rr) to hederagenin-glycosides[a]	Compound	0.98	1	ns
	Concentration	254.25	2	<0.001

[a] hederagenin cellobioside and α-hederin

results demonstrate that the major saponin in *Barbarea*, hederagenin cellobioside, is a feeding deterrent for all flea beetle genotypes, and that the biological activity depends on structure of the aglycone and on the presence of a carbohydrate moiety.

There were differences among lines in RCR on radish leaf discs treated with higher concentrations of hederagenin cellobioside (2 mM: $F=10.80$; $df=4$; $P<0.001$, and 0.5 mM: $F=11.37$; $df=4$; $P<0.001$). No significant differences among lines were found in responses to lower concentrations of this compound nor to oleanolic acid cellobioside or to the aglycones (data not shown). Differences among lines in RCR on leaf discs treated with hederagenin cellobioside could be attributed to a difference between the genotypes rr (ST females and males combined) and Rr (YE, AE, AK, and DE combined) (2 mM: $F=37.48$; $df=1$; $P<0.001$, and 0.5 mM: $F=25.80$; $df=1$; $P<0.001$). These results suggest that hederagenin cellobioside is an important defensive compound in *Barbarea* against susceptible genotypes, and that the R-genes in flea beetles might be adaptations to defenses in *Barbarea* mediated by this compound.

The importance of glycosylation pattern of saponins in defenses in *Barbarea* and counter-adaptations in flea beetles was illustrated further by beetle responses to the non-host compound α-hederin, which contains the same aglycon as hederagenin cellobioside, but differs in glycosylation pattern (Fig. 1). α-Hederin reduced RCR in all flea beetle lines (Tables 1, 2). Although hederagenin cellobioside and α-hederin influenced RCR in all lines, there were differences in the effects of the two compounds on susceptible and resistant genotypes. Hederagenin cellobioside and α-hederin had similar effects on RCR in the ST-line (rr), whereas α-hederin was significantly more inhibitory than hederagenin cellobioside to all the resistant lines (Table 2). Differential responses of resistant genotypes to hederagenin cellobioside and α-hederin strengthen the assumption that R-genes are adaptations to defenses in *Barbarea* mediated by saponins with a specific glycosylation pattern.

Discussion

The results presented here illustrate some of the chemical and metabolic changes that seem to have occurred in *Barbarea* and flea beetles as an outcome of evolutionary interactions between them.

Until now, *Barbarea* is the only genus of Brassicaceae known to contain saponins (Shinoda et al. 2002; Agerbirk et al. 2003a; Kuzina et al. 2009). The restricted distribution of saponins in the family suggests that they originated later than the glucosinolates, which have a wider distribution in the family. Hederagenin cellobioside was the most abundant saponin in the G-type and in hybrids from crosses between the G- and the P-type, whereas oleanolic acid cellobioside and other yet unidentified saponins were found in smaller quantities (Kuzina et al. 2009). There were significant, negative correlations between survival of flea beetle larvae and concentrations of hederagenin cellobioside, oleanolic acid cellobioside, and several minor saponins in a segregating F2 population from crosses between the G- and the P-type (Kuzina et al. 2009). The range of concentrations where hederagenin cellobioside was active in the present study was well within the range found for this compound in *B. vulgaris* (Shinoda et al. 2002; Nielsen et al. unpublished results). These results suggest that hederagenin cellobioside is an important part of the defenses in *Barbarea* against flea beetles, since it is both the most abundant and the most active compound. Further studies are necessary to unravel the role of less active oleanolic

acid cellobioside and the minor saponins in plant defenses against flea beetles. Since all these saponins are biosynthetically related, they tend to occur in the same plants, and it is still uncertain whether combinations of hederagenin cellobioside with oleanolic acid cellobioside and some of the minor saponins are more active than hederagenin cellobioside alone.

Both saponins isolated from *Barbarea* inhibit feeding in larvae of the diamond back moth, *Plutella xylostella* (Shinoda et al. 2002, unpublished results). This crucifer specialist readily accepts *B. vulgaris* for oviposition, but larvae do not survive on it (Badenes-Perez et al. 2006). *Barbarea* species also are less preferred hosts for several other crucifer specialists (Börjesdotter 2000; Renwick 2002), and some of these insects discriminate between G- and P-type plants (Renwick 2002; Nielsen, unpublished results). It is, therefore, likely that saponins contribute to defenses in *Barbarea* against several herbivorous insects, although in some cases other mechanisms also may be important (van Leur et al. 2008).

Mechanisms of the action of saponins on insects are not well understood. Saponins have been found to reduce feeding in adult flea beetles, but the present study did not allow any clear distinction between pre- and postingestive effects on food consumption (Glendinning 2002). *Barbarea* is acceptable for initial feeding by adult flea beetles and for mine initiation by larvae (Nielsen 1997a, b). The termination of feeding after a short meal might indicate that defensive compounds in *Barbarea* exert a toxic, post-ingestive effect that inhibits further feeding behavior. Behavioral as well as toxic effects of saponins have been demonstrated in other interactions between plants and insects (Adel et al. 2000; Golawska 2007).

This study demonstrates that glycosides are more active than aglycones, whereas the identity of the diglycosidic sugar moiety was less important against non-adapted flea beetles. Unfortunately, hederagenin-monoglycosides were not available to be tested. Saponin monoglucosides were not abundant in *Barbarea* (Kuzina et al. 2009), but they might be formed during degradation of the cellobiosides as part of a putative detoxification process in flea beetles (see below). Aglycones were more active than their parent glycosides from *Medicago* spp. against the generalist insect, *Spodoptera littoralis* (Adel et al. 2000) and against plant parasitic nematodes (Argentieri et al. 2008). Hederagenin glycosides with three sugar units had higher molluscicidal activity than saponins with only one sugar unit (Huang et al. 2003). These variable outcomes suggest that saponins may exert various effects on herbivores, and further studies are needed in order to understand the relationships between chemical structure and biological effects.

Presence of defensive compounds in plants may mediate counter-adaptations in herbivorous insects. Glucosinolates often are considered as a first generation of defensive compounds in members of Capparales (Feeny 1977; Nielsen 1978; Renwick 2002), and a number of specialist insects have evolved counter-adaptations to them and may use them as host specific cues (Renwick 2002). *Phyllotreta nemorum* is stimulated to feed by glucosinolates, and since all known host plants for the species contain glucosinolates (Nielsen 1978), it is likely that alleles that confer adaptations to glucosinolates have become fixed in the species.

Saponins belong to a second generation of defensive compounds in Capparales, and crucifer specialist insects have had a shorter time to adapt to them. The present study suggests that R-alleles in flea beetles represent specific adaptations to saponins in *Barbarea*. This is in agreement with previous observations that R-alleles confer improved performance on *Barbarea* but not on any other glucosinolate-containing plant (Nielsen 1999). R-alleles are, thus, considered to be derived traits, which have evolved in flea beetles as an outcome of natural selection imposed by the presence of defensive saponins in a potential, novel host plant, *Barbarea*. Different R-genes may have originated from independant mutations on different chromosomes, i.e., on an autosome in the gene(s) found in the AE, AK, and DE lines or on the Y-chromosome in the gene found in the YE line. Alternatively, they may have all originated from a single mutation on an autosome, and the Y-linked gene was later translocated to the Y-chromosome. The similarity of effects of R-alleles on feeding behavior on saponin-treated leaf discs suggests that their mode of action is similar, i.e., they may belong to the same gene family. *Phyllotreta nemorum* is polymorphic with respect to R-alleles, which are abundant in populations living on the G-type of *Barbarea*, but is relatively rare in populations that live on other host plants (Nielsen and de Jong 2005).

The present study indicates that enzymic degradation of hederagenin cellobioside might be a potential mode of action of R-alleles. The susceptible genotype (rr) of flea beetles is more sensitive to hederagenin cellobioside than the resistant genotype (Rr), whereas both genotypes are insensitive to the aglycone, hederagenin. This difference among genotypes could be explained if R-alleles encode for an enzyme (e.g., a β-glucosidase), which can cleave glycosidic bonds in hederagenin cellobioside. The partial sensitivity of resistant beetles to hederagenin cellobioside then could be explained as an effect of accumulation of the compound in the guts of flea beetles if consumption rates are higher than the rates of degradation. Resistant and susceptible genotypes of flea beetles were equally sensitive to the non-host compound, α-hederin. This observation provides further support for the idea that R-alleles encode for a specific enzyme, which can remove one or two glucose units from hederagenin cellobioside, but not

rhamnose or arabinose units from α-hederin. Deactivation of saponins by means of β-glucosidases or other glycohydrolase enzymes has not been demonstrated in any insect species, whereas detoxification of saponins by removal of sugars is an integral part of the infection process in many pathogenic fungi (Bowyer et al. 1995; Osbourn 1996; Morrissey et al. 2000; Morant et al. 2008).

This study has identified saponins as defensive compounds in *Barbarea* and as targets for counter-adaptations in flea beetles. Saponins may confer resistance in the plant against a number of herbivores, and no information is available yet on effects of individual herbivore species on evolution of defenses in *Barbarea*. The study identified R-alleles in flea beetles as derived traits that confer counter-adaptations in flea beetles to saponins in *Barbarea*. Improved knowledge of the chemical aspects of the interactions between *Barbarea* and flea beetles may provide further insight into the factors that influence the distribution and abundance of susceptible and resistant plants, and of the R-alleles in natural flea beetle populations that live on different host plants. Furthermore, there is geographic variation in host plant use by *P. nemorum* and in flea beetle abundance on local patches of *Barbarea* (Nielsen and de Jong 2005; de Jong et al. 2009). For this reason, the interaction between *Barbarea* and flea beetles offers excellent opportunities for investigations of several aspects of the geographic mosaic theory of coevolution (Thompson 2005).

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Biosynthesis of Phenolic Glycosides from Phenylpropanoid and Benzenoid Precursors in *Populus*

Benjamin A. Babst · Scott A. Harding · Chung-Jui Tsai

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Abstract Salicylate-containing phenolic glycosides (PGs) are abundant and often play a dominant role in plant-herbivore interactions of *Populus* and *Salix* species (family Salicaceae), but the biosynthetic pathway to PGs remains unclear. Cinnamic acid (CA) is thought to be a precursor of the salicyl moiety of PGs. However, the origin of the 6-hydroxy-2-cyclohexen-on-oyl (HCH) moiety found in certain PGs, such as salicortin, is not known. HCH is of interest because it confers toxicity and antifeedant properties against herbivores. We incubated *Populus nigra* leaf tissue with stable isotope-labeled CA, benzoates, and salicylates, and measured isotopic incorporation levels into both salicin, the simplest PG, and salicortin. Labeling of salicortin from [$^{13}C_6$]-CA provided the first evidence that HCH, like the salicyl moiety, is a phenylpropanoid derivative. Benzoic acid and benzaldehyde also labeled both salicyl and HCH, while benzyl alcohol labeled only the salicyl moiety in salicortin. Co-administration of unlabeled benzoates with [$^{13}C_6$]-CA confirmed their contribution to the biosynthesis of the salicyl but not the HCH moiety of salicortin. These data suggest that benzoate interconversions may modulate partitioning of phenylpropanoids to salicyl and HCH moieties, and hence toxicity of PGs. Surprisingly, labeled salicyl alcohol and salicylaldehyde were readily converted to salicin, but did not result in labeled salicortin. Co-administration of unlabeled salicylates with labeled CA suggested that salicyl alcohol and salicylaldehyde may have inhibited salicortin biosynthesis. A revised metabolic grid model of PG biosynthesis in *Populus* is proposed, providing a guide for functional genomic analysis of the PG biosynthetic pathway.

Keywords Poplar · Salicaceae · Isotope labeling · Phenolic glycosides · Herbivore defense · Carbon-13 · LC-MS

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B. A. Babst · S. A. Harding · C.-J. Tsai
School of Forest Resources and Environmental Science,
Michigan Technological University,
Houghton, MI 49931, USA

C.-J. Tsai
Department of Genetics, University of Georgia,
Athens, GA 30602, USA

Present Address:
B. A. Babst (✉) · S. A. Harding · C.-J. Tsai
Warnell School of Forestry and Natural Resources,
University of Georgia,
Athens, GA 30602, USA
e-mail: benbabst@uga.edu

Introduction

Salicylate-containing phenolic glycosides (PGs) are important mediators in ecological interactions of *Populus* and *Salix* species with their respective herbivore communities. While PGs function as chemical deterrents and toxicants against certain herbivores, their foliar concentration can be high (up to 25%) in some genotypes, posing a potential trade-off to growth (Osier and Lindroth 2001). Investigating the biosynthesis and turnover of PGs will be essential for understanding the growth-defense trade-off associated with PGs in these species.

Several studies suggest that PGs are derived from cinnamic acid (CA) (Fig. 1). For example, administering 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of phenylalanine ammonia lyase (PAL), the enzyme that catalyzes conversion of phenylalanine to CA, drastically reduced PG accumulation in shoot tips of *Salix pentandra*

Fig. 1 Salicylate-containing phenolic glycosides, putative precursors, and isotope labeling patterns

plantlets (Ruuhola and Julkunen-Tiitto 2003). Additionally, radioisotope-labeled CA and benzoic acid (BA) were incorporated into salicin, a salicyl alcohol glycoside (Fig. 1), based on administration to *Salix purpurea* leaf disks (Zenk 1967). Labeled benzyl alcohol and salicyl alcohol were converted to salicin as well, although administration of salicyl alcohol also resulted in formation of isosalicin, which was not present in untreated leaf disks (Zenk 1967). Isosalicin is not found in most *Populus* and *Salix* species (Palo 1984), but it has been observed in bark extracts of *Salix daphnoides* (Kammerer et al. 2005). Administering radiolabeled *o*-coumaric acid resulted in substantial radioactivity in salicyl alcohol, suggesting an alternate possible PG pathway, in which 2-hydroxylation of CA occurs prior to shortening of the propenyl side-chain. However, salicylic acid (SA), the well-known plant defense signal, was not readily converted to salicyl alcohol (Zenk 1967). Based on the ability of salicylaldehyde to compete with labeled CA for incorporation into salicin, Zenk (1967) suggested that salicylaldehyde is glucosylated to form helicin as a potential precursor for salicin and all other PGs. However, this hypothesis has not been tested.

The biosynthesis of higher-order PGs, such as salicortin, remains unclear, as well. Their potent biological activity has been attributed to the 6-hydroxy-2-cyclohexen-on-oyl (HCH) moiety, which is released by hydrolysis in the insect gut (Lindroth and Hwang 1996; Ruuhola et al. 2001). It is thought that salicin is an intermediate, which is conjugated

to the HCH precursor, 1-hydroxy-6-oxo-2-cyclohexene-1-carboxylic acid (HCC), to produce salicortin (Fig. 1). In the present study, we administered stable-isotope labeled CA, benzoates and salicylates to *Populus nigra* leaf disks, and measured incorporation of label into both salicin and salicortin, the two primary forms of PGs detected in this species. Salicortin is of particular interest, because it is nearly ubiquitous among *Salix* and *Populus* species, and because of its role in mediating ecological interactions in these species. Based on our findings, we propose revisions to the current model of the PG biosynthetic pathway.

Methods and Materials

Isotope Labeled Precursors All isotopic materials were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA), except salicylaldehyde and salicyl alcohol. All were uniformly labeled with ^{13}C (99 atom % ^{13}C) at each of the 6 phenyl carbons (e.g., [$^{13}C_6$]-CA; see Fig. 1), except [$^2H_5$]-BA and [$^2H_6$]-CA. [$^2H_5$]-BA was uniformly labeled with deuterium (99 atom % ^{2}H) at each of the five H positions on the phenyl ring, whereas [$^2H_6$]-CA was labeled similarly but has an additional ^{2}H at the β-carbon of the propenyl side-chain (98 atom % ^{2}H).

[$^{13}C_6$]-Salicylaldehyde was synthesized from [$^{13}C_6$]-phenol, using the modified Reimer-Tiemann reaction described by Mokle et al. (2006). Briefly, [$^{13}C_6$-]-phenol (99 atom % ^{13}C) was refluxed with 60% concentrated sodium hydroxide, 6% ethanol, and 34% chloroform at 65°C for 20 min. The solution was acidified with HCl, and then extracted with diethyl ether. Salicylaldehyde product was isolated by flash chromatography (normal phase), using hexane:ether (19:1, v/v) as the mobile phase. Fractions were collected and preliminarily evaluated by normal phase thin layer chromatography (TLC) with hexane:ether (9:1, v/v) mobile phase. Fractions containing a TLC band with Rf corresponding to authentic salicylaldehyde (Sigma, St Louis, MO, USA) were combined, and then roto-evaporated to remove solvent. The purified product was evaluated by HPLC-MS (negative ion mode) on a C18 reversed phase column (as described below), using authentic standard salicylaldehyde. The synthetic [$^{13}C_6$]-salicylaldehyde had the same retention time as the authentic standard, with the base peak at *m/z* 127, $[M-1+6]^-$ which is 6 units higher than the base peak of the natural abundance standard salicylaldehyde, *m/z* 121 $[M-1]^-$, as expected. Based on HPLC-MS, we calculated that the [$^{13}C_6$]-salicylaldehyde was 99 atom % ^{13}C.

[$^{13}C_6$]-Salicyl alcohol was synthesized by reducing [$^{13}C_6$]-salicylaldehyde in sodium borohydride, modified from (Setamdideh and Zeynizadeh 2006). We heated [$^{13}C_6$]-salicylaldehyde in 95% ethanol and sodium borohydride (1:1 molar ratio of salicylaldehyde:sodium borohydride) and refluxed for 20 min. The solution was cooled and concentrated under a stream of N_2 gas, and then ethanol was evaporated completely under vacuum centrifugation. The resulting powder was resuspended in water, acidified with 10% HCl, and extracted in methylene chloride. After evaporating the methylene chloride extract, the resulting salicyl alcohol was resuspended in ethanol, and checked by HPLC-MS. The synthetic [$^{13}C_6$]-salicyl alcohol at *m/z* 129, $[M-1+6]^-$ (99 atom % ^{13}C) co-eluted with the natural abundance authentic standard at *m/z* 123, $[M–1]^-$.

Plant Material Populus nigra NC5271 plants were propagated by rooted cuttings and then grown to a height of at least 0.5 m in a glasshouse before use in experiments. Supplemental lighting (600 μmole m^{-2} s^{-1} at mid canopy) was used to extend the day length in the fall and winter (14 :10 h light:dark). The leaf plastochron index (LPI) was used according to Larson and Isebrands (1971), with LPI 0 defined as the first fully unfolded leaf greater than 1 cm in length. LPI 3 leaves were used for the administration experiments because salicortin biosynthesis was active, based on preliminary testing, and the leaves provided ample, soft material for facile extraction of PGs.

Leaf Disk Administration Leaf disks were taken from LPI 3 leaves, avoiding the leaf margins and veins, where leaf tissues were less uniform. Typically, we tested 3 independent leaf disks per treatment group for each experiment. Each 6 mm diam leaf disk was submerged in 0.2% sodium bicarbonate solution with the appropriate isotope-labeled compound, and infiltrated by using gentle aspiration for 10 min. The concentration of labeled compounds varied depending on the experiment and is specified in the results section. Labeled compounds were administered at 99% isotopic purity. To aid infiltration, 0.01% Tween 20 and 1% ethanol were included. Following infiltration, the solution was replaced with 0.2% sodium bicarbonate and labeled compound, without Tween 20 or ethanol. Incubation under 100 μmole m^{-2} s^{-1} light was conducted for 6 h based on preliminary tests, thus indicating that a 4–6 h administration time was required for consistent detection of label incorporation from [$^{13}C_6$]-CA into salicin and salicortin. All experimental comparisons were made with similarly treated control leaf disks. In all samples analyzed (with or without phenolic compound administration), total salicin and salicortin levels detected in leaf disks were similar to those detected from intact tissues (i.e., tissues harvested directly into methanol or snap-frozen immediately), suggesting that the effect of cutting the leaf disks on PG accumulation, if any, was minimal during the short administration periods.

HPLC-MS Analysis Following incubation, each leaf disk was blotted dry, washed briefly with hexane, and then

extracted with cold methanol for 12 min in a 4°C ultrasonicator bath. An aliquot of the methanol extract was analyzed directly by reverse-phase using an Agilent 1100 HPLC equipped with DAD UV/VIS detector and single quadrapole mass spec (Agilent, Santa Clara, CA, USA). To discriminate between labeling of the salicylate ring and the HCH ring of salicortin, the remaining half of the methanolic extract was subjected to mild alkaline hydrolysis, and salicortin breakdown products were analyzed. The hydrolytic procedure was developed by using an authentic salicortin standard (kindly provided by R.L. Lindroth, University of Wisconsin). Briefly, the methanolic extract was mixed with 1:10 vol/vol 0.5 M sodium carbonate (pH 11.45) for hydrolysis at 60°C for 20 min. Under these conditions, all of the salicortin was hydrolytically cleaved, liberating salicin and HCH (Ruuhola et al. 2003). Since salicin also occurs naturally in *Populus* leaves, the amount of salicin prior to hydrolysis was subtracted from the amount of salicin after hydrolysis to estimate the amount of salicin specifically hydrolyzed from salicortin. Since an authentic HCH standard was not available, the data for putative HCH is presented in absorbance units for relative comparisons.

Each extract or hydrolysate (10 µl) was loaded onto an Eclipse XDB-C18 column (150 x 2.1 mm, Agilent, Santa Clara, CA, USA) equipped with an Eclipse XDB-C8 guard column (12.5 x 4.6 mm). Compounds were resolved using a 0.2 ml min^{-1} flow rate of mobile phase A (10 mM formic acid, pH 3.3) and mobile phase B (acetone), with the following gradient: 0–15 min (linear 0–70% B), 15–17 min (linear 70–100% B), and 17–19 min (100% B). Samples were analyzed by a DAD detector (recorded spectrum at 200–400 nm, and chromatographic signal at 215, 255, 270, 310, and 350 nm), as well as MS detection. The MS detector (negative ion mode) was set to API-ES ionization mode, *m/z* 90–800, with a drying gas temperature of 350°C, a flow rate of 10.0 l min^{-1}, and a voltage cap of 3,000 V. Due to the use of negative polarity, ions typically were detected as $[M–1]^-$. Additionally, some ions were observed at *m/z* 46 units higher than expected based on formula mass, due to formation of formide adducts $[M-H+HCOOH]^-$, from the formic acid mobile phase. Isotope labeled and unlabeled PG concentrations were determined from standard curves of authentic salicin and salicortin, using extracted ion chromatogram peak areas, and are presented as the concentration on a dry weight basis (µmol g^{-1} dry weight).

Statistical Analysis For each experiment, analysis of variance was performed using SigmaStat software version 3.5 (Systat Software, Inc., San Jose, CA, USA). When *ANOVA* indicated a significant effect of administration treatments or co-administration treatments, data were analyzed further by using a multiple comparison test. For labeled cinnamate, salicylate, and benzoate administration, all treatments were compared pairwise using a Fisher's protected *LSD* (α= 0.05). For co-administration experiments, a Holm-Sidak multiple comparison test was performed, with all treatments compared to the control, which was $[^{13}C]$-CA administration with no co-administration.

Results

Incorporation of Stable Isotope Labeled CA into PG We administered 0.5 mM $[^{13}C_6]$-CA to the leaf disks and evaluated isotope incorporation into PG. The [M+6] differential allowed us to distinguish clearly between the $[^{13}C_6]$-labeled and natural abundance forms of salicin and salicortin. When $[^{13}C_6]$-CA was administered to leaf disks for 6 h, a new ion indicating labeled salicin (*m/z* 337) $[M+6-H+HCOOH]$ appeared with the same retention time (11.5 min) as $[^{12}C]$-salicin (Fig. 2a and b). Authentic standard salicin was detected at *m/z* 331 $[M-H+HCOOH]^-$, consistent with detection of the formide adduct of salicin, due to formic acid in our HPLC solvent. This confirmed that CA is a precursor for salicin. Three ions of salicortin were detected at the same retention time (13.25 min) following administration (Fig. 2c). These included unlabeled salicortin at *m/z* 423 $[M–H]^-$, and two new ions that were 6 and 12 units larger (*m/z* 429 $[M-H+6]^-$ and *m/z* 435 $[M-H+12]^-$). Only the 423 ion was detected in extracts from control leaf disks (Fig. 2d). This indicated that the CA tracer was incorporated into salicortin, and that both the salicyl and HCH rings were derived from CA. Hereafter, the occurrence of the 429 ion is referred to as single-ring labeled salicortin, while the 435 ion is referred to as double-ring labeled salicortin.

A hydrolytic procedure was used to confirm tracer incorporation into the HCH or salicyl moieties of salicortin (see Methods and Materials). The hydrolysis of salicortin resulted in 2 major peaks on the HPLC chromatogram, including salicin (*m/z* 331) and an unidentified peak (*m/z* 187). The *m/z* 187 of the unidentified peak is consistent with the mass of 1-hydroxy-6-oxo-2-cyclohexene-1-hydroxymethane (HCH-alcohol) with a formide adduct $[M-H+HCOOH]$. Administering $[^{13}C_6]$-CA resulted in *m/z* 337 and *m/z* 193 following hydrolysis, consistent with labeled salicin and HCH-alcohol, respectively. For leaf extracts, there was an additional major peak with *m/z* 177 following hydrolysis, but the UV absorbance maxima (310 and 320 nm) suggested that this peak was derived from hydroxycinnamates. Hereafter, we treat the 187 ion as the putative HCH-derived hydrolysis product of salicortin, and the 193 ion as its $^{13}C_6$ labeled homolog.

Peak areas of labeled salicin and salicortin were smaller than those of the unlabeled forms (Fig. 2a and c). This was

Fig. 2 Salicylate glycoside labeling from [$^{13}C_6$]-cinnamic acid. Labeled (*m/z* 337, dashed line) and unlabeled (*m/z* 331, solid line) salicin, as well as single (*m/z* 429, dashed line) and double (*m/z* 435, dotted line) ring labeled salicortin and unlabeled salicortin (*m/z*=423, solid line) following **a**, **c** [$^{13}C_6$]-CA administration, or **b**, **d** control incubation. Chromatograms show overlays of mass-spec detector (MSD) extracted ion signals for the unlabeled ions on the primary y-axis, and for the labeled ions on the secondary y-axis (note the smaller scale on the secondary y-axes)

observed over a time course of 2, 4, and 6 h of administration, which showed a continual slow accumulation of labeled salicortin up to 0.7 µmol/g over 6 h, relative to 50–60 µmol/g unlabeled salicortin. Double-ring labeled salicortin was more abundant than single-ring labeled salicortin when [$^{13}C_6$]-CA was administered at 0.5 mM, but the reverse was true at the lower administration level of 0.1 mM (Fig. 3b). More double- than single-ring labeling at high administration levels may indicate that [$^{13}C_6$]-CA was present in the leaf cells at higher concentrations than the endogenous, unlabeled pool. This would suggest that the uptake of [$^{13}C_6$]-CA was not limiting, but the low percentage of labeled salicortin was due to slow biosynthesis.

Incorporation of Labeled Benzoates and Salicylates into PG Because they are putative intermediates in salicin biosynthesis, [$^{13}C_6$]-labeled benzyl alcohol, benzaldehyde, salicyl alcohol, and salicylaldehyde, as well as [$^2H_5$]-BA

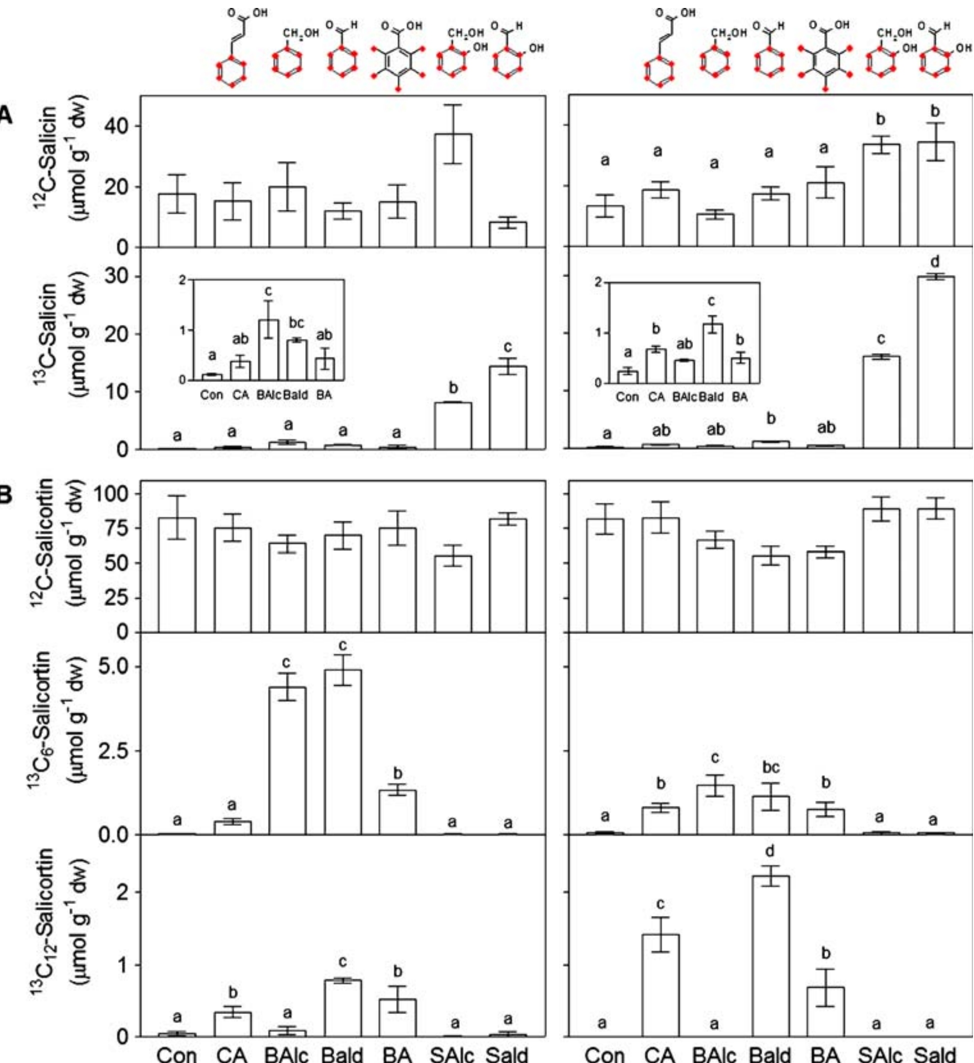

Fig. 3 Incorporation of stable isotope from labeled compounds into **a** salicin, and **b** salicortin. *Populus nigra* leaf disks were incubated in 0.2% sodium bicarbonate (Con) and with [$^{13}C_6$]-cinnamic acid (CA), [$^{13}C_6$]-benzyl alcohol (BAlc), [$^{13}C_6$]-benzaldehyde (Bald), [D_5]-benzoic acid (BA), [$^{13}C_6$]-salicyl alcohol (SAlc) or [$^{13}C_6$]-salicylaldehyde (Sald) administered at 0.1 mM (left panels) or 0.5 mM (right panels) for 6 h. Bars indicate means ± standard errors (N=3). Fisher's protected LSD was used to compare all treatments pairwise. Like letters above bars indicate a lack of significant difference. A separate analysis was conducted for insets

(Fig. 1) were administered to *P. nigra* leaf disks. Administering [$^2H_5$]-BA resulted in [$^2H_4$]-salicin (*m/z* 335, $[M-H+HCOOH+4]^-$), as well as [$^2H_4$]- and [$^2H_8$]-salicortin (*m/z* 427 $[M-H+4]^-$ and *m/z* 431 $[M-H+8]^-$, respectively). Administering labeled SA was not conducted, because preliminary administration of unlabeled SA yielded only SA glucoside (SAG) (Suppl. Fig. 1). We anticipated this result since transgenic knockdown of SA in *Populus* reduced SAG concentrations, but not those of salicin, salicortin, or tremuloidin (Morse et al. 2007).

Tracer from salicyl alcohol, salicylaldehyde, BA, benzyl alcohol, and benzaldehyde was incorporated into salicin, as was previously observed by Zenk (1967). Administration of labeled salicylaldehyde also resulted in trace levels of its labeled glucoside, helicin (RT 12.5 min; *m/z* 335) $[M-H+HCOOH+6]^-$, confirmed based on retention time and mass spectrum of authentic helicin. Unlabeled helicin was not detected in any other leaf samples. This indicates that salicylaldehyde is converted mostly to salicyl alcohol before conversion to salicin, with helicin as a minor artifact (via nonspecific glycosyltransferase activities). Alternatively, salicylaldehyde is glycosylated to form helicin first before being reduced rapidly to form salicin, thus leaving a small standing pool of helicin. Helicin has been suggested previously to be an intermediate in salicin biosynthesis (Zenk 1967). Accumulation of labeled salicin was 13–75 fold higher for salicyl alcohol and salicylaldehyde than for BA, benzyl alcohol, benzaldehyde or CA administration (Fig. 3a). Labeling of salicin from salicyl alcohol and salicylaldehyde increased with the administration concentrations from 0.1 mM to 0.5 mM, but remained constant and comparatively low from BA, benzyl alcohol, benzaldehyde, or CA at both administration concentrations. Administering salicylaldehyde and salicyl alcohol also resulted in accumulation of [$^{13}C_6$]-isosalicin (*m/z* 291) $[M-H+6]^-$, which was not present in the control leaf disks (Suppl. Fig. 2). The identity of isosalicin was based on comparison to the retention time and mass spectrum of authentic unlabeled isosalicin (*m/z* 285) $[M–H]^-$ (kindly provided by Dr. Bernd Schneider, Max Plank Institute for

Chemical Ecology). Although the molecular mass of isosalicin and salicin are the same, the difference in *m/z* (285 for isosalicin vs. 331 for salicin) indicates that electrospray ionization did not result in a formide adduct of isosalicin. Administration of benzyl alcohol and benzaldehyde also resulted in a new peak (not shown), putatively the labeled glucoside of benzyl alcohol, benzyl-glucoside, based on *m/z* 315 $[M - H + HCOOH + 6]^-$.

Label from salicyl alcohol and salicylaldehyde administered at either 0.1 mM or 0.5 mM was not incorporated into salicortin (Fig. 3b; Suppl. Fig. 2B and C). However, administration of labeled CA, BA, and benzaldehyde each resulted in double- and single-ring labeled salicortin. Interestingly, benzyl alcohol administration resulted in only single-ring labeled salicortin (Fig 3b). Analysis of hydrolyzed leaf extracts confirmed isotopic labeling of both the salicyl and HCH moieties of salicortin from labeled CA, BA, and benzaldehyde (Suppl. Fig 2B and C). On the other hand, tracer from benzyl alcohol was found only in the salicyl ring. With respect to salicortin biosynthesis, the data suggest that salicin and HCH may be derived from BA or benzaldehyde, but that enzymatic conversion of BA and/or benzaldehyde to benzyl alcohol could favor synthesis of the salicyl moiety.

Co-administration of Salicylates and Benzoates Affected PG Biosynthesis from $^{13}C_6$-cinnamate In preliminary co-administration experiments, $[^{13}C_6]$-CA was administered along with unlabeled salicylates, cinnamates, benzoates, and salicin (Suppl. Fig. 3). For preliminary testing, $[^{13}C_6]$-CA was administered at 0.5 mM, while the unlabeled compounds were provided at 0.5 mM and 1.0 mM. BA, 4-hydroxy-BA, *p*-coumaric acid, SA, and salicin had no effect on concentrations of labeled or unlabeled salicortin. In contrast, benzyl alcohol, benzaldehyde, salicyl alcohol, and salicylaldehyde had the most pronounced effects, reducing the labeling of salicin and salicortin (Suppl. Fig. 3). Therefore, dose response experiments were conducted for benzyl alcohol, benzaldehyde, salicyl alcohol and salicylaldehyde, to aid in the interpretation of the label incorporation experiments.

Unlabeled salicyl alcohol, salicylaldehyde, benzaldehyde, and benzyl alcohol, at concentrations ranging from 0.01 to 1 mM, were co-administered along with 0.5 mM $[^{13}C_6]$-CA for dose response experiments (Fig. 4). Unlabeled salicyl alcohol and salicylaldehyde resulted in a drastic increase in the concentration of unlabeled salicin *m/z* 331, but a decrease in the amount of salicin that was labeled *m/z* 337 (Fig. 4c and d). This is consistent with label dilution due to facile glucosylation of the administered salicylates. Additionally, a portion of the salicyl alcohol that was administered was also converted into unlabeled isosalicin (Suppl. Fig. 4C) *m/z* 285. Similarly, co-administering salicylaldehyde also resulted in a dose-dependent increase in unlabeled isosalicin *m/z* 285, but also resulted in labeled isosalicin (Suppl. Fig. 4D) *m/z* 291.

Salicyl alcohol and salicylaldehyde also reduced incorporation of label from CA into salicortin (Fig. 4g and h) *m/z* 429 and *m/z* 435. The response was weakly apparent at 0.1 mM, and the labeling was reduced further with increasing salicylaldehyde or salicyl alcohol concentrations.

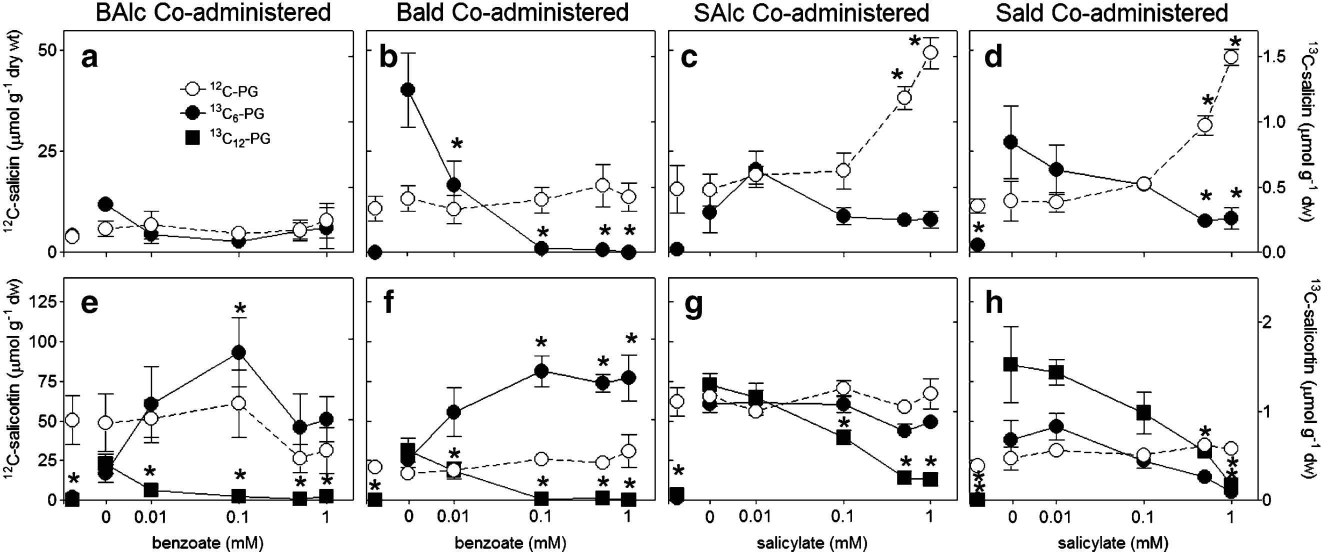

Fig. 4 Response of PG biosynthesis to co-administration of different concentrations of unlabeled BAlc, Bald, SAlc, and Sald. *De novo* PG biosynthesis was determined as $[^{13}C_6]$-CA conversion to $[^{13}C]$-PG. Values for no isotope controls are shown to the left of the response curves within each graph. Salicin is shown above **a**–**d**, and salicortin below **e**–**h**. Symbols and error bars indicate means and standard errors (N=3). Asterisks above symbols indicate significant differences from the no co-administration ^{13}C-CA control, using Holm-Sidak multiple comparison procedure

Analysis of hydrolysates indicated that labeling of both salicyl and HCH rings was decreased (Suppl. Fig. 4G, H, K and L). Together with the findings that label from salicyl alcohol and salicylaldehyde was not incorporated into salicortin (Fig. 3b), the reduction in [$^{13}C_6$]-CA-dependent labeling of salicortin in the co-administration experiment was consistent with inhibition or downregulation of *de novo* salicortin biosynthesis following salicyl alcohol and salicylaldehyde administration. Total salicortin concentrations did not decrease, probably because it is a relatively stable metabolite *in vivo*.

Co-administration of either unlabeled benzaldehyde or benzyl alcohol did not increase salicin levels or lead to accumulation of isosalicin (Fig. 4a, b; Suppl. Fig. 4A and B). However, both benzaldehyde and benzyl alcohol reduced incorporation of label from [$^{13}C_6$]-CA into salicin *m/z* 337, when co-administered at concentrations as low as 0.01 mM (Fig. 4a and b). Interestingly, formation of single-ring labeled salicortin *m/z* 429 from [$^{13}C_6$]-CA was stimulated (Fig. 4e and f), while double-ring labeled salicortin *m/z* 435 was reduced at 0.01 mM, and was essentially eliminated at 0.1 mM or higher concentrations of benzaldehyde and benzyl alcohol. The decreases in double-ring labeling of salicortin were due to decreased label incorporation into the salicyl moiety (Suppl. Fig. 4E and F). Thus, benzaldehyde and benzyl alcohol are both likely to be intermediates in the biosynthesis of the salicyl moiety. We observed a stimulation of HCH labeling that paralleled the increase in single-ring salicortin labeling, following benzyl alcohol and benzaldehyde co-administration (Suppl. Fig. 4I and J vs. Fig. 4e and f). At higher concentrations, benzyl alcohol appeared to have a negative effect as salicortin labeling decreased. However, benzaldehyde did not have the same effect, since salicortin labeling remained stable. These results are consistent with a sensitivity of the salicyl and HCH pathways to benzoate substrate availability.

Shortening the CA Propenyl Side Chain Deuterated (^{2}H) CA was administered to leaf disks to track the side-chain modification and hydroxylation reactions that lead to salicylate PGs. Each of the 5 hydrogen atoms at the phenyl ring, as well as the hydrogen at the β-carbon of the propenyl side-chain, was substituted with ^{2}H ([$^2H_6$]-CA; Fig. 1). The removal of the ^{2}H at the C-2 position of the phenyl ring is expected upon *O*-hydroxylation, based on the results of [$^2H_5$]-BA administration (see above). The removal of the ^{2}H on the β-carbon can indicate that the propenyl side chain shortening of cinnamate proceeds via a β-oxidative route (Jarvis et al. 2000). Upon administering [$^2H_6$]-CA, [$^2H_4$]-salicin *m/z* 335 $[M - H + HCOOH + 4]^-$ as well as $^2H_4$- and $^2H_8$-salicortin *m/z* 427 $[M - H + 4]^-$ and 431 $[M - H + 8]^-$ were detected (Supp Fig. 5), suggesting that a second deuterium was indeed removed from the β-carbon.

Discussion

Salicortin, a salicylate-containing phenolic glycoside (PG), plays an important role in ecological interactions and growth of *Populus* (Hwang and Lindroth 1997; Donaldson et al. 2006), but little is known about its biosynthetic pathway. Using stable isotope labeled prescursors, we provide the first direct empirical evidence that not only the salicyl moeity, but also the HCH moiety, of salicortin may be derived from CA, with benzoates as intermediates.

Salicin, the 2-*O*-glucoside of salicyl alcohol, has been considered a possible precursor of the salicyl moiety of salicortin (Ruuhola and Julkunen-Tiitto 2003). While administering labeled salicylaldehyde and salicyl alcohol resulted in accumulation of labeled salicin, it did not result in labeled salicortin at either concentration tested. Two possible explanations can be offered. First, salicylaldehyde and salicyl alcohol are precursors to salicortin, but exogenous salicylaldehyde and salicyl alcohol cannot gain entry to the salicortin biosynthetic pathway due to either metabolic channeling or abnormal sub-cellular localization. Metabolic channeling is known to be important for certain phenylpropanoid branch pathways (Winkel 2004). Exclusion of exogenous salicylaldehyde and salicyl alcohol from entering into PG pathways would not preclude glucosylation of salicylaldehyde or salicyl alcohol, since the glucosyltransferases that catalyze this type of reaction are known for their promiscuity in substrate affinity (Vogt and Jones 2000). Glucosylation of salicylaldehyde and salicyl alcohol in an abnormal sub-cellular context could have impeded their subsequent conjugation to HCH for salicortin synthesis, since glucosylation is known to alter sub-cellular localization of phenolics (Dean et al. 2003). If salicylaldehyde and salicyl alcohol were excluded from the salicortin biosynthetic pathway, the reduction of salicortin labeling from [$^{13}C_6$]-CA by co-administering unlabeled salicylaldehyde and salicyl alcohol cannot be explained by biosynthetic competition, but might be explained by down-regulation of the pathway or competition for a common pool of glucose or UDP-glucose. Second, it is plausible that salicylaldehyde and salicyl alcohol, and hence salicin, are not intermediates in salicortin biosynthesis. Since label from BA, benzaldehyde, and benzyl alcohol was incorporated into the salicyl group of salicortin, it is possible that salicortin biosynthesis may, instead, involve formation of a benzyl conjugate (e.g., benzyl-HCH or benzyl-benzoate) first, and then conversion of the benzyl moiety to a salicyl moiety by 2-hydroxylation (Fig. 5).

Among the benzoates, benzyl alcohol would be the most likely precursor of the salicyl ring of salicortin, since HCH is conjugated to the alcohol moiety of the salicyl group in salicortin (Pearl and Darling 1970, 1971). Consistent with this, co-administration of unlabeled benzaldehyde, benzyl alcohol, and, to a much lesser extent, BA, reduced labeling

Fig. 5 Revised working model of the salicortin biosynthetic pathway, or metabolic grid. Solid black arrows indicate pathways proposed previously in willow, tobacco, petunia and snapdragon (Zenk 1967; Jarvis et al. 2000; Ruuhola and Julkunen-Tiitto 2003; Boatright et al. 2004; Orlova et al. 2006; Long et al. 2009. Dashed black arrows indicate newly hypothesized pathways, including a CA/benzoate origin for 1-hydroxy-6-oxo-2-cyclohexene-1-carboxylic acid (HCC), the possible free acid precursor of the HCH moeity of salicortin (Pierpont 1994)

of the salicyl moiety of salicortin from [$^{13}C_6$]-CA. Administration of labeled BA and benzaldehyde resulted in labeled HCH and salicyl moieties, whereas labeled benzyl alcohol resulted only in a labeled salicyl moiety. Taken together, the results support a pathway in which CA is sequentially converted to BA and/or benzaldehyde, then to benzyl alcohol, and then to the salicyl moiety of salicortin. Further work will be necessary to resolve whether endogenous free salicylaldehyde and salicyl alcohol are intermediates in salicortin biosynthesis.

Isotope labeling suggests that BA and benzaldehyde, but not benzyl alcohol, may be intermediates in the conversion of CA to the HCH moiety of salicortin. However, co-administration of BA or benzaldehyde did not reduce label incorporation from [$^{13}C_6$]-CA into HCH. This suggests that benzoates might participate in a tangential, but interchangeable pathway, rather than as direct intermediates in the biosynthesis of HCH and its proposed immediate precursor, HCC (Fig. 5; Pierpont 1994). Emerging evidence from petunia suggests that benzoyl-CoA may be a precursor to

benzoic acid (grey box, Fig. 5). In petunia, CA may be converted to benzoyl-CoA via the β-oxidative pathway (Boatright et al. 2004; Orlova et al. 2006; Long et al. 2009). Most of the BA resulting from the β-oxidative pathway is actually derived from hydrolysis of benzyl-benzoate, itself the product of benzoyl-CoA conjugation with benzyl alcohol (Boatright et al. 2004; Orlova et al. 2006; Long et al. 2009). The benzoate pool also may be derived from two separate non-β-oxidative pathways via conversion of CA directly to benzaldehyde, or of phenylpyruvate directly to benzaldehyde (Boatright et al. 2004; Orlova et al. 2006; Long et al. 2009). In petunia, at least, both β-oxidative and non-β-oxidative pathways appear to be active simultaneously, although with differential flux and diurnal regulation (Boatright et al. 2004; Orlova et al. 2006). It is not yet clear whether either or both β-oxidative and non-β-oxidative pathways also exist in other distantly related species, such as *Populus*. The deuterium label at the β-carbon of [$^{2}H_{6}$]-CA administered to leaf disks was lost during the conversion to PGs, for both salicyl and HCH moieties, providing evidence in favor of a β-oxidative pathway in *Populus*. This result is similar to previous findings for tobacco (Jarvis et al. 2000), but we cannot rule out a contribution from a non-β-oxidative pathway, particularly under different environmental conditions (e.g., dark cycle).

The findings that both the salicyl and HCH moieties may be synthesized from the benzoates point to the importance of benzoate pool transformations in regulating salicortin biosynthesis. In *Salix pentandra* roots, which contained no PGs, administration of benzoic acid resulted in salicortin accumulation, providing further support for the central involvement of benzoates in the biosynthesis of both salicyl and HCH rings (Ruuhola and Julkunen-Tiitto 2003). Interestingly in this regard, co-administering unlabeled benzoic acid did not decrease HCH labeling from [$^{13}C_{6}$]-CA, while benzaldehyde and benzyl alcohol increased HCH labeling with a concomitant reduction in salicyl ring labeling. Unlabeled benzaldehyde and benzyl alcohol likely competed with the [$^{13}C_{6}$]-CA-derived pool for incorporation into the salicyl moiety, thereby reducing salicyl labeling, and shunting [$^{13}C_{6}$]-CA-derived metabolites toward the HCH pathway. If conversion to free benzoates is tangential to HCH biosynthesis, as discussed above, co-administering benzoates may be less likely to reduce HCH labeling directly. This would also be consistent with the structural complexity of HCH, implicating a multi-step conversion from the benzoate precursors, as opposed to the more facile conversion from benzoates to the salicyl moiety. On the other hand, if co-administration stimulated metabolism of benzoates through alternative pathways, the proportional labeling of the benzoate pools might have been higher than expected. Although it is unclear whether an alternative pathway was stimulated, benzaldehyde and benzyl alcohol administered to leaf disks were converted to benzyl-glucoside, indicating that at least one alternative pathway for benzoates was active. A synthesis of these scenarios is that there is great flexibility in the maintenance of salicyl:HCH balance due to the dynamic nature of benzoate and hydroxycinnamate pool size and complexity. The increased HCH labeling upon benzaldehyde and benzyl alcohol co-administration also suggests a possible signaling role of benzoates in maintaining the salicyl:HCH balance, which warrants further investigation.

PGs are diverse (Tsai et al. 2006), so the pathway is likely to be complex, and may vary in a species- and genotype-dependent manner. Ruuhola et al. (2003) suggested a metabolic grid for PG biosynthesis, rather than a strict pathway model. This would allow the possibility of multiple pathways of salicortin biosynthesis (e.g., salicyl alcohol-dependent and independent) that could function simultaneously or under different conditions. For example, administration of radiolabeled *o*-coumaric acid resulted in radioactive salicyl alcohol in *S. purpurea* (Zenk 1967). In our study, unlabeled *o*-coumaric acid appeared to compete with labeled CA for incorporation into both salicyl and HCH moieties of salicortin, although we cannot rule out that this result was due to inhibition rather than competition. Since 2-hydroxylation of benzoates can occur in *Populus*, as indicated by [$^{2}H_{5}$]-BA administration, *o*-coumaric acid might lie at the head of another pathway within the PG biosynthetic grid. Future studies will be necessary to test the extent to which 2-hydroxylation normally occurs before and after shortening of the propenyl side chain of CA.

By our revised model, the salicin biosynthetic pathway may need to be considered as a branch separate from the salicortin biosynthetic pathway, at least in some cases. Label from both salicylaldehyde and salicyl alcohol was incorporated into salicin. Co-administering unlabeled salicylaldehyde and salicyl alcohol reduced, but did not eliminate, label incorporation from CA into salicin, and it resulted in accumulation of high concentrations of unlabeled salicin, suggesting multiple pathways to salicin. However, administering unlabeled benzaldehyde or benzyl alcohol eliminated label incorporation from [$^{13}C_{6}$]-CA into salicin, indicating that all pathways to salicin required benzaldehyde and benzyl alcohol intermediates in our experimental system. Label from benzoates was also incorporated into salicin, but at a much lower rate than for salicylaldehyde and salicyl alcohol administration, suggesting that conversion from benzoates to salicylates may have been a rate limiting step. In addition, the proportion of salicortin that was labeled during benzoate administration was equal or greater than the proportion of salicin that was labeled. If salicin is an intermediate in

salicortin biosynthesis, then there may be more than one pool of salicin to account for the lack of labeling dilution in salicortin. A higher abundance, low turn-over pool (e.g., vacuolar storage) would explain the low percent labeling of salicin, while a smaller, high-turnover pool of salicin, that serves as intermediates for salicortin biosynthesis, could account for the higher than expected labeling in salicortin if salicin is an intermediate. Therefore, we conclude that a metabolic grid needs to be considered for salicin biosynthesis.

Salicin, while not incorporated into salicortin in the present study, cannot be excluded as a possible precursor for other PG derivatives, such as tremuloidin, that are not present in the experimental *P. nigra* clone. The HCH-devoid tremuloidin consists of a BA conjugated to the glucose moiety of salicin, and could be derived from a separate biosynthetic route than salicortin.

In summary, our results indicate that both the salicyl and the HCH rings of salicortin are of phenylpropanoid origin. CA is routed through benzyl alcohol in biosynthesis of the salicyl moiety, but is diverted upstream at benzaldehyde, BA, and/or benzoyl-CoA for synthesis of HCH. The sequenced *Populus trichocarpa* genome (Tuskan et al. 2006) and associated resources now offer the possibility of targeting specific gene families for functional genomic study (e.g., hydroxylases, glycosyltransferases) to begin identifying genes involved in the PG biosynthetic pathway. Future studies to identify PG-related genes should be designed that give consideration to all of the hypothetical pathways in our revised metabolic grid model. One novel addition to the metabolic grid of salicortin biosynthesis consists of addition of a benzoate group at the alcohol moiety of benzyl alcohol, followed by conversion of the benzoate moiety to HCH, 2-hydroxylation to form salicyl-HCH, and then glucosylation at the 2-hydroxyl group of the salicyl alcohol ring.

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Lichen Compounds Restrain Lichen Feeding by Bank Voles (*Myodes glareolus*)

Line Nybakken · Anne-Marit Helmersen · Yngvar Gauslaa · Vidar Selås

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Abstract Some lichen compounds are known to deter feeding by invertebrate herbivores. We attempted to quantify the deterring efficiency of lichen compounds against a generalist vertebrate, the bank vole (*Myodes glareolus*). In two separate experiments, caged bank voles had the choice to feed on lichens with natural or reduced concentrations of secondary compounds. We rinsed air-dry intact lichens in 100% acetone to remove extracellular compounds non-destructively. In the first experiment, pairs of control and rinsed lichen thalli were hydrated and offered to the bank voles. Because the lichens desiccated fast, we ran a second experiment with pairs of ground control and compound-deficient thalli, each mixed with water to porridge. Eight and six lichen species were tested in the first and second experiment, respectively. In the first, bank voles preferred compound-deficient thalli of *Cladonia stellaris* and *Lobaria pulmonaria*, but did not discriminate between the other thallus pairs. This was likely a result of deterring levels of usnic and stictic acid in the control thalli. When lichens were served as porridge, significant preference was found for acetone-rinsed pieces of *Cladonia arbuscula*, *C. rangiferina*, *Platismatia glauca,* and *Evernia prunastri*. The increased preference was caused mainly by lower consumption of control thalli. Grinding and mixing of thallus structures prevented bank voles from selecting thallus parts with lower concentration of secondary compounds and/or strengthened their deterring capacity. We conclude that some lichen secondary compounds deter feeding by bank voles.

Keywords Herbivory · Generalist · Grazing · Vertebrates · Lichen compounds · Lichenized fungi

L. Nybakken · A.-M. Helmersen · Y. Gauslaa · V. Selås
Department of Ecology and Natural Resource Management,
Norwegian University of Life Sciences,
Box 5003, NO-1432 Ås, Norway

Present Address:
L. Nybakken (✉)
Natural Products Research Laboratories, Department of Biology,
University of Eastern Finland,
P.O.Box 111, FIN-80101 Joensuu, Finland
e-mail: line.nybakken@uef.fi

Introduction

Lichens are photosynthetic organisms adapted to environments that are too low in nutrients, too cold, and/or too dry for vascular plants. Globally, as much as 8% of the land surface is dominated by lichens (Larson 1987). Lichen-dominated ecosystems are widespread in alpine and northern areas, but lichen herbivores are few. They include species of molluscs, oribatide mites, and moth larvae, as well as rodents, reindeer/caribou (*Rangifer tarandus*), and musk deer (*Moschus moschiferus*).

Few herbivores are adapted to lichen consumption. Compared with most forage plants, arboreal lichens regularly eaten by flying squirrels (*Glaucomys sabrinus*) and red-backed voles (*Myodes gapperi*) in Idaho, USA, are deficient in lipids, nitrogen, and several trace elements (Dubay et al. 2008). These animals have a low ability to digest and utilize nitrogen from lichens and are unable to maintain their N balance when feeding on lichens alone. In addition, most lichen species contain high amounts of carbon-based secondary compounds. Most of these lichen compounds are polyphenols that originate via the polyketide pathway (Culberson et al. 1977). They are produced by the fungal partner (Leuckert et al. 1990; Culberson and Armaleo 1992) by using the photosynthates delivered from the algae. More than 800 lichen compounds have been

identified (Huneck and Yoshimura 1996), and 0–5 compounds commonly are present in each lichen species. Many lichen secondary metabolites are biologically active and may protect the organisms against various pests (Huneck 1999; Romagni et al. 2000; Manojlovic et al. 2002) and environmental stresses (Bialonska and Dayan 2005). Some compounds also are known to deter grazing by invertebrates (Lawrey 1983a; Emmerich et al. 1993; Gauslaa 2005; Pöykkö et al. 2005). In a study of food choice by the slug *Pallifera varia*, Lawrey (1983a, b) observed that lichen species with the lowest concentrations of N and P were preferred. Lichens rich in N and P had a stronger chemical herbivore defense in terms of lichen compounds. In 2004, 400–500 free ranging elk (*Cervus elaphus*) developed paresis and died in Wyoming, USA, probably due to intoxification by the lichen *Xanthoparmelia chlorochroa* (Cook et al. 2007). Reindeer and caribou, on the other hand, survive on a winter diet that consists exclusively of lichens (Klein 1982; Aagnes and Mathiesen 1994; Terry et al. 2000; Storeheier et al. 2002). Recently, Sundset et al. (2008) identified a reindeer rumen bacterium that was able to tolerate and grow in the presence of various common lichen compounds, including the very common usnic acid. This suggests that reindeer are lichen feeding specialists, capable of detoxifying lichen compounds.

Earlier published experiments on the defensive role of lichen compounds have focused on invertebrates. Our aim was to test deterence against an herbivorous vertebrate, the bank vole (*Myodes glareolus*). The bank vole is a mixed granivorous-folivorous rodent. In Fennoscandia, it is most common in forests (Hansson 1978, 1983). In contrast to the central European bank vole that eats mostly easily digestible food and nutritious seeds, the Scandinavian bank vole feeds on forbs in summer, berries in late summer, fungi in summer-autumn, dwarf-shrubs (*Vaccinium* spp.) in autumn-winter, and lichens at least in winter-spring (Hansson 1985; Esseen and Renhorn 1998). Alectorioid lichens may constitute a significant part of the bank vole diet in old forests where they often occur in great abundance, but their dietary importance varies among years (Hansson and Larsson 1978; Hansson 1979).

By rinsing air-dry lichen thalli in 100% acetone, a substantial part of the secondary compounds can be extracted non-destructively (Solhaug and Gauslaa 1996, 2001). Thereafter, we can offer bank voles a choice between pairs of lichen pieces with and without assumed defensive compounds. This approach has been used in studies both with generalist (Gauslaa 2005) and specialist (Pöykkö et al. 2005) invertebrates, but not with vertebrates. By using pairs with control thalli and acetone-rinsed thalli of eight lichen species, we tested the hypothesis that reduced lichen compound concentrations lead to increased bank vole consumption of lichen material.

Methods and Materials

The Herbivore Ten adult bank voles, 4 males and 6 females, were captured in Vegårshei, Aust-Agder, S Norway, during 28 March–14 May 2005. The voles were named with letters A to J for identification, and were kept separately in 30×50×13 cm large cages. Between experiments they were fed every 2nd day with apples and bread. Water was always available in petri dishes.

Collection and Preparation of Lichens Eight species were collected in SE Norway in 2005 and used in the experiments from May to December 2005. *Cladonia arbuscula* (Wallr.) Flot. and *Cladonia rangiferina* (L.) F.H. Wigg were from a clear-cut in Siljan, Telemark, while *Cladonia stellaris* (Opiz.) Pouzar & Vězda was collected in an open pine forest in Atna, Hedmark. *Evernia prunastri* (L.) Ach. was sampled from oak trees near cultivated fields in Ås, Akershus. *Parmelia saxatilis* (L.) Ach. was picked from boulders in Ås, while *Platismatia glauca* (L.) W.L Culb. & C.F. Culb. originated from birch twigs in a spruce forest at Kittilbu, Gausdal, Oppland. *Lobaria pulmonaria* (L.) Hoffm. was collected on *Populus tremula* stems in Siljan, while *Umbilicaria spodochroa* (L.) Hoffm. was found on sea cliffs in Drøbak, Akershus.

After collection, sampled thalli were dried at room temperature for 48 h and then stored in a freezer (−20°C) until start of the experiment. One day before an experiment started, one species was taken from the freezer, all debris removed, and 20 randomly selected pieces of 1–2 g were prepared. They then were stored overnight, before dry weight was determined.

Ten randomly selected pieces were rinsed in 100% acetone. Each sample was put into a beaker placed in a fume cap. Acetone was added until it covered the entire lichen, by using either 100 or 200 ml, depending on the size of the thallus. Beakers were covered to avoid evaporation of acetone. After 20 min, the acetone with extracted compounds was transferred immediately to an empty glass flask closed by a lid. The procedure was repeated twice, and the supernatants were combined. An aliquot of 5 ml of the extract was transferred to a marked vial and left overnight to evaporate. Dried extracts were stored in freezer until HPLC analyses.

Before feeding lichens to the voles, all samples were weighed again, and moistened with water. Two different feeding experiments that used pairs of controls and compound-deficient lichens were done; 1) intact and hydrated thalli were offered to the voles, or 2) hydrated thalli finely ground in a ball mill (Retsch) for 3 min and mixed with more water were offered as porridge served in petri dishes. The latter approach was added because we observed that intact thalli dried within 2–3 h in the vole

cages, and desiccated thalli appeared less attractive than moist thalli. The porridge remained moist for 10–12 h. Two species, *L. pulmonaria* and *P. saxatilis*, were not served as porridge. We did not have enough material of *L. pulmonaria* from the same population, while the *P. saxatilis*-powder was highly hydrophobic and did not form porridge.

Feeding Experiments Between experiments, voles were fed with bread and apples. Before the start of a new experiment, all remaining food was removed from the cages. During the porridge experiments, we also removed sawdust and vole feces to avoid such debris from mixing with the fodder. The fodder was served in the cage in two permanently open traps so that the vole could go in and out, but the entrance was too small for the vole to drag the petri dish out of the trap and mix the two samples. One trap contained an acetone-rinsed sample, the other an unrinsed control. The feeding lasted from 3.30 PM to 08.00 AM the following day in experiments that used whole lichen thalli, and from 11.00 PM to 08.00 AM the following day in experiments with lichen porridge. Feeding experiments were run late evening and in the night when voles are most active, and disturbance by human activities is minimal. After the experiment, samples were collected from e cages and left to dry at room temperature for 1 D. All debris was removed before weighing. Each experiment was performed twice, as we had only 10 traps. This means that 5 voles were given the choice between treated and non-treated samples of one lichen species on one day, and the following day 5 new voles went through the same experiment ($N=10$).

High Performance Liquid Chromatography (HPLC) Analysis Dry lichen extracts from the acetone rinsing were redissolved in 500 µl of acetone and analysed on an 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) that included a 1040 M diode array detector. Separation was achieved on an ODS Hypersil 60×4.6 mm column. Oven and injector temperature were 25 and 22°C, respectively. The injection volume was 10 µl, and the flow rate 2 ml min^{-1}. Solvent A consisted of 0.25% orthophosphoric acid and 1.5% tetrahydrofuran in HPLC pure water, while solvent B was 100% methanol. The run started with 30% B. Within 15 min, solvent B was increased to 70% and further to 100% within15 min, and then isocratically in 100% B for a further 5 min. At the end of the run, solvent B was reduced to 30% within 1 min, and the column was flushed with 30% B for 10 min before the next run started.

The detection wavelength was 245 nm, and the identification of compounds was based on retention times, online UV-spectra, co-chromatography of commercial standards [atranorin, evernic acid, fumarprotocetraric acid (Apin Chemicals), usnic acid (Sigma)], and standards of tenuiorin, gyrophoric acid, salazinic acid, and stictic acid kindly provided by Dr. H.J. Sipman (Botanischer Museum

Table 1 Concentration of lichen secondary compounds (mg g^{-1} DW) rinsed with acetone from intact thalli and the percentage of all compounds rinsed compared with full extraction

	Cladonia arbuscula	*Cladonia rangiferina*	*Cladonia stellaris*	*Evernia prunastri*	*Lobaria pulmonaria*	*Parmelia saxatilis*	*Platismatia glauca*	*Umbilicaria spodochroa*
Atranorin		3.98±0.75		7.59±0.48		2.14±0.33	2.62±0.24	5.85±0.47
Chloratranorin						3.19±0.40		
Usnic acid	3.33±0.32		10.83±0.90	0.19±0.02				
Fumarprotocetraric acid	2.13±0.19	3.20±0.28						
Perlatolic acid			0.31±0.07					
Evernic acid				15.99±0.60				
Gyrophoric acid								19.05±2.83
Gyrophoric acid derivatives								27.37±2.73
Salazinic acid						12.04±0.95		
Constictic acid					1.68±0.12			
Peristictic acid					5.71±0.48			
Stictic acid					8.14±0.60			
Cryptostictic acid					0.64±0.05			
Norstictic acid					1.01±0.08			
Methylstictic acid					0.14±0.01			
Caperatic acid							0.76±0.10	
Unidentified compounds						1.01±0.12		
Sum	5.46±0.45	7.18±0.96	11.14±0.84	28.57±1.05	17.32±1.07	18.38±1.62	3.38±0.32	52.27±5.65
% rinsed out	82 %	57%	89 %	90 %	90 %	50 %	54 %	100 %

Berlin-Dahlem, Berlin, Germany). Compounds were quantified against response curves of the above-mentioned standards. We were not able to get standards of perlatolic acid and caperatic acid, and so response curves of usnic acid and atranorin, respectively, were used for quantification of these compounds. Constictic, peristictic, cryptostictic, norstictic, and methyl norstictic acids were all calculated from the response curve of stictic acid. Gyrophoric acid derivatives were calculated from the response curve of gyrophoric acid.

Efficiency of the Acetone Rinsing Method McEvoy et al. (2006) have shown that not all extractable secondary compounds are fully rinsed from whole thalli by acetone. We tested the efficiency of the extraction method by doing a full extraction of 3 thalli of each species. After the normal acetone-rinsing procedure, we ground the thalli to powder in a ball mill, took out subsamples of about 15 mg, added 500 µl of acetone, and vortexed the mixture for 30 sec. Samples then were left to stand for 10 min before they were centrifuged for 3 min at 15,000 rpm. The supernatants were collected, and the procedure repeated three times. Test extractions revealed that after 4 extractions, no HPLC-detectable secondary compounds were left in the lichen pellets. The combined supernatants were evaporated to dryness, 500 µl of acetone were added and analyzed by HPLC as described above.

Statistical Analysis The data were analysed in a pairwise *t*-test with the statistical software SYSTAT 10 (SPSS Inc., Chicago, U.S.A.).

Results

Lichen Compounds The studied lichen species contained 2–6 different secondary compounds, and the amounts removed by the original acetone rinsing varied from 3.38 mg g^{-1} DW in *P. glauca* to 52.3 mg g^{-1} DW in *U. spodochroa* (Table 1). A subsequent full extraction of previously acetone rinsed thalli documented species-specific rinsing efficiencies (Table 1). Acetone rinsing removed 100% of the secondary compounds from *U. spodochroa*, and 82–90% from *C. arbuscula*, *C. stellaris*, *L. pulmonaria,* and *E. prunastri*. In contrast, only 50–57% was removed from *C. rangiferina*, *P. glauca,* and *P. saxatilis*. We also checked whether or not the rinsing efficiency varied among different compounds and between positions in the lichen thallus (cortical or medullary compounds), but only small variations were found (data not shown).

The chemical composition of *C. arbuscula*, *C. rangiferina*, *C. stellaris*, *E. prunastri,* and *P. glauca* corresponded well with the literature for Nordic populations of the species (e.g.,

Table 2 Consumption of intact lichen thalli and lichen porridge, with and without lichen substances, in percent (%) and grams (g) ± S.E

Species	Whole lichen thalli						Porridge of lichen thalli					
	Herbivory, %			Herbivory, g			Herbivory, %			Herbivory, g		
	Rinsed	Control	*P*	Rinsed	Control	*P*	Rinsed	Control	*P*	Rinsed	Control	*P*
Cladonia arbuscula	23.3±3.5	24.3±4.3	0.868	0.49±0.08	0.46±0.08	0.782	30.5±5.4	27.0±4.6	**0.007**	0.51±0.08	0.26±0.08	**0.022**
Cladonia rangiferina	35.4±5.5	25.8±4.3	0.140	0.70±0.12	0.55±0.09	0.335	32.0±5.0	12.2±2.6	**0.007**	0.56±0.09	0.23±0.05	**0.010**
Cladonia stellaris	17.9±6.3	8.1±3.5	0.084	0.40±0.08	0.16±0.08	**0.043**	23.1±4.6	9.3±0.7	**0.009**	0.6±0.11	0.23±0.02	**0.005**
Evernia prunastri	23.7±5.9	16.1±4.5	0.376	0.42±0.37	0.33±0.29	0.572	60.5±8.6	14.0±4.2	**0.001**	0.87±0.12	0.20±0.06	**0.001**
Lobaria pulmonaria	30.8±10	3.9±1.8	**0.025**	0.25±0.08	0.03±0.01	**0.015**						
Platismatia glauca	24.4±5.5	19.6±5.4	0.463	0.29±0.06	0.24±0.09	0.613	31.0±5.6	12.9±3.0	**0.002**	0.39±0.07	0.18±0.04	**0.003**
Parmelia saxatilis	11.9±6.4	8.3±4.0	0.745	0.09±0.04	0.14±0.10	0.643						
Umbilicaria spodochroa	11.4±4.8	17.9±5.7	0.407	0.09±0.03	0.14±0.00	0.474	9.6±1.8	12.8±3.3	0.371	0.08±0.02	0.10±0.02	0.620

The level of significance ($P<0.05$) in bold is given according to a pairwise t-test of rinsed and unrinsed thalli.

Krog et al. 1994). Each contained two or three primary lichen substances. *Lobaria pulmonaria* contained six detectable compounds, all belonging to the stictic acid chemosyndrome (cf. Elix and Tønsberg 2006), with stictic acid itself being the most abundant. *Parmelia saxatilis* contained atranorin (minor) and salazinic acid (major) as reported by Krog et al. (1994), but chloroatranorin and one unidentified compound also were detected. *Umbilicaria spodochroa* contained gyrophoric acid (major) and atranorin (minor). Two minor unidentified compounds with spectra resembling gyrophoric acid may be lecanoric acid and hiasic acid, which can be expected in all characterized chemical races of the species (Posner et al. 1992).

Lichen Herbivory When intact lichens were served, acetone rinsing increased DW consumption only in *L. pulmonaria* and *C. stellaris* (Table 2). No significant preference was observed among rinsed and unrinsed pairs of the other species. However, when serving lichen as porridge (Fig. 1), acetone rinsing also increased consumption in *C. arbuscula*, *C. rangiferina*, *E. prunastri,* and *P. glauca*, but decreased consumption in *C. stellaris* (Table 2). *Lobaria pulmonaria* and *P. saxatilis* were not tested in the porridge experiment due to lack of material. The two species with the lowest total consumption (*U. spodochroa* and *P. saxatilis)* were the only species where no preference was found. In general, voles did not eat more lichen porridge than they did intact lichens (except for rinsed *E. prunastri* thalli). Grinding reduced the consumption of control thalli (Table 2).

Discussion

Many lichen species produce large amounts of secondary compounds that restrain grazing from invertebrates (Lawrey 1983a; Emmerich et al. 1993; Gauslaa 2005; Pöykkö et al. 2005). The present study is the first to show that lichen secondary compounds also may reduce grazing of a vertebrate herbivore (Fig. 1, Table 2).

When offered as porridge, the bank vole had a preference for lower concentration of lichen compounds in four of the tested lichen species. We expected that lichens would be more attractive and edible for voles in the moist state. Instead, either grinding or the addition of water made the porridge of control thalli less attractive in most of the species (Table 2). The reason for this is not clear. However, by quantifying the acetone rinsing efficiency, we showed that the extractability of secondary compounds in most lichen species is increased during grinding to powder. Since grinding releases and exposes the compound to crystalization, a bank vole may feel a stronger repulsiveness to the compounds. Furthermore, deterrent substances are not evenly distributed inside a lichen thallus. In the *Cladina*-species used in this experiment, the total concentration of lichen compounds was up to three times higher in the apical 10 mm of the podetia compared with other living parts (Nybakken and Julkunen-Tiitto 2006). When offered whole lichen thalli, voles had the option of selecting and eating the less toxic parts, while preparation of porridge may have reduced the variation in palatability within control thalli. Since lichen secondary compounds are not soluble in water, addition of water as such is not likely to enhance the smell or taste of lichen compounds.

Rinsing intact lichen thalli in acetone clearly reduced the concentration of secondary compounds, although extraction efficiency varied among species (Table 1). When lichens were served as intact thalli, the total concentration of compounds may have played a role. In both species where the voles preferred rinsed thalli (*C. stellaris* and *L. pulmonaria*), 90% of the secondary compounds were removed. However, acetone rinsing also was highly effective for other species (*C. arbuscula*, *E. prunastri,* and *U. spodochroa*) without influencing the vole's preference. This suggests that the presence and the concentration of

Fig. 1 The porridge experiment. Lichens were ground to powder, mixed with water and served to the bank voles in petri dishes. The photo shows petri dishes with *Evernia prunastri* after one night of feeding. Upper panel: Controls. Lower panel: acetone-rinsed thalli

specific compounds is important. Usnic acid is the most frequently studied lichen substance, and it has well documented deterrent effect in some herbivores (reviewed by Cocchietto et al. 2002). It occurred in high amounts in *C. stellaris* and in much lower concentrations in *C. arbuscula* and *E. prunastri* (Table 1). The low consumption of control thalli of *C. stellaris* compared with the two other species (Table 2) points at an antiherbivore role of usnic acid at high concentrations. *Lobaria pulmonaria,* the other species in which acetone-rinsing increased grazing in intact thalli, has a special chemistry (Table 1). In a study of snail herbivory on *L. pulmonaria* and *Pseudocyphellaria crocata*, Gauslaa (2008) observed a negative correlation between concentrations of stictic and constictic acids and frequency of grazing marks. Stictic acid was the compound rinsed out in highest amounts from our thalli, and it has been suggested previously as an antiherbivore compound (Lawrey 1980). Therefore, we postulate that stictic acid prevented the bank voles from feeding on the control thalli of *L. pulmonaria*.

Despite the fact that *U. spodochroa* contained the highest concentration of secondary compounds and that these were 100% extracted, no significant preference was measured in either of the two feeding experiments. The bank voles hardly sampled any of the served forms of this lichen (Table 2). Gyrophoric acid, the major compound, has been mentioned as a possible antiherbivore compound (Nimis and Skert 2006). However, as its removal did not increase feeding, this species probably uses other defense mechanisms (e.g., hard texture, un-extractable chemicals inside living cells, low nutritive value).

In summary, we have shown that some lichen secondary compounds function as deterrents against a generalist vertebrate herbivore. Some of these seem to deter herbivores across taxonomic groups, e.g., mammals, insects, and gastropods.

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Identification of the Sex Pheromone of the Spruce Seed Moth, *Cydia strobilella* L.

Hong-Lei Wang · Glenn P. Svensson · Olle Rosenberg · Marie Bengtsson · Erling V. Jirle · Christer Löfstedt

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Abstract The spruce seed moth, *Cydia strobilella* L., is a serious pest on cones of spruce (*Picea spp.*) in the Holarctic region. Previous studies from different parts of its area of distribution have reported conflicting results on the composition of its sex pheromone. By gas chromatography with electroantennographic detection, coupled gas chromatography-mass spectrometry, a Y-tube olfactometer bioassay, and field trials, the sex pheromone of Swedish populations of the species was identified as (8*E*,10*E*)-dodecadienyl acetate and (8*E*,10*Z*)-dodecadienyl acetate. About 0.5 pg of each pheromone component was extracted per female. The most attractive blend of *EE*- and *EZ*-isomers was about 6:4, respectively, and 0.3 µg of the blend per rubber septum was the most attractive dosage for field trapping. Monounsaturated components previously reported as sex pheromone components/attractants for *C. strobilella*, (*E*)-8-dodecenyl acetate in Canadian populations and (*Z*)-8-dodecenol in Polish and Dutch populations, did not attract any *C. strobilella* in this study. Large numbers of *C. jungiella* Clerck were trapped by using (8*E*,10*Z*)-dodecadienyl acetate alone, whereas (*Z*)-8-dodecenol attracted *Pammene splendidulana* Guenée and *P. rhediella* Clerck.

In memoriam of professor Jan Löfqvist and in recognition of his contributions to insect chemical ecology.

H.-L. Wang · G. P. Svensson · E. V. Jirle · C. Löfstedt (✉)
Department of Ecology, Lund University,
Sölvegatan 37, 223 62 Lund, Sweden
e-mail: Christer.Lofstedt@ekol.lu.se

O. Rosenberg
The Forestry Research Institute of Sweden, Skogforsk,
Uppsala Science Park,
751 83 Uppsala, Sweden

M. Bengtsson
Chemical Ecology, Swedish University of Agricultural Sciences,
Box 102, 230 53 Alnarp, Sweden

Keywords Sex pheromone · *Cydia strobilella* · Tortricidae · Lepidoptera · (8*E*,10*E*)-dodecadienyl acetate · (8*E*,10*Z*)-dodecadienyl acetate · Spruce seed orchard · Monitoring

Introduction

The spruce seed moth, *Cydia strobilella* L. [formerly *Laspeyresia strobilella* L., *C. youngana* Kearfott, and *L. youngana* Kearfott] (Lepidoptera: Tortricidae), is a serious cone pest of spruce in the Holarctic region; for instance Norway spruce, *Picea abies* (L.) H. Karst, in Europe; and white spruce, *P. glauca* (Moench) Voss, and black spruce, *P. mariana* (Miller) Britton, Sterns and Poggenburg, in North America. The flight phenology of this diurnal species is more or less synchronized with the pollination period of spruce hosts. Female calling and mating take place in the early photophase. The eggs are laid between the scales of female flowers, and the larvae feed on the maturing seeds, and cause great damage to the seed harvest without leaving any external evidence of infestation (e.g., Bakke 1963; Seifert et al. 2000). Studies have shown that *C. strobilella* is difficult to control as larvae (e.g., Annila 1973; Rosenberg and Weslien 2005). Thus, monitoring the flight of adults is a necessary element of integrated pest management strategies for control of this species.

Attempts to identify the sex pheromone of *C. strobilella* were initiated more than 30 years ago, but failed (C. Löfstedt, J.N.C Van der Pers, and J. Löfqvist, unpublished). Since then, several different compounds have been reported as sex attractants for the species. (*Z*)-8-Dodecenol (*Z*8–12:OH) was reported as an attractant in the Netherlands (Booij and Voerman 1984), and in Poland (Skrzypczynska et al. 1998). In Canada, (*E*)-8-dodecenyl acetate (*E*8–12:OAc) was demonstrated to be an attractant (Grant et al. 1989), and

later this compound was confirmed as a sex pheromone for *C. strobilella* (Bédard et al. 2002; Trudel et al. 2006). In China, where the species heavily infests cones of Korean spruce, *P. koraiensis* Nakai, field trapping tests revealed an attractant blend of *Z*8–12:OH, (*E*)-8-dodecenol (*E*8–12:OH), and (8*Z*,10*E*)-dodecadienol (*Z*8,*E*10–12:OH) (Sun et al. 2001).

In Sweden, as part of a large screening project of attractants for tortricid moths, a mixture of (8*E*,10*E*)-dodecadienyl acetate (*E*8,*E*10–12:OAc) and (8*E*,10*Z*)-dodecadienyl acetate (*E*8,*Z*10–12:OAc) was found to be attractive, but trap catches were low (Witzgall et al. 1996). More recently, mixtures of *E*8,*E*10–12:OAc and (8*E*,10*E*)-dodecadienol (*E*8,*E*10–12:OH), or *E*8,*Z*10–12:OAc and (8*E*,10*Z*)-dodecadienol (*E*8,*Z*10–12:OH), were reported to attract significant numbers of *C. strobilella* males (Witzgall et al. 2009).

A properly identified sex pheromone is still missing for European populations of *C. strobilella*, which has hampered the use of sex pheromones for monitoring and control of this moth in seed orchards. In the present paper, we report our identification of a two-component sex pheromone, which is highly efficient for trapping of *C. strobilella* in Sweden.

Methods and Materials

Insects Previous-year cones of Norway spruce were collected at Ålbrunna (59°30′N, 17°32′E), Uppland Province, Sweden, in March 2009. Cones were stored first at 6°C, 55% r.h. and a 18:6 L:D cycle. To trigger emergence of *C. strobilella*, cones were transferred later to 15°C, 60% r.h. and a 17:7 L:D cycle, and placed in small wooden boxes (16×16×15 cm) with a sealed glass exit tube inserted to facilitate collection of emerged insects as they moved towards the light. The boxes were checked every h during the photophase. Newly emerged moths were collected as soon as they appeared, separated by sex, and kept individually in small plastic tubes until used in experiments.

Chemicals Reference compounds of different origin and purity were available for the identification work from our laboratory collection of pheromone compounds. Compounds used for behavioral experiments included *E*8–12:OAc (chemical purity 99.4%), *Z*8–12:OH (chemical purity 98.1%), *E*8,*E*10–12:OAc (chemical purity 99.0%; isomeric purity 99.3%), and *E*8,*Z*10–12:OAc (chemical purity 95.8%; isomeric purity 96.3%). The dienic compounds were synthesized and purified as described in Witzgall et al. (1993).

Pheromone Gland Extraction Calling and mating behavior of *C. strobilella* were observed by putting newly emerged adults (6♀ × 6♂) into an outdoor transparent plastic cage (35×30×60 cm) with a spruce branch. Female calling (extrusion of the pheromone gland) was observed 2–5 h into the photophase, and mating was observed during this calling period. The pheromone glands of 0-to-2-d-old virgin females were dissected in the early photophase as soon as the calling behavior was observed. Up to 20–30 glands were combined and extracted together for 30 min in 20 µl of redistilled hexane. For quantification of pheromone titer by gas chromatography-mass spectrometry (GC-MS), combinations of 160, 222, and 230 female equivalent extracts were prepared and analyzed.

Electrophysiology Gas chromatography with electroantennographic detection (GC-EAD) was used to identify physiologically active compounds in female gland extracts. One-to-2-d-old males were analyzed. The head and both antennae, with their tips cut off, were used in preparations, and mounted to a PRG-2 EAG (10x gain) probe (Syntech, Kirchzarten, Germany) using conductive gel (Blågel, Cefar, Malmö, Sweden). Charcoal-filtered and humidified air passed over the antennal preparation from a glass tube outlet at 5 mm distance from the preparation. The GC effluent to the antennae passed through a heated transfer line set at 230°C.

Female pheromone gland extracts containing ≈20 calling females were placed in micro-vials with hexane before being used for GC-EAD screening. In addition, synthetic standards of putative pheromone compounds for *C. strobilella* that had been reported for other *Cydia* species also were used [*E*8–12:OAc, *Z*8–12:OAc, *E*8–12:OH, *Z*8–12:OH, *E*8,*E*10–12:OH, and a mixture of the isomers *Z*8,*E*10–12:OAc (34%), *E*8,*E*10–12:OAc (23%), *E*8,*Z*10–12:OAc (41%), *Z*8,*Z*10–12:OAc (2%)] at concentrations of 17 ng/µl, 2 ng/µl, and 0.2 ng/µl. Samples were injected into an Agilent 7890A GC (Agilent Technologies), equipped with either a medium-polar HP-INNOWax column (30 m×0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA) or a non-polar HP-5 column (30 m×0.32 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA). A split at the end of the column allowed a 1:1 division of the GC effluent to the flame ionization detector (FID) and to the antennal preparation. Hydrogen was used as carrier gas at a flow rate of 43 cm/sec, and injector temperature was 225°C. The column temperature was maintained at 80°C for 2 min after injection, and then was increased by 10°C/min to 220°C. Each antennal preparation was used for a single GC-EAD trial. Data were analyzed with the GC-EAD Pro Version 4.1 software (Syntech, Kirchzarten, Germany).

As the amounts of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc (the only EAD-active compounds found in female extracts, see results) were below the detection limit for the FID, a calibration curve was obtained to calculate the approximate relative ratio of the two compounds in female glands. Synthetic mixtures of the isomers (≈0.1 ng/µl) were

prepared in five different ratios, and GC-EAD analyses were performed to estimate the relative FID peak areas and corresponding EAD response amplitudes, for the isomers in these mixtures. These data were then fitted to a curve with the best correlation coefficient. By using this curve, the relative EAD amplitudes that corresponded to the isomers could be used to calculate the relative ratio of the compounds in seven female gland extracts to estimate mean and median *EE*/*EZ* ratios.

Gas chromatography-Mass Spectrometry The pheromone gland extracts were analyzed with a Hewlett-Packard 5975 mass-selective detector coupled to a Hewlett-Packard 6890 GC, equipped with a capillary column of either HP-INNOWax (30 m×0.25 mm i.d., and 0.25 μm film thickness; J&W Scientific, USA), or HP-5MS (30 m× 0.25 mm i.d., and 0.25 μm film thickness; J&W Scientific, USA). The HP-INNOWax column was programmed from 80°C for 1 min, 10°C/min to 200°C, hold for 10 min, and then to 230°C at 10°C/min, hold for 10 min. The HP-5MS column was programmed from 80°C for 1 min, 10°C/min to 150°C, then to 200°C at 4°C/min, hold for 10 min, and finally to 250°C at 10°C/min, holding for 10 min. Injector and transfer line temperatures were 250°C and 280°C, respectively, and helium was used as carrier gas.

Selected ion monitoring (SIM) was used to monitor the pheromone component candidates. The characteristic ions *m*/*z* 61($CH_3CO_2H_2^+$), 79, 81, 164($M\text{-}60^+$), and 224(M^+) were used for monitoring the 8,10-dodecadienyl acetate isomers, by comparing both retention time and ion ratios with those of the authentic synthetic compounds. The above SIM mode was also used for quantification when *E*8,*E*10–12:OAc was used as the external standard, with a concentration range of 0.01–0.1 ng. The absolute amounts of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc in female gland extracts were obtained by integrating the abundance of ion *m*/*z* 164 on the polar column and then calculating by the polynomial regression equation $y=6.37x^2 + 11.93x + 58.27$ ($r^2=0.997$), which was obtained based on the fragment abundance of ion *m*/*z* 164 in the external standard. Under these conditions, the limit of detection for the dodecadienyl acetate isomers is 4 pg with a signal/noise ratio (S/N) of 3:1.

Behavioral Experiments A glass Y-maze (15 cm length × 15 mm i.d.) (Scientific Glass, Löberöd, Sweden) was used to study the attraction of male moths to the *EE* and *EZ* isomers, individually or in a blend. The olfactometer was connected to a battery-driven air pump (GroTech, Gothenburg, Sweden), and the 200 ml/min was set to 20 ml/sec. The test stimulus was applied on a strip of filter paper inserted in a 10 ml plastic syringe and connected to the stimulus arm. The test stimulus always was applied at a volume of 10 μl. A second syringe loaded with 10 μl of hexane served as a control. Filters were replaced every 30 min. Four treatments were tested: the two isomers individually at a dose of 10 ng, and the binary blend (*EE*/*EZ* ratio of 4:3) at a total dose of 0.7 ng or 7 ng.

Experiments were performed 1–3 h into the photophase at 5,000 lux, 21°C and ≈45% r.h.. The olfactometer was placed in 45° angle in relation to the table, with the arms at 10 cm higher elevation compared to the area of the tube where the moths were introduced. This tilted position of the olfactometer triggered more walking behavior in moths compared to when the Y-tube was kept horizontally. One-to-3-d-old males were used for behavioral tests. After being transferred to the Y-tube, a male was given 5 min to respond; i.e., walk at least 1 cm into either of the two arms of the olfactometer. Moths not responding within the given time period were considered as non-responders and were excluded from the analysis. Each moth was used only once. For each experiment, the position of the stimulus and control arms was alternated to avoid position effects. Data on olfactory responses were analyzed with a binomial test (Siegel and Castellan 1998) against the null hypothesis that moths would be attracted equally to both arms of the olfactometer. Between experiments, Y-tubes were baked in an oven at 300°C for 2 h to remove traces of compounds.

Field Experiments Field trapping experiments were conducted in two spruce seed orchards in Sweden: Maltesholm, (55°54′N, 13°59′E), Skåne Province, and Ålbrunna (see above), in spring 2009. Custom-built Delta-traps or traps purchased from either PheroTech Inc. (Delta, BC, Canada) or AgriSense BCS Ltd (Pontypridd, UK), all with sticky inserts, were used. Synthetic blends were prepared in hexane, and 100 μl solution were added to red rubber septa (Catalogue no. 224100-020, Wheaton Science Products, Millville, NJ, USA) to be used as lures.

The first field trial was conducted in Maltesholm from 7 to 19 May, and in Ålbrunna from 7 May to 1 June. In this experiment, rubber septa were loaded with the *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc isomers, either individually (*EE*: 4 μg, *EZ*: 3 μg) or as a blend in a 4:3 ratio (same amounts of the isomers as when applied individually). In addition, lures were loaded with *E*8–12:OAc (3 μg) or *Z*8–12:OH (3 μg), which have been reported to be attractive to *C. strobilella* in Canada and Poland/The Netherlands, respectively. Septa loaded only with hexane served as controls. Five trap replicates were used.

The second field test was conducted from 13 to 29 of May in Maltesholm, and from 19 May to 1 June in Ålbrunna. In this experiment, different ratios of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc isomers (i.e., intended ratios of 100:0, 90:10, 75:25, 50:50, 25:75, 10:90, and 0:100) were applied to rubber septa at a total dosage of 3 μg per lure. Five trap replicates were used. The actual ratios of the

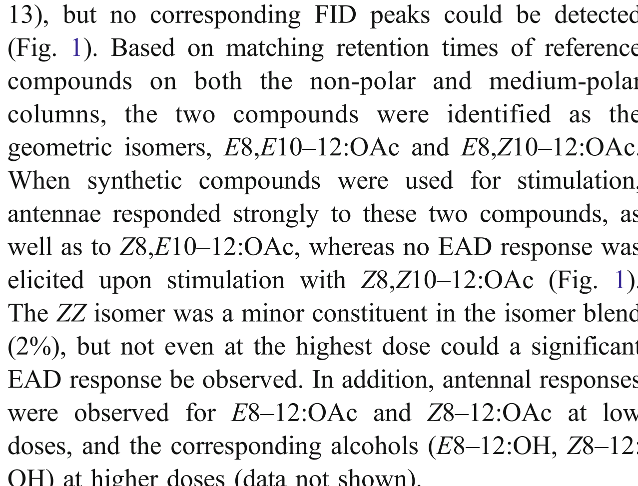
isomer blends were checked subsequently by both GC-MS on an HP-INNOWax column and GC-FID on an HP-1 column. To investigate the isomerization of compounds under field conditions, the 3 wk field-aged rubber septa were collected at the end of this experiment, and the wide-bore cavity of a septum was filled with 200 µl of hexane. The hexane was recovered from the septum after 5 min, concentrated to approximately 40 µl, and passed through a 1.5×0.5 cm column of Florisil (250 mg, 100–200 mesh) for purification. The column was washed with 1 ml of hexane, and the concentrated eluate was analyzed by GC-MS on an HP-INNOWax column (see above).

The third trial was conducted from 19 to 29 of May in Maltesholm and from 27 of May to 8 of June in Ålbrunna. It was designed to determine the optimal total dose of the two-component blend at an *EE*/*EZ* ratio of 60:40 (which was shown to give the highest trap catches during the first two checks in the ratio experiment in Maltesholm). Dosages of 0.1, 0.3, 1, 3, 10, and 30 µg per rubber septum were tested. Four trap replicates were used. The actual *EE*/*EZ* ratio used in this experiment was confirmed as 61:39 by both GC-FID (HP-1 column) and GC-MS (HP-INNOWax column).

Traps were suspended from spruce branches at 2–4 m height and at least 10 m apart within each replicate in Maltesholm, and at 1.3–2 m height and 7 m apart within each replicate in Ålbrunna. Traps were checked every 2nd day and redistributed, with exception of trial one and two in Ålbrunna that were not redistributed. The trapped tortricid moths were identified according to Svensson (2006) and Razowski (2003). Genital preparations from selected specimens were examined to confirm species identifications (courtesy of Ingvar Svensson, Österslöv, Sweden). A subsample of voucher specimens is kept in the laboratory for reference. Statistical analyses were based on the catches recorded on each occasion that a trap was checked. Traps then were redistributed, and the number of replicates thus was obtained as the number of physical replicates times the number of times the traps were checked. During the 1st and 2nd experiments in Ålbrunna, traps were not redistributed. Thus, the number of replicates was equal to the number of traps, and the total catch in a specific trap over the whole trapping period was treated as a replicate. Differences in trap catch among treatments were compared by using analysis of variance on log (x+1) transformed data, followed by multiple comparisons according to the Bonferroni *post hoc* test with adjusted levels for significance. All analyses were performed using SPSS ver. 16.

Results

GC-EAD Antennae from males responded exclusively and strongly to two compounds in female gland extracts (*N*=



13), but no corresponding FID peaks could be detected (Fig. 1). Based on matching retention times of reference compounds on both the non-polar and medium-polar columns, the two compounds were identified as the geometric isomers, *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc. When synthetic compounds were used for stimulation, antennae responded strongly to these two compounds, as well as to *Z*8,*E*10–12:OAc, whereas no EAD response was elicited upon stimulation with *Z*8,*Z*10–12:OAc (Fig. 1). The *ZZ* isomer was a minor constituent in the isomer blend (2%), but not even at the highest dose could a significant EAD response be observed. In addition, antennal responses were observed for *E*8–12:OAc and *Z*8–12:OAc at low doses, and the corresponding alcohols (*E*8–12:OH, *Z*8–12:OH) at higher doses (data not shown).

The FID-EAD curve, obtained from GC-EAD analyses of five synthetic blends with different *EE*/*EZ* ratios, showed a good correlation between the ratios for EAD responses and FID peaks for the two isomers ($y=0.32^{0.42x}$; $r^2=0.99$). Based on this relationship, we established the FID peak ratios of the isomers in seven female gland extracts by plotting onto the curve the observed ratios of EAD responses for the *EE* and *EZ* isomers. These FID peak ratios ranged from 0.6 to 2.3, but had the same mean and median value (1.3). Based on the median FID ratio obtained from the curve, an *EE*/*EZ* ratio of 4:3 was used in the olfactometer tests and the first field trapping experiment.

Chemical Analysis of Pheromone Gland Extract GC-MS analyses of pheromone gland extracts revealed high amounts of heneicosane, tricosane, and heptacosane (8.6 ng, 7.2 ng,

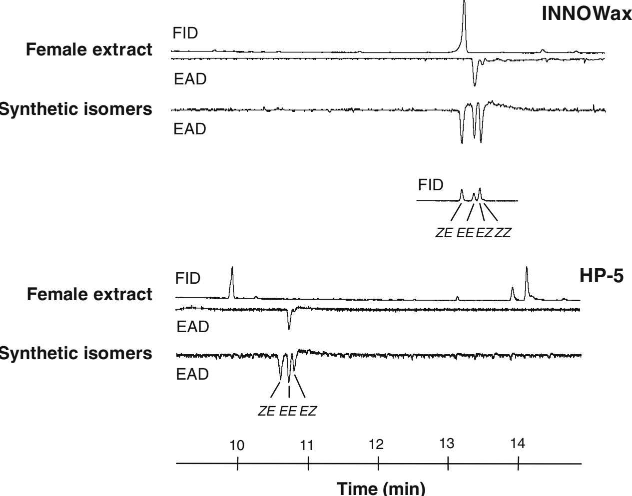


Fig. 1 Gas chromatography with flame ionization detection and electroantennographic detection (EAD) using male *Cydia strobilella* antennae responding to gland extracts of females or a synthetic mixture of *Z*8,*E*10–12:OAc (*ZE* 34%), *E*8,*E*10–12:OAc (*EE* 23%), *E*8,*Z*10–12:OAc (*EZ* 41%), and *Z*8,*Z*10–12:OAc (*ZZ* 2%). Two different column types were used (HP-INNOWax and HP-5)

and 18.3 ng per female gland, respectively), as well as small amount of hexadecanal and octadecanal (compounds identified based on matching mass spectra and retention times on both columns, data not shown). However, the EAD-active dodecadienyl acetates, *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc, were not detected in full-scan mode, but in SIM mode, both components were confirmed by using both the polar and the non-polar columns. The two insect-derived compounds eluted at the same retention times as the two authentic standards, and exhibited the diagnostic fragments at *m*/*z* 61, 79, 81, and 164 in the same ratios. The subsequent quantification showed an average titer of 0.42±0.07 pg of *E*8,*E*10–12:OAc, and 0.55±0.10 pg of *E*8,*Z*10–12:OAc per female equivalent (calculated from three extracts containing 160, 222, and 230 female equivalents, respectively). The average (±S.D.) *EE*/*EZ* ratio was found to be 43±3%, which differed somewhat from the ratio obtained from the FID-EAD curve (see above).

Bioassays Preliminary flight tunnel analyses revealed that male moths did not fly upwind to either the two-component blend at various doses or to calling females. In Y-tube experiments, males showed significant attraction to the two-component blend at both doses tested ($P<0.01$; Fig. 2). In addition, significantly more males chose the treatment arm vs. the control arm when the *EZ* isomer alone was used as stimulus ($P<0.05$; Fig. 2), but many males did not respond by walking upwind in the olfactometer in these experiments and, thus, were excluded from the analysis. No significant attraction was observed when using the *EE* isomer alone as stimulus, and many non-responders were observed in these experiments as well.

Field Trapping Results from the first field test (Fig. 3) showed that *E*8,*Z*10–12:OAc alone at a dosage of 3 µg per septum, or a 4:3 blend of *E*8,*E*10– and *E*8,*Z*10–12:OAc at a total dosage of 7 µg per septum attracted significant numbers of male *C. strobilella* at both locations, whereas the other treatments were unattractive to this species. Interestingly, the same two treatments also attracted another *Cydia* species; *C. jungiella* Clerck (formerly *Grapholita jungiella*). Trap catch of this species was much higher for *E*8,*Z*10–12:OAc alone compared to the binary blend (total catch Maltesholm: 31 (*EZ*) vs. 2 (*EE*+*EZ*); total catch Ålbrunna: 1,053 (*EZ*) vs. 43 (*EE*+*EZ*)). Traps baited with *Z*8–12:OH were not attractive to *C. strobilella*, but instead trapped significant numbers of two *Pammene* species, which were only attracted to this compound: *P. splendidulana* Guenée with a total catch of 67 at Maltesholm (no moths trapped at Ålbrunna), and *P. rhediella* Clerck with at total catch of 14 at Maltesholm and 45 at Ålbrunna. *E*8–12:OAc did not attract any moths in this study.

The optimal *EE*/*EZ* ratio for attraction of *C. strobilella* was investigated in a second experiment. In Maltesholm, all ratios except pure *EE* isomer attracted high numbers of males, with ratios ranging from 50% to 90% *EE* trapping the highest numbers (Fig. 4). In Ålbrunna, similar results were obtained. Analysis of the field-aged rubber septa at the end of the Maltesholm field test showed that after three weeks in the field, the percentage of *EE* isomer in the rubber septa had increased regardless of the initial ratio, except for one group in which the initial *EE* percentage was 99.3% but decreased to 97.5% after three weeks in the field (Fig. 5). Baits aimed to contain 0–75% *EE* after three weeks of aging in the field also contained a smaller but significant amount of the *ZE* isomer (3–13%), whereas this isomer could not be quantified reliably in the two mixtures with the highest original amount of *EE* isomer. This isomerization, plus the impurity of ca. 3.6% of the *EE* isomer in *E*8,*Z*10–12:OAc, makes it hard to carry out a strict test of precise ratios between the candidate pheromone components.

In the third experiment, the 6:4 blend of *EE* and *EZ* isomers attracted male *C. strobilella* at doses from 0.1 µg to 3 µg, whereas almost no males were attracted to the two highest doses (Fig. 6). The 0.3 µg dose attracted the highest numbers of males at both localities, whereas significantly fewer males were attracted to the two highest doses compared to the lower doses at Maltesholm. Trap catches at Ålbrunna were not significantly different because of too low total catches and high variation among traps within the same treatment. However the response profiles were identical in both places, revealing highest attraction to the lower doses.

Discussion

Our study demonstrates conclusively that a 6:4 mixture of *E*8,*E*10– and *E*8,*Z*10–12:OAc, a ratio of the components that

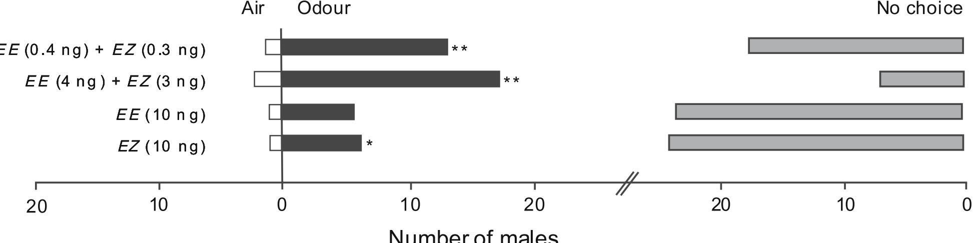


Fig. 2 Response of male *Cydia strobilella* to *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc, individually or in a 4:3 mixture, in Y-tube olfactometer tests (Binomial test; * $P<0.05$, ** $P<0.01$)

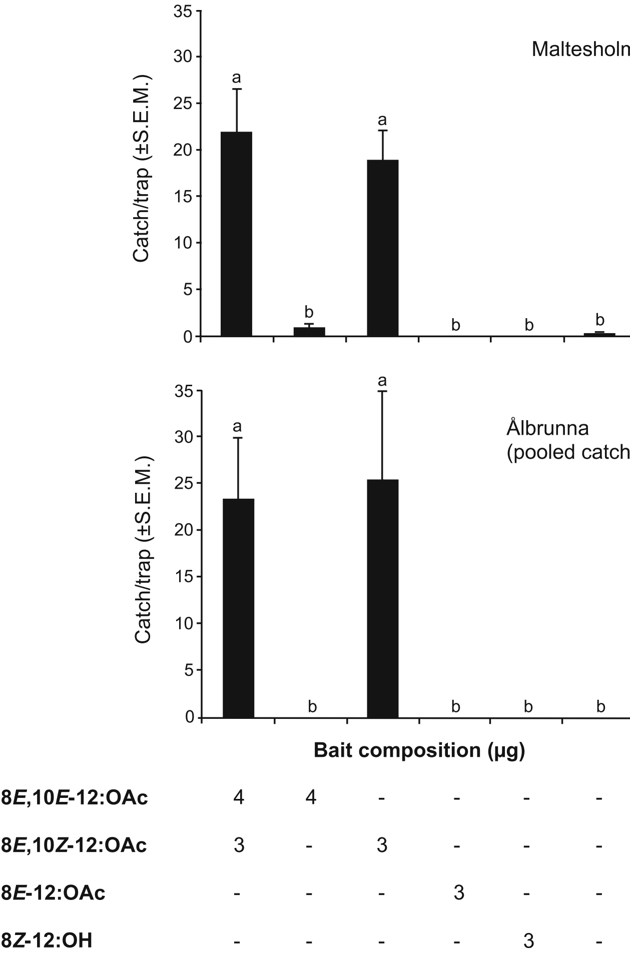


Fig. 3 *Cydia strobilella* males caught in traps using different pheromone candidate components at two field sites in Sweden. Analysis of data from Maltesholm is based on catch per trap per check (N=25), whereas the analysis of data from Ålbrunna is based on pooled catches per trap (N=5). *Bars with different letters* indicate significantly different catches (analysis of variance on log (x+1) transformed data, followed by multiple comparisons according to the Bonferroni *post hoc* test (α<0.0036))

comes close to that found in extracts of females, is an efficient attractant for male *C. strobilella* in Sweden. In a field screening of attractants for tortricids in Italy and Sweden, Witzgall et al. (1996) reported a few *C. strobilella* attracted to a 1:1 blend of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc at a dosage of 20 µg per lure (on average 5 males per replicate, N=8). Considering the fact that 0.3 µg was the most attractive dose for the species in our experiment, and that the catches using dosages above 3 µg were significantly lower, the low catches in the previous study can be explained by the excessive dosage used to bait the earlier traps.

In a more recent screening study, Witzgall et al. (2009) found that traps baited with a 1:1 blend of *E*8,*E*10–12:OAc and the corresponding alcohol *E*8,*E*10–12:OH, or *E*8,*Z*10–12:OAc and *E*8,*Z*10–12:OH, in a dosage of 20 µg per trap, attracted *C. strobilella* males, with the *E*,*Z*-combination being the most active. In our GC-EAD analyses of gland extracts of females, we did not record any responses at the expected retention times of those corresponding alcohols. In the GC-EAD experiments with synthetic standards, the antennae of males responded to three of the four dodecadienyl acetates (*Z*8,*E*10–12:OAc, *E*8,*E*10–12:OAc, and *E*8,*Z*10–12:OAc), and to two monounsaturated dodecenyl acetates (*E*8–12:OAc and *Z*8–12:OAc). However, males' antennae did not respond to the dodecadienol *E*8,*E*10–12:OH even when a relatively high dose of the synthetic compounds was used. Of course, this does not rule out the possibility that small amounts of the alcohols corresponding to the female-produced dienic acetates may still be released by calling females and act as behavioral synergists, but so far our results suggest that the female-produced sex pheromone of *C. strobilella* is a mixture of the two acetates.

When stimulated with gland extract of females, males' antennae responded exclusively to the *E*8,*E*10– and *E*8,*Z*10–12:OAc (Fig. 1), whereas there was no EAD response at the expected retention time of the *ZE* acetate isomer.

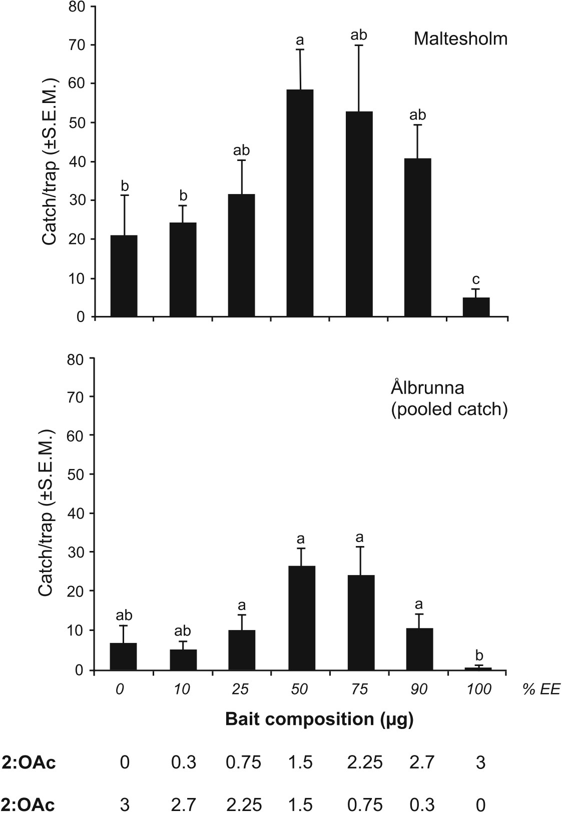


Fig. 4 Trap catch of *Cydia strobilella* males using different ratios of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc at two field sites in Sweden. Analysis of data from Maltesholm is based on catch per trap per check (N=38), whereas the analysis of data from Ålbrunna is based on pooled catches per trap (N=5). *Bars with different letters* indicate significantly different catches (analysis of variance on log (x+1) transformed data, followed by multiple comparisons according to the Bonferroni *post hoc* test (α<0.0025))

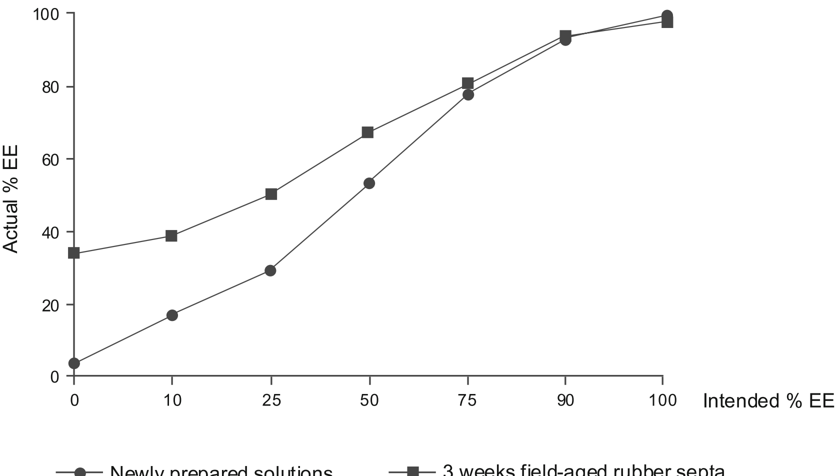


Fig. 5 Comparison of 8,10-dodecadienyl acetate isomer ratios (% *EE* isomer in a total amount of *EE* and *EZ* isomers) in newly prepared solutions for field tests (intended and actual) and in rubber septa aged for 3 wk in the field

Among *Cydia* species, the geometric isomers of 8,10-dodecadienyl acetate play different roles. For instance, in *C. jungiella*, which was observed flying in the course of our experiments with *C. strobilella, Z*8,*E*10–12:OAc was identified previously as a powerful attractant (Witzgall et al. 1996), whereas in the pea moth, *C. nigricana* F., *Z*8,*E*10–, *E*8,*Z*10–, and *Z*8,*Z*10–12:OAc isomers were not detected in gland extracts of females, and strongly inhibited male attraction to the sex pheromone compound *E*8,*E*10–12:OAc (Witzgall et al. 1993). We hypothesize that *Z*8,*E*10–12:OAc is a sex pheromone antagonist to *C. strobilella*, because of the strong GC-EAD response by antennae of males in spite of the absence of this compound in the female pheromone gland.

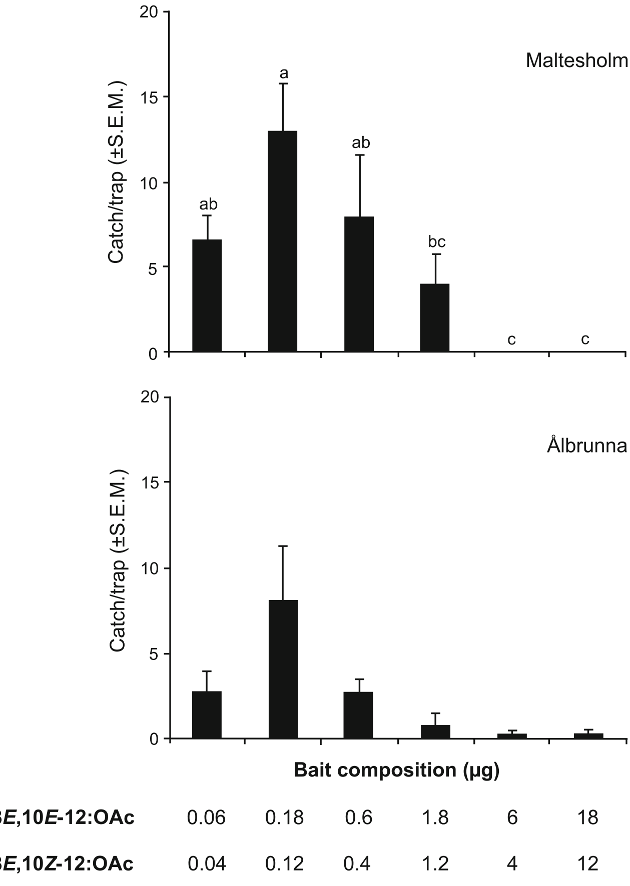


Fig. 6 Trap catch of *Cydia strobilella* males using different doses of a 6:4 blend of *E*8,*E*10–12:OAc and *E*8,*Z*10–12:OAc at two field sites in Sweden. Analyses of data from Maltesholm (*N*=23) and Ålbrunna (*N*=16) are based on catch per trap per check. *Bars with different letters* indicate significantly different catches (analysis of variance on log (x+1) transformed data, followed by multiple comparisons according to the Bonferroni *post hoc* test ($\alpha<0.0036$))

The composition of the identified sex pheromone for Swedish populations of *C. strobilella* in the present study differs from those previously reported for this species. Canadian populations of *C. strobilella* use only *E*8–12:OAc as a sex pheromone (Grant et al. 1989; Bédard et al. 2002). Although Brown and Miller (1983) synonymized the North American (formerly *C. youngana*) and European *C. strobilella* based on characters of the genitalia, it is tempting to suggest that, based on the marked difference in their sex pheromone composition, the two populations are, indeed, different species. This hypothesis could be tested by molecular phylogeographic analyses. The European situation should also be reexamined. Booij and Voerman (1984) and Skrzypczynska et al. (1998) reported *Z*8–12:OH as a sex pheromone for *C. strobilella*. In the present study, *P. splendidulana*, which is morphologically similar to *C. strobilella*, was attracted to traps baited with *Z*8–12:OH, but not to traps baited with any other compounds. Examination of genital preparations was required to confirm the species identity of trapped moths, and to show that *C. strobilella* and *P. splendidulana* were selectively attracted to different odor treatments in our field trials. However, at this point it cannot be ruled out that the pheromone composition of *C. strobilella* shows geographic variation.

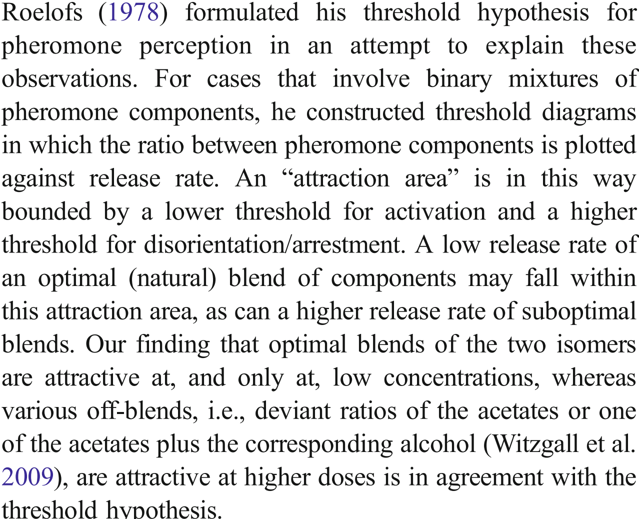
In the first field experiment, male *C. strobilella* were captured in traps that used both the binary blend and *E*8,

*Z*10–12:OAc alone, although GC-EAD analyses indicated that both components should be important for attraction (Fig. 3). Similar results were obtained in the indoor bioassays (Fig. 2). When checking possible isomerization of compounds after the second field experiment, we found that after three weeks in the field, the percentage of *EE* isomer in rubber septa generally increased except for the group in which the initial *EE*/*EZ* ratio was 99.3% (Fig. 5). This is in agreement with earlier findings of the isomerization of highly pure (99.4%) *EE* isomer on red rubber septa in the lab (Witzgall et al. 1993), and on polyethene dispensers in the field (Bengtsson et al. 1994) where in total 10% of the other three isomers were formed after ca. 20 days. It has been shown that the rate of isomerization of the *EE* isomer can be even faster in the field (25.1% *EZ*, 14.2% *ZE*, and 4.2% *ZZ* after four weeks; Davis et al. 1984). Isomerization, plus the impurity of ca. 3.6% of the *EE* isomer in *E*8,*Z*10–12:OAc, explains why this single component was attractive to *C. strobilella* in our first field test, as well as in our indoor bioassays. Similar extensive isomerization of 8,10-dodecandienyl acetate in rubber septa was found previously in the identification of the sex pheromone of the filbertworm, *C. latiferreanus* Walsingham (formerly *Melissopus latiferreanus*, Davis et al. 1984).

The third field test revealed that male *C. strobilella* are attracted to the sex pheromone at very low doses, which correlated well with the minute amounts of compounds produced by females. The most efficient dose for trapping was 0.3 µg per rubber septum, and the two highest doses tested (10 µg and 30 µg) trapped very few males. This sensitivity to the pheromone components in *C. strobilella* could explain the relatively low catches of males in the first experiment that used the 7 µg binary blend. A similar sensitivity in the pheromone communication system was observed in Canadian populations of *C. strobilella*, where the pheromone *E*8–12:OAc was produced in too small amounts by individual females to be detectable by FID, and where the optimal dose for attraction of males in field trials ranged from 0.3 µg to 3 µg (Grant et al. 1989; Bédard et al. 2002).

On the other hand, according to the sex pheromone identifications in tortricids to date, including the genus *Cydia*, the female-released sex pheromone titer in this family is normally at the nanogram level. For example, the codling moth *C. pomonella* (L.) produces the main sex pheromone component codlemone, *E*8,*E*10–12:OH (Roelofs et al. 1971), at an average amount of 8.7 ng per female (Witzgall et al. 2001). Among those tortricid moths for which the pheromone titer has been reported, only a few species, including the hickory shuckworm, *C. caryana* Fitch (McDonough et al. 1990) and *C. nigricana* (Greenway 1984), store the pheromone components in subnanogram levels.

It was observed earlier that male moths of some species were not always trapped in field tests by the ratio of pheromone components produced by the female moths.



Roelofs (1978) formulated his threshold hypothesis for pheromone perception in an attempt to explain these observations. For cases that involve binary mixtures of pheromone components, he constructed threshold diagrams in which the ratio between pheromone components is plotted against release rate. An "attraction area" is in this way bounded by a lower threshold for activation and a higher threshold for disorientation/arrestment. A low release rate of an optimal (natural) blend of components may fall within this attraction area, as can a higher release rate of suboptimal blends. Our finding that optimal blends of the two isomers are attractive at, and only at, low concentrations, whereas various off-blends, i.e., deviant ratios of the acetates or one of the acetates plus the corresponding alcohol (Witzgall et al. 2009), are attractive at higher doses is in agreement with the threshold hypothesis.

In summary, the lack of an efficient sex pheromone for European populations of *C. strobilella* has hampered the use of odor-based monitoring and control of this species in seed orchards, and our identified two-component sex pheromone hopefully may solve this problem. Mating disruption by means of synthetic pheromone may be an efficient way of controlling populations of *C. strobilella* in seed orchards. Because of the extremely high sensitivity of males to the pheromone, and the low female pheromone production, efficient mating disruption may be easier to obtain for *C. strobilella* compared to many other moth species. Trudel et al. (2006) used this technique in white spruce seed orchards in Canada to reduce cone damage by *C. strobilella*. Male captures in pheromone traps were reduced by up to 98%, and cone damage was reduced by up to 69% in pheromone-treated areas compared to control areas, thus showing the potential of the mating disruption technique to control populations of *C. strobilella* in European seed orchards as well.

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Sex Pheromone of *Agriotes acuminatus* (Stephens, 1830) (Coleoptera: Elateridae)

Till Tolasch · Maximilian von Fragstein · Johannes L. M. Steidle

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Abstract The click beetle species *Agriotes acuminatus* is distributed in open deciduous forests throughout a large area in Europe. In order to identify its sex pheromone, gland extracts of female beetles were investigated by using GC/MS. Neryl butanoate and 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate, in a ratio of approximately 1:5, were the only volatile compounds present in the extracts. Structures of both esters were confirmed by synthesis. Field experiments revealed a strong attraction of *A. acuminatus* males towards neryl butanoate, which could be synergistically enhanced by addition of 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate. The latter compound alone did not show any attractive effect. While all *Agriotes* spp. investigated to date use geranyl and/or (*E*,*E*)-farnesyl esters as sex pheromones, the nerol derivatives of *A. acuminatus* are the first (*Z*)-2-configurated pheromones within this genus.

Keywords *Agriotes acuminatus* · Coleoptera · Elateridae · Sex pheromone · Neryl butanoate · 2,6-Dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate · Monitoring

Introduction

Agriotes acuminatus is a fairly common click beetle species that inhabits a large part of Europe. Its distribution ranges from Spain in the west to the Caucasus in the east, and from Italy and Greece in the south up to Denmark and Great Britain in the north (Cate 2007).

With a body length of 6.5–7.5 mm, a light brown color with a dark longitudinal stripe in the middle of the elytra, the lighter colored hind angles of the pronotum and especially its much more slender form, the appearance of *A. acuminatus* clearly differs from the other Central European species of its genus. In contrast, it strongly resembles the closely related and very common *Dalopius marginatus* (L.), from which it can be distinguished in the field by the finer punctated and, therefore, shinier pronotum (cf. Lohse 1979). Furthermore, the larva of *A. acuminatus* shows a longitudinal wrinkled sculpturing on the abdominal tergites (Rudolph 1974; Klausnitzer 1994), similar to *Agriotes pallidulus* (Illiger), but unlike all other Central European *Agriotes* spp.

Adults of *A. acuminatus* occur in open deciduous forests, especially on their edges and on glades, from lowlands to the montane zone. In contrast to agriculturally important species such as *A. lineatus* (L.), *A. obscurus* (L.), and *A. sputator* (L.), which develop in open areas, the larvae of *A. acuminatus* inhabit forest soil, where they presumably feed on roots of various plants (Rudolph 1974). Since *A. acuminatus* usually occurs in much lower numbers than economically important species and is absent from open agricultural areas, it is not classified as a pest insect.

In this study, the identification and synthesis of the sex pheromone of *Agriotes acuminatus* is reported, and the results of the corresponding field experiments are shown. The aim of our investigation was to complete the knowledge concerning sex pheromones of the Central European members of the genus *Agriotes*, and to provide an effective monitoring tool for faunistic purposes where the species is rare.

T. Tolasch (✉) · M. von Fragstein · J. L. M. Steidle
Institut für Zoologie, Universität Hohenheim,
Tierökologie 220c, Garbenstraße 30,
70593 Stuttgart, Germany
e-mail: tolasch@uni-hohenheim.de

Methods and Materials

Insects Adult beetles of *Agriotes acuminatus* were collected in July 2004 by using a sweep net and a beating tray near Göppingen/Baden-Württemberg/Germany along a small river that cuts through an old beech forest. Beetles were sexed in the lab by observing the last abdominal segments, and a total of 22 female *A. acuminatus* were used for preparing extracts.

Extracts Beetles were frozen for 20 min, and allowed to thaw for another 20 min to room temperature. Subsequently, the ovipositor was extricated from the abdomen with forceps, and the now visible paired pheromone glands, similar to those described by Merivee and Erm (1993) for the click beetle *Agriotes obscurus*, were removed. Separate extracts were prepared for each beetle by transferring the glands to 300 µl CH_2Cl_2.

Chemical Analyses Analyses of natural extracts were carried out by coupled gas chromatography–mass spectrometry (GC/MS) in electron impact mode at 70 eV, 230°C, using a 6890 GC/5973 N MSD quadrupole instrument (Agilent Technologies, Santa Clara, CA, USA). Using helium as carrier gas, separations were achieved with a 30 m× 0.25 mm ID, 0.25-µm film HP5-MS fused silica column (Agilent), starting at 60°C for 3 min, then programmed to 300°C at a rate of 3°C /min, and finally held at 300°C for 40 min. Nuclear magnetic resonance (NMR) spectra of synthetic compounds were recorded with a Varian INOVA 500 instrument (Varian, Palo Alto, CA, USA).

Chemical Syntheses Chemicals and solvents were purchased from Aldrich or Merck and were of the highest purity available. Purification of synthetic products was carried out by flash chromatography on silica gel (silica 32–63, 60 Å, ICN-Biomedicals, Eschwege, Germany) at 1.3 bar using mixtures of ethyl acetate and hexane.

Neryl butanoate (**1**) and neryl hexanoate were prepared from nerol and the appropriate acid chlorides according to known methods (Tóth et al. 2003). Neryl hexanoate was oxidized to (*E*)-8-hydroxyneryl 1-hexanoate using SeO_2 and *tert*-butyl hydroperoxide, following Arm et al. (1986). Esterification of this product with hexanoyl chloride gave (*E*)-8-hydroxyneryl 1,8-dihexanoate (**2**) in a total yield of 57%.

Spectroscopic Data

*Neryl butanoate (***1***)* ^{1}H NMR (500 MHz, $CDCl_3$): *δ* (ppm)= 0.96 (t, J=7.4 Hz, 3H, 4′-H), 1.62 (s, 3H, *cis*-7-CH_3), 1.62–1.72 (m, 2H, 3′-H), 1.70 (s, 3H, *trans*-7-CH_3), 1.78 (s, 3H, 3-CH_3), 2.06–2.16 (m, 4H, 4-H und 5-H), 2.30 (t, J=7.4 Hz, 2H, 2′-H), 4.58 (d, J=7.1 Hz, 2H, 1-H), 5.11 (t, J=6.9 Hz, 1H, 6-H), 5.37 (t, J=7.1 Hz, 1H, 2-H).

^{13}C NMR (126 MHz, $CDCl_3$): *δ* (ppm)=13.64 (q, C-4′), 17.60 (q, cis-7-CH_3), 18.45 (t, C-3′), 23.46 (q, 3-CH_3), 25.63 (q, trans-7-CH_3), 26.63 (t, C-5), 32.13 (t, C-4), 36.23 (t, C-2′), 60.85 (t, C-1), 119.29 (d, C-2), 123.57 (d, C-6), 132.10 (s, C-7), 142.40 (s, C-3), 173.67 (s, C-1′).

MS (70 eV): *m/z* (%)=224 (0.25) [M$^+$], 155 (1) 154 (2), 139 (1) 137 (2), 136 (11), 135 (1), 123 (3), 122 (2), 121 (23), 120 (1), 119 (1), 111 (1), 109 (1), 108 (3), 107 (9), 106 (1), 105 (3), 97 (1), 95 (5), 94 (11), 93 (68), 92 (15), 91 (10), 89 (1), 85 (3), 84 (4), 83 (2), 82 (2), 81 (14), 80 (28), 79 (11), 78 (2), 77 (7), 73 (1), 72 (2), 71 (40), 70 (6), 69 (100), 68 (50), 67 (26), 66 (1), 65 (5), 63 (1), 60 (1), 59 (1), 57 (1), 56 (1), 55 (8), 54 (2), 53 (17), 52 (2), 51 (4), 50 (1), 45 (1), 44 (2), 43 (39), 42 (6), 41 (74), 40 (5), 39 (18), 38 (1).

Neryl hexanoate ^{1}H NMR (500 MHz, $CDCl_3$): *δ* (ppm)= 0.91 (t, J=6.9 Hz, 3H, 6′-H), 1.26–1.36 (m, 4H, 4′-H und 5′-H), 1.62 (s, 3H, *cis*-7-CH_3), 1.62–1.68 (m, 2H, 3′-H), 1.70 (s, 3H, *trans*-7-CH_3), 1.78 (s, 3H, 3-CH_3), 2.06–2.16 (m, 4H, 4-H und 5-H), 2.29 (t, J=7.6 Hz, 2H, 2′-H), 4.59 (d, J=6.9 Hz, 2H, 1-H), 5.11 (t, J=6.9 Hz, 1H, 6-H), 5.37 (t, J=7.1 Hz, 1H, 2-H).

^{13}C NMR (126 MHz, $CDCl_3$): *δ* (ppm)=13.82 (q, C-6′), 17.60 (q, *cis*-7-CH_3), 21.85 (t, C-5′), 23.42 (q, 3-CH_3), 24.61 (t, C-3′), 25.61 (q, *trans*-7-CH_3), 26.62 (t, C-5), 31.25 (t, C-4′), 31.60 (t, C-4), 34.34 (t, C-2′), 60.85 (t, C-1), 119.22 (d, C-2), 123.55 (d, C-6), 132.08 (s, C-7), 142.40 (s, C-3), 173.92 (s, C-1′).

MS (70 eV): *m /z* (%) = 252 (0.1) [M$^+$], 154 (1), 139 (1), 137 (2), 136 (13), 123 (4), 122 (4), 121 (35), 120 (1), 119 (2), 117 (1), 111 (2), 110 (1), 109 (1), 108 (4), 107 (13), 106 (1), 105 (6), 103 (1), 100 (2), 99 (19), 98 (1), 97 (2), 96 (1), 95 (6), 94 (17), 93 (95), 92 (26), 91 (23), 89 (1), 85 (1), 84 (3), 83 (1), 82 (2), 81 (20), 80 (39), 79 (21), 78 (4), 77 (19), 76 (1), 73 (1), 72 (1), 71 (16), 70 (6), 69 (100), 68 (56), 67 (35), 66 (3), 65 (8), 64 (1), 63 (2), 62 (1), 60 (1), 59 (1), 57 (2), 56 (1), 55 (13) 54 (3), 53 (20), 52 (3), 51 (4), 50 (2), 45 (1), 44 (2), 43 (26), 42 (7), 41 (69), 40 (6), 39 (25), 38 (1).

(E)-8-Hydroxyneryl hexanoate ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm)=0.91 (t, J=7.0 Hz, 3H, 6′-H), 1.26–1.36 (m, 4H, 4′-H und 5′-H), 1.60–1.68 (m, 2H, 3′-H), 1.68 (s, 3H, 7-CH_3), 1.78 (s, 3H, 3-CH_3), 2.14–2.19 (m, 4H, 4-H und 5-H), 2.30 (t, J=7.6 Hz, 2H, 2′-H), 4.00 (s, 2H, 8-H), 4.58 (d, J=7.2 Hz, 2H, 1-H), 5.36 (t, J=7.2 Hz, 1H, 2-H), 5.38–5.44 (m, 1H, 6-H).

^{13}C NMR (126 MHz, $CDCl_3$): *δ* (ppm)=13.62 (q, 7-CH_3), 13.87 (q, C-6′), 22.28 (t, C-5′), 23.35 (q, 3-CH_3), 24.64 (t, C-3′), 25.94 (t, C-5), 31.29 (t, C-4′), 31.75 (t, C-4), 34.32 (t, C-2′), 60.98 (t, C-1), 68.73 (t, C-8), 119.73 (d, C-

2), 124.87 (d, C-6), 135.54 (s, C-7), 141.64 (s, C-3), 173.98 (s, C-1′).

MS (70 eV): *m /z* (%)=183 (1), 182 (1), 169 (1), 152 (4), 151 (1), 150 (2), 139 (1), 138 (1), 137 (7), 136 (1), 135 (5), 134 (29), 133 (2), 125 (1), 124 (2), 123 (7), 122 (3), 121 (17), 120 (4), 119 (38), 118 (1), 117 (5), 116 (1), 115 (2), 111 (1), 110 (2), 109 (7), 108 (3), 107 (9), 106 (8), 105 (11), 104 (1), 103 (2), 100 (3), 99 (49), 98 (1), 97 (7), 96 (4), 95 (20), 94 (60), 93 (36), 92 (16), 91 (24), 89 (1), 87 (4), 86 (1), 85 (5), 84 (52), 83 (6), 82 (8), 81 (16), 80 (19), 79 (47), 78 (4), 77 (16), 75 (1), 74 (1), 73 (11), 72 (2), 71 (29), 70 (3), 69 (15), 68 (58), 67 (37), 66 (3), 65 (7), 63 (2), 62 (1), 61 (3), 60 (29), 59 (1), 58 (2), 57 (12), 56 (8), 55 (37), 54 (3), 53 (20), 52 (3), 51 (5), 50 (2), 45 (6), 44 (4), 43 (100), 42 (10), 41 (54), 40 (5), 39 (22), 38 (1).

2,6-Dimethyl-(Z,E)-2,6-octadien-1,8-diol dihexanoate (2) ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm)=0.91 (t, J=7.0 Hz, 6H, 6′-H und 6″-H), 1.26–1.36 (m, 8H, 4′-H, 4″-H, 5′-H und 5″-H), 1.61–1.69 (m, 4H, 3′-H und 3″-H), 1.68 (s, 3H, 7-CH_3), 1.79 (s, 3H, 3-CH_3), 2.14–2.20 (m, 4H, 4-H und 5-H), 2.28–2.38 (m, 4H, 2′-H und 2″-H), 4.48 (s, 2H, 8-H), 4.58 (d, J=7.2 Hz, 2H, 1-H), 5.38 (t, J=7.2 Hz, 1H, 2-H), 5.44–5.48 (m, 1H, 6-H).

^{13}C NMR (126 MHz, $CDCl_3$): δ (ppm)=13.89 (q, 7-CH_3), 13.90, 14.09 (2q, C-6′ und C-6″), 22.30, 22.68 (2t, C-5′ und C-5″), 23.44 (q, 3-CH_3), 24.68 (2t, C-3′ und C-3″), 26.34 (t, C-5), 31.31, 31.91 (2t, C-4′ und C-4″), 31.59 (t, C-4), 34.33 (2t, C-2′ und C-2″), 60.76 (t, C-1), 69.76 (t, C-8), 119.73 (d, C-2), 124.34 (d, C-6), 130.90 (s, C-7), 141.82 (s, C-3), 173.72, 173.83 (2 s, C-1′ und C-1″).

MS (70 eV): *m /z* (%)=251 (2), 183 (4), 182 (4), 153 (1), 152 (6), 151 (2), 137 (3), 136 (2), 135 (17), 134 (54), 133 (3), 124 (1), 123 (2), 122 (1), 121 (4), 120 (6), 119 (59), 117 (2), 115 (1), 110 (1), 109 (3), 108 (2), 107 (10), 106 (11), 105 (11), 100 (6), 99 (100), 97 (2), 96 (1), 95 (3), 94 (6), 93 (19), 92 (14), 91 (11), 89 (1), 85 (1), 84 (9), 83 (2), 82 (1), 81 (6), 80 (4), 79 (9), 78 (2), 77 (5), 73 (2), 72 (3), 71 (45), 70 (1), 69 (8), 68 (20), 67 (19), 66 (2), 65 (4), 60 (2), 59 (1), 57 (2), 56 (3), 55 (18), 54 (1), 53 (8), 52 (1), 51 (1), 45 (1), 44 (2), 43 (61), 42 (6), 41 (25), 40 (3), 39 (8).

In the NMR data, carbon atom numbers of the acid moieties in position 1 are indicated by one, in position 8 by two apostrophes.

Bait Dispensers and Traps Dispensers were prepared as described earlier (Tolasch et al. 2007) from 0.2 ml PCR tubes (ThermoTube™, Peqlab, Germany) that had been pierced with an insect pin 0.5 mm diam at the front side 2 mm below the lid. Dispensers were filled with three types of baits: **A**: 3 mg neryl butanoate (**1**), **B**: 15 mg 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate (**2**), and **C**: 3 mg **1** + 15 mg **2**, resembling the natural ratio of these compounds found in the glands (1:5). Under laboratory conditions, the odor dispensers had a stable mean release rate of approximately 8–10 µg per day, regardless of the amount in the tubes (determined by weighing, unpublished data). Due to their different physical properties, the particular release rates of **1** and **2** may be different in lure **C**; however, we assumed that the ratio is similar to that of the natural pheromone.

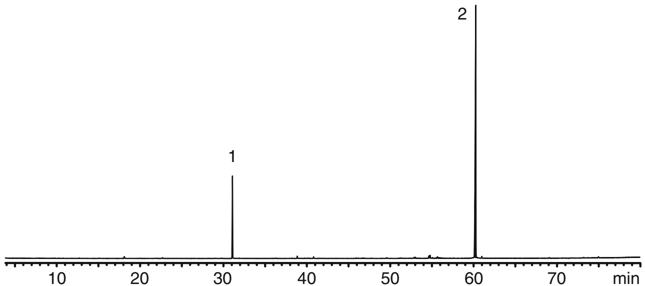

Fig. 1 Representative gas chromatogram (total ion current) of a gland extract from a female *Agriotes acumiatus* (30 m HP5-MS capillary column, 3 min at 60°C, then 3°C/min to 300°C)

Funnel traps were the same as described before (Tolasch et al. 2007). Each collecting bottle was filled with brine (200 ml) to preserve the beetles and minimize the possible attraction of further individuals to those already captured.

Field Experiments Two separate field tests were performed in 2006 and 2009. To check the general attractiveness of the synthetic blend of **1** and **2** resembling the natural ratio found in the glands, field experiments were carried out from 23 May to 13 June 2006 along a small river cutting through an old beech forest (*Fagus sylvatica* L.) near Göppingen/Baden-Württemberg/Southwest Germany. Sixteen traps were grouped into 8 sets, each set containing a trap baited with lure **C** and an unbaited control. Traps were suspended ca. 1.5–2.0 m above the ground from branches at the edge of the forest. The distance between traps within one set was about 5 m, while the distances between the sets were at least 50 m.

A second field bioassay, designed to examine the activity of the single compounds vs. the mixture, was performed

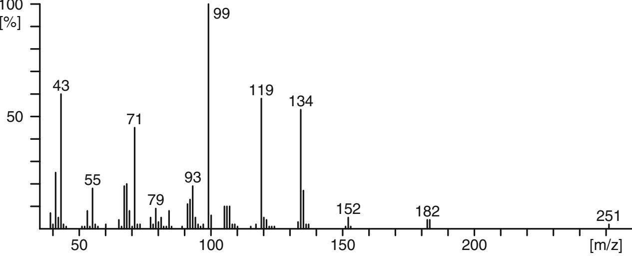

Fig. 2 70 eV–mass spectrum of the *Agriotes acuminatus* extract compound eluting after 60 min, subsequently identified as 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate (**2**)

Fig. 3 Structures of the two pheromone gland compounds identified in *Agriotes acuminatus*, neryl butanoate (**1**) and 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate (**2**)

from 9–30 June 2009 in a mixed lowland forest at the river Körsch near Plieningen/Baden-Württemberg/Southwest Germany. Predominant trees were black alder (*Alnus glutinosa* (L.) Gaertn.), willow (*Salix* spp.), poplar (*Populus* spp.), and black cherry (*Prunus serotina* Ehrh.). A total of 24 traps were grouped into 6 sets, each set comprised of three baited traps with lure **A**, **B**, **C**, respectively, and an empty control. Traps were suspended as described above, but the distances between the traps were larger (~15 m within one set, ~100 m between the sets). In both field tests, traps were checked weekly, captured beetles were removed and the brine was replaced. The numbers of male beetles were compared between traps by the Wilcoxon matched-pairs test using Statistica 6.1 (StatSoft, Tulsa, USA).

Results

GC/MS analysis revealed two compounds present in all gland extracts in a ratio of approximately 1:5, with only small quantitative variation between the samples of different females (Fig. 1). The minor compound, eluting at 31.1 min under the conditions described, was identified as neryl butanoate (**1**) by comparison with a synthetic sample. The major compound exhibited a mass spectrum resembling the spectrum of (*E*)-8-hydroxygeranyl diisovalerate (previously found in *Agriotes litigiosus* (Rossi), see Tóth et al. 2003), with the base peak shifted from *m/z* 85 to *m/z* 99 pointing to a dihexanoate (Fig. 2). A synthetic sample of (*E*)-8-hydroxygeranyl dihexanoate, prepared from geranyl hexanoate via the route described above (data not shown), eluted almost 1 min later than the natural product, however, the mass spectra were almost identical. Given that esters of nerol usually show very similar mass spectra but slightly shorter retention times than the corresponding geranyl esters, 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate (**2**) (= (*E*)-8-hydroxyneryl dihexanoate) finally was proposed, which turned out to be identical with the natural product. The structures of both gland compounds are depicted in Fig. 3. The absolute amount of neryl butanoate (**1**) was estimated to be 92.8±18.5 ng/female, and for 2,6-dimethyl-(*Z*,*E*)-2,6-octadien-1,8-diol dihexanoate (**2**) 427.8±62.3 ng/female (*N*=4, respectively).

In the first set of field tests, performed in 2006, a 1:5-mixture of **1** and **2** proved highly attractive for males of *A. acuminatus*. In traps baited with lure **C**, a total of 548 ♂♂ were caught, while only 6 ♂♂ were found in the unbaited controls, showing a highly significant difference ($P<0.001$, Fig. 4, left). The second field test (2009), designed to investigate the activity of the single compounds **1** and **2** vs. the mixture, revealed a strong attractiveness for neryl butanoate (**1**, lure **A**), which captured a total of 313 males. Catches with the mixture (lure **C**) were almost 3 times higher (902 ♂♂), showing a strong synergistic effect of **2**. Lure **B** attracted no beetles at all, while 9 ♂♂ were found in the control traps (Fig. 4, right).

Discussion

The results of our study show clearly that the sex pheromone of *Agriotes acuminatus* consists of neryl butanoate (**1**) and (*E*)-8-hydroxyneryl dihexanoate (**2**). While **1** is essential to provide a general attractiveness, **2** acts synergistically and strongly enhances the activity. Compound **2** alone did not show any attractive effect.

Interestingly, occasional catches of low but significant numbers of *A. acuminatus* males with traps for *A. brevis* and *A. sputator*, containing geranyl butanoate, have been reported in the literature (Tóth et al. 2003; Subchev et al. 2006). Most likely, this cross-attraction may be explained by traces of neryl butanoate as an impurity in the geranyl butanoate employed.

Neryl butanoate (**1**) has not been described as a component of a click beetle pheromone before. However,

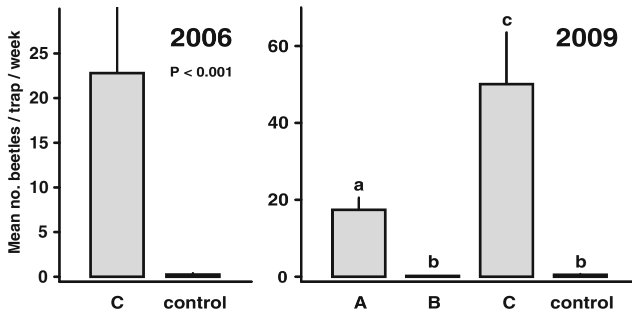

Fig. 4 Mean (+ SE) trap catches / week / trap of male *Agriotes acuminatus* in 2006 (*N*=24) and 2009 (*N*=18). Columns with different lowercase letters are significantly different at $P<0.001$ (Wilcoxon matched-pairs test)

it has been identified as a behaviorally inactive trace component among the volatiles of the pheromone gland of *A. sputator* (Tóth et al. 2003). (*E*)-8-Hydroxyneryl dihexanoate (**2**) represents an entirely new natural product.

Apart from the former subgenus *Ectinus* Eschscholtz (Tolasch 2008), the sex pheromones of the genus *Agriotes* are known to date form a relatively homogenous group with respect to their structural features: They are made up of one or two geranyl- and/or (*E*,*E*)-farnesyl esters of short fatty acids with equal chains (2–8 carbon atoms). If two compounds are involved, they differ either in the alcohol or in the acid moiety, but not in both (see Tóth et al. 2002, 2003). Representing acyclic monoterpene esters, the compounds reported here fit into this general scheme, however, there are two remarkabe differences: First, they show exclusively (*Z*)- instead of (*E*)- geometry at carbon 2 of the terpene unit. Second, they differ from each other in both the alcohol- and the acid moiety. This, along with the morphological features of the larvae and the adults mentioned above, points to a unique position of *A. acuminatus* within its genus, which is probably shared with the morphologically related *A. pallidulus* (Illiger) (pheromone hitherto unknown).

As mentioned in the introduction, *A. acuminatus* usually does not cause economical losses in agriculture or forestry. Nevertheless, the synthetic sex pheromone may serve as monitoring tool if the species should reach pest status. For the faunistic research, the pheromone could be useful for gathering records in places where the species is rare and/or reaches the limit of its distribution.

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Fatty Acid-amino Acid Conjugates Diversification in Lepidopteran Caterpillars

Naoko Yoshinaga · Hans T. Alborn · Tomoaki Nakanishi · David M. Suckling · Ritsuo Nishida · James H. Tumlinson · Naoki Mori

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Abstract Fatty acid amino acid conjugates (FACs) have been found in noctuid as well as sphingid caterpillar oral secretions; in particular, volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] and its biochemical precursor, *N*-linolenoyl-L-glutamine, are known elicitors of induced volatile emissions in corn plants. These induced volatiles, in turn, attract natural enemies of the caterpillars. In a previous study, we showed that *N*-linolenoyl-L-glutamine in larval *Spodoptera litura* plays an important role in nitrogen assimilation which might be an explanation for caterpillars synthesizing FACs despite an increased risk of attracting natural enemies. However, the presence of FACs in lepidopteran species outside these families of agricultural interest is not well known. We conducted FAC screening of 29 lepidopteran species, and found them in 19 of these species. Thus, FACs are commonly synthesized through a broad range of lepidopteran caterpillars. Since all FAC-containing species had *N*-linolenoyl-L-glutamine and/or *N*-linoleoyl-L-glutamine in common, and the evolutionarily earliest species among them had only these two FACs, these glutamine conjugates might be the evolutionarily older FACs. Furthermore, some species had glutamic acid conjugates, and some had hydroxylated FACs. Comparing the diversity of FACs with lepidopteran phylogeny indicates that glutamic acid conjugates can be synthesized by relatively primitive species, while hydroxylation of fatty acids is limited mostly to larger and more developed macrolepidopteran species.

N. Yoshinaga · J. H. Tumlinson
Center for Chemical Ecology, Department of Entomology,
The Pennsylvania State University,
University Park, PA 16802, USA

H. T. Alborn
Center of Medical, Agricultural, and Veterinary Entomology,
Agricultural Research Service, Chemistry Unit,
U. S. Department of Agriculture,
1600 Southwest 23rd Drive,
Gainesville, FL 32611-0620, USA

T. Nakanishi
Forestry and Fisheries Technology Center,
Fruit Tree Research Institute, Tokushima Prefectural Agriculture,
Katsuura-cho, Katsuura,
Tokushima 773-4301, Japan

D. M. Suckling
The New Zealand Institute for Plant and Food Research Limited,
PB 4704, Christchurch, New Zealand

N. Yoshinaga · R. Nishida · N. Mori (✉)
Division of Applied Life Sciences,
Graduate School of Agriculture, Kyoto University,
Sakyo,
Kyoto 606-8502, Japan
e-mail: mokurin@kais.kyoto-u.ac.jp

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Abbreviations

FACs fatty acid amino acid conjugates
VOC volatile organic compounds
ESI electrospray ionization

Introduction

It is well documented that constituents of insect oral secretions can trigger plant responses, such as elicitation of induced *de novo* synthesis and release of volatile organic compounds (VOCs) (Turlings et al. 1990; Paré et al. 1998; Kessler and Baldwin 2001). The best known and most studied of insect-produced elicitors are the fatty acid amino

acid conjugates (FACs), first identified from beet armyworm *Spodoptera exigua* larvae (Alborn et al. 1997). In a recent study of a range of plant species, FACs and especially volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] showed the broadest effects on plant hormone levels as well as induction of plant volatiles compared with caeliferin A16:0 and inceptin, two recently identified new classes of insect-produced elicitors of inducible plant defenses (Schmelz et al. 2006, 2009; Alborn et al. 2007). It also has been shown that application of volicitin to a mechanically wounded site selectively and transcriptionally activated genes for indole-3-glycerol phosphate lyase (*Igl*) and specific sesquiterpene cyclase (*stc1*), and that this activation also occurred systemically in undamaged leaves (Frey et al. 2000; Shen et al. 2000). However, previous studies (Truitt and Paré 2004; Truitt et al. 2004) showed that volicitin did not on its own serve as a mobile messenger for systemic VOCs emissions, but rather that a volicitin binding protein-ligand interaction may initiate plant defenses in response to herbivory.

Since glutamine-based FAC components initially were identified in oral secretions from *S. exigua* larvae, several other noctuid caterpillars have been reported to have the same glutamine-based FACs (Pohnert et al. 1999; Mori et al. 2001, 2003; De Moraes and Mescher 2004). In addition, oral secretions from some noctuid larvae such as *S. littoralis* also contain traces of glutamic acid-based FACs. Glutamine conjugates also are the major FACs in species of Geometridae, and one sphingid species (Pohnert et al. 1999; Mori et al. 2003). In contrast, glutamic acid conjugates are the dominant FACs in the sphingid tobacco hornworm, *Manduca sexta*, (Alborn et al. 2003) and tomato hornworm, *M. quinquemaculata* (Halitschke et al. 2001). Paré et al. (1998) showed that the fatty acid moiety of the FAC molecule originates from the diet of the caterpillar. Consequently, the fatty acid composition of the FACs roughly reflects the fatty acid composition in the host plant, although there seems to be a preference for linolenic and linoleic acid in the FAC synthesis (Aboshi et al. 2007).

Since the isolation and identification of FACs as elicitors of defensive reactions in plants, one intriguing question still remains to be answered: How do the insects benefit from producing FACs? Recently, we discovered that, at least for *S. litura*, glutamine-containing FACs play an active role in nitrogen assimilation by regulating the glutamine supply in the larval midgut (Yoshinaga et al. 2008). Enriching artificial diet with linolenic acid not only resulted in an increased FAC synthesis, but also promoted a 50% increased glutamic acid to glutamine conversion, ultimately resulting in a significantly increased amount of glutamine in the whole body. Thus, the ability to utilize FACs in the digestive system might be one way that lepidopteran larvae optimize their growth rate. However, it is not known if all lepidopteran larvae utilize FACs. Furthermore, differences in physiology or metabolism associated with glutamine-FACs vs. glutamic acid-FACs are not understood. These questions are not easy to answer, since only a limited number of insect species (mainly of agricultural interest) have been investigated for FAC content so far. Consequently, we decided to collect and analyze gut contents from an environmentally, taxonomically, and physiologically diverse group of lepidopteran larvae consisting of 29 species from16 families.

Materials and Methods

Caterpillar Source Colonies of *Spodoptera litura*, *Mythimna separate,* and *Hyphantria cunea* were maintained successively in the laboratory, and *Helicoverpa armigera* was supplied by Dr. Kenji Fujisaki, *Anadevidia peponis* by Dr. Tetsu Ando, *Samia cynthia ricini* by Dr. Masatoshi Ichida, and *Agrius convolvuli* by Dr. Masami Shimoda. Commercially available *Bombyx mori* were purchased from Mukin Yosan System Institute (Kyoto, Japan), *Agrotis ipsilon* eggs were purchased from Benzon Research Inc. (PA, USA), and *Manduca sexta* from North Carolina University, NC, USA. Another 18 wild species were collected in Kyoto, Osaka, Mie, and Tokushima prefectures in Japan. *Malacosoma americanum* was caught in Pennsylvania, USA, all identified by their morphological traits and food habitats. *Epiphyas postvittana* from New Zealand were from a recently-established colony fed on apple. *Hy. cunea* and *An. peponis* were fed on artificial diet (Insecta-LFS, Nihon Nosan Kogyo Ltd., Yokohama, Japan), while other species, including laboratory-reared species, were fed on their host plants. Last-instars of each species were frozen at −4°C to extract gut contents.

Gut Content Extraction and Sample Preparation At least three insects were used from one species (one insect per sample). The frozen gut contents were dissected out as earlier described (Mori et al. 2003), placed in plastic tubes, and immediately boiled for 20 min to avoid enzymatic decomposition of FACs (Mori et al. 2001). To each sample was added an equal volume of 50% water/acetonitrile solution (v/v) (an addition of 10–300 µl, dependent on the amount of gut content). The samples were then roughly homogenized with a plastic homogenizer and centrifuged at 14,000*g* for 5 min. Ten-fold dilutions of the supernatants with 50% acetonitrile solution were analyzed by LCMS.

LC/MS Analyses Mass spectral measurements were carried out with an LCMS-2010A instrument (Shimadzu, Kyoto, Japan) combined with an HPLC system (LC-10ADvp pump, CTO-10ACvp column oven, and SCL-10AVvp system

controller, Shimadzu). Three μl of sample solution were injected into a reversed-phase column (Cosmosil $5C_{18}$-AR-II, 50×2.0 mm I.D., Nacalai tesque, Kyoto, Japan) eluted for 15 min at (0.2 ml/min) with a solvent gradient of 40–95% CH_3CN containing 0.08% acetic acid, in water containing 0.05% acetic acid. The column temperature was maintained at 40°C (CTO-10Avp column oven, Shimadzu), and the column eluant was monitored by continuous MS total ion current trace. The CDL temperature was 250°C, the voltage was 1.5 kV, the nebulizer gas flow was 1.5 l/min, and the analytical mode was ESI negative scan from *m/z* 150–500. The negative ionization mass spectra gave characteristic $(M-1)^-$ ions for volicitin at *m/z* 421, *N*-hydroxylinolenoyl-L-glutamic acid *m/z* 422, *N*-hydroxylinoleoyl-L-glutamine *m/z* 423, *N*-hydroxylinoleoyl-L-glutamic acid *m/z* 424, *N*-linolenoyl-L-glutamine *m/z* 405, *N*-linolenoyl-L-glutamic acid *m/z* 406, *N*-linoleoyl-L-glutamine *m/z* 407, and *N*-linoleoyl-L-glutamic acid *m/z* 408. The position of the hydroxyl group in the hydroxylated FACs was not determined.

For some species, a few FACs were detected only in trace amounts. In such cases, we increased sample numbers, and determined an FAC was present only when it was detected in more than three replicates.

Results

Linolenic and linoleic acid were the dominant fatty acids in FACs. Although we also detected *N*-oleoyl-L-glutamine, and other less abundant fatty acids conjugated with glutamine reflecting the proportion of dietary fatty acids (Aboshi et al. 2007), we focused our analysis on conjugates based on these acids.

Figure 1 shows the reconstructed total ion chromatograms of gut contents of (a) *Locastra muscosalis* (Pyralidae), (b) *Phalera flavescens* (Notodontidae), (c) *Xanthodes transversa* (Noctuidae), (d) *Limantria dispar* (Lymantriidae), (e) *Agrotis ipsilon* (Noctuidae), and (f) *Acherontia styx* (Sphingidae),

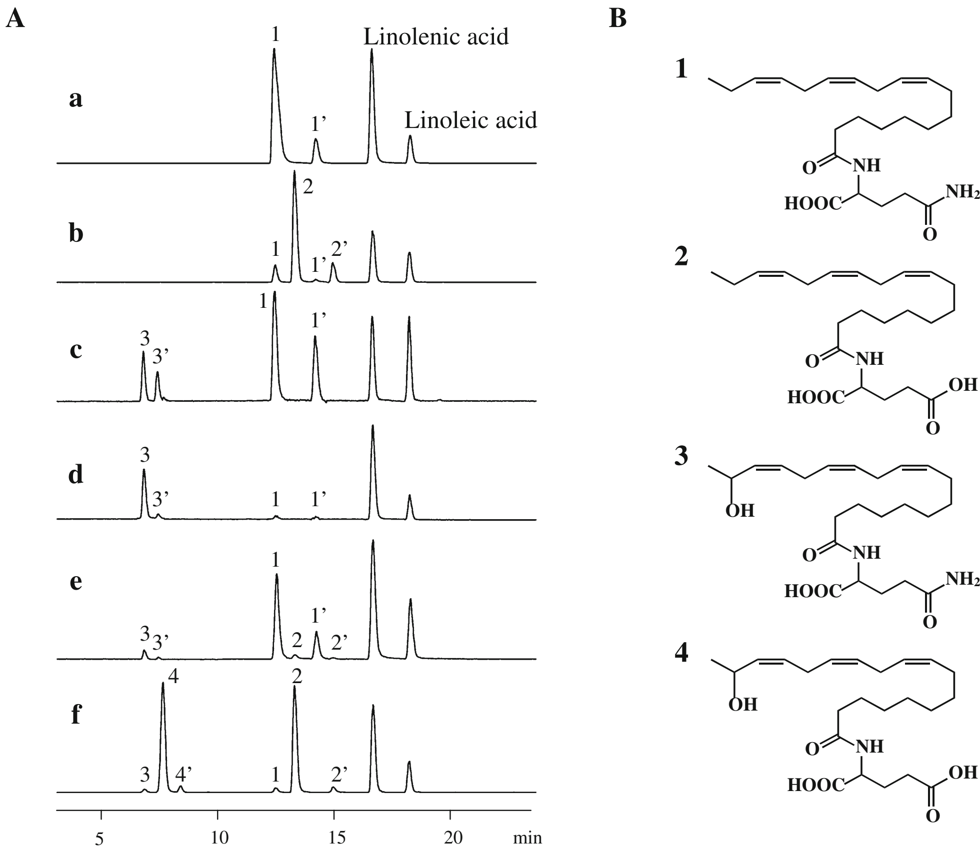

Fig. 1 (**A**) Typical extracted ion chromatograms of gut contents from (a) *Locastra muscosalis*, (b) *Phalera flavescens*, (c) *Xanthodes transversa*, (d) *Lymantria dispar*, (e) *Agrotis ipsilon*, and (f) *Acherontia styx*, and (**B**) chemical structures of compounds 1-4. A reversed-phase column was eluted for 15 min with a 40–95% solvent gradient of CH_3CN in water containing 0.05% acetic acid. ESI-negative MS scan at *m/z* 150–500. 1, *N*-linolenoyl-L-glutamine (*m/z* 405); 1′, *N*-linoleoyl-L-glutamine (*m/z* 407); 2, *N*-linolenoyl-L-glutamic acid (*m/z* 406); 2′, *N*-linoleoyl-L-glutamic acid (*m/z* 408); 3, *N*-hydroxylinolenoyl-L-glutamine (*m/z* 421); 3′, *N*-hydroxylinoleoyl-L-glutamine (*m/z* 423); 4, *N*-hydroxylinolenoyl-L-glutamic acid (*m/z* 422); 4′, *N*-hydroxylinoleoyl-L-glutamic acid (*m/z* 424)

and chemical structures of the major FACs. Noticeable differences in FAC composition among the species are first that *L. dispar* larvae (Fig. 1Ad) have more of the hydroxylated compounds (3, 3′) than glutamine conjugates (1, 1′), while for *X. transversa* (c) these ratios are reversed.

Second, *P. flavescens* (b), *Ag. ipsilon* (e) and *Ac. styx* (f) synthesize glutamic acid conjugates (2, 2′) as well as glutamine conjugates (1, 1′), but glutamine conjugates are the major compounds in *Ag. ipsilon,* while glutamic acid conjugates are dominant in *P. flavescens* and *Ac. styx*. Third, as seen in Fig. 1A, the linolenic/linoleic acid ratios in FACs are parallel to those corresponding free fatty acids as expected if the ratio simply depended on dietary fatty acid compositions rather than on an enzymatic substrate preference (Aboshi et al. 2007).

Table 1 shows the FAC components found in 29 lepidopteran species, including 10 species in which no FACs were found. Clearly, FACs are not specific to a certain lepidopteran group but synthesized by widely different families. As expected, closely related species in the same family tended to have the same or very similar FAC patterns. Interestingly, glutamine conjugates (1, 1′) were found commonly in all these 19 species. Based on this, all FAC patterns were classified into 4 types: ① glutamine only (Fig. 1Aa), ② addition of glutamic acid (b), ③ addition of hydroxylation (c, d), ④ addition of glutamic acid and hydroxylation (e, f) (Table 1).

Discussion

In this investigation, we were interested mainly in each species' ability to synthesize various FAC compounds regardless of diet. Considering the fact that linolenic acid-FACs are more active as plant volatile elicitors than linoleic acid-FACs, this ratio may have ecologically important

Table 1 Fatty Amino Acid Conjugate (FAC) components found in lepidopteran species

		FAC components				FAC
		1, 1′	2, 2′	3, 3′	4, 4′	Type
Papilionidae	*Atrophaneura alcinous*					–
Notodontidae	*Phalera flavescens*	+	+			②
Lymantriidae	*Lymantria dispar japonica*	+		+		③
Arctiidae	*Hyphantria cunea*					–
Noctuidae	*Arcte coerulea*					–
	Anadevidia peponis	+		+		③
	Xanthodes transversa	+		+		③
	Helicoverpa armigera	+		+		③
	Mythimna separata	+		+		③
	Spodoptera litura	+		+		③
	Agrotis ipsilon	+	+	+	+	④
Saturniidae	*Samia cynthia pryeri*	+		+		③
	Samia cynthia ricini	+		+		③
Sphingidae	*Clanis bilineata tsingtauica*	+	+			②
	Agrius convolvuli	+		+		③
	Acherontia styx	+	+	+	+	④
	Manduca sexta	+	+	+	+	④
	Cephonodes hylas	+		+		③
	Theretra oldenlandiae					–
Bombycidae	*Bombyx mori*					–
Lasiocampoidea	*Malacosoma americanum*					–
Pyralidae	*Locastra muscosalis*	+				①
Crambidae	*Notarcha derogata*					–
	Pyrausta panopealis					–
Limacodidae	*Parasa lepida lepida*					–
Zygaenidae	*Pryeria sinica*					–
Tortricidae	*Epiphyas postvittana*	+	+			②
Cossidae	*Cossus insularis*	+	+			②
Gelechiidae	*Brachmia triannulella*	+				①

meaning for each species (De Moraes and Mescher 2004). However, we found no indication that the ratio of linolenic/linoleic acid in insect FACs differed from the ratio of the free fatty acids in the gut contents. This result suggests that there is no appreciable bias for fatty acid selection during FAC synthesis. Consequently, we focused this investigation on the difference between glutamine and glutamic acid and the hydroxylation status, rather than the fatty acids. No species have glutamic acid-FACs (2, 2′) without glutamine-FACs, nor hydroxylated FACs (3, 3′) without glutamine-FACs. This suggests that glutamine-FACs are the evolutionarily older FACs, and that the presence of glutamic acid conjugates, as well as hydroxylated acyl conjugates, are two independent indicators of FAC diversification. For the hydroxylation, we found clear differences between species, but it is worth noting that the ratio of hydroxylation can be variable even within a species. Previously, we reported hydroxylated FACs (3, 3′) as the major FACs in *S. litura* (Mori et al. 2003), but recently we found that *S. litura* larval gut contents have more *N*-linolenoyl-L-glutamine (1) than volicitin (3) (Yoshinaga et al. 2005). Even within an individual larva feeding on the same diet we have seen variation depending on the time course after the meal (data not shown). We also have noticed that a high abundance of dietary lipid increased the relative abundance of hydroxylated compounds in *M. sexta* (data not shown). Thus, the hydroxylation/non-hydroxylation ratio might be, at least partially, diet related. Since glutamine conjugates (1, 1′) are precursors of hydroxylated FACs (3, 3′) (Yoshinaga et al. 2005), the composition also will be influenced by the kinetics of 3 enzymes: conjugase, hydrolase, and hydroxylase. The glutamine/glutamic acid ratio appears to be more stable. In this investigation, *P. flavescens* and *Ac. styx*, had the same FAC proportions as *M. sexta*, with substantial amounts of glutamic acid-FACs, and only minor glutamine-FACs peaks. At least for *M. sexta*, an insect with which we have worked for several years, this ratio has been consistent and independent of the diet. We have no idea of the decisive factor for this, but it indicates a critical regulation of the proportions of these two types of FAC compounds.

The data we present here provide a broad base of information about FAC diversification in lepidopteran caterpillars. Figure 2 shows the distribution on the lepidopteran family tree (based on the Tree of Life web project database: http://tolweb.org/tree/) of the four FAC types from the 29 lepidopteran species described in this paper and nine other species studied earlier: three Geometridae species (Pohnert et al. 1999); *S. frugiperda* (③), *S. littoralis* (④) (Pohnert et al. 1999); *Heliothis virescens* (③), *Helicoverpa zea* (③) (Mori et al. 2001); *Helio. subflexa* (③) (De Moraes and Mescher 2004) in Noctuidae; *M. quinquemaculata* (④) in Sphingidae (Halitschke et al. 2001). All FAC-containing species had glutamine-based FACs in common but, surprisingly, only two species were of group ①: *L. muscosalis* and *B. triannulella*. The fact that *B. triannulella* is evolutionarily the most ancestral species among these FAC-containing species supports our hypothesis that glutamine conjugates are the evolutionarily older FACs, although more data for ancestral species are essential to corroborate this conclusion. Equally surprising, group ②, characterized by glutamic acid conjugates, can be seen in relatively primitive lepidopteran families such as Cossidae and Tortricidae. In contrast, group ③ and ④, which include hydroxylated FACs, are limited to Macrolepidoptera, which is considered to be the most advanced lepidopteran group. These species typically are characterized by their relatively large body size. However, since there are some large-sized species that do not synthesize FACs at all, these compounds can not be correlated simply with size and weight. Alternatively, species in group ③ and ④ may be associated with a broad range of host plants. Li et al. (2003) suggested that host plant diversity can be related directly to P450 activity and inversely related to substrate specificity. It is, therefore, likely that a cytochrome P450 enzyme is responsible for the FAC hydroxylation as represented by volicitin (Ishikawa et al. 2009).

Possibly, there is an evolutionary interplay between a plant's ability to detect and respond to different FACs and the insect's dependence on these same compounds for maximized nitrogen assimilation. We have shown that glutamine-FACs are involved in this process, but the function of, and even the biosynthesis of, glutamic acid conjugates still remain unknown. In this study, glutamic acid conjugates were not limited to *M. sexta* or *M. quinquemaculata,* but were found also in relatively primitive lepidopteran families. Furthermore, we have already reported FACs in the gut content of crickets and larval fruit flies in a pattern similar to that of *M. sexta* or *Ar. styx* (Yoshinaga et al. 2007), in which glutamic acid conjugates are dominant, with only trace amounts of glutamine conjugates. It appears that glutamine, as well as glutamic acid FACs, play important roles in insects, but we do not know if the role is the same in all these different insect species.

Despite the apparent physiological benefit of FACs, we also found 10 lepidopteran species where the gut content did not contain detectable amounts of any FACs. If the main function for FACs is to maximize nitrogen uptake and subsequently maximize growth rate, then we would expect that these FAC-free insects all should be characterized by long developmental times, but this is not the case. *Bombyx mori* and *Ar. coerulea* grow fast and achieve large body size, while Cossidae species (which make glutamic acid conjugates) grow slowly, boring into the trunk of salixes or apples, and require years before pupation. Curiously, there is a tendency for FAC-free caterpillars to have specific defensive strategies: *Hyphantria cunera* and *Malacosoma americanum* are gregarious on a host tree and make nests. Two Crambidae species also make nests by using leaves to wrap themselves.

Fig. 2 FAC-pattern classification and the phylogenetic relationship of Lepidoptera. [The phylogenetic tree is based on the Tree of Life Web Project. 2003. Lepidoptera. Moths and Butterflies. Version 01 January 2003 (temporary). http://tolweb.org/Lepidoptera/8231/2003.01.01 in The Tree of Life Web Project, http://tolweb.org/.] ① gutamine conjugates only; ② glutamine and glutamic acid-type FACs; ③ hydroxylated glutamine-type FACs; ④ hydroxylated glutamine and glutamic acid type FACs; O no FACs detected

Atrophaneura alcinous, *Parasa lepida lepida*, and *Pryeria sinica* are all notoriously toxic. *Pryeria sinica* also has an escape strategy that involves releasing thread to hang down from a tree branch. *Theretra oldenlandiae* has a threatening bull's-eye pattern that mimics snakes. *Arcte coerulea*, which was the only FAC-free species found in the Noctuidae so far, is famous for its characteristic threatening behavior, vigorously shaking its upper body and spitting slimy gut contents. Only domesticated silkworms have no such means of protection, but they no longer live in nature. In contrast, most of the FAC-containing species show no direct defensive reactions, with a few exceptions as shown in the case of *E. postvittana,* which also uses thread to escape from parasitoids (Suckling et al. 2001). Noctuidae and Sphingidae

species seem to especially focus on feeding and sleeping to grow faster, which characterizes them as serious pests (Fig. 2).

In summary: in this investigation we found that volicitin-related compounds are more commonly synthesized in lepidopteran larvae than was previously known but also that not all Lepidoptera utilize FACs. We were able to classify the FAC pattern into 4 groups: 1, glutamine conjugates only; 2, glutamic acid variation; 3, hydroxylated glutamine-type FACs; and 4, hydroxylated glutamine and glutamic acid type FACs. All FAC-containing species had *N*-linolenoyl-L-glutamine and/or *N*-linoleoyl-L-glutamine, which might be evolutionarily older FACs. Although the data provided here are biased toward noctuid and sphingid species, a comparison of the diversity of FACs with lepidopteran phylogeny indicates that glutamic acid conjugates can be synthesized by relatively primitive species, while hydroxylation of fatty acids is mostly limited to larger and more developed macrolepidopteran species.

This paper has highlighted the need to intensify our studies of FACs in Lepidoptera as well as other insects in order to obtain a new perspective and understanding of the multifaceted functions of FACs in the insect world.

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RAPID COMMUNICATION

Rapid Communication: Experimental Evidence that Juvenile Pelagic Jacks (Carangidae) Respond Behaviorally to DMSP

Jennifer L. DeBose · Gabrielle A. Nevitt · Andrew H. Dittman

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Abstract Dimethylsulfoniopropionate (DMSP) is produced by marine algae and released during foraging activity by zooplankton and fish. Pelagic fishes depend on patchily distributed foraging opportunities, and DMSP may be an important signaling molecule for these events. We have previously shown that the abundance of carangid jacks is positively associated with elevated DMSP levels over coral reefs in the Gulf of Mexico, suggesting that these fishes may use spatial and temporal variation in DMSP to locate foraging opportunities. Here, we extend this work by demonstrating that juveniles of two species of pelagic jack, crevalle jack, *Caranx hippos*, and bluefin trevally, *C. melampygus*, detect and respond to DMSP in a flow-through tank in the laboratory. Juveniles of these species showed elevated swimming activity in response to ecologically relevant concentrations of DMSP (10^{-9} M). These results provide further evidence that this chemical may serve as a chemosensory cue for carangid species.

Keywords *Caranx hippos* · *Caranx melampygus* · Chemical ecology · Dimethylsulfoniopropionate (DMSP) · Foraging behavior · Pelagic fish · Sensory ecology

J. L. DeBose · G. A. Nevitt
Department of Neurobiology, Physiology and Behavior, University of California, Davis, CA, USA

J. L. DeBose (✉)
Currently at the Smithsonian Marine Station, Fort Pierce, FL, USA
e-mail: debosej@si.edu

A. H. Dittman
NOAA Fisheries–Northwest Fisheries Science Center, Seattle, WA, USA

Introduction

Dimethylsulfoniopropionate (DMSP) is a soluble compound produced by marine algae and released during planktivorous foraging events (reviewed by Simo 2004). High DMSP concentrations are recorded in seawater around areas of high primary productivity and foraging activity, such as frontal zones or seamounts where phytoplankton tend to aggregate. Foraging aggregations often are characterized by large numbers of plankton, including phytoplankton and zooplankton, surrounded by planktivorous fishes, which are then corralled by predatory fish. Planktivorous fishes aggregate in response to DMSP (DeBose et al. 2008), and increased numbers of predatory jacks are correlated with increases in combined DMSP and dimethylsulfide (DMS) over coral reefs (DeBose and Nevitt 2007). Laboratory studies further indicated that high concentrations of DMSP (10^{-2}–10^{-3} M) in food increased striking frequency and weight gain in two species of saltwater fish (Nakajima et al. 1990). Predatory fishes generally are assumed to rely on visual cues. Thus, we wanted to test whether or not they can detect ecologically relevant concentrations of dissolved DMSP, with the goal of understanding the use of chemical cues in driving foraging events, and ultimately, trophic cascades. Specifically, the aim of the current study was to use a simple behavioral assay to verify whether jacks can detect DMSP.

Methods and Materials

Experimental Animals We tested behavioral responses of juveniles of two species of jack, crevalle jack, *Caranx hippos*, and bluefin trevally, *C. melampygus*, to DMSP by using a flow-through Plexiglas tank (Hara 2006). Crevalle jack are reef-associated pelagic, schooling fish from the

Atlantic Ocean, and bluefin trevally are a commercially important Pacific Ocean species. Adult crevalle jack and bluefin trevally feed mostly on fish and crustaceans (Lieske and Myers 2001), although at the juvenile stage we tested, both species feed on plankton.

Juvenile bluefin trevally [~2-mo-old; size range: 45.65–82.15 mm total length (TL)] were hatched and originally reared at the Oceanic Institute (Waimanalo, HI, USA), and then transported to the Northwest Fisheries Science Center, NOAA-Fisheries, Mukilteo Research Station, Mukilteo, WA, USA, for this study. Fish were housed in circular tanks (1.3–2.0 m diam.) supplied with sand-filtered flow-through seawater (21–23°C). Juvenile crevalle (~1-2-mo-old; 57.30–72.80 mm TL), associated with Sargassum mats, *Sargassum natans*, were collected near Galveston, TX, USA, and held in rectangular seawater tanks (1.8×0.8×0.7 m) at the Moody Gardens Aquarium Quarantine facility in Galveston, TX, USA.

Flow Tank Specifications All experiments were conducted in a 51 l flow-through tank (157×30×14 cm) with a flow rate of 5–7 l/min and a water depth of 10.8 cm. Water flow was smoothed by a flow-straightener (FS) made of straws (length: 9 cm; diam: 1.1 cm) glued together to form a 'honeycomb' of channels, located 10 cm downstream from the intake. Stimuli were introduced into the tank upstream of the FS, where an airstone thoroughly mixed the injected stimuli prior to flowing into the experimental arena of the tank. An airstone is a piece of porous stone whose purpose is to gradually diffuse oxygen into aquaria, thus eliminating the noise and large bubbles of conventional air filtration systems. The entire tank was housed under black plastic to reduce visual cues, but the tank was illuminated by infrared lights to allow recording of fish movements with an infrared camera (Sony DCR-TRV-17).

Testing Protocol Behavioral trials for DMSP responses were conducted from June to September 2006 with a total of 10 crevalle and 8 bluefin trevally. Prior to each trial, a single fish was fasted for 24 h and then placed in the downcurrent end of the flow table for an 8 h acclimation period. After acclimation, fish movement patterns along the length of the flow table (see *Behavioral Scoring and Analysis* below) were recorded for 10 min immediately prior to stimulus release (DMSP or ASW). Stimulus water was metered into the flow table for 1 min, and behavior was recorded for an additional 10 min. After a 30 min recovery period, the process was repeated with the second stimulus. In pilot studies, 30 min was sufficient for the experimental stimulus to vacate the tank and fish to resume background levels of activity. Fish were used in only one trial, experiencing both stimuli. The order of stimulus delivery was switched every trial.

Chemical Stimuli Two stimuli were tested during each trial: 10^{-9} M DMSP and artificial saltwater (ASW, Instant Ocean®, Mentor, OH, USA). ASW or DMSP (1.0×10^{-7} M) were delivered via intravenous medical bags positioned outside the black plastic blind. Stimuli were injected at a rate of 60 ml/min into the pre-FS reservoir for 1 min. Complete mixing, confirmed by dye release studies (Bright Dyes®, Miamisburg, OH, USA), yielded a final concentration of 1.0×10^{-9} M DMSP in the reservoir. This concentration is within the natural range of DMSP measured over coral reefs (Jones et al. 2007: 0.43–27 nM). DMSP solutions were prepared in artificial saltwater (ASW) matched to background salinity (±1 ppt) with a YSI meter (MODEL 85, YSI Inc., Yellow Springs, OH, USA). Working DMSP solutions were prepared immediately before each trial from stock solutions of DMSP (10^{-3} M) (Chemische Laboratoria, Rijksuniversiteit Groningen, Groningen, The Netherlands) stored at −20°C. All solutions were equilibrated to background water temperature (22.0±0.10°C for bluefin trevally, 26.8±0.11°C for crevalle jack) before each experimental trial.

Behavioral Scoring and Analysis Recorded trials were scored with J-Watcher software (V1.0, Macquarie Univer-

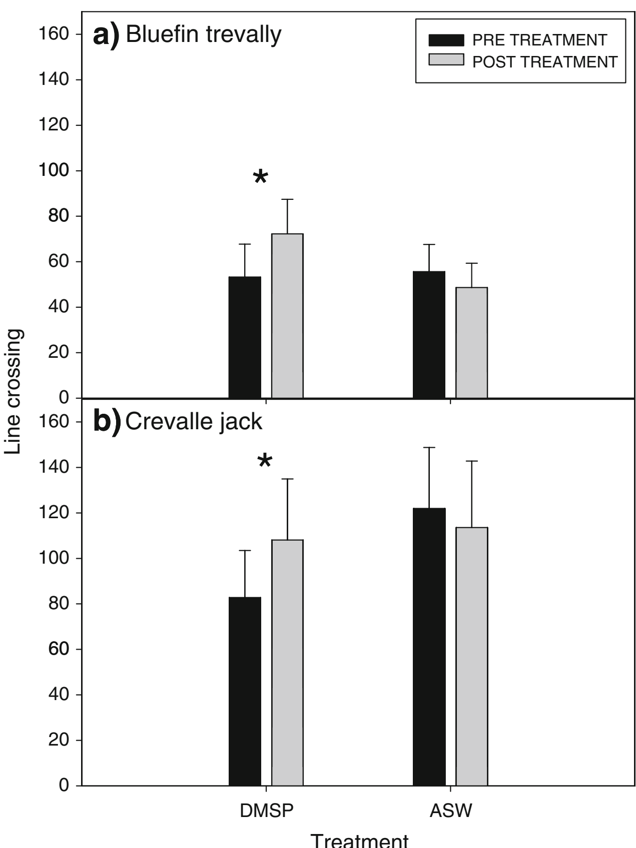

Fig. 1 Bluefin trevally (**a**) and crevalle jack behavioral response (**b**) to artificial salt water (ASW) and 10^{-9} M DMSP. Asterisk denotes significant difference in line crossing behavior between time periods associated with pre- (black bar) and post- (gray bar) treatment stimuli

sity and UCLA) by an observer blind to the treatment. The responses before and after stimuli exposure were defined as the number of lines crossed 10 min before and 10 min after stimuli presentation. The number of lines crossed was determined by using 10 cm grid lines on the bottom of the tank. Line crossings were defined by the entire head of the fish crossing the grid line. Data (before/after responses) were analyzed by paired *t*-test ($\alpha=0.05$) with before-stimulus responses as individual controls.

Results and Discussion

Both bluefin trevally and crevalle jack increased their number of line crosses in response to 10^{-9} M DMSP but not ASW (bluefin trevally: Fig. 1a, DMSP: $P<0.01$; ASW: $P=0.28$, $N=8$; crevalle: Fig. 1b, DMSP: $P<0.05$; ASW: $P=0.71$, $N=10$). Pre-stimulus activity levels were similar for both DMSP and ASW (bluefin trevally: $P=0.901$; crevalle: $P=0.353$), with fish moving throughout the test arena during the pre-stimulus period. Results indicate that *Caranx* spp. detected 10^{-9} M DMSP in a laboratory setting.

The ability to detect DMSP could be advantageous in finding food and/or habitat under laboratory and natural conditions; by increasing activity in the presence of this compound, jacks could use DMSP to locate foraging opportunities. DMSP was present in the commercial feeds fed to these jacks ($2.7–3.1\times10^{-7}$ M (extract); DeBose 2008) and wild crevalle jack used in these studies were captured under Sargassum mats, which also produce DMSP (Broadbent et al. 2002). In previous studies, we measured concentrations of total DMSP over coral reefs in the Gulf of Mexico and Caribbean (DMSPt, dissolved + particulate: $10^{-8}–10^{-9}$ M; DeBose and Nevitt 2007; DeBose 2008). Dissolved DMSP concentrations in the waters over the Great Barrier Reef are reported to be in the $10^{-7}–10^{-9}$ M range (Broadbent et al. 2002; Jones et al. 2007).

Our results provide evidence that two species of carangid jack detect ecologically relevant concentrations of DMSP, thus supporting the hypothesis that this compound, released during feeding interactions, might be an effective sensory cue for these fish in their natural environments. Overall, however, we are only beginning to understand the role of DMSP as a signal molecule in marine ecosystems. This study, combined with previous work (Nakajima et al. 1990; DeBose and Nevitt 2007; DeBose et al. 2008), provides intriguing evidence that pelagic fishes detect DMSP and may use this algal-produced compound as a foraging or habitat-associated cue in their natural environment. Reef-associated jacks must find habitat and foraging opportunities as they range over the reef and beyond into blue water, and most likely they use a suite of chemical and other sensory cues for orientation. By cueing to the release of DMSP associated with foraging activities of zooplankton and planktivorous fishes (Hill and Dacey 2006; DeBose et al. 2008), pelagic jacks could use this chemical to locate transient and localized foraging opportunities. The role of chemical cues in the behavior of pelagic fishes such as jacks is only beginning to be recognized. Although this study implicates DMSP as a compound of interest, others (e.g., amino acids, amines, etc.; Hara 2006) also are known feeding stimuli. Further work is needed to fully understand the multitude of ways that these fish may use chemical compounds to forage and navigate in their marine environment.

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Baicalin Released from *Scutellaria baicalensis* Induces Autotoxicity and Promotes Soilborn Pathogens

Shanshan Zhang · Yili Jin · Wenjie Zhu · Jianjun Tang · Shuijin Hu · Tongshui Zhou · Xin Chen

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Abstract Experiments were conducted to determine whether allelochemicals released by the important medicinal plant *Scutellaria baicalensis* Georgi help to explain why *S. baicalensis* performs poorly when continuously cropped. Based on high performance liquid chromatography, the concentration of baicalin (the major compound released by *S. baicalensis* roots) in the soil where *S. baicalensi* had been grown for 3 years was 0.97 μg.g^{-1}. Both the crude extracts from *S. baicalensis* roots and purified baicalin at 0.97 μg.g^{-1} increased the mortality of *S. baicalensis* seedlings in an autotoxicity test. This concentration stimulated the growth of two soilborne pathogens (*Pythium ultimum* and *Rhizoctonia solani*) on agar, and their growth and pathogenic activity in sand. Seedling mortality and damping-off caused by both pathogens were greater in sand where *S. canadensis* had previously grown than in sand where it had not previously grown. Mortality and damping-off of *S. baicalensis* seedlings also were significantly higher in soil collected from an *S. baicalensis* field than in soil collected from a *Nicotiana tabacum* L. field. The results are consistent with the hypothesis that allelochemicals released by *S. baicalensis* negatively affect *S. baicalensis* directly by inducing autotoxicity and indirectly by increasing pathogen activity in the soil.

Keywords *Scutellaria baicalensis* Georgi · Autotoxicity · Allelochemicals · Baicalin · Soilborn pathogen

S. Zhang · Y. Jin · W. Zhu · J. Tang · X. Chen (✉)
College of Life Sciences, Zijingang Campus, Zhejiang University,
No. 358 Zijinghua Road,
Hangzhou 310058, China
e-mail: chen-tang@zju.edu.cn

T. Zhou
College of Life Sciences, Fudan University,
Shanghai 200433, China

S. Hu
Department of Plant Pathology, North Carolina State University,
Raleigh, NC 27695, USA

Introduction

Autotoxicity, a form of intraspecific allelopathy, is defined as a process in which a plant species releases toxic chemicals that inhibit or delay germination and growth of other plants of the same species (Miller 1996; Singh et al. 1999). Autotoxicity occurs in natural ecosystems and is considered to have ecological significances in regulating plant population density (Canals et al. 2005; Sinkkonen 2007). Autotoxicity also is believed to be an obstacle to continuous cropping by causing regeneration failure in crop growth and yield in managed ecosystems (Chou and Lin 1976; Rice 1984; Batish et al. 2001; Hao et al. 2007; Liu et al. 2007; Fernandez et al. 2008).

Increasingly, studies have shown that root exudates can influence plant growth not only directly by acting as autotoxins (Mahall and Callaway 1992; Yu et al. 2000; Pramanik et al. 2001; Kong et al 2008; He et al. 2009) but also indirectly by affecting the soil microbial community (Christie et al. 1974; O'Donnell et al. 2001; Matamala et al. 2003; Bais et al. 2004; Yao and Allen 2006; Wu et al. 2009). Root exudates may affect soil microorganisms, which in turn influence the plant by initiating and manipulating biological and physical interactions between roots and other soil organisms (Kandeler et al. 2002; Bais et al. 2004); root exudates can, thus, affect the interactions between plants and soil microorganisms (Miethling et al.

2000). Although several studies have demonstrated that root exudates enhance soilborne pathogens by providing substrates for saprophytic growth (Nicol et al. 2003; Manici et al. 2004; Bonanomi et al. 2007), whether and how allelopathy influences soil microorganism communities to produce positive or negative feedback to the plants still is incompletely understood.

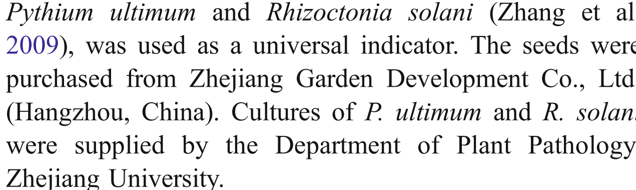
The dried root of the flowering plant *Scutellaria baicalensis* Georgi is used as a drug in Chinese traditional medicine (Yamamoto 1991), and the plant is widely grown as a crop in Eastern Asia (Qu et al. 2007). As a drug, the dry root of *S. baicalensis* is called Radix Scutellariae, and is officially listed in the Chinese Pharmacopeia (Kovacs et al. 2004). *Scutellaria baicalensis* contains large amounts of flavones, and the flavone baicalin (7-glucuronic acid, 5, 6-dihydroxy-flavone), which is one of the major effective components in dried roots of *S. baicalensis*, accumulates in the rhizosphere soil (Bochoráková et al. 2003). In the production of Radix Scutellariae, *S. baicalensis* suffers from a serious replant problem in that substantial yield reduction occurs after 2 years or 3 years of continuous cropping. Increasing evidence indicates that *S. baicalensis* suffers from diseases caused by the pathogens *Pythium ultimum* and *Rhizoctonia* spp. during continuous cropping (Benizri et al. 2005; Chang et al. 2007), suggesting that an increase in numbers of soilborne pathogens contributes to the replant problem.

We hypothesized that allelochemicals released by *S. baicalensis* negatively affect the source plant by inducing autotoxicity and by increasing pathogens in the soil. To test this hypothesis, we first quantified the concentration of baicalin in a field where *S. baicalensis* had been grown for 3 years. Based on that concentration of baicalin, we then conducted a series of experiments to determine: (1) whether baicalin directly reduces germination and growth of *S. baicalensis* seedlings; (2) whether baicalin promotes soilborne pathogens and thereby indirectly reduces germination and growth of *S. baicalensis* seedlings due to disease incidence.

Methods and Materials

Plants, Pathogens, and Chemicals Seeds of *Scutellaria baicalensis* Georgi were collected from the field site (see next section). For use as standards for quantitative analysis of flavonoids, high purity (>99%) baicalin, wogonoside, baicalein, and wogonin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Crude extracts of Radix Scutellariae (containing 16.7±1.3% baicalin) also were provided by the same institute.

Tomato (*Lycopersicon esculentum* Mill) variety Qianhong No.1, which is susceptible to the soilborne pathogens


Pythium ultimum and *Rhizoctonia solani* (Zhang et al. 2009), was used as a universal indicator. The seeds were purchased from Zhejiang Garden Development Co., Ltd. (Hangzhou, China). Cultures of *P. ultimum* and *R. solani* were supplied by the Department of Plant Pathology, Zhejiang University.

Study Site and Sampling The study was conducted in Wolong Town, Linqu County, Shandong Province, China (36°27′N, 118°38′E), where *S. baicalensis* is the major economic crop and has been grown by farmers for years. *Scutellaria baicalensis* had been grown on the study site, which is 5 ha, for the preceding 3 yr. The site is in the south temperate zone, and has a mean annual temperature of 12.4°C and a mean annual rainfall of 710 mm. The soil from the *S. baicalensis* field is a sandy loam with a pH of 6.89 (2.5:1, KCl aqueous solution:soil), an organic matter content of 17.17 $g{\cdot}kg^{-1}$, a total nitrogen content of 0.96 $g{\cdot}kg^{-1}$, an extractable phosphorus content of 60.90 $mg{\cdot}kg^{-1}$, and an extractable potassium content of 292.36 $mg{\cdot}kg^{-1}$. The field where *Nicotiana tabacum* had been grown had a pH of 7.07 (2.5:1, KCl aqueous solution: soil), an organic matter content of 20.15 $g{\cdot}kg^{-1}$, a total nitrogen content of 1.16 $g{\cdot}kg^{-1}$, an extractable phosphorus content of 31.55 $mg{\cdot}kg^{-1}$, and an extractable potassium content of 207.95 $mg{\cdot}kg^{-1}$.

For determination of the baicalin concentration in the study site soil, 30 soil cores (10 cm diam and 15 cm deep) in five sampling areas (2×2 m) were separately collected from the rhizospheres of *S. baicalensis* plants in November, 2008. In each of five sampling areas, six soil cores from one area were collected and combined to give one sample per area. The five soil samples were subjected immediately to slow air-drying, thus mimicking conditions that would occur during natural drying. Any obvious plant or animal debris was removed.

Quantification of Baicalin in the Soil For each 300-mg subsample per soil sample, baicalin was extracted with 30 ml of 70% MeOH for 60 min in an ultrasonic bath; the extraction procedure was repeated × 3 for each subsample. The methanolic extracts for each subsample were combined and evaporated to dryness *in vacuo*. The viscous residue was dissolved in 100 ml of MeOH and passed through a 0.45-μm nylon filter (Millex-HN, Ireland) before injection for HPLC analysis.

Standard curves were calibrated by using the linear least-squares regression equations derived from the peak areas of five injections. HPLC analysis was performed on an Aglient 1100 series system with a G1311A Quatpump, a G1314A variable wavelength detector, and a Chemstation (Rev. A. 10. 02) (Agilent Technologies, Waldbronn, Germany). The mobile phase was a gradient of (A) MeOH

and (B) H_2O-H_3PO_4 (99.9:0.1, v/v). The gradient was as follows: 0–42 min, 45–69% eluent A. Flow rate was 1.0 ml/min. Sample injection volume was 20 µl. Flavonoids were separated by using a Zorbax SB-C_{18} column (4.6×250 mm i.d., 5-µm particle), with a security guard column, Phenomenex C_{18} ODS (4×3.0 mm i.d.). Absorbance was recorded at 280 nm (Su et al. 2008).

Experiment 1: Germination of S. baicalensis Seedlings as Affected By Baicalin and Crude Extracts This experiment tested the toxicity of *S. baicalensis* and baicalin in Petri dish cultures. To separate phytotoxic effects from the carbon source effect of baicalin and to test whether baicalin is the major effective allelochemical, we designed four treatments (distilled water, glucose, baicalin, and crude extract). The final concentrations of glucose, baicalin, and crude extract were 0.97 $mg \cdot l^{-1}$, 0.97 $mg \cdot l^{-1}$, and 5.81 $mg \cdot l^{-1}$, respectively. The concentration of baicalin used in the experiment was based on the concentration found in the rhizosphere soil of *S. baicalensis* at the field site. The concentration of crude extract was based on the concentration of baicalin in the crude extract, which was 16.7±1.3%.

Seeds of *S. baicalensis* were surface-sterilized by soaking the seeds in 70% ethanol for 2.5 min, followed by four rinses in sterilized distilled water. They were then soaked in 2.5% sodium hypochlorite solution for 15 min, followed by five rinses in sterilized distilled water. Then 50 surface-sterilized seeds of *S. baicalensis* were dispersed on Whatman No. 40 filter paper in 9 cm Petri dishes (experimental unit), and 6 ml of each solution (distilled water, baicalin, glucose, or crude extract) were added. The Petri dishes were sealed with parafilm to prevent moisture loss and contamination. Seeds were germinated in incubators (Safe Experimental Instrument Company, Haishu, Ningbo, China) with a photoperiod of 16 h light and 8 h dark at 28°C (day) and 18°C (night) and 90% relatively humidity.

Seed germination was defined as when the radicle had extended 1 mm beyond the seed coat 5 d after sowing (Steinmaus et al. 2000). Recording ceased when there was no change in seed germination counts for more than 3 d.

The response index (RI), which indicates autotoxicity (Willamson 1988), was calculated by using germination rate. $RI = 1 - C/T$, where T is germination rate in treatments and C is germination rate of the control. RI values range from +1 to −1, RI>0 means stimulation, RI<0 means inhibition.

RI was analyzed with a one-way ANOVA by using the general linear model procedure in SPSS (V.10.0). LSD test at the 5% significance level was used to the compare the means.

Experiment 2: Growth of Pathogens in Agar Culture as Affected By Baicalin and Crude Extracts This experiment tested the effects of allelochemicals from *S. baicalensis* on the growth of pathogens in Petri dish cultures. We also designed four treatments (distilled water, glucose, baicalin, and crude extract) in this experiment as in experiment 1.

Agar media were prepared by mixing 9 ml of sterilized potato dextrose agar (PDA) at 60°C with 1 ml of each solution (distilled water, baicalin, glucose, and crude extract); these 10 ml were then added to each 9-cm diam Petri dish. Thus, the final concentrations in the Petri dishes were in accordance with the concentration of baicalin detected in the field soil. Before they were combined with agar, the solutions were sterilized by passing them through a 0.22-µm millipore filter.

Prepared inocula (5 mm diam agar plugs) were placed in the center of the Petri dishes, and the cultures were kept in a growth chamber (Safe Experimental Instrument Company, Haishu, Ningbo, China) in the dark at 28°C and 90% relatively humidity. The area of each colony was measured every 6 hs for 4 d (Fang 1998). The effect of the glucose, baicalin, and crude extract on growth of the pathogens was determined by calculating a PR (promotion rate): $PR = (At - Ac)/Ac$, in which At is the area of the colony treated with glucose, baicalin, or crude extract, and Ac is the area of the colony treated with distilled water.

There were six replicate Petri dishes for each solution. Because the main comparisons in this experiment were among the solution types within the *P. ultimum* or *R. solani* treatments, PR were analyzed with a one-way ANOVA for each pathogen. When an ANOVA was significant, an LSD test at the 5% significance level was used to the compare the means. SPSS V.10.0 was used for all statistical analyses in this study.

Experiment 3: Activity of Pathogens and Seedlings of S. baicalensis in Sand Culture as Affected By Baicalin and Crude Extract This experiment tested toxicity of *S. baicalensis* and the effects of baicalin and crude extract on activity of *P. ultimum* and *R. solani*. Both tomato (Rafin and Tirilly 1995; Yangui et al. 2008) and *S. baicalensis* were used to indicate pathogen activity.

A factorial experiment was designed with four solutions (distilled water, glucose, baicalin, and crude extract as in experiment 1) and with three pathogen treatments (no pathogen, *P. ultimum*, and *R. solani*). Each combination of solution and pathogen was applied to a 50-cm^3 plastic pot that contained sterilized fine sand (see next paragraph). There were six replicate pots per combination, giving a total of 72 pots.

Fine sand (0.45–1 mm diam) was soaked in a solution of NaClO for 24 h, washed with tap water, and sterilized at 120°C for 8 h in an oven. Each pot was filled with 150 g of sterilized sand. The solutions were diluted with distilled water as in experiment 1. Inoculum of *P. ultimum* and *R. solani* was prepared by growing the pathogens on cooked rice grains (Zhang et al. 2009). Immediately after a solution

was added to a pot, four rice grains colonized by *P. ultimum* or *R. solani* were added to the pot; the rice grains were buried 2 cm in the sand. For the pathogen-free treatment, pots received four rice grains treated in the same way as the colonized grains except that they were not exposed to the pathogens. After 1 wk at 22°C (to allow the pathogens to grow in the sand), 20 tomato or *S. baicalensis* seeds were planted in each pot. All pots were kept in a growth chamber with a photoperiod of 16 h light and 8 h dark at 22°C (day) and 18°C (night) and 90% relatively humidity. After seeds were sown, Hoagland's nutrient solution was added every 2 d to maintain normal seedling growth.

Numbers of germinated seeds and of damped-off seedlings were recorded daily for 21 d and 28 d. The mortality rate was calculated as follows: mortality rate (%)=(1−number of living seedlings/number of sown seeds)×100. The damping-off rate was calculated as follows: damping-off rate (%)=(number of damped-off seedlings/number of germinated seeds)×100 (Zhang et al. 2009).

The effects of the solution and pathogen on mortality rate and damping-off rate of tomato and *S. baicalensis* were determined with a two-way ANOVA (solution types and pathogens as factors). An LSD test at the 5% significance level was used to compare all treatment means for each combination of pathogen and plant species.

Experiment 4: Activity of Pathogens and S. baicalensis in Sand in Which S. baicalensis Had Grown Experiment 4 tested how sand in which *S. baicalensis* had grown affected seedlings and the pathogenic activity of *P. ultimum* and *R. solani*. A factorial experiment was designed with two plant levels (no plants and *S. baicalensis*) and three pathogens (no pathogen, *P. ultimum*, and *R. solani*). There were six replicate pots for each combination, giving a total of 36 pots.

The experiment was starting by growing *S. baicalensis* (four seeds per pot) or no plants in sterilized sand for 4 mo. The pots and sterilized fine sand used were the same as in experiment 2, except that each pot contained 500 g rather than 150 g of sterilized sand. Pots were placed in a growth chamber maintained at 30±1°C during the day and 25±1°C at night. The light was maintained at the photosynthetically active radiation flux (540 µmol m^{-2} s^{-1}) with a day length of 13 h. Air temperature, light density, and day length were similar to those experienced by *S. baicalensis* under the natural conditions that are optimum for its growth. Every 7 d, plants were fertilized with full-strength Hoagland's nutrient solution (Hoagland and Arnon 1950).

After 4 mo, the aboveground portion of each plant was removed, and pots were inoculated with *P. ultimum*, *R. solani*, or no pathogen using rice grains as described in experiment 2. After 1 wk (to allow the pathogens to grow into the sand). 20 tomato or *S. baicalensis* seeds, which had been surface sterilized with H_2O_2 (5%, v: v), were planted in each pot. All pots were kept in a growth chamber maintained as described for experiment 2. After seeds were sown, Hoagland's nutrient solution was added every 2 d. The experiment ended 21 d and 28 d after the seeds were added. Seed germination and seedling damping-off were quantified, and mortality rate and damping-off rate were calculated as in experiment 2.

The effect of plant and pathogen on mortality and damping-off rate of tomato and *S. baicalensis* was determined with a two-way ANOVA. Because the interaction between sand (no plant sand vs. *S. baicalensis* sand) and pathogen (no pathogen, *P. ultimum*, and *R. solani*) was significant (Table 1), an LSD test at the 5% significance levels was used to compare means for each combination of sand and pathogen treatment for each kind of plant.

Experiment 5: Seed Mortality and Seedling Damping-Off in Rhizosphere Soils from Fields Where S. baicalensis or N. tabacum Had Grown Experiment 5 tested whether *S. baicalensis* stimulates soilborne pathogens by comparing pathogenic activity in rhizosphere soils from fields where *S. baicalensis* or *N. tabacum* had grown for 3 yr.

Table 1 Significance of factors and factor interactions on mortality and damping-off based on two-way ANOVAs in experiments 3 and 4*

Experiment and variables	*d. f.*	Mortality rate		Damping-off rate	
		Scutellaria baicalensis	Tomato	*S. baicalensis*	Tomato
Experiment 3					
Solutions	3	F=63.41**	F=82.94**	F=605.24**	F = 271.36**
Pathogens	2	F=719.83**	F=1865.45**	F=19382.05**	F=10164.19**
Solutions × pathogens	6	F=7.18**	F=50.52**	F=154.03**	F=70.06**
Experiment 4					
Plants	1	F=348.06**	F=45.44**	F=1231.85**	F=127.41**
Pathogens	2	F=211.04**	F=1642.45**	F=1692.13**	F=4244.79**
Plants × pathogens	2	F=58.25**	F=6.55*	F=96.40**	F=32.92**

*Significance level: *P<0.05; **P<0.01

Soil cores were collected from the *S. baicalensis* and *N. tabacum* field sites (30 cores per site with 60-cm^3 of soil per core) and immediately transported to the laboratory. Each soil core was placed in a 50-cm^3 plastic pot. Each pot was inoculated with either *P. ultimum* or *R. solani* as described in experiment 2. After 10 d, 20 tomato seeds or and *S. baicalensis* seeds, which had been surface disinfected with H_2O_2 (5%, v:v) were planted in each pot. All pots were kept in a growth chamber with the same conditions as in experiment 2. The experiment ended 21 d and 28 d after the seeds were added. Seed germination and seedling damping-off were quantified, and mortality and damping-off rate were calculated as in experiment 2.

The effect of rhizosphere field soil (*S. baicalensis* vs. *N. tabacum*) on mortality and damping-off rate of tomato and *S. baicalensis* was determined with a one-way ANOVA. Following this analysis, we performed an independent *t*-test of mortality rate and damping-off rate of tomato and *S. baicalensis* growing in soil from the *S. baicalensis* field vs. in soil from the *N. tabacum* field.

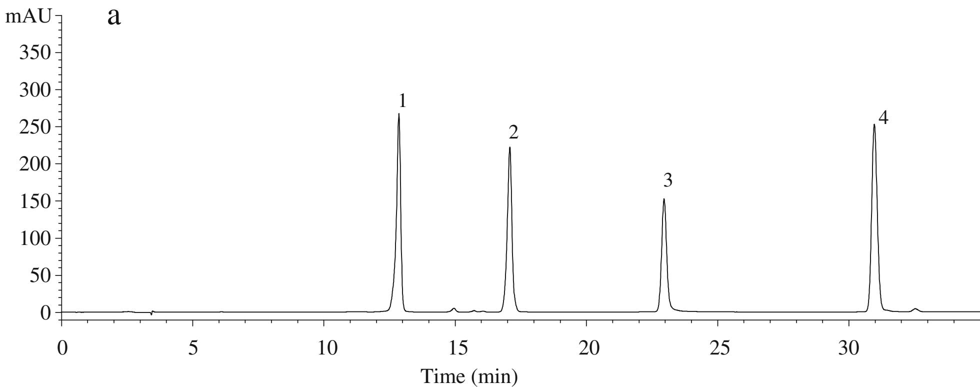
Results

Baicalin Concentration in Field Site Based on HPLC analysis, the soil extracts from the *S. baicalensis* field site contained 0.000097±0.000012% baicalin, or 0.97 μg of baicalin per g of soil (Fig. 1).

Experiment 1 Germination of S. baicalensis Seedlings as Affected By Baicalin and Crude Extracts In experiment 1, baicalin, glucose, or the crude extracts significantly impact germination of *S. baicalensis* (Fig. 2, $P<0.05$). The response index (RI) showed that both baicalin and the crude extracts inhibited the germination of *S. baicalensis*, whereas glucose stimulated the germination.

Experiment 2: Growth of Pathogens in Agar Culture as Affected By Baicalin and Crude Extract In experiment 2, the growth of both pathogens was significantly greater in agar containing baicalin, glucose, or the crude extract than in the nonamended agar distilled water. For both pathogens, the increase in growth relative to the nonamended agar was greater with baicalin and with the crude extract than with glucose (Fig. 3). The increase in growth did not differ between the baicalin and the crude extract treatments.

Experiment 3: Activity of Pathogens and S. baicalensis in Sand Culture as Affected By Baicalin and Crude Extract In experiment 3, baicalin, glucose, and the crude extract significantly affected seed mortality in sand (Table 2). When no pathogens were present, the mortality rate of both *S. baicalensis* and tomato seedlings was higher with



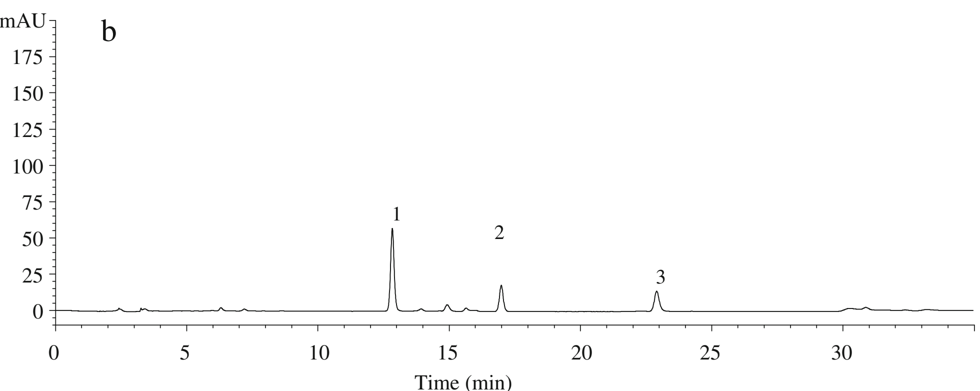


Fig. 1 Typical HPLC chromatograms of flavonoids. *a* Mixture of standards, *b* Soil samples of *Scutellaria baicalensis* on an Agilent Zorbax SB-C_{18} column (250 mm×4.6 mm i.d., 5 μm particle) and precolumn (3.0 mm×4.0 mm i.d.; Phenomenex C_{18} ODS). A gradient prepared from mixtures of MeOH and H_2O-H_3PO_4 (99.9:0.1, v/v) was used as mobile phase (0–42 min. 45–69% eluent A); the flow rate was 1.0 mL/ min and UV detection was performed at 280 nm (1 = baicalin; 2 = wogonoside; 3 = baicalein; 4 = wogonin)

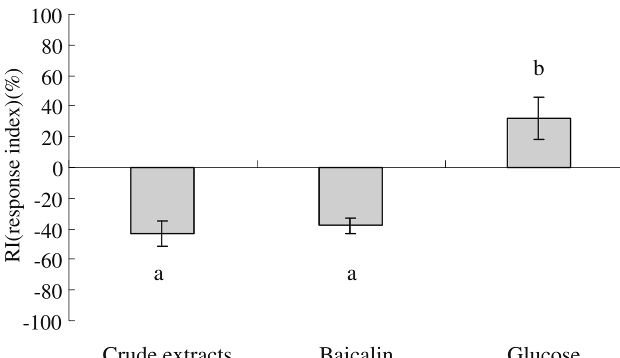


Fig. 2 Response index of germination of *Scutellaria baicalensis* affected by baicalin, crude extracts of Radix Scutellaria from *S. baicalensis*, and glucose in Petri dishes. Values indicate the response in germination relative to the control. Values are means±SE of four replicate Petri plates. For each treatment, values followed by the different letter are significantly different from the control at $P<0.05$

baicalin and crude extract than with water or glucose; in addition, *S. baicalensis* seeds incurred substantially higher mortality than tomato with baicalin and crude extract. Mortality of both plants was substantially greater with the pathogens than without, and the effect of the pathogens on mortality was enhanced by baicalin and crude extract (Table 2). There also was a tendency for glucose to enhance the mortality of *S. baicalensis* seeds but not the mortality of tomato seeds (Table 2).

No damping-off occurred in experiment 3 unless pathogens were added (Table 3). The damping-off rate caused by the pathogens was greater with baicalin and crude extract than with water and glucose, but the rate did not differ between baicalin and crude extract (Table 3).

Experiment 4: Activity of Pathogens and S. baicalensis in Sand in Which S. baicalensis Had Grown When no pathogen had been added to the sand in experiment 4, the mortality and damping-off of *S. baicalensis* seedlings but not of tomato seedlings was greater in soil previously planted with *S. baicalensis* than in soil that was not previous planted with *S. baicalensis* (Table 4). When pathogens were added, the mortality and damping-off of both *S. baicalensis* and tomato seedlings were greater in soil previously planted with *S. baicalensis* than in soil that was not previous planted with *S. baicalensis* (Table 4).

Experiment 5: Seed Mortality and Seedling Damping-Of in Rhizosphere Soils from Fields Where S. baicalensis or N. tabacum Had Grown In experiment 4, mortality rates of both *S. baicalensis* and tomato seedlings were significantly higher in the field soil collected from the rhizosphere of *S. baicalensis* than in the field soil collected from rhizosphere of *N. tabacum* (Table 5). Damping-off did not occur with tomato but damping-off of *S. baicalensis* was greater in the *S. baicalensis* soil than in the *N. tabacum* soil (Table 5).

Discussion

Experiments have shown that the roots of some plant species release allelochemicals that can accumulate in the soil and directly inhibit the same plant species (Ben-Hammouda et al. 2002; Liu et al. 2007). Our study demonstrated that baicalin (7-glucuronic acid, 5, 6-dihydroxy-flavone) released from the plant *S. baicalensis* accumulated in the soil. After *S. baicalensis* had been grown three consecutive years, the soil contained 0.97 $\mu g \cdot g^{-1}$ of baicalin (Fig. 1) measured in November when *S. baicalensis* has been harvested for 1 month. This concentration of baicalin might be lower than that before November since allelochemicals in the soil could be degraded by soil microorganisms (Inderjit 2005). However,

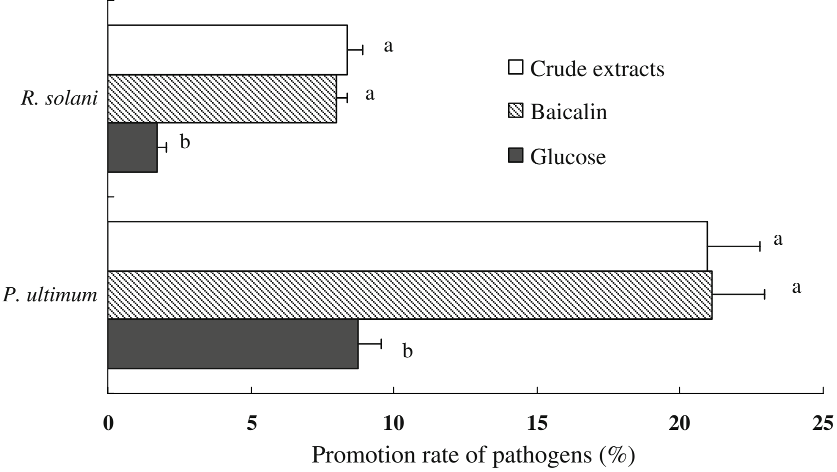


Fig. 3 Promotion rate of pathogen growth by baicalin, crude extracts of Radix Scutellaria from *Scutellaria baicalensis*, and glucose in agar culture. Values indicate the increase in growth relative to the control. Values are means±SE of six replicate Petri plates. For each pathogen, values followed by the different letter are significantly different at $P<0.05$

Table 2 Mortality of tomato and *Scutellaria baicalensis* seeds caused by pathogens (*Pythium ultimum* and *Rhizoctonia solani*) in sand culture as affected by baicalin and crude extract of Radix Scutellaria (experiment 3)[a]

Plant and treatment	Mortality of seeds (%)		
	No pathogen	*P. ultimum*	*R. solani*
S. baicalensis			
Distilled water	32.5±3.2a	76.3±2.4a	71.3±1.3a
Glucose	31.3±2.0a	85.0±3.2b	82.5±2.0b
Baicalin	47.5±4.8b	90.0±3.1bc	88.8±2.0bc
Crude extract	53.8±3.1b	93.8±4.8b	90.0±1.3b
Tomato			
Distilled water	11.3±3.1a	88.8±1.3a	85.0±0.0a
Glucose	13.8±2.4a	88.8±1.3a	86.3±2.4a
Baicalin	32.5±3.2b	96.3±1.3b	93.8±2.4b
Crude extract	31.3±3.1b	96.3±1.3b	93.8±2.4b

[a] Values are the means±SE of six replicates. For each species, values followed by the same letter within a column are not significantly different at $P<0.05$

we still used this concentration to test the baicalin effects. The reason for that is that baicalin may remain somewhat constant from November to March due to the low temperatures during this period at our study site (major production site of *S. baicalensis* in China). Qiu et al. (2004) have reported baicalin is stable in solution with a pH 6.8 and temperatures below 20°C. *Scutellaria baicalensis* usually is planted around April when baicalin in soil (having a similar concentration to that in November) may begin to have effects on germination and seedling growth directly and indirectly through affecting soilborne pathogens. As hormesis is common with antifungal compounds (Calabrese and Baldwin 2003), we also tested whether the concentrations of baicalin at lower or higher concentration than 0.97 $\mu g \cdot g^{-1}$ led to different effects (promotion at lower concentrations or inhibition at higher concentration). We measured the effects with four concentrations 0.097 $\mu g \cdot g^{-1}$, 0.97 $\mu g \cdot g^{-1}$, 5, and 10 $\mu g \cdot g^{-1}$ on pathogens growth in Petri-dish cultures. We did not find inhibition within these concentrations (data not shown). Thus, we used the baicalin concentration (0.97 $\mu g \cdot g^{-1}$) that we measured in November.

Both baicalin at 0.97 $\mu g \cdot g^{-1}$ in a pure solution and a crude extract of *S. baicalensis* roots that contained this same baicalin conentration inhibited the germination of *S. baicalensis* in both the Petri dish culture and sand culture. Experiments 2 and 3 demonstrated that baicalin and crude extracts both stimulated the growth on agar of two common soilborne pathogens and enhanced damping-off of *S. baicalensis* and tomato seedlings growing in sand. Even in the absence of pathogens in experiment 3, the baicalin and crude extract solution inhibited germination of *S. baicalensis* and tomato, suggesting both intraspecific allelopathy (autotoxicity) and interspecific allelopathy. There were no differences between the effects of baicalin and crude extract, suggesting that baicalin may be the principal active component in the crude extract.

It is not clear that whether the effects of root exudates are due to their serving as carbon sources (Nicol et al. 2003). Glucose was included in experiments 2 and 3 to determine whether the effects of baicalin and crude extract on the pathogens and seedlings may have resulted from a general increase in carbon availability. In most cases, the effects of baicalin and crude extracts on the pathogens and on the seedlings were much greater than those of glucose, suggesting that the *S. baicalensis* root secretions did more than simply provide carbon. The mechanism by which secretions from *S. baicalensis* stimulate pathogens, however, requires further investigation.

In this regard, we designed experiment 4 to separate direct allelopathic effects from indirect effects by growing *S. baicalensis* in sterilized sand with and without *R. solani* and *P. ultimum*. Results showed that even in the absence of the pathogens, seed germination was reduced and seedling damping-off was increased when *S. baicalensis* was grown in sand in which *S. baicalensis* had previously grown rather than in sand in which *S. baicalensis* had not previously grown, i.e., there was an effect of a previous *S. baicalensis* soil on subsequent *S. baicalensis* plants. When pathogens were present, seed germination usually was reduced and seedling damping-off increased when either *S. baicalensis* or tomato was grown in sand in which *S. baicalensis* had

Table 3 Damping-off of tomato and *Scutellaria baicalensis* seeds caused by pathogens (*Pythium ultimum* and *Rhizoctonia solani*) in sand culture as affected by baicalin and crude extract of Radix Scutellaria (experiment 3)

Plant and treatment	Damping-off (%)		
	No pathogen	*P. ultimum*	*R. solani*
S. baicalensis			
Distilled water	0.0±0.0a	32.0±2.2a	28.2±2.3a
Glucose	0.0±0.0a	40.8±3.4a	34.2±6.1a
Baicalin	0.0±0.0a	58.8±5.9b	56.7±4.1b
Crude extract	0.0±0.0a	68.8±2.1b	60.4±6.3b
Tomato			
Distilled water	0.0±0.0a	43.3±1.3a	32.5±0.0a
Glucose	0.0±0.0a	43.3±1.3a	39.6±2.4a
Baicalin	0.0±0.0a	72.9±1.3b	64.6±2.4b
Crude extract	0.0±0.0a	75.0±1.3b	66.7±2.4b

*Values are the means±SE of six replicates. For each species, values followed by the same letter within a column are not significantly different at $P<0.05$

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Table 4 Mortality and damping-off of tomato and *Scutellaria baicalensis* caused by pathogens (*Pythium ultimum* and *Rhizoctonia solani*) and as affected by growing the plants in sand in which *S. baicalensis* had previously grown (*S. baicalensis* sand treatment) or in sand in which no plant had grown (no plant sand treatment) (experiment 4)

Plant and treatment	No pathogen		*P. ultimum*		*R. solani*	
	Mortality rate (%)	Damping-off rate (%)	Mortality rate (%)	Damping-off rate (%)	Mortality rate (%)	Damping-off rate (%)
S. baicalensis						
Sand without plant grown	26.7±3.3a	0.0±0.0a	51.7±2.1a	19.6±2.1a	52.0±3.4a	13.7±1.8a
Sand conditioned by *S. baicalensis*	52.5±5.3b	8.8±4.7b	66.0±0.9b	35.6±2.6b	59.2±3.7a	24.3±3.4b
Tomato						
Sand without plant grown	18.3±3.3a	0.0±0.0a	84.2±1.5a	32.1±9.2a	93.3±2.1a	46.7±19.1a
Sand conditioned by *S. baicalensis*	26.7±2.1a	0.0±0.0a	95.0±1.3b	45.5±16.8b	96.7±2.5b	66.7±21.1b

*Values are the means±SE of six replicates. For each species, values followed by the same letter within a column are not significantly different at $P<0.05$

previously grown rather than in sand in which *S. baicalensis* had not previously grown. In other words, chemicals released into the rhizosphere by *S. canadensis* had an indirect effect on other plants of the same or different species in that they enhanced disease incidences (Table 4).

The soilborne pathogens *R. solani* and *P. ultimum* commonly infect *S. baicalensis* tissues (Benizri et al. 2005; Chang et al. 2007). Although we did not quantify the populations of *R. solani* and *P. ultimum* in the *S. baicalensis* or *N. tabacum* field soils in this study, disease severity of both *S. baicalensis* and tomato were greater when in soil collected from the *S. baicalensis* field than in soil from the *N. tabacum* field (experiment 5). This finding is consistent with the idea that *S. baicalensis* promotes soilborne pathogens by releasing allelochemicals into the rhizosphere. The fields, of course, could have differed in other ways that could affect pathogen numbers and activities.

Our study increases the understanding of how plant-conditioned soils affect other plants of the same or different species through allelochemicals. It is known that plants and plant products can dramatically affect the structure, size, and activity of the soil microbial community (Bastida et al. 2008; Qu and Wang 2008), and that plants and plant products are primary drivers of soil microbial dynamics (Janssens et al. 1998; Larkin 2008). The effects of plants on soil microbial communities are presumed to result primarily from the release of different secondary compounds from roots and also from the breakdown of plant residues (Curl and Truelove 1986; Grayston et al. 1998; Pedrol et al. 2006). Van der Putten et al. (1993) and Bever (2003) have suggested that well-established plant succession may depend more on the accumulation of harmful (Bonanomi et al. 2005; Eppinga et al. 2006) and beneficial (Bezemer et al. 2006) rhizosphere organisms than on altered abiotic soil properties because biotic plant-soil feedback can be highly specific (Packer and Clay 2000; van der Putten 2003). Previous research has documented that root-derived phytochemicals promote soil pathogens (Ye et al. 2004; Mangla et al. 2008), which mediate the growth and survival of plants (Beckstead and Parker 2003; Ridenour and Callaway 2003). In this study, *S. baicalensis* stimulated soil pathogen accumulations apparently by releasing baicalin into the soil, thus generating negative feedback to *S. baicalensis* itself. This result provides additional supporting evidence that allelochemicals are important in mediating plant-soil feedbacks.

Our study also contributes to the understanding of problems associated with continuous cropping. For many crops, autotoxicity is believed to be a major cause of poor

Table 5 Mortality and damping-off of tomato and *Scutellaria baicalensis* plants growing in rhizosphere soils from fields where *S. baicalensis* or *Nicotiana tabacum* had grown

Plant	Mortality (%)		Damping-off (%)	
	N. tabacum soil	*S. baicalensis* soil	*N. tabacum* soil	*S. baicalensis* soil
Tomato	19.0±6.4a	35.0±0.7b	0.0±0.0a	0.0±0.0a
S. baicalensis	35.0±1.1a	52.0±0.3b	0.0±0.0a	17.0±2.9b

*Values are the means±SE of five replicates. For each species, and for mortality or damping off, values followed by the same letter are not significantly different at $P<0.05$

performance when the same crop is repeatedly grown in the same soil (Rice 1984; Batish et al. 2001; Liu et al. 2007). Our study suggests that secretions accumulating in the soil have profound effects on pathogen dynamics, thus inducing high disease incidences, which may contribute to problems with continuous cropping.

In summary, we found that baicalin released from the medicinal plant *S. baicalensis* accumulates in soil. In addition to directly inhibiting the growth of *S. baicalensis* seedlings (autotoxicity), baicalin also stimulates soil pathogens and may thus increase diseases of *S. baicalensis*. Continuous cropping of *S. baicalensis* may result in poor performance of this crop because baicalin is released from *S. baicalensis* roots and accumulates in the soil.

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Fatty Acids Released by *Chlorella vulgaris* and Their Role in Interference with *Pseudokirchneriella subcapitata*: Experiments and Modelling

Marina DellaGreca · Armando Zarrelli · Paolo Fergola · Marianna Cerasuolo · Antonino Pollio · Gabriele Pinto

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Abstract The role of extracellular fatty acids in the interference between two algae, *Chlorella vulgaris* Beijerink and *Pseudokirchneriella subcapitata* (Korshikov) Hindak, was assessed by the co-cultivation of the two selected strains, as well as by the chemical analysis of exudates from the culture media of single strain cultures. The effect of culture age and phosphate limitation was evaluated. The experiments showed that the composition and amount of fatty acids, released by *C. vulgaris* and by *P. subcapitata*, both in a batch and in a continuous monoculture, depend on the culture age and on the phosphate concentration in the culture medium. We also found that the amount of chlorellin generated in the two algae co-culture increased and was almost exclusively constituted by a mixture of C18 fatty acids. By using the evaluated concentrations of these fatty acids, an artificial chlorellin was prepared. The toxicity of this mixture to *P. subcapitata* appears to be similar to that of the natural chlorellin. For both algae, a stimulation of growth was observed at low concentrations of the natural chlorellin, whereas higher concentrations produced inhibitory effects on both species. However, *P. subcapitata* was much more sensitive than *C. vulgaris*. By using some of these new experimental results, two new mathematical models have been used to describe the toxicity of chlorellin to *C. vulgaris* and to the interference between *C. vulgaris* and *P. subcapitata*, respectively.

Keywords *Chlorella vulgaris* · *Pseudokirchneriella subcapitata* · Chlorellin · Fatty acids · Allelopathy · Mathematical model

M. DellaGreca (✉) · A. Zarrelli
Dipartimento di Chimica Organica e Biochimica,
Università degli Studi di Napoli Federico II,
Via Cinthia 4,
80126 Naples, Italy
e-mail: dellagre@unina.it

P. Fergola · M. Cerasuolo
Dipartimento di Matematica e Applicazioni "R. Caccioppoli",
Università degli Studi di Napoli Federico II,
Via Cinthia 4,
80126 Naples, Italy

M. Cerasuolo
Soil Science Department, Rothamsted Research,
Harpenden, Hertfordshire AL5 2JQ, UK

A. Pollio · G. Pinto
Dipartimento delle Scienze Biologiche, Sezione di Biologia Vegetale, Università degli Studi di Napoli Federico II,
Via Foria 223,
80139 Naples, Italy

Introduction

The green unicellular coccoid alga *Chlorella vulgaris* Beijerink was the first plant species in which a release of substances affecting other organisms was observed (Pratt and Fong 1940). Further investigations revealed that not a single compound, but a mixture of fatty acids and hydrocarbons (Spoehr et al. 1949), named chlorellin, was responsible for the toxicity towards bacteria and algae (McCracken et al. 1980). Such a joint action seems to be a widespread phenomenon in allelopathy and, according to Einhellig (1996), all allelopathic activities are due to a mixture of two or more compounds. Fatty acid mixtures frequently have been reported toxic in aquatic environments (Ikawa 2004). Chiang et al. (2004) found that blooms of the green unicellular alga *Botryococcus braunii* Kützing produce high amounts of fatty acids, which are severely toxic

to fish. Furthermore, Wu et al. (2006) demonstrated that the cytotoxicity of free fatty acids towards green algae and cyanobacteria affects primarily the plasma membrane.

Generally speaking, it is difficult to assess the role of allelopathy in the interference between two algal populations in the field, because it is difficult to evaluate the bioactive concentration of allelopathic substances, and also because too many environmental factors may influence their release from the donor organism (Blum et al. 1999). Furthermore, the sensitivity of the target species may vary, depending on the mode of action of the allelochemicals and environmental conditions (Inderjit and Duke 2003).

It is therefore more appropriate to study allelopathic phenomena under controlled laboratory conditions, where many variables can be kept constant, provided that the laboratory environment can mimic the natural environment sufficiently. This applies, for instance, for many open aquatic ecosystems, which often can be quite well simulated with chemostat laboratory cultures (Smith and Waltman 1995). This approach has been used previously by Fergola et al. (2007), and is used in the present paper. Fergola et al. (2007) found that *Pseudokirchneriella subcapitata* (Korshikov) Hindak became extinct in mixed cultures with *C. vulgaris* grown under phosphate-limiting conditions. A constant release of chlorellin-like material by *C. vulgaris* was observed during the time course of those experiments, and preliminary analyses confirmed that the exudates were composed primarily of various fatty acids. Here, by employing some new experimental results, two new mathematical models were used to describe the toxicity of chlorellin to *C. vulgaris* and to the interference between *C. vulgaris* and *P. subcapitata*, respectively.

Mathematical modelling is making increasingly significant contributions to many research areas. Mathematical models of biological and biochemical processes often represent a successful tool to better understand complex phenomena. Analysis (mathematical and numerical) can permit a deeper understanding both by predicting new scenarios and by suggesting new experiments. In the context of population dynamics, the influence of toxicants on the growth of populations and on competition among species has been widely studied in the last few years (Hallam et al. 1983; Hallam and Ma 1986, 1987a, b; Freedman et al. 1989; Smith and Waltman 1995; Hsu and Waltman 1998; Mukhopadhyay et al. 1998, 2003; Braselton and Waltman 2001). In particular, importance has been given to the competition that takes place in a chemostat-type environment. Such competitions have been mathematically represented essentially according to two different types of modelling approaches, adopted by Chattopadhyay (Mukhopadhyay et al. 1998, 2003), and Waltman (Smith and Waltman 1995; Braselton and Waltman 2001; Fergola et al. 2004, 2006), respectively. The two models used here follow this latter approach and give a representation of the dynamics of the interference taking place between the two algal species.

The main issue addressed in this study was to assess whether the age of culture, phosphate depletion, or co-occurrence of another species affected the composition of chlorellin and its release by *C. vulgaris*. Moreover, the biological activity of the natural exudate mixtures isolated from the co-culture of *C. vulgaris* and *P. subcapitata*, was compared to artificial chlorellin added to the chemostat culture, obtained by mixing the four main fatty acids in the proportion found in the exudates. On the basis of these results, a data analysis was performed. Additionally, some numerical simulations were performed by assigning to the parameters of the two considered models, numerical values obtained from some of the laboratory experiments described both in this paper and in Fergola et al. (2007).

Methods and Materials

Selection of Strains and Culture Conditions Two axenic strains were used: *C. vulgaris* CCAP 211/11b and *P. subcapitata* UTEX 1648 from ACUF (Algal Collection of the University Federico II, Naples). The algal strains were cultivated for 2 wk on Bold Basal Medium (BBM) (Nichols 1973) with an inorganic phosphate (Pi) concentration of 25 g l^{-1}. For *P*-limited cultures, the Pi concentration was reduced to 0.5 mg l^{-1} (Chen et al. 1997). Cultures were maintained in 100 ml Erlenmeyer flasks, at 23°C on shaking incubators at 40 rpm. Continuous illumination, with an irradiance of 80 μE m^{-2} sec^{-1}, was supplied by cool white fluorescent tubes (Philips TLD 39 w/55). Batch experiments, either with each single species or with mixed cultures of both species, were performed under the same conditions. The growth of population of *C. vulgaris* or *P. subcapitata* was followed using a spectrophotometer (Secoman 250) at λ=550 nm. When the experiments were carried out with mixed culture of the two algae, cells were counted by using a Burker hemocytometer, under an optical microscope (Nikon Eclipse 800).

Chemostat Cultures Algal competition for a limiting nutrient (phosphate) was examined in a chemostat culture system. The culture vessel was a standard chemostat containing 1,200 ml of culture media. Nutrient was pumped at a constant rate into the culture flasks by means of a peristaltic pump (GILSON–MINIPLUS 3) at a rate of 600 ml d^{-1} to give a washout rate of 50% a day. Experiments were carried out either on unialgal or mixed cultures. Continuous illumination (80 μE m^{-2} sec^{-1}) was provided by cool fluorescent lamps (Philips TLD 39 w/55). Sterile humidified air was bubbled through the cultures.

The air flow produced by an air compressor was controlled by a flow-meter and conveyed into two flasks of sterile water. Each culture was stirred continuously with a mixer (Heidolph). All experiments were performed using a BBM medium with a Pi concentration of 0.5 mg l^{-1}, as suggested by Chen et al. (1997). For the competition experiments, the two species were grown initially in different flasks to give a fixed cell number of 300,000 cells ml^{-1} for both strains. Successively, samples of both algae were withdrawn from 4-d-old cultures, and aseptically inoculated in the chemostat.

Analysis of Chlorellin Batch cultures either of *C. vulgaris* or *P. subcapitata* were carried out in 5,000 ml cylinders under continuous light at (80 μE m^{-2} sec^{-1}) at 23°C. The algal cultures were stirred continuously and air bubbled through the cells that were grown under *P*-sufficient (25 g l^{-1}) or *P*-limiting (0.5 mg l^{-1}) conditions. Aliquots of the cultures (2,500 ml) were collected and filtered during the mid-exponential phase (6,650,000 cells ml^{-1}) and at the beginning of the stationary phase of growth (14,000,000 cells ml^{-1}). In the co-cultures grown in the chemostat, the outflow was collected daily and stored at −20°C. All culture media were concentrated with an evaporator to 200 ml, and exhaustively extracted with methylene chloride (3×150 ml; recovery rate ~90%, 8%, <1%, respectively). The organic fraction was concentrated to dryness to obtain a lipoid fraction. This was derivatized with ethereal diazomethane and analyzed with a GC (Gas Chromatography) by using an RTX-5 capillary column (Restek; 30 m×0.25 mm inside diam, flow rate 10 ml min^{-1}, N_2 used as carrier gas). Analysis was performed with the following temperature program: 140°C for 5 min, from 140°C to 240°C at 4°C min^{-1}, and 240°C for 60 min. Fatty acid methyl esters (FAMEs) were identified by comparing their retention times with those of 19 commercial fatty acid standards purchased from SUPELCO, with the Limit of Quantitation of 14 ppb. Fatty acids yield and composition were studied also in co-cultures of *C. vulgaris* and *P. subcapitata* grown in *P*-limiting cultures. Individual algae from continuous cultures (300,000 cells ml^{-1}) were mixed at a 1:1 ratio, and after 4 d, when *C. vulgaris* was at a concentration of 1,200,000 cells ml^{-1} and *P. subcapitata* at 100,000 cells ml^{-1}, the composition of the medium was analyzed.

Toxicity Bioassays In order to analyze the composition and evaluate the toxicity of natural chlorellin, the culture medium (4 l) of *C. vulgaris* and *P. subcapitata* co-cultures was collected, concentrated with an evaporator to 200 ml, and exhaustively extracted with methylene chloride (3× 150 ml). The mixture of fatty acids extracted was dried and re-dissolved in 5 ml of dimethyl sulfoxide (DMSO). The solution was stirred for 2 h in the dark at room temperature. Test solutions were prepared by mixing the appropriate volumes of the DMSO stock solutions with culture media. The concentration of DMSO in culture media was 2 ml l^{-1} at the highest concentration of chlorellin tested (52 mg l^{-1}). Liquid growth inhibition tests against this extracted, natural mixture of fatty acids were performed with *P. subcapitata* or *C. vulgaris*. Inocula corresponding to 10,000 cells ml^{-1} (ISO protocol 1987) from cultures of each strain in mid exponential phase (4-d-old) were grown in 100 ml Erlenmeyer flasks containing 50 ml of BBM. Liquid inhibition tests also were carried out with chemostat cultures of *P. subcapitata* with the same initial cell concentration (300,000 cells ml^{-1}) against chlorellin at a constant concentration of 1.90 mg l^{-1}, and number of cells, photosynthetic rate, and Chlorophyll *a* concentration were monitored daily. For this purpose, pre-cultures of *P. subcapitata* were grown on *P*-sufficient BBM medium (concentration mg l^{-1}). When the algae were in mid-exponential growth phase (4-d-old), an inoculum (10 ml) was centrifuged, washed two times in sterile 0.9% NaCl solution, and then added to a culture vessel of a standard chemostat containing 1,200 ml of *P*-limited BBM (Pi 0.5 mg l^{-1}). Algae were grown in the chemostat according to the conditions described above (section *Chemostat Cultures*). Preliminary tests carried out on *P. subcapitata* gave evidence that cells that have been treated according to this protocol and re-suspended in *P*-sufficient BBM grew at rates comparable to those of control cultures. To assess the relationship between fatty acid toxicity and cell concentrations at very low cell densities, *P. subcapitata* cultures at an initial inoculum concentration ranging from 500 to 5,000 cells ml^{-1} were exposed to 1.90 mg l^{-1} of chlorellin isolated from co-cultures of *C. vulgaris* and *P. subcapitata*.

Fatty acids extracted from the medium of co-cultures of *C. vulgaris* and *P. subcapitata* were tested at concentrations ranging from 0.40 to 52 mg l^{-1}. A mixture of the four main fatty acids found in the natural chlorellin extract also was prepared by using stearic acid (C18:0, >99%), oleic acid (C18:1n9c, >98%), linoleic acid (C18:2n6c, 97%), and linolenic acid (C18:3n3c, 99%) (Sigma–Aldrich, Germany). It was dissolved in DMSO to give the same ratio found in a natural extract (linoleic acid 46.4%, linolenic acid 21.0%, oleic acid 19.2%, and stearic acid 13.2%). All bioassays and BBM analyses, controls and positive controls (containing only 2 ml l^{-1} DMSO) were carried out in triplicate in axenic conditions at 23°C with continuous illumination of 80 μE m^{-2} sec^{-1}. At the end of the incubation, the final pH was also measured. No difference in growth was observed among controls. Chlorophyll *a* was measured according to Lazzara et al. (1990), and photosynthetic rate was measured according to Pollio et al. (1993).

Statistical Evaluation For liquid growth inhibition tests, the effect of concentration, which differs significantly from that of the control, was determined by multi comparison Dunnett's tests, after verifying the Shapiro–Wilk's test for normality and the Hartley's test for homogeneity of variance ($P>0.5$). Calculations were performed using TOXSTAT 3.0 software (Gulley et al. 1989).

Mathematical Modelling Here, we introduce two deterministic mathematical models constituted by Ordinary Differential Equations, which represent the influence of chlorellin on *C. vulgaris* and *P. subcapitata* both in mono- and mixed culture. The two models are based on data obtained with several experiments performed in our laboratories. Our experiments gave us the opportunity to understand and quantitatively represent the following four crucial features (Fergola et al. 2007): a) nutrient uptake rates; b) yields (ratios between the amounts of produced biomass and absorbed nutrient); c) rate of allelochemical production; and d) the inhibitory effects of allelochemicals.

Mathematical Modelling of Inhibition Effects of Chlorellin We tested two mathematical models, both based on experimental data, to represent the inhibition effects of chlorellin on *P. subcapitata* and *C. vulgaris*. For the inhibitory effects on the growth rate of *P. subcapitata*, Eq. (1) was a suitable model fitting the experimental data (Fergola et al. 2007):

$$f_1(S)e^{-\gamma p} \tag{1}$$

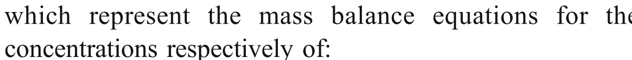
where $f_1(S)$ is the growth function in the absence of toxicant, p is the concentration of chlorellin in the environment, and γ is a measure of the inhibiting effect of chlorellin $\gamma=7.81$ $\mathrm{l^2\ mg^{-2}}$). In Fergola et al. (2007) the influence of chlorellin on *C. vulgaris* was not included. Here, on the basis of new experiments, this process is taken into account and Eq. (2) is proposed as a suitable representation of the growth rate of *C. vulgaris* (LAB Fit software):

$$f_2(S)e^{-\alpha p^2} \tag{2}$$

where $f_2(S)$ is the growth function in absence of toxicant and computed at a given value of the nutrient concentration, and α is a measure of the inhibiting effect of chlorellin $\alpha>0$).

The estimate of the parameter α is chosen to minimize the χ^2 merit function given by the sum of squared residuals $\sum_i e_i^2$. This iterative fitting procedure is based on a modified Levenberg–Marquardt algorithm (Meyer and Roth 1972).

The Effect of Chlorellin on C. vulgaris in Chemostat Culture In order to describe the growth of *C. vulgaris* we considered three Ordinary Differential Equations (ODE), which represent the mass balance equations for the concentrations respectively of:



the nutrient,

$$\frac{dS}{dt} = \left(S^0 - S\right)D - f(S)e^{-\alpha p^2}\frac{N}{\eta} \tag{3}$$

the algae,

$$\frac{dN}{dt} = N\left[(1 - kN)f(S)e^{-\alpha p^2} - D\right] \tag{4}$$

and the chlorellin,

$$\frac{dp}{dt} = kf(S)e^{-\alpha p^2}N^2 - Dp \tag{5}$$

where d/dt denotes the time derivative, $S = S(t)$ is the phosphate concentration at time t, $N = N(t)$ is the biomass of *C. vulgaris* at time t, $p = p(t)$ is the concentration of chlorellin in the environment at time t, η is the constant yield of the population (Smith and Waltman 1995), S^0 is the constant input rate of the limiting nutrient concentration ($S^0>0$), D is the constant washout rate ($D>0$), $f(S) = \frac{mS}{a+S}$ is the functional response of *C. vulgaris* (Michaelis–Menten model) with the semi-saturation constant a ($a>0$), and m ($m>0$) the maximal specific growth rate of *C. vulgaris*, and α is a measure of the inhibiting effect of chlorellin ($\alpha>0$).

Finally we note that, according to Fergola et al. (2007), the term $kNf(S)$ is the rate of allelochemical production and is represented by the fraction of potential growth devoted to produce the chlorellin ($0<kN<1$). As in Fergola et al. (2007) we assume that the overall energy available to *C. vulgaris* arises only from nutrient consumption. Moreover, it is not completely available for the algae growing process, but is devoted partially to the production of chlorellin. In other words, we assume that a sort of energy conservation law holds, and furthermore, that the production of allelochemicals is proportional to the concentration of algae and thus represented by a linear function, which includes the constant k.

By performing the following scaling of the parameters (Smith and Waltman 1995)

$$\begin{aligned} &S = \overline{S}S^0, N = \overline{N}\eta S^0, p = \overline{p}S^0\eta, m = \overline{m}D, t = \frac{\tau}{D}, \\ &a = \overline{a}S^0, \alpha = \frac{\overline{\alpha}}{S^{02}\eta^2}, f(S) = \overline{f}\left(\overline{S}S^0\right), \overline{k} = k\eta S^0 \end{aligned} \tag{6}$$

we obtain from the Eqs. (3)–(5) a dimensionless system for the state variables S, N, p, which can be written, by removing the bars, in the form

$$\begin{cases} \frac{dS}{dt} = 1 - S - f(S)e^{-\alpha p^2}N \\ \frac{dN}{dt} = N\left[(1 - kN)f(S)e^{-\alpha p^2} - 1\right] \\ \frac{dp}{dt} = kf(S)e^{-\alpha p^2}N^2 - p \end{cases} \tag{7}$$

By standard techniques, we can prove the following two lemmas:

Lemma 1 Any solution of (7), with positive initial conditions, remains positive whenever it exists.

Lemma 2 Any solution of (7) is bounded (See proof in Appendix).

In view of the results concerning the growth of species in a chemostat-like environment (Smith and Waltman 1995), we suppose $m_i>1$, $i=1, 2$ and state the following theorem without the proof.

Theorem 1 System (7) admits the steady state solution E=(1, 0, 0) for all values of the parameters.

Theorem 2 If $\frac{m}{a+1} > 1$ then system (7) admits one positive equilibrium $E^*=(S^*, N^*, p^*)$. (See proof in Appendix)

Local Stability The stability analysis of the generic equilibrium $\overline{E} = (\overline{S}, \overline{N}, \overline{p})$ can be performed by means of the characteristic equation associated to the linearized system of (7)

$$\begin{cases} \dot{x}_1 = \left(-1 - f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}\right)x_1 - f(\overline{S})e^{-\alpha\overline{p}^2}x_2 + 2\alpha f(\overline{S})\overline{p}\overline{N}e^{-\alpha\overline{p}^2}x_3 \\ \dot{x}_2 = f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}(1 - k\overline{N})x_1 + \left[(1 - 2k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} - 1\right]x_2 - 2\alpha\overline{p}\overline{N}(1 - k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2}x_3 \\ \dot{x}_3 = f'(\overline{S})\overline{N}^2ke^{-\alpha\overline{p}^2}x_1 + 2k\overline{N}f(\overline{S})e^{-\alpha\overline{p}^2}x_2 + \left(-2\alpha\overline{p}\overline{N}kf(\overline{S})e^{-\alpha\overline{p}^2} - 1\right)x_3 \end{cases} \tag{8}$$

whose characteristic equation can be written as follows

$$\det\begin{bmatrix} \left(-1 - f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}\right) - \rho & -f(\overline{S})e^{-\alpha\overline{p}^2} & 2\alpha f(\overline{S})p\overline{N}e^{-\alpha\overline{p}^2} \\ f'(\overline{S})\overline{N}e^{-\alpha\overline{p}^2}(1 - k\overline{N}) & \left[(1 - 2k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} - 1\right] - \rho & -2\alpha\overline{p}\overline{N}(1 - k\overline{N})f(\overline{S})e^{-\alpha\overline{p}^2} \\ f'(\overline{S})\overline{N}^2ke^{-\alpha\overline{p}^2} & 2k\overline{N}f(\overline{S})e^{-\alpha\overline{p}^2} & -2\alpha\overline{p}\overline{N}kf(\overline{S})e^{-\alpha\overline{p}^2} - 1 - \rho \end{bmatrix} = 0. \tag{9}$$

We can prove that:

Theorem 3 The following statements hold true: (See proof in Appendix)

(i) If $m < a + 1$, then the equilibrium E_0 turns out locally asymptotically stable;
(ii) If E^* exists, then it turns out locally asymptotically stable.

A New Model for the Interference between C. vulgaris and P. subcapitata In the system (3) of Fergola et al. (2007) the influence of chlorellin on *C. vulgaris* was not included. Here, according to the previous section, we assume that chlorellin also can act on *C. vulgaris*, and we propose to model the interference between the two algae with the following new system

$$\begin{cases} \frac{dS}{dt} = (S^0 - S)D - f_1(S)e^{-\gamma p}\frac{N_1}{\eta_1} - f_2(S)e^{-\alpha p^2}\frac{N_2}{\eta_2} \\ \frac{dN_1}{dt} = N_1[f_1(S)e^{-\gamma p} - D] \\ \frac{dN_2}{dt} = N_2\left[(1 - kN_2)f_2(S)e^{-\alpha p^2} - D\right] \\ \frac{dp}{dt} = kf_2(S)e^{-\alpha p^2}N_2^2 - Dp \end{cases} \tag{10}$$

where the inhibition effects of chlorellin on the two algae have been modeled in different ways and, according to what was suggested in the Discussion in Fergola et al. (2007), the two functions $f_1(S)$ and $f_2(S)$ are both of the Michaelis–Menten type.

Results

Concentration and Composition of Fatty Acids Released by C. vulgaris under Different Culture Conditions Experiments evaluated whether the chlorellin production of *C. vulgaris* is affected by age of culture, phosphate concentration in the culture medium, or co-occurrence of another algal strain. The lipoidic material recovered from the medium (*P*-sufficient) of *C. vulgaris* was 0.41 mg l^{-1} 10^{-6} cells in mid-exponential phase cultures and 0.70 mg l^{-1} 10^{-6} cells in stationary phase cultures. From *P. subcapitata* cultures, 0.08 mg l^{-1} 10^{-6} cells in the mid-exponential phase and 0.11 mg l^{-1} 10^{-6} cells in the stationary phase of growth were recovered (Table 1).

The GC analysis of the exudates showed the presence of fatty acids, identified by comparison with commercial standards. Under *P*-sufficient conditions, *C. vulgaris* batch cultures released fatty acids, ranging from C8 to C18, during their exponential growth phase, but a significantly

Table 1 Amounts of fatty acids (mg/l/10^6 cells) recovered from culture medium of *Chlorella vulgaris* Beijerink and *Psuedokirchneriella subcapitata* (Korshikov) Hindak. a) exponential growth phase, batch culture, *P*-sufficient; b) stationary growth phase, batch culture, *P*-sufficient; c) exponential growth phase, chemostat culture, *P*-limiting conditions; d) a mixed culture of *C. vulgaris* and *P. subcapitata* (initial ratio 1:1) in chemostat under *P*-limiting conditions, exponential growth phase

C. vulgaris			*P. subcapitata*			*C. vulgaris P. subcapitata*
a	b	c	a	b	c	d
0.41	0.70	0.85	0.08	0.11	0.25	1.90

changed fatty acid composition during their stationary phase that showed a higher degree of polymerization (C14 to C22 acids). *Pseudokirchneriella subcapitata* cultures produced, during the mid-exponential growth phase, a mixture whose major components were C12, C18, C20, and C22 fatty acids; the same compounds, except C20 and C22, also were found in the medium collected from the stationary phase of growth.

These data indicate that the amounts and kinds of fatty acids were different for the two algae cultures (Tables 1, 2). The experiments on a single culture of the selected algae also were carried out in a chemostat, under *P*-limiting conditions. Fatty acids isolated from *C. vulgaris* under these conditions yielded 0.85 mg l^{-1} 10^{-6} cells (Table 1). Their composition was compared with that found in *P*-sufficient cultures during the exponential and stationary growth phases. The comparison showed an increase of acid compounds ranging from C8 to C18 (Table 2). Additionally, in the *P. subcapitata* culture, an increase of fatty acid concentrations was observed (0.25 mg l^{-1} 10^{-6} cells) (Table 1) in respect to the values obtained from batch cultures, and the fatty acid composition was represented by different compounds ranging from C8 to C20 (Table 2).

Fatty acid yield and composition also were studied in co-cultures of *C. vulgaris* and *P. subcapitata* grown in *P*-limiting cultures and with an initial inoculum of 300,000 cell ml^{-1} each. After four days, the composition of the medium was analyzed. At that time, we observed an increased growth of *C. vulgaris* (1,200,000 cells ml^{-1}) and a partial extinction of *P. subcapitata* (100,000 cells ml^{-1}). The isolated fatty acid mixture yielded 1.90 mg l^{-1} 10^{-6} cells ml^{-1}, which was the highest fatty acid concentration observed in our experiments (Table 1). The co-culture of the two algae grown under phosphorus deficiency also influenced the fatty acid composition of the mixture found in the culture medium (Table 2), which in this case

Table 2 Qualitative composition of the fatty acid mixture recovered from different culture media and growth conditions of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*. a) exponential growth phase, batch culture, *P* sufficient; b) stationary growth phase, batch culture, *P* sufficient; c) exponential growth phase, chemostat culture, *P*-limiting conditions; d) a mixed culture of *C. vulgaris* and *P. subcapitata* (initial ratio 1:1) in chemostat under *P*-limiting conditions; (x = present; ± = in trace)

Fatty acids	*C. vulgaris*			*P. subcapitata*			*C. vulgaris P. subcapitata* (ratio 1:1)
	a	b	c	a	b	c	d
C8:0	x		x			x	
C10:0			x				
C12:0			x	x	x	x	
C13:0	x		x				
C14:0		x				x	
C14:1n9c	x		x	±	±		
C15:0			x	±	±	x	
C16:0				±	±	x	
C16:1n9c		x	x		±		
C17:0	x	x	x	±		x	x
C18:0		x	x	±	±		x
C18:1n9c		x	x	±	x		x
C18:1n9t		x	x	±	x		x
C18:2n6c		x	x	x	x	x	x
C18:3n3c		x	x				x
C20:0		x					x
C20:1n9c		x	x	x		x	
C22:0			x			x	
C22:1n9c		x	x	x			

x=>14 ppb, ±=<14 ppb

was constituted almost exclusively by the following C18 fatty acids: stearic acid (13.2%), oleic acid (19.2%), linoleic acid (46.4%), and linolenic acid (21.0%).

Effect of Fatty Acids Mixture on C. vulgaris and P. subcapitata Growth The toxicity of chlorellin isolated from the co-culture of the two algae was assayed separately either on *P. subcapitata* or *C. vulgaris* grown in batch cultures. The range of concentrations tested was 0.4–52 mg l^{-1}. Figure 1 shows the concentration-response relationship of the two algae following 96 h exposure to the fatty acid mixtures. A stimulation of growth at low concentrations of chlorellin was observed for both algae. However, *P. subcapitata* was much more sensitive than *C. vulgaris* to chlorellin concentration. In the case of this latter organism, fatty acids elicited slight growth stimulation at concentrations ranging from 0.4 to 13.0 mg l^{-1}, and exerted a toxic action only at the highest concentrations tested, whereas *P. subcapitata* growth was appreciably stimulated only at the lowest concentrations tested and was strongly inhibited from 6.5 mg l^{-1} onward.

Although chlorellin isolated from algal co-cultures contained traces of other unidentified substances, we prepared a synthetic mixture of the four commercially available C18 fatty acids only, at the same ratio found in natural exudates. The mixture was assayed against *P. subcapitata* and *C. vulgaris*, giving the results reported in Fig. 2. For both species, we observed a growth stimulation at low concentrations and an inhibitory effects at high concentration. Kruskal–Wallis one way analysis of variance on ranks showed that there was no significant difference between the toxicity of chlorellin and the artificial fatty acid mixtures (P=0.171). The results shown in Figs. 1 and 2 also indicate that, under the experimental conditions of batch assays, a concentration of 1.90 mg l^{-1} of the fatty acid mixture was not toxic to *P. subcapitata* (1.90 mg l^{-1} is the amount found in co-culture). Two further experiments were carried out to better understand whether or not even lower cell densities are affected by such a concentration of fatty acids. In the first, the relationship between fatty acid toxicity and cell concentration showed no inhibition of growth in any of the inocula tested. In the second, the influence of *P*-limiting conditions together with a chlorellin concentration of 1.90 mg l^{-1} was evaluated on *P. subcapitata* grown in a chemostat. Although the experimental environment was equivalent to that described for previous experiments of co-culture with *C. vulgaris*, no inhibition was observed. The results of the experiments were used to develop a revised mathematical model that describes the nature of interference between *C. vulgaris* and *P. subcapitata*.

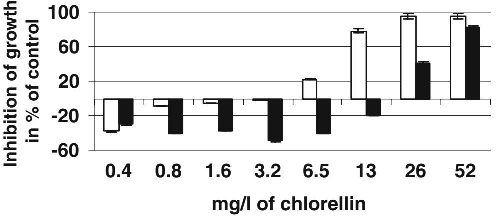


Fig. 1 Effects of chlorellin isolated from co-cultures of the two algae on *Chlorella vulgaris* or *Psuedokirchneriella subcapitata* growth (after 96 h exposure), grown in chemostat under *P*-limiting conditions. (black square *C. vulgaris*; white square *P. subcapitata*); *error bars* indicate the standard deviation

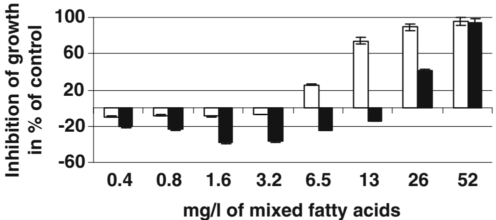


Fig. 2 Effects of fatty acids mixture (stearic, oleic, linoleic and linolenic acids) on *Chlorella vulgaris* or *Pseudokirchneriella subcapitata* growth (after 96 h exposure), grown in chemostat under *P*-limiting conditions; (black square *C. vulgaris*; white square *P. subcapitata*); *error bars* indicate the standard deviation

Mathematical Modelling The model (2) was fitted to the experimental data contained in Table 3 by using a non-linear minimization function (Nonlinear Regress) of the software package Mathematica (Wolfram-Research 1988). In this way, we computed α=4.38 l^2 mg^{-2} with an asymptotic standard error of 0.75 (Fig. 3). The variance was estimated to be 0.2×10^{-2}.

With respect to other models quoted in literature, the two considered here [(1), (2)] were selected mainly because of the advantage of mathematical tractability, simplicity, and the lowest residual standard error. The numerical simulation obtained from Eqs. (3)–(5) is given in Fig. 4. It shows good agreement between the experimental data of *C. vulgaris* growth in the chemostat and the numerical curve.

In a previous study (Fergola et al. 2007), we performed simulations of co-cultures of *C. vulgaris* and *P. subcapitata*, grown in a chemostat under phosphorus limitation. We found that *P. subcapitata* was excluded from the culture within nine days. The model developed to describe the interference between the two algae suggested that both competition for nutrients and toxic action of fatty acids, excreted by *C. vulgaris*, were responsible for the final exclusion of *P. subcapitata*.

For this investigation, some numerical simulations were performed. However, by comparing the numerical simulations obtained through system (10) of this paper with the experimental data of the laboratory interference, currently collected, there was not much difference from that obtained using system (3) of the Fergola et al. (2007).

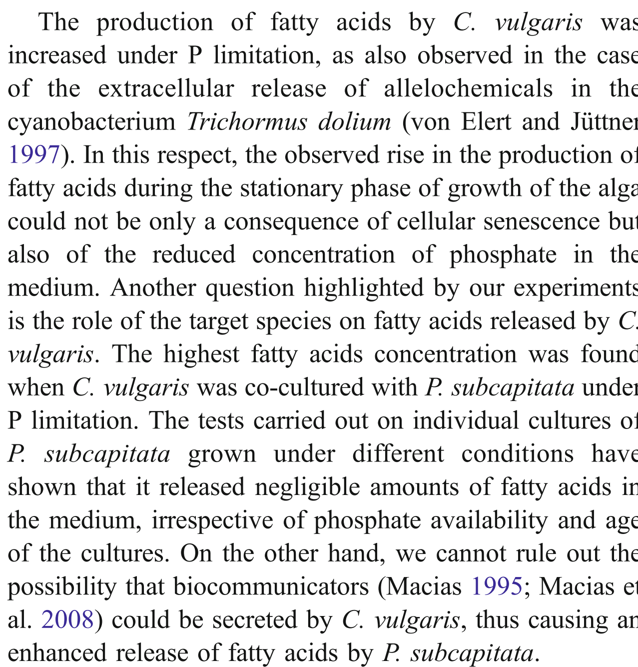
Table 3 The experimental *Chlorella vulgaris* growth rates in correspondence of different toxicant concentrations

Chlorellin (mg/l 10^2)	0	0.002	0.004	0.0078	0.0156	0.03125	0.0625	0.125	0.25	0.52
Growth rate (d^{-1})	0.64	0.70	0.67	0.70	0.71	0.72	0.72	0.69	0.41	0.19

In other words, the effect of chlorellin on *C. vulgaris* in system (10) does not seem to play an important role on the interference between the two algae, unless, once again, the amount of chlorellin produced throughout the experiment was so small that it was not sufficient to produce an observable inhibitory effect. It should be noted, however, that in contrast to the outcome of the laboratory experiments in mono-culture with synthetic chlorellin, the mathematical model predicts, in the experiment in co-culture, an inhibition of the *P. subcapitata* growth by the naturally produced chlorellin.

Discussion

One of the points addressed in this study deals with the chemical characterization of chlorellin. Since the works of Spoehr et al. (1949) and Scutt (1964), only few contributions to the characterization of this mixture have been attempted (Sushchik et al. 2001). Moreover, little information is available on environmental factors that influence the production and release of chlorellin. Sushchik et al. (2003) reported that the composition of extracellular free fatty acids released by *C. vulgaris* did not vary when the algae were grown at different temperatures. The results of our study suggest that the production and the composition of released fatty acids by *C. vulgaris* and *P. subcapitata* are influenced by *P*-supply and/or growth phases, and also by the presence and absence of a competitor.



The production of fatty acids by *C. vulgaris* was increased under P limitation, as also observed in the case of the extracellular release of allelochemicals in the cyanobacterium *Trichormus dolium* (von Elert and Jüttner 1997). In this respect, the observed rise in the production of fatty acids during the stationary phase of growth of the alga could not be only a consequence of cellular senescence but also of the reduced concentration of phosphate in the medium. Another question highlighted by our experiments is the role of the target species on fatty acids released by *C. vulgaris*. The highest fatty acids concentration was found when *C. vulgaris* was co-cultured with *P. subcapitata* under P limitation. The tests carried out on individual cultures of *P. subcapitata* grown under different conditions have shown that it released negligible amounts of fatty acids in the medium, irrespective of phosphate availability and age of the cultures. On the other hand, we cannot rule out the possibility that biocommunicators (Macias 1995; Macias et al. 2008) could be secreted by *C. vulgaris*, thus causing an enhanced release of fatty acids by *P. subcapitata*.

The profile of the fatty acid mixture was shown to be dependent on cultural conditions. Our data indicate that there was a shift towards C18 fatty acids when *C. vulgaris* was cultured under stress conditions. Furthermore the fatty acids mixture isolated from *C. vulgaris* culture under *P*-depleting conditions and in the presence of the competitor *P. subcapitata* was composed mainly of four C18 fatty acids with different degrees of unsaturation. These compounds have been found to be major components of the toxic mixtures released by a number of microalgae

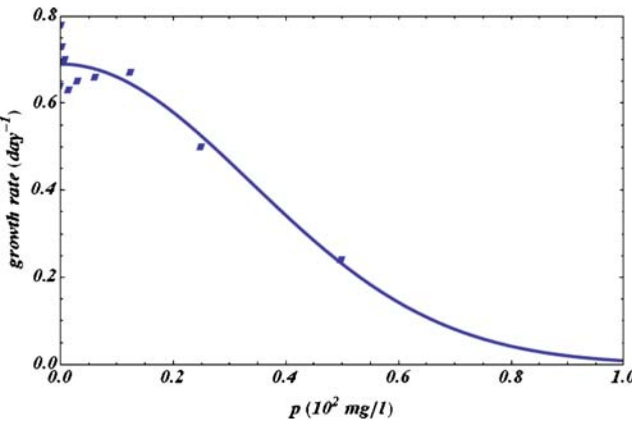


Fig. 3 Dependence of *Chlorella vulgaris* growth rate on the chlorellin concentration p. Comparison between experimental data and the exponential function $f_2\left(S\right)e^{-\alpha p^2}$ where α=4.38 (l^2/mg^2)

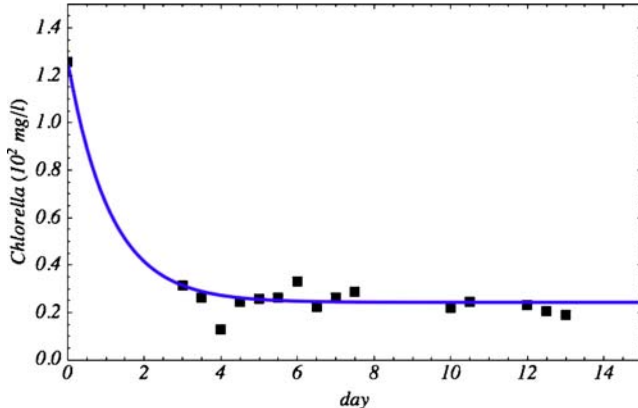


Fig. 4 In the picture, the *points* represent the chemostat experimental data and the *continuous line* is obtained through numerical simulations of Eqs. (3)–(5) with the following parameter values: D=0.9, S^0=0.52, m=1.16, a=0.004, k=0.4, η=0.5, α=4.38

belonging to Chlorophyta, such as *Chlamydomonas reinhardtii* (McCracken et al. 1980), *Chlorococcus* sp., and *Dunaliella primolecta* (Ohta et al. 1995).

The toxicity of fatty acids often has been attributed to their degradation products derived through photoxidation (Spoehr et al. 1949; Murata et al. 1989), but Aliotta et al. (1990) showed that an inhibitory activity of free fatty acids cannot be ruled out. Recently, Bosma et al. (2008) reported that the growth of the Xanthophyte *Monodus subterraneus* was inhibited severely by palmitoleic and oleic acids.

There is increasing evidence of the ecological role played by allelopathic interference in aquatic ecosystems and particularly by fatty acids and their products of photoxidation (Gross 2003, Ianora et al. 2006). However, their effective concentration under natural conditions remains of pivotal importance. The concentration of the fatty acid mixture isolated from single species culture of *C. vulgaris* varied from 0.4 to 0.85 mg l^{-1} in our experiments. In the mixed culture (*C. vulgaris* and *P. subcapitata*), the concentration was much higher (1.90 mg l^{-1}), which is close to those found in some freshwater lakes, where fatty acids are found within a range of 1 and 2 mg l^{-1} (Ikawa 2004). Concentrations of about 0.5 mg l^{-1} were inhibitory to *Phormidium tenue* (Yamada et al. 1993), but it is known that different species exhibit different sensitivities to fatty acid mixtures (Figueredo et al. 2007). In our experiments, chlorellin at 1.90 mg l^{-1} did not significantly influence the growth of *P. subcapitata*. However, in the field, a long exposure to sub-lethal concentrations of fatty acids could affect microalgal species succession. As demonstrated for other classes of compounds (Ianora et al. 2006), there is a high species-specific variability in the effects of allelochemicals on microalgae. Low concentrations of a metabolite can stimulate the growth-rate of some species, but also can be slowly accumulated within the cell, leading to adverse effects in other less resistant species (Ikawa 2004).

The experiments have shown that in the presence of chlorellin the growth of the two algae is modified. To be more precise, we observed that it is slightly stimulated at low concentrations and strongly inhibited as the chlorellin concentration increases. In the models, we did not include the stimulating effect of chlorellin on the two algae, since related experiments are currently in progress.

The simulations obtained on the basis of the suggested mathematical models predicted the experimental results well. Therefore, we reasonably conclude the following:

1) Chlorellin at concentrations above 6.5 mg l^{-1} is autotoxic for *C. vulgaris*. The autotoxic action of a free fatty acid mixture also should be evaluated in the field because, for diatom-derived unsaturated aldehydes, it has been demonstrated that they can act as a diffusible bloom-termination signal (Ianora et al. 2006).
2) *Pseudokirchneriella subcapitata* does not produce sufficient amounts of chlorellin in single species cultures to be ineffective in the competition of the two algal species under chemostat-like conditions. However, experiments are necessary to exclude the idea that biocommunicators produced by *C. vulgaris* or other parameters could enhance the productivity of chlorellin by *P. subcapitata* in co-cultures.
3) The inhibitory effects of a given concentration of natural chlorellin on *P. subcapitata* are similar and qualitatively equivalent to those produced by the same concentration of synthetic chlorellin, prepared as a mixture of the four C18 fatty acids (stearic, oleic, linoleic, and linolenic acids). This is relevant for future experiments, because it allows the use of artificial chlorellin, which is available in large amounts, instead of natural chlorellin, which is usually produced in very small quantities (Fergola et al. 2007).

Appendix

Proof (Lemma 2) If we set

$$z(t) = S(t) + N(t) + p(t) \tag{11}$$

due to system (7), we get

$$\dot{z}(t) = 1 - S - N - p = 1 - z(t), \tag{12}$$

and by integrating from 0 to t, we obtain

$$z(t) = z(0)e^{-t} + 1 - e^{-t},$$

and then

$$\lim_{t \to +\infty} z(t) = 1 \tag{13}$$

thus proving, for any initial condition, the boundedness of the solutions of (7).

Proof(Theorem 2) In order to prove the theorem, we look for positive steady state solutions ($S^*>0$, $N^*>0$, $p^*>0$) and we solve the system obtained by setting the right-hand side of system (7) equal to zero. In this way, we get

$$N^* = \frac{1 - S^*}{k(1 - S^*) + 1}, \qquad p^* = \frac{k(1 - S^*)^2}{k(1 - S^*) + 1} \tag{14}$$

and

$$\left(1 - S^*\right)\left[1 - \frac{f(S^*)}{k(1 - S^*) + 1} e^{-\alpha\left(\frac{k(1-S^*)^2}{k(1-S^*)+1}\right)^2}\right] = 0 \tag{15}$$

with a few calculations from (14) we find that $N^*>0$, $p^*>0$, if and only if $S^*<1$. We have to exclude the value $S^*=1$

because it makes both p^* and N^* equal to zero. Therefore (by omitting the star), we study the solutions of the equation

$$k(1-S)+1-f(S)e^{-\alpha\left(\frac{k(1-S)^2}{k(1-S)+1}\right)^2}=0 \quad (16)$$

if we define

$$h(S)=k(1-S)+1 \quad \text{and} \quad g(S)=f(S)e^{-\alpha\left(\frac{k(1-S)^2}{k(1-S)+1}\right)^2} \quad (17)$$

then the problem of finding the solution of (15) is changed into finding the solution of $h(S)=g(S)$. Remembering that $S<1$, it is easy to show that in the interval [0, 1], $h(S)$ is strictly decreasing whereas $g(S)$ is strictly increasing.

Therefore, because $h(1)=1$ and $g(1)=\frac{m}{a+1}$ the two curves admit only one intersection point with abscissa $S^*<1$ (Fig. 5).

Proof (Theorem 3)

(i) The roots of Eq. (9) computed in $E_0=(1, 0, 0)$ are

$$\rho_1=-1,\ \ \rho_2=f(1)-1,\ \ \rho_3=-1.$$

Therefore, the steady state E_0 turns out asymptotically stable if

$\rho_2<0 \Rightarrow f(1)<1$ that is $m<a+1$.

(ii) In $E^*=(S^*, N^*, p^*)$, Eq. (9) can be written as follows

$$\begin{aligned}&\rho^3+(a_{11}+a_{22}+a_{33})\rho^2\\&\quad+(a_{12}a_{21}+a_{11}a_{22}-a_{13}a_{31}+a_{23}a_{32}+a_{11}a_{33}+a_{22}a_{33})\rho+\\&\quad+a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}\\&\quad-a_{13}a_{22}a_{31}-a_{13}a_{21}a_{32}=0\end{aligned} \quad (18)$$

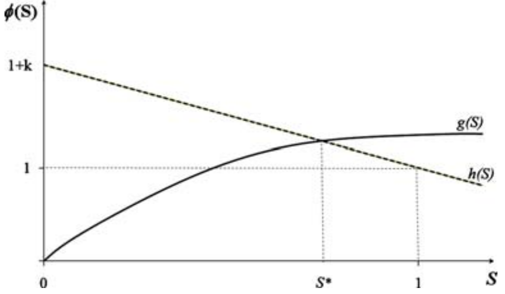


Fig. 5 Here we represent the plots of the two functions $h(S)$ and $g(S)$ defined in (12) in the proof of Theorem 4. These *curves*, as shown, admit only one intersection point with abscissa $S^*<1$

where

$$\begin{aligned}&a_{11}=-\frac{a+S^{*2}}{S^*(a+S^*)},\ a_{12}=-\frac{1-S^*}{N^*},\ a_{13}=2\alpha p^*\left(1-S^*\right),\\&a_{21}=\frac{aN^*}{S^*(a+S^*)},\ a_{22}=-k\left(1-S^*\right),\ a_{23}=-2\alpha p^*N^*,\\&a_{31}=\frac{ap^*}{S^*(a+S^*)},\ a_{32}=2k\left(1-S^*\right),\ a_{33}=-1-2\alpha p^{*2}\end{aligned}$$

are positive constants. In order to study the stability properties of the equilibrium, we can use the Routh–Hurwitz criterion. We observe that, being

1. $a_{11}+a_{22}+a_{33}>0$
2. $a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}-a_{13}a_{22}a_{31}-a_{13}a_{21}a_{32}=\dfrac{-\left(1-S^*\right)\left(-\frac{a+ak+kS^{*2}}{S^*\left(a+S^*\right)}-2kp^*\left(p^*+2N^*\right)\right)}{\alpha}>0$
3. $(a_{11}+a_{22}+a_{33})(a_{12}a_{21}+a_{11}a_{22}-a_{13}a_{31}+a_{23}a_{32}+a_{11}a_{33}+a_{22}a_{33})-(a_{12}a_{21}a_{33}+a_{11}a_{22}a_{33}-a_{12}a_{23}a_{31}+a_{11}a_{23}a_{32}+-a_{13}a_{22}a_{31}-a_{13}a_{21}a_{32})>0$

then all the hypotheses of the Routh–Hurwitz criterion are satisfied and the local stability of the equilibrium E^* follows.

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Arbuscular Mycorrhizal Fungi Protect a Native Plant from Allelopathic Effects of an Invader

Kathryn Barto · Carl Friese · Don Cipollini

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Abstract The allelopathic potential of the Eurasian invasive plant *Alliaria petiolata* has been well documented, with the bulk of the effects believed to be mediated by arbuscular mycorrhizal fungi (AMF). We exposed the herbaceous annual *Impatiens pallida*, which is native to North America, to fractionated *A. petiolata* extracts at four developmental stages (germination, presymbiosis growth, symbiosis formation, and symbiosis growth) by using exposure levels expected to be similar to field levels. Surprisingly, we found strong direct effects on *I. pallida* germination and growth, but no indirect effects on *I. pallida* growth mediated by AMF. We also observed strong synergistic effects with a complete *A. petiolata* extract that inhibited *I. pallida* germination and presymbiosis root growth more than either a glucosinolate or flavonoid enriched fraction alone. In fact, the flavonoid enriched fraction tended to stimulate germination and presymbiosis root growth. In contrast to these strong direct effects, *I. pallida* plant growth during both the symbiosis formation and symbiosis growth phases was unaffected by *A. petiolata* extracts. We also found no inhibition of AMF colonization of roots or soils by *A. petiolata* extracts. We show that AMF can actually ameliorate allelopathic effects of an invasive plant, and suggest that previously observed allelopathic effects of *A. petiolata* may be due to direct inhibition of plant and fungal growth before symbiosis formation.

K. Barto · D. Cipollini
Department of Biological Sciences, Wright State University,
3640 Colonel Glenn Hwy,
Dayton, OH 45435, USA

C. Friese
Department of Biology, University of Dayton,
300 College Park,
Dayton, OH 45469, USA

Present Address:
K. Barto (✉)
Freie Universität Berlin, Institut für Biologie, Plant Ecology,
Altensteinstraße 6,
14195 Berlin, Germany
e-mail: barto@zedat.fu-berlin.de

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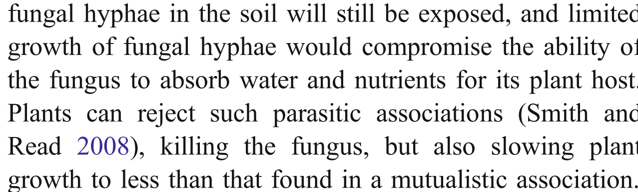
Introduction

Allelopathy has historically been defined as direct inhibition of plant growth by organic compounds produced by a donor plant (Choesin and Boerner 1991; Barkosky et al. 1999). These compounds act through several mechanisms that include causing DNA mutations (Hashimoto et al. 1984; Hashimoto and Shudo 1996; Wu et al. 2000), blocking photosynthesis (Einhellig et al. 1993; Gonzalez and Estevez-Braun 1997), and/or triggering programmed cell death (Bais et al. 2003). While these mechanisms clearly act directly on the target plants, many potential mechanisms of indirect inhibition exist as well (Inderjit and Weiner 2001). For example, compounds released by a donor plant can influence nutrient availability in a way that is more beneficial for the donor plant (Inderjit and Mallik 1999). Also, microbial populations supported by a donor plant can selectively inhibit growth of surrounding plants (Kaminsky 1981), or microbes that benefit surrounding plants can be inhibited by a donor plant (Stinson et al. 2006). Such beneficial microbes include the arbuscular mycorrhizal fungi (AMF), with which more than 80% of plants surveyed associate (Smith and Read 2008). AMF are obligately symbiotic and provide their host plants with

mineral nutrients and water in exchange for photosynthate (Smith and Read 2008). Many plants depend on AMF for normal growth, and any inhibition of AMF, therefore, would also indirectly slow plant growth.

Alliaria petiolata (M. Bieb.) Cavara and Grande (garlic mustard), a Eurasian native that is invasive in North America, can inhibit seed germination and growth of native plant species, and AMF and ectomycorrhizal germination and growth (Vaughn and Berhow 1999; Roberts and Anderson 2001; Prati and Bossdorf 2004; Stinson et al. 2006; Callaway et al. 2008; Wolfe et al. 2008; Barto and Cipollini 2009b), although inhibition does not always occur (McCarthy and Hanson 1998; Cipollini et al. 2008; Barto and Cipollini 2009a). Many of these results are based on seed and spore germination assays, or exposures that began before seed and spore germination. Stinson et al. (2006) observed reductions in colonization of woody species exposed to *A. petiolata* during the symbiosis formation phase, but a herbaceous plant exposed during the symbiosis formation phase was unaffected (Barto and Cipollini 2009a). Allelopathic effects of *A. petiolata* have been attributed to glucosinolates and isothiocyanates (Vaughn and Berhow 1999), but extracts enriched in alliarinoside and several flavonoid glycosides, known anti-herbivory compounds from *A. petiolata* (Haribal and Renwick 1998; Haribal et al. 2001), reduced AMF spore viability more than a glucosinolate enriched fraction (Callaway et al. 2008). Plant responses to alliarinoside and the flavonoid glycosides before symbiosis formation have not yet been determined.

We explored the effects of *A. petiolata* extracts on the four major developmental stages of a plant-mycorrhizae association (1. germination, 2. presymbiosis growth, 3. symbiosis formation, and 4. symbiosis growth), each in a separate experiment. The germination phase includes any stratification requirement up to and including emergence of the radicle from the seed. Growth of the plant before colonization by AMF is included in the presymbiosis growth phase. Although the non-mycorrhizal condition is usually rare in the field, sites with a long invasion history are likely to have low AMF colonization potential (Roberts and Anderson 2001), and this experiment demonstrates how well a re-introduced native grows when exposed to *A. petiolata* extracts without the benefit of AMF. During the symbiosis formation phase, contact between AMF and the plant is initiated and fungal structures begin to form inside the plant root. Allelopathic compounds could interfere with signaling between host plants and AMF, thereby limiting the formation of the symbiosis. Finally, the symbiosis continues to operate throughout the remainder of the plant's lifetime in the symbiosis growth phase. Once a symbiosis is formed, fungal structures inside the root are likely to be somewhat insulated from allelopathic effects. However,



fungal hyphae in the soil will still be exposed, and limited growth of fungal hyphae would compromise the ability of the fungus to absorb water and nutrients for its plant host. Plants can reject such parasitic associations (Smith and Read 2008), killing the fungus, but also slowing plant growth to less than that found in a mutualistic association.

The objectives of this experiment were to assess the effects of glucosinolate and flavonoid glycoside enriched fractions of *A. petiolata*, alone and in concert, on growth of a North American native plant and its associated AMF. These effects were assessed across multiple life stages in order to determine the importance of AMF in mediating any observed allelopathic effects. We expected glucosinolate and flavonoid enriched fractions to inhibit growth of *I. pallida*, primarily indirectly through inhibition of AMF in later life stages.

Methods and Materials

Extracts were prepared from first year *A. petiolata* plants randomly collected from the Wright State University Nature Preserve (39°48.0′N, 84°1.0′W) during late Spring, when the establishment of mycorrhizal symbioses with host plants is occurring actively. Plant tissues, leaves and roots, either were extracted immediately after collection, or flash frozen and stored at −20°C to maintain a standard lot of material for extract preparation. It is unclear whether secondary metabolites produced by *A. petiolata* enter the environment as root exudates, leaf leachates, or both, so we used both leaves and roots to prepare our extracts. Glucosinolates were separated from the flavonoid glycosides by using a butanol/water fractionation as described in Callaway et al. (2008), yielding glucosinolate enriched and flavonoid enriched fractions. The glucosinolates and flavonoids were by far the most abundant compounds in their respective fractions, but it was not our intention to purify these compounds because we wanted the combined treatment of glucosinolate and flavonoid enriched fractions to represent the complete *A. petiolata* phytochemical profile. All experiments were dosed at a rate equivalent to 3.3 mg *A. petiolata* tissue equivalents per g assay media, a dose that was estimated by assuming equivalent transfer rates of glucosinolates from plants to soils for *A. petiolata* as reported for Brassicaceae used as biofumigants (Callaway et al. 2008).

Impatiens pallida Nutt. (pale jewelweed) was chosen as the target plant because it grows in the same woodland habitats invaded by *A. petiolata* and is dependent on AMF for normal growth (K Barto, personal observation). While AMF generally are not thought to be host specific, certain plant-fungus associations are more effective than others (Stampe and Daehler 2003; Johnson et al. 2004), so we used naturally associated AMF of *I. pallida*. Mycorrhizal inocu-

lum consisted of finely chopped *I. pallida* roots collected from a population in Yellow Springs, Ohio (39 °47.0′N, 83 °52.5′W). *Impatiens pallida* seed were collected from the same population for all experiments. Inoculum was prepared in the fall and stored at 5°C until needed.

The seed germination experiment was conducted in small glass dishes (9 cm diam), which were stored at 3°C throughout the experiment. The presymbiosis growth experiment and the symbiosis formation and symbiosis growth experiments were conducted in root-viewing chambers made of 13×30×0.15 cm glass plates held ½ cm apart by silicon on 3 sides (Friese and Allen 1991). All experimental chambers, glass dishes, and root-viewing chambers, were filled with a 1:1 mix of field soil and sterile coarse sand or the same mix with activated carbon (AC) added at a rate of 20 ml/l soil, as has been used in other experiments with *A. petiolata* (Prati and Bossdorf 2004). Activated carbon sorbs organic compounds and serves as an additional experimental control to verify that effects are due to allelochemicals (Inderjit and Callaway 2003). Field soil, collected from an *A. petiolata* free area of the Wright State University Nature Preserve, has a high proportion of clay, and forms impenetrable bricks in the chambers without the addition of sand (K. Barto, personal observation). Root-viewing chambers were covered with foil and stored at a slight angle to encourage root and hyphal growth along one glass plate (Friese and Allen 1991). With the exception of the seed germination experiment, all chambers were placed under fluorescent grow-lights (130 μmol PAR/m^2/sec) at ~22°C in a growth room. Four separate experiments were conducted, examining one developmental stage per experiment.

Germination We examined the effects of treatments on germination by concurrently adding test fractions and ungerminated seeds to glass dishes. Seed germination was assessed by placing 5 *I. pallida* seeds on the soil surface, and applying doses every other week. Dishes were covered but not sealed to allow oxygen to circulate. Germination was scored weekly for 6 mo. We used 4 extracts (glucosinolate enriched fraction, flavonoid glycoside enriched fraction, combined fraction, and water control) crossed with 2 activated carbon treatments (with or without). There were 5 replicate chambers for each treatment combination. Final percent germination was analyzed using ANOVA with activated carbon and extract as fixed factors, followed by Tukey's HSD test where indicated. Data did not need to be transformed to meet assumptions of normality and homogeneity of variances, and were analyzed using R 2.7.1 (R Development Core Team 2008).

Presymbiosis Plant Growth We examined the presymbiosis growth stage by simultaneously adding test fractions and germinated seeds to root-viewing chambers. *Impatiens pallida* seeds were germinated in sterile water at 3°C (Leck 1979). Preliminary experiments showed that seedlings quickly became colonized with AMF in field soils, so chambers were treated with 190 mg/l of the fungicide chlorothalonil (Daconil®, Syngenta Crop Protection, Inc, Greensboro, NC, USA) every other day to maintain the uncolonized status of seedlings in this experiment. Chlorothalonil can inhibit plant growth at high does, so we conducted preliminary experiments and determined that this dosage schedule would block mycorrhizal colonization of seedlings without directly impacting *I. pallida* growth (K. Barto, unpublished data). Presymbiosis seedling growth was assessed by placing one newly germinated *I. pallida* seed on the soil surface while adding test compounds. Doses were applied once a week throughout the experiment by injecting 5 ml of extract into the chamber through each of three injection sites, located 7.5, 15, and 23 cm from the top of the chamber, along the 30.5 cm side. This volume was sufficient to wet the entire soil volume without forcing water out of the base of the chamber. The roots were traced onto transparency film and digitized every 3 d until a root reached the bottom of the chamber.

Total root length and area of the root system were quantified using ImageJ (NIH: http://rsb.info.nih.gov/ij/). We also measured the box-counting fractal dimension (FD), which quantifies exploration efficiency of the system independently of rhizosphere size (Walk et al. 2004), using the FracLac plugin for ImageJ (http://rsb.info.nih.gov/ij/plugins/frac-lac.html) (Barto and Cipollini 2009b). Height of the aboveground portion of *I. pallida* also was recorded once a week. Many plants died before the end of the experiment, so we also recorded life span. Growth rates for plant height, root length, and rhizosphere area, along with the box-counting fractal dimension of the root system and the life span of the plants, were analyzed using PROC GLM and ANOVA with activated carbon and extract as fixed factors followed by Tukey's HSD test where indicated. Data were transformed as necessary to meet assumptions of ANOVA and analyzed with SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA).

Symbiosis Formation We examined the symbiosis formation stage by injecting compounds into inoculated root-viewing chambers with one growing *I. pallida* seedling. *Impatiens pallida* seeds were stratified in water at 3°C to stimulate germination, then planted in chambers. Mycorrhizal inocula, which consisted of chopped roots of field-collected *I. pallida*, was mixed with the soil at a rate of 1 g inocula/100 g soil before filling chambers. Injections began as soon as seeds were added, and doses were applied once a week throughout the experiment. Mycorrhizal structures were identified non-destructively in the chambers by their

fluorescence after excitation with 460 nm light (Friese and Allen 1991). Observations were made with an Eclipse TE 2000-S microscope with a B-2E/C filter cube.

Chambers were observed along horizontal 6 cm transect lines centered at the middle of the chamber and spaced vertically 5 cm apart. The first transect line was 2.5 cm below the top of the chamber, and additional lines were observed as the roots grew down through the chamber. In order to quantify AMF abundance inside roots, we scanned along each transect line and scored uncolonized and colonized roots. Then, we calculated the percentage of colonized roots observed in each chamber. In order to quantify AMF abundance outside of plant roots, we also scanned along each transect line, but due to time constraints we could not quantify hyphal abundance along the entire transect line. Instead, in every third field of view that did not contain a root, we scored for presence of absence of AMF hyphae. Only non-septate hyphae were counted as AMF. Then we calculated the percentage of fields of view containing AMF hyphae.

Height of the above ground portion of *I. pallida* also was recorded each week. Plants were harvested at the end of the experiment, dried at 30°C to constant mass, and root and shoot dry mass were measured. There were 4 treatments (glucosinolate enriched fraction, flavonoid glycoside enriched fraction, combined fraction, and water control) for each of two carbon amendments (with or without). There were ten replicate chambers per treatment combination yielding 80 chambers total. Data were analyzed as described for the presymbiosis growth experiment, excluding life span. Also, dry mass of the root, shoot, and the root to shoot ratio were analyzed by ANOVA with activated carbon and extract as fixed factors followed by Tukey's HSD test where indicated. Data did not need to be transformed and were analyzed with SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). In addition, percent colonization of the root and soil (untransformed) were analyzed using a repeated measures ANOVA with activated carbon and extract as fixed factors using STATISTICA Version 8.0 (StatSoft, Inc.).

Symbiosis Growth We examined the symbiosis growth stage by injecting compounds into chambers with already established symbioses between *I. pallida* and its associated mycorrhizal fungi. Established symbioses were generated by growing *I. pallida* seedlings in root-viewing chambers in field soil containing mycorrhizal inoculum. When seedlings were 4 wk old, colonization was verified by fluorescence microscopy before beginning injections of compounds. Injections were repeated weekly thereafter until the end of the experiment. Fungal development characteristics were monitored as in the symbiosis formation experiment. Plates were monitored weekly with observations beginning when plants were 3 wk old. Plants were harvested at the end of the experiment, dried at 30°C to constant mass, and root and shoot dry mass were measured. There were 4 treatments (glucosinolate enriched fraction, flavonoid glycoside enriched fraction, combined fraction, and water control) for each of 2 carbon amendments (with or without). There were 10 replicate chambers per treatment combination yielding 80 chambers total. Growth rate for plant height, percent colonization of roots and soil, dry mass of the root, shoot, and the root to shoot ratio were analyzed as for the symbiosis formation experiment.

Results

Germination Impatiens pallida seeds began to germinate after 20 weeks of stratification, and no additional seed germinated after 24 weeks. Exposure to a flavonoid or glucosinolate enriched fraction alone had no significant effect on germination. However, germination was lower in dishes dosed with a combined extract than either control dishes or those receiving a flavonoid enriched fraction (Fig. 1, $F_{3,32}$ = 3.71, P=0.021). Activated carbon did not affect germination rates ($F_{1,32}$ = 0.08, P=0.779), and there was no interaction between AC and extract ($F_{3,32}$ = 1.36, P=0.273).

Presymbiosis Growth Impatiens pallida plants in chambers with AC grew faster than plants in chambers without AC (Fig. 2a, Table 1). *Alliaria petiolata* extracts had no effect on *I. pallida* height (Table 1). Total root length of *I. pallida* plants increased more quickly in plants grown in soil containing AC than in plants grown without AC (Fig. 2b,

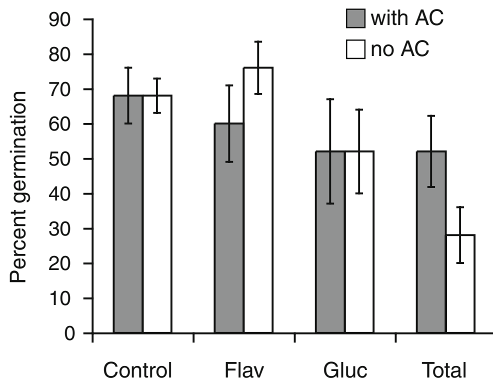


Fig. 1 Percent germination of *Impatiens pallida* seeds exposed to *Allairia petiolata* extracts during stratification, means ± 1 SE. Control—Chambers treated with water only. Flav—Chambers treated with a flavonoid enriched fraction. Gluc—Chambers treated with the glucosinolate enriched fraction. Combined—Chambers treated with both flavonoid and glucosinolate enriched fractions. with AC—Chambers contained activated carbon. no AC—Chambers did not contain AC. (N=5)

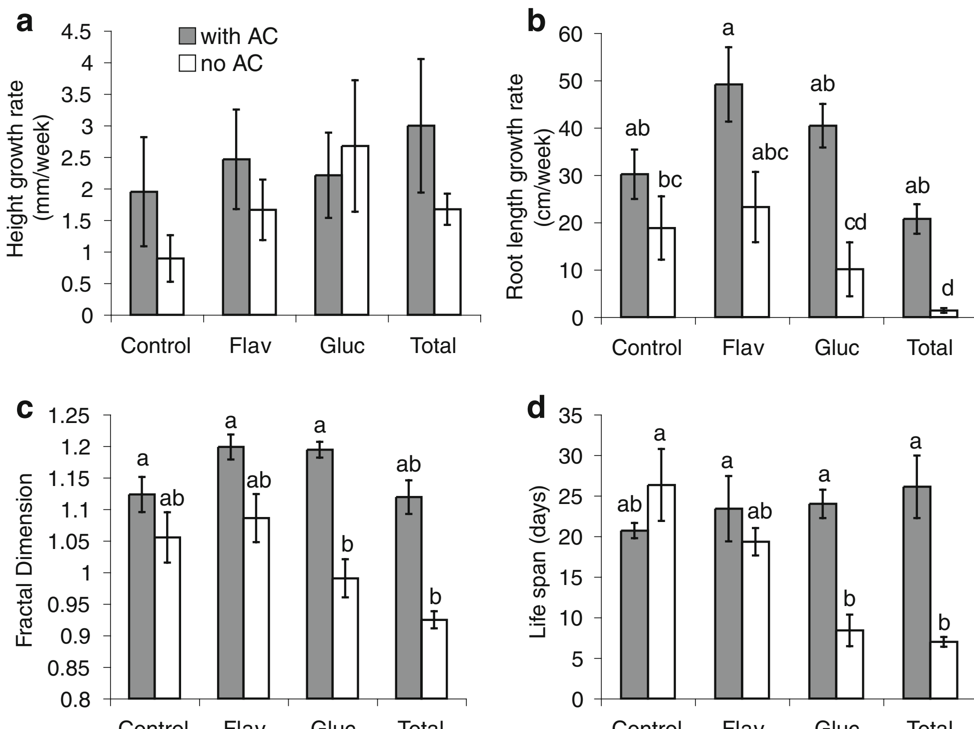


Fig. 2 Responses of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the presymbiosis growth phase, means ± 1 SE. Control—Chambers treated with water only. Flav—Chambers treated with a flavonoid enriched fraction. Gluc—Chambers treated with the glucosinolate enriched fraction. Combined—Chambers treated with both flavonoid and glucosinolate enriched fractions. with AC—Chambers contained activated carbon. no AC—Chambers did not contain AC. Different letters indicate significant differences by Tukey HSD at α=0.05. **a** Height growth rate (N=9–10). **b** Root length growth rate (N=10). **c** Box counting fractal dimension of the root system (N=10). **d** Life span of *I. pallida* plants (N=10)

Table 1). Root growth varied with extract treatment, and only the combined extract significantly reduced root growth (Fig. 2b, Table 1). Since these plants were not yet colonized by AMF, these impacts represent direct effects of *A. petiolata* extracts on root growth. Patterns for growth of rhizosphere area followed those of root length (Table 1).

Root structure of *I. pallida* plants also was affected by *A. petiolata* extracts. The planar box-counting fractal dimension, which quantifies the exploration efficiency of the root system, was significantly higher in chambers with AC than in chambers without (Fig. 2c, Table 1). As for root length and rhizosphere area, the fractal dimension was highest in chambers that received a flavonoid enriched fraction and lowest in chambers treated with a combined extract (Fig. 2c, Table 1). The interaction between AC and extract also was significant (Table 1), with AC increasing the fractal dimension significantly only in chambers dosed with a glucosinolate enriched fraction (Fig. 2c).

Impatiens pallida plants grown in soil with AC lived longer than plants in soil without AC (Fig. 2d; Table 1). The glucosinolate enriched fraction and the combined *A. petiolata* extract significantly shortened *I. pallida* life span, while a flavonoid enriched fraction had no effect (Fig. 2d; Table 1). This inhibition was seen only in chambers without AC (Fig. 2d; Table 1).

Symbiosis Formation During the symbiosis formation phase, growth rates of *I. pallida* height, root, and rhizosphere area were unaffected by AC or *A. petiolata* extract (Table 2). Root structure of *I. pallida* plants was unaffected by AC (Table 2). However, regardless of AC treatment, root systems of plants exposed to a glucosinolate

Table 1 Results of ANOVA for growth rates of height, root length, rhizosphere area, and final box-counting fractal dimension, and life span of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the presymbiosis phase, with AC (activated carbon) and extract as factors. Bold text indicates P<0.05

Source		Height		Root length		Rhizosphere area		FD		Life span	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AC	1	6.12	**0.016**	59.49	**<0.001**	51.10	**<0.001**	54.51	**<0.001**	43.43	**<0.001**
extract	3	2.12	0.108	7.12	**<0.001**	6.71	**<0.001**	6.35	**<0.001**	7.64	**<0.001**
AC*extract	3	1.28	0.292	2.56	**0.062**	1.68	0.180	2.79	**0.047**	12.35	**<0.001**
		Error *df*: 54		Error *df*: 68		Error *df*: 68		Error *df*: 68		Error *df*: 68	

enriched fraction had significantly higher fractal dimensions (mean ± SE; 1.24±0.015) than those exposed to a combined fraction (1.18±0.020; Table 2). Root systems of control plants (1.21±0.011) or those exposed to a flavonoid enriched fraction (1.19±0.013) had fractal dimensions indistinguishable from either extreme. Shoot and root dry masses of plants at the end of the experiment, and the root to shoot ratio, were unaffected by AC or *A. petiolata* extracts (Table 2).

Arbuscular mycorrhizal colonization of *I. pallida* roots was higher in chambers containing AC than those without throughout the experiment, even though colonization declined with time in all chambers (Fig. 3a, Table 3). Hyphal abundance in the soil was unaffected by *A. petiolata* extract, but increased with time in chambers containing AC while remaining relatively constant in chambers without AC (Fig. 3b, Table 3).

Symbiosis Growth During the symbiosis growth phase, plant height increased more quickly in chambers without AC (mean ± SE; 6.63±0.42 cm/week) than in chambers with AC (5.41±0.25 cm/week; Table 4). Growth rate was unaffected by *A. petiolata* extracts, and there was no interaction between AC and extract (Table 4). Since *I. pallida* were given a month in the chambers before dosing began, roots had filled the chambers to such an extent that it would have been extremely difficult to track further development. Root morphology data, therefore, was not collected.

Arbuscular mycorrhizal fungal colonization was higher in roots of plants grown in soils containing AC than in soils without (Fig. 4a, Table 5), but fewer hyphae were found in soils containing AC than in soils without (Fig. 4b, Table 5). Root colonization declined over time (Fig. 4a, Table 5). Root dry mass was higher in chambers without AC (mean ± SE; 0.19±0.026 g) than in chambers with AC (0.15±0.032 g), as was shoot dry mass (without AC: 0.11±0.0076 g, with AC: 0.08±0.0047 g; Table 4). Both were unaffected by *A. petiolata* extracts (Table 4). The root to shoot ratio was not affected by *A. petiolata* extracts (Table 4).

Discussion

As expected, glucosinolate and flavonoid enriched fractions from *A. petiolata* extracts inhibited the growth of *I. pallida*. Synergistic effects of the flavonoid and glucosinolate enriched fractions were observed in the germination and presymbiosis growth phases, but not in either symbiosis phase. The flavonoid-enriched fraction had no effect alone, but appeared to enhance the slight toxicity of the glucosinolate enriched fraction, leading to the very low germination or growth rates observed after exposure to a combined fraction. These effects likely are due to the glucosinolates and flavonoids themselves, given their abundance in the extracts, but it is possible that unidentified compounds in the extracts also contributed to effects. Synergistic interactions among allelopathic compounds also occur in *Triticum*, where phenolic compounds increase cell permeability in root tips, allowing greater uptake of mutagenic benzoxazinoids (Blum et al. 1992; Hashimoto and Shudo 1996). Synergistic mechanisms also operate in *Desmodium* and *Sorghum,* which block parasitism by *Striga* plants by concurrently stimulating germination of *Striga* seeds while inhibiting further root growth (Chang et al. 1986; Weston et al. 1989; Yoder 2001; Tsanuo et al. 2003). While the specific mechanism of action remains to be elucidated, synergistic effects among compounds produced by *A. petiolata* clearly limited plant and AMF growth during the early stages of development.

We frequently observed declines in root colonization over time, potentially because as the plants aged and their roots filled the soil volume, assistance from AMF became less necessary. Plants have some control over intraradical fungal growth (Smith and Read 2008) and could have limited fungal development as the plants became root bound. This is supported by the decline in root colonization values in chambers containing AC, as well as control chambers receiving only water. Soil colonization values remained relatively constant throughout the symbiosis growth phase, and even increased in chambers containing AC during the symbiosis formation phase, suggesting that extraradical fungal structures are less dependent on the age

Table 2 Results of ANOVA for growth rates of height, root length, and rhizosphere area, final box-counting fractal dimension (FD), root and shoot dry masses, and root to shoot ratio of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the symbiosis formation phase, with AC (activated carbon) and extract as factors. Bold text indicates $P<0.05$

Source		Height		Root length		Rhizosphere area		FD		Shoot		Root		Root:Shoot	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AC	1	0.57	0.454	0.22	0.637	1.55	0.217	0.22	0.642	0.35	0.555	0.30	0.584	0.05	0.819
extract	3	0.82	0.486	2.59	0.059	0.06	0.982	3.11	**0.032**	0.76	0.522	0.13	0.942	0.40	0.750
AC*extract	3	0.91	0.442	0.40	0.756	0.82	0.489	0.41	0.744	0.13	0.944	0.81	0.493	0.95	0.423
		Error *df*: 72		Error *df*: 72		Error *df*: 72		Error *df*: 72		Error *df*: 72		Error *df*: 72		Error *df*: 72	

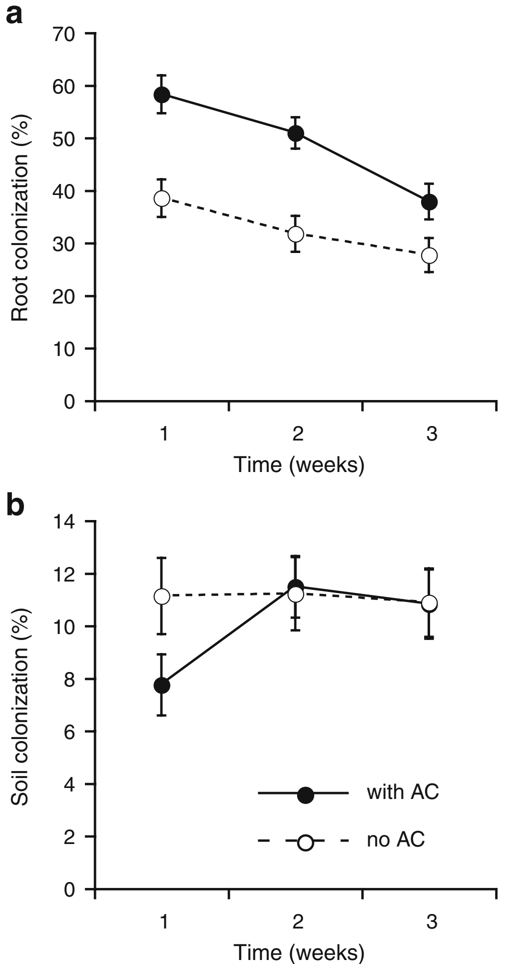


Fig. 3 Responses of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the symbiosis formation phase, means ± 1 SE. with AC—Chambers contained activated carbon. no AC—Chambers did not contain AC. **a** Percentage of root length colonized by AMF over time (N=9–10). **b** Percentage of soil area containing AMF hyphae over time (N=9–10)

or status of the symbiosis than intraradical fungal structures. The plant likely has more control over intraradical fungal structures than extraradical, and the continued growth of extraradical structures may represent growth by the fungus away from its host in the face of decreasing support, via intraradical structures.

Activated carbon sorbs organic compounds and should rescue plants from allelopathic inhibition when incorporated into soils (Inderjit and Callaway 2003). Surprisingly, AC had no effect on germination. Extracts were added to test soils every other week since *A. petiolata* metabolites generally are not stable in non-sterile soils (Gimsing et al. 2006, 2007; Barto and Cipollini 2009c), but sinigrin has a half-life in excess of 120 d in non-sterile soil water (Tsao et al. 2000). Although low temperatures did not limit degradation of flavonoids (Barto and Cipollini 2009c), it is possible that compounds accumulated over the course of the six month experiment to levels that saturated the AC present in the soils. As expected, AC additions in the presymbiosis experiment rescued plants exposed to *A. petiolata* extracts. Plants exposed to extracts that were also growing in soils containing AC were not distinguishable from control plants receiving only water. Activated carbon effects during the symbiosis formation and symbiosis growth stages were less consistent, further suggesting that allelopathic inhibition was not as important in these life stages.

We found extensive evidence of inhibition of seed germination and presymbiosis plant growth in the absence of AMF. The lack of these non-AMF mediated inhibitory effects during the symbiosis formation and growth phases suggests that the AMF may actually be protecting the plant from the allelopathic compounds. This is not simply an age effect, where a larger plant is more able to resist inhibition than a small plant, because the presymbiosis and symbiosis formation experiments both began with germinated seed. The only difference was that the soil for the symbiosis formation experiment also contained AMF inocula with no previous exposure to *A. petiolata*.

In contrast to prior work that demonstrated inhibition of AMF by *A. petiolata* (Roberts and Anderson 2001; Stinson et al. 2006; Callaway et al. 2008; Barto and Cipollini 2009b), we found no evidence for impacts on AMF. However, earlier research monitored AMF that were not associated with a host plant (Roberts and Anderson 2001; Stinson et al. 2006; Callaway et al. 2008), or began dosing AMF in the presymbiosis phase (Barto and Cipollini 2009b), where we saw inhibition also. When exposures began in the symbiosis formation phase, Barto and Cipollini (2009a) did not see inhibition of *I. pallida* growth

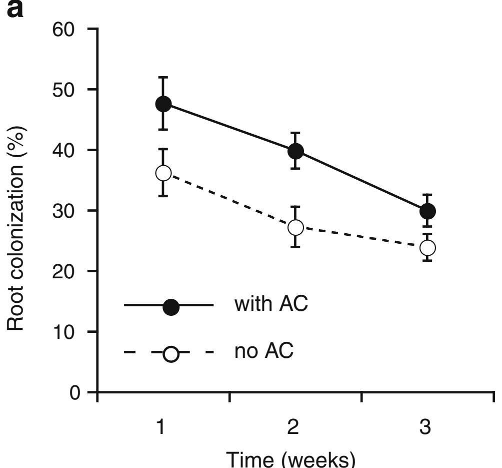
Table 3 Results of repeated measures ANOVA for percent root and soil AMF colonization in chambers exposed to *Alliaria petiolata* extracts during the symbiosis formation phase, with AC (activated carbon), and extract as factors. Bold text indicates P<0.05

Source		Root		Soil	
	df	*F*	*P*	*F*	*P*
AC	1	19.61	**<0.001**	0.29	0.589
extract	3	0.53	0.661	0.60	0.619
AC*extract	3	0.78	0.508	1.25	0.298
Error	72				
time	2	14.65	**<0.001**	4.23	**0.016**
time*AC	2	1.58	0.209	3.66	**0.028**
time*extract	6	0.71	0.644	0.96	0.453
time*AC*extract	6	0.35	0.907	0.38	0.891
Error	144				

Table 4 Results of ANOVA for height and dry mass of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the symbiosis growth phase, with AC (activated carbon), and extract as factors. Bold text indicates $P<0.05$

Source		Height		Shoot		Root		Root:Shoot	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AC	1	5.41	**0.023**	5.83	**0.018**	4.42	**0.039**	2.87	0.095
extract	3	1.89	0.139	1.80	0.155	0.73	0.536	1.44	0.239
AC*extract	3	0.30	0.822	0.35	0.786	1.02	0.390	0.84	0.592
		Error *df*: 70		Error *df*: 71		Error *df*: 71		Error *df*: 70	

by the same *A. petiolata* extracts used here. Roberts and Anderson (2001) relied on spore and seed germination assays, and used a dose about eight times higher than that used in our study, so results cannot be compared directly. Stinson et al. (2006) used doses of *A. petiolata* extracts twice as high as those used here, which could account for the inhibition observed in that study. Ideally, allelopathy studies should use exposure levels equivalent to those found in the field. However, *A. petiolata* metabolites degrade rapidly, and rarely reach detectable levels in natural soils (Barto and Cipollini 2009c). This likely means that the actual bioactive compounds are degradates of the compounds produced by the plant. We, thus, used doses estimated to be realistic field levels (Callaway et al. 2008). Soil levels under naturally occurring plants likely are even lower than our estimated values, and any impacts not seen at these doses are unlikely to be important in the field.



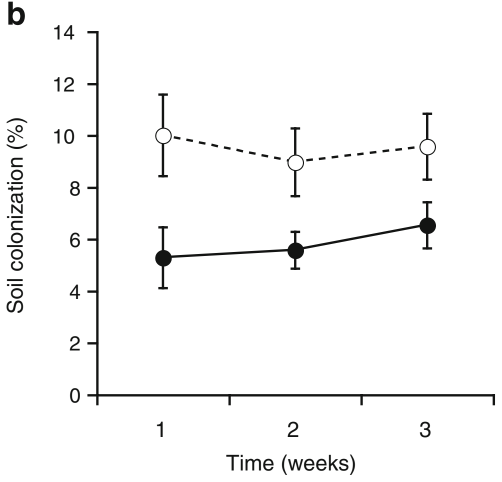


Fig. 4 Responses of *Impatiens pallida* plants exposed to *Alliaria petiolata* extracts during the symbiosis growth phase, means ± 1 SE. with AC—Chambers contained activated carbon. no AC—Chambers did not contain AC. **a** Percentage of root length colonized by AMF over time (N=8–10). **b** Percentage of soil area containing AMF hyphae over time (N=8–10)

Stinson et al. (2006) also observed reduced AMF colonization of tree seedlings in conditioned soils, which could be a legacy effect of very low inoculum potential due to direct suppression of spore viability and germination (Roberts and Anderson 2001; Stinson et al. 2006; Callaway et al. 2008), rather than allelopathic inhibition of colonization in the plant. Callaway et al. (2008) used a soil conditioning approach, and found reduced AMF spore viability and infectivity, and reduced emergence of plant seedlings in soil conditioned by *A. petiolata*. However, all of these endpoints occur before the symbiosis is established. The final biomass of plants grown in conditioned soil was lower than that of plants grown in control soil, suggesting that even after the beneficial mycorrhizal

Table 5 Results of repeated measures ANOVA for percent root and soil AMF colonization in chambers exposed to *Alliaria petiolata* extracts during the symbiosis growth phase, with AC (activated carbon), and extract as factors. Bold text indicates $P<0.05$

Source		Root		Soil	
	df	*F*	*P*	*F*	*P*
AC	1	8.90	**0.004**	5.99	**0.017**
extract	3	1.11	0.350	1.11	0.350
AC*extract	3	0.58	0.629	0.69	0.564
Error	72				
time	2	11.59	**<0.001**	0.83	0.439
time*AC	2	1.17	0.315	1.57	0.211
time*extract	6	1.47	0.191	1.21	0.306
time*AC*extract	6	1.28	0.269	1.92	0.081
Error	144				

symbiosis becomes established, the direct inhibition incurred during early life stages cannot be overcome.

We suggest that observed allelopathic inhibition by *A. petiolata* is due to legacy effects of direct inhibition of plant and fungal partners before the symbiosis is established, rather than to inhibition of colonization during an established symbiosis. This may seem like a purely mechanistic distinction because the end result of reduced AMF colonization and reduced plant growth is still the same, but direct inhibition is more easily addressed during restoration, especially when healthy AMF can ameliorate allelopathic effects. The strongest allelopathic effects occur during seed and spore germination and presymbiosis growth, thus suggesting that bypassing these life stages during restorations will be more successful than traditional methods of sowing ungerminated seed. More work is needed to determine whether or not other plant species, especially the woody species used by Stinson et al (2006), also are more sensitive to allelopathic inhibition before associating with mycorrhizal fungi then after the symbiosis has been initiated. Field studies that incorporate germinated seed and fresh AMF inocula also could be used to distinguish between legacy effects and ongoing allelopathic inhibition.

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Subterranean Herbivore-induced Volatiles Released by Citrus Roots upon Feeding by *Diaprepes abbreviatus* Recruit Entomopathogenic Nematodes

Jared G. Ali · Hans T. Alborn · Lukasz L. Stelinski

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Abstract Herbivore-induced volatile emissions benefit plant hosts by recruiting natural enemies of herbivorous insects. Such tritrophic interactions have been examined thoroughly in the above-ground terrestrial environment. Recently, similar signals have also been described in the subterranean environment, which may be of equal importance for indirect plant defense. The larvae of the root weevil, *Diaprepes abbreviates*, are a serious pest of citrus. Infestations can be controlled by the use of entomopathogenic nematodes, yet the interactions between the plant, insect and nematode are poorly understood and remain unpredictable. In bioassays that used a root zone six-arm olfactometer, citrus roots ('Swingle citrumelo' rootstock) recruited significantly more entomopathogenic nematodes (*Steinernema diaprepesi*) when infested with root weevil larvae than non-infested roots. Infested plants were more attractive to nematodes than larvae alone. Roots damaged by weevil larvae attracted more nematodes than mechanically damaged roots and sand controls. By dynamic *in situ* collection and GC-MS analysis of volatiles from soil, we determined that four major terpene compounds were produced by infested plant roots that were not found in samples from non-infested roots or soil that contained only larvae. Solvent extracts of weevil-infested roots attracted more nematodes than extracts of non-infested roots in a two choice sand-column bioassay. These findings suggest that Swingle citrus roots release induced volatiles as an indirect defense in response to herbivore feeding, and that some of these induced volatiles function as attractants for entomopathogenic nematodes.

Key Words Entomopathogenic nematodes · *Diaprepes abbreviatus* · Herbivore induced volatiles · Below-ground tritrophic interactions · *Steinernema diaprepesi*

J. G. Ali · L. L. Stelinski (✉)
Entomology and Nematology Department,
Citrus Research and Education Center, University of Florida,
700 Experiment Station Road,
Lake Alfred, FL 33850, USA
e-mail: stelinski@ufl.edu

H. T. Alborn
Center for Medical, Agricultural, and Veterinary Entomology,
Agricultural Research Service, U.S. Department of Agriculture,
Gainesville, FL 32608, USA

Introduction

Plants produce an array of signals with diverse roles, thus providing them with responses necessary to survive in their dynamic environment. Examples of plants luring organisms and thereby facilitate their reproductive requirements are ubiquitous and often taken for granted (Pichersky and Gershenzon 2002). Less acknowledged is the ability of a plant to manipulate the behavior of organisms to serve defensive roles (Turlings and Wäckers 2004). However, examples of such tritrophic interactions, among plants, herbivores, and natural enemies are common (Agrawal and Rutter 1998; Agrawal and Karban 1999; Baldwin and Preston 1999; Dicke et al. 2003).

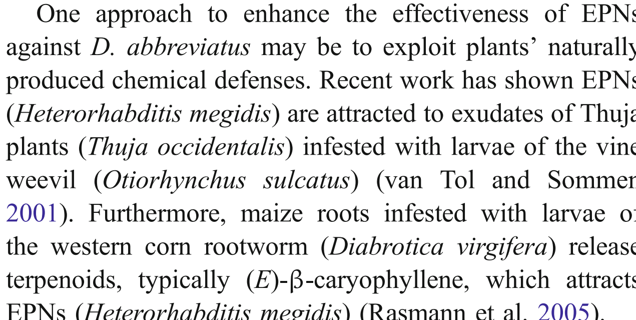
Herbivore feeding on plants results in release of volatile compounds, which may attract arthropod predators and/or parasitoids. For instance, lima bean plants (*Phaseolus lunatus*), release volatiles when infested with spider mites (*Tetranychus urticae*), which attract the predatory mite *Phytoseiulus persimilis* (Takabayashi and Dicke 1996). Oviposition can also stimulate plant exudates that are attractive to egg parasitoids; the legume, *Vicia faba* emits volatiles that attract the egg parasitoid, *Trissolcus basalis* after oviposition by the Pentatomid, *Nezara viridula*

(Colazza et al. 2004). Specific compounds from both the plant and salivary elicitors from the herbivore have been shown to mediate these interactions (Alborn et al. 1997). For example, methyl salicylate attracts herbivore predators (e.g., De Boer and Dicke 2004). Volicitin, found in oral secretions of caterpillars (*Spodoptera exigua*), has been well characterized and induces volatile production in maize (Alborn et al. 2000; Turlings et al. 2000). As the details of above-ground tritrophic interactions have become substantially resolved (Vet et al. 1991; Vet and Dicke 1992), recent attention has focused on analogous communication systems in the subterranean environment.

Volatile signaling by plant roots can contribute to belowground defense by acting as antimicrobial or anti-herbivore substances (Tumlinson et al. 1992, 1999; Neveu et al. 2002; Bais et al. 2006). Plants also can benefit by releasing herbivore-induced volatile emissions that recruit natural enemies of subterranean herbivores, as shown by van Tol and Sommen (2001), Aratchige et al. (2004), and Rasmann et al. (2005). The pressure from belowground pests of plants is significant and likely imparts selection pressure for evolution of induced plant responses.

Diaprepes abbreviatus (L.) is a significant belowground pest of plant roots on more than 290 plant species including citrus, sugarcane, vegetables, potatoes, strawberries, woody field-grown ornamentals, sweet potatoes, papaya, guava, mahogany, containerized ornamentals, and non-cultivated wild plants (Simpson et al. 2000). *Diaprepes abbreviatus* was first introduced into Florida in 1964 (Beavers and Selhime 1975). Over the past 40 years, it has contributed significantly to the spread of disease and damage to citrus, ornamental plants, and other crops causing approximately $70 million in damage annually (Weissling et al. 2002). *Diaprepes abbreviatus* damage the vegetative portion of plants by notching young leaves (Fennah 1940). Mature adults lay eggs between older leaves and emerging first instars drop to the soil where they develop and feed on roots causing the most severe damage to plants (Fennah 1940; Schroeder 1992). Currently, the most effective method for controlling the larval stage is with entomopathogenic nematodes (EPN), from the genera *Heterorhabditis* or *Steinernema* (Downing et al. 1991; Schroeder 1992).

EPNs are obligate parasites that kill their host with the aid of a symbiotic bacterium (Poinar 1990). Mass-produced EPNs have been used for control of *D. abbreviatus* by citrus growers for over 20 years (Duncan et al. 1999). Mass release of EPNs can effectively reduce larval populations of *D. abbreviatus* (Downing et al. 1991; Schroeder 1992; Bullock et al. 1999). However, the reported efficacy of EPNs against *D. abbreviatus* ranges from 0% to >90% suppression (Adair 1994; Bullock et al. 1999; McCoy et al. 2000), and thus improved consistency of this tactic is desired.



One approach to enhance the effectiveness of EPNs against *D. abbreviatus* may be to exploit plants' naturally produced chemical defenses. Recent work has shown EPNs (*Heterorhabditis megidis*) are attracted to exudates of Thuja plants (*Thuja occidentalis*) infested with larvae of the vine weevil (*Otiorhynchus sulcatus*) (van Tol and Sommen 2001). Furthermore, maize roots infested with larvae of the western corn rootworm (*Diabrotica virgifera*) release terpenoids, typically (*E*)-β-caryophyllene, which attracts EPNs (*Heterorhabditis megidis*) (Rasmann et al. 2005).

In this investigation, we quantified the behavior of the entomopathogenic nematode, *Steinernema diaprepesi* Nguyen & Duncan, in response to citrus plants damaged by larval *D. abbreviatus*. We show that EPNs are attracted to weevil-damaged roots, but not so to mechanically damaged roots, undamaged roots or larvae alone. We also identified volatile compounds induced by weevil feeding and showed that EPN response is specifically mediated by solvent extracts of infested roots.

Methods and Materials

Insects *Diaprepes abbreviatus* larvae were obtained from a culture at University of Florida's Citrus Research and Education Center (CREC) in Lake Alfred, FL, USA. This culture was periodically supplemented from a large culture maintained at the Division of Plant Industry Sterile Fly Facility in Gainesville, FL, USA. Larvae were reared on an artificial diet developed by Beavers (1982) using procedures described by Lapointe and Shapiro (1999). Larvae used in experiments were 3rd to 6th instars.

Nematodes *Steinernema diaprepesi* were isolated from *D. abbreviatus* larvae buried in a commercial citrus orchard in Florida. The nematodes were then reared in last-instar greater wax moth larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), at approximately 25°C according to procedures described in Kaya and Stock (1997). Infective juveniles (IJs) that emerged from insect cadavers into White traps (White 1927) were stored in shallow water in transfer flasks at 15°C for up to 2 wk prior to use.

Plants 'Swingle citrumelo' (*Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf.) rootstock is prominent in commercial citrus production. The prevalence of this genotype is due to its tolerance to blight, citrus tristeza virus, plant parasitic nematodes, and *Phytophthora* spp., as well as cold tolerance (Stover and Castle 2002). The extensive use of this rootstock in commercial citrus production justified its use in this investigation. All plants were grown and maintained at the CREC in Lake Alfred, FL, USA in a greenhouse at 26°C, and 60–80% RH.

Olfactometer EPN response to *D. abbreviatus*-infested roots was tested with a root zone olfactometer (Analytical Research Systems, Gainesville, FL, USA) according to the design described in Rasmann et al. (2005). The olfactometer consists of a central glass chamber (8 cm diam and 11 cm deep) attached by six side arms to six glass pots (5 cm diam and 11 cm deep) in which various plants/ treatments were tested. The side arms are joined to the six treatments pots with Teflon connectors fitted with a fine mesh filter impervious to nematodes (2300 mesh, Smallparts, Inc., Miramar, FL, USA). For all tests, the olfactometer was filled with sand that had been autoclaved for 1 hr at 250°C and then adjusted to 10% moisture (dry wt. sand:water volume; W/V). In tests involving plants, seedlings were given 3 d to adjust to their sand filled olfactometer for each experiment.

In the first experiment, we tested nematode response to weevil-infested plants vs. non-infested controls. Infested plants were subjected to 3 d of feeding by 3rd–6th instar weevil larvae. Non-infested plants were not exposed to weevils. Three of the arms of the olfactometer were assigned randomly to a weevil-infested plant, while the remaining three received the non-infested control. IJ nematodes (2,500) were released into the central olfactometer chamber. Twenty-four hours after nematode release, the olfactometer was disassembled, and nematodes from each connecting arm were recovered from soil using Baermann extractors; extracted nematodes were collected and counted with a dissection scope. The tests were replicated with ten nematode releases for each treatment.

In the second experiment, we compared the response of EPNs to weevil-infested plants with larvae alone in sand. The bioassay consisted of three chambers with plants infested with six larvae each (as above) and three chambers containing six larvae in sand only. The experimental protocol and sampling procedures were otherwise identical to experiment 1.

In a third experiment, EPN response was assayed to weevil-infested plants (as above) vs. mechanically damaged roots. The treatments compared consisted of two mechanically damaged plants, two infested plants, and two sand only control arms. Treatments were assigned randomly to chambers. Plant roots were damaged mechanically by stabbing them five times daily with a metal corkborer for 3 d prior to nematode release (7 mm diam). This damage procedure was used because it visually resembled the type of damage inflicted by feeding *D. abbreviatus* larvae after 72 h. All other experimental and sampling procedures were identical to those described for experiment one.

Volatile Collections The objective of this experiment was to identify volatiles emitted by citrus roots damaged by weevil larvae. Volatiles were collected from 1) sand alone (negative control), 2) larvae alone in sand, 3) non-infested plant roots, and 4) weevil-infested roots. Each treatment was prepared within a chamber and connecting arm of the 6-chambered olfactometer and filled with the same 10% moistened sand as in the bioassays. Larvae, non-infested plants, and infested plants were maintained for 3 d before sampling. All plants were maintained in the olfactometer chambers for 3 d prior to weevil infestation. Thereafter, each chamber of the olfactometer containing a treatment was connected to a vacuum pump (ARS, Gainesville, FL, USA) for 24 hr with a suction flow of 0.8 ml/min. Compounds emitted from chambers were collected on adsorbent traps filled with 50 mg Super-Q,800–1000 mesh (Alltech Deerfield, IL, USA) held in glass fittings between the chamber and vacuum pump. Thereafter, Super-Q traps were rinsed with 150 µl of dichloromethane into individual 2.0 ml clear glass vials (Varian, Palo Alto, CA, USA, part number: 392611549 equipped with 500 µl glass inserts).

GC-MS Analysis A 1 µl aliquot of each dichloromethane extract was injected onto a GC-MS gas chromatograph (HP 6890) equipped with 30 m×0.25-mm-ID, 0.25 µm film thickness DB-5 capillary column (Quadrex, New Haven, CT, USA), interfaced to a 5973 Mass Selective Detector (Agilent, Palo Alto, CA, USA), in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10±°C/min to 260°C. The carrier gas used was helium at a flow average velocity of 30 cm/sec. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C in CI and 220°C in EI. EI Spectra library search was performed using a floral scent database compiled at the Department of Chemical Ecology, Göteborg Sweden, the Adams2 terpenoid/natural product library (Allured Corporation, Adams 1995) and the NIST05 library. When available, mass spectra and retention times were compared to those of authentic standards.

EPN Response to Root Extracts The objective of this experiment was to compare EPN response to solvent extracts of citrus roots before and after weevil feeding. Citrus plants were placed individually into chambers of the 6-arm olfactometer for 3 d as previously described. Thereafter, volatiles were collected from chambers for 24 hr as described above in the volatile collections procedure. Six larvae were placed into each chamber containing a plant and allowed to feed for 3 d. Thereafter, volatiles were collected a second time from the intact feeding system for 24 hr. The adsorbent Super-Q traps from both treatments (before and after feeding) were extracted by rinsing with 150 µl of dichloromethane directly after their 24 hr collections as described above.

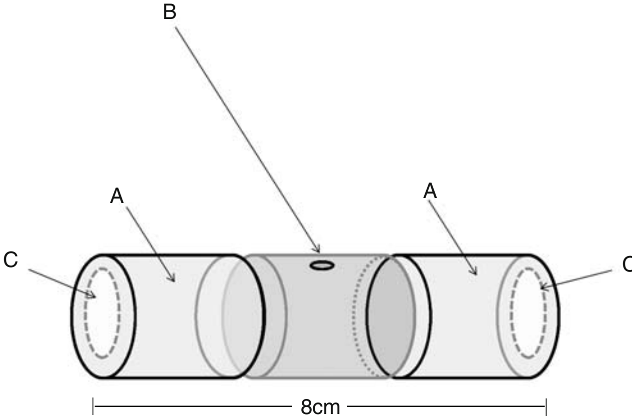


Fig. 1 Schematic diagram of sand column assay unit. Glass jar (17 ml) with samples at base (**a**), connecting tube (3 cm) with hole for nematode application (**b**), extracts placed on filter paper (**c**), arena was filled with heat sterilized sand at 10% moisture for all assays

To quantify EPN response to the root extracts collected, a two choice sand-filled olfactometer was used (Fig. 1). The olfactometer consists of three detachable sections: two opposing glass jars (Fig. 1a) (16 ml BTL, sample type 111, CLR, SNAPC, Wheaton, Millville, NJ, USA), which contained treatments and a central connecting tube 3 cm in length (Blue Max™ 50 ml polypropylene conical tube 30×115 mm, Becton Dickinson Labware, Becton Dickinson Company, Franklin Lakes, NJ, USA), with an apical hole into which nematodes were applied (Fig. 1b). Extracts were placed on filter paper, which was allowed to dry 30 sec for solvent evaporation. Thereafter, filter papers were placed on the bottoms of each glass jar (Fig. 1c), which subsequently were filled with 10% saturated, sterilized sand as described above. The central chamber connecting the two jars (arms of the olfactometer) also was filled with sterilized and moistened sand. The entire olfactometer was 8 cm in length when assembled with two possible extract treatments at opposite ends of the nematode release point. Nematodes (200 IJs) were applied into the central orifice of the connecting tube and given 8 hr to respond. Thereafter, the column was disassembled and the contents of the two collection pots were sampled using Baermann extractors; extracted nematodes were collected and counted. The experiment was replicated ten times.

Statistical Analysis Paired *t*-tests were used to compare nematode response in experiments testing root extracts in the two-choice olfactometers (df=9). Data from experiments using the six-arm olfactometer were analyzed with a log-linear model. Given that these data did not conform to simple variance assumptions implied in using the multinomial distribution, quasi-likelihood functions were used to compensate for the over dispersion of nematodes within the olfactometer (Turlings et al. 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (R Development Core Team 2004).

Results

Olfactometer Bioassays Significantly more EPNs were found attracted to *D. abbreviatus*-infested roots than non-infested control roots (F=12.76, df=1, 58, P<0.001) (Fig. 2a). Infested roots attracted more EPNs per arm than those containing larvae alone (F=13.78, df=1, 58, P<0.001) (Fig. 2b). More EPNs were attracted to *D. abbreviatus*-infested roots than to either mechanically damaged roots or the sand control (F=12.34, df=2, 57, P<0.001) (Fig. 2c).

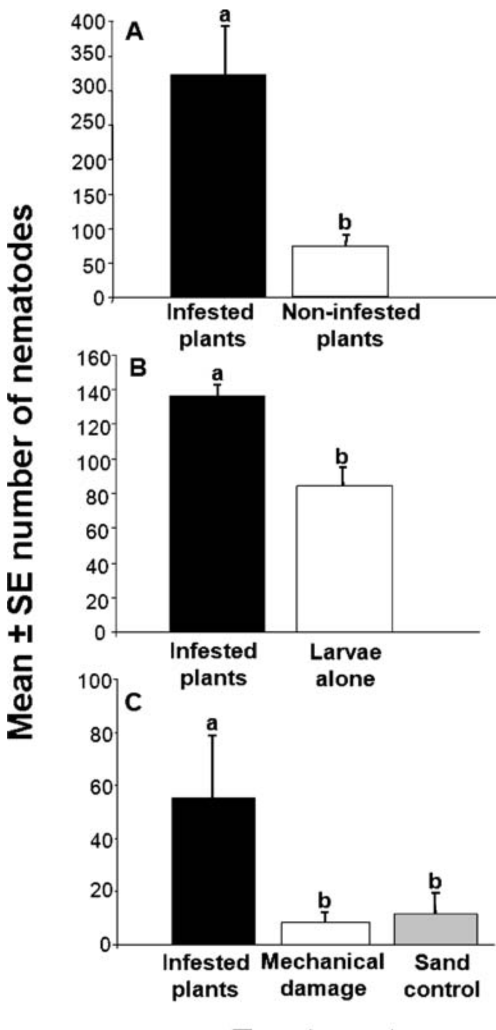


Fig. 2 Mean number of *Steinernema diaprepesi* attracted to chambers containing weevil-infested plants vs. non-infested control plants (**a**), weevil-infested plants versus larvae alone (**b**), weevil-infested plants, mechanically damaged plants or sand control (**c**). Each panel represents a separate experiment (N=10) conducted in a 6-arm olfactometer. Different letters indicate statistical significance at P<0.001

Table 1 GC-MS identification of volatiles from Swingle citrumelo rootstock (*Citrus paradise* × *Poncirus trifoliate*)

Peak #	RT	Name	CAS#	Infested root	Non-infested root	Larvae only
1	7.50	α-pinene[a,b]	000080-56-8	+	+	−
2	8.08	β-pinene[a,b]	000127-91-3	+	+	−
3	10.81	Geijerene[b]	006902-73-4	+	−	−
4	12.93	Pregeijerene[b]	020082-17-1	+	−	−
5	14.75	α-Santalene[b]	000512-61-8	+	−	−
6	14.93	α-Z-Bergamotene[b]	018252-46-5	+	−	−

[a] Synthetic standard comparison. [b] Indentification was based on comparisons of retention times with standard and spectral data from Adams, EPA, and Nist05 Libraries

There was no significant attraction to mechanically damaged roots as compared with the sand control (P=0.34) (Fig. 2c).

GC-MS Analysis Both α-pinene and β-pinene were identified in non-infested and infested plant roots by GC-MS (Table 1). *Diaprepes abbreviatus*-infested roots released four additional unique compounds that were not present in non-infested roots (Table 1). Two C_{12} terpenes were the most abundant, and they were consistently present in infested roots. These were geijerene and its precursor pregeijerene (Fig. 3). On-column GC/MS analyses showed significantly less geijerene and a comparable increase of pregeijerene, strongly suggesting a thermal degradation of pregeijerene to geijerene during GC analyses with splitless injection. It is therefore an open question how much geijerene might actually be released by the infested roots. The above six compounds were absent from pots containing larvae alone (Table 1).

EPN Response to Root Extracts Significantly more EPNs were found in arms containing solvent extracts of *D. abbreviatus*-infested roots than non-infested roots (P=0.03) (Fig. 4).

Discussion

Interactions between EPNs and their host insects, competitors, and natural enemies are well documented, but the degree to which herbivore-induced plant signals alter EPN orientation is largely unknown (Jaffee and Strong 2005; Duncan et al. 2007). Carbon dioxide has long been known

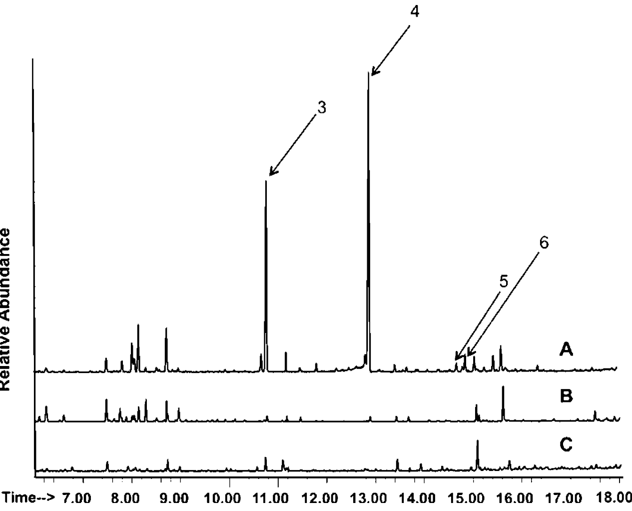


Fig. 3 Example chromatograms showing volatile profiles of *Diaprepes abbreviatus*-infested plants, non-infested plants and larvae alone. Volatile profile of infested *Citrus paradise* × *Poncirus trifoliate* rootstock (**a**) Volatile profile of non-infested *Citrus paradise* × *Poncirus trifoliate* rootstock (**b**) Volatile profile of *D. abbreviatus* alone in sand (**c**). All samples were collected for a 24 hr. Geijerene (3), Pregeijerene (4), α-Santalene (5), α-Z-Bergamotene (6). (Compound numbers correspond to Table 1)

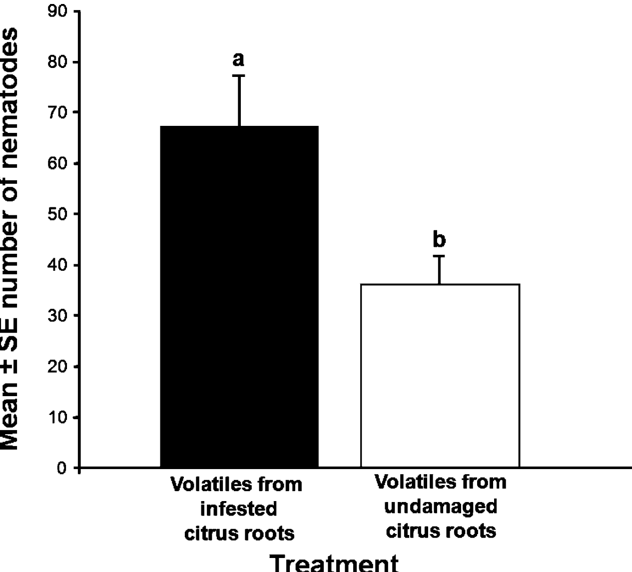


Fig. 4 Mean number of nematodes attracted to volatiles from *Diaprepes abbreviatus*-infested roots compared with volatiles from undamaged roots. Different letters indicate statistical significance at P=0.03

to attract nematodes to plant roots (Gaugler et al. 1980; Prot and Van Gundy 1981). However, functioning alone, such an ambiguous signal might not allow efficient host location by EPNs. van Tol and Sommen (2001) postulated that plants produce induced compounds that attract EPNs; this hypothesis has been confirmed in two systems (Boff et al. 2002; Rasmann et al. 2005). Furthermore, (*E*)-β-caryophyllene has been identified as the specific EPN recruitment signal emitted by maize roots damaged by corn rootworms (Rasmann et al. 2005).

The current results indicate that Swingle citrumelo rootstock releases herbivore induced volatiles that recruit EPNs. Our results also suggest that 'geijerenes' mediate this response. These C_{12} terpenes have not been described for citrus previously; however, they are known for insecticidal, antifeedant, and oviposition deterrent effects in leaves of other rutaceous plant species (Kiran et al. 2006; Kiran and Devi 2007). Geijerenes also have been described in hairy root cultures of *Pimpinella anisum* (Santos et al. 1998). Although these compounds were consistently present in infested root samples and are presumed candidate attractants for *S. diaprepesi*, we have yet to confirm the behavioral activity of the individual compounds. Solvent extracts of infested roots attracted *S. diaprepesi* suggesting that one or a blend of these compounds may be active. Fractionation studies of the induced compounds via preparative gas chromatography in concert with two choice bioassays of the partitioned profile may enable us to resolve the role of individual compounds on EPN behavior.

Recent identification of an EPN recruitment chemical is in the initial stages of application for crop protection and has been promising (Degenhardt et al. 2003, 2009; Turlings and Ton 2006). Direct application of (*E*)-β-caryophyllene to soil has been shown to reduce rootworm damage through enhanced action of their EPNs (Rasmann et al. 2005). Furthermore, recent advances in biochemistry/molecular genetics have made it possible to engineer cultivated maize that releases (*E*)-β-caryophyllene and recruits EPNs, thus protecting roots from herbivore damage (Degenhardt et al. 2003, 2009; Hiltpold et al. 2010). The currently investigated citrus rootstock system is different from the annual maize cropping system for which EPN recruitment is already being developed for corn rootworm management. Perennial systems characterized by fewer disturbances are believed to support more effective biological control than annually disturbed crops (Southwood and Comins 1976). Thus, augmenting the impact of *S. diaprepesi* in a perennial tree fruit system by application of recruitment chemicals may prove even more effective than in annual crops.

It also will be informative to investigate the parent lines of the Swingle rootstock, *Citrus paradisi* and *Poncirus trifoliata* to determine if either or both lines exhibit the herbivore-induced EPN recruitment seen in the hybrid. We plan also to investigate if other non-citrus hosts of *D. abbreviatus* release induced recruitment signals. Given the wide host range of *D. abbreviatus,* it will be important to determine the breath of this EPN recruitment response among its diverse host plants.

Several nematode species attack *D. abbreviatus*. *Steinernema glaseri*, *S. carpocapsae*, and *Heterorhabditis bacteriophora* were investigated initially as possible control agents (Downing et al. 1991; Schroeder 1992). Of the species evaluated in laboratory bioassays and greenhouse trails, *S. riobrave* and a Florida isolate of *H. indica* were the most effective (Shapiro-Ilan and McCoy 2000). Currently, *S. riobrave* and *H. indica* are formulated for commercial application against *D. abbreviatus* in Florida citrus. These two EPN species, in addition to *S. diaprepesi*, will be evaluated and compared in similar future studies to determine whether the tentatively identified EPN recruitment signals are specific to the natively occurring EPN associated with the weevil or whether these signals function more broadly for other EPN species.

We report here for the first time an *in situ* method for sampling subterranean herbivore-induced volatiles during real time insect feeding. Previously used methods involve freeze-drying and crushing root samples (Rasmann et al. 2005), which will affect and misrepresent volatile production from intact roots. The currently described method allows identification of belowground volatiles as they are released over time without disturbance to the system.

The current results indicate that a commercially used citrus rootstock emits induced volatile chemicals in response to herbivore feeding that attract beneficial nematodes. Identification of the specific active compounds may lead to the development of an augmentive EPN recruitment tactic that improves biological control of *D. abbreviatus*. Also, such identification would be the first step towards development of genetically-engineered citrus rootstocks for enhanced recruitment of EPNs. Alternatively, it is possible that engineering plants for increased release of terpenes in general may prove effective (Schnee et al. 2006).

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Induction of Phenolic Glycosides by Quaking Aspen (*Populus tremuloides)* Leaves in Relation to Extrafloral Nectaries and Epidermal Leaf Mining

Brian Young · Diane Wagner · Patricia Doak · Thomas Clausen

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Abstract We studied the effect of epidermal leaf mining on the leaf chemistry of quaking aspen, *Populus tremuloides*, during an outbreak of the aspen leaf miner, *Phyllocnistis populiella*, in the boreal forest of interior Alaska. *Phyllocnistis populiella* feeds on the epidermal cells of *P. tremuloides* leaves. Eleven days after the onset of leaf mining, concentrations of the phenolic glycosides tremulacin and salicortin were significantly higher in aspen leaves that had received natural levels of leaf mining than in leaves sprayed with insecticide to reduce mining damage. In a second experiment, we examined the time course of induction in more detail. The levels of foliar phenolic glycosides in naturally mined ramets increased relative to the levels in insecticide-treated ramets on the ninth day following the onset of leaf mining. Induction occurred while some leaf miner larvae were still feeding and when leaves had sustained mining over 5% of the leaf surface. Leaves with extrafloral nectaries (EFNs) had significantly higher constitutive and induced levels of phenolic glycosides than leaves lacking EFNs, but there was no difference in the ability of leaves with and without EFNs to induce phenolic glycosides in response to mining. Previous work showed that the extent of leaf mining damage was negatively related to the total foliar phenolic glycoside concentration, suggesting that phenolic glycosides deter or reduce mining damage. The results presented here demonstrate that induction of phenolic glycosides can be triggered by relatively small amounts of mining damage confined to the epidermal tissue, and that these changes in leaf chemistry occur while a subset of leaf miners are still feeding within the leaf.

Key Words *Populus tremuloides* · *Phyllocnistis populiella* · Epidermal leaf mining · Induced defense · Phenolic glycosides · Extrafloral nectaries · Alaska

B. Young · D. Wagner · P. Doak
Institute of Arctic Biology and Department of Biology & Wildlife, University of Alaska Fairbanks,
Fairbanks, AK 99775-7000, USA

T. Clausen
Department of Chemistry and Biochemistry,
University of Alaska Fairbanks,
Fairbanks, AK 99775-6160, USA

Present Address:
B. Young (✉)
Department of Forest Sciences, University of Alaska Fairbanks,
Fairbanks, AK 99775-7200, USA
e-mail: bdyoung@alaska.edu

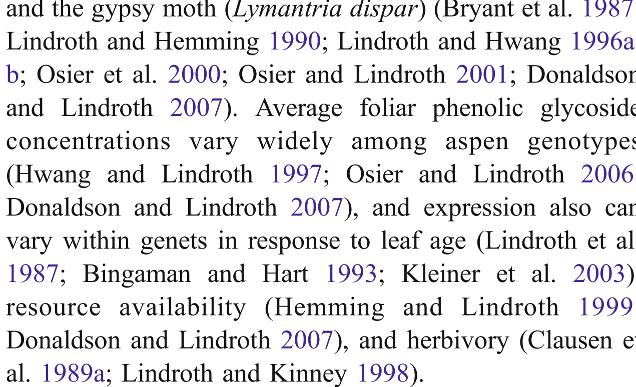
Introduction

Some plants respond to herbivore damage by increasing production of chemical, physical, or biotic defenses, responses that can help protect the remaining tissue against further damage (Karban and Baldwin 1997). Induction of resistance traits may have evolved in response to the costs of resistance. Such costs may include tradeoffs in resource allocation between growth and resistance and enhanced apparency to specialist herbivores that use resistance traits to locate hosts (Rhoades 1979; Strauss et al. 2002). For trees such as immature *Populus* spp., which must grow quickly to avoid being shaded by competitors or consumed by browsers (Bokalo et al. 2007), cost savings may allow a greater proportion of resources to be invested in early growth and survival.

Quaking aspen (*Populus tremuloides* Michx.) is a fast-growing forest tree subject to attack by a variety of vertebrate and invertebrate herbivores, including several

insect species subject to large-scale population outbreaks (Mattson et al. 2001). Since the late 1990s and continuing through 2009, aspen in Alaska and western Canada have experienced a severe outbreak of the aspen leaf miner (*Phyllocnistis populiella* Chambers, Lepidoptera: Gracillariidae) (Fig. 1a) (U.S. Forest Service 2006, 2007, 2008). Unlike most leaf miner taxa, which feed on the photosynthetic tissue of the mesophyll, *P. populiella* feeds only on the cells of the leaf epidermis, removing very little leaf biomass (Condrashoff 1964) (Fig. 1b). Although feeding damage is restricted to the epidermis, at high density *P. populiella* can reduce photosynthesis and slow the growth of aspen ramets (Wagner et al. 2008).

The leaves of aspen trees produce phenolic glycosides, which can provide resistance against some herbivores (Philippe and Bohlmann 2007). Four types of phenolic glycosides appear in aspen leaves, salicin, salicortin, tremuloidin, and tremulacin, with salicortin and tremulacin having greater biological activity than the others (Lindroth et al. 1987). Phenolic glycosides can reduce the growth rate and survivorship of several of aspen's most devastating pests, including the large aspen tortrix (*Choristoneura conflicta*), the forest tent caterpillar (*Malacosoma disstria*),



and the gypsy moth (*Lymantria dispar*) (Bryant et al. 1987; Lindroth and Hemming 1990; Lindroth and Hwang 1996a, b; Osier et al. 2000; Osier and Lindroth 2001; Donaldson and Lindroth 2007). Average foliar phenolic glycoside concentrations vary widely among aspen genotypes (Hwang and Lindroth 1997; Osier and Lindroth 2006; Donaldson and Lindroth 2007), and expression also can vary within genets in response to leaf age (Lindroth et al. 1987; Bingaman and Hart 1993; Kleiner et al. 2003), resource availability (Hemming and Lindroth 1999; Donaldson and Lindroth 2007), and herbivory (Clausen et al. 1989a; Lindroth and Kinney 1998).

In addition to phenolic glycosides, aspen also produce extrafloral nectaries (EFNs). Studies of other plant species indicate that EFNs can function as an indirect defense by attracting arthropod predators and parasitoids of herbivores to the plant (Bentley 1977; Koptur 1992; Röse et al. 2006). In some populations, EFNs increase plant fitness by reducing tissue loss to herbivores (Heil et al. 2004; Rudgers et al. 2004). EFN expression in aspen is extremely variable. EFNs occur on only a subset of aspen leaves, and the frequency of EFN expression varies among shoots, ramets, and genets (Doak et al. 2007). At the level of the leaf, the expression of EFNs and phenolic glycoside concentrations co-vary (Young et al. 2010). After controlling for mean differences among sites, ramets, and shoots, concentrations of foliar phenolic glycosides are about 30% higher in leaves with EFNs than in leaves lacking them (Young et al. 2010). On short aspen ramets, leaves bearing EFNs sustain lower levels of epidermal leaf mining than leaves lacking EFNs (Doak et al. 2007), but this difference may be caused by the higher phenolic glycoside content of EFN-bearing leaves, rather than by the indirect defense of EFN-bearing leaves by the leaf miner's natural enemies (Mortensen 2009; Young et al. 2010).

In this study, we tested whether leaf mining damage restricted to the epidermis induces the production of phenolic glycosides salicortin and tremulacin in leaves with and without EFNs. By comparing the phenolic glycoside concentrations of leaves collected serially from ramets with natural and reduced levels of leaf mining, we tracked the time course of phenolic glycoside expression in young aspen leaves and investigated whether induction has the potential to impact the performance of *P. populiella*.

Fig. 1 **a** Aspen leaf miner moth and eggs on a leaf with two EFNs at the petiole. **b** Epidermal leaf mining by the aspen leaf miner on a leaf without EFNs

Methods and Materials

Natural History Quaking aspen is widely distributed in North America (Mitton and Grant 1996) and reproduces both sexually and asexually, resulting in the formation of clonal stands. In the interior of Alaska, it tends to grow on south facing hillsides and along ridgelines.

The EFNs of *P. tremuloides* are located at the junction of the petiole and the leaf (Trelease 1881). The number of EFNs on a leaf can vary from 0 to 6, with most leaves possessing 0 or 2 (Doak et al. 2007). Among the preformed leaves, the first 5 to 8 leaves to emerge in spring (Critchfield 1960), EFNs are consistently expressed on the most proximal leaf, with frequency of expression decreasing distally along the shoot (Doak et al. 2007). In contrast, the neoformed leaves, which emerge later and extend the growing shoot, typically all possess EFNs (Doak et al. 2007). We focused on the preformed leaves in this study.

The aspen leaf miner *P. populiella* overwinters as an adult and emerges in early to mid May, prior to bud break. Oviposition begins as soon as the protective bud scales are shed, exposing the furled aspen leaves, and continues for about 10 d. Eggs are laid singly on both the upper and lower surfaces of young leaves. The eggs sink into the leaf tissue, and about a week after oviposition the larvae hatch directly into the epidermal tissue. The developing larvae feed on the cells of the leaf epidermis, leaving obvious tracks or mines. Larvae remain on the leaf surface on which they hatched and feed for 9–15 d (Condrashoff 1964). In the final instar, which lasts ≤2 d, larvae cannot feed and form leaf folds in which to pupate (Condrashoff 1964). During the years of this study (2006 and 2007), the median preformed leaf at the study site hosted a single leaf fold (Doak and Wagner, unpublished survey data).

Field Method To investigate the effects of epidermal leaf mining on aspen phenolic glycoside concentrations, we first (2006) experimentally reduced leaf miner densities on a set of aspen ramets by using an insecticide, and we contrasted the mean foliar phenolic glycoside concentration of insecticide-sprayed and naturally-mined ramets at a single point in time. The following year (2007), we measured the concentration of phenolic glycosides on insecticide-sprayed and naturally-mined ramets at intervals during the period of active feeding by the leaf miner.

In early June of 2006, we randomly chose a set of 12 small ramets (0.8–1.2 m in height) near the summit of Ester Dome (64°52′56″N, 148°03′57″W, elevation 720 m) near Fairbanks, Alaska. Ramets were located within a 20× 20 m area and exhibited similar physical characteristics; however, we cannot be certain that they all belonged to a single clone. Of the 12 ramets, half were assigned at random to receive an insecticide treatment. Just as the *P. populiella* eggs began to hatch, the treatment ramets were sprayed with the insecticide spinosad (Conserve; Dow AgroSciences, Indianapolis, IN.USA; concentration 1.56 ml l^{-1}; applied with a hand-powered pump sprayer until runoff). The insecticide was reapplied 6 d later. When the treatment ramets were sprayed with the insecticide, the remaining ramets were sprayed with an equal quantity of water.

This experiment relied on the assumption that the application of insecticide does not prevent aspen from producing an induced response to damage. To test this assumption, we took advantage of the ability of aspen leaves to mount a rapid, localized induction of phenolic glycosides in response to mechanical leaf damage (Clausen et al. 1989a). One day before we planned to harvest the insecticide-sprayed and naturally-mined leaves, we damaged 6 randomly-chosen leaves, from leaf positions 1–5 from two separate shoots, on each of the ramets that had been sprayed with insecticide by cutting them in half diagonally across the mid vein with scissors.

We harvested all leaves (naturally-mined, sprayed, and sprayed and mechanically damaged) 11 d following the approximate onset of leaf mining. At this point, 80–90% of the leaves on the naturally-mined ramets possessed one or more pupal folds, indicating that most of the aspen leaf miner larvae had ceased to feed (Condrashoff 1964). We sampled preformed leaves from the 5 most proximal leaf positions from 3 randomly-chosen shoots per ramet. Leaves were snipped at the petiole and immediately immersed in 10 ml of 50% aqueous MeOH . Cold 50% aqueous MeOH effectively extracts phenolic glycosides from aspen leaves (Bryant et al. 1987; Lindroth and Pajutee 1987). We maintained the leaf samples in solution at 2°C±1°C for 24 h. The leaves were then removed from the extract solution and pressed in a plant press for subsequent analysis of herbivore damage. The extract was stored at −40°C until analysis was conducted.

For each leaf, we counted the number of EFNs and visually estimated leaf mining on the top and bottom surfaces of the leaf and the percent of leaf tissue missing and/or damaged by other herbivores to the nearest 5.0 %. Our visual estimates of mining correlated strongly with measurements made using image analysis software (R^2> 0.9, Doak et al. 2007). For simplicity, we report % total leaf surface mined, calculated as the average of % mining on the top and bottom surfaces. Leaves were scanned using a desktop scanner and leaf area was measured using the image analysis program Scion Image (Fredrick, MD, USA). Leaves were dried at 60°C for 1 wk and weighed to the nearest 0.1 mg.

To determine how foliar phenolic glycoside concentrations changed on a finer time scale during the period of feeding by *P. populiella*, we conducted a second experiment at the same study area in May 2007. We chose 42 ramets ranging in size from 0.5 to 1.6 m in height; all located within a 25×25 m area adjacent to the 2006 site. Again, the genetic identity of the plants was not known. We assigned half of the ramets at random to receive an insecticide treatment. On the day that it appeared that the majority of

leaf miner eggs had hatched, we applied insecticide to treatment ramets and water to the others. The method of insecticide application was identical to the previous year. To accommodate repeated sampling while avoiding the removal of excessive numbers of leaves from individual ramets, we used a larger number of ramets than in 2006 and sampled each ramet on only two occasions. Leaf samples were collected on days 1, 2, 4, 5, 6, 9, and 12 after the onset of leaf mining. On each sampling day, we sampled preformed leaves from leaf positions 2 and 3 on a single shoot from 6 naturally-mined and 6 insecticide-sprayed ramets, chosen at random from the set of ramets that had not yet been sampled. Leaves were again sampled by cleanly snipping leaves at the petioles. Mattson and Palmer (1988) did not find a chemical response of the ramet when the leaf was sampled in this manner. When each ramet had been sampled once, we resampled each ramet a second time, using a different shoot. All leaf samples were handled and processed as in the previous year.

Chemical Analysis We prepared the aspen leaf extracts for analysis by filtering a 1.0 ml aliquot of extract through a 0.45 μm pore poyproplyene membrane syrine filter (Acrodisc, Thermo Fisher Scientific, Waltham, MA, USA). Samples were injected onto a 4.6×250 mm XDB-C8 column (Agilent) attached to a High Performance Liquid Chromatography (HPLC) (Agilent 1100) equipped with a UV/VIS diode array detector (Agilent) and analyzed at 230 nm. The phenolic glycosides were separated using a mobile phase gradient of acetonitrile (CH_3CN) and H_20 with a constant flow rate of 1.0 ml/min. The gradient elution was: 1% CH_3CN (0–4 min), 1–60% CH_3CN (4–10 min), 60–80% CH_3CN (10–15 min), 100% CH_3CN (15–22 min), and 100–1% CH_3CN (22–27 min). This was followed by a 10 min period at 1% CH_3CN prior to the injection of next sample. Salicortin and tremulacin were quantified using purified reference standards (Clausen et al. 1989b).

Statistical Analyses For both 2006 and 2007 data sets, we compared the % leaf mining damage, and the % leaf damage caused by other herbivores, on insecticide-sprayed vs. naturally-mined ramets using Wilcoxon tests.

Preliminary data analysis showed that concentrations of salicortin and tremulacin were highly correlated within leaves (R=0.8, df=166, P<0.01), responded similarly to treatment, and changed in similar ways over time. For simplicity, we therefore report total phenolic glycoside concentrations, calculated as the sum of salicortin and tremulacin.

To test whether insecticide treatment prevented induction of phenolic glycosides in response to damage, we compared the phenolic glycoside concentration of experimentally cut and uncut leaves (2006 data) using a mixed model analysis of covariance (ANCOVA) with mechanical damage as a fixed effect, leaf position as a covariate, and ramet and shoot as random effects.

To test the effect of leaf mining on foliar phenolic glycoside concentration (2006 data, leaves mined for 11 d) we again used ANCOVA, with insecticide treatment, EFNs (dichotomous: absence vs. presence), and their interaction as fixed effects, leaf position as a covariate, and shoot and ramet as random effects.

We tested when induction occurred (2007 data) by comparing the phenolic glycoside concentrations of leaves from naturally-mined and insecticide-sprayed ramets within each sampling date. We first ran a mixed model ANCOVA with insecticide treatment, day sampled, and their interaction as fixed effects, leaf position as a covariate, and shoot and ramet as random effects. We then conducted planned contrasts of the mean foliar phenolic glycoside concentration of naturally-mined and insecticide-sprayed ramets within each sampling day.

Phenolic glycoside concentrations were log transformed to meet parametric assumptions. Mixed model analyses applied the restricted maximum likelihood method and were conducted using JMP IN version 5.1.2 using the Kenward and Roger (1997) method to calculate denominator degrees of freedom (SAS Institute, Cary, NC, USA).

Results

The insecticide treatment prevented all detectable leaf mining damage by *P. populiella* on the treated ramets during both 2006 and 2007 (Table 1). Across all experimental trees, leaf mining represented by far the greatest source of leaf damage, although a small amount of chewing, skeletonizing, and galling damage were also observed (Table 1). Leaf damage due to herbivores other than *P. populiella* was not reduced significantly by the insecticide treatment (Table 1).

Foliar phenolic glycosides were induced in insecticide-sprayed leaves in response to mechanical leaf damage. One day after leaves on sprayed ramets were cut with scissors, the cut leaves had higher concentrations of phenolic glycosides than un-cut leaves ($F_{1,\ 113}$=6.45, P=0.012). The average phenolic glycoside concentration of the cut leaves was 37% higher then the undamaged leaves (back-transformed least square means; damaged leaves=27.3 mg/g, undamaged leaves=19.9 mg/g).

Leaf mining by *P. populiella* induced foliar phenolic glycosides. Eleven days after the approximate onset of leaf mining in 2006, the foliar phenolic glycoside concentration was, on average, 125% higher for ramets that had sustained natural levels of leaf mining damage than for ramets

Table 1 Percent leaf damage (mean±SE) by *Phyllocnistis populiella* and other herbivore taxa after experimental reduction in leaf miner abundance 11 days post treatment. "Other damage" is the sum of chewing, skeletonizing, and galling

	2006		2007	
	Naturally-mined	Insecticide-sprayed	Naturally-mined	Insecticide-sprayed
Top mining	60.7±3.6	0.0±0.0 ***	18.1±3.2	0.0±0.0 ***
Bottom mining	43.9±3.6	0.0±0.0 ***	17.3±4.1	0.0±0.0 ***
Other damage	0.2±0.1	0.3±0.1 n.s.	0.2±0.2	1.1±0.7 n.s.

Mean values for each ramet were compared with Wilcoxon signed rank tests

n.s. $P>0.05$, *** $P<0.001$

sprayed with insecticide (Fig. 2; $F_{1,\ 10}=7.58$, $P<0.001$). Across all naturally-mined and insecticide-sprayed ramets, leaves with EFNs contained on average 26% higher concentrations of phenolic glycosides than leaves lacking EFNs (Fig. 2; $F_{1,\ 148}=5.40$; $P=0.021$). EFN presence and treatment did not interact ($F_{1,\ 145}=0.08$; $P=0.77$), indicating that leaves with and without EFNs were similar in their ability to induce phenolic glycosides in response to mining. Foliar phenolic glycoside concentrations increased along the shoot, with the most distal leaves having the highest concentrations (Fig. 3; $F_{1,\ 144}=6.14$; $P<0.01$).

By monitoring the time course of induction during the next growing season (2007), we found that induction occurred approximately 6–9 days after the onset of mining. Because on each collection day we harvested many fewer leaves ($N=12$) than in the 2006 experiment ($N=180$), we had less statistical power to detect differences between treatments. In the overall ANCOVA model, the mean phenolic glycoside content of leaves varied over time ($F_{6,\ 50}=2.85$, $P=0.01$), but there was no statistically significant effect of insecticide treatment ($F_{1,\ 37}=2.30$, $P=0.14$) or interaction between treatment and time ($F_{6,\ 50}=1.12$, $P=0.36$). However, the results of planned contrasts within days provided information about the timing of induction. During the first six days, average phenolic glycoside concentrations in both naturally-mined and insecticide-sprayed leaves rose, peaking at day 4, and then declined (Fig. 4a). Contrasts within sampling day revealed no significant differences in phenolic glycoside concentration between naturally-mined and insecticide-sprayed leaves up to and including day 6 ($P>0.05$). However, after day 6 the two groups diverged, and the average phenolic glycoside concentration was higher in naturally-mined leaves than in insecticide-treated leaves on day 9 (Fig. 4a, $F_{1,\ 67}=4.42$, $P=0.04$). The average phenolic glycoside concentration of both insecticide-treated and naturally mined ramets increased somewhat between days 9 and 12. While average phenolic glycoside concentration of the mined trees still exceeded that of insecticide-treated trees on day 12, the difference was no longer statistically significant (Fig. 4a, $F_{1,\ 69}=3.32$, $P=0.07$). Leaf mining damage extended over approximately 5% of the leaf surface at the time induction was noted (Fig. 4b).

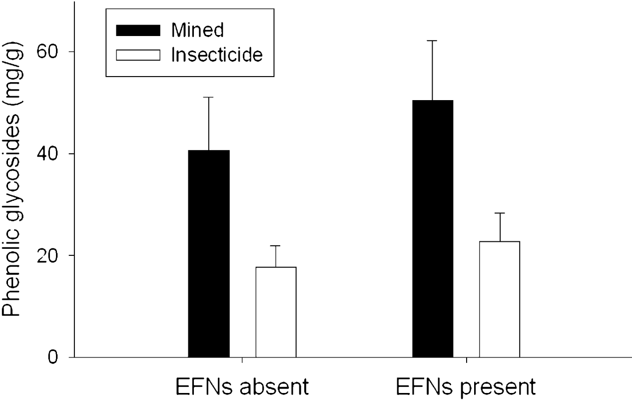


Fig. 2 Concentrations of phenolic glycosides from naturally-mined and insecticide-sprayed leaves with and without EFNs during the summer of 2006 eleven days after the approximate onset of leaf mining. Values are back transformed least squared means±SE. There was a significant effect of insecticide treatment ($P<0.001$) and EFN presence ($P<0.05$), and no interaction between insecticide treatment and EFNs ($P>0.05$)

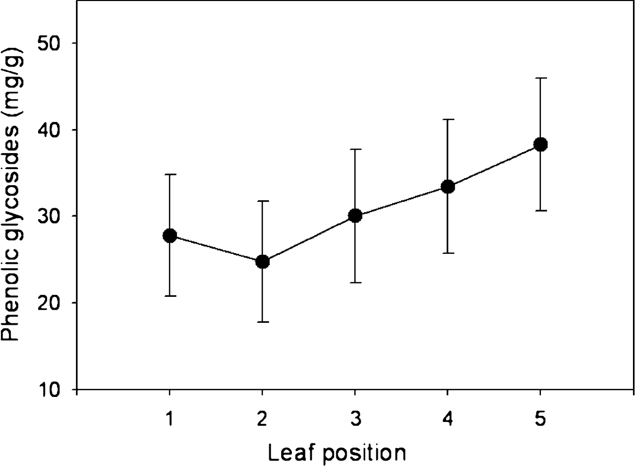


Fig. 3 Concentrations of foliar phenolic glycosides from leaf positions 1 through 5 during the summer of 2006. Values are back transformed least squared means±SE

Although some leaf miner larvae had ceased to feed by the time induction was detected, a subset of larvae apparently ingested leaves containing induced levels of phenolic glycosides. About 25% of sampled leaves possessed at least one pupal leaf fold (indicating that feeding had ceased) on day 6, and this percentage rose to 42% by day 9 (Fig. 4c), the day we observed induced levels of phenolic glycosides (Fig. 4a). On day 12, 92% of leaves had at least one pupal fold, indicating that approximately half of the leaves still contained feeding larvae on day 9 (Fig. 4c). Moreover, a substantial amount of feeding damage occurred post-induction (Fig. 4b), indicating that some larvae continued to feed following the damage-induced increase in phenolic glycoside concentration between days 9 and 12.

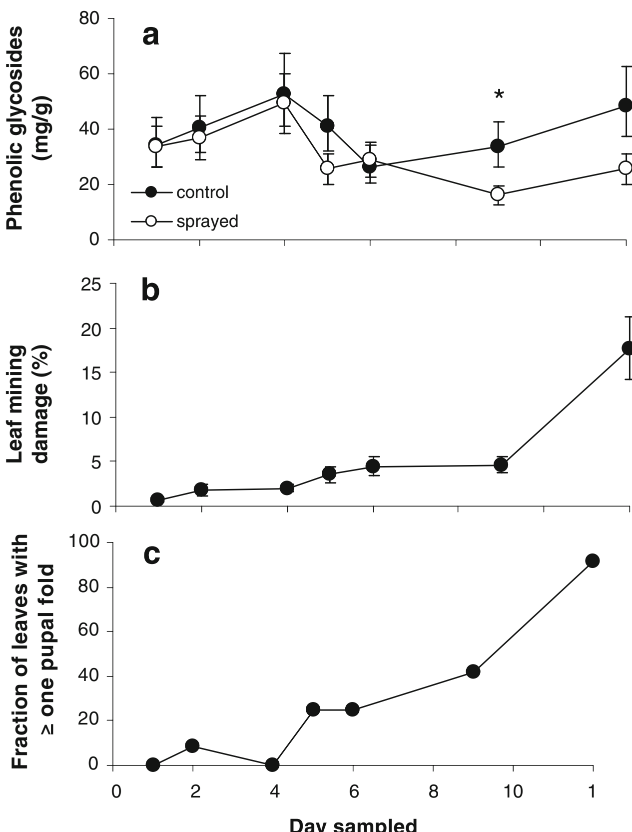


Fig. 4 Variation over time in **a** total foliar phenolic glycoside concentration (back transformed least squared means±SE) of naturally-mined (*filled symbols*) and insecticide-sprayed (*open symbols*) ramets; **b** mining damage (means±SE) on naturally-mined ramets and (**c**). the fraction of leaves sampled from naturally-mined ramets on which at least one leaf miner had ceased to feed and formed a pupal fold. Data are from summer 2007. The asterisk indicates that, on this sampling date, the contrast between average phenolic glycosides in naturally-mined and insecticide-sprayed leaves was statistically significant at $P<0.05$

Discussion

Damage to the epidermis caused by the mining activity of *P. populiella* led to the induction of phenolic glycosides in aspen leaf tissue. Epidermal leaf mining, while geographically widespread and ecologically important, is taxonomically restricted to a small subset of leaf miner taxa (Hering 1951). Consequently, few studies have investigated the physiological consequences of this form of herbivory for plants. In contrast, the vast majority of leaf mining species feed primarily on the photosynthetic cells of leaf mesophyll (Hering 1951). Studies investigating the effect of mesophyll mining on the chemistry of a variety of plant species report that mining can cause induced responses (Stout et al. 1994; Karban and Adler 1996; Inbar et al. 1999), although investigations of the induction of phenolic compounds in particular generally have reported negative results (Fisher et al. 2000; Ramiro et al. 2006).

Our results suggest that phenolic glycosides were induced while many leaf miner larvae were still feeding and growing and before the majority of the mining damage was inflicted on leaves (Fig 4). Phenolic glycosides are expressed in the epidermal tissue of aspen (Kao et al. 2002), thus it appears that *P. populiella* larvae ingest phenolic glycosides as they feed. Phenolic glycosides have negative effects on growth and survivorship of several generalist lepidopteran herbivores of aspen (Bryant et al. 1987; Hemming and Lindroth 1995; Hwang and Lindroth 1997; Osier and Lindroth 2001), but specialist herbivores, such as *P. populiella*, often are resistant to the effects of host plant secondary chemistry. However, natural patterns of leaf mining on leaves varying in phenolic glycoside concentration support the hypothesis that phenolic glycosides reduce *P. populiella* feeding damage (Young et al. 2010).

Treatment with insecticide did not prevent aspen leaves from inducing phenolic glycosides in response to mechanical damage. One day after mechanical damage was imposed, insecticide-sprayed leaves that were cut had 37% higher phenolic glycoside concentrations than sprayed leaves that were not cut. Clausen et al. (1989a) found that mechanically damaged aspen leaves contained 15% more phenolic glycosides (salicortin and tremulacin combined) than undamaged leaves one day post-damage. The data suggest that the difference in foliar phenolic glycoside concentration between aspen ramets sprayed with insecticide and ramets naturally damaged by leaf miners reflects induction caused by leaf mining, rather than an artifact of the insecticide treatment.

The concentration of phenolic glycosides was 26% higher in leaves with EFNs than in leaves lacking EFNs, in both the damaged and undamaged states. There was no

evidence that leaves with EFNs induced phenolic glycosides more strongly than leaves without EFNs; rather, leaves with and without EFNs both increased in phenolic glycoside concentration by approximately 125% in response to leaf mining (Fig. 2). High levels of phenolic glycosides in EFN-bearing leaves may help to explain the previously reported pattern of lower mining on these leaves (Doak et al. 2007). The association between EFNs and phenolic glycosides is addressed in greater detail elsewhere (Young et al. 2010).

In this study, approximately 9 days of mining damage accumulated before phenolic glycoside induction was observed. The newly-unfurled leaves may have been too young to mount an induced response. Alternatively, a threshold amount of damage may be required before aspen leaves respond by up-regulating phenolic glycoside expression (Underwood 2000). At the onset of induction in our study, approximately 5% of the total leaf epidermis was mined. While low, this level of damage falls within the range of damage levels documented to trigger induced resistance among species (Karban and Baldwin 1997).

Aside from the induction response, we observed considerable ontogenetic variation in the phenolic glycoside content of leaves. This also has been observed in previous studies (Lindroth et al. 1987; Osier et al. 2000). Foliar concentrations of phenolic glycosides varied more than 2-fold during the first six days of sampling and prior to the herbivore-induced response (Fig 4a). It is possible that the generally high concentrations of phenolic glycosides observed during the first four sampling days were induced by damage caused by eggs or newly hatched leaf miner larvae prior to insecticide application. However, we find this unlikely because the phenolic glycoside concentration subsequently declined between days 4 and 6 on both insecticide-sprayed and naturally-mined trees. More likely, the increase in concentration between days 1 and 4 reflected up-regulation of phenolic compounds during early leaf development, and the subsequent decrease in the concentration of phenolic glycosides after sampling day 4 reflected dilution of phenolic glycosides as the leaf gained mass (Jones and Hartley 1999). Regardless of the mechanism, high constitutive concentrations of phenolic glycosides in these newly-developing leaves, albeit short-lived, could present a challenge to some early spring herbivores.

Induced defenses can be costly to deploy (Agrawal et al. 1999). For aspen, detection of a cost of allelochemical expression depends upon the environmental conditions (Stevens et al. 2007). However, tradeoffs between aspen allelochemical expression and growth have been reported by several studies (Hwang and Lindroth 1997; Osier and Lindroth 2006; Stevens et al. 2007) suggesting that the production of phenolic glycosides may be costly. Aspen ramets naturally mined by *P. populiella* grew more slowly than those treated with insecticide to reduce mining damage (Wagner et al. 2008). In addition to the direct costs of herbivory that stem from decreased photosynthesis and early leaf abscission in mined leaves (Wagner et al. 2008), the cost of induction of phenolic glycosides in response to *P. populiella* mining damage may have contributed to the slow growth of naturally-mined ramets.

In summary, our results demonstrate that *P. populiella* mining damage to a small fraction (<10%) of the epidermis of aspen leaves induced the production of phenolic glycosides. The induction response did not occur immediately upon initiation of mining, suggesting that a threshold amount of damage was necessary to trigger the induction response. The onset of induction occurred while many of the leaf miners were still feeding; hence, induction of phenolic glycosides may increase aspen resistance to *P. populiella* herbivory. Higher constitutive and induced concentrations of phenolic glycosides on leaves with EFNs, relative to those without EFNs, may contribute to previously-reported lower mining damage on leaves bearing EFNs (Doak et al. 2007).

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Species by Environment Interactions Affect Pyrrolizidine Alkaloid Expression in *Senecio jacobaea*, *Senecio aquaticus*, and Their Hybrids

Heather Kirk · Klaas Vrieling · Eddy Van Der Meijden · Peter G. L. Klinkhamer

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Abstract We examined the effects of water and nutrient availability on the expression of the defense pyrrolizidine alkaloids (PAs) in *Senecio jacobaea* and *S. aquaticus*. *Senecio jacobaea*, and *S. aquaticus* are adapted to different natural habitats, characterized by differing abiotic conditions and different selection pressures from natural enemies. We tested if PA concentration and diversity are plastic over a range of water and nutrient treatments, and also whether such plasticity is dependent on plant species. We also tested the hypothesis that hybridization may contribute to PA diversity within plants, by comparing PA expression in parental species to that in artificially generated F_1 hybrids, and also in later generation natural hybrids between *S. jacobaea* and *S. aquaticus*. We showed that total PA concentration in roots and shoots is not dependent on species, but that species determines the pattern of PA diversification. Pyrrolizidine alkaloid diversity and concentration are both dependent on environmental factors. Hybrids produce a putatively novel PA, and this PA is conserved in natural hybrids, that are backcrossed to *S. jacobaea*. Natural hybrids that are backcrossed several times to *S. jacobaea* are with regard to PA diversity significantly different from *S. jacobaea* but not from *S. aquaticus*, while F_1 hybrids are in all cases more similar to *S. jacobaea*. These results collectively suggest that PA diversity is under the influence of natural selection.

H. Kirk (✉) · K. Vrieling · E. Van Der Meijden ·
P. G. L. Klinkhamer
Plant Ecology Section, Institute of Biology, Leiden University,
P.O. Box 9505, 2300 RA Leiden, The Netherlands
e-mail: hkirk10@hotmail.com

Key Words Hybridization · Secondary metabolite diversity · Plant resistance · Alkaloid composition · Phenotypic plasticity

Introduction

Plants mediate interactions with herbivores and pathogens by production and distribution of secondary metabolites among various plant tissues. Herbivores are either deterred by (van Dam et al. 1995; Macel et al. 2005) or attracted to (Rauscher 2001) secondary metabolites. The community of soil organisms in the plant rhizosphere also is often determined by the composition of secondary metabolites in plant roots (Hol et al. 2004; Kowalchuk et al. 2006), and such metabolites can decrease growth of particular microbial species, and stimulate the growth of others (Hol and van Veen 2002). A single plant species often possesses many structural variations within single classes of secondary metabolites, and often it has been hypothesized that diversity of secondary metabolites is partially explained by selection pressure by herbivores and pathogens (Hol and van Veen 2002; Adler and Kittelson 2004; Macel et al. 2005; Albrechtsen et al. 2007). In many cases, environmental factors also are correlated with large variations in concentration, allocation, and diversity of secondary metabolites (Holton et al. 2003; Close et al. 2005; Loney et al. 2006).

Pyrrolizidine alkaloids (PAs) occur in a number of families including Apocynaceae, Asteraceae, Boraginaceae, and Orchidaceae (Hartmann and Witte 1995). They are toxic to generalist mammalian (Cheeke 1988) and insect (van Dam et al. 1995; Macel et al. 2005) herbivores, and may play a role in pathogen resistance (Hol and van Veen 2002; Kowalchuk et al. 2006). Pyrrolizidine alkaloid

composition, including concentration and diversity, exhibits some plasticity, and can alter in response to plant developmental cues (Schaffner et al. 2003), abiotic environment (Macel and Klinkhamer 2009), and interactions with natural enemies (Hol et al. 2004).

The *Senecio* genus (Asteraceae) contains more than 1500 species and, the production of PAs is widespread. In *Senecio*, PAs are synthesized as senecionine *N*-oxide in plant roots (Hartmann and Toppel 1987). Senecionine *N*-oxide is transported through the phloem to the shoots (Hartmann and Dierich 1998) where diversification into a number of related PA structures occurs. PA diversification is species specific, such that the suite of PAs within a plant species is generally unique (e.g., Pelser et al. 2005). Furthermore, significant variation in PA composition and concentration within *Senecio* species has been observed (Trigo et al. 2003; Macel et al. 2004).

We investigated the effects of both the abiotic environment, and of plant hybridization on the concentration and diversity of PAs expressed in the roots and shoots of *Senecio aquaticus* and *Senecio jacobaea*. The habitats of *S. jacobaea* and *S. aquaticus* are largely differentiated based on the organic and water content of the soil (Kirk et al. 2005b). Additionally, selection pressure by herbivores differs between the two species, partially because the wet soils found around natural *S. aquaticus* populations do not support the lifecycles of many invertebrate herbivores (see below).

We expect that differences in PA expression among species are due to different selection pressures over time. Because the environments of *S. jacobaea* and *S. aquaticus* are different, we also expect that these species may differentially alter production, allocation, and diversification of PAs according to environmental cues (e.g., Hol et al. 2003). For example, seasonal inundation excludes some root-associated herbivores from the habitat of *S. aquaticus*, and we thus expect that PAs associated with those herbivores are regulated differently in the roots of *S. aquaticus* from in *S. jacobaea*. Several studies have shown that environmental factors have an impact on PA expression in *S. jacobaea* (Macel 2003; Hol et al. 2003; Macel and Klinkhamer 2009).

Furthermore, *S. jacobaea* hybridizes in nature with *S. aquaticus* (Kirk et al. 2004). Natural hybrids between *S. jacobaea* and *S. aquaticus*, first observed at the Zwanenwater nature reserve in The Netherlands in 1979 (Ruud van der Meijden, personal communication), can be found in a narrow zone spanning a bank at the edge of a lake, which is intermediate to parental sites with regards to soil organic content and humidity (Kirk et al. 2005b). A previous study showed that natural hybrids are similar to *S. jacobaea* with regard to growth characteristics when subject to drought and flood-like conditions, while F_1 hybrids possess the drought resistant characteristics of *S. jacobaea*, and the flood resistant characteristics of *S. aquaticus* (Kirk et al. 2005b).

It has been postulated that hybridization may provide a mechanism for rapid evolution of resistance to parasites, including herbivores and pathogens. Hybridization creates a greater range of phenotypic variation than is found in parental species (Rieseberg et al. 1999; Kirk et al. 2005a; Wissemann 2007). If resistance traits exhibit higher variation among hybrid individuals than among parental species, hybrids may be more responsive to selection pressure than pure parental species. This hypothesis has been supported empirically (though not in relation to parasite resistance) in *Drosophila*, for which several studies have shown that variation in abiotic stress resistance can be higher in hybrid lines than in parental lines (Hercus and Hoffman 1999), such that hybrids may evolve more quickly in response to stress than parental species. Additionally, epistatic interactions among genes combined from both parents may create unique resistance traits, such as novel secondary metabolites (Fritz 1999; Orians 2000), and hybrid lines or hybrid genes may be selectively favoured.

Here, we investigated the effects of species by environment interactions on quantitative and qualitative expression of PAs in *S. jacobaea*, *S. aquaticus*, and their hybrids. We asked: i) Are there quantitative and qualitative differences in the expression of PAs between *S. jacobaea*, *S. aquaticus*, and their hybrids? ii) Do environmental factors affect PA expression in *S. jacobaea*, *S. aquaticus*, and their hybrids? iii) Is there evidence that novel PAs are produced in hybrids?

Methods and Materials

Study System Viable hybrids between *S. jacobaea* L. and *S. aquaticus* Hill have been reported from a number of locations including the United Kingdom (Stace 1975), Germany (Christian Düring, personal communication), and The Netherlands (Kirk et al. 2004). In this investigation, we studied *S. jacobaea*, *S. aquaticus*, and natural hybrids individuals sampled from the Zwanenwater reserve, located immediately to the South of Callantsoog, The Netherlands (52˚50′00”N, 004˚41′00”E). Composed mostly of sand dunes, the Zwanenwater reserve contains a small lake around which a hybrid population exists (see Kirk et al. 2005b for diagram). *Senecio jacobaea* is abundant in the dunes surrounding the lake, while *S. aquaticus* occurs infrequently at the lake fringe.

Selection pressure by specialist herbivores on *S. jacobaea* may be quite high, since a number of specialist herbivores, including *Tyria jacobaeae* and *Longitarsus jacobaeae*, cause extreme damage to above ground and

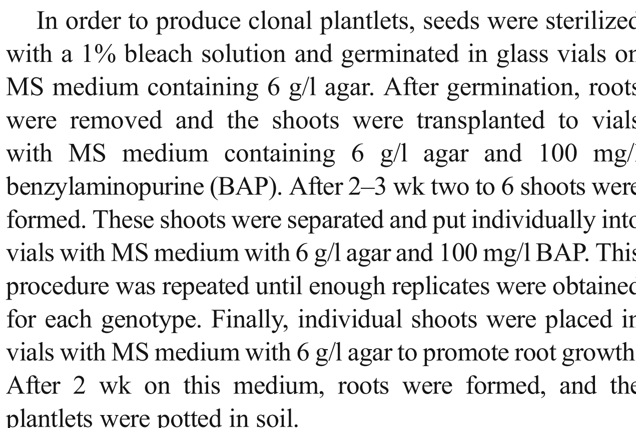
below ground plant parts. *Senecio aquaticus* is not subject to attack by *T. jacobaeae*, and other specialists that pupate in the soil around the plant, because pupae do not survive in the moist environments where *S. aquaticus* is found (personal observation). Thrips appear to be common in the *S. aquaticus* population (personal observation). Preliminary tests showed that in climate chamber experiments, *S. aquaticus* and *S. jacobaea* were equally susceptible to *T. jacobaeae* (Macel et al. 2002), and the generalist lepidopteran herbivore *Spodoptera exigua* (personal observation), but *S. aquaticus* was more resistant to the generalist thrips species *Thrips tabaci* than *S. jacobaea* (Kirk et al. 2005a).

Senecio aquaticus usually is found in seasonally waterlogged, humic, chalk-poor, and nutrient-rich soils (Weeda and Van Deursen 1994), while *S. jacobaea* prefers sunny environments with sparse vegetation (Weeda and Van Deursen 1994). *Senecio jacobaea* is able to elongate its roots rapidly in response to drought, in order to reach water sources that are unavailable to *S. aquaticus*, and *S. aquaticus* is resistant to flooding, while *S. jacobaea* is not (Kirk et al. 2005b).

Seeds of *S. jacobaea*, *S. aquaticus*, and natural hybrids were collected from plants in the field during 2001 and 2002. Putative hybrids were identified based on leaf lobe and flower morphology, and were later confirmed to be hybrids based on diagnostic amplified fragment length polymorphism (AFLP) markers (Kirk et al. 2004).

F_1 hybrids were produced by collecting from the field second year rosettes of parental plants that exhibited the development of flowering stems. Second year rosettes were collected because parental plants generally do not flower until their second year of growth, after vernalization (Kirk et al. 2005c). To minimize chances that introgressive genes were present in experimental parents, *S. aquaticus* individuals were collected from a marshy agricultural grassland approximately 500 m from the hybrid zone, and *S. jacobaea* individuals were collected from dunes located approximately 300 m from the hybrid zone. Plants from both species were placed in a greenhouse, allowed to flower, and were crossed in pairs of *S. jacobaea*×*S. aquaticus* by rubbing flower heads together. Both species are self-incompatible (Kirk et al. 2005c). Seeds were harvested from both parental plants to be certain that maternal effects are not involved in patterns of PA expression in F_1 hybrid offspring.

Effects of Environmental Factors on PA Expression We selected five *S. aquaticus* genotypes, five *S. jacobaea* genotypes, and five natural hybrid genotypes for experimental use. We also included 10 F_1 genotypes, including five pairs of full-sibs. Each pair of sibs was comprised of one genotype harvested from a *S. aqaticus* mother, and one genotype harvested from a *S. jacobaea* mother.



In order to produce clonal plantlets, seeds were sterilized with a 1% bleach solution and germinated in glass vials on MS medium containing 6 g/l agar. After germination, roots were removed and the shoots were transplanted to vials with MS medium containing 6 g/l agar and 100 mg/l benzylaminopurine (BAP). After 2–3 wk two to 6 shoots were formed. These shoots were separated and put individually into vials with MS medium with 6 g/l agar and 100 mg/l BAP. This procedure was repeated until enough replicates were obtained for each genotype. Finally, individual shoots were placed in vials with MS medium with 6 g/l agar to promote root growth. After 2 wk on this medium, roots were formed, and the plantlets were potted in soil.

One equal sized clonal plantlet from most genotypes was transplanted into each of 6 experimental columns (1 m length, 15 cm diam), yielding a total of 136 experimental plants. We aimed for 150 plants, but due to variance in sizes and difficulties with cloning, only 136 plants were used, giving an average of 22 plants per experimental treatment. For each treatment, we aimed to include 5 genotypes of each parental species, 5 natural hybrid genotypes, and 10 F_1 hybrid genotypes.

The experiment was established to test a combination of two nutrient and three water treatments. One meter tall columns were filled with sieved dune sand. In half the columns, the dune sand was mixed with 'Osmocote' slow release fertilizer (N:P:K = 15:11:13+2 MgO) at a concentration of 1.3 g/l sand to provide a nutrient rich medium. After establishment of seedlings, the bottoms of the columns were placed in water of three different depths: 5, 50, and 100 cm. A previous study (Kirk et al. 2005b) showed that columns placed in 5 cm of water created drought-like conditions. Columns placed in 50 cm of water created conditions, which did not subject plants to water-related stress, and columns were placed into 100 cm of water mimicked flooding (soil saturation). Columns were placed in a climate chamber for the duration of the experiment (light 16 h, temperature 20/15°C, relative humidity 70%).

All columns were given sufficient water at the beginning of the experiment to allow for seedling establishment. At the beginning of the experiment, soil throughout the total length of the column was moist. Experimental conditions were established 2 wk after seedlings were transplanted to columns, and the experiment was subsequently continued for 10 wk. Plants were not watered from the top during this period. Roots and shoots were harvested separately, and were dried in an oven for 3 d at 50°C.

PA Extraction and Identification All dried leaves and roots from each plant were separately milled to a fine powder and homogenized. Milled samples were stored in a freezer at −0°C until use. For extraction, approximately 15 mg of

plant material were extracted according to a modified version (de Boer 1999) of the acid-base extraction method (Hartmann and Zimmer 1986). Extractions were dissolved in methanol containing heliotrine at a concentration of 1 μg/ml(Latoxan, France) as an internal standard and analyzed by gas chromatography (GC). Final concentrations of each PA within the sample were calculated by integrating the GC peaks and converting these areas to concentration by using the known concentration of the internal standard. Conditions (injector 250°C, temperature program 220°C–250°C 5°C/min, split mode 1:30, carrier gas N_2 0.9 ml min^{-1}, pressure 56 kPa; detector NPD) were controlled by a Hewlett Packard gas chromatographer (model 6890). GC peaks were compared with known references to identify sample composition. Identities of PAs from known reference samples were previously confirmed by using GC-MS (Macel et al. 2002). PA_1 and PA_2 were confirmed to be PAs, and florosenine was identified by GC-MS according to Witte et al. (1992).

Statistics We tested PA concentrations and diversity separately for roots and shoots, since PAs may interact both with root and with shoot pathogens and herbivores. We analyzed diversity in two ways. First, we counted the absolute number of PAs produced in shoots and roots of each plant group across differing treatments. This is biologically relevant because the production of each PA requires the activation of at least one new enzymatic pathway within the plant (e.g., Pelser et al. 2005). We refer to this measure of diversity as PA richness throughout the remainder of the text. Differences in both PA richness and total PA concentration were analyzed with ANOVAs, including interaction terms, for which we identified the fixed factors as water treatments, nutrient treatments, and taxa. Taxa denote parental species, F_1 hybrids, and natural hybrids. Shoot to root ratio was used as a covariate because it was assumed that root biomass is proportional to PA production, and because the size of the shoots is not related to PA production (Hol et al. 2003). Relatively large shoots may lead to a dilution of PAs. We first tested for maternal effect on PA expression in F_1 hybrids, by including only F_1 hybrids in the analysis. Previous research has shown that maternal effects can affect both growth (Kirk et al. 2005b) and reproductive fitness (Kirk et al. 2005c) of F_1 hybrids between *S. jacobaea* and *S. aquaticus*. Since we found that the maternal parent was never a significant factor in the analyses, we treated all F_1 hybrids as one group for subsequent ANOVAs by using the entire data set.

We also analyzed PA diversity by applying principal component analysis (PCA) to quantitative PA data, which is likely most ecologically relevant from the perspective of plant defense. We applied ANOVAs to each principal component (PC) to test whether nutrient and water treatments and plant taxa had significant effects on PA composition. We then identified individual PAs that were highly correlated with each PC. All tests were performed with SPSS 8.0 (SPSS Inc. 1998).

Results

Eleven PAs were identified during our analyses (Table 1). We found eight PAs that commonly occurred in *S. jacobaea* (eight in the shoots, and eight in the roots). Additionally, PA_1 was present in low amounts in two plants of *S. jacobaea* and florosenine (Fig. 1) in one plant. To our knowledge, neither of these PAs has been reported previously from *S. jacobaea* individuals, and may represent PAs introgressed from the Zwanenwater hybrid zone. Ten PAs were present in *S. aquaticus*, of which the rare PA otosenine (Fig. 1) was found only in *S. aquaticus*. In contrast to findings for *S. jacobaea*, PA_1 almost always was present in *S. aquaticus* shoots. The reverse was true for jacobine. All eleven PAs were present among both natural hybrid and artificial hybrid genotypes. Florosenine appeared to be specific to hybrids, although trace amounts were expressed by one *S. jacobaea* plant.

There was no significant effect of, or interaction involving plant taxa on either shoot or root PA concentrations (Table 2 Fig. 2). In shoots, PA concentration was affected by both water, and an interaction between water and nutrient treatments. Pyrrolizidine alkaloid concentrations were approximately equal in all nutrient-rich treatments (regardless of water treatment), while in sand without nutrients, concentrations were high in the intermediate water treatment compared to other treatments. In roots, PA concentration was affected by both water and nutrient effects (Table 2). PA concentration increased with increasing soil wetness, and was higher in sand without nutrients than in sand with nutrients (Fig. 2).

PA richness in the shoots differed significantly among taxa (Table 3). Averaged over all treatments, the number of PAs per plant (± SE) were, respectively, 3.96±0.35, 3.59± 0.33, 4.13±0.33, 4.80±0.24 for *S. jacobaea*, *S aquaticus*, natural hybrids, and F_1 hybrids. In shoots, PA richness also was affected by an interaction between nutrient and water treatments and interactions between nutrient treatment and taxa (Table 3). The latter interaction is caused mainly by low PA richness of *S. aquaticus* in medium and dry nutrient rich conditions. In the roots, water and taxa were both significant factors; PA richness was higher in wetter conditions, and *S. aquaticus* and F_1 hybrids exhibited higher PA richness than *S. jacobaea*, with variable PA richness in natural hybrids (Fig. 3).

PCA yielded 2 components (Table 4) for PAs in plant shoots, which cumulatively explained 85.6% of the varia-

Table 1 Mean concentration (mg/g)±se of 11 pyrrolizidine alkaloids in the roots, and shoots of *senecio jacobaea*, *senecio aquaticus*, natural hybrid, and f_1 hybrid genotypes. data shown is pooled over all treatments

RT[1]	Alkaloid		S. jacobaea	*S. aquaticus*	Natural Hybrid	F_1 Hybrid
3.97	PA_1	Shoots	$3.43 \times 10^{-3} \pm 2.39 \times 10^{-3}$	$1.61 \times 10^{-1} \pm 1.85 \times 10^{-2}$	$1.07 \times 10^{-1} \pm 2.60 \times 10^{-2}$	$6.39 \times 10^{-2} \pm 1.36 \times 10^{-2}$
		Roots	$4.20 \times 10^{-3} \pm 4.21 \times 10^{-3}$	$2.29 \times 10^{-2} \pm 2.03 \times 10^{-2}$	$1.73 \times 10^{-3} \pm 1.73 \times 10^{-3}$	$4.65 \times 10^{-3} \pm 4.65 \times 10^{-3}$
7.07	Senecivernine	Shoots	0	0	$2.40 \times 10^{-3} \pm 1.77 \times 10^{-3}$	$4.20 \times 10^{-4} \pm 4.20 \times 10^{-4}$
		Roots	$3.70 \times 10^{-4} \pm 2.64 \times 10^{-4}$	$1.06 \times 10^{-3} \pm 6.3 \times 10^{-4}$	0	$1.35 \times 10^{-3} \pm 9.9 \times 10^{-4}$
8.015	Senecionine	Shoots	$1.85 \times 10^{-1} \pm 4.12 \times 10^{-2}$	$1.85 \times 10^{-1} \pm 3.71 \times 10^{-2}$	$1.56 \times 10^{-1} \pm 4.20 \times 10^{-2}$	$1.22 \times 10^{-1} \pm 2.35 \times 10^{-2}$
		Roots	$7.21 \times 10^{-1} \pm 1.53 \times 10^{-1}$	$2.06 \times 10^{-1} \pm 3.54 \times 10^{-2}$	$3.07 \times 10^{-1} \pm 6.02 \times 10^{-2}$	$3.76 \times 10^{-1} \pm 4.36 \times 10^{-2}$
8.257	Seneciphylline	Shoots	$2.61 \times 10^{-1} \pm 6.39 \times 10^{-2}$	$3.26 \times 10^{-1} \pm 6.55 \times 10^{-2}$	$3.49 \times 10^{-1} \pm 7.82 \times 10^{-2}$	$3.32 \times 10^{-1} \pm 5.58 \times 10^{-2}$
		Roots	$1.95 \times 10^{-1} \pm 3.76 \times 10^{-2}$	$3.35 \times 10^{-1} \pm 9.08 \times 10^{-2}$	$2.48 \times 10^{-1} \pm 5.50 \times 10^{-2}$	$3.41 \times 10^{-1} \pm 4.17 \times 10^{-2}$
8.874	Integerrimine	Shoots	$3.46 \times 10^{-2} \pm 9.14 \times 10^{-3}$	$3.49 \times 10^{-2} \pm 9.93 \times 10^{-3}$	$3.07 \times 10^{-2} \pm 1.08 \times 10^{-2}$	$2.62 \times 10^{-2} \pm 6.02 \times 10^{-3}$
		Roots	$9.76 \times 10^{-2} \pm 1.83 \times 10^{-2}$	$4.65 \times 10^{-2} \pm 1.61 \times 10^{-2}$	$6.06 \times 10^{-2} \pm 1.20 \times 10^{-2}$	$7.74 \times 10^{-2} \pm 1.06 \times 10^{-2}$
10.386	Jacobine	Shoots	$3.06 \times 10^{-1} \pm 6.63 \times 10^{-2}$	$3.91 \times 10^{-3} \pm 3.63 \times 10^{-3}$	$1.87 \times 10^{-1} \pm 5.98 \times 10^{-2}$	$2.36 \times 10^{-1} \pm 4.80 \times 10^{-2}$
		Roots	$8.96 \times 10^{-2} \pm -3.10 \times 10^{-2}$	0	$5.32 \times 10^{-2} \pm 2.80 \times 10^{-2}$	$1.09 \times 10^{-1} \pm 2.47 \times 10^{-2}$
10.852	Jacoline	Shoots	$7.19 \times 10^{-3} \pm 4.41 \times 10^{-3}$	$1.06 \times 10^{-2} \pm 7.05 \times 10^{-3}$	$5.33 \times 10^{-2} \pm 1.92 \times 10^{-2}$	$2.23 \times 10^{-2} \pm 7.14 \times 10^{-3}$
		Roots	$6.74 \times 10^{-2} \pm 1.87 \times 10^{-2}$	$1.47 \times 10^{-1} \pm 4.97 \times 10^{-2}$	$8.70 \times 10^{-2} \pm 2.11 \times 10^{-2}$	$9.44 \times 10^{-2} \pm 1.70 \times 10^{-2}$
11.30	PA_2	Shoots	$7.10 \times 10^{-4} \pm 4.11 \times 10^{-4}$	$4.00 \times 10^{-4} \pm 2.81 \times 10^{-4}$	$2.70 \times 10^{-4} \pm 1.85 \times 10^{-4}$	$1.29 \times 10^{-2} \pm 1.05 \times 10^{-2}$
		Roots	$1.12 \times 10^{-4} \pm 1.12 \times 10^{-4}$	$4.55 \times 10^{-3} \pm 2.69 \times 10^{-3}$	$3.30 \times 10^{-4} \pm 2.30 \times 10^{-4}$	$2.19 \times 10^{-3} \pm 1.48 \times 10^{-3}$
12.01	Erucifoline	Shoots	$2.45 \times 10^{-2} \pm 1.21 \times 10^{-2}$	$2.91 \times 10^{-2} \pm 1.21 \times 10^{-2}$	$2.33 \times 10^{-2} \pm 1.10 \times 10^{-2}$	$4.80 \times 10^{-2} \pm 1.47 \times 10^{-2}$
		Roots	$9.39 \times 10^{-3} \pm 6.27 \times 10^{-3}$	$1.28 \times 10^{-2} \pm 8.06 \times 10^{-3}$	$3.99 \times 10^{-3} \pm 3.81 \times 10^{-3}$	$2.01 \times 10^{-2} \pm 6.91 \times 10^{-3}$
12.50	Otosenine	Shoots	0	0	0	0
		Roots	0	$6.40 \times 10^{-4} \pm 3.54 \times 10^{-4}$	$1.77 \times 10^{-4} \pm 1.77 \times 10^{-4}$	$1.98 \times 10^{-3} \pm 1.71 \times 10^{-3}$
14.31	Florosenine	Shoots	0	0	$1.42 \times 10^{-4} \pm 1.42 \times 10^{-4}$	$1.37 \times 10^{-2} \pm 9.10 \times 10^{-3}$
		Roots	$1.50 \times 10^{-4} \pm 1.50 \times 10^{-4}$	0	0	$6.07 \times 10^{-3} \pm 3.88 \times 10^{-3}$

[1] Indicates retention time of GC-NPD peaks

Fig. 1 Chemical structures of otosenine and florosenine

Otosenine R = H
Florosenine R = Ac

tion in PA expression. The first component (PC1) mostly explained variation in the least metabolically derived alkaloids identified in this experiment [i.e., was significantly correlated with senecionine (*r*= *0.68*), seneciphylline (*r*= *0.99*), and integerrimine (*r*= *0.74*), and to a lesser extent variation in jacobine (*r*= *0.21*), jacoline (*r*= *0.47*), and erucifoline (*r*= *0.38*) (Table 4)]. The second component (PC2) explained variation in jacobine (*r*= *0.98*), florosenine (*r*= *0.44*), PA_1 (*r*= *-0.29*), senecionine (*r*= *−0.31*) (Table 4). PC1 was affected significantly by all abiotic conditions and their interaction. Scores for PC1 were higher in sand without nutrients, and were low in wet and high in intermediate (moisture) conditions. PC2 was affected significantly by taxa with the parents that showed the most extreme scores (*S. jacobaea*: 0.34±0.17a, *S. aquaticus*: −0.61±0.17b). Mean hybrid scores were intermediate to those of parental species, but natural hybrids were statistically equal to *S. aquaticus*, while F_1 hybrids were statistically equal to *S. jacobaea* (natural hybrids −.04±0.17b, F_1 hybrids 0.16±0.12a; different letters indicate significant differences). Another significant main effect was found for nutrients with scores for nutrient rich conditions (0.31±0.11) higher than for sandy soils (−0.38±0.11).

Two principal components (PCs) accounted for 88.9% of the variation in root PA expression. As in the shoots, the first component (PC1) mostly explained variation in the least metabolically derived alkaloids [i.e., senecionine (*r*= *0.95*), seneciphylline (*r*= *0.66*), and integerrimine (*r*= *0.77*), and to a lesser extent variation in jacoline (*r*= *0.47*), and erucifoline (*r*= *0.25*) (Table 4)]. The second component (PC2) explained variation in senecionine (*r*= *−0.31*), seneciphylline (*r*= *0.75*), integerrimine (*r*= *0.26*), jacoline (*r*= *0.6*), PA_2 (*r*= *0.5*), and erucifoline (*r*= *0.48*). All three main effects (taxa, water, nutrients) were significant for PC1. Again, parents showed the most extreme scores (*S. jacobaea*: 0.53±0.18a, *S. aquaticus*: −0.31± 0.17b). Natural hybrids statistically were equal to *S. aquaticus*, while F_1 hybrids had intermediate mean scores, but statistically were equal to both parents (natural hybrids: −.21±0.17b, F_1 hybrids: 0.01±0.12ab; different letters indicate significant differences). Scores for PC1 decreased with dryer conditions and were lower in nutrient rich soils. PC2 showed significant mains effects of taxa and nutrients and a significant interaction between water and nutrients. Parental taxa showed the most extreme scores (*S. jacobaea*: −0.84±0.17a, *S. aquaticus*: 0.42±0.16b). Hybrid means

Table 2 Effect of nutrient and water treatment, and plant taxa (fixed factors) on pyrrolizidine alkaloid concentration in the roots and shoots of *senecio jacobaea*, *senecio aquaticus*, natural hybrids, and f_1 hybrids. shoot to root ratio (sr ratio) of the plant was used as a covariate

Source	Type III Sum of Squares	*df*	Mean Square	*F*	*P*
Shoots					
Covariate SR ratio	.640	1	.640	1.528	.219
Taxa	.726	3	.242	.578	.631
Nutrient	1.465	1	1.465	3.497	.063
Water	6.632	2	3.316	7.917	.001
Taxa * nutrient	.408	3	.136	.325	.807
Taxa * water	4.152	6	.692	1.652	.140
Nutrient * water	10.123	2	5.062	12.085	<.001
Taxa * nutrient * water	2.846	6	.474	1.132	.348
Error	46.490	111	.419		
Roots					
Covariate SR ratio	.794	1	.794	1.292	.258
Taxa	4.059	3	1.353	2.202	.092
Nutrient	6.884	1	6.884	11.204	.001
Water	6.549	2	3.275	5.329	.006
Taxa * nutrient	1.900	3	.633	1.031	.382
Taxa * water	1.507	6	.251	.409	.872
Nutrient * water	.639	2	.320	.520	.596
Taxa * nutrient * water	7.858	6	1.310	2.132	.055
Error	68.203	111	.614		

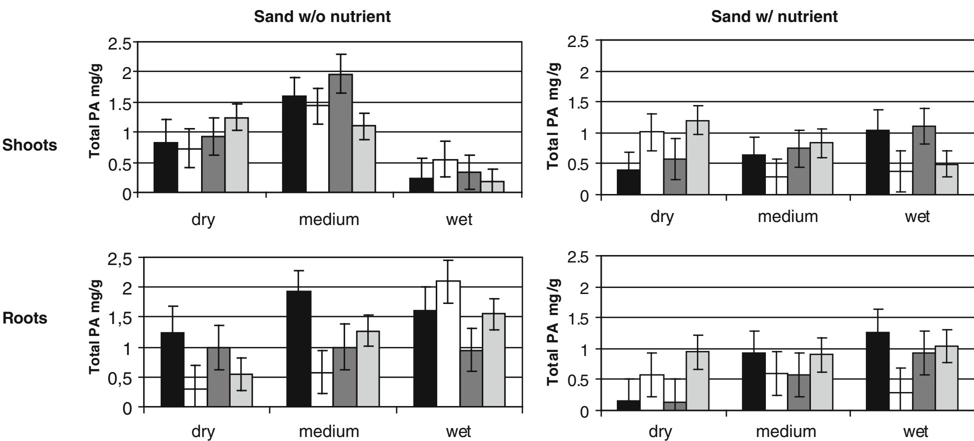


Fig. 2 Pyrrolizidine alkaloid (PA) concentration in the shoots and roots of *Senecio jacobaea* (*black*), *Senecio aquaticus* (*white*), natural hybrids (*dark grey*), and F_1 hybrids (*light grey*) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error

were intermediate to parental means, but both hybrids groups were statistically equal to *S. aquaticus* (natural hybrids: −.01±0.16b, F_1 hybrids: 0.15±0.11b; different letters indicate significant differences).

Discussion

We found that both environmental and taxa effects mediate the expression of PAs in the roots and shoots of the plant species presented here.

Environmental Factors Total PA concentration in roots and shoots is influenced by both soil moisture and nutrient availability (Salmore and Hunter 2001; Hol et al. 2003). According to the resource availability hypothesis, which postulates that plants should make higher investments in defense in the absence of abundant resources (Coley et al. 1985), PA concentrations are expected to be higher when nutrient availability is low. This was the case in our study; trends showed higher PA concentrations in sand without nutrients compared to sand with nutrients. This finding corroborates evidence presented by Hol et al. (2003) that PAs were diluted when *S. jacobaea* plants were grown on nutrient-rich media, due to rapid accumulation of biomass in relation to PA production.

The influence of soil moisture and nutrients on PA richness in the shoots was relatively small. In the roots, PA richness increased with increasing soil moisture. This suggests that differences in PA richness may play a large

Table 3 Effect of nutrient and water treatment, and plant taxa (fixed factors) on pyrrolizidine alkaloid richness in the roots and shoots of *senecio jacobaea*, *senecio aquaticus*, natural hybrids, and f_1 hybrids. shoot to root ratio (sr ratio) of the plant was used as a covariate

Source	Type III Sum of Squares	*df*	Mean Square	*F*	*P*
Shoot					
Covariate SR ratio	1.758	1	1.758	.577	.449
Taxa	30.530	3	10.177	3.339	.022
Nutrient	.439	1	.439	.144	.705
Water	5.421	2	2.711	.889	.414
Taxa * nutrient	31.988	3	10.663	3.499	.018
Taxa * water	24.382	6	4.064	1.333	.248
Nutrient * water	67.319	2	33.660	11.044	<.001
Taxa * nutrient * water	8.652	6	1.442	.473	.827
Error	338.292	111	3.048		
Root					
Covariate SR ratio	2.364	1	2.364	.806	.371
Taxa	28.433	3	9.478	3.233	.025
Nutrient	1.727	1	1.727	.589	.444
Water	42.868	2	21.434	7.312	.001
Taxa * nutrient	22.180	3	7.393	2.522	.061
Taxa * water	21.237	6	3.539	1.207	.308
Nutrient * water	1.975	2	.988	.337	.715
Taxa * nutrient * water	7.419	6	1.236	.422	.863
Error	325.386	111	2.931		

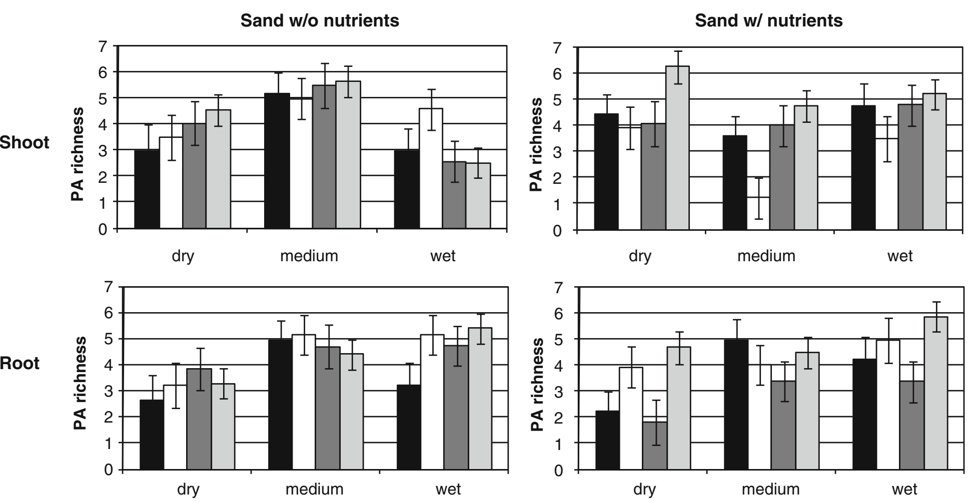


Fig. 3 Pyrrolizidine Alkaloid (PA) richness, defined as the number of PAs detected within each sample, in the shoots and roots of *Senecio jacobaea* (*black*), *Senecio aquaticus* (*white*), natural hybrids (*dark grey*), and F_1 hybrids (*light grey*) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error

role in below-ground plant defense because pathogenic pressure will rise with increasing soil moisture (e.g., Shafer and Kotonan 2003). Based on limited data, below-ground defense seems to be more important for some species than others; Brassicaceous plants produce greater concentrations and a higher diversity of glucosinolates in roots than in shoots, and at least one glucosinolate has higher toxicity below-ground than above-ground (van Dam et al. 2009). In contrast, Milkweed allocates more resources to above-ground plant defense compared to below-ground defense (Rassman et al. 2009).

Nutrients and water conditions had a strong effect on PA expression in shoots and roots for both PCAs. In the shoots seneciphylline (PC1) and jacobine (PC2), and in the roots senecionine (PC1) and seneciphylline (PC2), were correlated strongly with PCA scores suggesting that the concentrations of these PAs are most influenced by environmental conditions. Concentration of jacobine in the shoots more than doubled when nutrient concentrations increased, although total PA concentration decreased. This might have implications for plant defense because jacobine has been shown to be more effective against thrips than all other PAs found in *S. jacobaea* (Leiss et al. 2009).

Taxa Plant taxa (including hybrid groups) never showed differences in *total* PA concentration in plant shoots or

Table 4 Effects of nutrient treatment, water treatment, and plant taxa on principal components (pcs) that are correlated with pyrrolizidine alkaloids (pas) expression in roots and shoots of *senecio*

	Principal Component	% variation[1]	Correlated PAs[2]	Significant Factors[3] ($P < .050$)
Shoots	1	53.0	(+)Senecionine	Nutrient
			(+)Seneciphylline	Water
			(+)Integerrimine	Water*Nutrient
			(+)Jacobine	
			(+)Jacoline	
			(+)Erucifoline	
	2	32.6	(−)PA1	Taxa
			(−)Senecionine	Nutrient
			(+)Jacobine	
			(+)Florosenine	
Roots	1	64.7	(+)Senecionine	Taxa
			(+)Seneciphylline	Water
			(+)Integerrimine	Nutrient
			(+)Jacoline	
			(+)Erucifoline	
	2	24.2	(−)Senecionine	Taxa
			(+)Seneciphylline	Nutrient
			(+)Integerrimine	Water*Nutrient
			(+)Jacoline	
			(+)PA2	
			(+)Erucifoline	

[1] Indicates percent of total PA variation explained by each PC

[2] Indicates PAs that are significantly correlated with each PC ($P<0.05$)

[3] Significant factors represent single factors and/or interactions that had significant effects on PC values in ANOVA analyses

roots. This suggests that selective pressure by different natural enemies does not play a large role in determining total PA concentration, because *S. aquaticus* and *S. jacobaea* grow in different environments (nutrient rich and moist vs. nutrient poor and dry), with a different host of herbivores and soil bacteria and fungal communities (Kirk et al. 2005b; Singh et al. 2009). In contrast to total PA concentration, PA *richness* (diversity) was significantly different between taxa for shoots and roots. PA richness tended to be higher in shoots and roots of the F_1s compared to the parental species, and the natural hybrids and the F_1s contained PAs from both *S. aquaticus* and *S. jacobaea*. PA expression was affected significantly by taxa in both shoots and roots. Based on principal component analysis, we found that taxa was a significant factor with regard to PC2 in the shoots, which is explained mostly by variation in jacobine content. For PC1, which was highly correlated with seneciphylline in the shoots, there was no effect of taxa. In plant roots, taxa was a significant factor for both principal components. The effects of selective pressures by natural enemies on secondary metabolite composition have been difficult to demonstrate experimentally (i.e., Macel et al. 2005; Albrechtsen et al 2007), and the comparative method used here is a promising avenue of further research.

Novel Compounds With regard to hybridization between *S. jacobaea* and *S. aquaticus*, both F_1 and natural hybrids produce the PA florosenine, which may be a novel product of hybridization in the Zwanenwater population. Florosenine has been reported previously from the South American *Senecio* species *S. glaber* (Reina et al. 1993) and *S. leptolobus* (Mendez et al. 1990), as well as from a Swiss population of *S. aquaticus* (Pelser et al. 2005). Florosenine was never found in *S. aquaticus* from the Zwanenwater nature reserve during this current study, or during previous ones (Kirk et al. 2004). Trace amounts of florosenine were found in one *S. jacobaea* individual here, but this occurrence may represent introgression, since this PA has never been reported from other *S. jacobaea* populations, and was not found in either Zwanenwater *S. jacobaea* genotypes, or control populations from a variety of European locations analyzed in a previous study (Kirk et al. 2004). Florosenine is the O-acetyl derivative of otosenine. Mechanistically, it is possible that hybridization combines the ability of *S. jacobaea* to acetylate PAs, and the ability of *S. aquaticus* to synthesize otosenine. If such inter-specific epistatic interactions between enzymes and substrates can result in the production of unique PA structures, then hybridization within the genus *Senecio* may be a mechanism for structural PA diversification.

This study system is useful for studying variation in PA expression. Environmental conditions play an important regulatory role in PA concentrations in above-and below-ground plant parts, and these interactions seem mainly to be governed by senecionine, seneciphylline, and jacobine. A general problem with these types of experiments is that it is not yet possible to distinguish between the direct effects of the treatments and the indirect effect of the treatments caused by changes in the microbial community in the soil. Although the induced changes in PA expression in richness are likely to be relevant for plant defense, this still remains to be tested experimentally. The evaluation of natural selection on different hybrid genotypes that possess different combinations of PAs may be extremely useful for elucidating the role of PA diversity or expression of other secondary metabolites in plants (e.g., Lexer et al. 2003).

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The Tea Weevil, *Myllocerinus aurolineatus*, is Attracted to Volatiles Induced by Conspecifics

Xiao-Ling Sun · Guo-Chang Wang · Xiao-Ming Cai · Shan Jin · Yu Gao · Zong-Mao Chen

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Abstract The tea weevil, *Myllocerinus aurolineatus* (Voss) (Coleoptera: Curculionidae), is a leaf-feeding pest of *Camellia sinensis* (O.Ktze.) with aggregative behaviors that can seriously reduce tea yield and quality. Although herbivore-induced host plant volatiles have been shown to attract conspecific individuals of some beetle pests, especially members of the Chrysomelidae family, little is known about the volatiles emitted from tea plants infested by *M. aurolineatus* adults and their roles in mediating interactions between conspecifics. The results of behavioral bioassays revealed that volatile compounds emitted from tea plants infested by *M. aurolineatus* were attractive to conspecific weevils. Volatile analyses showed that infestations dramatically increased the emission of volatiles, (*Z*)-3-hexenal, (Z)-3-hexenol, (*E*)-β-ocimene, linalool, phenylethyl alcohol, benzyl nitrile, indole, (*E*, *E*)-α-farnesene, (*E*)-nerolidol, and 31 other compounds. Among the induced volatiles, 12 chemicals, including γ-terpinene, benzyl alcohol, (*Z*)-3-hexenyl acetate, myrcene, benzaldehyde, (*Z*)-3-hexenal, and (*E, E*)-α-farnesene, elicited antennal responses from both sexes of the herbivore, whereas (*E*)-β-ocimene elicited antennal responses only from males. Using a Y-tube olfactometer, we found that six of the 13 chemicals, γ-terpinene, benzyl alcohol, (*Z*)-3-hexenyl acetate, myrcene, benzaldehyde, and (*Z*)-3-hexenal, were attractive to both males and females; two chemicals, (*E/Z*)-β-ocimene and (*E, E*)-α-farnesene, were attractive only to males; and four chemicals, (*E*)-4,8-dimethyl-1,3,7-nonatriene, phenylethyl alcohol, linalool, and (Z)-3-hexenol, were attractive only to females. The findings provide new insights into the interactions between tea plants and their herbivores, and may help scientists develop new strategies for controlling the herbivore.

Key Words Attraction · Volatiles · *Camellia sinensis* · *Myllocerinus aurolineatus* · Bioassay

Xiao-Ling Sun and Guo-Chang Wang contributed equally.

X.-L. Sun · G.-C. Wang · S. Jin · Y. Gao · Z.-M. Chen (✉)
Tea Research Institute,
Chinese Academy of Agricultural Sciences,
Hangzhou 310008, China
e-mail: zmchen2006@163.com

G.-C. Wang
Henan Institute of Science and Technology,
Xinxiang 453000, China

X.-M. Cai
Zhejiang Academy of Agricultural Sciences,
Hangzhou 310008, China

Introduction

Herbivore-induced plant volatiles (HIPVs) have many functions: they can act as an indirect defense by attracting natural enemies of the herbivores (Vet and Dicke 1992; Dicke and Vet 1999; Turlings and Wäckers 2004), as a direct defense by deterring attack of the subsequent herbivores (Delphia et al. 2007), as a synergistic agent with sex or aggregation pheromones (Wakefield et al. 2005; Erbilgin et al. 2007), or as an attractant for conspecific or non-conspecific herbivores (Loughrin et al. 1996; Bolter et al. 1997; Kalberer et al. 2001; Otálora-Luna1 et al. 2009). Many species of Coleoptera beetles with aggregative behaviors attract conspecifics (Loughrin et al. 1996; Bolter et al. 1997; Kalberer et al. 2001), but the active chemicals involved have not been identified.

In Curculionidae beetles, some species use host plant volatiles as pheromone synergists or to locate host plants (Ndiege et al. 1996; Perez et al. 1997; Wang and Kays

2002; Wakefield et al. 2005). The addition of volatiles from sugarcane stalks to pheromone lures, for example, enhances lure effectiveness for trapping the West Indian sugarcane weevil *Metamasius hemipterus sericeus* (Oliv.) (Perez et al. 1997). Carob volatiles not only acted synergistically with aggregation pheromones in 3 weevil species, *Sitophilus zeamais* (Motsch.), *S. granarius* (L.), and *S. oryzae* (L.) but also directly attracted *S. zeamais* and *S. oryzae* (Wakefield et al. 2005). Volatile extracts from storage roots and aerial parts of the sweet potato were attractive to female sweet potato weevils (Wang and Kays 2002). Surprisingly, few studies have determined the role of HIPVs in mediating conspecific interactions in Curculionidae beetles. To date, only *Diaprepes abbreviatus* (L.) has been verified to be attracted to the volatiles from host plants infested by conspecific weevils. Otálora-Luna et al. (2009) discovered that (±)-linalool and carvacrol were emitted from citrus plants infested by the tropical root weevil *D. abbreviatus*; combined with a green leaf volatile (Z)-3-hexenol at a dose of 25:2.5:25 μg, these compounds attracted conspecific females, but, were repellent for males.

Myllocerinus aurolineatus (Voss) (Coleoptera: Curculionidae) is one of the main leaf-feeding insects on tea plantations (Zhu et al. 1988). Native to China, adult weevils feed on young, tender leaves, seriously deteriorating the yield and quality of tea. Although chemical control is effective against *M. aurolineatus*, it poses health and environmental risks associated with the residues that remain on tea leaves and the resistance that herbivores may develop, which in turn may lead to the resurgence of primary pests and outbreaks of secondary pests. Alternative strategies for controlling this weevil are needed.

The composition of volatiles from tea plants infested by *M. aurolineatus* adults and their roles in mediating interactions between conspecifics have not been investigated. To elucidate the role of tea volatiles induced by feeding of *M. aurolineatus* adults in host-searching behavior, we first measured the weevil response to intact plants, *M. aurolineatus* frass, and *M. aurolineatus* adults. Then, we collected and identified the volatiles released by plants infested with *M. aurolineatus* adults and the volatiles released by control plants. Finally, we screened and tested the attractiveness of the active components from the collected volatiles to *M. aurolineatus* adults.

Methods and Materials

Tea Plants One year before the experiment commenced, 1-yr-old Longjing tea plants were individually planted in plastic pots (14×15cm), irrigated every other day, and fertilized once a month. Plants were kept in a controlled climate room at 26±2°C and 70–80% relative humidity with a 12-h light cycle to provide plants with about 450 μmol photons/m^2/sec.

Insects Adult weevils of mixed age and sex and of unknown mating status were collected from damaged plants on the Tea Experimental Plantation (Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou Xihu Longjing First-degree Reserve, China). The insects were kept in a box for 2 d, then separated into males and females according to morphological characters and maintained in separate plastic containers (11.5×13 cm) with fresh tea leaves. Containers were put into a controlled climate chamber at 25±2°C and 70±5% relative humidity, with a photoperiod of 13 hL:11 h D. Two weeks later, weevils were starved for 2 h and used in the experiments.

Olfactometer Test Responses of *M. aurolineatus* adults to odors released from different sources or synthetic compounds were measured in a Y-tube olfactometer consisting of a Y-shaped glass tube (2.5 cm diam) consisting of a 12 cm base tube and two 12 cm arms at a 75° angle. Each arm was connected in series to an odor source container, a humidifier bottle, a tube with activated charcoal, and a flow meter. Both arms were connected to the exhaust of a common vacuum pump that controlled the air flow through the system at 800 ml min^{-1}. Weevils were allowed to walk through the base tube towards the odor sources. The Y-tube olfactometer was placed in a green box and each edge of the top was illuminated by a single 25-W lamp. All bioassays were conducted between 15:00 and 20:00 h in a room that was maintained at 25–27°C.

Each weevil was introduced individually into the base tube. Choice for an odor source was defined as a weevil crossing a line 8 cm after the division of the base tube and remaining there for at least 1 min. If a weevil did not make a choice within 5 min, it was considered a non-responding individual and recorded as “no choice”. After 2 weevils were tested, the olfactometer tube was washed with 98% acetone and then heated at 100°C for 5 min. The position of odor source containers in relation to each arm was reversed after each replication to eliminate directional bias. To avoid contamination, the connections of the odor chamber to the flow meter were rinsed with 98% acetone and then heated at 100°C for 5 min after each replication.

Response to Individual Components of the Plant—Weevil Complex In this experiment, the following odor sources were prepared:

1. Intact plants (IP). Potted plants were washed with running water and then put in glass chambers, which were the same as those in which volatiles were collected (see below). Four days later, the plants were used as an odor source for bioassays.

2. Plant-*M. aurolineatus* adult complex (PMA). Plants were prepared as in the above treatment (1) and 3 d later were infested individually with 100*M. aurolineatus* adults (female:male=1:1). One day later, the plants carrying 100*M. aurolineatus* adults were used as an odor source.
3. *M. aurolineatus* adults (MA). One hundred *M. aurolineatus* adults (female:male=1:1) were collected and used as an odor source.
4. Frass: Plants were individually infested with 100*M. aurolineatus* adults (female:male=1:1). One day later, frass was collected carefully with a clean brush, and 2 g were placed on a small Petri dish (5 cm diam) as an odor source.
5. Chemical standards. Silicon rubber septa were used as odor dispensers by adding 10 μl of one chemical to a septum, airing for 5 h, and storing the loaded septum at −20°C until it was individually placed in an odor container. Each septum was used for experiments for less than 3 h. Within that time, the release rates of the various chemicals from the septa were stable. All chemicals used were at least 98% pure and were obtained from the commercial suppliers listed in Table 1.

The behavioral responses of the weevils to the following pairs of odors were tested in the olfactometer: (1) purified air vs. IP, frass, MA, and PMA, respectively; (2) IP vs. PMA; (3) MA vs. PMA; (4) purified air vs. (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, (*Z*)-3-hexenal, benzaldehyde, myrcene, benzyl alcohol, β-ocimene, γ-terpinene, linalool, phenylethyl alcohol, (*E*, *E*)-α-farnesene, or (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), respectively. The sex ratios of the weevil adults tested in (1), (2), and (3) were 1:1 (female:male). The weevil adults used in (4) were sexed according to their morphological characteristics.

Collection and Identification of Tea Volatiles The collection system (Fig. 1) consisted of a vertical cylindrical glass tube (18 cm i.d.×25 cm height) resting on a glass plate with a hole in its center. A halved rubber stopper with a 0.6 cm i.d. hole allowed the stem of the plant to pass through the hole in the plate, so that the living aerial part of the plant in the cylinder was separated from the pot (Fig. 1). At the top of the cylinder, another glass plate with a hole in the center was closed with a rubber plug that had two holes (0.8 cm i.d.). The inlet port was a glass tube (0.6 cm i.d.×30 cm) that was inserted through the stopper to about 1.5 cm above the bottom plate. The outlet port was another glass tube (0.6 cm i.d.×6.5 cm) inserted just to the bottom edge of the rubber plug. Purified air was pulled into the cylinder over the plant at a rate of 1.36 $l min^{-1}$. Incoming air was filtered through a silica gel for dryness, an activated charcoal filter for purification, a flow meter for measuring and regulating the airflow, and another activated charcoal filter for secondary

Table 1 Relative amounts (% of internal standard peak area) of volatiles released by tea plants infested by *Myllocerinus aurolineatus* adults for 1 day

Compound	mean ± SE (%)
1. (*Z*)-3-hexenal [a]	6.6±1.1
2. (*E*)-2-hexenal [c]	3.2±0.6
3. (Z)-3-hexenol [c]	8.4±1.7
4. Unknown 1	4.3±1.0
5. Unknown 2	2.9±0.6
6. benzaldehyde [b]	1.3±0.3
7. β-myrcene [c]	0.4±0.04
8. (*Z*)-3-hexenyl acetate [a]	1.7±0.4
9. cymene (*para*) [d]	0.3±0.03
10. limonene [c]	0.2±0.1
11. benzyl alcohol [d]	0.8±0.2
12, (*E*)-β-ocimene [ch]	27.5±5.0
13. terpinene (*gamma*) [e]	0.2±0.03
14. linalool oxide (*E*)- furanoid [h]	1.0±0.1
15. linalool oxide (*Z*)- furanoid [h]	0.4±0.03
16. linalool [e]	5.5±0.3
17. 3,7-octadiene-2,6-diol, 2,6-dimethyl- [i]	0.3±0.03
18. phenylethyl alcohol [e]+DMNT [g]	23.5±4.0
19. 1,3,8-p-menthatriene [h]	0.4±0.1
20. benzyl nitrile [e]	7.2±1.0
21. linalool oxide (*E*) (pyranoid) [h]	0.5±0.048
22. linalyl oxide (*Z*)-(pyranoid) [h]	0.2±0.01
23. (*Z*)-3-hexenyl butyrate [a]	1.9±0.1
24. (*Z*)-3-hexenyl-2-methyl butyrate [b]	0.9±0.036
25. (*Z*)-3-hexenyl-3-methyl butyrate [b]	0.2±0.028
26. Unknown3	1.1±0.2
27. Unknown4	0.9±0.1
28. indole [b]	17.1±0.8
29. phenyl ethane(1-nitro-2-) [h]	1.4±0.2
30. (*Z*)-3-hexenyl hexanoate [b]	0.4±0.03
31. (*E*)-2-hexenyl hexanoate [b]	0.2±0.01
32. Unknown5	0.2±0.02
33. (*E*)-caryophyllene [d]	0.9±0.2
34. (*E*)-β-farnesene [f]	0.1±0.02
35. Muurolene(*gamma*) [h]	0.2±0.03
36. phenyl ethyl 2-methylbutanoate [h]	0.3±0.02
37. Muurola-4(14),5-diene (*trans*) [h]	0.3±0.1
38. (*E*, *E*)-α-farnesene [f]	15.1±3.2
39. cadinene(*delta*) [h]	0.1±0.004
40. (*E*)-nerolidol [f]	4.7±0.5

The names of the compounds followed by different letters indicate different methods for confirming identities: [a–g] comparison of retention times and mass spectra with those of authentic standards as follows: [a] Roth; [b] Sigma-Aldrich; [c] Fluka; [d] TCI; [e] Acros; [f] Pherotech; [g] gift from Taro Maeda; [h] comparison of Kovats Indices (KI) on DB-5, [i] comparison of mass spectra with the database. Data represent the mean of four replications ± SE.

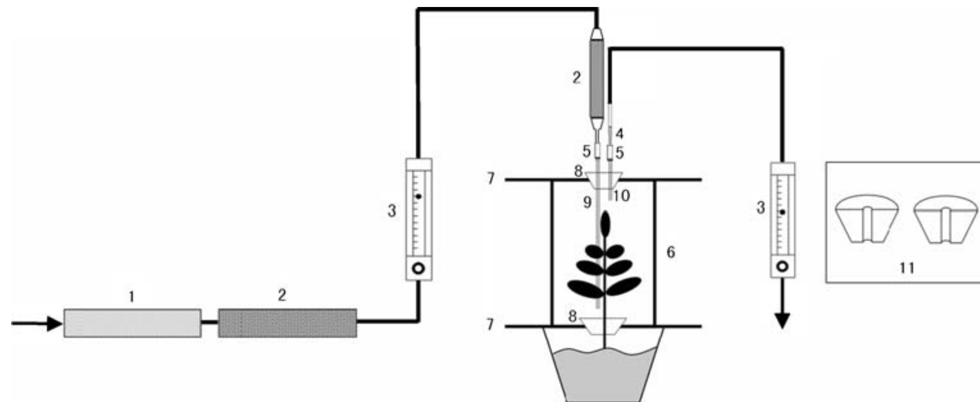


Fig. 1 Apparatus for collection of volatiles. 1. silica gel filter, 2. activated charcoal filter, 3. flow meter, 4. superQ trap, 5. teflon tubing, 6. cylindrical glass tube, 7. glass plate, 8. rubber plug, 9. inlet port, 10. outlet port, 11. details of rubber plug

purification. The blend of volatiles was trapped in a glass tube (0.4 cm i.d.×10 cm) that contained 35 mg of 80/100 mesh SuperQ absorbent (Alltech Associates., Inc., Deerfield, IL, USA). Teflon tubing was used to connect the flow meter to the inlet port, the outlet port to the trap, and the other end of the trap to a flow meter and vacuum pump. During collection, room temperature was 27±1°C, and the system was illuminated by two fluorescent lamps with a photoperiod of 13 hL:11 h D.

Volatiles emitted from intact plants and plants that were infested with 100*M. aurolineatus* adults (female:male=1:1) were collected. We also collected volatiles from a pot of soil without plants to determine whether the system was clean. Collection started 24 h after treatment and lasted 1 h. After each collection, traps were rinsed with 500 µl methylene dichloride, and 50 ng of ethyl decanoate were added to each sample as an internal standard. Each treatment was replicated four times. The amounts of volatile compounds were expressed as percentages of peak areas relative to the internal standard.

Analyses were carried out with a coupled gas chromatograph-mass spectrometer QP2101 (Shimadzu, Japan) equipped with an automated on-column injection system and a DB-5 MS capillary column (60 m×0.25 mm i.d., 0.25 µm film J&W Scientific, Folsom, CA, USA). The injection port was operated in splitless mode with a constant helium flow of 1 ml min^{-1}. The injector temperature was 250°C, the ionization potential was 70 eV, and the scan frequency was 2 sec^{-1}. Following injection, the column temperature was maintained at 45°C for 2 min, ramped at 5°C min^{-1} to 210°C, ramped at 25°C min^{-1} to 260°C, and held for 10 min. Data were collected with Shimadzu GC ChemStation software. The limitation of detection (LOD) is approximately 2.0 ng±0.4 ng.

For data analysis, GC-MS solution software (Shimadzu, Japan) was used, including the mass spectra library NIST 02 (National Technical Information Services). Library matches revealed candidate compounds. Identities were confirmed by comparing retention times and mass spectra with those of authentic standards (when available) or by comparing Kovats Indices (KI) on DB-5 if the chemical standards were not available.

Electrophysiological Recording and Chemical Analysis To distinguish the active components of weevil-induced volatiles, a GC-EAD instrument was used. An Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-5 capillary column (30 m×0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Folsom, CA, USA) was coupled to electro-antennographic detection (GC-EAD system). Samples used for the EAD tests were collected as described above from the plants carrying 100*M. aurolineatus* adults (female: male=1:1). Each sample was concentrated under nitrogen (99.999%) to about 2 µl for injection. The injection port was operated in splitless mode. The temperature program was the same as that described for GC-MS analyses. The column effluent was split 1:1, with one part going to the flame ionization detector of the GC and another to the EAD through a heated (230°C) transfer line (Syntech, Hilversum, Netherlands). The EAD outlet was introduced into a glass tube with a constant air flow (0.5 ml min^{-1}). The humidified and purified supplemental airstream for the EAD was supplied continuously at 400 ml min^{-1} and directed at an excised tea weevil antenna. Two glass capillary recording Ag-AgCl electrodes filled with 0.5 M KCl solution were brought into contact with the distal and basic segments of the antenna, the end of which was cut carefully. Antennal signals were amplified and converted by using an interface box (Auto Spike, IDAC 2/3, Syntech), Krichzarten, Germany) before they were recorded. The FID and EAD signals were monitored simultaneously and stored on a personal computer for subsequent analysis with Electro-AntennoGraphy software (professional version, Syntech).

Statistical Analysis All statistical analyses were performed using the Statistica program (Statistica, SAS, Institute Inc., Cary, NC, USA). The differences between electrophysiological responses from the antennae of male and female weevil adults were analyzed for significance by *t*-test. The differences between the number of *M. aurolineatus* males and females entering each arm of the olfactometer for each paired treatment were analyzed by the *Kruskal-Wallis* test (χ^2 approximation).

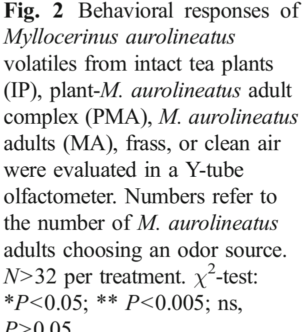
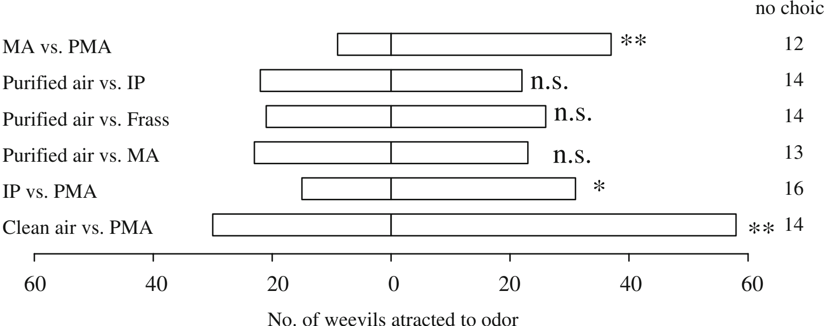


Fig. 2 Behavioral responses of *Myllocerinus aurolineatus* volatiles from intact tea plants (IP), plant-*M. aurolineatus* adult complex (PMA), *M. aurolineatus* adults (MA), frass, or clean air were evaluated in a Y-tube olfactometer. Numbers refer to the number of *M. aurolineatus* adults choosing an odor source. $N>32$ per treatment. χ^2-test: *$P<0.05$; ** $P<0.005$; ns, $P>0.05$



Results

Responses of M. aurolineatus Adults to Different Odors Volatiles emitted from the tea plant-weevil complex were significantly more attractive to the weevils than were clean air or the volatiles from weevils, frass, or intact tea plants (Fig. 2).

Volatile Analysis When plants were infested by adult weevils for one day, the quantity and number of volatiles dramatically increased compared to the volatiles released from intact plants (Table 1, Fig. 3). More than 40 compounds were emitted from plants infested with *M. aurolineatus* adults, including 9 fatty acid derivatives, 7 amino acid derivatives, 20 terpenoids, and 5 unknown compounds (Table 1, Fig. 3).

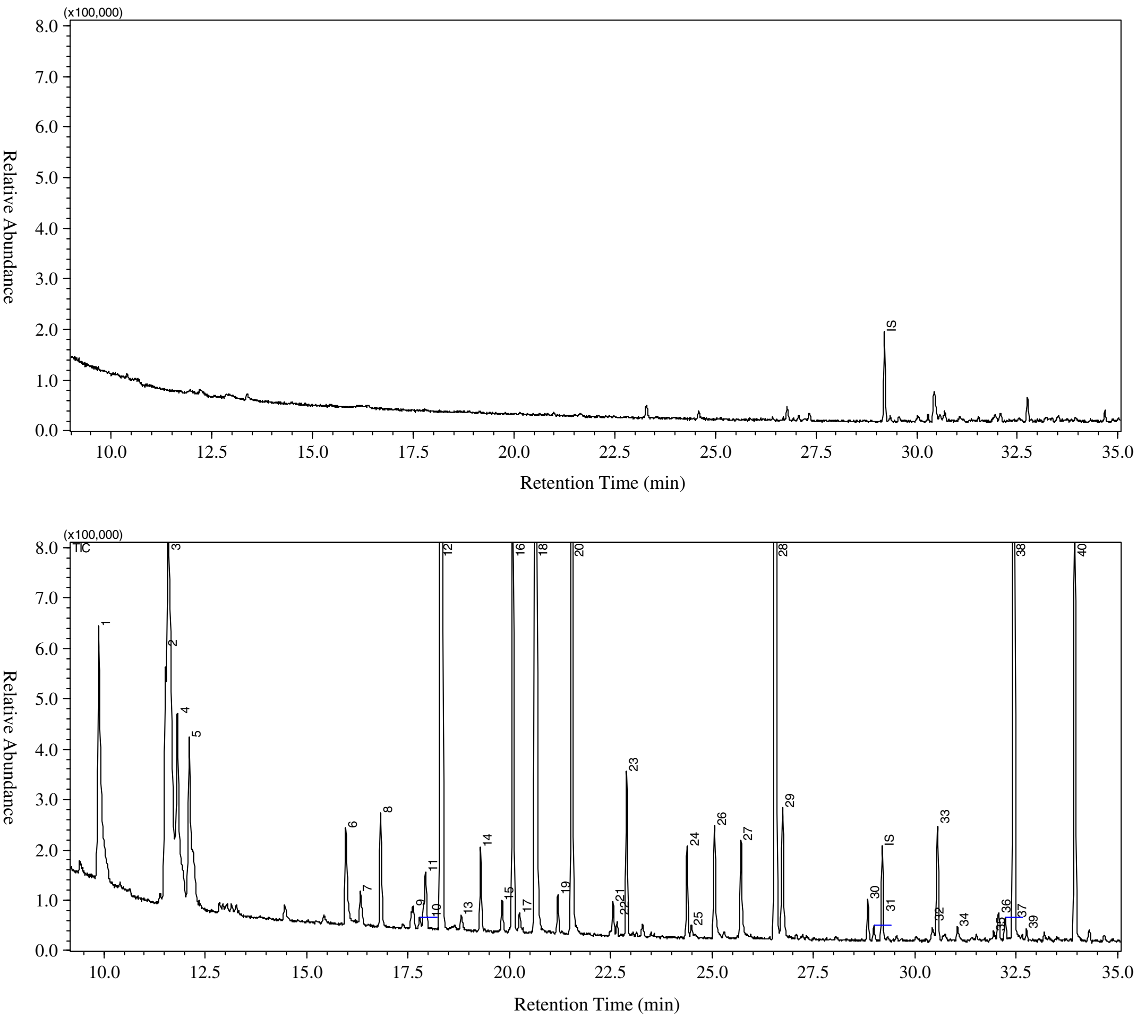


Fig. 3 Typical chromatograms obtained from headspace collections of volatiles from intact tea plants (IP) and plant-*Myllocerinus aurolineatus* adults complex (PMA)

Table 2 Electrophysiological activity (mean ± se) during gas chromatography-electroantennography (GC-EAD) recording of male and female *Myllocerinus aurolineatus* antenna exposed to volatiles collected from conspecific weevils on tea plants

Peak	Identification[a]	Female antennae		Male antennae	
		EAG responses in 5 runs[b]	EAG intensity (μV)	EAG responses in 5 runs[b]	EAG intensity (μV)
1	(*Z*)-3-hexenal *	5	92±20.6	5	136±8.9
2	(Z)-3-hexenol	5	96±11.7	5	92±4.9
3	Unhnown	5	44±9.8	5	28±8
4	benzaldehyde	2	8±4.9	3	12±4.9
5	myrcene	2	8±4.9	2	8±4.9
6	(*Z*)-3-hexenyl acetate	2	16±9.8	5	28±4.9
7	benzyl alcohol	5	20±0	4	16±4
8	(*E*)-*β*-ocimene	0	0	1	4±4
9	γ-terpinene	5	24±4	4	24±7.5
10	linalool	5	24±4	4	24±7.5
11	phenylethyl alcohol	5	36±4	5	32±4.9
12	DMNT	2	8±4.9	4	20±6.3
13	(*E*, *E*)-α-farnesene	4	16±4	2	8±4.9

[a] Identification is based on comparing mass spectra and retention times of authentic standards with the volatiles induced by weevils.

[b] Based on five different volatile samples.

* *t*-test comparing male response to female response, $P<0.05$.

(*E*)-β-Ocimene, phenylethyl alcohol, or DMNT were the most abundant. These volatiles were not detected in the headspace from intact plants.

Gas Chromatography-electroantennography Analysis In the blend of volatiles, there were 13 and 12 compounds that elicited antennal responses from male and female weevils, respectively (Table 2, Fig. 4). Among these, two compounds, (*Z*)-3-hexenal and (Z)-3-hexenol (peaks 1 and 2) elicited strong and consistent electrophysiological responses from the antennae of both sexes of *M. aurolineatus* adults, and 7 chemicals (peaks 3–5, 7, 9–11) elicited weak electrophysiological responses from the antennae of both sexes (Table 2 , Fig. 4). Among these, only (*Z*)-3-hexenal (peak 1) elicited significantly different electrophysiological responses from the antennae of male and female weevil adults.

Behavioral Responses of M. aurolineatus Adults to EAD-Active Compounds Twelve EAD-active compounds were tested in a Y-tube olfactometer (Fig. 5). Compared with clean air, both males and females were significantly attracted to γ-terpinene, benzyl alcohol, (*Z*)-3-hexenyl acetate, myrcene, benzaldehyde, and (*Z*)-3-hexenal. Two chemicals, (*E, E*)-α-farnesene and (*E/Z*)-β-ocimene, were attractive only to males, and 4 compounds, DMNT, phenylethyl alcohol, linalool, and (*Z*)-3-hexenol, were attractive only to females.

Discussion

Our results show clearly that the tea weevil, *M. aurolineatus,* was attracted to tea plants infested by conspecifics but not to undamaged tea plants, conspecific herbivores, or frass

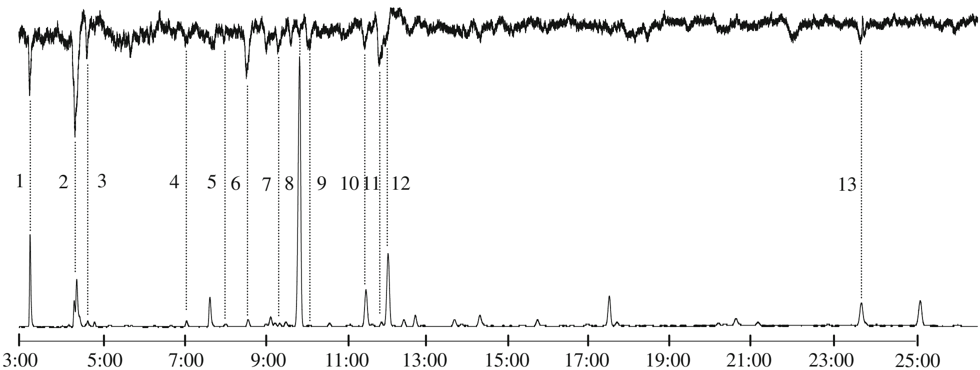
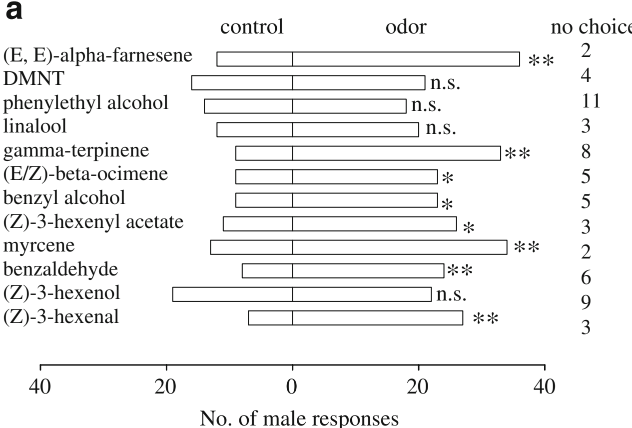


Fig. 4 Simultaneous responses of flame ionization detector (FID) and electroantennographic detection (EAD) recording of female *Myllocerinus aurolineatus* adult antennae response to the volatiles collected from the headspace of tea plants infested by *M. aurolineatus*. Thirteen EAD active compounds were identified as follows: 1. (*Z*)-3-hexenal, 2. (*Z*) -3-hexen-1-ol, 3. Unknown, 4. Benzaldehyde, 5. Myrcene, 6. (*Z*)-3-hexenyl acetate, 7. Benzyl alcohol, 8. (*E*)-*β*-ocimene, 9. γ-terpinene, 10. Linalool, 11. Phenylethyl alcohol, 12. DMNT, 13. (*E*, *E*)-α-farnesene



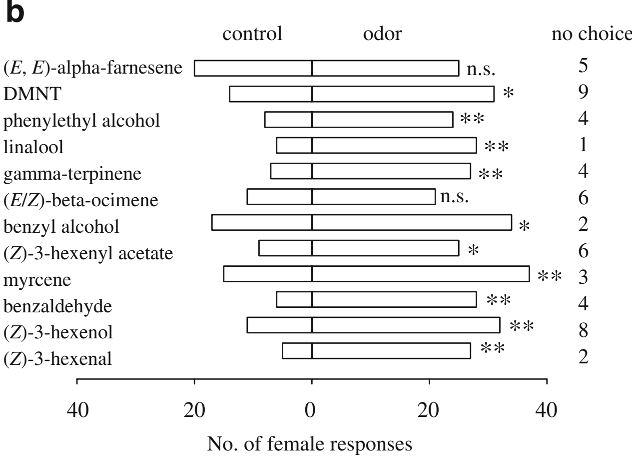


Fig. 5 Behavioral responses of *Myllocerinus aurolineatus* (A) males and (B) females to twelve synthetic compounds *vs*. clean air evaluated in a Y-tube olfactometer. Numbers refer to the number of *M. aurolineatus* adults choosing an odor source. $N>32$ per treatment. χ^2-test: *$P<0.05$; ** $P<0.005$; ns, $P>0.05$

from conspecifics. This suggests that *M. aurolineatus* adults use volatiles induced by conspecific feeding as olfactory cues when foraging for hosts, and it may in part explain the aggregation behavior of these herbivores in the field. Similar findings have been reported for other species of beetles in the Chrysomelidae (e.g., the Colorado potato beetle, *Leptinotarsa decimlineata* (Say) (Bolter et al. 1997) and the leaf beetle, *Oreina cacaliae* (Schrank) (Kalberer et al. 2001)), Scarabaedae (e.g., *Popillia japonica* (Newman) (Loughrin et al. 1996)); and in the Curculionidae (e.g., *D. abbreviatus* (Otálora-Luna et al. 2009)), as well as in the larvae of some lepidopteran families (Carroll et al. 2006).

The tea weevil may benefit from its response to volatiles from conspecific-damaged tea plants. First, the behavioral response may result in mass attacks that may decrease or even overwhelm the host plant's resistance. This has been reported in other beetles, especially the bark beetle (Lieutier 2002). Second, such behavior results in aggregations of both sexes on individual plants, which may in turn increase the probability of mating and the number of mates from which to choose, as shown in *O. cacaliae* by Kalberer et al. (2001), *Popillia japonica* Newman by Loughrin et al. (1996), and *L. decemlineata* by Bolter et al. (1997). Finally, the aggregation behavior may dilute the effect of natural enemies, as shown for the female diamondback moth *Plutella xylostella* (Linnaeus) (Shiojiri and Takabayashi, 2003). Our study suggests that the volatiles emitted from tea plants damaged by *M. aurolineatus* provide weevils with information about the host and conspecifics. These chemical signals may enhance host-searching efficiency, thus facilitating the survival and reproduction of *M. aurolineatus* under natural conditions.

After infestation with *M. aurolineatus* adults, tea plants released more than 40 chemicals, including green leaf volatiles, amino acid derivatives, and terpenoids. Not all of these compounds are unique to the induction of adult *M. aurolineatus* infestation, as (*Z*)-3-hexenol, benzaldehyde, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, ocimene, and linalool have been reported as components of tea shoot–aphid complexes (Han and Chen 2002). Using GC-EAD analysis combined with Y-tube olfactometer bioassays, we found 6 chemicals, γ-terpinene, benzyl alcohol, (*Z*)-3-hexenyl acetate, myrcene, benzaldehyde, and (*Z*)-3-hexenal, which were attractive to both male and female *M. aurolineatus* adults; 2 chemicals, (*E*/*Z*)-β-ocimene and (*E, E*)-α-farnesene, which were attractive only to males; and 4 chemicals, DMNT, phenylethyl alcohol, linalool, and (*Z*)-3-hexenol, which were attractive only to females. Nine of the 12 compounds identified in this study have been reported previously as attractants for other insects. Campbell et al. (1993) found that the damson-hop aphid *Phorodon humuli* (Schrank) was attracted by (*Z*)-2-hexenal and β-caryophyllene, and deterred by methyl salicylate, and that these chemicals are emitted by conspecifics feeding on the hop host, *Humulus lupulus* L. Faccoli et al. (2008) found that myrcene, a component of HIPVs, modulates the host-seeking behavior of *Tomicus destryens* (Wollaston).

Unique combinations of odor components may provide a substantial degree of specificity for the host-seeking process of phytophagous insects. For instance, the Colorado potato beetle is attracted by a blend of (*Z*)-3-hexenyl acetate, (±)-linalool, and methyl salicylate (Dickens 2000). The blend of (±)-linalool, (Z)-3-hexenol, and carvacrol (25:25:2.5 μg) is attractive to the female tropical root weevil *D. abbreviatus*, but repellent to males (Otálora-Luna et al. 2009). However, linalool released from *Zea mays* plants infested by sixth-instars of *Spodoptera frugiperda* (Smith) was attractive to conspecific larvae (Carroll et al. 2006). An attractive function has been described in a number of insects for the following four compounds: DMNT for *S. frugiperda* (Carroll et al. 2008), phenylethyl alcohol for mosquito *Culex pipiens pipiens* Linnaeus (Jhumur et al. 2007), benzaldehyde for *Chrysopa sinica*

Tjeder, *Aphidius sp*., (*E, E*)-α-farnesene for the codling moth, *Cydia pomonella* (L.) (Yan et al. 2003; Landolt and Guédot 2008) and *Coccinella septempunctata* Linnaeus (Han and Chen 2002). To our knowledge, γ-terpinene, (Z)-3-hexenal, and benzyl alcohol have not been found to attract any insects.

Our results suggest that herbivore-induced host plant volatiles may have potential application in the monitoring and control of *M. aurolineatus* adults. Therefore, future research should be carried out in the field to assess the potential trapping effectiveness of the semiochemicals identified here.

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Isolation and Phytotoxicity of Terpenes from *Tectona grandis*

Francisco A. Macías · Rodney Lacret · Rosa M. Varela · Clara Nogueiras · Jose M. G. Molinillo

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Abstract A study was carried out on the allelopathic potential of four forest species, *Tectona grandis*, *Aleurites fordii*, *Gliricidia sepium*, and *Maytenus buxifolia*. The most active species, *T. grandis*, was selected to perform a phytochemical study. A new compound, abeograndinoic acid, was isolated, and elucidation of its structure showed that this compound has an unusual carbon skeleton. A further 21 known terpenoids—including 4 sesquiterpenoids, 8 diterpenes and 9 triterpenes—also were isolated. A biosynthetic scheme for the presence of the new compound is proposed. Bioactivity profiles that used etiolated wheat coleoptiles and phytotoxicity bioassays on the isolated compounds were conducted. The compounds that presented the highest phytotoxic activity are the diterpenes **9** (2-oxokovalenic acid) and **12** (19-hydroxyferruginol).

Key Words Allelopathy · Verbenaceae · *Tectona grandis* · Terpene · Clerodane

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F. A. Macías (✉) · R. Lacret · R. M. Varela · J. M. G. Molinillo
Grupo de Alelopatía, Departamento de Química Orgánica,
Facultad de Ciencias, Universidad de Cádiz,
C/ República Saharaui s/n, Apdo. 40,
11510 Puerto Real, Cádiz, Spain
e-mail: famacias@uca.es

C. Nogueiras
Centro de Estudios de Productos Naturales,
Departamento de Química Orgánica, Facultad de Química,
Universidad de la Habana,
La Habana, Cuba

Introduction

Exotic plant species represent the core of agricultural production in many countries. Cuba is no exception, since most of the agricultural species grown in the country are exotic, having been introduced at some point in the past. A number of exotic timber trees were introduced into Cuba from tropical and subtropical regions. The scientific literature supports the ethnobotanical use of such species as sources of bioactive substances of both pharmacological and agrochemical interest. Examples of this phenomenon include neem (*Azadirachta indica* A. Juss) (Atawodi and Atawodi 2009), chinaberry (*Melia azedarach* L.) (Charleston et al. 2006), tamarind (*Tamarindus indica* L.) (Khanzada et al. 2008), gliricidia (*Gliricidia sepium* Jack) (Ramamoorthy and Paliwal 1993), wild indigo (*Indigofera suffruticosa* Mill) (Barros and Teixeira 2008), and sugar-apple (*Annona squamosa* L.) (Naik et al. 2008).

Better utilization of non-wood forest products (NWFPs) requires knowledge of the ecological relationships of introduced alien species and their potential as sources of bioactive compounds, especially agrochemical and allelopathic potential. The families *Celastraceae*, *Fabaceae*, *Euphorbiaceae*, and *Verbenaceae* have a wide variety of tree species with a number of biological activities. Several species within these families are distributed in Cuba, and these include *Tectona grandis* (Verbenaceae), *Aleurites fordii* (Euphorbiaceae) (Fozdar et al. 1989), *Gliricidia sepium* (Fabaceae) (Herath et al. 1997), and *Maytenus buxifolia* (Celastraceae) (González et al. 1997). These species are economically important and have been used in agroforestry systems or for various ethnobotanical applications. However, the possible environmental impact that arises from the introduction of these species has not been

evaluated, nor has the possible use of these species as a source of natural chemicals.

Herein, we describe a study of the allelopathic potential of these forest species. The species with the highest allelopathic activity, i.e., teak (*T. grandis) (Verbenaceae),* was selected for a phytochemical study with the aim of isolating and characterizing the metabolites responsible for the biological activity. Few compounds have been isolated from teak, and most of these are quinones and triterpenes (Rimpler and Christiansen 1977). The family Verbenaceae is characterized in chemical terms by the presence of low levels of diterpenes, with tectograndinol as the only example previously isolated from the genus *Tectona* (Joshi et al. 1977; Marwani et al. 1997).

The characterization of chemical components from *Tectona grandis* and the assessment of their activity are likely to provide information to explain the success of this tree culture where it has been used in agroforestry (taungya system). This system involves combining crops such as corn, cotton, cassava, ginger, peanut, soybean, upland rice, and beans with young teak plantations, an approach that results in higher crop yields and better control of the weeds (Wiersum 1982). From an ecological point of view, a move towards sustainable agriculture where weeds can be controlled to some extent by forest species such as *T. grandis* is desirable. The increased forest area is not only as a source of wood, but as a new potential tool for pest control.

Previously, we reported the isolation of seven apocarotenoids from *T. grandis* and elucidated the structures of two of them, tectoionols A and B (Macias et al. 2008). As a continuation of our study of this species, a bioassay-guided fractionation of extracts from *T. grandis* was carried out in order to isolate and identify the chemical constituents and to characterize their potential phytotoxic activities. We report here the isolation and structure elucidation of a new compound, abeograndinoic acid, which has an unusual carbon skeleton. A further 21 known terpenoids, including 4 sesquiterpenoids, 8 diterpenes, and 9 triterpenes, also were isolated. The bioactivity profiles of the isolated compounds were studied.

Methods and Materials

General IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000 or a Matton 5020 spectrophotometer. NMR spectra were run on Varian INOVA-400 and Varian INOVA 600 spectrometers. Chemical shifts are given in ppm with respect to residual ^{1}H signals of $CHCl_3$-d_1 and acetone-d_6 (δ 7.25 and 2.04, respectively), and ^{13}C signals are referenced to the solvent signal (δ 77.00 and 29.80, respectively). Optical rotations were determined by using a Perkin-Elmer model 241 polarimeter (on the sodium D line). HRMS were obtained on VG AUTOESPEC mass spectrometer (70 eV). HPLC was carried out on a Merck-Hitachi instrument, with RI detection, using three different Merck LiChrospher columns: RP-18 (10 µm, 250×10 mm), SI 60 (5 µm, 250×4 mm), and SI 60 (10 µm, 250×10 mm).

Plant Material and Aqueous Extracts Leaves and bark of *Tectona grandis*, leaves and flowers of *Gliricidia sepium*, and leaves of *Maytenus buxifolia* and *Aleurites fordii* were collected between the months February and March (2003) in Ciudad de La Habana and were identified by MsC. Lutgarda González. Voucher specimens of each species (80613, 80614, 80921, and 80922, respectively) were deposited at the Jardín Botánico de Cuba.

Vegetal material was dried at room temperature in the shade. Dried materials of each species (50 g) were extracted with water (450 ml) for 24 h at room temperature in the dark—except for leaves of *G. sepium* and *A. fordii*, which were extracted with 900 ml of water.

Extraction and Isolation Dried leaves of *Tectona grandis* (5 kg) were extracted with water (35 l) for 24 h at room temperature in the dark. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc at room temperature. Details of the extraction procedure and the bioassays on the extract have been described previously (Macias et al. 2008). DCM/H_2O and DCM extracts were the most active.

The DCM/H_2O extract (8.8 g) was chromatographed on SiO_2 using mixtures of hexane/EtOAc of increasing polarity, acetone and methanol to afford twelve fractions: A_1–L_1.

Fraction E_1 (0.750 g, hexane/EtOAc, 17:3–1:4) was subjected to CC on Sephadex LH-20 using mixtures of n-hexane/chloroform/methanol (3:1:1) in order to remove chlorophylls and other high molecular weight compounds. The residue was separated by CC and HPLC on silica gel using mixtures of chloroform/acetone and hexane/EtOAc to yield compounds **1** (5.0 mg), **2** (0.4 mg), and **3** (4.8 mg). Fraction F_1 (0.800 g, hexane/EtOAc, 1:4) was subjected to CC on Sephadex LH-20 using a mixture of hexane/chloroform/methanol (3:1:1). Further purification by C-18 HPLC (water/methanol, 7:13) yielded compound **4** (2.8 mg). Fraction I_1 (0.750 g) was subjected to CC on silica gel using mixtures of chloroform/acetone of increasing polarity and methanol. The largest fraction, $I_1$6 (0.300 g), was purified by CC on silica gel using mixtures of hexane/acetone to yield compound **21** (1.1 mg).

The DCM (120 g) extract was chromatographed on silica gel (2.0 kg) using hexane/ethyl acetate mixtures of increasing polarity to yield eight fractions: A_2–H_2.

Fraction C_2 (15.00 g) was subjected to CC on silica using 1.5 l of each solvent (hexane, chloroform, ethyl

acetate, acetone, and methanol) to afford five fractions: C_21–C_25. Fraction C_22 (0.906 g, $CHCl_3$) was chromatographed by CC on silica gel, using mixtures of hexane/chloroform and chloroform/acetone. Purification by silica gel HPLC yielded **15** (2.3 mg), **5** (2.8 mg), and **13** (60 mg). Fraction C_24 (4.900 g, acetone) was subjected to CC on silica gel, using mixtures of hexane/chloroform and chloroform/acetone. Further purification by CC using hexane/ethyl acetate mixtures on silica gel and hexane/chloroform/methanol (3:1:1) on Sephadex LH-20 , that was the stationary phase that best separated these compounds, afforded compounds **12** (12.5 mg), **16** (5.0 mg), **14** (3.2 mg), and **6** (3.3 mg).

Fraction D_2 (hexane/EtOAc, 3:2, 7.46 g) was subjected to CC on silica gel, using mixtures of n-hexane/chloroform and chloroform/acetone. Fraction D_22 (0.930 g) was chromatographed using mixtures of n-hexane/chloroform and chloroform/acetone to afford, after further purification by HPLC, compounds **17** (3.0 mg), **8** and **10** (3.6 mg). Fraction D_23 (1.800 g, n-hexane/chloroform 1:3) was subjected to CC on silica gel using mixtures of n-hexane/chloroform and acetone to afford six fractions D_23A–D_23F. Fractions D_23B (0.485 g, chloroform) and D_23C (0.492 g, chloroform) were purified by CC on silica gel using mixtures of n-hexane/acetone and by HPLC to yield compounds **9** (18 mg) and **7** (15.5 mg). Fraction D_24 (1.200 g, chloroform/acetone 19:1) was subjected to CC on silica gel, using mixtures of n-hexane/acetone and chloroform/acetone. Compound **4** (3.6 mg) and an isomeric mixture of **18** and **19** (16 mg) were isolated from fraction D_24C after silica gel CC chromatography and C-18 HPLC. Fraction D_24E (0.059 g) was subjected to CC on silica gel using mixtures of n-hexane/chloroform and chloroform/acetone to yield compound **20** (4.9 mg) after purification by silica gel HPLC. Further chromatography of fraction D_24F (0.110 g) by CC and HPLC on silica gel, using mixtures of chloroform/acetone and acetone, afforded **11** (0.7 mg).

Abeograndinoic Acid (**11**), colorless oil; $[\alpha]_D^{25}$ −12.9 (*c* 0.01, $CHCl_3$); IR (KBr) ν_{max} 3410 (OH), 1735 (C=O), 1696 (C=O), 1650 (C=C) cm^{-1}. ^1H NMR data (400 MHz) $CHCl_3$-d_1: δ 9.78 (d, 1.2, H-3), δ 5.67 (br s, H-14), δ 4.13 (dd, 10.2, 4.4, H-2), δ 2.39 (ddd, 10.2, 10.2, 18.0, H-1), δ 2.36 (dd, 10.2, 10.2, H-10), δ 2.16 (d, 3H, 1.2, H-16), δ 2.13 (ddd, 12.9, 12.9, 4.2, H-12), δ 2.05 (ddd, 12.9, 12.9, 4.8, H-12′), δ 1.76 (ddd, 18.0, 10.2, 4.4, H-1′), δ 1.69 (ddd, 12.9, 12.9, 4.8, H-11), δ 1.60 (m, H-8), δ 1.40 (dddd, 13.4, 11.0, 11.0, 4.4, H-7), δ 1.31 (dddd, 13.4, 3.5, 3.5, 3.5, H-7′), δ 1.27 (ddd, 13.4, 4.4, 3.5, H-6′), δ 1.24 (m, H-11), δ 1.19 (m, H-6), δ 1.10 (s, 3H, H-18), δ 1.05 (s, 3H, H-19), δ 0.88 (s, 3H, H-20), δ 0.82 (d, 3H, 6.8, H-17); ^{13}C NMR data (100 MHz) $CHCl_3$-d_1: δ 208.3 (CH, C-3), δ 164.3.0 (C, C-15), δ 164.1 (C, C-13), δ 114.1 (CH, C-14), δ 78.0 (CH, C-2), δ 65.0 (C, C-4), δ 46.9 (CH, C-10), δ 46.8 (C, C-5), δ 36.7 (C, C-9), δ 36.0 (CH_2, C-12), δ 35.9 (CH, C-8), δ 35.2 (CH_2, C-1), δ 31.8 (CH_2, C-11), δ 30.9 (CH_3, C-6), δ 26.2 (CH_2, C-7), δ 25.7 (CH_3, C-20), δ 20.2 (CH_3, C-19), δ 19.4 (CH_3, C-16), δ 15.3 (CH_3, C-17), δ 12.2 (CH_3, C-18); HREIMS *m/z* 336.2311 (calc. for $C_{20}H_{32}O_4$ *m/z* 336.2300).

Coleoptile Bioassay Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diam Petri dishes misted with water and grown in the dark at 22±1ºC for 3 d (Hancock et al. 1964). Roots and caryopses were removed from the shoots. Shoots were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for the bioassay. All manipulations were performed under a green safelight (Nitsch and Nitsch 1956). Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls with water and DMSO at the same concentration also were run.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. Assays were carried out in duplicate. Phosphate-citrate buffer (2 ml) containing 2% sucrose (Nitsch and Nitsch 1956) at pH 5.6 was added to each test tube. Five coleoptiles were placed in each test tube (3 tubes per dilution), and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22ºC in the dark. Coleoptiles were measured following digitalization of their images. Data were statistically analyzed using Welch's test (Martín Andrés and Luna del Castillo 1990). Data are presented as percent difference from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Phytotoxicity Bioassays The selection of target plants was based on an optimization process developed by us in our search for a standard phytotoxicity bioassay (Macías et al. 2000). Several Standard Target Species (STS) were proposed, including the monocot *Allium cepa* L. (onion) and dicots *Lycopersicon esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce), which were assayed for this study.

Bioassays were conducted using Petri dishes (50 mm diam) with one sheet of Whatman No.1 filter paper as a support. Germination and growth were conducted in aqueous solutions at controlled pH using 10^{-2}M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.2, 0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5 µl DMSO solution/ml buffer) so

that test concentrations for each compound (10^{-3}, $5{\cdot}10^{-4}$, 10^{-4}, $5{\cdot}10^{-5}$, and 10^{-5}M) were reached. This procedure facilitated the solubility of the assayed compounds. The number of seeds in each Petri dish depended on the seed size. Twenty seeds were used for tomato, lettuce, cress, and onion. Treatment, control or internal reference solution (1 ml) was added to each Petri dish. A similar procedure was used for wheat in 90 mm diam Petri dishes with 10 seeds. Four replicates were used for tomato, cress, onion, and lettuce (80 seeds).

After adding seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25ºC in a Memmert ICE 700 controlled environment growth chamber in the dark. Bioassays took 4 d for cress, 5 d for lettuce, tomato, and wheat, and 7 d for onion. After growth, plants were frozen at −10ºC for 24 h to avoid subsequent growth during the measurement process.

The commercial herbicide Logran®, a combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2yl)amino]carbonyl} benzenesulfonamide (triasulfuron, 0.6%), was used as an internal reference in accordance with a comparison study reported previously (Macías et al. 2000). This herbicide was used at the same concentrations (10^{-3}, $5{\cdot}10^{-4}$, 10^{-4}, $5{\cdot}10^{-5}$, and 10^{-5}M), and under the same conditions as the compounds reported here. Control samples (buffered aqueous solutions with DMSO and without any test compound) were used for all of the plant species assayed.

Evaluated parameters (germination rate, root length, and shoot length) were recorded using a Fitomed© system (Castellano et al. 2001), which allowed automatic data acquisition and statistical analysis by use of the associated software. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percent difference from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.

IC_{50} values were obtained after adjusting phytotoxicity data to concentration (logarithmic scale), to a sigmoidal dose-response curve, defined by equation:

$$Y = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + 10^{\log EC50 - X}}$$

Where X indicates the logarithm of concentration, Y indicates the response (phytotoxicity), and $Y_{\max}$ and $Y_{\min}$ are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r^2). The adjustment and r^2 values were obtained using GraphPad Prism® software v. 4.00 (GraphPad Software Inc.).

Results and Discussion

Six aqueous extracts were obtained from leaves and bark of *T. grandis* (**TL**, 6 g; **TB**, 4 g), leaves and flowers of *G. sepium* (**GL**, 13 g; **GF**, 11.5 g), leaves of *A. fordii* (**AL**, 8.5 g) and leaves of *M. buxifolia* (**ML**, 1.2 g). The extracts were subjected to a bioassay of etiolated wheat coleoptiles. Five dilutions were used in the assay (1,000, 500, 250, 125, and 75 ppm) and these were prepared from the dried aqueous extracts.

The results obtained in the bioassay are shown in Fig. 1, where activity levels correlate with the concentration expressed in ppm. Extracts that showed higher activity levels were **TL**, **ML**, **TB,** and **GF**, which showed the highest inhibition values at 1,000 ppm, with values of −69, −66, −63, and −60%, respectively. The aqueous extracts of leaves from *A. fordii* (**AL**) and *G. sepium* (**GL**) at the highest concentrations showed lower inhibitory activity (−41 and −36%, respectively). Among the most active extracts, only the extract from leaves of *T. grandis* retained activity levels at lower concentrations, and these were close to those observed at the highest concentration. Thus, the activities of these extracts at 500 and 250 ppm were **TL** (−56 and −53%), **ML** (−48 and −45%), **TB** (−45 and −33%), and **GF** (−38 and −28%). In light of the observed values; its successful use in agroforestry systems; its bioavailability; and the possibility of exploiting non-timber forest products, the leaves of *T. grandis* were selected for further phytochemical study.

The study of the allelopathic potential of *Tectona grandis* was initiated with the study of the DCM extract obtained from the aqueous extract and the DCM extract

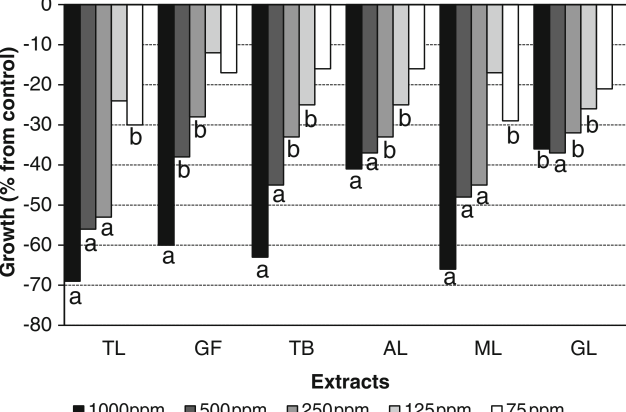

Fig. 1 Effect of aqueous extracts on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage from the control and are not significantly different with $P>0.05$ for the Mann–Whitney's test. **a** Values significantly different with $P<0.01$. **b** Values significantly different with $0.01<P<0.05$. **TL** (leaves of *Tectona grandis*), **TB** (bark of *T. grandis*), **GL** (leaves of *Gliricidia sepium*), **GF** (flowers of *G. sepium*), **AL** (leaves of *Aleurites fordii*) and **ML** (leaves of *Maytensus buxifolia*)

Fig. 2 Terpenes isolated from *Tectona. grandis*

obtained by direct maceration of the dry leaves. Both extracts were selected on the basis of the bioactivity levels shown in the wheat coleoptile bioassay (Macías et al. 2008).

Spectroscopic data for sesquiterpenes **1** (Gutierrez and Herz 1988), **2** (Zhang et al. 2003), **3** and **4** (Niwa et al. 1978), diterpenes **5** (Zulueta et al. 1995), **6** (Urones et al. 1987), **7** (Marsaioli et al. 1975), **8** (Dekker et al. 1988), **9** (Hasan et al. 1982), and **12** (Cambie et al. 1984), as well as triterpenes **13** (Dellagreca et al. 1990), **14** (Aliotta et al. 1991), **15** (Wenkert et al. 1978), **16** (Brandao et al. 1992), **17** (Seebacher et al. 2003), **18**, **19** (Pungitore et al. 2005), **20** (Biessels et al. 1974), and **21** (Takahashi et al. 1974) were identical to those reported previously (see Fig. 2 for structures). This is the first time that sesquiterpenes have been described for the genus *Tectona* and the first time that compounds **2**–**6**, **8**–**10**, **12,** and **21** have been isolated from

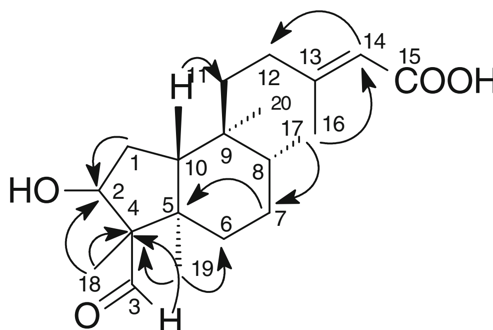

Fig. 3 ^{1}H-^{13}C correlations observed in the g-HMBC experiment on compound **11** (Abeograndinoic acid)

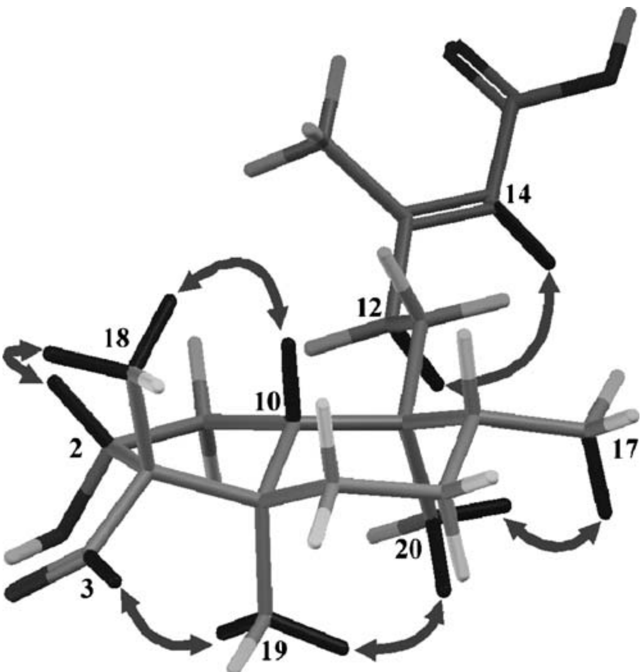

Fig. 4 nOe effects observed for the most stable conformer of **11** (abeograndinoic acid) using PM3 calculations

Fig. 5 Proposed biosynthetic scheme for compound **11** (abeograndinoic acid)

the family *Verbenaceae*. Compound **11** is also described for the first time in the literature.

Compound **11** was isolated as a colorless oil from the DCM extract. The mass spectrum of **11** shows a molecular ion at *m/z* 336.2311, corresponding to a formula $C_{20}H_{32}O_4$. The IR spectrum shows a band at 3,410 cm^{-1} due to the hydroxyl group, which along with the intense band at 1,696 cm^{-1} establishes the existence of an acid group in the molecule. Signals at 1,735 and 1,650 cm^{-1} are assigned to a carbonyl group and a double bond, respectively.

In the ^{1}H-NMR spectrum a characteristic set of signals is observed for the angular methyl groups present in the clerodane diterpene: two signals at δ 0.88 (s, 3H) and 1.05 (s, 3H), corresponding to the methyl groups attached to a quaternary carbon, are assigned to H-20 and H-19, a doublet methyl signal at δ 0.82 (d, 3 H, 6.8) corresponds to H-17 and a signal due to a methyl on a double bond with an *E* geometry at δ 2.16 (d, 3 H, 1.2, H-16). Moreover, the ^{1}H-NMR spectrum of **11** is similar to that of solidagonal (**10**) (Bohlmann et al. 1985), with a broad singlet at δ 5.67 (bs, H-14), suggesting the presence of a trisubstituted double bond, and a signal due to an aldehyde group at 9.78 (d, 1.2). These data suggest that this compound could have an abeoclerodane skeleton. The most significant differences observed in the spectrum in comparison to that of **10** are the signal at δ 1.10 (s, 3H), which corresponds to the H-18 methyl located on a quaternary carbon instead of a double bond, and the presence of a signal at δ 4.13 (dd, 10.2, 4.4) due to a proton geminal to a hydroxyl group (g-HSQC C-2 at δ 78.0).

The ^{1}H-NMR-2D-COSY experiment allowed us to establish the following correlations: the signal at δ 4.13 (dd, 10.2, 4.4, H-2) shows coupling with signals at δ 2.39 (ddd, 10.2, 10.2, 18.0, H-1) and δ 1.76 (ddd, 4.4, 10.2, 18.0, H-1′), corresponding to two geminal protons, which in turn are coupled with the signal at δ 2.36 (dd, 10.2, 10.2, H-10). These data imply that the formyl group cannot be attached at C-3, as it is in solidagonal (**10**).

The presence in the ^{1}H-NMR spectrum of a singlet assigned to H-18 and the correlations observed in the g-HMBC spectrum (Fig. 3) between the signals of H-18 at δ 1.10 and the quaternary carbon at 65.0 (C-4), as well as between C-3 and the aldehydic proton, allow us to place the aldehyde group geminal to C-18. These correlations, together with the previous data, lead us to suggest an unusual carbon skeleton for this compound. The correlations observed in the g-HMBC spectrum (see Fig. 3) allowed us to assign unambiguously all signals in the ^{1}H-NMR and ^{13}C-NMR spectra.

In order to determine the relative stereochemistry of the molecule, a series of 1D NOESY-NOESY experiments was carried out. The observed nOe effects are shown in Fig. 4. The nOe effect between H-14 and H-12 confirms an *E* stereochemistry for the double bond (Fig. 4).

The spectroscopic data for **11** suggest that this compound has the structure presented in Fig. 2, and we have named this abeograndinoic acid. This diterpene has a rearranged clerodane skeleton. Some furoclerodane compounds with a analogous carbon skeleton and very different functionalization have been described from the family

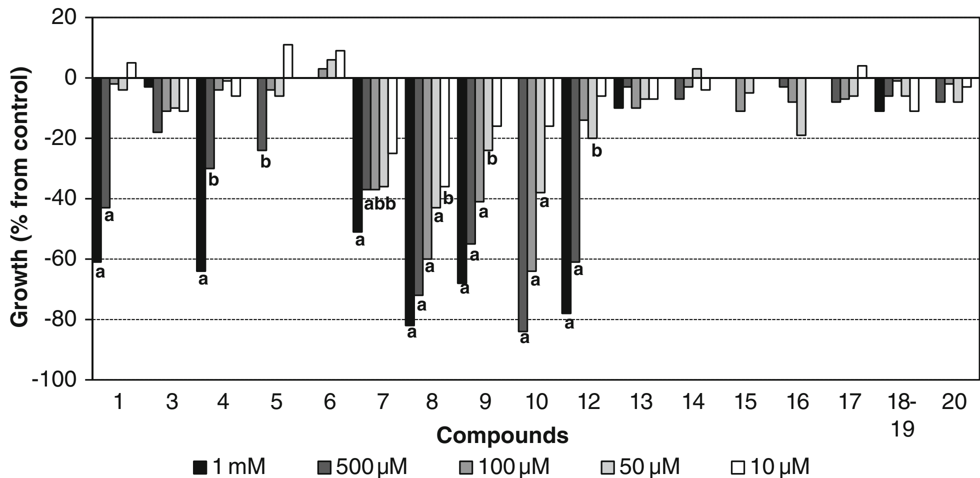

Fig. 6 Effects of compounds **1**–**20** on etiolated wheat coleoptiles. Values are expressed as percentage from the control and are not significantly different with $P > 0.05$ for the Mann–Whitney's test. **a** Values significantly different with $P < 0.01$. **b** Values significantly different with $0.01 < P < 0.05$

Asteraceae (Jakupovic et al. 1986; Bohlmann and Mungai 1990; Zdero et al. 1990a, b). A biosynthetic route through a pinacolinic rearrangement from the corresponding 3,4-dihydroxy derivative was proposed for these compounds. It also has been proposed that the biosynthetic origin of **10** is from a clerodane with a double bond between C-4 and C-5, after subsequent oxidative rupture, cyclization, and dehydration (Bohlmann et al. 1985). In the case of **11**, the biosynthesis could start with a clerodane skeleton, which has a double bond between C-2 and C-3. Oxidative rupture of the double bond and subsequent aldolic cyclization would afford compound **11** (Fig. 5).

Coleoptile Bioassay Results Etiolated wheat coleoptiles bioassay is a fast test (24 h), which is sensitive to a wide range of bioactive substances (Cutler et al. 2000) including plant growth regulators, herbicides (Cutler 1984), antimicrobials, mycotoxins, and assorted pharmaceuticals.

Fig. 7 Effects of compounds **7**–**9** and **12** on growth of standard target species. Values are expressed as percentage from the control and are not significantly different with $P>0.05$ for the Mann–Whitney's test. **a** Values significantly different with $P<0.01$. **b** Values significantly different with $0.01<P<0.05$

(Jacyno and Cutler 1993). The bioactivities on coleoptiles of these compounds were evaluated when sufficient quantities were avaliable (i.e., all cases except **2**, **11**, and **21**). The highest concentration tested for **1**, **3**, **4**, **7**–**9**, **12**, **13**, and **18**–**19** was 10^{-3} M, whereas **5**, **6**, **10**, **14**–**17**, and **20** were tested from $5{\cdot}10^{-4}$ M. It can be seen from Fig. 6 that sesquiterpenes **1** and **4** show inhibitory activity greater than −50% at the maximum concentration.

The results show that compounds **8**, **9**, and **12** inhibit coleoptile elongation by more than −60% at 10^{-3} M. In particular, **8** and **12** present values close to −80% at this concentration. It should be pointed out that compound **10** shows an inhibition value of −84% at $5{\cdot}10^{-4}$ M, which is the highest concentration tested. On the other hand, **5** and **6** show activity levels that are not particularly significant.

The most active compounds were the diterpenes **7**, **8**, **9**, **10**, and **12**. The activity of **12** decreases rapidly on dilution. The profile of the activity with dilution suggests that the presence of this α,β-unsaturated carbonyl group in the bicyclic system is influential in the activity, whereas the double bond and the carboxylic acid group on the lateral chain are less important. Thus, compound **7** shows an inhibition of −37% at $5{\cdot}10^{-4}$ M, whereas **8**, **9,** and **10** inhibit the elongation by −72, −55, and −84%, respectively, at the same concetration. Furthermore, the inhibition values are higher than −60% in the cases of **8** and **10** at 10^{-4} M.

Neither triterpenes nor steroids isolated in this work show relevant activity in this bioassay and, as a consequence, they were not selected for evaluation of phytotoxic activity.

In order to compare compound activities, IC_{50} values were calculated using the sigmoidal dose-response model. This approach allows comparison of the inhibitory activity of active compounds even when the starting concentrations are different. The order of increasing activity of the tested compounds in this bioassay is: **10** (IC_{50}=0.078 mM, R^2=0.984)>**8** (IC_{50}=0.31 mM, R^2=0.965) > **12** (IC_{50}=0.41 mM, R^2=0.983)>**9** (IC_{50}=0.65 mM, R^2=0.97) > **1** (IC_{50}=0.67 mM, R^2=0.998)>**4** (IC_{50}=0.96 mM, R^2=0.976)>**7** (IC_{50}=2.1 mM, R^2=0.975).

Phytotoxicity Bioassay Results The most active compounds were selected for phytotoxicity evaluation. Compounds **1**, **4,** and **10** were not included in the phytotoxicity bioassay due to the small amounts available (Fig. 7). The concentrations tested were identical to those in the previous bioassay. Standard target species (STS) were *Lactuca sativa* (lettuce), *Lycopersicum esculentum* (tomato), *Lepidium sativum* (cress), and *Allium cepa* (onion).

Regarding the dicotyledonous species, the tested compounds did not cause significant effects on germination or growth of *Lepidium sativum*. The behavior of the evaluated compounds in the germination and growth of *Lactuca sativa* is inhibitory in almost all cases. The highest inhibitory effects on the germination were caused by **9**, which shows activity values similar to the herbicide Logran® at the highest concentrations (10^{-3} M, −89%; $5{\cdot}10^{-4}$ M, −86%; 10^{-4} M, −68%) and higher values at lower concentrations ($5{\cdot}10^{-5}$ M, −48%; 10^{-5} M, −36%). With regard to the third dicotyledonous species, *Lycopersicum esculentum*, the effects on germination were of low significance. Root growth of tomato was stimulated by diterpenes **9** and **12**, with values higher than 20% for all concentrations, whereas the effects on shoots were not relevant.

In the monocotyledon species *Allium cepa*, the most affected parameter was root growth. Compounds **9** (10^{-3} M, −55%; $5{\cdot}10^{-4}$ M, −33%) and **12** (10^{-3} M, −44%) were inhibitory, with **9** being the most active. The effects on germination and shoot development were not significant, with **12** being the most active compound with values around −40% at the highest concentration.

In summary, the compounds that present the highest phytotoxic activity are the diterpenes **9** (2-oxokovalenic acid) and **12** (19-hydroxyferruginol). In addition, the general activity of the diterpene **10** (solidagonal acid), with an IC_{50} value of 78 μM, has to be highlighted, although due to the small amount isolated its phytotoxic activity could not be evaluated.

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Comparison of the Phytotoxic Effects of Usnic Acid on Cultures of Free-Living Alga *Scenedesmus quadricauda* and Aposymbiotically Grown Lichen Photobiont *Trebouxia erici*

Martin Bačkor · Katarína Klemová · Miriam Bačkorová · Veneta Ivanova

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Abstract The phytotoxic effects of the lichen secondary metabolite—usnic acid on cultures of free living alga—*Scenedesmus quadricauda* (UTEX 76) and aposymbiotically grown lichen photobiont *Trebouxia erici* (UTEX 911) were assessed. We found a relatively strong inhibition effect of usnic acid on the growth of alga *Scenedesmus*, accompanied by an increase of cell size, an alteration of assimilation pigment composition, followed by strong degradation of chlorophyll *a*, a decrease of chlorophyll *a* fluorescence, and an increase of reactive oxygen species in the cells. The content of soluble proteins remained a stable parameter. Phytotoxicity of usnic acid on cultures of *Trebouxia* photobiont was significantly lower. Usnic acid in lichens may act as an allochemical that controls the division of photobiont cells, thereby regulating the balance between the photobiont and mycobiont forming thallus. Higher tolerance to usnic acid in *Trebouxia* cultures may be an adaptation resulting from the long term co-evolution of these algae with fungi that produce secondary metabolites.

Key Words Algae · Lichens · Secondary metabolites · Toxicity · Usnic acid

M. Bačkor (✉) · K. Klemová
Department of Botany, Institute of Biology and Ecology,
Faculty of Science, Šafárik University,
Mánesova 23,
041 67 Košice, Slovak Republic
e-mail: martin.backor@upjs.sk

M. Bačkorová
Department of Cell and Molecular Biology, Institute of Biology and Ecology, Faculty of Science, Šafárik University,
Moyzesova 11,
040 01 Košice, Slovak Republic

V. Ivanova
The Stephan Angeloff Institute of Microbiology,
Bulgarian Academy of Sciences,
Acad. G. Bonchev Str. 26,
1113 Sofia, Bulgaria

Introduction

Lichens are a stable and self-supporting association between fungi (mycobionts) and photoautotrophic algae or cyanobacteria (photobionts). Interactions within a lichen thallus are, however, complex, and lichens may be considered more like an ecosystem or community than a typical organism (Fahselt 2008).

Lichens produce many unique compounds that are considered to have important biological and ecological roles. These roles include antimicrobial activity, allelopathy, antiherbivory, chelating of heavy metals, light screening, and other proposed functions not yet sufficiently supported by experimental evidence under laboratory conditions (Lawrey 1986; Pöykkö et al. 2005; Latkowska et al. 2006; Hauck et al. 2009; Solhaug et al. 2009). Secondary metabolites may be responsible for the ecological success of lichenization, allowing lichens frequently to be the dominant organisms in environments characterized by extreme ecological conditions. Lichen compounds are secreted by the fungal partner, and they are deposited on the surface of hyphae, as well as on lichen algae, typically constituting 0.1 to 5.0% (w/w) of thallus dry weight (Fahselt 1994).

Usnic acid, a yellowish pigment and dibenzofuran derivative, is a common lichen metabolite, especially abundant in members of the genera *Alectoria*, *Usnea*, or *Xanthoparmelia*. The antibiotic, antiviral, antimycotic, antiprotozoal, antiherbivoral, antiproliferative, anti-inflammatory, analgesic, antipyretic, allelopathic, and UV protecting effects of usnic acid have been stated previously (for review see Cocchietto et al. 2002).

There is little doubt that the presence of secondary metabolites, including usnic acid, has significant influence on lichen survival. Lichens are slow growing and long lived organisms, and due to the toxic effect of usnic acid to a wide range of organisms, their thalli are relatively well protected against pathogens and herbivores. However, usnic acid potentially can be toxic to cells that come in direct contact with it, even the algal partner of the lichen that produces it. Crystals of lichen cortical secondary metabolites may be deposited directly on the surface of photobiont cells (Sarret et al. 1998). Previous studies have suggested that the presence of usnic acid may regulate photobiont populations (Bačkor et al. 1998; Buďová et al. 2006). Production of usnic acid may be a critical step of maintaining the required ratio of mycobiont to photobiont biomass in thalli for mutualistic symbiosis.

The mechanisms of the phytotoxic effects of usnic acid on plants, including on its own photobionts are not understood sufficiently; however, inhibition of chlorophyll *a* fluorescence (F_V/F_M), decrease of carotenoid and chlorophyll levels in the cells, and decrease of viability have been demonstrated as responses of plants to the presence of usnic acid (Cardarelli et al. 1997; Endo et al. 1998; Buďová et al. 2006). Decrease of biomass production also has been demonstrated (Bačkor et al. 1998; Buďová et al. 2006; Lechowski et al. 2006).

Concentrations of secondary metabolites around photobiont cells are high, and lichen photobionts are in direct contact with cortical metabolites produced by the mycobiont (Takahagi et al. 2008). Although lichen compounds may act as allelochemicals and are responsible for maintaining balance between bionts inside lichen thalli, photobionts must be protected from the toxicity of these compounds. We hypothesized that lichen photobionts have evolved protective mechanisms against phytotoxicity of secondary metabolites through co-evolution with lichen mycobionts. To test this hypothesis, we evaluated the potential phytotoxic effect of usnic acid on the aposymbiotically grown lichen photobiont *Trebouxia erici*. For assessment of the potential differences in phytotoxicity of usnic acid to free-living algae without evolutionary experience with the presence of lichen secondary metabolites, *Scenedesmus quadricauda* also was chosen.

Methods and Materials

Organisms and Culture Conditions Free-living alga *Scenedesmus quadricauda* (Turp.) Bréb. (UTEX 76) and aposymbiotically grown lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) were used. Both species belong to Chlorophyta, a division of green algae. Algae were cultivated on agar medium (1.5%) previously developed for cultivation of lichen algae by Ahmadjian (1993). This was Bold's Basal Medium (BBM 3N) plus 10 g casein acid hydrolyzate and 20 g glucose per liter with the pH adjusted to 6.5 (Bačkor et al. 2004). Cultures were maintained at 22°C under a 16:8 hr, L:D photoperiod and 30 $\mu mol.m^{-2}.s^{-1}$ artificial irradiance ("cool white" tubes).

Toxicity Assay Algae were cultivated on the surface of Whatman CF/C filters (glass fiber filter disks, 25 mm diam). Glass fiber filter disks were used instead of the cellulose-acetate disks, previously used for lichen photobiont cultivation (Bačkor et al. 2003, 2004). Glass fiber filter disks may be used directly for disruption in the mortar of algal cells grown on their surfaces. Crystals of usnic acid on the surface of fibers of the disk simulate the situation in natural lichens, where extracellular secondary metabolites located on the surface of hyphae are in the contact with algal cells in the photobiont layer. Briefly, cells from both algal species (approximately two inoculation loops) grown on stock solid *Trebouxia* media were transferred into 50 ml of liquid *Trebouxia* media in an Erlenmeyer flask. Cells were suspended by gentle stirring on a magnetic stirrer for 1 hr. Cultures were maintained for 5 d in a cultivation room under conditions described previously, with daily stirring on a magnetic stirrer for about 1 hr. Homogeneity of the algal suspension was verified microscopically, and the number of cells was calculated by using a standard haemocytometer. Cell density of cultures was adjusted to approximately 10^6 cells ml^{-1} of medium before quantitative cultivation of algae.

For cultivation of both algal species, sterilized Whatman CF/C filters (25 mm diam) were subjected to three different pretreatments. Usnic acid (0.01 and 0.1 mg / disk) dissolved in acetone (volume 30 µl) was applied by automatic pipette on the surface of disks, while the same volume of acetone was applied to control disks. We used (+)-usnic acid enantiomer (Sigma-Aldrich). After evaporation of acetone for 4 hr, one disk was transferred to the surface of solid *Trebouxia* medium in a separate Petri dish, (6 cm diam), and 20 µl of algal suspension were inoculated into the center of each disk. Disk pores allowed supplemental nutrient media to pass through the disk and permitted growth to be determined easily from changes in biomass (Bačkor et al. 2003, 2004). The total mass of cultures was calculated by subtracting the mean fresh weight (fw) of a

Whatman CF/C disk saturated by identical medium, from the fw of a disk supporting algal cultures after 14 d. Each treatment was replicated eighteen times for both algal species assessed.

Pigment Analysis and Measurement of Chlorophyll a Integrity The influence of usnic acid on the algae was determined by using cultures grown on Whatman CF/C disks. Weighed disks were extracted in the dark for 1 hr at 65°C in 5 ml of dimethyl sulfoxide (DMSO). To maximize chlorophyll extraction, cell aggregates were homogenized with a mortar, and glass fibers of disks facilitated disruption of cell walls of algae. After cooling to ambient temperature, the absorbance of the extract, as a reflection of turbidity, was determined at 750 nm with a spectrophotometer to be certain that it was always less than 0.01. Absorbance of extracts was read at 665, 649, 435, and 415 nm to assess chlorophyll content and the possibility of chlorophyll *a* degradation (Barnes et al. 1992; Wellburn 1994). To utilize the linear portion of the response curve, extracts from disks with very high cell densities (absorbance at 665 nm higher than 0.8) were diluted with fresh DMSO to fall into the absorbance range 0.2–0.8. To determine the content of “total” carotenoids, absorbance was read at 480 nm. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and total carotenoids were calculated by using equations derived from specific absorption coefficients for pure chlorophyll *a* and chlorophyll *b* in DMSO. Chlorophyll *a/b* was used to assess the physiological competence of algal cells.

The ratios of absorbances at 435 and 415 nm (A 435/A 415), termed the phaeophytinization quotient, were calculated as a reflection of the ratio of chlorophyll *a* to phaeophytin *a* and to provide an indication of integrity of photobiont chlorophyll. Each treatment was replicated three times.

Activity of Photosystem II Chlorophyll *a* fluorescence was measured in algae grown on Whatman CF/C disks on the surface of *Trebouxia* agar medium. While still on the surface of the medium in Petri dishes (to minimize desiccation), algae were dark-adapted for 30 min prior to measurement. The potential quantum yield of photosystem II (PSII) was measured with a Plant Stress Meter (PSM Mark II, Biomonitor, SCI AB), with a sensor 5 mm diam, and results were expressed as F_V/F_M calculated as the maximal fluorescence (F_M) less the minimal fluorescence (F_0), divided by F_M of dark adapted plants: $(F_M - F_0) / F_M = F_V/F_M$. Chlorophyll fluorescence parameters were taken from three separate positions on each disk, and the mean value used as one observation. Each treatment was replicated three times.

Content of Soluble Proteins Algae grown on Whatman CF/C disks (controls, as well as with addition of usnic acid on the surface of disks) were homogenized with disks in an ice-cold mortar in phosphate buffer (50 mM). After centrifugation at 15,000 g at 4°C for 20 min, the water-soluble protein content of supernatants was measured using the Bradford (1976) method. Supernatants (100 µl) were pipetted into 900 µl of Bradford assay kit (Biorad) in a spectrophotometric cuvette and mixed. After 10 min, absorbance of samples was spectrophotometrically measured at 595 nm. Bovine serum albumin was used as calibration standard. Each treatment was replicated three times.

Detection of Oxidative Status Hydrogen peroxide and superoxide were measured in homogenates with potassium phosphate buffer prepared for assay for determination of soluble proteins. Hydrogen peroxide was quantified by the $TiCl_4$ method (Kováčik and Bačkor 2007). Superoxide was estimated according to Elstner and Heupel (1976) by monitoring formation of nitrite from hydroxylamine at 530 nm. Reaction mixture contained 0.27 ml of potassium phosphate buffer, 0.03 ml of 10 mM hydroxylamine, 0.3 ml of supernatant, 0.3 ml of 17 mM sulphanilamide, 0.3 ml of 7 mM α-naphthylamine, and 0.3 ml of diethyl ether. Each treatment was replicated three times.

Cell Size Algae grown on Whatman CF/C disks from each treatment were separated gently from the surface of the disks by a scalpel. Cells were suspended immediately in 5 mM HEPES buffer, pH 6.5 and viewed under a light microscope at 800×. Cell size parameters (width and length in the case of *S. quadricauda* cells, or length in the case of *T. erici* spherical cells) were measured with an eyepiece micrometer. Twenty individual cells from each treatment and both algal species were evaluated.

Statistical Analysis The one-way analysis of variance and Tukey’s pairwise comparisons (MINITAB Release 11, 1996) were applied to determine significance ($P<0.05$) of differences in all measured parameters. In addition, two-way ANOVA with interactions (SPSS version 16, 2008) was applied to evaluate species effect (species), usnic acid effect (UA), and interactions between species effect and UA effect (species × UA) where applicable.

Results

Biomass production of the control *Scenedesmus* cultures was almost five times higher after 2 weeks of cultivation when compared to control *Trebouxia* cultures (Table 1). Presence of usnic acid on the disks significantly affected biomass production of both algal cultures in a dose dependent manner. However, a 0.01 mg/disk of usnic acid decreased biomass production of *Scenedesmus* cultures by a factor of 4,

Table 1 Biomass production (mg fw/disk), chlorophyll integrity (435/415), chlorophyll *a/b* ratio (A/B), total carotenoid/total chlorophyll ratio (CAR/CHL) and chlorophyll *a* fluorescence (F_V/F_M) of 2 week old *Secenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	Biomass (mg fw/disk)	435/415	A/B	CAR/CHL	F_V/F_M
Scenedesmus	0	246±39.9**a**	1.45±0.02**a**	3.57±0.22**a**	0.25±0.02**a**	0.65±0.02**a**
	0.01	66±25.5**b**	1.40±0.03**a**	3.22±0.29**a**	0.33±0.06**a**	0.51±0.04**b**
	0.1	22±8.5**b**	0.94±0.09**b**	1.17±0.38**b**	0.22±0.05**a**	0.14±0.07**c**
	F value	*F*=54.93	*F*=75.17	*F*=54.35	*F*=4.11	*F*=84.69
	P value	*P*<0.001	*P*<0.001	*P*<0.001	*P*=0.075	*P*<0.001
Trebouxia	0	55±4.0**a**	1.40±0.04**a**	2.82±0.21**a**	0.27±0.01**a**	0.60±0.04**a**
	0.01	45±9.7**ab**	1.36±0.08**a**	2.66±0.06**a**	0.27±0.02**a**	0.57±0.01**ab**
	0.1	25±6.1**b**	1.32±0.07**a**	3.24±0.78**a**	0.29±0.02**a**	0.52±0.01**b**
	F value	*F*=12.29	*F*=1.22	*F*=1.25	*F*=0.87	*F*=8.50
	P value	*P*=0.008	*P*=0.36	*P*=0.351	*P*=0.468	*P*=0.018

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test (*P*<0.05)

and a 0.1 mg/disk decreased biomass production by a factor of 11, while in *Trebouxia* culture biomass production was decreased only at the highest tested usnic acid dose of a 0.1 mg/disk by a factor of approximately 2.2 (Table 1). Two-way ANOVA for biomass production: species (*F*=63.25, *P*<0.001), UA (*F*=52.98, *P*<0.001), species × UA (*F*=40.98, *P*<0.001).

The highest tested usnic acid doses significantly decreased chlorophyll *a* integrity in *Scenedesmus* cultures, while in *Trebouxia* cultures none of the tested usnic acid concentrations significantly altered chlorophyll integrity (Table 1). Two-way ANOVA for chlorophyll *a* integrity: species (*F*=40.75, *P*<0.001), UA (*F*=10.84, *P*=0.006), species × UA (*F*=23.75, *P*<0.001).

Similarly, the highest tested usnic acid doses significantly decreased chlorophyll *a/b* of *Scenedesmus* cultures. In the *Trebouxia* cultures, we did not observe significant changes of chlorophyll *a/b* under any of the tested concentrations (Table 1). Two-way ANOVA for chlorophyll *a/b*: species (*F*=10.26, *P*=0.002), UA (*F*=1.88, *P*=0.194), species × UA (*F*=24.11, *P*<0.001).

Total carotenoid/total chlorophyll ratio was stable in both algal cultures regardless of usnic acid application (Table 1). Two-way ANOVA for total carotenoid/total chlorophyll ratio: species (*F*=2.32, *P*=0.139), UA (*F*=0.13, *P*=0.716), species × UA (*F*=4.81, *P*=0.03).

When calculated per unit of fresh weight (mg/g fw) of algal biomass, we observed a significant decrease of chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, and total carotenoids in *Scenedesmus* cultures with the presence of a 0.1 mg/disk of usnic acid (Table 2). In the *Trebouxia* cultures, we did not observe any decrease in these parameters when calculated per unit fresh weight (Table 2). Two-way ANOVA for chlorophyll *a*: species (*F*=11.04, *P*=0.002), UA (*F*=20.69, *P*<0.001), species × UA (*F*=6.73, *P*=0.01). Two-way ANOVA for chlorophyll *b*: species (*F*=3.91, *P*=0.049), UA (*F*=14.81, *P*=0.002), species × UA (*F*=0.44, *P*=0.651). Two-way ANOVA for chlorophyll *a*+*b*: species (*F*=8.74,

Table 2 Chlorophyll *a* content (A, mg/g fw), chlorophyll *b* content (B, mg/g fw), chlorophyll *a*+*b* content (A+B, mg/g fw) and content of total carotenoids (TOT CAR, mg/g fw) of 2 week old *Secenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	A (mg/g fw)	B (mg/g fw)	A+B (mg/g fw)	TOT CAR (mg/g fw)
Scenedesmus	0	0.43±0.06**a**	0.12±0.02**a**	0.54±0.08**a**	0.14±0.02**a**
	0.01	0.42±0.08**a**	0.13±0.03**a**	0.55±0.11**a**	0.19±0.06**a**
	0.1	0.06±0.01**b**	0.06±0.01**b**	0.12±0.01**b**	0.03±0.01**b**
	F value	*F*=40.57	*F*=7.98	*F*=29.03	*F*=13.58
	P value	*P*<0.001	*P*=0.02	*P*=0.001	*P*=0.006
Trebouxia	0	0.52±0.06**a**	0.18±0.01**a**	0.71±0.07**a**	0.19±0.02**a**
	0.01	0.49±0.16**a**	0.19±0.06**a**	0.68±0.23**a**	0.18±0.04**a**
	0.1	0.46±0.07**a**	0.15±0.05**a**	0.61±0.11**a**	0.18±0.02**a**
	F value	*F*=0.24	*F*=0.57	*F*=0.31	*F*=0.35
	P value	*P*=0.796	*P*=0.594	*P*=0.746	*P*=0.720

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test (*P*<0.05)

Table 3 Content of soluble proteins (PROT, mg/g fw), content of hydrogen peroxide (PEROX, µmol/g fw), content of superoxide (SUPEROX, µg/g fw) and cell size (WIDTH and LENGTH, µm) of 2 week old *Secenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	PROT (mg/g fw)	PEROX (µmol/g fw)	SUPEROX (µg/g fw)	WIDTH µm	LENGTH µm
Scenedesmus	0	8.5±0.7**a**	1.5±0.37**b**	0.65±0.12**b**	5.1±1.4**b**	14±3.7**b**
	0.01	10±3.6**a**	2.1±0.31**ab**	0.69±0.11**b**	10.5±2.9**a**	22±3.1**a**
	0.1	4.8±2.3**a**	2.9±0.32**a**	1.12±0.21**a**	9.6±2.7**a**	20±5.3**a**
	F value	F=3.45	F=13.06	F=8.51	F=14.72	F=10.96
	P value	P=0.101	P=0.007	P=0.018	P<0.001	P<0.001
Trebouxia	0	12.2±1.8**a**	1.35±0.25**a**	0.66±0.15**b**	10±3.7**a**	–
	0.01	13.5±2.3**a**	1.50±0.21**a**	0.72±0.18**ab**	9.7±5.3**a**	–
	0.1	16.6±3.1**a**	2.0±0.40**a**	1.25±0.27**a**	13±2.8**a**	–
	F value	F=2.63	F=4.07	F=4.07	F=1.95	–
	P value	P=0.151	P=0.076	P=0.076	P=0.162	–

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test (P<0.05)

P=0.004), UA (F=19.48, P<0.001), species × UA (F=4.15, P=0.042). Two-way ANOVA for total carotenoids: species (F=9.08, P=0.004), UA (F=14.93, P=0.002), species × UA (F=8.14, P=0.005).

Usnic acid decreased the F_V/F_M ratio of *Scenedesmus* cultures. Presence of a 0.1 mg/disk decreased the F_V/F_M ratio more than four times, while in *Trebouxia* cultures the F_V/F_M ratio decreased only 1.15 times at the highest tested usnic acid dose of 0.1 mg/disk (Table 1). Two-way ANOVA for F_V/F_M: species (F=91.20, P<0.001), UA (F=55.38, P<0.001), species × UA (F=49.48, P<0.001).

Increase of usnic acid on the disk did not cause a significant decrease in the soluble protein content of algal cultures calculated per unit fresh weight according to one-way ANOVA (Table 3). Two-way ANOVA for soluble protein content: species (F=0.492, P=0.623), UA (F=29.12, P<0.001), species × UA (F=5.60, P=0.019). According to two-way ANOVA, significant UA, as well as species × UA effects were observed.

The highest tested usnic acid dose of 0.1 mg/disk caused a significant increase of hydrogen peroxide content in *Scenedesmus* cultures (Table 3). In the cultures of *Trebouxia*, we also observed an increase of hydrogen peroxide content at a dose of 0.1 mg/disk, however, the increase was not strong enough to be significant (Table 3). Two-way ANOVA for hydrogen peroxide content: species (F=16.16, P<0.001), UA (F=11.80, P=0.004), species × UA (F=1.96, P=0.183).

The highest tested usnic acid dose of 0.1 mg/disk caused a significant increase of superoxide in cultures of both algal species tested (Table 3). Two-way ANOVA for superoxide content: species (F=15.49, P<0.001), UA (F=0.42, P=0.526), species × UA (F=0.21, P=0.815).

Both tested usnic acid doses caused significant increases of width and length of *Scenedesmus* cells. In *Trebouxia* cultures, we did not observe any significant increase of cell size at any of the tested usnic acid concentrations (Table 3). Two-way ANOVA for width: species (F=6.70, P=0.002), UA (F=8.53, P=0.005), species × UA (F=3.86, P=0.026).

Discussion

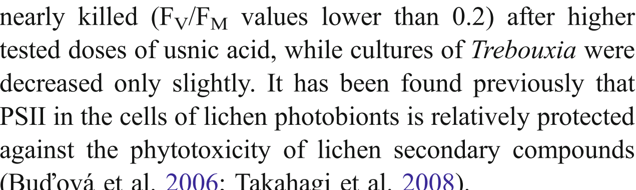
Influence of Usnic Acid on Algal Growth Usnic acid appears to have phytotoxic effects on vascular plants (Cardarelli et al. 1997; Lechowski et al. 2006) as well as on the algal partner of lichens (Bačkor et al. 1998; Buďová et al. 2006). In the present study, we found that control cultures of free-living alga *Scenedesmus* grew better when compared to aposymbiotically grown control cultures of *Trebouxia*. Presence of usnic acid on the surface of fibers caused a dose-dependent decrease of the growth of both algal cultures, however, the decrease was more pronounced in cultures of *Scenedesmus* compared to cultures of *Trebouxia*. Lower toxicity of usnic acid for photobiont cells may be a result of co-evolution of *Trebouxia* photobionts with filamentous fungi over a long period of time, perhaps hundreds of millions of years (Yuan et al. 2005). Usnic acid possesses antimitotic effects that have been demonstrated in taxonomically diverse organisms (Cardarelli et al. 1997). Surprisingly, presence of usnic acid in this study caused a significant increase of cell size of *Scenedesmus* cultures. This may be a result of the effect of usnic acid on the spindle apparatus during mitosis (Al-Bekairi et al. 1991). Although a similar trend was observed in the *Trebouxia* cells, the effect was not strong enough to be significant.

Influence of Usnic Acid on Algal Metabolic Processes The mechanisms of phytotoxic effects of usnic acid still are mostly unknown. It has been demonstrated that viability of the protoplasts of *Nicotiana tabacum* decreases due to the presence of usnic acid (Cardarelli et al. 1997). Buďová et al. (2006) demonstrated that viability of the *Trebouxia erici* photobiont cells decreased due to increased doses of usnic acid.

Changes in composition of assimilation pigments, as well as in chlorophyll *a* fluorescence of aposymbiotically grown lichen photobionts previously have been found to be excellent markers for assessment of the influence of xenobiotics, including metals (Bačkor et al. 2003). One of the most frequently used parameters in lichen stress physiology is chlorophyll degradation, expressed as the phaeophytinization quotient, which reflects the ratio of chlorophyll *a* to phaeophytin *a* (Bačkor and Loppi 2009). This is defined as the ratio of optical densities at 435 nm and 415 nm (OD_{435}/OD_{415}). In healthy lichens and photobionts, the OD_{435}/OD_{415} ratio is about 1.4, and the presence of xenobiotics, including heavy metals, may cause a marked decrease of this value (Ronen and Galun 1984; Bačkor and Loppi 2009). In the present study, none of the tested usnic acid concentrations caused a significant decrease of phaeophytinization quotient in *Trebouxia* cultures, while the highest tested dose of 0.1 mg/disk caused a significant decrease of the phaeophytinization quotient in the cultures of *Scenedesmus*. An identical trend was observed for the ratio of chlorophyll *a/b*, where only the highest tested dose of usnic acid in the cultures of *Scenedesmus* caused a significant decrease; chlorophyll *a* of *Scenedesmus* cells was more sensitive than chlorophyll *b*. However, when we compared changes of total chlorophyll content to total carotenoid content due to presence of usnic acid, we did not find significant differences in either algal species.

Irrespective of the previously noted parameters, we observed a decrease in the content of chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, and total carotenoids of *Scenedesmus* calculated per unit of fresh weight. Although chlorophyll *a* and chlorophyll *b* differ only in the composition of side chain (chlorophyll *a* contains a methyl group and chlorophyll *b* a formyl group), decrease of chlorophyll *a* was notable. Previous studies suggested that plants exposed to pollutants, including acid rain, heavy metals, and herbicides respond by decrease of chlorophyll *a* rather than chlorophyll *b* (Hendry et al. 1987). Degradation of chlorophyll *a* here was accompanied by increased phaeophytinization. Han et al. (2004) demonstrated that usnic acid is toxic to cultured hepatocytes and disrupts electron transport in mitochondria and induces oxidative stress in the cells. A previous study suggested that production of the reactive oxygen species in the cells of the lichen photobiont *Trebouxia erici* caused increase of membrane lipid peroxidation (Bačkor et al. 2007).

The reduced F_V/F_M values measured in our usnic acid treated cultures indicate damage to PSII. Endo et al. (1998) demonstrated a decrease of chlorophyll *a* fluorescence in spinach leaves due the presence of lichen-derived secondary metabolites. However, we found a differential sensitivity of algae to usnic acid as cultures of *Scenedesmus* were



nearly killed (F_V/F_M values lower than 0.2) after higher tested doses of usnic acid, while cultures of *Trebouxia* were decreased only slightly. It has been found previously that PSII in the cells of lichen photobionts is relatively protected against the phytotoxicity of lichen secondary compounds (Buďová et al. 2006; Takahagi et al. 2008).

Xenobiotics, including metals, may alter the composition of soluble proteins in cultures of lichen photobionts (Bačkor and Fahselt 2008). Although we observed a significant decrease of protein content due to usnic acid when analyzed for individual disks (data not shown), we did not observe significant changes in soluble protein content calculated per unit of algal biomass either in *Scenedesmus* or in *Trebouxia* cultures. Caviglia et al. (2001) demonstrated that usnic acid in lichen *Parmelia soredians* may act as an antioxidant that detoxifies reactive oxygen species produced by the application of the herbicide Paraquat. However, Han et al. (2004) demonstrated that usnic acid itself may induce oxidative stress in the cells. In the present study, we found that the highest tested dose of usnic acid significantly increased hydrogen peroxide content in cultures of *Scenedesmus*, as well as superoxide radicals in cultures of both tested algal species.

In summary, we confirmed that usnic acid has a phytotoxic effect on algae, but that algae are differentially sensitive, and that the lichen photobiont *Trebouxia* is significantly less affected than *Scenedesmus*. These conclusions are based on analyses of physiological processes including biomass production, composition of assimilation pigments (mostly by decrease of chlorophyll *a* content and increase of its phaeophytinization), chlorophyll *a* fluorescence, soluble proteins, and reactive oxygen species. Higher tolerance of *Trebouxia* cultures to usnic acid may be an adaptation resulting from the long term co-evolution of these algae with fungi that produce secondary metabolites. These also may act as allelochemicals that control cell division of photobiont cells inside lichen thalli, thus regulating balance between symbionts forming lichens.

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Cryptic Color Change in a Crab Spider (*Misumena vatia*): Identification and Quantification of Precursors and Ommochrome Pigments by HPLC

Mickaël Riou · Jean-Philippe Christidès

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Abstract Mimicry is used widely by arthropods to survive in a hostile environment. Often mimicry is associated with the production of chemical compounds such as pigments. In crab spiders, the change of color is based on a complex physiological process that still is not understood. The aim of this study was to identify and quantify the ommochrome pigments and precursors responsible for the color change in the mimetic crab spider *Misumena vatia* (Thomisidae). A modified high performance reverse phase ion-pair chromatography technique enabled us to separate and quantify the ommochrome pigments, their precursors, and related metabolites in individual spiders. Compounds such as tryptophan, kynurenine, and kynurenic acid occurred only or mainly in white crab spiders. In contrast, compounds such as 3-hydroxy-kynurenine, xanthommatin, and ommatin D occurred only or mainly in yellow crab spiders. Factor analysis ranked the different color forms in accordance with their metabolites. The biochemical results enabled us to associate the different phases of formation of pigment granules with specific metabolites. Yellow crab spiders contain many unknown ommochrome-like compounds not present in white crab spiders. We also found large quantities of decarboxylated xanthommatin, whose role as precursor of new pathways in ommochrome synthesis needs to be assessed. The catabolism of ommochromes, a process occurring when spiders revert from yellow to white, warrants further study.

Keywords Crypsis · 3-Hydroxy-kynurenine · Xanthommatin · High Performance reverse phase ion-pair chromatography technique

M. Riou · J.-P. Christidès
IRBI, Institut de Recherche de la Biologie des Insectes,
UMR CNRS 6035, Université François-Rabelais de Tours,
Parc de Grandmont,
F-37200 Tours, France

J.-P. Christidès
e-mail: christides@univ-tours.fr

Present Address:
M. Riou (✉)
UE 1277 Plate-Forme d'Infectiologie Expérimentale (PFIE),
Centre – INRA,
F-37380 Nouzilly, France
e-mail: mickael.riou@tours.inra.fr
e-mail: mickaelriou@hotmail.com

Introduction

Color changes play many roles in the behavioral ecology of animals (Morse 2007; Brechbühl et al. 2009), but neither the biochemical and physiological mechanisms of color production nor the underlying genetic basis of this phenomenon have been studied thoroughly. The crab spider, *Misumena vatia*, can change its color reversibly from white to yellow, matching the color of the flowers on which it perches, which supposedly helps increase prey capture (Morse 2007; Ings and Chittka 2008; Théry and Casas 2009). In butterflies, color changes also play an important role in camouflage against predators, reproduction, and sensory physiology (Reed et al. 2008). The biochemistry and nutrient budget that underlie color changes in crab spiders is not well understood. This work investigated the relationship in a crab spider between ommochromes and visual crypsis.

Color changing crab spiders of the family Thomisidae, in particular *Misumena vatia* and *Thomisus onustus*, have been studied with respect to their pigmentation for well over a hundred years (Heckel 1891). These spiders are

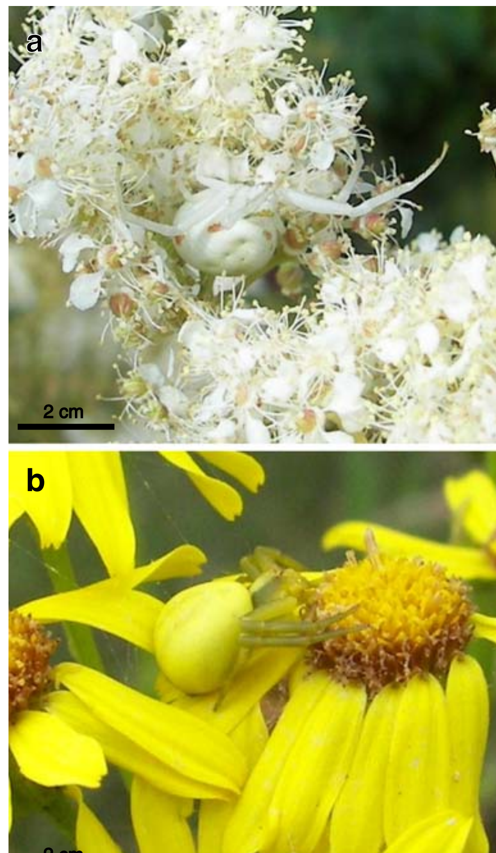


Fig. 1 White and yellow adult females of *Misumena vatia* on flowers of a fruit tree (*white form*) and *Rudbeckia* spp. (*yellow form*)

unusual, as they are able to change color reversibly, within a few days, from white to yellow (Fig. 1). The crypsis of these spiders is astonishing. For example, bees often are deceived with fatal consequences (Chittka 2001; Théry and Casas 2002; Chittka and Osorio 2007; Théry 2007; Ings and Chittka 2008; Taylor and Pfannenstiel 2008). This form of mimicry also may protect the spiders from detection by birds and other predators (Chittka and Osorio 2007; Théry and Casas 2009). Although the physiological and ecological mechanisms for color change in cryptic spiders have been discussed in detail previously (Insausti and Casas 2008, 2009), the biochemistry of color changes in spiders and other organisms has not been pursued vigorously. Older work assumed that the yellow color of *Misumena vatia* was due to carotenoids (Millot 1926), but ommochromes later were found to be the pigments responsible for this color change (Seligy 1972; Insausti and Casas 2008). Most research on ommochrome pigments occurred in the 1970's and 80's (Linzen 1974; Needham 1974; Fuzeau-Braesch 1985; Kayser 1985). Since then, there has been some related research on the developmental biology of butterfly wing patterns (Koch 1993; Nijhout 1997; Reed and Nagy 2005), the physiology of mosquito eyes (Beard et al. 1995; Rasgon and Scott 2004), and tryptophan metabolism (Kato et al. 2006; Han et al. 2007). Ommochrome pigments are the main chromogenic class in the pathway from tryptophan to catabolic compounds (Scheme 1). These pigments range from yellow (xanthommatin), red (ommatin), purple, violet, to black. The chemically characteristic property of ommochromes is their reduction-oxidation (redox) behavior, absorption of ultraviolet and visible light, and low solubility in aqueous medium. The reduced (bathochromic) forms of ommochromes are red, while the corresponding oxidized form is usually yellow. Redox properties of ommochromes enable them to act not only as functional pigments (e.g., in the eyes and integument), but also as an electron accepting or donating system, and as metabolic end products (Scheme 1; Linzen 1974; Needham 1974).

Ommochrome pigments can be divided into three groups whose criteria are based on structure (Linzen 1974): ommins, ommatins, and ommidins and cryptommidins (only found in the eyes of migratory locust *Locusta migratoria*) (Scheme 1).

We hypothesize that the ommochrome biochemical pathway is responsible for the yellow/white reversible color change in *M. vatia*. However, the biochemical pathway involved is more complex than that described by Linzen (1974). In *M. vatia*, tryptophan is derived from digested protein, mainly of insect origin. Tryptophan is the precursor of the first catabolic pathway leading to the production of ommochromes but it does not accumulate (Grob K. and Linzen B. unpublished), rather it is catabolized. In insects, one pathway leads to the synthesis of kynurenine and 3-hydroxy-kynurenine metabolites needed for ommochrome synthesis. According to the physiological state of the crab spider, biosynthesis can favor production of kynurenic acid, xanthurenic acid, anthranilic acid, 3-hydroxyanthralinique acid, or alanine. Kynurenine is a precursor of ommochromes; however, it also may function as a yellow pigment (Seligy 1972; Oxford and Gillespie 1998). 3-Hydroxy-kynurenine also can serve as a pigment (Seligy 1972; Koch 1993; Oxford and Gillespie 1998), and this compound is the major route of the metabolism of tryptophan. 3-Hydroxy-kynurenine is labile, readily transforming into xanthurenic acid (which is stable), or 3-hydroxyanthralinic acid and alanine, and may produce the first ommochrome (xanthommatin) in the chain reaction. Xanthommatin can be metabolized into two other ommochrome pigments: rhodommatin (dihydro-xanthommatin-O-beta-D-glucoside) and ommatin D (dihydro-xanthommatin sulfate, or OmD). These pigments are red and resistant to oxidation in air (Linzen 1974).

The aim of this work was to identify and quantify, in the same run and on the same animals of varying coloration, the relative amounts of ommochrome precursors and pigments associated with color change in crab spiders. In

Scheme 1 Simplified and synthetic ommochrome pathway in insects, based on Phillips et al. 1973; Linzen 1974; Needham 1974 and Naya et al. 1991. * others energetic metabolic or excreta pathways, ** hypothetical pathway for ommin synthesis, *** hypothetical involvement of dihydro-xanthommatin in rhodommatin/ommatin D syntheses

Tryptophan
TDO
Formylkynurenine
Excreta *
Energetic metabolism and excreta *
Kynurenic acid
Kynurenine
Anthranilic acid + alanine
KMO
Xanthurenic acid
3-hydroxy-kynurenine
3-hydroxyanthranilic acid + alanine
PXS
Energetic metabolism and excreta *
Physiological development and excreta *
Xanthommatin
Ommins
?**
?***
?***
Rhodommatin
Dihydro-xanthommatin ***
Ommatin D

this process, we developed a simple and sensitive high performance liquid chromatography method for these types of compounds.

Methods and Materials

Crab Spiders White and yellow *M. vatia* adult female crab spiders (Thomisidae) were collected on flowers in the surroundings of Tours, France, during spring and summer (Fig. 1). Upon capture, they were maintained individually in plastic vials with water (cotton plug) for 3 d (without changing color) and fed a fly every day (*Lucillia* spp.). They then were separated in two groups on the basis of color appearance, and were left 2 d without food before being frozen at −80°C. Yellow crab spiders were rare in the field compared to white crab spiders. The *M. vatia* group corresponding to white with red strips was not used for this study.

Chemicals and Standard Solutions All chemicals were of analytical grade. Acetonitrile, heptanesulphonate, methanol, and hydrochloric acid were obtained from VWR International (Pessac, France). Tryptophan, kynurenine, 3-hydroxy-kynurenine, kynurenic acid, xanthurenic acid, sodium phosphate monobasic, and orthophosphoric acid were obtained from Sigma-Aldrich (St. Quentin, France).

Xanthommatin and the ommatin D were prepared by the method of Butenandt and Schaefer (1962), and verified by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS). An HP1946B quadripole MS (Agilent 66 Technologies, SantaClara, CA, USA) equipped with an electrospray ionization source working in positive mode was used. Standard solutions were prepared from 1 mg of the synthesized product dissolved in 2 ml of acidified methanol (hydrochloric acid at 0.5%, pH 2), giving a 0.5 mg/ml solution. A standard mixture was made from the precursors (7 µg/ml) and ommochromes (35 µg/ml).

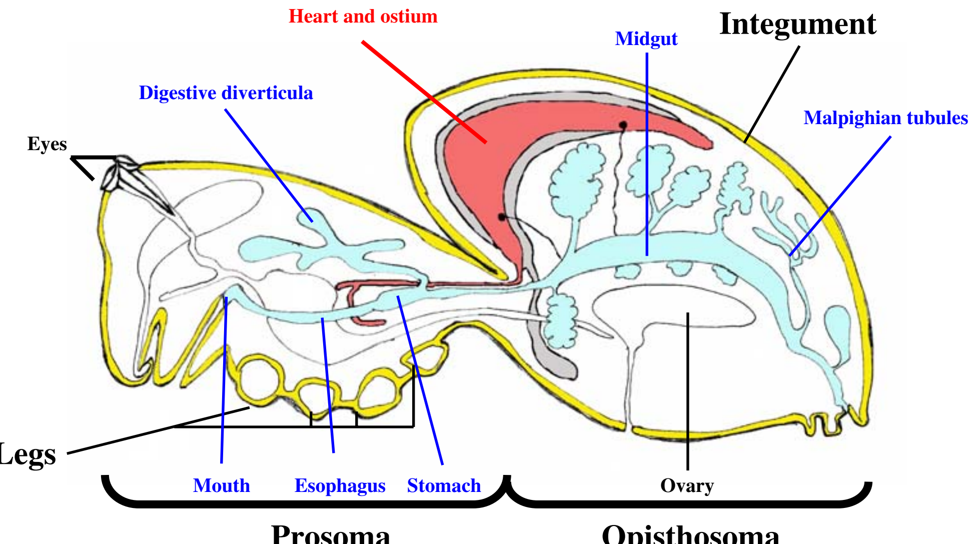
Tissue Preparation and Extraction of Precursors and Ommochromes Four whole yellow and 4 whole white spiders were analyzed. Each was dissected into the legs and the entire opisthosomal hypodermis (Scheme 2), and these tissues were frozen at −80°C until use. For analysis, each sample was crushed in 2 ml of acidified methanol using a Wheaton glass tissue grinder (Fisher-Bioblock, Elancourt, France), transferred to a 2 ml Eppendorf tube, incubated overnight at room temperature, and centrifuged for 15 min at +4°C at 10,000 g. The supernatant was passed through a 0.22 µm filter (Millex GV®, Millipore, Saint-Quentin, France), and condensed by lyophilization (Hyophilisateur, Christ-Alpha, Bioblock Scientific, Illkirch, France).

HPLC Analysis The chromatographic method was based on an ion-pairing technique described previously (Iwahashi and Ishii 1997; Arnault et al. 2003). A Waters HPLC system (Milford, MA, USA) was used, consisting of a 600E quaternary gradient pump, a 996 diode array detector, and a 717 autoinjector. The UV absorbance spectral data were collected from 200 to 500 nm, and the data were acquired and processed using Millenium 3.1 software. Compounds were separated on a Hypurite Elite reversed phase C18 column (150 mm length × 3 mm i.d., 3 µm particle size; Thermo Hypersil, Keystone, Bellafonte, PA, USA). The elution gradient was based on a binary solvent system (time in min [% eluant A/ % eluant B]: 0 min [100/0]; 5 min [70/30]; 25 min [46/54]; 26 min [0/100]; 28 min [0/100]; 30 min [100/0], and 40 min [100/0]). Solvent A consisted of 20 mM sodium phosphate monobasic + 10 mM heptanesulphonate (pH 2.1 adjusted with 85% orthophosphoric acid), and solvent B consisted of 50% acetonitrile / 50% solvent A (v/v), at pH 2.1. Before use, the mobile phase was filtered as above. The column temperature was set at 38°C, and the flow rate was constant at 0.4 ml/min. The detection limit was defined as three times the background noise (American Chemical Society 1980); only areas exceeding this background noise were kept for further analysis.

Statistical Analyses Statistical analyses were performed by using XLstat software, 2007 version (Addinsoft, New-York, USA). Comparisons of means among different groups of crab spiders were carried out by the Mann-Whitney test for small samples (non-parametric test). The correlation between the metabolites and the color of the individual was carried out by using a multivariate principal component analysis (PCA).

Results

Identification and Quantification of Ommochrome Pigments and Precursors The chromatogram in Fig. 2 shows



Scheme 2 Diagrammatic longitudinal section of a spider

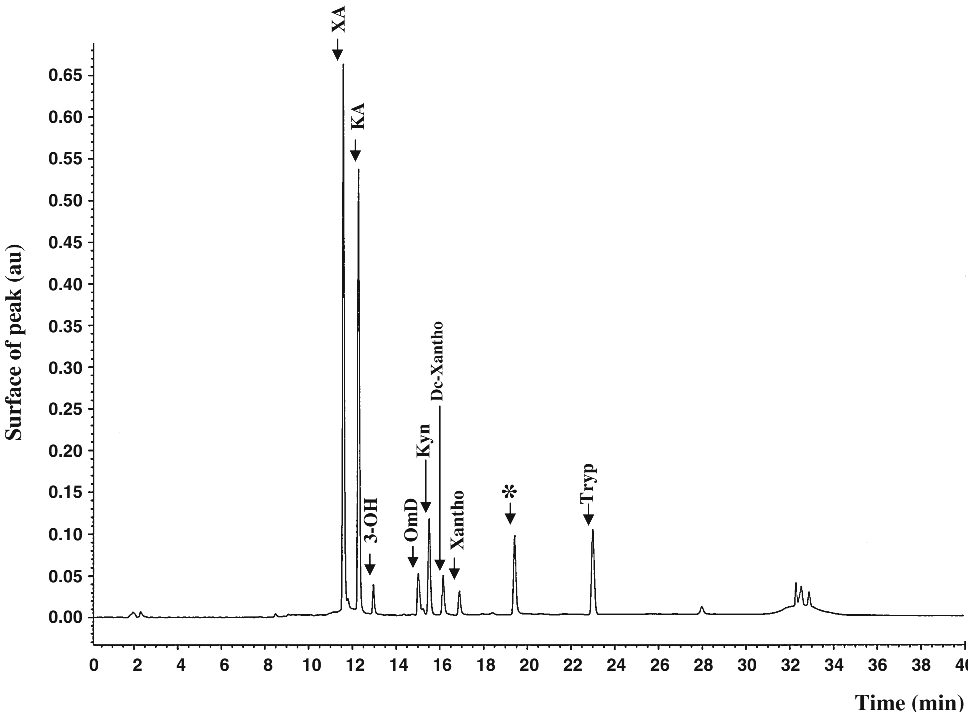


Fig. 2 Chromatogram of the 10–24 min range of the standard solution of eight synthetic ommochromes and metabolites (*degradation product)

the five precursors (Tryptophan “Tryp”, Kynurenine “Kyn”, 3-OH-Kyn-kynurenine “3-OH”, kynurenic acid “KA”, and xanthurenic acid “XA”), and three ommochromes (xanthommatin “Xantho”, ommatin D “OmD”, and decarboxy-xanthommatin “Dc-Xantho”) in the standard solution. One other compound (*; RT 19.50) also was detected. Retention times, absorbance spectra, and maximum absorbance wavelengths for standards are shown in Table 1.

Chromatograms from extracts of whole white (A) and yellow (B) crab spiders exhibited the same substances, but in different quantities (Fig. 3). Table 2 shows the mean quantities of each detected metabolite in each group of four crab spiders. Both precursors and ommochromes were detected but with strong variability among individuals, particularly in yellow crab spiders. Tryp, Kyn, and KA were statistically more abundant in white crab spiders; 3-OH and OmD were more abundant in yellow crab spiders ($P<0.05$, Table 2). Xantho and XA were similar in the two groups. Dc-Xantho was detected in both white and yellow crab spiders, sometimes in large quantities. Many non-identified substances (NIS) with UV spectra and retention times similar to ommochromes were detected in yellow crab spiders; NIS are expressed as a percentage of each substance compared to the total of all extracted substances (Table 3). The NIS with similar chemical properties to Xantho represent approximately 15% of the metabolites in yellow crab spiders, and only 3% in white spiders. Some metabolites are typical of a specific color group, such as the NIS-1 for white spiders or NIS-5, NIS-7 and NIS-10 for yellow spiders ($P<0.05$, Table 3).

Relationship between Spider Color and Constituents Figure 4 illustrates the multivariate PCA for eight crab spiders for the known metabolites (A) or all the compounds detected (B). Two PCE groups were distinguished clearly (Pearson correlation test) on the basis of a match with the visual color appearance (F1: color of individuals) and the different metabolites (F2: time of retention, spectra, and the chemical nature of compounds) (Fig. 4a). The nature of the substance is related directly to the color of the individual (Fig. 5b); however, the variability of the yellow crab spiders was higher than that for white crab spiders. The relationship between color of the crab spider and substance was 66% and, if only the known substances were taken into account in the PCA analysis, this relationship reached 81% correlation (data not shown).

Localization of Ommochrome Metabolites In whole spiders, Tryp was found exclusively in white individuals (Fig. 5), while OmD was found only in yellow spiders (see also Table 1, $P<0.05$). Kyn was the most abundant metabolite, not only in the whole body but also in the integument and legs of white crab spiders (Fig. 5, $P<0.05$). In contrast, 3-OH was present in much larger amounts in yellow spiders, both in whole animals and in their integument and legs. Xantho was found in the whole body of both types of crab spider, but was restricted to the legs and integument in yellow crab spiders.

Table 1 Characteristics of the crab spider ommochrome metabolites (standard solution; spectral range of 200–500 nm)

Peak number	Time of retention (min)	Name of metabolite	λ max (nm)	Spectra Y: arbitrary unit X: λ max (nm)
1	11.59	Xanthurenic acid	348	
2	12.24	Kynurenic acid	332	
3	12.93	3-hydroxy-kynurenine	373	
4	14.85	Ommatin D	486	
5	15.52	Kynurenine	363	
6	16.15	Dc-xanthommatin	453	***
7	16.89	Xanthommatin	459	
8	22.99	Tryptophan	286	

*Footnotes: ***: The spectrum of Dc-xanthommatin was identical to that xanthommatin.*

Discussion

Color change in crab spiders is a complex and still poorly understood process. Earlier research showed that compounds such as the 3-hydroxy-kynrenine and the xanthommatin are responsible for the visual color *M. vatia* crab spiders, and play a role in cryptic mimicry (Heckel 1891; Millot 1926; Seligy 1972; Linzen 1974; Needham 1974; Brown and Nickla 1977; Théry and Casas 2002). Xanthommatin also seems to play a role in optical perception of ultraviolet light in crab spiders (Bhaskara et al. 2009), but metabolic pathways by which these pigments are produced and degraded are not known precisely. The ability to change color is rare in crap spiders; according to Linzen (1974), only 1/4 of crab spiders change color. Reversible color change seems to be a strategy that camouflages crab spiders from both their predators and their potential prey (Théry and Casas 2009). The HPLC technique developed here allowed us to identify and quantify several ommochrome precursors and metabolites in a single run on small samples. Our results are in agreement with the sub-cellular localization of compounds described by Insausti and Casas (2008, 2009).

By using our HPLC procedure, we confirmed the presence of ommochrome pigments and their precursors in crab spiders, first identified by Seligy (1972). In addition, we detected new ommochrome metabolites (particularly in yellow crab spiders), many of which remain unidentified. Several of these metabolites might be pigments associated with ommochromes. First, the extraction technique, based on extremely acid pH, is rather selective for the ommochromes. It is, therefore, unlikely that other

Fig. 3 Chromatograms of white (**a**) and yellow (**b**) adult female *Misumena vatia*. [Tryp (tryptophan); Kyn (kynurenine); 3-OH-Kyn (3-hydroxy-kynurenine); XA (xanthurenic acid); KA (kynurenic acid); Dc-Xantho (decarboxylated-xanthommatin), Xantho (Xanthommatin) and OmD (Ommatin D).]

pigments such as pterins, carotenoids, guanine, or uric acid were present in our samples. Whole pterins and carotenoid pigments were not found in another study of crab spiders (Oxford and Gillespie 1998). Guanine and uric acid crystals have been recovered in white and yellow crab spiders (Oxford and Gillespie 1998; Insausti and Casas 2008), and the presence of guanine crystals in the opisthosoma and uric acid crystals in the prosoma have been associated with a white color (Insausti and Casas 2008). Second, the 450–520 nm spectral profiles of these unidentified compounds are reminiscent of those for known ommochromes. It is unclear if Dc-xanthommatin is an artifact of processing the samples or a true metabolite present in living spiders. Vogliardi et al. (2004) suggest that this compound could be a precursor of new pathways to ommochrome production. In this study, the principal component analysis revealed that the amounts of Dc-xanthommatin are independent of the amounts of other metabolites that give the yellow colora-

Table 2 Quantification of ommochrome metabolites in whole adult female crab spiders

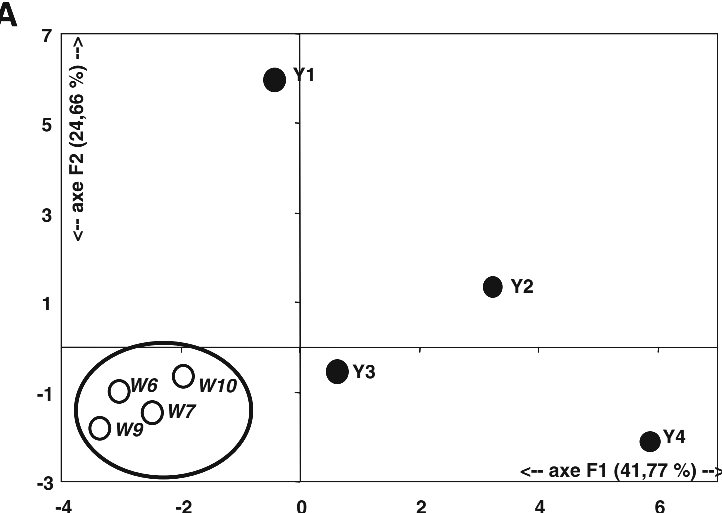
		Mean quantity of metabolites (μg / crab spider)		
		Color of *Misumena vatia*		
Type of metabolites	Name of metabolites	White means (*N*=4)	Yellow means (*N*=4)	Significant level correlation of Pearson
Precursors	Tryptophan	4.17±0.34	0.00±0.00	*P*<0.05
	Kynurenine	35.02±6.40	4.32±1.48	*P*<0.05
	3-hydroxy-kynurenine	8.84±1.19	52.42±6.14	*P*<0.05
	Xanthurenic acid	0.49±0.16	0.66±0.22	NS
	Kynurenic acid	0.15±0.03	0.00±0.00	*P*<0.05
Ommochromes	Dc-xanthommatin	4.26±0.71	9.41±3.78	NS
	Xanthommatin	2.90±0.95	2.74±0.42	NS
	Ommatin D	0.00±0.00	2.42 ± 0.30	*P*<0.05

Mean values correspond to the results of four independent assays for each color of crab spider (means ± S.D.).

Table 3 Conditions of detection and quantification of all detected metabolites in whole white and yellow crab spiders

				Relative proportion of ommochromes (%)		
	Name of metabolites	Retention Time (min)	λ (nm)	White *N*=4	Yellow *N*=4	Significance level (Mann Whitney Correlation)
Precursors and derivatives	XA	11.59	348	1.70±0.48	2.35±1.33	NS
	KA	12.24	331	0.82±0.25	0.00±0.00	*P*<0.05
	3-OH	12.93	373	8.60±1.75	70.34±13.92	*P*<0.05
	Kyn	15.65	363	83.51±23.02	14.21 ± 8.23	*P*<0.05
	Tryp	22.99	286	2.13±0.26	0.00±0.00	*P*<0.05
Ommochromes and non identified substances (NIS)	NIS1	12.48	265,367,445	1.00±0.48	0.00±0.00	*P*<0.05
	NIS2	12.81	365, 489	0.00±0.00	0.42±0.35	NS
	OmD	14.85	487	0.00±0.00	0.76±0.16	*P*<0.05
	NIS3	15.74	373, 420	0.00±0.00	0.12±0.10	NS
	Dc-Xantho	16.15	453	1.68±0.24	4.48±3.04	NS
	NIS4	16.23	351, 441, 463	0.00±0.00	0.11±0.09	NS
	Xantho	16.89	459	0.41±0.22	0.56±0.15	NS
	NIS5	17.60	405,489	0.00±0.00	1.09±0.15	*P*<0.05
	NIS6	18.13	402	0.00±0.00	0.27±0.13	NS
	NIS7	18.65	402,489	0.06±0.04	0.66±0.16	*P*<0.05
	NIS8	19.00	400,465,484	0.00±0.00	0.41±0.35	NS
	NIS9	19.44	299,405,484	0.00±0.00	0.70±0.34	NS
	NIS10	19.80	298,404,481	0.00±0.00	1.55±0.46	*P*<0.05
	NIS11	20.24	326,404,486	0.00±0.00	0.66±0.25	NS
	NIS12	20.66	325,404,494	0.00±0.00	0.50±0.31	NS
	NIS13	20.95	404	0.00±0.00	0.08±0.07	NS
	NIS14	21.56	400,484	0.00±0.00	0.29±0.14	NS
	NIS15	23.16	323,424,487	0.00±0.00	0.12±0.10	NS
	NIS16	23.81	323,400,459	0.10±0.04	0.10±0.08	NS
	NIS17	24.52	386,463,484	0.00±0.00	0.12±0.10	NS
	NIS18	25.13	371,400,459	0.00±0.00	0.11±0.09	NS

The amount of each metabolite is expressed in the percentage of total amount of ommochromes and precursors measured. Data are means (means ± S.D.) of four crab spiders in each group. *XA* Xanthurenic Acid; *KA* Kynurenic Acid; *3-OH* 3-Hydroxy-Kynurenine; *Kyn* Kynurenine; *Tryp* Tryptophan; *OmD* Ommatin D; *NIS* Non Identified Substances; *Dc-Xantho* decarboxylated xanthommatin; *Xantho* Xanthommatin.



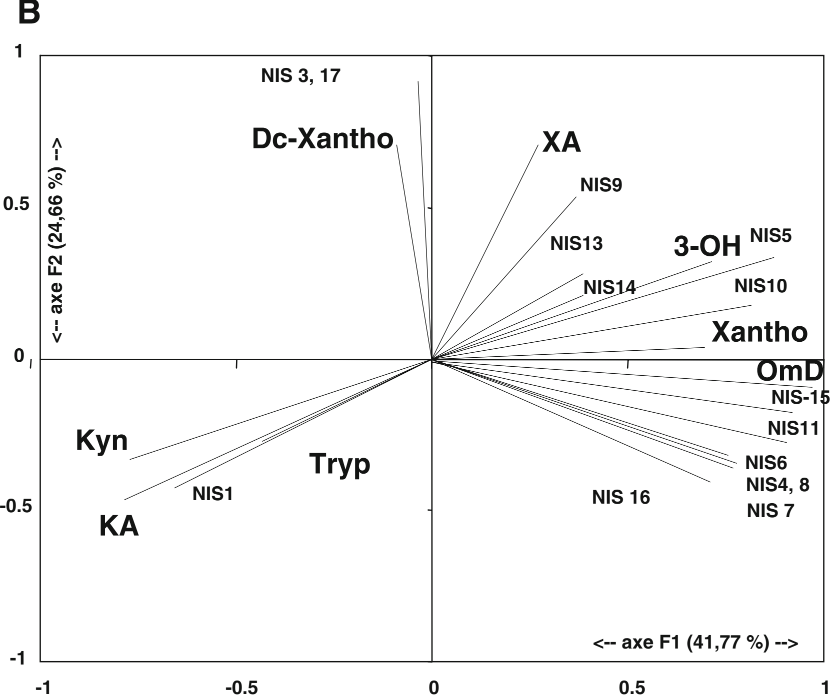


Fig. 4 Multivariate principal component analysis of eight spiders on the basis of their ommochrome-related metabolites (F1: color of individuals and F2 nature of compounds). **a** Statistical analysis based on different metabolites showed two groups of crab spiders (W: white, group 1 and Y: yellow, group 2). **b** Statistical analysis showed a significant difference between the two groups of crab spiders of color of compounds and the color intensity of crab spiders: Colorless or translucent compounds (Tryp, kyn, KA), pale yellow compounds (3-OH, XA), and ommochromes (xantho and Dc-Xantho: yellow/brown and OmD: orange / red). Statistical analysis showed a significant correlation between the color of the crab spiders and the color of compounds, with a well grouped cluster of uncolored compounds in white crab spiders, and diverse ommochrome pigments in the yellow crab spiders

tion, suggesting that the presence of this compound is not due to degradation of xanthommatin.

After separation of compounds by HPLC, the distribution of metabolites between white and yellow crab spiders was different. Small quantities of tryptophan were found exclusively in white crab spiders, which is consistent with prior knowledge. Tryptophan is toxic in its free form (Linzen 1974) and, in the crab spider we studied, it is found in the integument and legs. Tryptophan precursors (kynurenine and 3-hydroxy-kynurenine) were found in abundance, both in the integument and in the legs, and the presence of tryptophan is inversely proportional for white and yellow crab spiders. Xanthommatin is a yellow-brown pigment made from condensation of two molecules of 3-hydroxy-kynurenine catalyzed by phenoxazinone synthetase (Phillips and Forrest 1980). Xanthommatin was found in yellow and white crab spiders, but in different locations in the two groups as described by Insausti and Casas (2008, 2009). Taking into account Dc-xanthommatin and the many other less abundant compounds reminiscent of ommochromes, we conclude that there is twice the amount of ommochrome-related products in yellow as compared to white spiders. Ommatin D, a red ommochrome pigment produced from xanthommatin by the addition of a sulphate group, was found only in yellow crab spiders. Our technique may, however, have

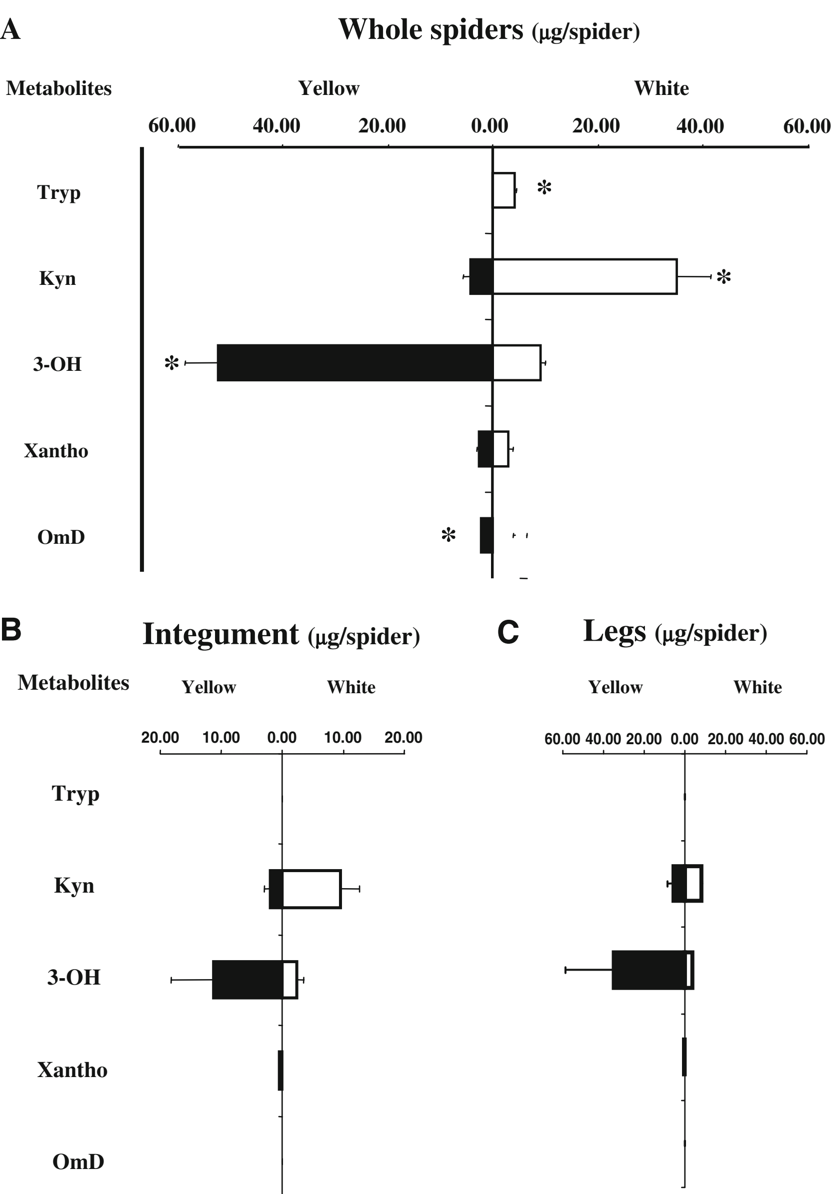


Fig. 5 Quantification of the main identified metabolites of whole spiders (**a**), integument (**b**) and legs (**c**), in white and yellow spiders (only known ommochrome pigments and precursors were analysed; abbreviations as in Table 3)

underestimated the amount of this metabolite present because, at the acid pH used for extraction, some ommatin D may have precipitated in the cytosol. Thus, the role of ommatin D in crab spider coloration is still uncertain, although its water solubility suggests that it is more easily removed than xanthommatin and, therefore, may be part of an elimination pathway.

The complete congruence of crab spider color and their color classification by using PCA of chemical composition is powerful evidence that ommochrome precursors and end products give rise to the yellow coloration. The PCA also highlights the chemical variability of the yellow coloration vs. that for white spiders. The hues of varying intensities might originate in the varying stages of color change in these spiders. The observed reversal in the ratio kynurenine/3-hydroxy-kynurenine is correlated positively with the ultra-structural changes observed in the opisthosoma and prosoma during color change (Insausti and Casas 2008, 2009), and conclusively associates the granule morphology identified by these authors with biochemical composition. We interpret *Type III* granules as ommochromes and *Type I* granules as kynurenine. 3-Hydroxy-kynurenine might be part of the heterogeneous *Type II granules*. The distribution of different metabolites in tissues was heterogenic whatever the color of crab spiders, and may be explained by the presence of ommin-binding proteins capable of transporting ommochromes and precursors, as described in insects (Sawada et al. 2006). It is presently unclear whether ommochromes are synthesized in the intestine and transported to the integument, or whether

tryptophan is transported and converted to ommochromes in the integument.

In summary, the pathway of either tryptophan degradation or ommochrome synthesis plays an important role in visual color of *M. vatia* crab spiders. However, it is not clear what triggers cryptic color change (Théry and Casas 2002; Brechbühl et al. 2009). The catabolic pathway of tryptophan also is involved in others physiological processes in arthropods, such as production of ATP and the regulation of ecdysone synthesis (Linzen 1974; Naya et al. 1991; Okech et al. 2006). In this study, 3-hydroxy-kynurenine is most abundant in yellow crab spiders, and may lead to precursors of xanthommatin synthesis. We hypothesise that the early degradation of kynurenine or 3-hydroxy-kynurenine (yellow precursors) to xanthralinic acid, 3-hydroxy-xanthralinic acid, and alanine (precursors without visible coloration) may explain the reversion of color in *M. vatia* crab spiders (yellow to white), without xanthommatin synthesis. In contrast, the synthesis and the catabolism of xanthommatin might produce degradation products of similar spectral and chemical behavior such as the numerous unknown metabolites reminiscent of ommochromes that are observed in yellow spiders. In this case, the yellow color of crab spiders seems be due to the summation of yellow ommochrome precursors (from yellow to brown according to concentration and pH).

In summary, as in insects, the metabolic pathway of tryptophan catabolism seems to play an important role in the physiology and behavior of crab spiders. *Misumena vatia* crab spiders appear able to control this metabolic pathway at different levels, thus utilizing these metabolic products for optimal crypsis.

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Regulation of Reproduction in the Primitively Eusocial Wasp *Ropalidia marginata*: on the Trail of the Queen Pheromone

Anindita Bhadra · Aniruddha Mitra · Sujata A. Deshpande · Kannepalli Chandrasekhar · Dattatraya G. Naik · Abraham Hefetz · Raghavendra Gadagkar

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Abstract Queens and workers are not morphologically differentiated in the primitively eusocial wasp, *Ropalidia marginata*. Upon removal of the queen, one of the workers becomes extremely aggressive, but immediately drops her aggression if the queen is returned. If the queen is not returned, this hyper-aggressive individual, the potential queen (PQ), will develop her ovaries, lose her hyper-aggression, and become the next colony queen. Because of the non-aggressive nature of the queen, and because the PQ loses her aggression by the time she starts laying eggs, we hypothesized that regulation of worker reproduction in *R. marginata* is mediated by pheromones rather than by physical aggression. Based on the immediate loss of aggression by the PQ upon return of the queen, we developed a bioassay to test whether the queen's Dufour's gland is, at least, one of the sources of the queen pheromone. Macerates of the queen's Dufour's gland, but not that of the worker's Dufour's gland, mimic the queen in making the PQ decrease her aggression. We also correctly distinguished queens and workers of *R. marginata* nests by a discriminant function analysis based on the chemical composition of their respective Dufour's glands.

Key Words *Ropalidia marginata* · Queen pheromone · Potential queen · Dufour's gland · Reproductive monopoly

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A. Bhadra · A. Mitra · S. A. Deshpande · K. Chandrasekhar · R. Gadagkar (✉)
Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012, India
e-mail: ragh@ces.iisc.ernet.in

R. Gadagkar
Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India

D. G. Naik
Agharkar Research Institute, G. G. Agarkar Road, Pune, India

A. Hefetz
Department of Zoology, Tel Aviv University, Ramat Aviv, 69978 Tel Aviv, Israel

Introduction

The differentiation of adult colony members into fertile queens and functionally sterile workers is an important feature of insect societies of ants, bees, and wasps. In these highly eusocial insect species, which generally have large colony sizes (>>100 individuals), queens and workers are morphologically differentiated, with queens maintaining their reproductive monopoly with pheromones. On the other hand, in primitively eusocial species, colony sizes are usually small (<100 individuals), and queens and workers are morphologically indistinguishable, with queens believed to suppress worker reproduction by physical aggression (often referred to as dominance behavior) (Wilson 1971). *Ropalidia marginata*, the subject of this study, is classified as a primitively eusocial species, because of the absence of morphological differentiation between queens and workers. However, the queen in *R. marginata* is a strikingly non-aggressive and non-interacting individual that, nevertheless, maintains complete reproductive monopoly. Since the queen does not use physical aggression to suppress worker reproduction (Gadagkar 2001), we hy-

pothesized that *R. marginata* queens may use pheromones for this purpose.

If the queen is lost or removed from a colony, one of the workers becomes highly aggressive within a few minutes; if the queen is not returned, this individual develops ovaries and becomes the new queen of the colony within a few days. We have designated this individual as the potential queen (PQ; Premnath et al. 1996). However, if the queen is returned to the colony within a day of removal, the PQ decreases her aggression and swiftly returns to being a typical worker (Premnath et al. 1996; Kardile and Gadagkar 2002; Sumana and Gadagkar 2003; Deshpande et al. 2006). This suggests that the PQ perceives the absence of the queen within a few minutes. When workers are separated from their queen by a wire mesh screen, one of the workers establishes itself as the PQ and will lay eggs if the wire mesh is not removed (Sumana et al. 2007/2008). How then do workers perceive the presence or absence of their queen? One possibility is through physical interactions with the queen. However, we have shown that the rates of interaction of the PQ with the queen (both direct and indirect, via interactions with other workers) are not frequent enough to explain the rapidity with which the queen's absence is perceived (Bhadra et al. 2007). Another possibility is that the queen applies her pheromone to the nest surface. This possibility is supported by the observation that the queen, but not the workers, frequently rubs the ventral side of her abdomen on the nest surface (Bhadra et al. 2007). Because the Dufour's gland, which opens into the tip of the abdomen, is believed to be a source of the queen signal in honeybees (Katzav-Gozansky et al. 1997; Katzav-Gozansky et al. 2002; Dor et al. 2005), we examined the possibility that the queen pheromone of *R. marginata* is produced in the Dufour's gland.

Here, we demonstrated that a crude macerate of the queen's Dufour's gland mimics her presence in a bioassay, and that queens and workers can be classified correctly by a discriminant function analysis that uses the hydrocarbon profiles of their respective Dufour's glands.

Methods and Material

We collected post-emergence nests of *R. marginata* from various localities in Bangalore (13° 00' N and 77° 32' E), India, and transplanted them to the Vespiary at the Centre for Ecological Sciences, Indian Institute of Science, Bangalore. The nests were maintained in closed cages made of wood and fine mesh, and provided with food, water, and building material, ad libitum. All adults were uniquely color-coded with small spots of Testors® enamel paints (Gadagkar 2001). The queen was identified by egg-laying behavior prior to beginning the experiment.

Bioassay

We developed a bioassay for the queen pheromone based on the observation that the PQ immediately reduces her aggression when the queen is returned to the colony. Thus, we tested whether applying crude macerate of the Dufour's gland of the queen but not of the workers mimics the return of the queen by a similar reduction in the aggression of the PQ. The bioassay consisted of three observation sessions lasting 36 min. each. Each session consisted of six observation periods of 5 min. each, interspersed with a 1 min. break. After observing the normal queen-right colony in the first session (Queen-right Session), the queen and a randomly chosen worker were removed, and the queen-less colony was observed in the second session (Queen-less Session). Before beginning observations in the third session (Treatment Session), the contents of the Dufour's gland of the removed queen or a worker (each crushed in 30 µl of Ringer's solution) or 30 µl of Ringer's solution, were applied to the nest with a micropipette. The choice of treatment was decided by drawing random numbers and was unknown to the observer. A total of 25 nests were used in the experiment (8 queen extract; 9 worker extract, and 8 Ringer's solution). All statistical analyses were performed using the software package STATISTICA 7.

We used Ringer's solution because we observed that organic solvents, such as pentane or acetone, had an adverse effect when applied to the nest, with the wasps becoming agitated or even dying after contact with these solvents. Later, we validated the use of Ringer's solution by chemical analysis of crude macerates of glands crushed in Ringer's solution and found a similar chemical profile to that obtained by extracting glands in pentane.

Chemical Analysis

Preparation of Sample Seven additional colonies were used for chemical analysis. The queens of each colony and a total of 18 workers (2 to 5 per colony, depending on colony size) had their Dufour's glands dissected in distilled water under a stereomicroscope, after gently pulling out the sting. The gland was placed in a vial (chilled with dry ice and acetone), containing 5 µl pentane, and crushed with a needle.

Gas Chromatographic-Mass Spectrometric Analysis After adding another 5 µl pentane to the vial containing the crushed gland, 2 µl extract was injected into an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass selective detector. A fused-silica capillary column, coated with 100% dimethyl polysiloxane (Agilent HP-1, 60 m×250 µm×0.25 µm), was used for the gas chromatography-mass spectrometry (GC-MS) analyses. The injector port and transfer line were set at 250°C, and

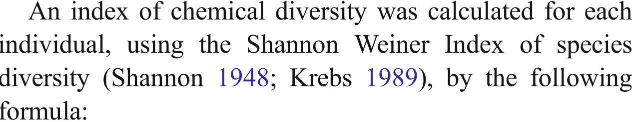
helium was the carrier gas. The column oven was programmed from 100–275°C at 7°C min^{-1}, held for 5 min, and then to 280°C at 5°C min^{-1} held for 33 min. Analyses were performed in split mode (split ratio 10:1). Straight-chain and methyl-branched hydrocarbons were identified from their characteristic mass spectral fragmentation patterns, produced by electron impact ionization at 70 eV. The compounds identified were either straight-chain or branched hyrdrocarbons, the spectra of which are very clear and can be interpreted unambiguously. For example, branching at carbon 11 (11-methylHC) gives a distinct peak fragment at m/z 168 with a complementary fragment depending on the chain length of the compound. Likewise, 14-methylHC has a pronounced peak fragment at m/z 196, etc. For dimethyl compounds, we have two branching points and therefore 4 complementary peak fragments, the m/z of which are characteristic to the compound. The spectra of most of these compounds have been previously published with a list of the ions characteristic to the branching points. A blank run with 2 µl pentane confirmed that none of the detected compounds was present as impurities in the solvent.

Statistical Analyses Both multivariate and univariate analyses were carried out. To reduce the number of peaks used in the multivariate analyses, only those present in at least 70% of all individuals were considered. Before applying any multivariate test, the areas under each peak were transformed by √(X=+0.5) to eliminate zero values (Zar 1999). Before applying the transformation, we confirmed that group variances were proportional to the means. The transformed data were subjected to the further transformation:

$$Z_{p,j} = \ln\left[\frac{A_{p,j}}{g(A_j)}\right]$$

where, $A_{p,j}$ is the area of the peak p for individual j, $g(A_j)$ is the geometric mean of all peaks considered for analysis in individual j, and $Z_{p,j}$ is the transformed area of peak p for individual j (Reyment 1989). These transformed areas then were subjected to principal components (PC) analysis, followed by stepwise discriminant function analysis. All peaks had communality <0.8 on PC1 and were considered subsequently for discriminant analysis. The significance of Wilk's λ (for canonical discriminant function), and the percentage of correct assignments (for classification discriminant functions) were used to evaluate the validity of the discriminant functions. For univariate analysis, the areas under each peak were transformed into percentages of total area under all peaks for each individual, and Mann-Whitney *U* tests were carried out to determine whether queens and workers differed for each compound.

An index of chemical diversity was calculated for each individual, using the Shannon Weiner Index of species diversity (Shannon 1948; Krebs 1989), by the following formula:



$$H' = -\left(\sum_{i=1}^{S} p_i \ln p_i\right)$$

where H' is the index of chemical diversity, S is the total number of peaks in the respective individual, p_i is the relative abundance of each peak (i.e., the area under the respective peak divided by total area under all peaks in that individual). A Mann-Whitney *U* test was carried out to test whether queens and workers differed with respect to indices of chemical diversity.

Squared Euclidean distances were calculated to estimate the chemical distances between all possible pairs of individuals, using the standardized percentages of area under each peak (percentages calculated out of total area under all peaks as mentioned earlier). Z scores for the matrices were used to standardize the percentages, and the differences between groups were analyzed by Mann-Whitney *U* tests. Statistical analyses used the software packages StatistiXL, version 1.7 and Mystat 12.

Validation of Bioassay

Because we carried out the bioassay using Ringer's solution, and the chemicals we identified from the Dufour's gland are insoluble in Ringer's, we determined whether the Ringer's solution macerates used in the bioassays actually contained the Dufour's gland compounds or not. Each gland was crushed in 30 µl Ringer's solution, as in the bioassay, and the contents transferred to a clean glass vial. The Ringer's solution was evaporated from the vial in an oven at 30°C, the dry vial chilled, and 10 µl pentane added to it. Two microliters of this extract were analyzed by GC using an Agilent 6890 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column coated with 5% phenyl methyl siloxane (Agilent HP-5, 30 m × 320 µm × 0.25 µm). General GC conditions were the same as those in the GC-MS analyses. The Ringer's solution was analyzed similarly to rule out any organic compounds that might be present as impurities. Peaks were identified by analyzing a sample of 21 glands in 20 µl pentane by both GC-MS and GC.

Further, to rule out the possible involvement of other compounds in the Dufour's gland-Ringer's solution that are insoluble in pentane, we analyzed glands crushed in acetone. GC conditions were identical to those used in the pentane extracts, and blank runs were also carried out to exclude solvent-based impurities.

Results

The Bioassay

In all nests, a PQ was obvious after queen removal, due to a significant increase in its aggression from the Queen-right Session to the Queen-less Session (Wilcoxon matched pairs signed-ranks test: $P=0.012$ for queen-gland and Ringer's-solution treatments, and $P=0.007$ for worker-gland treatment). There was a significant reduction in the PQ's aggression from the Queen-less Session to the Treatment Session (Fig. 1) in nests treated with the queen's Dufour's gland macerate ($P=0.012$). However, in nests treated with worker-gland macerate or Ringer's solution, the PQ did not show any change in aggression from the Queen-less Session to the Treatment Session ($P=0.593$ for worker and $P=0.401$ for Ringer's).

Chemical Analysis

Of 18 workers, two were eliminated from statistical analyses because they had poorly developed glands, and most compounds in their glands were below the detectable limit of the GC-MS analysis. All queens had well developed glands. The Dufour's gland contained a series of linear, monomethyl and dimethyl branched alkanes with 21 to 33 carbon atoms in the main chains. We did not find any unsaturated compounds. Considering all individuals, we found about 30 different compounds (Fig. 2 and Table 1). We did not find any compound or set of compounds present exclusively in queens or in workers.

We found that: 1) queens had a significantly greater percentage peak area than workers for three compounds (peaks #16, #24, and #29; Mann-Whitney U test, $U=96$, $P=0.003$ for peak #16; $U=91$, $P=0.009$ for peak #24; and $U=96.5$, $P=0.002$ for peak #29); 2) a significantly lower percentage peak area than workers for one compound (peak #13; $U=83$, $P=0.038$; and 3) they were indistinguishable from workers in percentage peak areas for the remaining compounds ($P>0.05$). When the queens and workers of each colony were examined separately, the differences based on these compounds were not consistent. There was no compound that was consistently greater or lower in any queen compared to all the workers tested from her colony. The differences between queens and workers became significant only when all queens were pooled together and compared with all workers pooled together. However, when we added up the percentage areas under peaks 16, 24, and 29, for which queens had higher percentage areas than workers, queens of each colony were greater than workers from their respective colonies, except for one colony, which was a small colony with only three workers, out of which two were newly eclosed. Queens and workers did not differ with respect to total area under all peaks ($U=59$, $P=0.871$) or total number of peaks present ($U=64.5$, $P=0.579$).

We differentiated all queens and workers by a stepwise discriminant analysis (Wilk's $\lambda=0.214$, $P<0.001$, classification analysis: 100% correct classification; Fig. 3). Peak

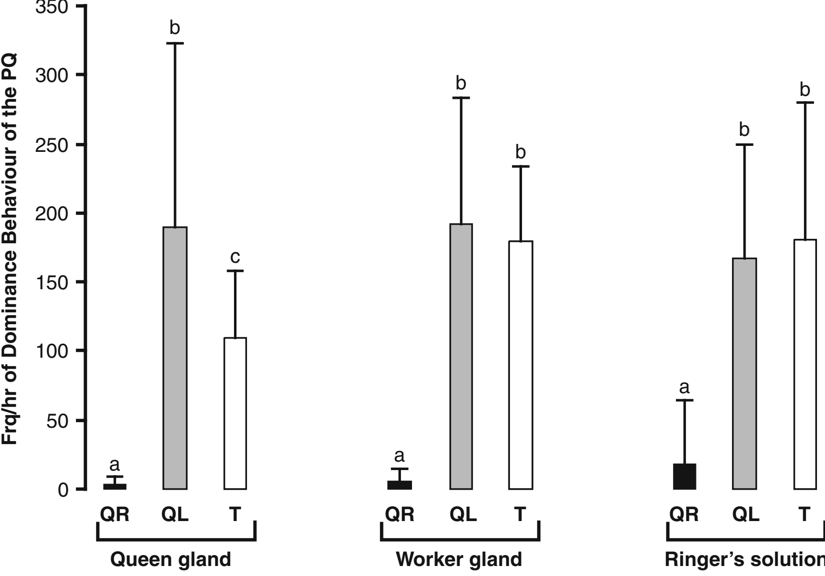


Fig. 1 Mean and standard deviation of the frequency per hour of dominance behavior exhibited by the potential queen from *Ropalidia marginata* nests in the three sessions of the bioassay (Queen-right, Queen-less and Treatment; $N=8$, 9, and 8, respectively) when the nest was exposed to queen Dufour's gland macerate, worker Dufour's gland macerate or Ringer's solution (control). Comparisons are by Wilcoxon matched pairs signed-ranks test among the three sessions within each treatment. Different letters denote significant difference among bars ($P<0.05$)

Fig. 2 Total ion mass chromatograms of a *Ropalidia marginata* worker and a queen from the same colony. Asterisk signifies contaminant present in blank run. Peak numbers correspond to compounds identified in Table 1

numbers 8, 13, 14, 15, 16, 21, and 22 were selected in the discriminant analysis. Analysis of index of chemical diversity showed that queens had higher indices of diversity than workers (Mann Whitney U=82, P=0.044), but the difference was not universal. There were two colonies in which the queen had a lower diversity index than a worker from the respective colony. Both of these colonies were small, one had three workers, out of which two were newly eclosed (see above), and the other with four workers out of which three were newly eclosed. Analysis of chemical distances showed that queen-worker distances were significantly greater than worker-worker distances (Mann-Whitney U=8237, P=0.001). Queen-queen distances were, however, not different from queen-worker distances (U=1266, P=0.583), but were greater than worker-worker distances (U=1660, P=0.01).

Validation of Bioassay

Glands crushed in Ringer's solution gave similar GC-MS profiles to those obtained using pentane extracts of glands (Supplementary material S1 and S2). GC analysis of glands crushed in acetone also gave profiles similar to those obtained using pentane (Supplementary material S3).

Discussion

We have shown that applying an extract of the queen's Dufour's gland onto the nest surface results in the PQ behaving as if the queen has returned to the nest. We also have shown that queens and workers can be classified

Table 1 Peak numbers, retention times, identity and average percent areas and standard deviations of dufour's gland compounds of queens and workers in *Ropalidia marginata*

Peak #	Retention Time (min)	Identity of compound	% area of each compound mean±standard deviation	
			workers	queens
1	20.83	Heneicosane	0.16±0.46	0.0008±0.002
2	21.431	11-Methylheneicosane	0.6±0.99	0.04±0.08
3	23.504	Tricosane	0.54±1.51	0.03±0.07
4	23.963	11-Methyltricosane	0.37±0.774	0.007±0.012
5	25.914	Pentacosane	1.85±3.06	1.12±1.96
6	26.323	Mixture of 11- and 13-methylpentacosane	3.99±3.58	3.28±4.40
7	28.267	Heptacosane	0.63±1.14	1.63±2.78
8	28.714	Mixture of 11- and 13-methylheptacosane	1.77±3.08	1.90±1.99
9	29.288	3-Methylheptacosane	2.19±2.754	1.062±2.069
10	30.187	Mixture of 12-, 14-, 16- and 18-methyloctacosane	0.341±0.59	0.25±0.33
11	30.614	4-Methyloctacosane	0.56±1.16	0.27±0.53
12	31.29	Nonacosane	1.63±3.10	6.74±10.40
13	31.915	Mixture of 11-, 13- and 15-methylnonacosane	41.87±30.06	16.95±14.97
14	32.279	5-Methylnonacosane	3.24±4.81	7.30±9.29
15	32.463	11-, 15-Dimethylnonacosane	7.814±9.753	4.54±4.09
16	32.712	3-Methylnonacosane	2.13±2.24	16.43±8.95
17	32.859	2-Methylnonacosane	0.25±0.91	0.10±0.20
18	33.395	8-Methyltriacontane	2.70±4.23	0.73±1.20
19	33.79	14-, 16-Dimethyltriacontane	1.92±2.26	1.02±1.95
20	34.377	2-Methyltriacontane	0.02±0.06	0.05±0.09
21	35.984	Mixture of 11-, 13- and 15-methylhentriacontane	12.03±17.53	14.98±17.40
22	36.2	Mixture of 7- and 9-methylhentriacontane	0.60±1.68	1.58±2.15
23	36.698	Mixture of 11-, 17- and 13-, 17-dimethylhentriacontane	11.10±11.09	9.99±8.40
24	37.029	3-Methylhentriacontane	0.02±0.07	1.39±2.37
25	37.253	Mixture of 5-, 21-, 5-,19- and 5-, 17-dimethyldotriacontane	0.02±0.07	1.11±2.04
26	37.896	14-Methyldotriacontane	0.17±0.32	0.66±0.76
27	38.056	8-Methyldotriacontane	1.84±3.52	0.38±0.7
28	38.643	14-, 18-Dimethyldotriacontane	0.04±0.12	0.63±1.45
29	41.8	Mixture of 13-, 15- and 17-methyltritriacontane	0.43±1.42	5.31±7.22
30	42.813	13-, 19-Dimethyltritriacontane	0.08±0.15	0.55±1.03

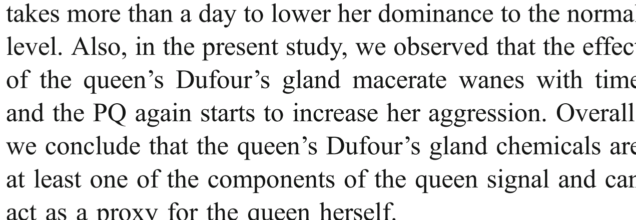
correctly based on the contents of their Dufour's glands. These results are consistent with the hypothesis that, in *R. marginata*, the workers (at least the PQ) perceive the presence of their queen through contents of the Dufour's gland that she applies to the nest surface. It should be noted that our ability to classify queens and workers correctly required that we consider the effect of several compounds simultaneously with no single compound being adequate for this purpose. We do not rule out the role of other compounds or indeed of other mechanisms contributing to queen recognition by colony members. We do not have direct evidence that the compounds tested here are responsible for the lack of ovarian development of the workers in the presence of the queen. However, if workers use an honest signal of their queen's fertility to refrain from reproduction, as suggested by Keller and Nonacs (1993), the compounds we identified also may be involved in reproductive regulation in *R. marginata,* thus permitting the queen to maintain reproductive monopoly in spite of her docility.

Analysis of chemical diversity showed that queens, in general, had higher indices than workers, implying that queens have either greater numbers of compounds, or a more even distribution (i.e., less variable ratios, within individuals) of compounds. Since we did not find any difference between queens and workers with respect to numbers of compounds present, it is likely that the distribution of relative proportions of compounds is more even in the case of queens and less even in the case of workers. The two

Fig. 3 Scores on discriminant function analysis for gas chromatographic analyses of Dufour's glands of *Ropalidia marginata* queens and workers (n_{queens}=7, $n_{workers}$=16, Wilk's λ=0.214, P<0.001, classification analysis: 100% correct classification)

colonies in which the queen did not have the highest index value were small colonies with only one mature worker, with the rest of the workers being newly eclosed. Chemical profiles of old, well established queens could be different from those of newly established queens, as queens are known to be behaviorally dominant during the nest initiation period or pre-emergence phase (Gadagkar 2001). Also, in this species, the old queen sometimes gets overthrown with a worker becoming the PQ and ultimately taking over as the new queen of the colony, a phenomenon known as queen turnover in natural colonies (Gadagkar 2001). It is possible that queens that are on the verge of being overthrown have a declining queen signal and are chemically different from normal queens. Since we do not have any information on the history of each colony, and also did not measure the ovarian development of the queens used in our GC-MS analyses, we cannot make any conclusive statement as to why these colonies were different from the others. Chemical distances show that queen-worker distances are higher than worker-worker distances, indicating that queens and workers are more different from each other than workers are from other workers. However, queen-queen distances are different from worker-worker, but not queen-worker distances, implying that more variability in peak area (relative amount) occurs among queens (between individuals).

Suppression of the PQ's dominance is not complete after applying the queen's Dufour's gland extract to the nest, although the level of dominance is significantly higher than that observed in the queen-right condition. It should be noted that in earlier studies (Sumana and Gadagkar 2003) we saw that, after keeping the queen away from her colony for a day followed by queen reintroduction on the next day, the dominance of the PQ remained significantly higher than in the normal queen-right condition, suggesting that the PQ



takes more than a day to lower her dominance to the normal level. Also, in the present study, we observed that the effect of the queen's Dufour's gland macerate wanes with time and the PQ again starts to increase her aggression. Overall, we conclude that the queen's Dufour's gland chemicals are at least one of the components of the queen signal and can act as a proxy for the queen herself.

Our results add to the growing evidence for chemical communication between queens and workers in primitively eusocial wasps. For example, Downing (1991) demonstrated that the contents of the Dufour's gland in *Polistes fuscatus* act as an egg-marking pheromone. Sledge et al. (2001) showed that queens and workers in *Polistes dominulus* differ in their cuticular hydrocarbon signatures. Dapporto et al. (2007) suggested that the contents of the Van der Vecht's gland may function as a queen pheromone in *Polistes gallicus*. Unlike *Polistes* queens, that are also aggressive in addition to producing a chemical cue signaling their presence, *R. marginata* queens appear to rely more heavily on pheromones for communication with workers, as they are non-aggressive and interact directly with other members of their colonies relatively infrequently. Our results are similar to the situation in honeybees in which queens are non-aggressive and where there is good evidence for the contents of Dufour's glands as signals of fertility (Katzav-Gozansky et al. 2002).

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Variation in and Responses to Brood Pheromone of the Honey Bee (*APIS mellifera* L.)

Bradley N. Metz · Tanya Pankiw · Shane E. Tichy · Katherine A. Aronstein · Robin M. Crewe

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Abstract The 10 fatty acid ester components of brood pheromone were extracted from larvae of different populations of USA and South African honey bees and subjected to gas chromatography-mass spectrometry quantitative analysis. Extractable amounts of brood pheromone were not significantly different by larval population; however, differences in the proportions of components enabled us to classify larval population of 77% of samples correctly by discriminant analysis. Honeybee releaser and primer pheromone responses to USA,Africanized and–European pheromone blends were tested. Texas-Africanized and Georgia-European colonies responded with a significantly greater ratio of returning pollen foragers when treated with a blend from the same population than from a different population. There was a significant interaction of pheromone blend by adult population source among Georgia-European bees for modulation of sucrose response threshold, a primer response. Brood pheromone blend variation interacted with population for pollen foraging response of colonies, suggesting a self recognition cue for this pheromone releaser behavior. An interaction of pheromone blend and population for priming sucrose response thresholds among workers within the first week of adult life suggested a more complex interplay of genotype, ontogeny, and pheromone blend.

B. N. Metz (✉) · T. Pankiw
Department of Entomology, Texas A&M University,
College Station, TX, USA
e-mail: bmetz@tamu.edu

S. E. Tichy
Agilent Technologies Incorporated,
Santa Clara, CA, USA

K. A. Aronstein
USDA-ARS-KSARC, Honey Bee Research Unit,
Weslaco, TX, USA

R. M. Crewe
Department of Zoology and Entomology, University of Pretoria,
Pretoria, South Africa

Key Words Brood pheromone variation · Honey bee · Pollen foraging · Sucrose response threshold · Africanized honey bees · African honey bees

Abbreviations
BP brood pheromone

Introduction

Honey bees, *Apis mellifera* L., are distributed naturally throughout Africa, Asia, Europe, and the Middle East (Ruttner 1988). European honey bees in the US are a mixture of approximately seven subspecies introduced by English and Spanish settlers in the 1600s and by bee breeders in the late nineteenth and early twentieth centuries (Sheppard 1989). The gene pool reflects these introductions in both the commercial and feral populations (Schiff et al. 1994; Schiff and Sheppard 1995). In 1956, a South African sub-species, *A. m. scutellata*, was introduced to Brazil, and queens from this sub-species mated with European males producing the Africanized honey bee, a genetic admixture of *A. m. scutellata* and European honey bees (Pinto et al. 2005). The first Africanized colony was discovered in the USA in 1990 (Pinto et al. 2005). As a consequence, there are two behaviorally different types of honey bees found in the Americas, referred to as Africanized and European honey bees.

The comparative study of differences in pollen foraging behavior of these two types (Winston 1987) can help us understand the proximal mechanisms that regulate foraging activity. When fostered together in the same European colony, Africanized bees are more likely to forage for pollen and water than European bees (Danka et al. 1987; Pesante et al. 1987; Fewell and Bertram 2002; Pankiw 2003).

Along with other factors (reviewed in Page and Erber 2002), a mixture of larval chemicals, called brood pheromone (BP), influences various pheromone releaser responses in honey bee colonies, including the proportion of pollen foragers, pollen load weight, and pollen foraging trip frequency (Pankiw and Page 2001; Pankiw 2004a, 2007; Sagili 2007). Brood pheromone also elicits primer responses (Pankiw 2004b) such as, for example, affecting the age of first foraging; bees in colonies treated with a relatively low amount of BP forage at significantly younger ages than bees in colonies treated with a relatively high amount of BP (Le Conte et al. 2001; Sagili 2007).

Africanized bees forage at younger ages than European bees in common colony-rearing studies (Pankiw 2003). First foraging at a younger age and a greater probability to forage for pollen is associated with a greater sensitivity to sucrose (Page and Erber 2002; Pankiw et al. 2002; Pankiw 2003). Africanized bees have significantly lower response thresholds compared to European bees, when newly emerged or prior to any feeding experience (Pankiw 2003). Sucrose-response thresholds are modulated by BP, such that a relatively low amount of BP increases sucrose sensitivity and a relatively high amount decreases sucrose sensitivity (Pankiw and Page 2001; Pankiw 2004b; Pankiw et al. 2004). Treatment with a low dose of BP for 30 days increases the amount of brood area reared by colonies vs. controls, probably as a result of the combined suite of primer and releaser effects outlined above (Pankiw et al. 2004; 2008b; Sagili 2007).

Differences in a suite of pollen foraging behaviors between European and Africanized honey bees results in the Africanized bees collecting more pollen, rearing more brood, and their colonies reproducing more frequently in a tropical and subtropical environment (Winston 1992). This has led to the domination of the Africanized mitotype in Texas and much of the southern USA (Pinto et al. 2005; USDA-ARS 2008). Given that BP infleunces this suite of pollen foraging behaviors, a possible mechanism regulating differential pollen foraging between Africanized and European is variation in BP produced by the respective larvae, or differential response to BP by the respective adults.

Brood pheromone is composed of a blend of the methyl and ethyl esters of palmitic (16:0), stearic (18:0), oleic (18:1 Δ^{9}), linoleic (18:2 $\Delta^{9,12}$), and linolenic (18:3 $\Delta^{9,12,15}$) acids; the pheromone blend and extractable amounts vary with larval age and caste (reviewed in Pankiw 2004b and references therein). To date, all published studies on the effects of synthetic BP have been performed using the blend characterized from larvae of France (Le Conte et al. 1994; Pankiw 2004b), with most studies using colonies of European or mixed European lineage in the USA (Le Conte et al. 2001; Pankiw and Page 2001; Pankiw 2004a, b, 2007; Pankiw et al. 2004, 2008b). To date, there have been no published studies on variation in blend or amount of BP among different populations of same-stage larvae. Furthermore, the consequent behavioral or physiological effects of different blends of BP have been explored only superficially by using whole hexane extracts of larvae (Pankiw and Rubink 2002) or by not separating the effects of blend and dose (Pankiw and Page 2001).

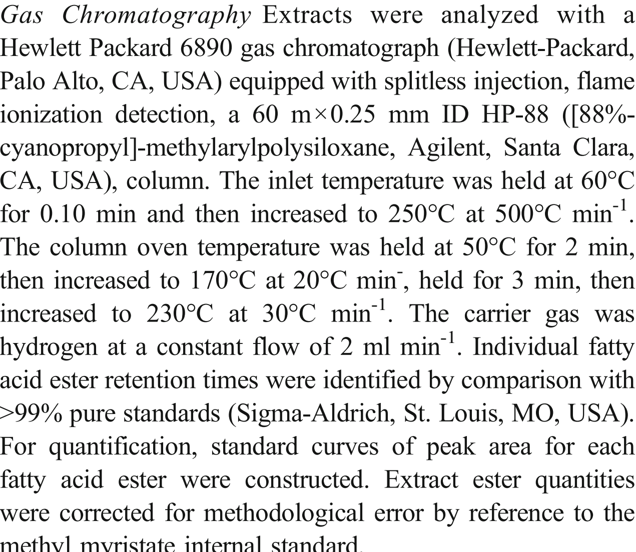
The goal of this study was to examine BP blend variation and behavioral responses to any variation in three populations of bees characterized by geographical location and mitochondrial lineage: honey bees having European mitochondrial DNA from Texas and Georgia, USA, and bees having African mitochondrial DNA from Texas, USA and Pretoria, South Africa. We characterized BP blends by chromatography-mass spectrometry (GC-MS), and we examined differences in blend by using a discriminant analysis model. Because releaser and primer responses are modulated by different physiological systems (Pankiw 2004b), the effects of BP blends were cross-tested among the different populations of bees in a pollen foraging bioassay (releaser response) and a sucrose response threshold bioassay (a primer response).

Methods and Materials

Bee/Larval Sources Four bee populations were used. European queens and package bees were purchased from a breeder in Moultrie, Georgia, USA where no record of the African mitotype has been reported (USDA-ARS 2008). Africanized colonies were collected from Mission, Texas, USA. Texas-European honey bees were from Navasota, Texas, USA and had European mitochondrial DNA. The prevalence of Africanized bees in Texas suggested that introgression of African nuclear DNA in colonies with European mitochondrial DNA was likely (Pinto et al. 2005), meaning that this population may be intermediate. European and African mitochondrial lineage was confirmed by the method of Pinto et al. (2003). Texas-Africanized, Texas-European, and Georgia-European colonies were all maintained in a College Station, Texas apiary (30° 6′ N; 96° 32′ W). African (*A. m scutellata*) colonies were maintained in an apiary at the University of Pretoria, Pretoria, South Africa (25° 45’ S; 28° 14’ E). All the queens in this study were unrelated to each other and naturally mated.

Collection of Larvae for Chemical Analysis Larvae were collected from 8 Texas-Africanized, 8 Georgia-European colonies, 11 Texas-European colonies, and 7 South African colonies. Single frames containing larvae were removed from the colonies and brought within 30 min. to the laboratory, where larvae were removed from the cells with a gentle stream of water and collected into 1 mm mesh cloth. Larvae were lightly shaken dry and 10 fifth instars selected by weight and morphology (Rembold et al. 1980; Michelette and Soares 1993). Larvae were pooled in a glass beaker and soaked for 1 min. in 2 ml of 95% *n*-hexane containing 1 μg methyl myristate as an internal standard and 0.05% (w/w) *t*-butyl hydroquinone as an antioxidant. Addition of this antioxidant has been found to prevent significant chemical change of a mixture of fatty acid esters stored at room temperature for 72 wk (Pankiw et al. 2008a). Extracts were filtered through a Buchner funnel to remove particulate matter, and the larvae and glassware were washed further with 2 ml of 95% *n*-hexane, which was added to the extract. Extracts were stored in 7-ml glass, screw-top vials, sealed with aluminum foil and paraffin, and stored for up to 2 wk at -20°C before further processing. South African extracts were shipped by air from Pretoria to College Station, but in other respects were treated identically.

Extracts were concentrated to 1 ml by using a nitrogen stream in a water bath at 55°C and vortexed for 30 sec to wash down any chemicals on the glass. Extracts were fractionated by liquid chromatography using a column of 0.5 g of silica gel (70–230 mesh/60 Å; Sigma-Aldrich, St. Louis, MO, USA) in a Pasteur pipette plugged with a piece of dust-free paper cloth (Kimberly-Clark, Neenah, WI, USA). Before use, columns were rinsed with 10 ml each of 99% dichloromethane and 95% *n*-hexane. Extracts were placed on freshly prepared columns, and two fractions were eluted: the first, containing long and short-chain hydrocarbons, but no esters, with 10 ml of 95% *n*-hexane, and the second, containing the esters, with 10 ml of 99% dichloromethane. The dichloromethane fraction was evaporated to apparent dryness using a nitrogen stream in a water bath at 50°C, and then immediately reconstituted in 1 ml of 95% *n*-hexane and vortexed for 30 sec. This was concentrated to ca 100 μl and transferred to an autosampler vial containing a 250-μl glass insert; the vial previously containing the fraction was rinsed 3 times with 50 μl hexane and the rinse added to the insert. Solvent was removed from the fraction by heating to 50°C, after which the vial was cooled and 20 μl hexane containing 2 μg of n-octadecane (Sigma-Aldrich, St. Louis, MO, USA) added to the fraction as a reference. Fractionated extracts were stored at -20°C for up to 7 d until analysis by gas chromatography, using flame ionization or mass spectrometric detection.



Gas Chromatography Extracts were analyzed with a Hewlett Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with splitless injection, flame ionization detection, a 60 m×0.25 mm ID HP-88 ([88%-cyanopropyl]-methylarylpolysiloxane, Agilent, Santa Clara, CA, USA), column. The inlet temperature was held at 60°C for 0.10 min and then increased to 250°C at 500°C min^{-1}. The column oven temperature was held at 50°C for 2 min, then increased to 170°C at 20°C min^{-}, held for 3 min, then increased to 230°C at 30°C min^{-1}. The carrier gas was hydrogen at a constant flow of 2 ml min^{-1}. Individual fatty acid ester retention times were identified by comparison with >99% pure standards (Sigma-Aldrich, St. Louis, MO, USA). For quantification, standard curves of peak area for each fatty acid ester were constructed. Extract ester quantities were corrected for methodological error by reference to the methyl myristate internal standard.

GC-MS GC-MS analyses were performed on a 6890 GC coupled with a 5975C Inert XL quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). The GC was equipped with a 30 m×0.25 mm HP- 5MS [(5%-phenyl)-methylpolysiloxane], fused silica capillary column (Agilent, Santa Clara, CA, USA), and a splitless injector using helium as carrier gas at a constant flow of 1.0 ml min^{-1} and an inlet temperature of 300°C. The column temperature was programmed from 150° to 320°C at 20°C min^{-1}. The MS was operated in electron ionization mode (EI) at 70-eV electron energy, using selected ion monitoring (SIM) mode for the following *m/z* (indicative of): 74, 242 (methyl myristate), 74, 270 (methyl palmitate), 88, 284 (ethyl palmitate, 81, 294 (methyl linoleate), 84, 264 (methyl oleate), 79, 292 (methyl linolenate), 74, 298 (methyl stearate), 81, 308 (ethyl linoleate), 98, 310 (ethyl oleate), 79, 306 (ethyl linolenate), 88, 312 (ethyl stearate), and 85, 254 (octadecane).

Comparison of Total Amount of Brood Pheromone Extractable from Larvae of Four Different Populations Quantitated amounts of fatty acid esters were summed to provide a total amount of BP for each sample. Total amounts were transformed to a proportion of mean larval weight (ng BP/g larvae) to account for the expected significant differences between the races ($F_{3,114}$=22.01; P<0.001; data not shown). Total amount of BP/gram of larvae was not normally distributed (Kolmogorov-Smirnov; Z=3.9; df=115; P< 0.001;SPSS 2007) and was therefore transformed by natural log. Total amount was tested by ANOVA for homogeneity of variance (Sokal and Rohlf 1995; SPSS 2007).

Comparison of Brood Pheromone Blends among Four Larval Populations Amount of individual esters was transformed to proportion of total BP, natural log-transformed

and classified by predictive discriminant analysis (Huberty 1994; SPSS 2007). The natural log-transformed ester proportions were normally distributed, meaning that the assumption of multivariate normality was likely met (Huberty 1994). Group covariance matrices were not homogenous as tested by Box's M (M=486.1; $F_{110,15764}$= 3.7, P<0.001; Huberty 1994; SPSS 2007). A linear model was applied despite the potential differences in covariance matrices among larval populations for two reasons: first, Box's M is known to be overly sensitive, and second, the ratio of number of groups (n_g=3) to total samples (p=88) was low (n_g/p=0.03) (as per Huberty 1994). Under these conditions and using external classification through the leave-one-out method, linear discriminant functions perform as well or better than their quadratic counterparts (Huberty 1994). A total of 34 blends from Texas-Africanized colonies, 31 blends from Georgia-European colonies, and 23 blends from South African colonies were analyzed; colonies were sub-sampled 3–6 times. Because colonies are comprised of several patrilines (Tarpy et al. 2004), it was reasonable to include colony-level subsamples to better represent the genetic diversity of the colony. The leave-one-out classification scheme was utilized for external validation, meaning that each data point was categorized based on classification functions generated from all other points (Huberty 1994). Having no *a priori* hypothesis for unequal probabilities of group membership, equal probabilities were used (Huberty 1994). One condition of the discriminant analysis is that members of a group be assigned conclusively to a single category (Huberty 1994). We did not consider this condition true in the case of the Texas-European population, because a possibility existed for Africanized nuclear DNA introgression (Pinto et al. 2005). Therefore, BP blends from the Texas-European larvae were not categorized by the analysis, and rather were classified as ungrouped samples.

Synthetic Pheromone Preparation The synthetic blends of BP derived from Texas-European, Texas-Africanized, and Georgia-European populations were formulated from mean ester percentages obtained using GC and GC/MS, while unpublished percentages of the French blend were personally communicated by Y. Le Conte (Table 1). Synthetic esters were measured by mass and added to an amber glass vial (Grace-Davison, Columbia, MD, USA) with 0.05% of t-butyl hydroquinone (Sigma-Aldrich, St. Louis, MO, USA) added as an antioxidant. The synthetic BP blends were diluted in 95% n-hexane to a concentration of 0.5 μg/μl for application in the bioassays described below.

Pollen Foraging Response of Texas-European Colonies to France and Texas-European Derived Brood Pheromone Blends The pollen foraging bioassay was performed in 12 similarly-sized, Texas-European colonies, installed 1 wk earlier. Each colony was comprised of approximately 1 kg of bees, 1 queen, and few very young larvae (< 2 cm^2). Colonies received 0.6 mg of France or Texas-European derived BP characterized as above or an equal volume of solvent as blank control. This dose was selected through a series of dose-response trials performed prior to experimentation (as per Pankiw and Page 2001). Pheromone was applied on glass plates measuring 14×7 cm. Solvent was allowed to evaporate prior to placement in colonies. A single plate then was hung on metal wire between two frames in the middle of the colony. One hour after placement of glass plates the number of pollen and non-pollen foragers entering colonies was counted for a 5 min period (as per Pankiw et al. 1998). Pollen foragers are distinguishable visually from non-pollen foragers by the pollen pellets on their hind legs. Counts were performed between 08:00–10:00 h. All colonies received all treatments on subsequent days in a random, non-repeating manner. Counts were analyzed by 3×2 *chi-square* contingency table analysis for the effect of pheromone treatment on pollen to non-pollen forager ratio (Sokal and Rohlf 1995; SPSS 2007). Pairwise comparisons were performed using 2x2 *chi-square* contingency table analysis with the Dunn-Šidák method of correcting for possible Type I errors (Sokal and Rohlf 1995).

Pollen Foraging Responses to Texas-Africanized and Georgia-European Derived Brood Pheromone Blends by Texas-Africanized and Georgia-European Colonies The pollen foraging bioassay was performed as described above with 6 Texas-Africanized and 6 Georgia-European colonies each treated with 1.0 mg of the synthetic blend of Texas-Africanized or Georgia-European BP, or an equal volume of solvent control. Texas-Africanized and Georgia-European colony responses to pheromone treatments were statistically analyzed separately.

Modulation of Sucrose Response Thresholds by Texas-Africanized and Georgia-European Derived Brood Pheromone Bblends Frames of pupae collected from 6 Texas-Africanized and 8 Georgia-European colonies were taken to the lab and adults were allowed to emerge for 24 h in an incubator at 30°C, 55% RH. Three plexiglass/wire-mesh cages (1,400 cm^3) each received 300 of either Texas-Africanized or Georgia-European newly emerged bees. Cages were provisioned with 30 ml of 30% sucrose solution and 30 ml of water. Pheromone was applied on 7×7 cm glass plates hung by metal wire in the center of each cage. Each cage received one of three treatments: 1.8 mg of either Texas-Africanized or Georgia-European derived BP (Table 1), or an equivalent volume of solvent control. Solvent was allowed to evaporate prior to inserting

Table 1 Blend formulations of synthetic brood pheromone (%)

Fatty acid ester	European mtDNA			African mtDNA	
	France	Texas, USA	Georgia, USA	Texas, USA	South Africa
Methyl palmitate	3.0	6.9	2.9	4.1	6.0
Ethyl palmitate	3.0	3.9	3.9	4.7	8.8
Methyl stearate	17.0	15.6	6.5	14.9	25.6
Ethyl stearate	7.0	6.4	8.1	10.3	14.5
Methyl oleate	25.0	16.8	20.6	9.0	11.5
Ethyl oleate	8.0	15.4	13.8	15.9	12.9
Methyl linoleate	2.0	2.9	4.2	9.3	4.5
Ethyl linoleate	1.0	5.4	12.1	6.4	4.9
Methyl linolenate	21.0	13.8	16.9	11.7	2.8
Ethyl linolenate	13.0	12.9	11.0	13.7	8.5

glass plates into cages. Cages were stored in a dark incubator at 30°C, 55% RH. Treatment plates, sucrose, and water were changed daily. Bees were reared thus for 5 d so that they were tested within the first week of adult life (as per Pankiw and Page 2001). Mortality over the course of treatment was less than 15 bees per cage. On the sixth day, a subset of 60 bees was collected from each cage and their sucrose response thresholds were measured using the proboscis extension reflex assay.

Proboscis Extension Reflex Bioassay Honey bees reflexively respond by extending the proboscis when a sufficiently concentrated solution of sucrose is applied to the antennae (Pankiw and Page 2001). The response threshold of an individual may be estimated by the lowest concentration that elicits proboscis extension when bees are presented with an ascending concentration series (Pankiw and Page 2001; Pankiw and Rubink 2002; Pankiw et al. 2002; Pankiw 2003). The proboscis extension reflex bioassay followed previously reported methodologies (Pankiw and Page 2001; Pankiw et al. 2004). Briefly, bees were restrained by thin strips of tape such that their heads and mouthparts moved freely. Bees were allowed 1 h to acclimate to restraint before testing. All bees were tested for proboscis extension to antennal stimulation with water; those responding were allowed to drink water to satiation. Bees then were presented with a logarithmically ascending series of sucrose concentrations sucrose (0.1, 0.3, 1.0, 3.0, 10, and 30%). A single droplet of sucrose concentration was touched to each antenna for <3 sec; ascending concentrations were applied at least 5 min apart. A positive response was recorded if the bee extended her proboscis. Positive responses to sucrose were summed to yield scores that were analyzed by Kruskal-Wallis (Sokal and Rohlf 1995; SPSS 2007). A high score corresponded to high sensitivity or a low response threshold to sucrose and a low score to low sensitivity or a high response threshold to sucrose. Mann-Whitney *U* was performed to assess pairwise differences in sucrose response threshold by treatment using the Dunn-Šidák method as above (Sokal and Rohlf 1995; SPSS 2007).

Results

Comparison of Total Amount of Brood Pheromone Extractable from Larvae of Four Different Populations Total amount of BP extractable from the larval cuticle did not differ among the four honey bee populations ($F_{3,114}$=0.9; P=0.45; SPSS 2007). Mean total extractable amounts were 662±166 ng pheromone/g larvae.

Comparison of Brood Pheromone Blends among Four Larval Populations Two discriminant functions were generated. Means of the functions were different among populations (χ^2=117.5, 20 *df*, P<0.001; SPSS 2007). Squared canonical correlation coefficients showed that function 1 explained 57.2% of the variation among the groups, while function 2 explained 37.2%. Overall, 77% of the samples were correctly classified. That is, 66.7% of the Georgia-European blends categorized correctly, 30% of this group were categorized as Texas-Africanized, and 3.3% as South African. Among the Texas-Africanized blends, 76.5% were correctly categorized, 11.8% were categorized as Georgia-European, and 11.8% as South African. The categorization of South African blends was 91.3% correct, while 4.3% were categorized as Georgia-European and 4.3% as Texas-Africanized. Of the 18 Texas-European blends, 39% were classified as Georgia-European, 33% as Texas-Africanized, and 28% as South African (Fig. 1). Function 1 was the primary discriminator between Texas-Africanized and Georgia-European blends and the South African blends, while function 2 was the primary discriminator

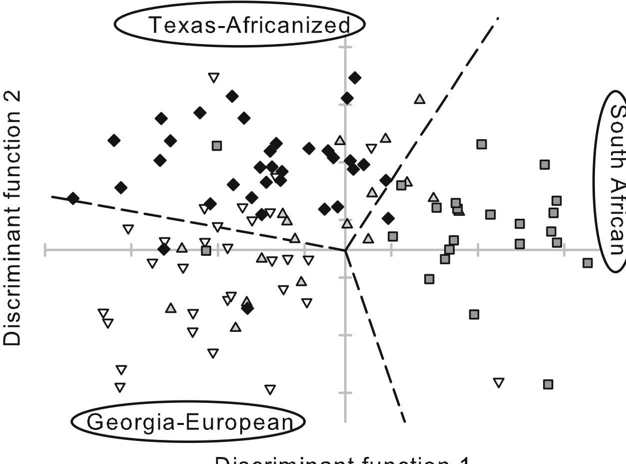


Fig. 1 Scatterplot of the discriminant scores for brood pheromone ester proportions extracted from larvae of colonies of Texas-Africanized and Georgia-European honey bees and South African. Each point represents a single sub-sample. Circled labels indicate the classification of all sub-samples within the territories demarcated by the dashed lines. Georgia-European samples are represented by open, upside-down triangles, Texas-European by grey triangles, Texas-Africanized by black diamonds, and South African samples are represented by dark grey squares

between the Georgia-European and Texas-Africanized blends (Fig. 1).

Plotting the structure matrix coefficients of the transformed ester proportions to the discriminant functions revealed the relative importance of each ester to the classification of the blends (Fig. 2). Vectors were plotted at an angle, the tangent of which was the ratio of function 2 to function 1. The method of calculating the magnitude of the vectors was adapted from Overall and Klett (1972) as the ratio of the standard error of the group means for each ester proportion to the mean standard error of the ester proportions within each group. The ethyl and methyl esters of palmitic (16:0) and stearic (18:0) acids were highly positively correlated to function 1, while methyl linolenate (Me-18:3$\Delta^{9,12,15}$ was highly negatively correlated (Fig. 2). Palmitic and stearic acids are both saturated, while linolenic acid is triple-unsaturated. This means that the South African blends were more likely to exhibit a high proportion of unsaturated fatty acid esters and a relatively low proportion of poly-unsaturated fatty acid esters, particularly methyl linolenate. Both methyl and ethyl linolenate were highly positively correlated with function 2, while methyl oleate (Me-18:1Δ^{9} and ethyl linoleate (Et-18:2 $\Delta^{9,12}$ were highly negatively correlated. Georgia-European blends were more likely to exhibit a higher proportion of methyl oleate and ethyl linoleate, and a lower proportion of the esters of linolenic acid.

Pollen Foraging Response of Texas-European Colonies to France and Texas-European Derived Brood Pheromone Blends Proportion of pollen to non-pollen foragers differed by pheromone treatment (χ^2=64.0, 2 *df*, *P*<0.001; Fig. 3a; SPSS 2007). Pairwise comparisons indicated that all treatments were different from each other, with the greatest ratio of pollen to non-pollen foragers in response to the Texas-European BP blend compared to the French blend (χ^2=15.1, 1 *df*, *P*<0.001) or the solvent control (χ^2=64.2, 1 *df*, *P*< 0.001). Pollen to non-pollen forager ratios in response to the French blend was greater than control (χ^2=22.1, 1 *df*, *P*< 0.001), which was consistent with previous studies (Pankiw et al. 1998; Pankiw and Page 2001; Pankiw 2004a).

Pollen Foraging Responses to Texas-Africanized and Georgia-European Derived Brood Pheromone Blends by Texas-Africanized and Georgia-European Colonies Proportions of pollen to non-pollen foragers also were different by pheromone treatment among Georgia-European colonies (χ^2=121.4, 2 *df*, *P*<0.001; Fig. 3b). Pollen to non-pollen forager ratios were greatest in response to treatment with the Georgia-European derived BP blend compared to either the Texas-Africanized blend (χ^2=3.3, 1 *df*, *P*=0.038) or solvent control (χ^2=109.1, 1 *df*, *P*<0.001). Pollen to non-pollen forager ratios in response to the Texas-Africanized blend was greater than control (χ^2=72.6, 1 *df*, *P*<0.001).

Proportions of pollen to non-pollen foragers differed by treatment among Texas-Africanized colonies (χ^2=59.8,

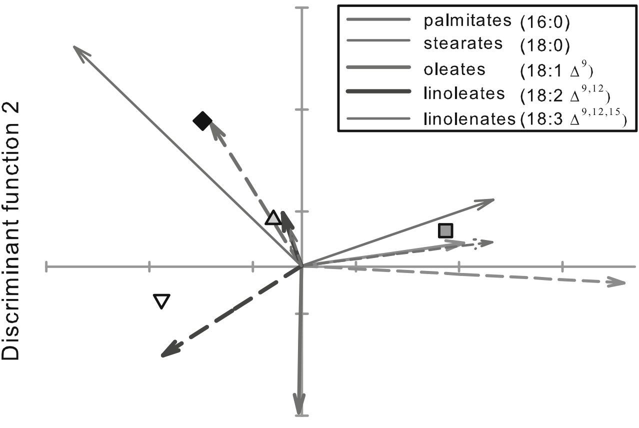


Fig. 2 Strength of the relationship of the proportions of brood pheromone esters to the discriminant functions. Solid lines represent methyl esters and dashed lines ethyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids. Points represent the discriminant score of the group centroid: Georgia-European by an open, upside-down triangle, Texas-European by a grey triangle, Texas-Africanized by a black diamond, and South African by a dark grey square. Vector angles represent the ratio of the correlation of the ester proportion to the discriminant function and magnitudes represent the ratio of between- to within-groups standard error

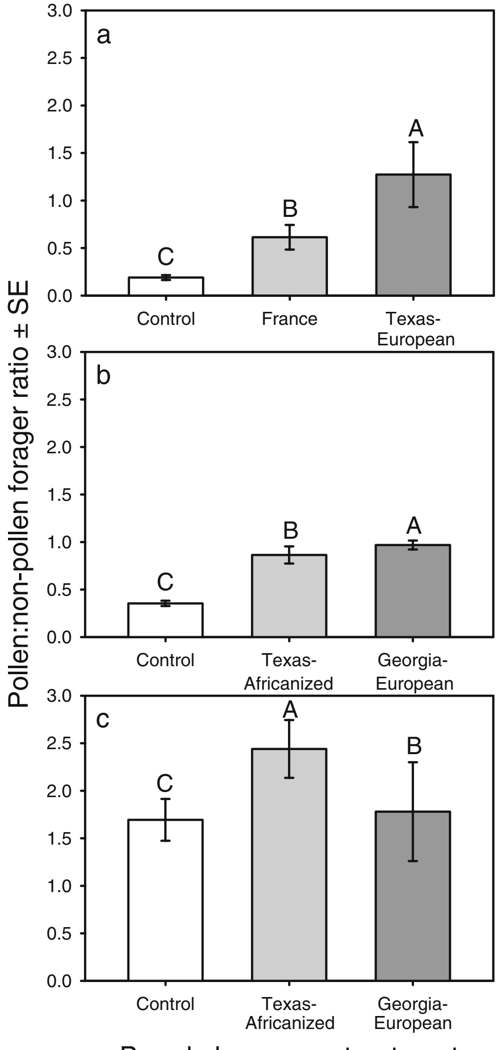


Fig. 3 Pollen to non-pollen forager ratio of **a** Texas-European colonies treated with French or Texas-European derived synthetic brood pheromone blends or solvent control, **b** Georgia-European honey bee colonies in response to Texas-Africanized and Georgia-European synthetic brood pheromone blends, and **c** Texas-Africanized honey bee colonies in response to Texas-Africanized and Georgia-European synthetic brood pheromone blends. Each letter denotes a statistically different subset (*Chi-square*; $P<0.017$)

2 *df*, $P<0.001$; Fig. 3c). Pollen to non-pollen forager ratios were highest in response to the Texas-Africanized blend when compared to the Georgia-European blend ($\chi^2=58.8$, 1 *df*, $P<0.001$) or solvent control ($\chi^2=26.0$, 1 *df*, $P<0.001$). Pollen to non-pollen forager ratios in response to the Georgia-European blend were greater than control ($\chi^2=7.1$, 1 *df*, $P=0.004$).

Modulation of Sucrose Response Thresholds by Texas-Africanized and Georgia-European Derived Brood Pheromone Georgia-European bees exhibited different proboscis extension reflex score distributions between pheromone treatments ($\chi^2=77.2$, 2 *df*, $P<0.001$: Fig. 4a). In Mann-Whitney comparisons, scores were higher when bees were treated with Texas-Africanized blend compared to control, but non-significantly ($Z=-2.3$, $P=0.023$). Scores were lower when bees were treated with Georgia-European blend when compared to control ($Z=-6.5$, $P<0.001$). Georgia-European bees exhibited higher scores when treated with Texas-Africanized derived BP blend compared to those treated with the Georgia-European blend ($Z=-8.3$, $P<0.001$). Proboscis extension reflex scores were not significantly different by pheromone treatment in Texas-Africanized bees ($\chi^2=1.4$, 2 *df*, $P=0.51$; Fig. 4b).

Discussion

Brood pheromone component proportions, blends, were significantly and sufficiently different to correctly classify the population of origin with 77% accuracy. The South African BP blends were characterized by higher proportion of esters of the saturated palmitic and stearic acids, both unsaturated fatty acids. Esters of unsaturated fatty acids oxidize more readily and are therefore more labile than saturated fatty acid esters (Kotz et al. 2006). It is reasonable to hypothesize that the South African blends may be more

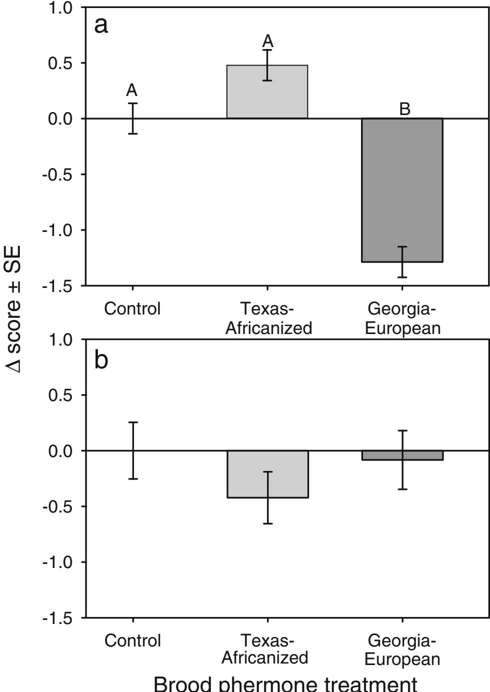


Fig. 4 Proboscis extension reflex scores of **a** Georgia-European or **b** Texas-Africanized honey bees in cages treated with Georgia-European or Texas-Africanized synthetic brood pheromone blends and compared as a mean difference from solvent control treated bees. Letters denote statistically different scores (Mann-Whitney *U*; $P<0.017$)

stable over time relative to the European blends and represent a more persistent pheromone cue. Proportions of methyl oleate were highest in the European blends compared to those extracted from populations with African maternal lineage and suspected Africanized DNA introgression. Methyl oleate has been demonstrated as an attractant for young adult bees in queen retinue bioassays (Keeling et al. 2003). Texas-Africanized blends were characterized by relatively higher proportions of ethyl oleate. Ethyl oleate increases the protein extractable from the hypopharyngeal glands after 14 days when fed to caged bees in the presence of an unmated queen (Mohammedi et al. 1996). Hypopharyngeal glands produce proteinaceous secretions of brood food fed to larvae (Winston 1987). Combined, these differences may be indicative of differential nursing behaviors among the different populations. Interestingly, the Texas BP blends from larvae with European *mt*DNA were evenly categorized among Texas-Africanized, Georgia-European, and South African categories, meaning that these blends may be viewed as intermediate. This result is consistent with our hypothesis of nuclear DNA introgression of African genes in this Texas-European population. Geographic isolation and subsequent selection of naturally and human distributed honey bee races are potential contributory factors that affect pheromone variation. Differential apicultural selection pressures on colonies for pollination or honey production in the USA, France, and South Africa, also may have changed BP blend. Further studies of BP blend variation among single patrilines of larvae will be necessary to determine the genetic and environmental components of this variation.

Contrary to our hypothesis, the Africanized blend did not release a greater proportion of pollen foragers in all colony types; instead, colonies responded with the greatest proportion of pollen foragers when treated with their own synthetic blend of pheromone. Thus, pollen foraging behavior cannot be explained through differences in extractable amounts or blend of BP. In contrast, Pankiw and Rubink (2002) found no race-by-pheromone interaction when testing pollen foraging response to whole hexane extracts of larvae. In our discriminant analysis, Texas-European blends of BP were intermediate between Georgia-European and Texas-Africanized blends. Because Pankiw and Rubink (2002) used Texas-European colonies, it is possible that the differences between Texas-Africanized and Texas-European BP blends were not sufficient to elicit a differential pollen foraging response. As shown previously, (Pankiw and Rubink 2002), Texas-Africanized colonies fielded a higher proportion of pollen foragers relative to Georgia-European colonies. Population differences in pheromone blend and response to blend may be the result of genetic isolation, however, BP blends varied within each population and even within colonies (personal observations). Considering that colonies consist of multiple patrilineal genotypes (Tarpy et al. 2004), forager genotype may interact with pheromone blend to act on response thresholds to BP that regulate division of foraging labor and contribute to intracolony variation in foraging ontogeny and specialization (*reviewed in* Winston 1987). Careful measures of intracolony variation in BP blend and responses of individuals to different blends will be necessary to explore this supposition further.

Modulation of sucrose response thresholds, a primer response, also showed a pheromone blend by population-dependent response interaction. The BP blend derived from Georgia-European colonies significantly decreased sucrose responsiveness in bees of the same population within the first week of adult life vs. the control response. However, Texas-Africanized bees appeared to be insensitive to the modulating effects of BP on sucrose sensitivity regardless of the BP blend environment in which they were reared. This study is the first to measure sucrose response threshold modulation by blends of BP other than the French blend and among individuals with African mitochondrial DNA. Only the Georgia-European blend modulated sucrose responsiveness among bees of USA origin that have a European mitotype in a manner predicted from previous studies (Pankiw and Page 2001; Pankiw et al. 2004). The highly responsive baseline of Africanized bees, consistent with that observed in previous studies (Pankiw and Rubink 2002), may be indicative of a more mature sucrose sensitivity status associated with a faster rate of foraging ontogeny (Pankiw 2003). If BP modulates sucrose responsiveness in pre-foraging Africanized bees, then we predict that modulation should be measurable in bees that are ontogenetically younger than those tested here, given that the French synthetic blend does not modulate USA European ontogenetically mature foragers (Metz and Pankiw unpublished). Brood pheromone modulated sucrose responsiveness is likely a complex interaction of baseline responsiveness, amount, and blend of BP.

This study has shown that BP blend changed among same-stage larvae from different populations. A pheromone comprised of at least 10 compounds presents an enormous challenge to our understanding of how honey bees process mixtures of pheromone compounds. The pattern observed for pollen foraging responsiveness to variation in blends of BP suggests BP may serve as a recognition cue. That is, in each case, there was a positive and significant response to a synthetic blend of BP that was characterized from a source of larvae from the same population as the colony. Pheromone modulated sucrose response threshold did not follow the same pattern, however, primer and releaser responses are not necessarily coordinated but rather uncoupled generating a complex interaction to variation in BP at various levels within a colony.

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Nestmate Recognition and the Role of Cuticular Hydrocarbons in the African Termite Raiding Ant *Pachycondyla analis*

Abdullahi A. Yusuf · Christian W. W. Pirk · Robin M. Crewe · Peter G. N. Njagi · Ian Gordon · Baldwyn Torto

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Abstract Cuticular hydrocarbons (CHCs) are used for chemical communication among nestmates in many ant species, and they may play a role in the discrimination of nestmates and non-nestmates. Using the mandible opening response (MOR) bioassay, we tested the response of the African termite raiding ant, *Pachycondyla analis*, to CHC extracts of nestmates and non-nestmates. The ants were able to distinguish control chemical cues, from nestmate CHCs, and from non-nestmate CHCs, and, based on a CHC recognition threshold, aggression was demonstrated toward non-nestmates. Gas chromatography (GC) and GC-mass spectrometric analyses showed that CHC components of different ant colonies had chain lengths ranging from C_8 to C_{31}, comprising mainly *n*-alkanes, alkenes, and methyl branched alkanes, with the *n*-alkanes occurring in the same proportions among all colonies. The ants were grouped successfully according to their colonies of origin by using discriminant analysis of CHCs. We demonstrate that nestmate recognition occurs in *P. analis*, and that some of the cues involved are evidently alkenes and methyl-branched alkanes.

Key Words Nestmate recognition · Mandible opening response bioassay · *Pachycondyla analis* · Cuticular hydrocarbons · Ponerine ant · Formicidae · Ponerinae

A. A. Yusuf · P. G. N. Njagi · I. Gordon · B. Torto
ICIPE,
P. O. Box 30772-00100, GPO, Nairobi, Kenya

A. A. Yusuf (✉) · C. W. W. Pirk · R. M. Crewe
Social Insects Research Group,
Department of Zoology and Entomology, University of Pretoria,
0002 Pretoria, South Africa
e-mail: ayusuf@icipe.org
e-mail: aayusuf@zoology.up.ac.za

Introduction

Communication is a common and important phenomenon in all biological organisms. Among social insects, nestmate recognition enables integration within a colony and prevents non-colony members (both conspecifics and heterospecifics) from exploiting the colony's resources (Crozier and Pamilo 1996). The presence of non-nestmates (intruders) usually elicits active defensive behaviors (Hölldobler and Wilson 1990; Vander Meer and Morel 1998). Nestmate recognition in social insects can be adaptive because workers obtain benefits from aiding nestmates and discriminating against non-nestmates provided that the nestmates are more closely related to one another than to members of other conspecific colonies (Hölldobler 1995). The cues involved can be of genetic or environmental origin, and can differ among populations and seasons (Pirk et al. 2001), even in species that form super-colonies like *Formica exsecta* (Katzerke et al. 2006). The primary cues of communication in most insects are chemical in nature (Wyatt 2003) and are perceived by olfaction or contact chemoreception (Breed 1998).

Ants are among the dominant social insects in the world, and they employ complex forms of chemical communication. Over 100 exocrine glands have been described in social insects with more than half of these found in ants (Billen 2004). An array of signals and information on an individual's species, sex, age, caste, status, and relatedness, as well as alarm and trail pheromones are encoded in the secretions from these glands (Howard and Blomquist 2005).

Fielde (1901) advocated that nestmate discrimination signals are encoded in cuticular lipids, particularly those hydrocarbons that coat all insects. Since then, the role of cuticular hydrocarbons (CHCs) has been a subject of much debate, and various studies have attempted to determine

their functions in insect chemical communication. Examples of these roles include recognition at various levels, such as the individual (e.g., D'Ettore and Heinze 2005), nestmate (e.g., Wagner et al. 2000; Akino et al. 2004, Martin et al. 2008a,b), species (e.g., Neems and Butlin 1995; Dapporto 2007), kin (Arnold et al. 1996), and as cues for reproduction and division of labor (e.g., Dietemann et al. 2003; Martin and Drijfhout 2009). Most recently CHCs have been found to be responsible for enforcing altruism in ants (Smith et al. 2009). In adult insects, CHCs are synthesized internally in the oenocytes (Blomquist and Dilwith 1985) and, hence, are under a strong genetic influence reflecting an insect's genetic makeup (Lockey 1991). After synthesis, they are transferred to the cuticle by lipophorin (Schal et al. 2001). CHCs are made up of a homologous series of long, straight-chained saturated alkanes that can be modified by addition of methyl groups or the introduction of double bonds (Jackson and Morgan 1993).

The ant *Pachycondyla analis* (Formicidae: Ponerinae) is a specialized termite predator, which is widely distributed in sub-Saharan Africa (Lévieux 1966). This species, commonly referred to as 'Matabele ants', organizes group raids on termite species that mainly belong to the sub-family Macrotermitinae (Longhurst et al. 1978). There is no information on CHCs of *P. analis*, nor the role they play in nestmate recognition. We recently initiated a comprehensive investigation of the chemical ecology of this ant species, and in the present paper we report the results from a study of CHCs of different colonies of *P. analis* and the role they play in nestmate recognition.

Methods and Materials

Study Species Three colonies of *P. analis* were excavated from Mpala Research Centre (0°17′N, 37°52′E) central Kenya, 250 km north of Nairobi. The colonies were kept in artificial nest boxes (20×20×20 cm) made of aluminium, which were connected to a foraging arena (1.5×1.0 m) made of Perspex. The nests were maintained at 25±1°C, with about 50–60% relative humidity and a 12L: 12D photoperiod. Ants were fed twice daily on live termites (mainly from the subfamily Macrotermitinae) collected around the Duduville campus of *ICIPE* in Nairobi, Kenya.

Extraction of CHCs for Bioassay CHCs from 5 ants per colony were extracted for use as sources of chemical stimuli in bioassays (Guerrieri and d'Ettorre, 2008). Ants previously in contact with their own colony odor were selected for extraction of CHCs. The ants were first killed by placing them on ice for 15 min, and CHCs were extracted by washing them in 500 µl of pentane for 10 min. Solvent was evaporated under a gentle stream of nitrogen, the residue was dissolved in 50 µl of pentane, and stored at −20°C until analysis. Twentyfour extracts were prepared from each of the 3 colonies making a total of 72 extracts. A solvent control (pentane) also was subjected to the same evaporation procedure. An average quantity corresponding to the extract of one ant (10 µl) was applied to the tip of a Pasteur pipette by using a Hamilton syringe. The pipette tip was held downwards until the solvent evaporated from the tip, thus leaving the residue of the extract around the lower and outer part of the pipette.

Mandible Opening Response (MOR) Bioassay Ants were removed from their colonies and transferred to 20 ml glass vials, and immobilized by placing them on ice. They then were harnessed using methods previously described (Guerrieri and d'Ettorre 2008), and were kept undisturbed for 2 hr to recover and habituate to the harness.

Aggression responses were quantified by presenting four different types of stimuli to the test ants from colonies 1, 2, and 3: a) solvent extract only (CTRL); b) extract from colony 1 (C1); c) extract from colony 2 (C2); d) extract from colony 3 (C3). For a test ant, extracts from individuals of its own colony served as nestmate stimuli, while extracts from individuals of other colonies served as non-nestmate stimuli. All ants were tested with all extracts and the control.

In each trial, one stimulus was presented to a harnessed individual ant. A test individual was removed from its resting place and allowed to habituate for 2 min prior to presenting it with the test stimulus. After habituation, its antennae were touched gently for 5 sec with the tip of the stimulating pipette. When the test ant opened its mandibles continuously (i.e., displacing them from the resting position), the behavior was recorded as aggression (score=1). If the individual did not open its mandibles, and instead antennated continuously, the response was recorded as non-aggressive (score=0) following the protocol of Guerrieri and d'Ettorre (2008). After presenting a stimulus, the test ant was returned to its resting place. Stimuli were presented at random to individual ants after an interval of 20 min to allow for the recovery of antennal receptors. From each of the three colonies studied, 24 ants chosen at random were tested with each of the 4 stimuli, thus a total of 72 ants were tested.

Extraction of Cuticular Hydrocarbons (CHCs) for Chemical Analyses Cuticular hydrocarbons were extracted in a way similar to those used for the MOR bioassay, but in this case each ant was extracted in 1 ml of pentane. The solvent was evaporated under a gentle stream of nitrogen, dissolved in 100 µl of pentane, and stored at −20°C until analysis. Twentyfour extracts were prepared from each of the three colonies, making a total of 72 extracts. A pentane control also was subjected to the same evaporation procedure.

Chemical Analyses Gas chromatography (GC) was carried out on an HP 5890 series II GC equipped with a flame ionization detector (FID) and an HP-5 column (30× 0.25 mm ID×0.25 µm film thickness). Nitrogen was used as carrier gas, with a column pressure of 46 psi and injection temperature of 250°C. One µl of sample was injected in the splitless mode, with the oven temperature programmed at 60°C for 5 min to 280°C at 10°C/min, and held at this temperature for 13 min. GC-mass spectrometry (MS) analysis was carried on an Agilent Technologies 7890A GC equipped with an HP-5 MS capillary column (30×0.25 mm ID×0.25 µm film thickness) coupled to 5795C MS. One microliter of each sample was injected in the splitless mode, and helium was used as carrier gas at 1.0 ml min^{-1}. The oven temperature was 35°C for 5 min, increased to 280°C at 10°C min^{-1} and then held at this temperature for 15 min. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode. All the n-alkanes, 2-methylheptadecane, 1-heptadecene, (*Z*)-9-tricosene, and squalene were identified by GC-MS co-injection, and comparison of MS data with those of authentic standards. Other methyl-branched alkanes and alkenes were tentatively identified by using EI diagnostic ions (El-Sayed 2009).

Chemicals *n*-Undecane, *n*-Dodecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, and *n*-heptadecane with the purity of >99% were obtained from Aldrich Chemical Company (Gillingham, Dorset, UK). *n*-Octadecane, *n*-nonadecane, *n*-eicosane, *n*-heneicosane, *n*-docosane, *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-hexacosane, *n*-heptacosane, *n*-nonacosane, and *n*-hentriacontane were provided by Dr. Peter Teal, USDA/ARS-CMAVE, Florida, USA. 1-Heptadecene, (*Z*)-9-tricosene, squalene, and 2-methylheptadecane were provided by Dr. Antony Hooper, Rothamsted Research, Harpenden, UK.

Statistical Analyses Logistic regression was performed on the dichotomous data (1 vs. 0) on the aggressive responses of ants. We tested the differences in aggression response of ants to the solvent control, nestmate and non-nestmate extracts. The levels of aggression between colonies were tested using *Kruskal Wallis* ANOVA. The relative areas of the peaks of the individual compounds in the CHC profile for each ant were standardized to 100%. The standardized peak areas were then transformed following the method proposed by Aitchinson (1986):

$$Z_{ij} = \ln\left[Y_{ij}/g\left(Y_j\right)\right]$$

where Z_{ij} is the standardized peak area i for individual ant j, Yij is the observed peak area i for individual ant j, and g (Yj) is the geometric mean of all peak areas for ant j included in the analyses. We performed a stepwise discriminant function analysis (stepwise DA) on the transformed variables followed by canonical discriminant analysis on the selected peaks to determine whether the colonies could be separated on the basis of their CHC profiles. Pairwise generalized square distances between colonies and classification error rates were also calculated. All statistical analyses were carried out using SAS 9.1.2 statistical software.

Results

MOR Bioassay The number of ants that opened their mandibles when presented with the solvent control was lower compared to an extract (*Wald's* χ^2=58.34, *df*=1, *P*<0.001). There was less mandible opening when ants were presented with a nestmate extract compared to an extract from a non-nestmate (*Wald's* χ^2=101.24, *df*=6, *P*<0.001). In general, the levels of aggression (i.e., MOR) increased

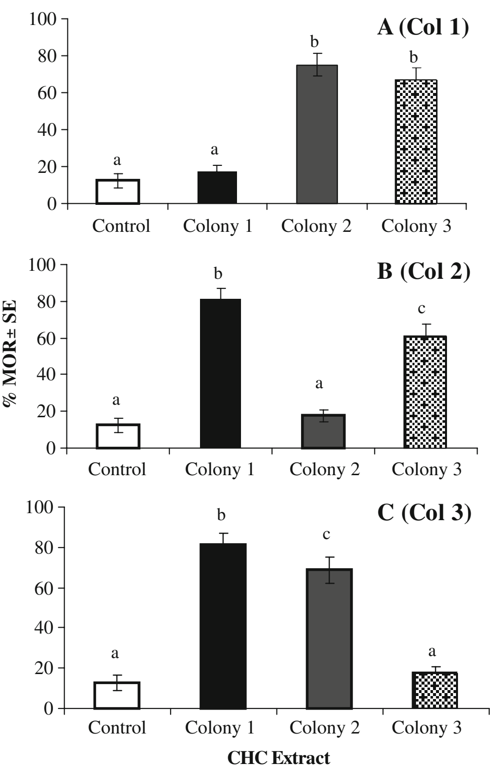


Fig. 1 Mandible opening response (MOR)±SE for, Pachycondyla ants (**A**) from colony 1, (**B**) from colony 2, and (**C**) from colony 3 to the presented extracts. □=Control solvent (pentane), ■=CHC extract from colony 1, ■=CHC extract from colony 2 and = CHC extract from colony 3. Ants responded significantly different to test stimulus and control (*Wald's* χ^2=58.34, *P*<0.001). Response of ants to the extract of nestmate and those of non-nestmate also differed significantly (*Wald's* χ^2=101.24, *df*=6, *P*<0.001). The same letters on bars represent means that are not significantly different

when an ant was presented with a non-nestmate stimulus compared to a nestmate extract or a solvent control (*Wald's* χ^2=132.19, *df*=2, *P*<0.001, Fig. 1). Ants from colony 1 were slightly more aggressive than those from colonies 2 and 3 (Fig. 1), although aggression between colonies was not significantly different (*Kruskal Wallis ANOVA*, χ^2= 5.08, *df*=2, *P*=0.082).

CHC Profiles of P. analis GC-MS analysis revealed that the CHCs of *P. analis* were a complex mixture of alkanes, alkenes, and methyl-branched alkanes ranging from C_8 to C_{31} (Fig. 2, Table 1). The *n*-alkanes, 2-methylheptadecane, 1-heptadecene, (*Z*)-9-tricosene, and squalene had retention times and mass spectra that matched those of authentic standards. The identities of these compounds in the extracts were confirmed further by GC-MS co-injection. Other components in the extracts, including methyl-branched alkanes and alkenes, were identified tentatively from their characteristic mass spectral diagnostic ions (El-Sayed 2009). The major components varied between colonies, with (*Z*)-9-tricosene being present in varying proportions in all the colonies. The proportions of alkanes in the extracts remained constant, while there was variation in the proportions of the alkenes and the methyl-branched alkanes between colonies (Fig. 3).

CHC Differentiation Among Colonies Ants from the different colonies could be distinguished by using the transformed peak areas of the 35 identified compounds (Fig. 2) that differed among the colonies. Using the stepwise DA, 17 variables clustered the ants according to their colonies of origin (*Wilk's* λ=0.0007, *df*=34, 10, *P*< 0.001). Discriminating compounds selected by the stepwise DA were: undecane, 3-methylundecane, 3,6-dimethylundecane, 3,8-dimethyl decane, pentadecane, heptadecane, 3-methylheptadecane, 2,6,10,14-tetramethylpentadecane, octadecane, nonadecane, heneicosane, tricosane, 1-nonadecene, 9-nonadecene, 9-methylnonadecane, squalene, and hentriacontane.

Using the 17 compounds selected by the stepwise DA, ants were grouped into their colony of origin (*Wilk's* λ= 0.0000, *df*=34, 10, *P*<0.001), with function 1 explaining 88.07 % of the variation separating colony 3 from both colonies 1 and 2, and function 2 explaining 11.93 % of the variation further separating colony 3 from 2 and 1 (Fig. 4). All the ants were grouped into their colonies correctly based on their CHC profiles.

Discussion

In this study, we demonstrated the use of a 'yes or no' aggression bioassay by using mandible opening as a measure of aggression between different colonies of *P. analis*. The results show that *P. analis* discriminate between nestmates and non-nestmates, since they were significantly more aggressive toward extracts of non-nestmates. These results

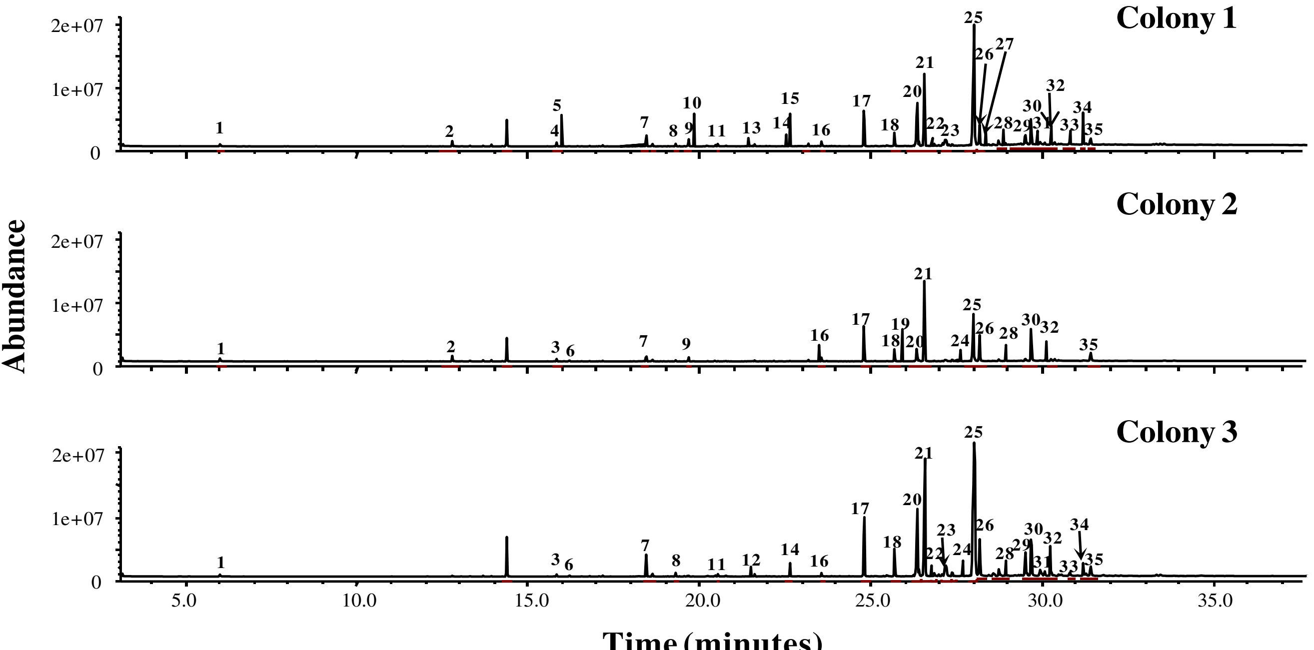


Fig. 2 Total ion chromatograms for the cuticular hydrocarbons of Pachycondyla ants from the colonies studied. Colony 1=CHC extracts from colony 1 ants, Colony 2=CHC extracts from colony 2 ants, Colony 3=CHC extracts from colony 3 ants (see Table 1 for the list of identified compounds)

Table 1 Compounds identified from the cuticular hydrocarbon profiles of *Pachycondyla analis,* along with retention indices and diagnostic ions

No[a]	Compound	RI[b]	Diagnostic EI-MS ions(m/z)
1	*n*-Octane	800	114
2	*n*-Undecane	1100	156
3	5-Methylundecane	1154	43, 57, 71, 85, 99, 112
4	3-Methylundecane	1169	43, 57, 71, 85, 99, 112, 141, 170
5	3,8-Dimethyldecane	1063	57, 71, 85, 99, 113, 141, 155, 170
6	*n*-Tridecane	1300	184
7	*n*-Pentadecane	1500	212
8	3-Methylpentadecane	1572	43, 57, 71, 85, 99, 113, 127, 141, 155, 168, 197, 226
9	2-Methylheptadecane	1765	43, 57, 71,85, 99, 113, 127, 141, 155, 169, 183, 195, 211, 239, 254
10	1-Heptadecene	1679	83,97,111,125,196, 210,239
11	8-Heptadecene	1679	41, 55, 69, 83, 97, 111, 125, 140, 238
12	5-Octadecene	1789	43, 55, 69, 83, 97, 111, 125, 139, 166, 180, 195, 224, 252
13	*n*-Octadecane	1800	254
14	9-Nonadecene	1875	43, 55, 69, 83, 97, 111, 125, 139, 153, 167, 238, 266
15	*n*-Nonadecane	1900	268
16	*n*-Eicosane	2000	282
17	*n*-Heneicosane	2100	296
18	1-Docosene	2195	43, 57, 69, 83, 97, 111, 125, 280,308
19	*n*-Docosane	2200	310
20	*(Z)*-9-Tricosene	2270	43, 55, 69, 83, 97, 111, 125, 139, 153, 223, 237, 294, 322
21	*n*-Tricosane	2300	324
22	Unidentified		
23	1-Tetracosene	2396	43, 57, 69, 85, 97, 113, 309, 338
24	*n*-Tetracosane	2400	338
25	Cyclotetracosane	2445	43, 57, 69, 83, 97, 111, 125, 139, 153, 207, 392
26	9-Pentacosene	2465	43, 57, 69, 85, 97, 113, 141, 169, 197, 326, 350
27	*n*-Pentacosane	2500	352
28	*(Z)*-12-Pentacosene	2496	43, 57, 69, 83, 97, 125, 236, 257, 290, 322, 350
29	1-Hexacosene	2593	43, 57, 69, 83, 97, 111, 125, 139, 336, 364
30	*n*-Hexacosane	2600	366
31	*n*-Heptacosane	2700	380
32	*n*-Octacosane	2800	394
33	Squalene	2663	41, 55, 69, 81, 95, 109, 121, 136, 148, 341, 367, 410
34	*n*-Nonacosane	2900	408
35	*n*-Hentriacontane	3100	436

[a] No=Peak numbers referring to Fig. 2

[b] *RI* Retention Index

are in agreement with those previously reported for the invasive Argentine ant (*Linepithema humile*) workers by Vásquez et al. (2008), where they showed evidence that CHCs are used in queen adoption, confirming that the MOR is a sensitive assay that can be used effectively to determine recognition or aggression thresholds in ants. Recognition thresholds usually are based on a template odor that is characteristic of a given colony, with ants deciding to accept or reject an individual when it smells greater than a minimum similarity threshold or below a dissimilarity threshold (Reeve 1989).

Aggression towards nestmates of similar CHC profiles could be due to errors arising while reacting to recognition cues, as shown by Vásquez et al. (2008) in *L. humile,* or due to a lower threshold to avoid false-positive identification. In the present study, the MOR bioassay was used successfully to measure inter-colony aggression at the individual and colony levels in *P. analis*.

We identified 35 different compounds in the CHCs of different *P. analis* colonies. These compounds were mainly alkanes, alkenes, and methyl-branched alkanes, as previously reported for other ant species (Dietemann et al. 2003;

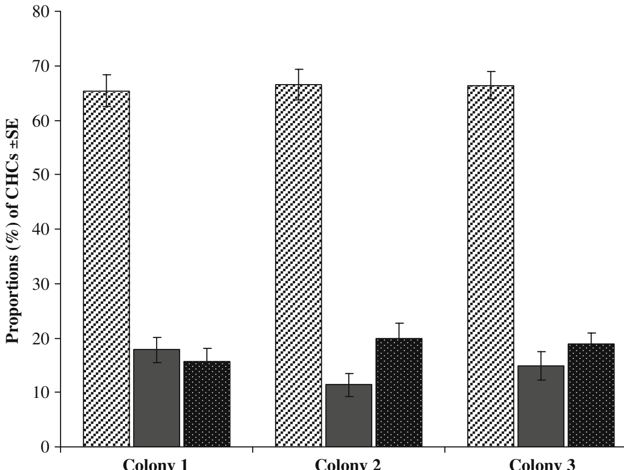


Fig. 3 Proportions±SE of the different groups of hydrocarbons (▨=*n*-Alkanes, ■=Alkenes and ▩=Methyl-branched alkanes) in the cuticular hydrocarbon profiles of *Pachycondyla analis* ants from colony 1, 2 and colony 3 (*error bars* represent mean proportions±SE)

Lucas et al. 2005; Martin et al. 2008b), with (*Z*)-9-tricosene occurring in variable proportions between colonies. In *P. analis, n*-alkanes occurred roughly in the same proportion in all colonies, with the alkenes and methyl-branched alkanes present in different proportions between the colonies, unlike in *Formica japonica* where *n*-alkanes and (*Z*)-9-alkenes varied between colonies (Akino et al. 2004) and in *F. exsecta* where (*Z*)-9-alkenes are colony specific (Martin et al. 2008b). In the genus *Pachycondyla,* species

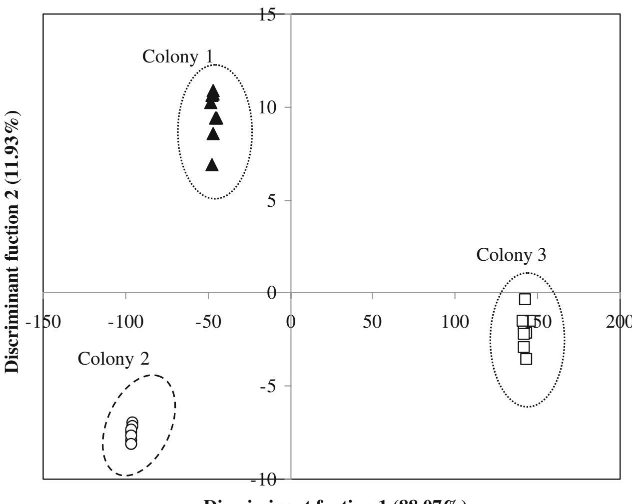
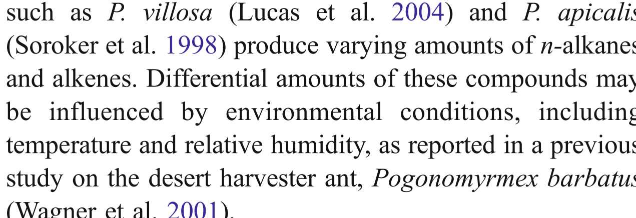


Fig. 4 Discriminant function analysis of ants from the three colonies of *Pachycondyla analis* based on relative proportions of 17 cuticular hydrocarbons determined in stepwise fashion. Colony 1 (*filled triangles*), Colony 2 (*circles*) and Colony 3 (*squares*). Circles around individual points are arbitrarily drawn to denote each colony. All individuals were clearly grouped into their respective colonies based on their CHC profiles



such as *P. villosa* (Lucas et al. 2004) and *P. apicalis* (Soroker et al. 1998) produce varying amounts of *n*-alkanes and alkenes. Differential amounts of these compounds may be influenced by environmental conditions, including temperature and relative humidity, as reported in a previous study on the desert harvester ant, *Pogonomyrmex barbatus* (Wagner et al. 2001).

The alkenes and methyl-branched alkanes in the CHCs could constitute nestmate recognition cues in *P. analis*. (*Z*)-9-Alkene has been reported as a nestmate recognition and aggression cue in *Formica* ants (Akino et al. 2004; Martin et al. 2008a,b), and as a recognition cue in the desert ant, *Cataglyphis niger* (Lahav et al. 2001). Nestmate recognition hydrocarbons identified in this study for *P. analis* might serve two purposes, colony defense (the traditional role of nestmate recognition), and recognition of nestmates when foraging. The roles played by the alkenes and methyl-branched alkanes in nestmate recognition and aggression in *P. analis* need to be further investigated by manipulating the CHC profiles of ants using synthetic compounds to see whether ants respond differently to the manipulated nestmate or non-nestmate CHCs.

The results from the discriminant function analysis show clearly that differences exist in the CHC profiles between colonies of *P. analis*. The implicated compounds (undecane, 3-methylundecane, 3,6-dimethylundecane, 3,8-dimethyldecane, pentadecane, heptadecane, 3-methylheptadecane, 2,6,10,14-tetramethylpentadecane, octadecane, nonadecane, heneicosane, tricosane, 1-nonadecene, 9-nonadecene, 9-methylnonadecane, squalene, and tetratriacontane) can be used effectively to correctly group the ants into their respective colonies. The colony-specific nature of CHCs in *P. analis* is consistent with findings in other ant species (e.g., Lahav et al. 2001; Akino et al. 2004; Lucas et al. 2004; Denis et al. 2006; Martin et al. 2008a,b). We demonstrated that these clear-cut groupings, based on CHC profiles, can explain the degree of aggression between different colonies. Colony 1 was further away from colonies 2 and 3, and exhibited higher aggression toward the latter colonies; likewise, colony 2 and 3 were closer together than colony 1, and were less aggressive toward each other's workers. Thus, these CHC differences could explain the differential acceptance of workers from other colonies competing for the same resources. Intruders or encroachers are usually killed upon encounter (AAY personal observation). By contrast the invasive Argentine ant displays minimal nestmate discrimination and individual non-nestmates are often integrated into an alien colony (Vásquez et al. 2008).

Colony odor recognition cues in ants are phenotypic and are derived either from the environment (diet, nesting sources) or produced endogenously (genetically determined or both) (Vander Meer and Morel 1998); the relative

importance of environmental and genetic differences can vary between populations (Pirk et al. 2001). Whatever the source of CHCs is, it is predicted that each colony will display a uniform odor that constitutes a gestalt. However, in some studies, it has been shown that this is not always the case because different castes within a colony may possess different CHC profiles that code different information within the colony (Dietemann et al. 2003; Martin and Drijfhout, 2009). In the present study, we showed that CHCs in *P. analis* are colony specific. However, a further investigation of workers in a colony based on the roles they play and their body sizes (major vs. minor workers) might reveal differences in the CHCs as is the case in some other ant species.

In summary, the MOR bioassay was used successfully to measure differential responses of *P. analis* workers based on colony of origin. Aggression was found to be associated with colony odor, mainly in the CHCs. As in other ant species, CHCs in *P. analis* comprise three main groups; *n*-alkanes, alkenes, and methyl-branched alkanes. The *n*-alkanes were consistent between colonies, with the alkenes and methyl alkanes serving as possible nestmate recognition cues.

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RAPID COMMUNICATION

Flight Initiation by Male *Rhodnius prolixus* is Promoted by Female Odors

Claudia A. Zacharias · Gina B. Pontes · Marcelo G. Lorenzo · Gabriel Manrique

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Abstract Several triatomine bug species utilize chemical cues for sexual communication. We tested whether female or male *Rhodnius prolixus*, a vector of Chagas disease, produce volatile chemicals that elicit flight responses from conspecifics, and then isolated the source of the chemical. Males confronted with an airstream containing female odors showed a significantly greater take-off frequency compared to a blank airstream or an airstream with male odors. In contrast, females exhibited similar take-off frequencies to male or female odor as to a clean airstream. Occlusion of female metasternal glands with paraffin wax resulted in a significant decrease in male take-off frequency compared to that of intact females. Additionally, excised female metasternal glands elicited a similar take-off frequency from males as did intact females, both significantly greater than the take-off frequency to clean air. These results show that *R. prolixus* females release a pheromone from their metasternal glands that causes upwind flight in conspecific males.

Key Words *Rhodnius prolixus* · Flight · Sexual behavior · Communication · Metasternal glands · Pheromones · Chagas disease · Trypanosoma · Hemiptera · Reduviidae · Triatominae

Claudia A. Zacharias and Gina B. Pontes contributed equally.

C. A. Zacharias · G. Manrique (✉)
Laboratorio de Fisiología de Insectos, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,
C1428EHA Buenos Aires, Argentina
e-mail: gabo@bg.fcen.uba.ar

G. B. Pontes · M. G. Lorenzo
Laboratory of Triatomines and Chagas Disease Epidemiology.
René Rachou Institute/FIOCRUZ,
30190002 Belo Horizonte, Minas Gerais, Brazil

Introduction

Rhodnius prolixus Stål (Hemiptera: Reduviidae: subfamily Triatominae) transmits *Trypanosoma cruzi*, the etiological agent of Chagas disease, in northern South America and parts of Central America. In Latin America, Chagas disease afflicts 15 million people, with roughly a further 90 million at risk from potential transmission.

Triatomines are capable of sustained flight, dispersing over considerable distances (Gringorten and Friend 1979; Schweigmann et al. 1988). Flight is influenced by temperature and nutritional state of the insect (Lehane et al. 1992; Gurevitz et al. 2006). Adults are attracted strongly to light sources (Noireau and Dujardin 2001; Minoli and Lazzari 2006).

It is known that male triatomine bugs are attracted to and aggregate around mating pairs of adults (Baldwin et al. 1971; Manrique and Lazzari 1995). Triatomine bugs have paired Brindley's (BGs) and metasternal glands (MGs) that emit volatile compounds. The compounds emitted from the MGs, in particular, appear to mediate triatomine sexual communication (Crespo and Manrique 2007; Pontes et al. 2008). Pontes et al. (2008) demonstrated that female *R. prolixus* emit MG odors at dusk and in greater quantities than males.

We evaluated whether male or female odor added to an airstream promotes an increase in flight initiation or oriented take-off in adult *R. prolixus*. Also, we demonstrated that the source of the odor responsible for this effect are the female MGs.

Methods and Materials

Rhodnius prolixus were obtained from the Servicio Nacional de Chagas, Argentina, and reared in the laboratory

at 26±1°C, 30–50% RH, 12:12 h L:D, with live hens as a blood meal source. Hens were handled according to the biosafety rules from the Servicio de Higiene y Seguridad, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Bugs were sexed before final ecdysis, and the two sexes were kept separately until use. One to 2-months-old virgin adults, starved for 20±5 d, were used in the experiments.

We used a cage constructed of voile fabric (based on Minoli and Lazzari 2006), with a circular acrylic arena (15 cm diam.×5 cm high) hung 1 m above the center of the floor. A vertical wooden rod (1.5 cm diam.×15 cm high), fixed in the center of the arena, functioned as a platform for flight take-off. Bug movement was recorded with an infrared video camera (Videoman, JCC-300, Korea), with an infrared LED light source illuminating the platform. The video monitor was divided into eight 45° sectors, centered on the image of the top of the take-off platform, allowing determination of the angular direction of take-off. A Vaseline® film, applied to the interior surface, allowed only flying insects to leave the arena. A fan fixed inside an 85-cm-long PVC tube (12 cm i.d.) generated an airstream of 12±0.9 cm sec^{-1} (measured with a Testo 405-V1 anemometer, Germany) at the take-off platform. Insects used as odor sources were placed inside mesh-covered, open-ended flasks that permitted airflow. Groups of 20 males or females (four replicates per treatment) were released onto the arena at the start of the scotophase and left for 8 hr. We recorded the number of take-offs and their angular direction.

In the first experiment, we tested the responses of males and females, separately, to: a) clean air, b) air with odors from females, and c) air with odors from males. Ten insects per flask were used in all treatments using insects as odor sources. Next, we occluded the orifices of female glands with paraffin wax (Crespo and Manrique 2007) 24 hr prior to testing the responses of males to the following treatments: a) BG-occluded females, b) MG-occluded females, c) MG- and BG-occluded females, and d) sham females that had wax applied between the prosternum and metasternum that did not occlude the glands. Finally the take-off frequency and orientation of males was also tested to airstreams with: a) excised female MGs (ten glands), b) intact females, and c) control consisting of ten pieces of tissue and cuticle from the coxae of the first legs of females.

Take-off frequency data were analyzed by linear parametric statistics, after normality (P-Plot) and homoscedasticity (Levene's test) were verified. A two-way ANOVA, assuming fixed factors (gender and stimulus type), or a one-way ANOVA was applied, as appropriate. A significant ANOVA was followed by a *post-hoc* comparison (Tukey test). The MGs odor data set did not meet normality assumptions; therefore, data were log-transformed. Take-off frequencies presented in the results section are means (±SEM) from four replicates.

Results and Discussion

Typically, responsive insects climbed the take-off platform, moved their antennae, and took flight. There was a significant interaction (two-way ANOVA, $F_{2,18}$=12.63, P<0.001) between gender and stimulus type, indicating that males and females responded differently to stimuli. Male take-off frequency varied with stimulus (Simple effect, $F_{(2,18)}$= 11.94, P<0.001), with the greatest response being to female odor. There were no significant differences in responses of females to any of the odor treatments (Fig. 1a).

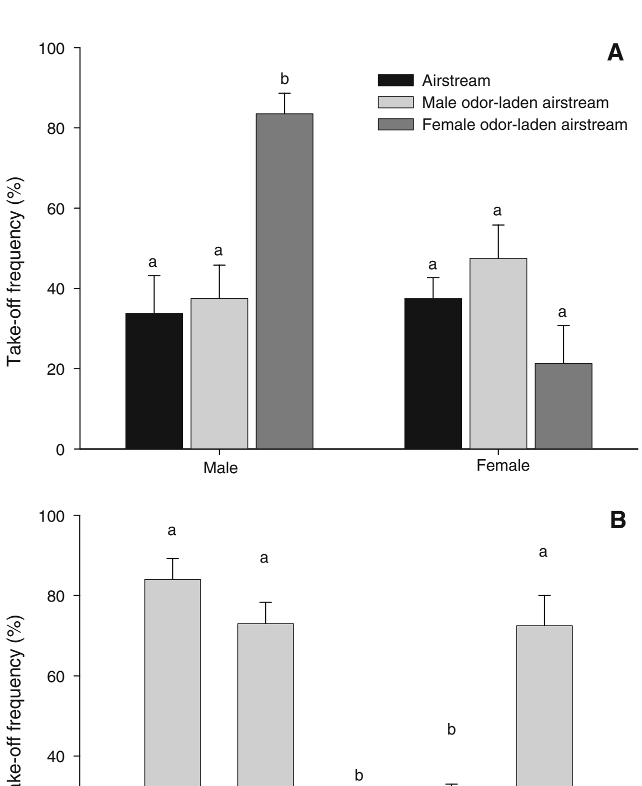

Fig. 1 Take-off frequencies (mean+SEM) of: **a** *Rhodnius prolixus* males or females to airstreams with intact males, intact females or clean air (control), and **b** *R. prolixus* males to various odor-laden airstreams; Intact = intact females, BG = females with Brindley glands occluded with paraffin wax, MG = females with metasternal glands occluded, MG+BG = females with metasternal and Brindley glands occluded, Sham = females that had paraffin applied to the cuticle without occluding any glands. Different letters atop bars denote statistically different means (Tukey, P<0.05). Each treatment consisted of four replicates

In the second experiment, flight frequencies showed a significant (one-way ANOVA, $F_{(4,15)}=19.95$, $P<0.001$) effect of occlusion treatment, with females with occluded MG or MG+BG eliciting significantly lower ($P<0.05$) take-off responses from males than did intact, sham-operated, or BG-occluded, females (these three treatments elicited similar take-off frequencies; Fig. 1b). In the final experiment, male take-off frequency was different depending on treatment (one-way ANOVA, $F_{(2,9)}=13.75$, $P=0.002$). Excised glands elicited a similar take-off frequency as intact females (60±7% vs. 84±5%; $P>0.05$, Tukey test). However, odors from control tissues elicited a lower male take-off frequency compared to odors from intact females (34±7% vs. 84±5%; $P<0.05$) or from excised MGs (34±7% vs. 60±7%; $P<0.05$, Tukey test).

The take-off direction of insects had a non-uniform distribution for all treatments tested. Insects tended to orient toward the direction of the airstream, regardless of odor.

The present work demonstrates that the flight of adult male *R. prolixus* can be triggered by volatile chemical cues released by conspecific females. In contrast, females did not show significant flight responses to male chemical cues. The source of the volatiles that elicit male flight appear to be the MGs of females, because when this gland was occluded with paraffin wax, the flight responses of males decreased significantly. In contrast, occlusion of another gland of females, the BG, or sham operation, did not affect flight responses of males. This sex-specific response suggests that this behavior is part of a mate location mechanism involving a sex pheromone emitted by females (Pontes et al. 2008).

Now that we have established that female *R. prolixus* produce a pheromone that elicits flight from males, chemical identification can proceed. Indeed, the chemical (s) may already have been identified in the study of Pontes et al. (2008). Identification of the pheromone will allow us to determine whether male behavior can be manipulated for controlling vectors of Chagas disease.

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Preparation of an Acid Butanol Standard from Fresh Apples

Chunmei Li · John D. Trombley · Michael A. Schmidt · Ann E. Hagerman

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Abstract We have developed a simple method for preparing and verifying suitable standards for the acid butanol assay from a readily available source. Phenolics were extracted from fresh apples with methanol, and sugars were removed from the crude extract by treatment with Amberlite resin before fractionating the proanthocyanidins into ethyl acetate. The ethyl acetate fraction was chromatographed on Toyopearl TSK HW-50F to yield about 50 mg of procyanidin dimer and 35 mg of trimer from 1 kg fresh apple fruit. The purity and identity of the standards was easily confirmed by using ESI-MS. In the acid butanol assay, the pure dimer, trimer and purified *Sorghum* procyanidin had similar color yields on a mass basis, and produced about three times more color than purified quebracho tannin. This new standard overcomes problems associated with overestimation of tannin due to use of the unreactive quebracho tannin standard. Use of the new standard will enable accurate comparisons of tannin levels between laboratories and will standardize comparisons between species, thus promoting our understanding of the role of condensed tannins in plants.

Key Words Acid butanol assay · Proanthocyanidin · Procyanidin · Condensed tannin · Polyphenolic

C. Li
College of Food Science and Technology,
Huazhong Agricultural University,
Wuhan 430070, China

J. D. Trombley · M. A. Schmidt · A. E. Hagerman (✉)
Department of Chemistry and Biochemistry, Miami University,
Oxford, OH 45056, USA
e-mail: hagermae@muohio.edu

Introduction

Research in chemical ecology relies on accurate methods for quantitative determination of metabolites. Polyphenolics (tannins) comprise one of the most intensively studied classes of secondary products, because they are widely distributed among plants and they have potent bioactivities. One of the most widely employed methods for quantitative analysis of the subclass of polyphenolics known as proanthocyanidins (condensed tannins) is the acid butanol assay (Swain and Hillis 1959) (Fig. 1). The acid butanol assay, in which proanthocyanidins are oxidatively depolymerized to yield colored anthocyanidins that are measured spectrophotometrically, is simple, specific, and reproducible if the optimized reaction conditions described by Porter et al. (1986) are used. Although specific compound analysis by HPLC is attractive for hydrolyzable tannins, HPLC methods for determining individual proanthocyanidins have not been developed adequately (Yarnes et al. 2006). Instead, the acid butanol method is the most widely used assay for selective determination of condensed tannins (Hagerman and Butler 1989; Yu and Dahlgren 2000).

Despite the widespread use of the acid butanol method, there is no generally accepted chemical standard for the assay. Standard curves used to convert absorbance to tannin concentration are generated by using a variety of materials, each with a unique color yield. Lack of a single standard hinders comparison of results between and within labs, and generates confusion about the quantities of tannins found in biological samples (Schofield et al. 2001). Porter et al. (1986) developed the assay by using cyanidin as a standard, but the expense of cyanidin precludes its routine use as a laboratory standard. Catechin, the nominal monomer of the procyanidins, is readily available, but it cannot be used as a

Fig. 1 Reaction of procyanidin dimer (I) with acid butanol to form cyanidin (II) from the extender (*top*) unit and the flavan-3-ol (III) from the terminal (*bottom*) unit. The apple dimer preparation was predominantly epicatechin-(4β->8)-epicatechin (dimer B-2) but contained some epicatechin-(4β->8)-catechin (dimer B-1). Epicatechin, *R*= OH, catechin, *R*= OH

standard because it does not react with acid butanol to yield an anthocyanidin. Quebracho tannin is a commercially available condensed tannin that has been recommended as a standard (Hagerman and Butler 1989). However, purification of the commercial product to remove nontannin materials is labor intensive and has low yield (Nelson et al. 1997; Hagerman 2002). Furthermore, quebracho is a 5-deoxy proanthocyanidin that is resistant to oxidative depolymerization (Schofield et al. 2001), so color yields are low, and tannin contents are typically overestimated (Wolfe et al. 2008), leading to an underestimation of biological potency. *Sorghum* procyanidin is well characterized (Gupta and Haslam 1978) and highly reactive with acid butanol, but it is not available commercially and is tedious to purify (Hagerman and Butler 1980; Hagerman 2002). Furthermore, there is no simple method to verify that the purified product is similar to that produced in other labs from the same plant material.

It has been recommended that the most suitable standard may be a purified proanthocyanidin prepared from the species of interest. The general strategy for purification uses sorption to Sephadex LH20 or similar resins to separate tannin from other phenolics (Mole et al. 1989; Rehill et al. 2006). The tannin that is produced, which contains the particular mixture of the condensed and the hydrolyzable tannins found in the plant, is generally used without further characterization or purification. Variable reactivity of "standards" prepared this way is not surprising given the variation in tannin composition across plant species, season, and environmental conditions. The condensed tannin "standards" prepared in this way from the same species in two different labs, or from different batches of tissue within the same lab, may have significantly different color yields in the acid butanol assay because of differences in the plant source and differences in the methods of purification. Extensive chemical characterization of unique plant standards by each laboratory where they are prepared could overcome these difficulties, but because the tannins produced by these methods are complex mixtures of complex polymeric natural products, characterization is daunting. We propose that adoption of a single, well characterized, chemically defined standard is a simpler solution that can be implemented uniformly in all labs.

Apple fruit is widely available, and comprises abundant proanthocyanidins but not hydrolyzable tannins, thus making it a good potential source for a condensed tannin standard (Guyot et al. 2001; Shoji et al. 2003). HPLC has been used for analytical separation of flavan-3-ol monomers and some small oligomers from apple (Perez-Ilzarbe et al. 1992; Yanagida et al. 2000), but these methods are not modified easily to preparative scale. Yanagida et al. (1999) fractionated apple procyanidins on Toyopearl TSK-gel HW by using a mixture of acetone and 8 M urea as an eluent, but the detection method was complicated, and removal of the urea from the product was laborious. Suitable methods for preparing large quantities of oligomeric proanthocyanidins from apple or other convenient sources have not been published.

Adoption of a single, well characterized standard for the acid butanol assay would improve our understanding of tannins not only in ecological systems, but also in food science, medicine, and nutrition (Hummer and Schreier 2008). With a more uniform basis for reporting tannin content, studies from different laboratories will be more easily compared. The main object of the work described here was to develop a simple method for preparing an acid butanol standard from readily available materials, and to provide simple parameters for validating the standard.

Methods and Materials

Chemicals All solvents were analytical or HPLC grade, and were obtained from Fisher Chemicals (Fair Lawn, NJ, USA), as were trifluoroacetic acid (TFA) and phenylmethane thiol (benzyl mercaptan). Catechin, epicatechin, cyanidin, and delphinidin, and the chromatographic supports Toyopearl HW50F, Sephdex LH 20, and Amberlite XAD7HP were from Sigma Chemical Co. (St. Louis, MO, USA).

Sample Preparation About 1.5 kg fresh Granny Smith apples (*Malus domestica*) were purchased from a local super market (Oxford, OH, USA), to be initially processed in seven batches. About 200 g of the fruit were cut into 1–2 cm cubes, transferred to a 1 l round bottom flask, and refluxed in 800 ml methanol at 70°C for 1.5 h, using a heating mantle to control the temperature and a water jacketed condenser to return the vapor to the pot. The extract was filtered, and the residue was extracted two more times. As the extracts were prepared, they were evaporated under reduced pressure to about one tenth the original volume and combined, yielding a somewhat viscous, sugar-rich extract. The sample (~250 ml) was loaded onto a column (3.8×50 cm) packed with Amberlite XAD7HP resin equilibrated with water, and sugars were eluted with 500 ml deionized water at a flow rate of 3 ml/min. When the eluate was sugar-free, as indicated by lack of reactivity with phenol-sulfuric acid (Dubois et al. 1956), 95% ethanol was used to elute polyphenolics at a flow rate of 2 ml/min. The eluate was periodically checked with the Prussian blue spot test (Hagerman 2002), and when all the phenolics had been eluted (~200 ml), the sample was evaporated under reduced pressure, and the residue was redissolved in about 50 ml water. This extraction process was repeated × 7 to obtain about 350 ml of aqueous sample from the 1.5 kg of fresh fruit. The aqueous sample was partitioned against an equal volume of ethyl acetate × 4, and the ethyl acetate extracts were combined and evaporated under reduced pressure to remove the solvent before redissolving the sample in about 100 ml water and freeze drying to yield about 0.7 g crude phenolics from 1,360 g fresh apple (Table 1).

Column Chromatography About 0.2 g of the freeze dried phenolic fraction was dissolved in 1 ml of methanol and applied onto a Toyopearl HW-50F column (3.0×50 cm) equilibrated with methanol. The column was washed with methanol at 1.5 ml/min, monitoring the eluate at 280 nm to identify fractions to combine. Each pooled fraction was diluted with an equal volume of water and evaporated under reduced pressure to remove the methanol before freeze drying to yield products for further analysis (Fig. 1, Table 1).

Table 1 Yield of fractions obtained by Toyopearl chromatography of crude phenolic extract from apple fruit

Fraction	Yield (mg)
Crude phenolics (ethyl acetate fraction)	704
Toyopearl fractions 1–3	184
Toyopearl fraction 4 (dimers)	51.2
Toyopearl fraction 5 (trimers)	36.8
Toyopearl fraction 6 (trimers, tetramers)	15.2
Toyopearl fraction 7 (tetramers)	24.6
Toyopearl fraction 8 (tetramers)	19.5

Values are typical values for a preparation from 1,360 g fresh apple fruit. Fractions were freeze dried and weighed to determine yields

HPLC Analysis Proanthocyanidin oligomers in each fraction were analyzed by HPLC using an Agilent 1050 system (Santa Clara, CA, USA) equipped with a diode array detector and controlled with Agilent ChemStation software (A.09.03). The system was equipped with two tandem 5 μm Hypersil ODS2 C_{18} cartridge columns (30×2.1 mm) with a guard column of the same materials (Grace Davison, Deerfield IL, USA). Separation was achieved with a gradient of 0.13% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B) at 0.5 ml/min in a 45 min program as follows: 0–10 min, 5% B, 10–15 min, increase to 20% B, 20–25 min, increase to 55% B, 25–35 min, 55% B, 35–40 min decrease to 5% B, 40–45 min, re-equilibrate at 5% B. The samples were filtered through a 0.22 μm cellulose acetate spin filter before injecting 10 μl, and the eluate was monitored at 280 nm.

ESI-MS Analysis Mass spectra were obtained on an Esquire LC ion trap instrument (Bruker Daltonics, Billerica, MA, USA). The electrospray ionization source (ESI) was operated in negative mode. A nominal target mass was set to 1,000 before fine tuning. The capillary, skimmer 1, and trap drive voltages were 3,500, −51.8, and 72.9 V, respectively. The ion charge control was on with a target of 30,000. The 300°C nitrogen dry gas flow rate was 4 l/min, and the nebulization gas pressure was 11 psi. A syringe pump was used to infuse the sample at 10 μl/min. Each data point in the spectrum consisted of an average of eight scans over a mass range of 50–2,000 m/z.

Gel Permeation Chromatography (GPC) GPC was carried out on the Agilent series 1050 HPLC equipped with a Varian PL Gel 5.0 μm MIXED-D, 300×7.5 mm column (Fisher Scientific, Fair Lawn, NJ, USA). An isocratic mobile phase of 1% water in tetrahydrofuran was delivered at 1 ml/min. The samples were dissolved at 1 mg/ml in tetrahydrofuran containing 1% water, and filtered through a 0.22 μm nylon spin filter before injecting 10 μl. The eluent was monitored at 280 nm.

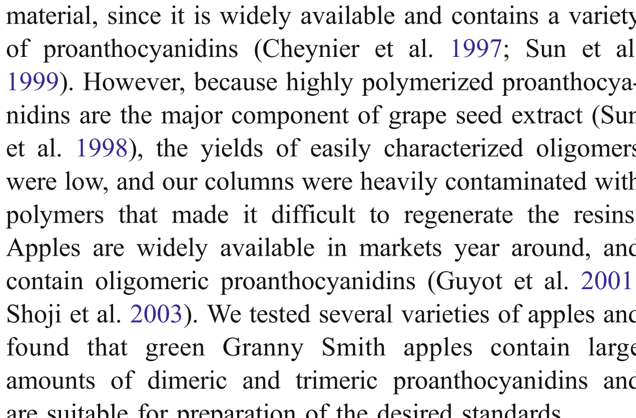
Thiolysis Freeze dried samples were dissolved in methanol at 1 mg/ml and were mixed with an equal volume of thiolysis reagent (5% (v/v) phenylmethanethiol in methanol containing 0.2 M HCl) (Gu et al. 2003). The mixture was heated at 40°C for 30 min, placed in the freezer for 5 min to stop the reaction, and immediately analyzed using an Agilent series 1100 HPLC (Santa Clara, CA, USA) equipped with a diode array detector. Separation was on a 5 µm Agilent Eclipse XDB-C_8 column (4.6 × 150 mm) with a gradient of 0.13% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B) in a 57 min program as follows: 0–3 min, 15% B, 3–8 min, 15–20% B, 8–10 min, 20–25% B, 10–30 min, 25–35% B, 30–34 min, 35–70% B, 34–44 min, 70% B, 44–47 min, 20% B, 47–52 min, 20–15% B, 52–57 min, re-equilibrate with 15% B. Thiolysis products were filtered through a 0.22 µm cellulose acetate spin filter before injecting 10 µl, and monitoring at 220 nm. Products were identified by comparison of elution times to the elution times of catchin and epicatechin obtained commercially, and epicatechin benzylthioether obtained by thiolysis of *Sorghum* procyanidin (Gupta and Haslam 1978). Peak areas were determined using Agilent Chem Station software (A.09.03) and then were converted to moles relative to the flavan-3-ol terminal using standard curves, and the average degree of polymerization (mDP) was calculated from the molar ratio of all the flavan-3-ol units (thioether adducts plus terminal units) to terminal units.

NMR Analysis Samples were dissolved at about 13 mg/ml in 90% H_2O/10% D_2O with DSS as the internal reference for NMR analysis at 500 MHz (proton) or 125 MHz (carbon). The Bruker (Billerica, MA, USA) spectrometer was equipped with a 5 mm probe and temperature was set at 15°C. HSQC was carried out with 128 experiments each with 256 scans and 16 dummy scans.

Acid Butanol Assay Samples were dissolved in methanol at 0.25 mg/ml for reaction with 3.0 ml acid butanol (5% (v/v) hydrochloric acid in n-butanol) and 0.1 ml of ferric ammonium sulfate (2% (m/v) in 2 M HCl) (Hagerman 2002). After 50 min of reaction in a boiling water bath, the samples were cooled, and absorbance was recorded at 550 nm in 1 cm cells, using an Agilent 8453 spectrophotometer (Santa Clara, CA, USA).

Results and Discussion

Starting Material Our goal was to establish a method to isolate a well defined standard for the acid butanol assay from a readily available material. We initially considered using commercial grape seed extract as our starting material, since it is widely available and contains a variety of proanthocyanidins (Cheynier et al. 1997; Sun et al. 1999). However, because highly polymerized proanthocyanidins are the major component of grape seed extract (Sun et al. 1998), the yields of easily characterized oligomers were low, and our columns were heavily contaminated with polymers that made it difficult to regenerate the resins. Apples are widely available in markets year around, and contain oligomeric proanthocyanidins (Guyot et al. 2001; Shoji et al. 2003). We tested several varieties of apples and found that green Granny Smith apples contain large amounts of dimeric and trimeric proanthocyanidins and are suitable for preparation of the desired standards.



Isolation and Characterization of Dimers and Trimers We exhaustively extracted the phenolics from diced apples with methanol, and removed sugars by a rapid, simple treatment with Amberlite resin. Methanol was removed from the phenolic extract, and smaller phenolics including oligomeric proanthocyanidins were selectively extracted with ethyl acetate and separated on Toyopearl into eight fractions (Fig. 2, Table 1). Reversed phase (RP)-HPLC and mass spectrometry (MS) indicated that fractions 1, 2, and 3 contained small phenolics but not procyanidins (data not shown).

RP-HPLC revealed that fraction 4 contained two major components with retention times of 4.21 min and 12.6 min (Fig. 3), but ESI-MS indicated that the fraction comprised compounds with a common molecular mass and fragmentation pattern (Fig. 4a), suggesting a pair of isomers. The molecular ion $[M-H]^-$ at *m/z* 577 is consistent with procyanidin dimer(s) while the *m/z* 289 peak is consistent with interflavan bond breakage to yield the monomer (epi) catechin. The fragment at m/z 425 corresponds to loss of 152 upon C ring cleavage of the terminal unit (Zeeb et al.

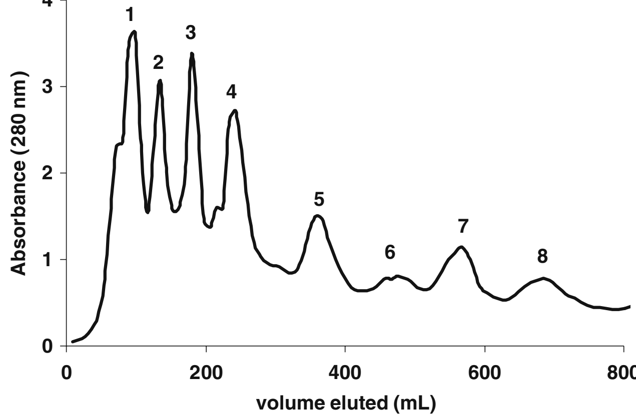


Fig. 2 The ethyl acetate soluble fraction of the apple extract was chromatographed on Toyopearl TSK-HW50 (F) using methanol at 1.5 ml/min as the eluent, and monitoring at 280 nm. The eluate was pooled as indicated to give 8 fractions for further characterization

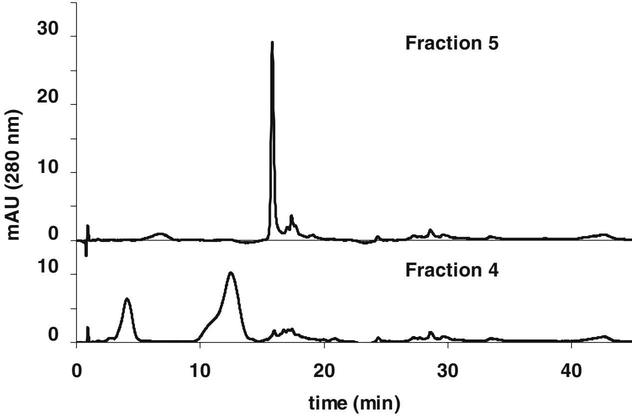
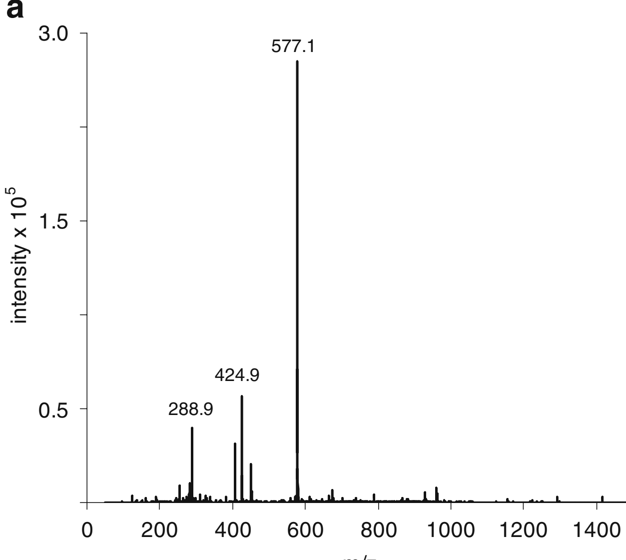


Fig. 3 Fractions 4 (*bottom panel*) and 5 (*top panel*) from the Toyopearl chromatography were analyzed by C-18 RP-HPLC using a gradient of water to acetonitrile with trifluoroacetic acid modification. The eluent was monitored at 280 nm to detect phenolics, which included procyanidin dimers B-1 and B-2 (fraction 4) and trimers (fraction 5)

2000). The major signals in the 2D NMR were consistent with dimer B-2 (Table 2) although weaker signals suggested that the sample also contained dimer B-1 (Tarascou et al 2006). The products of thiolysis of fraction 4 were catechin (6.34 min), epicatechin (7.84 min), and epicatechin benzylthioether (31.4 min) in molar ratios of 0.3:0.7:1.4, respectively. The mean degree of polymerization based on thiolysis was 2.4, consistent with the MS determination. We concluded that fraction 4 comprises two procyanidin dimers, B-1 (epicatechin-(4β->8)-catechin) and B-2 (epicatechin-(4β->8)-epicatechin) (Fig. 1). About 50 mg of this mixture of dimers were obtained from about 1 kg of fresh apple (Table 1). Based on RP-HPLC, NMR signal strength, and thiolysis products we estimated that about 70–80% of the dimer was B-2, similar to previous reports for apple procyanidins (Guyot et al. 2001). We recommend that the ESI-MS is a simple, reproducible way to confirm the identity of this fraction. For individuals without access to MS instrumentation, many commercial facilities (e.g., http://www.ccic.ohio-state.edu/MS/) will perform this analysis for a fee.

RP-HPLC indicated that fraction 5 was homogeneous (retention time 15.8 min, Fig. 3), as did ESI-MS (Fig. 4b). The molecular ion $[M-H]^-$ at *m/z* 865 indicated that the fraction contains procyanidin trimer(s), and fragments corresponding to dimer (m/z 577), monomer (*m/z* 288) and typical fragmentation of the C ring on the terminal unit to yield *m/z* 425 confirm that conclusion. The major products resulting from the thiolysis of fraction 5 were quite similar to those for fraction 4. The release of two terminal units, catechin and epicatechin, and one extension unit, epicatechin, in the molar ratio 0.2:0.8:2.6 indicated that fraction 5 was comprised of a mixture of two trimers, epicatechin$_2$-catechin and epicatechin$_2$-epicatechin, with a mean degree of polymerization of 3.6. As expected for higher oligomers, the NMR spectra were broadened for the trimer. Because we did not attempt to purify the various isomers to purity, exact structural assignments cannot be made, but the major peaks in the 2-D NMR (Table 2) correspond to the peaks assigned for trimer C-1 (epicatechin$_2$-epicatechin) and its isomer epicatechin$_2$-catechin (Shoji et al. 2003). About 35 mg of the mixture of trimers were obtained from 1 kg of fresh apple (Table 1). It is likely that trimer C-1 is the major component from this Toyopearl fraction, as in other examinations of apple procyanidins (Guyot et al. 2001), and the identity of this fraction was confirmed easily by ESI-MS.



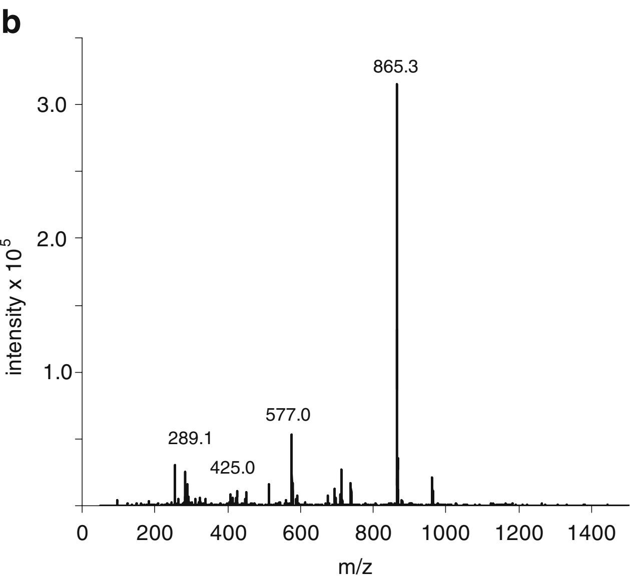


Fig. 4 ESI-MS of fraction 4 (**a**) and 5 (**b**) from the Toyopearl chromatography. The molecular ion in a (*m/z* 577.1) is characteristic of procyanidin dimers, and in b (*m/z* 865.3) is trimers

Table 2 ^{1}H and ^{13}C NMR data for authentic B-2[1], dimer (fraction 4) and trimer (fraction 5) from apple

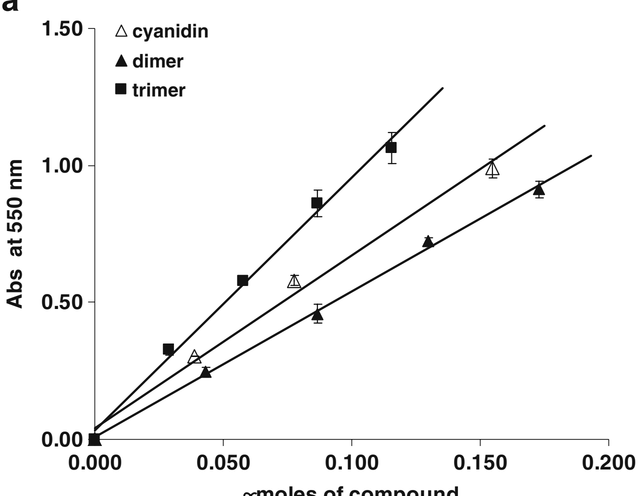
Position	B-2 [1]H	B-2 [13]C	Toyopearl fraction 4 [1]H	Toyopearl fraction 4 [13]C	Toyopearl fraction 5 [1]H	Toyopearl fraction 5 [13]C
2 extender	5.42	78.1	5.27	77.8	4.86	80.52
3 extender	4.06	74.6	4.02	73.9	4.09	74.1
4 extender	4.47	38.7	4.40	38.0	4.60	38.3
2 terminal	4.32	81.3	4.33	81.0	5.28	78.4
3 terminal	4.02	68.4	4.02	73.9	4.32	68.1
4 terminal	2.86	31.1	2.81	30.4	2.87	30.6

[1] Data for the compact conformer of authentic B-2 from Tarascou et al. 2006

HPLC and ESI-MS indicated that fraction 6 was a mixture of procyanidin trimers and tetramers, fractions 7 was procyanidin tetramers, fraction 8 was a mixture of tetramers and pentamers, and fraction 9 (not shown in Fig. 2) was a procyanidin pentamer. The tetramers and pentamers were not fully characterized, but the molecular size homogeneity of fractions 4, 5, 7, and 9 was confirmed by GPC. Each fraction eluted as a single peak with a characteristic retention time (catechin, 8.33 min; Fractions 4, 5, 7, and 9, t_R=8.11, 7.99, 7.91, and 7.87 min, respectively). The retention times were tightly correlated with the assigned molecular masses (mol mass=9E+14$e^{-3.46x}$, where x is retention time in min; R^2=0.996). Although high molecular weight procyanidins have been found in some varieties of apple (Guyot et al. 2001), none were detected in the extracts of Granny Smith apples. As a consequence the chromatographic supports were reconditioned easily for reuse in subsequent purifications, a major advantage for a preparative method.

Acid Butanol Assay In the acid butanol assay, the inter-flavan bond of proanthocyanidins is oxidatively cleaved to yield extender units as the colored anthocyanidin, and terminal units as the flavan-3-ol (Porter et al. 1986) (Fig. 1). Although each anthocyanidin (cyanidin, delphinidin, etc.) has a unique λ_{max}, the absorbance peak is broad, and all the anthocyanidins absorb strongly at 550 nm, with molar extinction coefficients in the acid butanol reagent of 24,500 M^{-1} cm^{-1} and 21,200 M^{-1} cm^{-1} for cyanidin (Fig. 5a) and delphinidin (data not shown), respectively. Thus, standardizing the assay with a well defined compound that yields only cyanidin will give reasonable results even for mixed proanthocyanidins.

The dimers and trimers that we isolated from apple are very reactive in the acid butanol assay. The molar yield of cyanidin from dimer was 84% of the expected yield, and from the trimer was 73% (Fig. 5a), similar to the yields from a variety of oligomers reported by Porter et al. (1986). On a mass basis, the color yield of dimer or trimer was 70–80% that from the well-known polymeric procyanidin from *Sorghum*, and 2.5–3 times higher than the color yield from



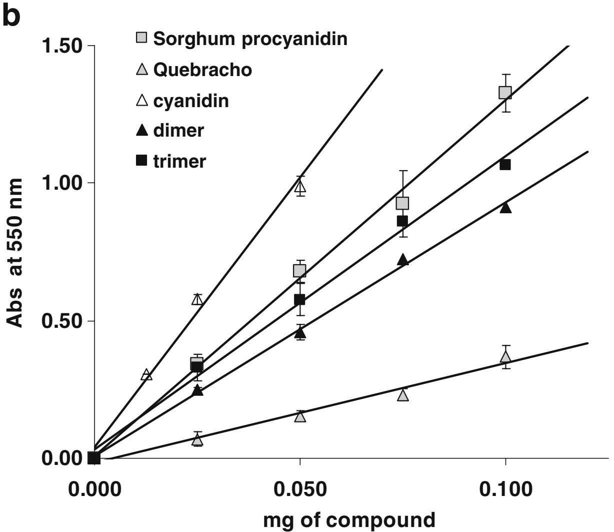


Fig. 5 Acid butanol assay. The absorbance is shown as a function of μmoles of compound (**a**) or mass of compound (**b**) for the purified apple dimer and trimer. The color yield is compared to that of cyanidin, Sorghum procyanidin, and purified Quebracho tannin. Each point is the mean of three determinations, and the error bars show standard deviation. The lines are fit by linear regression, and for every line the R^2 value is >0.99

purified quebracho tannin (Fig. 5b). Because the apple dimer and trimer react with high color yield, similar to the proanthocyanidins found in many common plants, these standards will provide realistic estimates of tannin levels for a wide variety of plants. The problems of gross overestimation of tannin content typically encountered when using quebracho tannin as a standard (Wolfe et al. 2008) will not be encountered with the apple standards.

We recommend that proanthocyanidin concentrations be reported as mass % equivalents of procyanidin dimer. In a recent study of *Ficus* tannins, we reported a range of 0.15 to 6.40% dry matter of proanthocyanidin, using the acid butanol assay standardized with our *Sorghum* procyanidin standard (Kendrick et al. 2009). If we had used the apple dimer, we would have reported values from 0.21 to 8.96% dry matter procyanidin dimer equivalents. The ranking and relative amounts of tannin in the species examined are the same whether the *Sorghum* standard or the dimer standard is used to calculate the tannin level. The important feature of reporting based on the apple dimer is that using that standard, our data could be reliably reproduced for these samples, and compared directly to data from other samples, by any other laboratory. The values calculated with the *Sorghum* tannin standards can be reproduced only by an individual who has access to this particular batch of our *Sorghum* tannin standard, since for complex polymeric tannin mixtures there is no convenient way to confirm that one preparation is identical to another. Since the apple dimer and trimer are defined chemical compounds, inter-laboratory comparisons of acid butanol analysis based on these standards will be more meaningful than comparisons based on poorly defined, polymeric standards such as quebracho or tannins isolated from a specific plant of interest.

In some cases, more biologically meaningful data might be obtained by using a standard purified from a plant of interest. For example, *Acacia* tannins are primarily 3-deoxyproanthocyanidins, which have low color yields in the acid butanol assay (Ishida et al. 2005). The apple procyanidin dimer would provide an unrealistic overestimation of tannin in this case, and the best standard would be one isolated from *Acacia*. In those cases, the apple procyanidin dimer would provide a very useful external reference for characterizing the reactivity of the purified *Acacia* proanthocyanidin for inter-study comparisons.

In summary, we found that the dimer and trimer from fresh apple are suitable standards for the acid butanol assay. Our fractionation and isolation procedure is fast, inexpensive, and yields pure dimeric and trimeric fractions whose identity is easily confirmed by ESI-MS. We believe that wide adoption of these standards will improve the quality of data reporting quantities of proanthocyanidins in a variety of plant tissues.

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Dynamics of the Volatile Defense of Winter "Dormant" Balsam Poplar (*Populus balsamifera*)

Thomas P. Clausen · Janice Chen · John P. Bryant · Frederick D. Provenza · Juan Villalba

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Abstract 6-Hydroxycylohex-2-en-1-one (6-HCH) has been reported as a major chemical defense of the winter-dormant internodes of balsam poplar (*Populus balsamifera*) against feeding by herbivores such as the snowshoe hare (*Lepus americanus*). We report that the concentration of 6-HCH in the fall internodes is triggered by a single hard frost, and then undergoes an exponential decline through volatilization over the winter that results in barely detectable quantities by early spring. We conclude that the role of 6-HCH in the defense of mature balsam poplar is more complex than simply acting as a toxin. Rather, 6-HCH's role as a defensive agent must evolve over the course of the winter from being a co-toxin to a cue for a conditioned flavor aversion (CFA) to finally having no role by late spring.

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T. P. Clausen (✉)
Department of Chemistry & Biochemistry,
University of Alaska Fairbanks,
Fairbanks, AK 99775-6160, USA
e-mail: tpclausen@alaska.edu

J. Chen
Department of Psychology, Stanford University,
Stanford, CA 94305, USA

J. P. Bryant
Institute of Arctic Biology, University of Alaska Fairbanks,
Fairbanks, AK 99775-7000, USA

F. D. Provenza · J. Villalba
Department of Wildland Resources, Utah State University,
Logan, UT 84322-5230, USA

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Introduction

The role of chemical defenses in the consumption of winter-dormant *Populus balsamifera* (balsam poplar) by hares has been studied for over two decades (Bryant 1981a, b; Sinclair and Smith 1984; Mattes et al. 1987; Jogia et al. 1989; Reichardt et al. 1990; Clausen et al. 1992; Schmitz et al. 1992). When eating the twigs of either the juvenile or mature ontogenetic phase of poplar, hares eat the woody internodes and reject the highly resinous foliar buds (see Online video clip). Internodes of the mature phase are moderately palatable, while juvenile internodes are unpalatable. Here, we were concerned with the chemical defense of internodes of the mature phase.

Three volatile and potentially toxic shikimates {Fig. 1; salicaldehyde, 6-hydroxycyclohex-2-en-1-one (6-HCH), 1,2-cyclohexadione; Mattes et al. 1987} have been implicated as internode defenses against snowshoe hares (Reichardt et al. 1990). In the case of mature poplar, 6-HCH has been implicated as the primary secondary metabolite that regulates internode consumption (Mattes et al. 1987). In this paper, we consider the possibility that chemical defense of the internodes of the mature phase may change over winter because of the volatility of 6-HCH. Since it is unlikely that 6-HCH is produced throughout the winter, it follows that volatilization would deplete the 6-HCH pool and complicate its role in defense.

The chemistry of the poplar internode shikimates involves complex dynamics (Fig. 2). Phenolic glycosides such as salicortin undergo enzymatic conversion to toxic

Fig. 1 Known volatile defensive secondary metabolites of *Populus balsamifera*. Taken from Mattes et al. 1987 and Jogia et al. 1989

derivatives such as optically active 6-HCH and corrosive cyclohexadione upon tissue damage (Fig. 2; Mattes et al. 1987; Reichardt et al. 1988; Ruuhola et al. 2003). Both esterase and β-glucosidase activity lead to these transformations although, in our previous studies, *in vitro* β-glucosidase treatment of salicortin also leads to a potentially toxic benzyl substituted analog of 6-HCH along with enzyme deactivation (Clausen et al. 1990; Jianjun et al. 1998). The facile conversion of 6-HCH to the toxins phenol under acidic conditions and catechol under basic/oxidative conditions (Clausen et al. 1989; Miyoshi et al. 2001; Ruuhola et al. 2003) is consistent with a toxic mode of action of both 6-HCH and its immediate precursor, salicortin. In addition, substances such as benzyl gentisate, which is enzymatically generated from a biogenetically related glycoside, trichocarpin, affects internode consumption (Mattes et al. 1987). The enzymatic transformations of salicortin and trichocarpin are complete within minutes of tissue damage, similar to what must occur when an internode is masticated by a hare.

The importance of salicortin and related glycosides such as tremulacin in deterring foraging has been widely reported (Lindroth et al. 1988; Reichardt et al. 1988, 1990; Kelly and Curry 1991; Julkunen-Tiitto et al. 1994; Hemming and Lindroth 2000; Haruta et al. 2001; Miyoshi et al. 2001; Ruuhola et al. 2001; Kleiner et al. 2003). Many of these studies have stressed the facile conversion of salicortin to toxic phenol or catechol via 6-HCH as the driving mechanism. In addition, the importance of free 6-HCH in deterring hare selection of mature balsam poplar internodes was shown clearly by Reichardt et al. (1990).

There remain two different hypotheses regarding the mechanisms for why hares are deterred by free 6-HCH

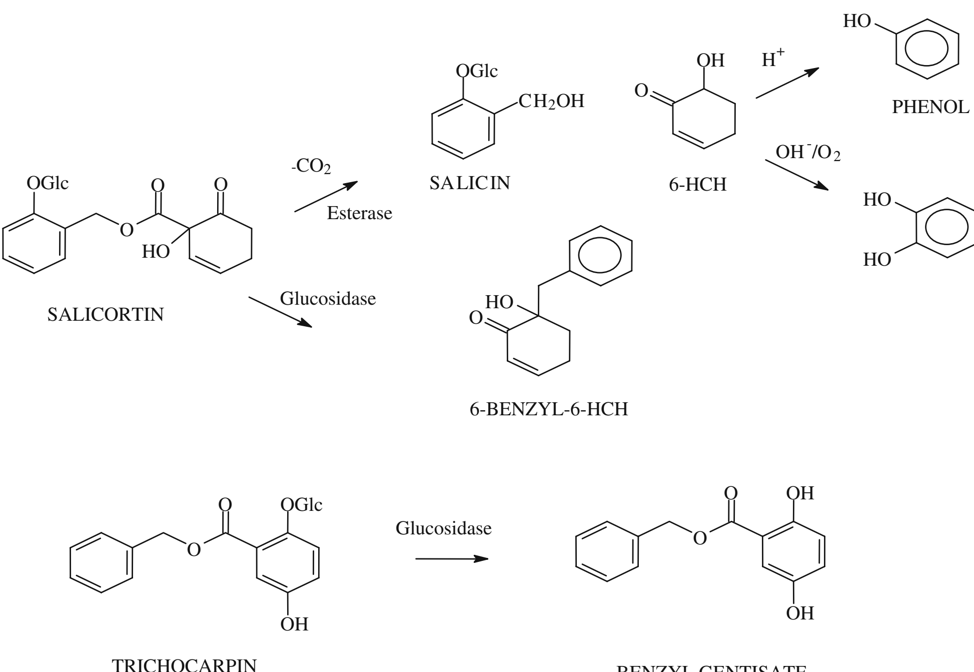

Fig. 2 Facile transformations reported for *Populus balsamifera's* internode secondary metabolites

found in mature balsam poplar internodes. The first is based on the toxicity of 6-HCH, but would be valid only if its concentrations in the plant were above toxic thresholds of ingestion. The other is based on free 6-HCH acting as a warning to hares regarding the presence of additional 6-HCH-producing toxins in the plant. This latter hypothesis is attractive given Tahvanainen et al.'s (1985) observation that hares choose salicaceae browse low in phenol glycosides based on olfactory and not taste stimuli; 6-HCH is volatile and hence has an odor, while its phenol glycoside precursors such as salicortin are not volatile or odiferous.

Both of these mechanisms, however, have an inherent weakness. Over the winter-dormant season, it is unlikely that 6-HCH lost through volatilization will be replenished through metabolic processes. If the concentration of 6-HCH decreases as the winter progresses, then either the toxicity of the browse must decrease (hypothesis 1) or hares must continually adjust their perception of warning signals (hypothesis 2). Thus, further understanding the dynamics of the hare-mature poplar internode browsing system first requires answering the question posed in this study: Does the concentration of mature poplar internode 6-HCH decline over the winter?

Methods and Materials

Starting in late October in 1996, this study focused on the chemistry of mature balsam poplar internodes through mid-January for two consecutive years. About every 1–4 d over the course of the study, a mature (3–4 m tall) poplar tree was selected haphazardly from a stand growing on the University of Alaska Fairbanks campus. A voucher specimen (Clausen 2) has been deposited at the University of Alaska, Fairbanks Museum. Current season growth twigs (diam<4 mm) were collected randomly and immediately processed upon return to the laboratory. Buds were removed, and about 1 g of internode was accurately weighed and extracted for 24 hr in 10 ml MeOH (VWR HPLC grade) containing 0.1% 1-hexadecanol (internal standard; Aldrich-Sigma) at −20°C. Samples were filtered, diluted with MeOH, and analyzed by GC/MS (1 µl splitless injection) using a 5% phenyl dimethyl siloxane (0.25 µm thickness) column (30 m×0.25 mm; Alltech). The following parameters were used. Gas (He) flow rate=1 ml/min; injector and detector temperatures of 275°C and 300°C, respectively; oven temperature program: 50°C for 6 min, then raised to 85°C at 4°C/min, held at 85°C for 2 min, and then ramped to 275°C at 20°C/min to recondition the column. Synthetic 6-HCH from an earlier study (Mattes et al. 1987) was used to determine the retention time of 6-HCH to be 10.5 min.

Results

We did not detect 6-HCH in mature poplar internodes until the first hard frost of the fall at which time 6-HCH internode concentrations were estimated to be about 1% (dry wt). During the 1996/1997 dormant season, its concentrations then exponentially declined (r^2=0.8) to about 0.01% by mid January (Fig. 3). A similar trend was observed over the 1997/1998 dormant season (r^2=0.59; Fig. 3).

Discussion

The volatile phenolic glycoside derivative 6-HCH is absent in poplar in summer presumably because of autotoxicity, as leaf biomass is rapidly killed by contact with 6-HCH (Clausen unpublished). Our observation of a spike in the concentration of 6-HCH after the first hard frost, while possibly coincidental, is not surprising given that freeze-

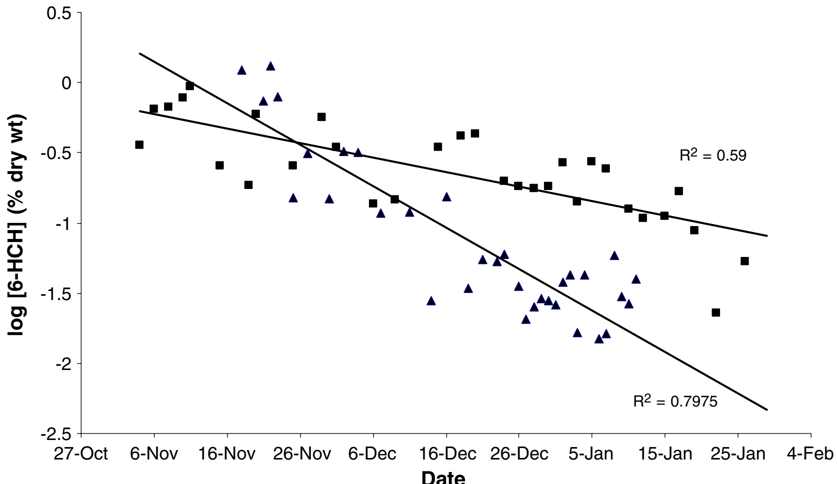

Fig. 3 A logarithmic plot of 6-HCH concentrations in *Populus balsamifera* internodes through the early half of the dormant season of years 1996/1997 (triangles) and 1997/1998 (squares)

thawing of plant material containing salicortin results in its rapid enzymatic breakdown to 6-HCH (Julkunen-Tiitto and Tahvanainen 1989; Orians 1995; Lindroth and Koss 1996; Julkunen-Tiitto and Sorsa 2001).

While our reported early winter concentrations of 6-HCH, as well as the 1.4% reported by Reichardt et al. (1990), may have toxic consequences for browsing hares, the mid-winter amounts found in late January probably have fallen below a toxic threshold. By late January 1997, for instance, the concentration of 6-HCH in mature internodes is projected to be less that 0.01%. Thus, if 6-HCH is to remain an effective antibrowsing defense over the entire winter it must be part of a Conditioned Flavor Aversion (CFA; *vide infra*). CFA involving volatiles such as 6-HCH have been documented in other woody plant–mammals interactions (e.g., Provenza 1996; Lawler et al. 1999; Provenza et al. 2000; Moore et al. 2004, 2005). It is conceivable that even at near zero concentrations free 6-HCH could warn hares of the possibility of phenolic glycoside poisoning following internode consumption. However, such a volatile-containing CFA defense is unlikely as the winter progresses far beyond January. Near Fairbanks, Alaska there are about 80 days of winter after January, and over these 80 days, the 6-HCH concentration will continue to decline towards the zero level found in summer growth. In addition, we would expect hare use of twigs to increase as free 6-HCH concentrations decline if the consequence (toxicity) is linked closely with the volatile cue (free 6-HCH).

Toxic compounds like 6-HCH or salicortin with a generalized defensive function have the potential to deter herbivory through the ability of mammalian herbivores to integrate food cues (e.g., taste, odor, texture) with the food's postingestive consequences (e.g., secondary compounds) (Provenza 1995, 1996). This cue-postingestive feedback interplay enables herbivores to regulate intake of foods that contain secondary compounds in amounts that do not cause toxicity. Strong food aversions occur when a physiochemical agent causes nausea after a food (secondary compound) has been consumed in excess (Provenza et al. 1990). CFA, thus, results from the stimulation of the emetic system of the midbrain and brain stem (Mitchelson 1992). After ingesting a food containing a toxin, afferent impulses to the central nervous system (CNS) cause nausea, which in turn causes the animal to decrease intake of food as a function of the dose of the toxin ingested (du Toit et al. 1991). Consistent with this mechanism, antiemetic drugs, which decrease the stimulation of the emetic system, attenuate food aversions in mammals (Provenza et al. 1994; Lawler et al. 1998). Animals respond strongly to taste in CFA, as afferents for taste converge directly with afferents from throughout viscera in the brain stem (Provenza 1995), but they also respond strongly to other food cues such as odor. Indeed, taste potentiates odor during food ingestion, and it is the tremendous plasticity in the sense of olfaction and its linkages in the brain with the amygdala and the hippocampus that enables animals to learn and remember quite subtle discriminations among vastly diverse food flavors (Provenza 1995, 1996).

Herbivores associate novel odors with aversive post-ingestive consequences from a toxin associated with odor inhalation (Provenza et al. 2000). Thus, odors are important cues in conditioned food aversions, though the odor need not be the cause of the aversion, merely a signal consistently associated with the toxicant in the plant (Lawler et al. 1999; Provenza et al. 2000). For instance, marsupials develop CFA to high concentrations of volatile terpenes because their flavor (cue) is consistently associated with the negative postingestive consequences of ingesting formylated phlor-oglucinol compounds, which are toxic (Lawler et al. 1998, 1999). Thus, 6-HCH may act as a cue and as a trigger for stimulating the emetic system (and conditioning an aversion) when concentrations in the plant are high. 6-HCH also may serve as a cue that causes avoidance, if the aversion is strong enough, even when concentrations in the plant decline over time, as herbivores generalize aversions based on familiar flavor cues (Launchbaugh and Provenza 1993).

The results of Reichardt et al. (1990), which show 6-HCH plays a dominant role in hare utilization of mature balsam popular internodes, and our study that demands 6-HCH play a diminishing role as winter progresses until it fades away completely by early to late spring, are in stark contrast. It is apparent that the role of "free" 6-HCH in the internodes is dynamic and must evolve from being a co-toxin (along with salicortin) early in the dormant season, to perhaps becoming a warning signal for other toxins, to eventually having no consequence as summer approaches.

The evidence is persuasive that snowshoe hares regulate use of mature balsam poplar through a feedback mechanism that prevents toxicity, and that salicortin (along with perhaps trichocarpin) is the primary toxin. Depending on the temperature (Pease et al. 1979), the mass of fresh browse required for hare survival in winter is between 300–400 g/hare/day (Bookhout 1965). Yet, when captive snowshoe hares are fed only internodes of mature poplar growing near Fairbanks, they limit intake of internode biomass to about 100–150 g fresh mass (Bookhout 1965), which is well below their need for survival. Tannins can be ruled out as the cause for lower intake, as they are absent in balsam poplar twigs (Reichardt et al. 1990). Similarly, 6-HCH can be ruled out in the late winter as our study shows its concentrations approach zero. The secondary metabolites of buds, while complex and in abundant amounts, are absent in the internodes (Clausen et al. 1992), which hares selectively consume (see Online video clip). The only other secondary metabolites present in

significant quantities in mature balsam poplar internodes are salicortin and trichocarpin (Reichardt et al. 1990; Clausen et al. 1992). As stated, salicortin is a labile compound that is rapidly converted to toxic 6-HCH through tissue maceration and conditions typically found in the gut (Mattes et al. 1987). In addition, numerous studies show experimentally that salicortin and its benzoyl derivative, tremulacin, have toxic consequences to invertebrate herbivores (Lindroth et al. 1988; Kelly and Curry 1991; Julkunen-Tiitto et al. 1994; Ayres et al. 1997). Finally, hares (Tahvanainen et al. 1985) and moose (Stolter et al. 2009) prefer willows with low concentrations of phenol glycosides.

There is circumstantial evidence that 6-HCH can be associated with a conditioned flavor aversion for hares browsing poplar. The secondary chemistries of many willow species are similar to that of balsam poplar (salicortin is a common major phenol glycoside), and hares appear to use olfaction to select food low in phenolic glycosides (Tahvanainen et al. 1985). Other evidence comes from a study by Schmitz et al. (1992) wherein a nearly two-fold increased consumption of balsam poplar by free-ranging hares was accomplished by drying fresh-clipped twigs at 30°C for 48 hr, thus reducing twig water content by 33% and decreasing the wet-mass ratio from 1.72 to 1.40±0.06. Drying browse sufficient to reduce twig water content would also greatly reduce the concentrations of a low molecular weight volatile such as 6-HCH, which would contribute to the increase in intake of poplar twigs by hares observed in their study.

Based on our findings, we propose that following a hard frost, 6-HCH reaches high concentrations (>1% dry weight) that form part of a toxic basis for herbivore selection of browse. However, as the winter-dormant season progresses, the concentration of 6-HCH drops below a toxic threshold (where it acts as a warning signal) and eventually approaches the zero concentration found during the growing season (that has negligible effect on hare browsing). If and how the changing concentration and shifting role of 6-HCH affects the consumption of mature balsam poplar internodes by snowshoe hares goes beyond the goals and experimental design of this study. In light of our results and discussion, there is a need for future studies to establish if hare usage of balsam poplar significantly changes over the dormant season and to establish the roles of 6-HCH as a CFA factor and that of balsam poplar's major phenolic glycosides (salicortin and trichocarpin).

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Plant Volatiles Influence Electrophysiological and Behavioral Responses of *Lygus hesperus*

Livy Williams III · Jacquelyn L. Blackmer · Cesar Rodriguez-Saona · Su Zhu

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Abstract Previous laboratory studies have shown that the mirid *Lygus hesperus* is attracted to volatiles emitted from alfalfa; feeding damage increases the amounts of several of these volatiles, and visual cues can enhance attraction further. The present study tested single plant volatiles in electrophysiological and behavioral trials with *L. hesperus*. Electroantennogram (EAG) analyses indicated that antennae responded to most plant volatiles included in the test, and that when gender differences were observed, males usually were more responsive than females. Antennal responses to the alcohols (*(E)*-3-hexenol*, (Z)*-3-hexenol, 1-hexanol), the acetate *(E)*-2-hexenyl acetate, and the aldehyde *(E)*-2-hexenal were among the strongest. Moderate responses were observed for *(E)*-β-ocimene, *(E,E)*-α-farnesene, (±)-linalool, and methyl salicylate. A dose dependent response was not observed for several terpenes (β-myrcene, β-caryophyllene, (+)-limonene, or both (*R*)-(+)- and (*S*)-(−)-α-pinenes). EAG responses, however, were not always consistent with behavioral assays. In Y-tube bioassays, males did not exhibit a positive behavioral response to any of the compounds tested. Instead, males were repelled by (*E*)-2-hexenyl acetate, (±)-linalool, *(E,E)*-α-farnesene, and methyl salicylate. In contrast, female *L. hesperus* moved upwind towards *(R)*-(+)-α-pinene, *(E)*-β-ocimene, and *(E,E)*-α-farnesene, and showed a negative response towards (*Z*)-3-hexen-1-ol, *(S)*-(−)-α-pinene, and methyl salicylate. This study emphasizes the use of multiple approaches to better understand host plant finding in the generalist herbivore *L. hesperus*.

Key Words Electroantennography · Y-tube olfactometer · Behavior · Plant volatiles · Heteroptera · Miridae · *Lygus hesperus*

Introduction

Plant chemistry plays an important role in the mediation of arthropod behavior (Bernays and Chapman 1994). In particular, plant odors help guide insects to food sources (Byers et al. 1985; Blight et al. 1995; Stensmyr et al. 2001; Dobson 2006), oviposition sites (Showler 2001; Broad et al. 2008), and often that work in concert with insect-derived odors to facilitate mate finding (Dickens 1989; Landolt and Phillips 1997; van Tol et al. 2004; Frati et al. 2009). Furthermore, herbivory usually alters the blend of volatiles emitted by plants (Dicke and van Loon 2003; Tumlinson and Engelberth 2008), thereby providing additional information to insects about the potential suitability of the plant for colonization (De Moraes et al. 2001; Meiners et al.

Jacquelyn L. Blackmer: Deceased

L. Williams III (✉)
USDA-ARS Exotic and Invasive Weeds Research Unit,
920 Valley Rd.,
Reno, NV 89512, USA
e-mail: livy.williams@ars.usda.gov

J. L. Blackmer
USDA-ARS Pest Management
and Biological Control Research Unit,
21881 N. Cardon Lane,
Maricopa, AZ 85138, USA

C. Rodriguez-Saona
Department of Entomology, Philip E. Marucci Blueberry
and Cranberry Research Center, Rutgers University,
125a Lake Oswego,
Chatsworth, NJ 08019, USA

S. Zhu
Department of Chemical, Biological, and Materials Engineering,
University of Oklahoma,
Norman, OK 73019, USA

2005; Choh et al. 2008). The central role of plant volatiles in insect life history has been exploited to manipulate the behavior of economically important species in monitoring and management programs (Smart et al. 1996; Piñero and Prokopy 2003; De Camelo et al. 2007; Martel et al. 2007). Several monophagous and oligophagous species have shown behavioral responses to single plant compounds (Leskey et al. 2001; Coracini et al. 2004; Meagher and Landolt 2008), suggesting that this approach deserves consideration when biologically-based pest control strategies are being developed.

Lygus hesperus Knight (Heteroptera: Miridae) is an important pest of cotton and other crops in western North America (Leigh et al. 1988; Wheeler 2001), and improved understanding of *Lygus*—plant interactions might lead to sustainable crop protection strategies. Studies have demonstrated that *L. hesperus* perceives and responds to plant odors, and that composition of plant odor is altered after damage by this insect. Rodriguez-Saona et al. (2002) showed that feeding and oviposition by *L. hesperus* females induced emission of significant amounts of volatiles from cotton, and the level of emission was positively associated with the number of eggs laid on a plant. Damage to alfalfa by *L. hesperus* increased emissions of several compounds found in the headspace of undamaged plants, and led to production and emission of additional compounds not emitted by undamaged plants (Blackmer et al. 2004). Fifth instar and adult female *L. hesperus* were attracted to volatiles emitted from undamaged alfalfa and from alfalfa infested by onspecifics (Blackmer et al. 2004) This response was enhanced by the presence of a green light-emitting diode (Blackmer and Cañas 2005). Williams et al. (2005) reported that feeding by virgin females of *L. hesperus* induced 2.6-fold higher emission of cotton volatiles than feeding by mated females, and treatment of maize plants with *L. hesperus* salivary gland extracts and artificial mechanical injury resulted in 2.7-fold higher emission of volatiles than artificial mechanical injury alone.

The neurological basis for reception of odors by heteropterans is not well understood, in part because these insects are difficult subjects for electrophysiological studies relative to other groups, such as Lepidoptera (but see Chinta et al. 1994; Groot et al. 1999). The goal of the present study was to evaluate the response of *L. hesperus* adults to individual plant odors by using electrophysiological (electroantennogram, EAG) and behavioral studies in the laboratory. Our specific questions were: 1) Do antennae of female and male *L. hesperus* respond differentially to plant volatiles?, 2) Does *L. hesperus* respond to individual plant volatiles in a behavioral assay?, and 3) Does the insect's electrophysiological response reflect its behavioral response to plant volatiles?

Methods and Materials

Insect Rearing and Maintenance Lygus hesperus nymphs and adults were collected from alfalfa fields located at The University of Arizona-Maricopa Agricultural Center, Maricopa, AZ, USA. To maintain genetic diversity, feral individuals were added to the colony 3–4 times per year. Green beans, carrots, pink bollworm eggs (*Pectinophora gossypiella* [Saunders]) and 10% sucrose solution were provided as food to adults and nymphs. Artificial diet packets, as described by Debolt (1982), were included as part of the feeding regime. Green beans and agar-filled parafilm packets served as oviposition substrates. Diet and oviposition substrates were replaced every other day, and oviposition substrates were placed in paper cartons (8.5× 12.5 cm) where the center of each lid was replaced with nylon organdy to allow air circulation. Newly emerged first instars were transferred to fresh paper cartons and provided with the diet mix indicated above. Containers were maintained in an incubator until adults began to emerge, at which time they were placed in mesh-covered plastic rearing containers (28 cm^3) and held at 26±1°C, 30±10% RH, and 14:10h L:D photoperiod until behavioral bioassays were conducted (7–17-d-old adults). Insects to be used in electroantennography trials were shipped to the laboratory (USDA-ARS Southern Insect Management Research Unit, Stoneville, MS, USA) in paper cartons with green beans. These bugs were transferred to Plexiglass cages (26×26× 20-cm) and held with green beans at 25±1°C, 60–85% RH, and 14:10h L:D photoperiod until experimentation (10–14-d-old adults).

Electroantennography Test Chemicals Fifteen compounds included in this study (Table 1) were tested individually as olfactory stimuli. Plant volatiles comprising green leaf volatiles, monoterpenes, and other groups were chosen based on previous studies that demonstrate their emission from undamaged and insect-infested plants (Takabayashi et al. 1994; Loughrin et al. 1995; Röse et al. 1996; Paré and Tumlinson 1998; Rodriguez-Saona et al. 2002; Blackmer et al. 2004; Williams et al. 2005). For the EAG study, serial dilutions (0.1, 1.0, 10, and 100 μM) of each compound were made with paraffin oil (E. Merck, Darmstadt, Germany). Stimulus applicators were prepared by pipetting 25 μl of a test solution onto a 6 cm by 0.5 cm strip of Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, Kent, UK), after which the filter paper was placed inside a 14.5-cm long glass Pasteur pipette. Fresh stimulus applicators were prepared after 2 h of use. Three controls were used: 1) an empty pipette, 2) a pipette containing 25 μl paraffin oil only on filter paper, and 3) a pipette containing 25 μl 100 μM octanal in paraffin oil on filter paper (octanal standard).

Table 1 Amounts and emission rates of chemicals used in electroantennography and Y-tube olfactometer

Plant volatile	Purity (%)	Source	Y-tube bioassay		EAG bioassay	
			Volume (μl)[1]	Emission Rate[2]	Volume (μl)[3]	Emission Rate[4]
Green leaf volatiles						
(E)-2-hexenal	98	AlfaAesar	6	2.2±0.9	25	16±3.2
1-hexanol	98	Aldrich.	6	274.2±147.5	25	9.9±1.6
(Z)-3-hexen-1-ol	98	AlfaAesar	6	113.7±46.9	25	11.6±0.9
(E)-3-hexen-1-ol	97	Aldrich	6	103.8±22.5	25	11.5±1.4
(Z)-3-hexenyl acetate	99	AlfaAesar	6	61.2±31.6	25	29.1±1.5
(E)-2-hexenyl acetate	98	AlfaAesar	6	116.9±42	25	36.2±4
Monoterpenes						
(R)-(+)-α-pinene	98	Acros	6	4.1±2.2	25	41.3±3.9
(S)-(−)-α-pinene	98	Aldrich	6	4.1±1.3	25	40.6±7.7
β-myrcene	>99	Aldrich	6	19.3±9.5	25	24.2±2.9
(+)-limonene	96	AlfaAesar	25	556.3±119.8	25	28.1±3.2
(−)-limonene	90	Acros	ND[5]	–	ND	–
(E)-β-ocimene	>90	Fluka	25	309.5±102.8	25	20.6±1.7
(±)-linalool	97	Fluka	12	49.9±6.5	25	22.9±7.9
Sesquiterpenes						
β-caryophyllene	>80	Sigma	6	6.5±0.5	25	1.5±1.5
(E,E)-α-farnesene	mixture	Bedoukian	6	0.4±0.1[6]	25	UD[7]
Methyl salicylate	>99	Sigma-Aldrich	6	41.1±10.3	25	5.3±2.7

[1] Amount of undiluted compound

[2] mean ± SE (μg/min)

[3] Amount of a 100 μM solution

[4] mean ± SE (ng/min)

[5] ND = not determined

[6] Based on highest GC peak

[7] UD = undetected

Electroantennography: Dose Dependent Response The electroantennography apparatus (Syntech Ltd., Hilversum, The Netherlands) was linked to a desktop computer (with IDAC-02 data acquisition interface board) on which recording, storing, and quantifying EAG responses were performed. The recording and indifferent electrodes were silver wires enclosed in drawn glass capillary tubes filled with phosphate buffered saline (NaCl, 4 g; Na_2HPO_4, 0.57 g; KH_2PO_4, 0.1 g; KCl, 0.1 g in 500 ml distilled water; pH 7.4). Antennal preparations were made by cutting off the head with a scalpel and mounting it on the indifferent electrode. Care was taken not to damage the antennae or mouthparts. Both antennae remained intact, and the recording electrode was placed on the tip of one randomly chosen antenna. The antennal preparation was bathed continuously by a stream of charcoal-filtered and humidified air at a flow rate of 1 l/min. Air temperature and relative humidity was measured ca. 15 cm from the antennal preparation (overall ranges for all trials: 18.4–25.6°C, 15–56% RH).

EAG recording began 6 min after the antennal preparation was set up. At this time, the following test protocol was used for each recording trial. The controls were tested in the following order (1, 2, 3, 2), after which six randomly chosen chemical treatments were tested. For each chemical, order of delivery of the four doses was random. Delivery of controls (2, 3, 2) was made after each four-dose-series of a chemical treatment. The same chemical was tested at the beginning and end of each recording trial, allowing correction for decrease of antennal response over the course of the trial. After the final chemical treatment for each recording, controls were presented in the following order (2, 3, 2, 1). Presentation of controls throughout the recording session permitted standardization of antennal responses, and correction for antennal fatigue. Test compounds and controls were applied (0.5 sec pulse) at 30 sec intervals separated by a purge of filtered and humidified air via an aluminum tube ca. 5 mm from the antenna. EAGs were measured as maximum amplitude of depolarization

(mV). Each chemical was tested on ≥20 individuals of each gender.

Olfactometry Bioassays were conducted in a, glass Y-tube olfactometer (40 mm diam by 36-cm long) that had a 50° inside angle (Blackmer et al. 2004). Incoming air was filtered through activated charcoal and humidified with doubly distilled, deionized water. The filtered air was split between two 2-l holding chambers; one chamber served as a control (clean air) and the other held the test material. From each holding chamber, air passed into the respective arms of the Y-tube, and then through a fine-meshed nylon organdy screen before entering the main tube of the olfactometer. Airflow through the system was maintained at 4.0 l/min (=3.2 m/min inside the tube) by an inline flowmeter (Gilmont Instr., Barnant Co., Barrington, IL, USA). A smoke test demonstrated laminar airflow throughout the olfactometer. Modifications of the Y-tube setup allowed us to examine the insect's response to both synthetic volatile compounds and a plant visual cue. A light emitting diode (Green LED, NSPG520S, Nichia America Corp., Mountville, PA, USA) was used to simulate a visual plant cue. The light emitted a narrow wavelength in the range of 565±10 nm, and power was supplied by a universal adapter that provided 6 V DC. An LED was inserted behind the organdy screen at the far upwind end of one arm of the Y-tube setup and was flush with the side wall of the tube to eliminate turbulence in the air flow. A 60-cm long, wide-spectrum fluorescent lamp (GE, F20T12-PL/AQ) was positioned 22 cm above the arms of the Y-tube. Before each trial, light intensity over each arm was measured with a light meter (ExTech Instr. Model 401025, Zefon International, St. Petersburg, FL, USA), and the tube was adjusted until intensity was the same in both arms. Light intensity averaged 686.5±3.5 (mean ± S.E.) lux. The Y-tube setup was surrounded by a 50×70×60-cm wooden enclosure painted flat black, and the holding chambers containing the treatments were placed outside the enclosure.

Bioassays A 'trial' refers to all of the behavioral assays conducted each day. Approximately 30–60 min before a trial was initiated, 7 to 17-d-old adult *L. hesperus* were placed into individual tubes as described previously (Blackmer et al. 2004; Blackmer and Cañas 2005). In each day's trials, 20–30 insects were bioassayed. One adult was placed inside the tube, and the end where the bulb tip had been removed was sealed with a cork. Tubes containing bugs were then placed into a separate holding container, so they would not be exposed to test odors before their release. Male and female bugs were included in each trial. For each individual, a clean Y-tube was used. Previous experiments (Blackmer et al. 2004) showed that the responses of experienced and 'naïve' (insects reared in the same manner as the present study) *L. hesperus* to plant volatiles were similar, thus only naïve individuals were used in the present study. Also, Blackmer and Cañas (2005) demonstrated previously that visual and volatile cues in combination resulted in the strongest upwind response by *L. hesperus*. Therefore, in the present study, all synthetic compounds were tested in combination with the LED. At the beginning of each individual assay, the cork was removed from the release tube, and the open end was placed at the downwind end of the Y-tube. Each insect was given 5 min to respond to the treatment, and a choice for the left or right arm of the olfactometer was recorded when the insect walked 1 cm beyond the Y-junction. The following measurements were recorded for all individuals: time required for bug to exit the release tube, percentage exiting release tube, response time to first choice, percentage walking upwind and selecting an arm of the Y-tube, and percentage that went 'all the way' (ATW) to the end of the Y-tube (i.e., exhibited a complete response). Temperature and RH in the olfactometer were maintained at 25.6±0.1°C and 29.9±0.9%, respectively.

Assays were continued until a minimum of 40 individuals made a choice between arms. This sometimes required the testing of more than 100 individuals for each chemical treatment. Bioassays were conducted between 10.00–18.00 h, and the same treatment usually was run on consecutive days until it was completed. Y-tube trials were setup with treatments of a synthetic plant volatile in combination with the green LED cue vs. a humidified blank air control without an LED cue. Additional trials were setup with a humidified blank air control with the green LED vs. a humidified blank air control without an LED cue. Trials that used the LED in the absence of synthetic volatile compounds were included to facilitate comparison of the response to the visual cue only vs. visual plus volatile cues. Sixteen volatiles were tested, which included the following undiluted synthetic compounds: six green leaf volatiles (GLVs), seven monoterpenes, two sesquiterpenes, and methyl salicylate (Table 1). The volume (6 μl) used was selected based on preliminary observations of the insect's upwind response to the test compounds. Higher volumes were used for (+)-limonene, (*E*)-β-ocimene, and (±)-linalool to mimic the pattern of emissions from *Lygus*-induced cotton (Rodriguez-Saona et al. 2002) and alfalfa (Blackmer et al. 2004) (compare Table 1).

Relationship Between Volatile Emission Rates and Lygus Response Although the same amounts were used for most volatiles in our experiments, their emission rates may vary depending on volatility. Thus, a study was conducted to determine the release rates of individual compounds used in olfactometer and EAG assays. For this, a known amount of each volatile compound was applied to a filter paper strip following the methods described above. Filter papers were

placed under a hood for 5 min. Each filter paper then was placed separately inside a 20 ml (internal volume) glass vial for volatile collections. The lid of the vial had two openings, one of these fitted an adsorbent trap containing 30 mg of Super-Q (Analytical Research Systems, Inc., Gainesville, FL, USA), while the other fitted a Pasteur pipette containing ca. 1 g of activated charcoal (Alltech, Deerfield, IL, USA) to clean the incoming air. Volatiles from inside the vials were collected in traps at a constant rate of 2 ml/min, measured with the aid of a flow meter (Watchman II, Kontes Glass Co., Vineland, NJ, USA) connected to a 12 V vacuum pump (Sensidyne, Clearwater, FL, USA). Volatiles were sampled for 5 min under standard laboratory conditions. For each synthetic volatile, the emission rate was recorded from 3 independent replicates. Because the emission rates were measured in closed, small glass vials (20 ml), they may not necessarily reflect the emission rates encountered by insects in the main tube of the olfactometer. The same may be true for EAG assays.

The volatiles trapped in Super-Q adsorbent were eluted using 150 µl dichloromethane, and 400 ng of *n*-octane were added as an internal standard. One µl of each sample was injected onto a Hewlett Packard 6890 gas chromatograph equipped with an HP-1 column (10 m×0.53 mm×2.65 µm; Agilent Technologies, Palo Alto, CA, USA), using a splitless mode with helium as the carrier gas, and a temperature program of 40°C for 1 min, increased gradually at 14°C/min to 180°C (held for 2 min), then at 40°C/min to 200°C (for 2 min), and finally to 220°C (for 5 min). The amount of each volatile compound was calculated in relation to the area of the internal standard.

Data Analyses Maximum EAG responses were control-adjusted with the paraffin oil only control, and expressed as proportional responses relative to the octanal standard. These data then were square root-transformed ($0.5(\sqrt{x} + \sqrt{x+1})$) (Zar 1996), and analysis of variance, PROC MIXED (SAS Institute 2003), was used to compare maximum EAG deflection between gender and plant volatile concentrations. Regression analysis was also used to assess the influence of gender and plant volatile treatment on EAG amplitude (PROC REG and PROC MIXED) (SAS Institute 2003). Due to heteroscedasticity over concentrations, a weighted regression (reciprocal of the variance) was calculated.

For behavioral trials, the null hypothesis that *L. hesperus* showed no preference for either olfactometer arm (a response of 50:50) was analyzed with a Chi-square goodness of fit test after correcting for continuity with Yates' correction factor (Zar 1996). Occasionally, an insect would move back and forth between arms of the Y-tube and when this happened the final choice was considered to be the arm the insect spent the most time in. When the insect went all the way (ATW) to the end of the tube, this was considered to be the final choice and was recorded as 'complete response'. Time required for male and female bugs to exit the release tube, percentage of bugs exiting the release tube, percentage walking upwind into an arm, response time to first choice, and percentage walking upwind to the end of an arm (ATW) for controls vs. treatments were compared by two-way ANOVAs (SigmaStat Software version 3.5). For each gender, time spent in each arm of the arena, number of individuals walking upwind into an arm, and ATW responses between volatile treatments+LED and control+LED were compared by Pearson's goodness of fit Chi-square test for independent samples. Percent data were transformed by the arcsine function, and exit time and time to first choice data by the logarithmic function when needed to meet the requirements of normality and homogeneity of variance before analyses. Means and standard errors were calculated for volatile release data.

A Pearson correlation analysis was conducted to determine if the effects of plant volatiles on *L. hesperus* EAG and behavioral responses were due to the amount of chemical used. First, we correlated emission rates with EAG responses for the highest concentration tested (100 µM) for all compounds. Another analysis was conducted to correlate emission rates with the percent response to each compound in behavioral assays (percentage walking upwind into an arm). Analyses were performed for males and females separately.

Voucher specimens of *L. hesperus* are deposited in the National Entomological Collection, National Museum of Natural History, Smithsonian Institution, Washington, D.C.

Results

Electroantennography Dose-Dependent Responses Average EAG responses by males were greater than by females (F=4.86; df=1, 54.3; P=0.032) (Fig. 1). This difference in EAG response was observed for 1-hexanol at 1 µM (F=2.48; df=1, 1344; P=0.013), for *(Z)*-3-hexenol and *(E)*-3-hexenol at 100 µM (F=3.36 and 3.18, respectively; df=1, 1316 and 1329, respectively; P<0.001 and P=0.002, respectively), for *(Z)*-3-hexenyl acetate at 10 µM (F=2.96; df=1, 1362; P=0.003), and for *(E)*-2-hexenyl acetate at 10 and 100 µM (F=3.89 and 5.55, respectively; df=1, 1315; P<0.001). In contrast, EAG response by females was greater than by males for methyl salicylate at 100 µM (F=2.63; df=1, 1295; P<0.009) (Fig. 1).

Regression analysis of the impact of gender and host plant volatiles on antennal response indicated a linear response between antennal sensitivity (y) and plant volatile concentration (log10×) (Table 2). Contrasts of slopes between the

Female
Male

(E)-3-hexen-1-ol **

(Z)-3-hexen-1-ol ***

1-hexanol *

(E)-2-hexenal

(E)-2-hexenyl acetate *** ***

(Z)-3-hexenyl acetate **

(R)-(+)-α-pinene

(S)-(-)-α-pinene

β-myrcene

(+)-limonene

(E)-β-ocimene

(±)-linalool

β-caryophyllene

(E,E)-α-farnesene

Methyl salicylate **

EAG Amplitude (mV ± SE)

Concentration in Pipette Cartridge (μM)

◀ **Fig. 1** EAG dose response curves of *Lygus hesperus* to 15 synthetic plant volatiles. EAG amplitudes (mean ± SE) are control-adjusted and presented as relative responses to the standard, 100 µM octanal. Each compound was tested on ≥20 individuals of each gender; concentrations (0.1, 1.0, 10, and 100 µM) of each compound in paraffin oil. Significant differences between genders are noted by asterisks (*P= 0.05−0.01, **P=0.01−0.001, ***P<0.001). P values correspond to ANOVA

genders revealed greater (P<0.05) slopes for males for 3 of the 15 plant volatiles (Table 2). Ranked in order of P values (most to least significant), the three significant compounds were: *(E)*-3-hexenol < *(E)*-2-hexenyl acetate < *(Z)*-3-hexenol (Table 2). For methyl salicylate, the slope for females was marginally greater than for males (P=0.056) (Table 2).

Olfactometer Bioassays Females remained longer in their release tube than did males (F=13.31; df=1, 31; P<0.001; 37.1±2.1 vs. 24.8±1.8 sec), but the percentage of females and males exiting the release tubes was not significantly different (F=3.32; df=1, 31; P=0.080; 88.3±1.5 vs. 93.0± 1.6%). Of the individuals that exited the release tube, significantly more males than females progressed upwind to the Y-junction (F=9.11; df=1, 31; P=0.005; 83.6±1.7 vs. 76.0±1.7%), and males were faster than females in making a choice between arms (F=17.15; df=1, 31; P<0.001; 76.6± 3.4 vs. 104.0±3.3 sec). A similar percentage of females and males walked ATW to the end of the Y-tube, regardless of treatment (F=2.33; df=1, 25; P=0.113; 23.7±1.9 vs. 19.8± 1.2% for treatment and F=1.92; df=1, 27; P=0.218; 28.5± 1.4 vs. 18.8±1.0% for control, respectively). There was no difference in ATW response between treatment+LED vs. control+LED (t=1.12; df=1, 62; P=0.268; 21.8±1.2 vs. 23.6±1.2%).

In terms of response to the treatments vs. controls, females exhibited significant (P<0.05) movement upwind into the volatile+LED arm when *R)*-(+)-α-pinene, *(E)*-β-ocimene, or *(E,E)*-α-farnesene was tested (Fig. 2a). Females exhibited significant (P<0.05) movement upwind into the control +LED arm when *(Z)*-3-hexenol, *(S)*-(−)-α-pinene, or methyl salicylate was offered in the volatile+LED arm (Fig. 2a). Significant ATW response by females was observed to control+LED when 1-hexanol and β-myrcene was tested (Fig. 2a). Males exhibited significant movement upwind into control+LED arm when offering *(E)*-2-hexenyl acetate, (±)-linalool, *(E,E)*-α-farnesene, or methyl salicylate in the volatile+LED arm (Fig. 2b). Significant ATW response by males was observed to control+LED when methyl salicylate was used in the volatile+LED arm (Fig. 2b).

Table 2 Regression equations, F values, and significance levels for contrasts of slopes (female versus male) describing the effects of plant volatiles on *Lygus hesperus* antennal response

Plant volatiles	Female		Male		Denominator df[2]	F	P
	Equation[1]	R^2	Equation[1]	R^2			
Green leaf volatiles							
(E)-3-hexen-1-ol	1.62+0.011(×)	0.434	1.43+0.019(×)	0.534	116	12.18	<0.001
(E)-2-hexenyl acetate	1.63−0.001(×)	0.004	1.58+0.010(×)	0.229	116	8.47	0.004
(Z)-3-hexen-1-ol	1.37+0.013(×)	0.673	1.52+0.017(×)	0.419	112	7.03	0.009
1-hexanol	1.57+0.011(×)	0.534	1.75+0.012(×)	0.515	96	0.21	0.647
(Z)-3-hexenyl acetate	1.32+0.004(×)	0.138	1.62+0.004(×)	0.103	88	0.06	0.805
(E)-2-hexenal	1.35+0.010(×)	0.293	1.39+0.010(×)	0.282	179	0.00	0.957
Monoterpenes							
(R)-(+)-α-pinene	1.34−0.002(×)	0.050	1.41−0.002(×)	0.107	116	0.56	0.455
(S)-(−)-α-pinene	1.24+0.002(×)	0.109	1.28+0.000(×)	0.003	180	2.56	0.111
β-myrcene	1.48−0.001(×)	0.017	1.61+0.001(×)	0.017	132	0.00	0.973
(+)-limonene	1.33+0.002(×)	0.040	1.44−0.002(×)	0.027	128	1.97	0.163
(E)-β-ocimene	1.64+0.003(×)	0.061	1.60+0.002(×)	0.026	100	1.77	0.187
(±)-linalool	1.70+0.005(×)	0.185	1.63+0.005(×)	0.222	116	0.01	0.913
Sesquiterpenes							
β-caryophyllene	1.42+0.001(×)	0.007	1.67+002(×)	0.056	76	1.22	0.273
(E,E)-α-farnesene	1.83+0.002(×)	0.014	1.80+0.001(×)	0.003	124	0.31	0.577
Methyl salicylate	1.53+0.004(×)	0.154	1.48+0.001(×)	0.020	180	3.71	0.056

[1] Antennal response = a + b (log10 concentration)

[2] Numerator df = 1

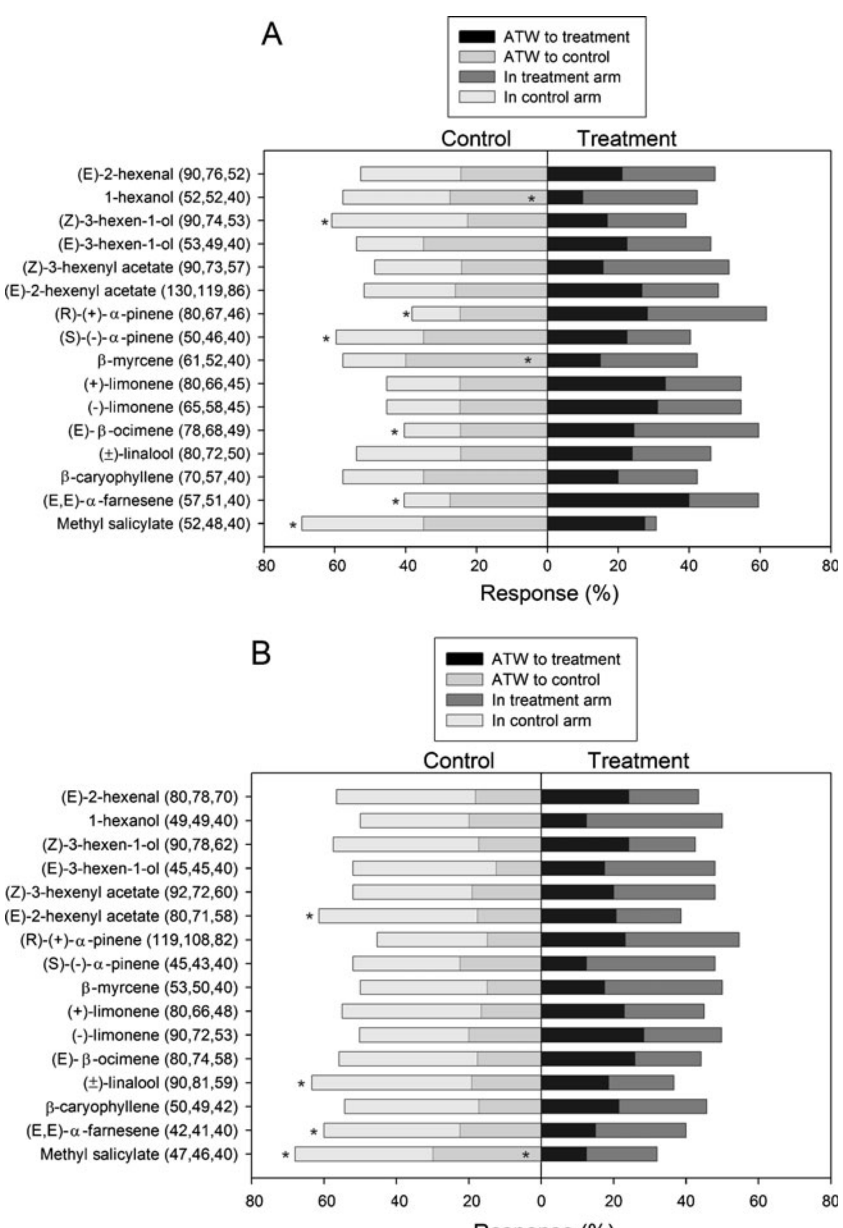

Fig. 2 Behavioral response by female (**a**) and male (**b**) *Lygus hesperus* adults to synthetic plant volatiles + green light-emitting diode (LED) and blank air control + green LED. Asterisk *inside* a column denotes significant difference ($P<0.05$) in locomotion ATW ('all the way') to the end of a Y-tube arm; asterisk *outside* a column denotes significant difference ($P<0.05$) in individuals walking upwind into a Y-tube arm (Pearson's goodness of fit *Chi-square* test for independent samples). Numbers in parentheses indicate total number of insects released, number of insects that exited release tube, and number of insects that moved upwind, respectively

Relationship Between Volatile Emission Rates and Lygus Response Emission rates of plant volatiles in EAG experiments varied from 1.5 ng/min for β-caryophyllene to 41.3 ng/min for (*R*)-(+)-α-pinene (Table 1). Female EAG responses were negatively correlated with emission rates (Pearson correlation = −0.545; P=0.036), indicating that *Lygus* female antennae exhibited stronger responses to highly volatile compounds (e.g., GLVs and monoterpenes). Although the trend was the same for males, the correlation between emission rates and EAG responses in male *L. hesperus* was not significant (Pearson correlation=−0.3; P= 0.278). Emission rates for the behavioral study were several orders of magnitude higher than for the EAG study and varied from 0.4 μg/min for (*E*,*E*)-α-farnesene to 556.3 μg/min for (+)-limonene (Table 1). There was, however, no correlation between emission rates and behavioral responses (percentage walking upwind into an arm) for males (Pearson correlation = 0.054; P=0.849) or

females (Pearson correlation = 0.25; *P*=0.368), indicating that the response of *L. hesperus* to a volatile in our Y-tube experiments was independent of the volatile's emission rate.

Discussion

This study is the first to document the EAG and behavioral responses of *L. hesperus* to host plant volatiles. EAG analyses clearly revealed that *L. hesperus* perceived the compounds tested. Although male antennae were generally more responsive than female antennae, both genders responded to most plant volatiles. For green leaf volatiles, alcohols generally elicited stronger responses by both genders than did the aldehyde or acetates. A strong response to plant alcohols also was reported for *L. lineolaris* by Chinta et al. (1994), although differences between genders were only observed for *(E)*-2-hexenal, where female responses were greater. In the present study, *L. hesperus* showed no gender effect to *(E)*-2-hexenal, but male response was greater than female for *(E)*-3-hexenol, *(Z)*-3-hexenol, and *(E)*-2-hexenyl acetate. However, *L. lineolaris* showed no gender differences in response to *(E)*-2-hexenyl acetate (Chinta et al. 1994). Another mirid, *Lygocoris pabulinus* (L.), also showed no gender differences for *(E)*-2-hexenyl acetate or *(Z)*-3-hexenyl acetate, but female antennae showed greater EAG response to 1-hexanol than did male antennae (Groot et al. 1999).

Previous studies with other mirids have shown that their EAG response to most monoterpenes is relatively low compared to other chemical groups, but when gender differences occur, females usually are more responsive than males (Chinta et al. 1994; Groot et al. 1999). Our results with *L. hesperus* are consistent with these studies in that *L. hesperus* showed low EAG responses to all the monoterpenes and sesquiterpenes tested; however, no gender differences were found for any of the terpenes. For example, female *L. pabulinus* and *L. lineolaris* antennae were more sensitive to (±)-linalool than were male antennae (Chinta et al. 1994; Groot et al. 1999), although *L. hesperus* showed no gender effect for this monoterpene. *Lygus hesperus* was least responsive to the monoterpenes β-myrcene, β-caryophyllene, (+)-limonene, and both enantiomers of α-pinene. Gender differences for methyl salicylate were observed for both *L. hesperus* (in the present study) and *L. pabulinus* (Groot et al. 1999); female antennae were more sensitive than male antennae in both cases. EAG responses to *(E)*-β-ocimene, *(E,E)*-α-farnesene, and methyl salicylate were moderate relative to GLVs, and had similar dose—response curves, except for farnesene, which had a somewhat elliptical shape. Both genders of *Lygus rugulipennis* Poppius showed EAG responses to several GLVs (*(Z)*-3-hexenol, 1-hexanol, and *(Z)*-3-hexenyl acetate) emitted by healthy plants, and to methyl salicylate and β-caryophyllene emitted by *Lygus*-damaged plants (Frati et al. 2009).

In Y-tube behavioral assays of ATW movement and number of bugs in the treatment arm, 13 of the 64 comparisons (of behavioral response, gender, and volatile combinations) were significant. When considering these 13 comparisons, the bugs showed movement toward the treatment only in three of them. These were for female bugs tested with *(R)*-(+)-α-pinene, *(E)*-β-ocimene, and *(E,E)*-α-farnesene for number of individuals in the treatment arm. The results are consistent with our previous studies that show that female *L. hesperus* are attracted to plant odors (Blackmer et al. 2004; Blackmer and Cañas 2005). Blackmer et al. (2004) showed that female *L. hesperus* are attracted strongly to flowering alfalfa with feeding damage and vegetative alfalfa with conspecifics. They also found that vegetative alfalfa damaged by conspecifics (nymphs and adults) emits large quantities of *(E)*-β-ocimene and *(E,E)*-α-farnesene, while flowering alfalfa damaged by adults emits large quantities of *(E)*-β-ocimene. Thus, ours and previous findings indicate that *(E)*-β-ocimene and *(E,E)*-α-farnesene may play key roles in the attraction of female *L. hesperus* to host plants. The role of *(R)*-(+)-α-pinene is less clear because this compound was emitted in low quantities both constitutively and after *Lygus* feeding damage in alfalfa (Blackmer et al. 2004). In the 10 remaining significant comparisons, more bugs were found in the control arm, thus suggesting repellency. Males either did not respond to plant volatiles or were repelled by them, e.g., by (*E*)-2-hexenyl acetate, (±)-linalool, *(E,E)*-α-farnesene, and methyl salicylate. This also is consistent with previous studies that show that males *L. hesperus* are repelled by volatiles from vegetative and flowering alfalfa (Blackmer et al. 2004). Our results unlikely are due simply to the amount of compound used in the behavioral experiments but instead to the way the insect perceives each volatile because emission rates were not correlated with the insect's response. Likewise, *L. rugulipennis* females generally were more responsive than males to host plant and conspecific odors in a wind tunnel and vertical open Y-track olfactometer (Frati et al. 2008). Although we refer to the bugs used in this study as 'naïve', they were, in fact, reared on hosts (green beans and carrots) that might have produced volatiles to which the bugs became conditioned prior to the olfactometer trials. Although Blackmer et al. (2004) demonstrated no difference in *L. hesperus* behavior between experienced and 'naïve' bugs, it is not known if *L. hesperus* held without food prior to the trials would have behaved differently from bugs used in the present study. However, because *L. hesperus* usually is found on host plants where it can feed, it is likely that behavioral

responses exhibited by the bugs in our study are representative of those exhibited under field conditions.

Results of the behavioral study did not always reflect the results of the EAG measurements. For example, EAG response to (*S*)-(−)-α-pinene was weak for both genders, but a strong behavioral response to this monoterpene was observed for both male and female bugs. Conversely, both genders responded strongly to 1-hexanol in EAG trials, but neither gender exhibited a behavioral response to this chemical. Finally, male antennae responded more strongly to *(E)*-2-hexenyl acetate than did female antennae, but females exhibited a behavioral response to this chemical, whereas males did not. In general, although males showed stronger responses than females, both *L. hesperus* genders showed strong EAG responses to the highly volatile GLVs, whereas behavioral responses to plant volatiles varied widely between genders and among different chemical classes, and, thus, was uncorrelated to the volatiles' emission rates. Inconsistencies between electrophysiological and behavioral results are not uncommon (Mayer et al. 1984; Suckling et al. 1996; Wee et al. 2008), and illustrate the importance of using multiple research approaches. Here, we provide three possible explanations for these discrepancies. First, in the present study we tested only single plant compounds. It is likely that more than one plant volatile is needed to elicit a behavioral response in *L. hesperus*. For instance, although male *L. hesperus* has a stronger EAG response than females to the plant alcohols and acetates, its behavioral response towards these compounds may depend on their concentration in the presence of other host plant odors. Second, the present study used only EAG recording to compare with behavioral studies. Electrophysiological studies that use the single-cell technique may show more correspondence with behavioral trials than EAG recordings, which measure the summation of responses by all neurons on the antenna (Anderson et al. 1993; Bjostad 1998). Finally, our current understanding of host plant finding by *Lygus* suggests that several distinct steps, involving long-range and close-range orientation, are involved. Thus, behavioral studies may require a variety of approaches that use different arenas in the lab (e.g., Y-tube olfactometers for walking, and wind tunnels for flight) and field to understand the hierarchical nature of orientation behaviors. Inconsistencies between EAG and behavioral trials notwithstanding, our results draw attention to important points in *Lygus*—plant interactions. Attraction was observed in *L. hesperus* females towards several constitutive and inducible plant volatiles. These chemical cues may help females locate plants that are suitable hosts for feeding, oviposition, and feeding of offspring (Groot et al. 1999; Innocenzi et al. 2004, 2005; Frati et al. 2008). In comparison, repellency was observed commonly in *L. hesperus* males in response to several plant volatiles. Males may use these volatiles to avoid plants that harbor potential competing males (e.g., Pallini et al. 1997), or attract greater numbers of natural enemies (e.g., Turlings et al. 1990; De Moraes et al. 1998).

Recently, there has been increased awareness of the importance of multimodal cues in host-finding by insects. While our results showed that single compounds can be used to attract *L. hesperus* in Y-tubes, both visual and volatile information is important in the orientation behaviors of this insect, and probably need to be incorporated into any trapping system to maximize trap catch. In fact, the addition of a plant visual cue (green LED) in combination with plant volatile cues enhanced the upwind responses of both female and male *L. hesperus* (Blackmer et al. 2004; Blackmer and Cañas 2005). In the future, electrophysiological studies that use single-cell recordings coupled with GC might identify additional compounds detected by *L. hesperus*. Also, further bioassays that use blends of synthetic plant volatiles with experimentally altered ratios and concentrations, and volatile blends emitted by plants and concomitant collection of the volatiles for GC-MS, might give a clearer picture of the relationship between plant volatiles and *L. hesperus* behavior.

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The Herbivore-Induced Plant Volatile Methyl Salicylate Negatively Affects Attraction of the Parasitoid *Diadegma semiclausum*

Tjeerd A. L. Snoeren · Roland Mumm · Erik H. Poelman · Yue Yang · Eran Pichersky · Marcel Dicke

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Abstract The indirect defense mechanisms of plants comprise the production of herbivore-induced plant volatiles that can attract natural enemies of plant attackers. One of the often emitted compounds after herbivory is methyl salicylate (MeSA). Here, we studied the importance of this caterpillar-induced compound in the attraction of the parasitoid wasp *Diadegma semiclausum* by using a mutant Arabidopsis line. *Pieris rapae* infested *AtBSMT1-KO* mutant Arabidopsis plants, compromised in the biosynthesis of MeSA, were more attractive to parasitoids than infested wild-type plants. This suggests that the presence of MeSA has negative effects on parasitoid host-finding behavior when exposed to wild-type production of herbivore-induced Arabidopsis volatiles. Furthermore, in line with this, we recorded a positive correlation between MeSA dose and repellence of *D. semiclausum* when supplementing the headspace of caterpillar-infested *AtBSMT1-KO* plants with synthetic MeSA.

Key Words Herbivore-induced plant volatile · SABATH methyl transferase · *BSMT1* · Methyl salicylate · Parasitoid host-location behaviour · Herbivory · Volatile emission

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T. A. L. Snoeren (✉) · R. Mumm · E. H. Poelman · M. Dicke
Laboratory of Entomology, Wageningen University,
P.O. Box 8031, 6700 EH Wageningen, the Netherlands
e-mail: tjeerd.snoeren@wur.nl

Y. Yang · E. Pichersky
Department of Molecular, Cellular and Developmental Biology,
University of Michigan,
Ann Arbor, MI 48109, USA

Present Address:
R. Mumm
Plant Research International B.V.,
P.O. Box 619,
6700 AP Wageningen, the Netherlands

Present Address:
Y. Yang
Department of Plant Biology, Michigan State University,
East Lansing, MI 48824-1312, USA

Introduction

Plants effectively combat herbivorous insects through direct and indirect defenses (Kessler and Baldwin 2002). Direct defense comprises the production and storage of metabolites that negatively influence herbivore performance (Wittstock and Gershenzon 2002). In contrast, indirect defense encompasses the production of metabolites that benefit the natural enemies of herbivores (Dicke et al. 1999; Wäckers et al. 2001; Halitschke et al. 2008; Kost and Heil 2008). Natural enemies such as parasitoids use herbivore-induced plant volatiles (HIPVs) to locate their herbivore host and thereby indirectly aid plants in their combat against herbivorous insects. HIPVs are complex blends of compounds and mainly comprise green leaf volatiles (GLV) (C6 aldehydes, alcohols, and derivatives), terpenoids, and phenolics (Paré and Tumlinson 1997; Dicke 1999b). Green leaf volatiles originate from linolenic and linoleic acid, which are released particularly when cells are damaged (Bate and Rothstein 1998). Terpenoids are synthesized via the mevalonic acid (MVA) or methylerythritol phosphate (MEP) pathway (Dudareva et al. 2006; Gershenzon and Dudareva 2007). Finally, aromatic compounds, such as

methyl salicylate (MeSA) and indole, are formed via the shikimic acid pathway (Paré and Tumlinson 1997). The induced volatile production is orchestrated by at least three main signal-transduction pathways: the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways (Dicke and Van Poecke 2002; Kessler and Baldwin 2002). These pathways can be induced differentially by different herbivore species (Heidel and Baldwin 2004; De Vos et al. 2005; Schmidt et al. 2005), leading to the emission of a volatile blend that is specific for an herbivore species (Vet and Dicke 1992; Ozawa et al. 2000; Walling 2000; Leitner et al. 2005). These herbivore-specific volatile blends can provide foraging natural enemies of herbivores, such as predators and parasitoid wasps, with detectable and reliable information to locate their prey or host respectively (Dicke et al. 1990; Turlings et al. 1991; Vet and Dicke 1992; Du et al. 1998; Dicke 1999a).

Variation in attraction of carnivorous arthropods towards host or non-host infested plants is ascribed mainly to the presence and relative abundance of attractive compounds within the HIPV blend (D'Alessandro et al. 2009). An intriguing question is which components of the complex HIPV blend affect parasitoid attraction most. This will be different for different parasitoid species. Up to now, several approaches have been applied to study the relative importance of certain HIPVs in the attraction of carnivorous arthropods, e.g., by offering synthetic compounds alone or in mixtures, inducing certain subsets of the HIPV blend with elicitors, or manipulating signal-transduction or biosynthetic pathways through a molecular genetic approach (for review see D'Alessandro and Turlings 2006; Snoeren et al. 2007; Schroeder and Hilker 2008). One HIPV component for which biological relevance for carnivorous arthropod attraction has been addressed is methyl salicylate (MeSA) (De Boer and Dicke 2004; De Boer et al. 2004; James and Price 2004; Zhu and Park 2005; Ishiwari et al. 2007). This methyl ester of the plant hormone salicylic acid (SA) has been reported in the HIPV blends emitted by several plant species, e.g., lima bean (Dicke et al. 1990), tomato (Ament et al. 2004), cabbage (Geervliet 1997; Poelman et al. 2009), and Arabidopsis (Van Poecke et al. 2001; Chen et al. 2003; Snoeren et al. 2009). Its role in the attraction of carnivorous arthropods has been addressed by investigating the response towards synthetic MeSA in field (James and Price 2004; Zhu and Park 2005) and in laboratory studies (Dicke et al. 1990; De Boer and Dicke 2004; De Boer et al. 2004; Ishiwari et al. 2007). However, so far, no studies have addressed the effects of the absence of MeSA from an otherwise complete HIPV blend on the behavior of carnivorous arthropods.

In this study, we focused on the function of MeSA within the HIPV blend through a molecular ecological approach that involves the elimination of MeSA. MeSA is synthesized by SA carboxyl methyltransferase (SAMT), a member of the SABATH methyl transferase family, to which jasmonic acid, indole-acetic acid and cinnamic/*p*-coumaric acid methyltransferases also belong (Seo et al. 2001; Chen et al. 2003; Zubieta et al. 2003; Kapteyn et al. 2007). Related enzymes that methylate benzoic acid (BA) to give MeBA also have been reported (Murfitt et al. 2000). Some SABATH enzymes can methylate both SA and BA with roughly equal efficiencies, and have been designated as Benzoic Acid and Salicylic Acid Methyl Transferases (BSMTs). One such example is the *Arabidopsis thaliana* gene designated *BSMT1* (Chen et al. 2003).

While SAMT or BSMT enzymes have been identified in a number of plant species, including fairy fans (*Clarkia breweri*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), Arabidopsis, and jasmine (*Stephanotis floribunda*), we selected Arabidopsis for further work on the physiological significance of MeSA emission because of the availability of molecular genetic tools and because this species is a valuable stepping stone towards other brassicaceous plants for studying the role of HIPVs in plant–insect interactions (Snoeren et al. 2007). We addressed the role of MeSA in the foraging behavior of the parasitoid wasp *Diadegma semiclausum* Hellén (Hymenoptera, Ichneumonidae) that attacks caterpillars feeding on brassicaceous plants including Arabidopsis. Caterpillar feeding is known to induce MeSA in Arabidopsis (Van Poecke et al. 2001; Chen et al. 2003; Snoeren et al. 2009). We used wild-type Arabidopsis plants and a knock-out mutant that does not have a functional BSMT and, thus, no MeSA biosynthesis. We addressed the effects of the mutation on parasitoid- and herbivore behavior, and on headspace composition.

Methods and Materials

Plants and Insect Material An Arabidopsis line with an insertion in the *AtBSMT1* gene was obtained from the Torrey Mesa Institute collection, and the position of the insertion was verified by sequencing (Fig. 1, see also supporting information Fig. S1). Arabidopsis seeds (*A. thaliana* wild-type Columbia (Col-0) and *AtBSMT1-KO* in a Col-0 background) were germinated in sandy Arabidopsis soil (Lentse potgrond BV, Lent, Netherlands), and cultivated in a growth chamber at 21±2°C, 50 to 60% relative humidity (RH), and 8:16 hr, L:D photoperiod with 80 to 110 μmol m^{-2} sec^{-1} PPFD. The soil was heated to 90°C for at least 2 hr prior to sowing of the plants. Two-wk-old seedlings were transferred to plastic containers (5 cm diam) filled with the same soil type. Plants were watered twice a week. To prevent infestation by root-feeding sciarid flies, the soil was treated weekly with the entomopathogenic nematode *Steinernema feltiae* (Koppert Biological Systems,

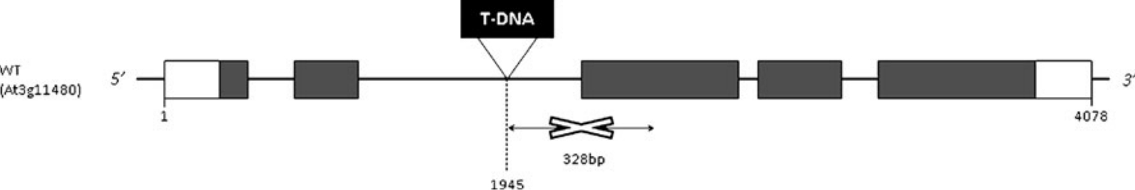

Fig. 1 Illustration of the *AtBSMT* T-DNA mutant (GARLIC_776_B10 Line) from The Torrey Mesa Institute's T-DNA insertion mutant collection. The exons are indicated as grey boxes and nucleotide numbers are given below. The position of the approximately 200-nt T-DNA insertion, as well as a deletion of 328-nt extending downstream from the right border of the T-DNA element and including the end part of intron 2 and the beginning part of exon 3 also are shown. Both insertion and deletion were determined by sequencing. Furthermore, we determined that transcripts from the mutant gene were still produced in the plant, and the processed mRNA had a similar size to that of the wild type (WT) mRNA. However, when the mutant transcript was amplified by RT-PCR and sequenced, it was seen that in the absence of the original 3′ end of intron 2, a new 3′ splice site was used within the remains of exon 3. This led to a protein that is much shorter and does not have SAMT activity (See also Fig. S1)

Berkel en Rodenrijs, the Netherlands). Fully grown vegetative plants were used for the experiments, i.e., at 6–8 wk after sowing.

The small cabbage white butterfly, *Pieris rapae* L. (Lepidoptera, Pieridae), was reared on Brussels sprouts plants (*Brassica oleracea* var. gemmifera, cv Cyrus) in a growth chamber (16:8 hr, L:D; 20±2°C and 70% RH) as described in detail in Fatouros et al. (2005).

The parasitoid wasp *D. semiclausum* was reared on *Plutella xylostella* on Brussels sprouts in a climatized room (16:8 hr, L:D; 20±2°C and 70% RH). Emerging wasps were provided *ad libitum* with water and honey, and are referred to as 'naïve' wasps, as they had received neither exposure to plant material, nor obtained an oviposition experience. This parasitoid is known to be attracted to the volatiles emitted by *P. rapae*-infested Arabidopsis Col-0 plants (Loivamäki et al. 2008).

Plant Treatments Plants were infested by equally distributing 20 first-instar *P. rapae* larvae per plant over the fully expanded leaves. Uninfested plants that otherwise received a treatment similar to the infested plants were used as controls. In all experiments, plants from both genotypes that required an infestation were treated 24 hr before the experiments and kept in a climate room (21±2°C, 50–60% RH; 8:16 hr, L:D photoperiod, and 80–110 μmol m^{-2} sec^{-1} PPFD).

Y-tube Olfactometer Behavioral Assays The effects of HIPVs from wild-type Arabidopsis Col-0 and transgenic *AtBSMT1-KO* plants on parasitoid behavior were tested in a closed-system Y-tube olfactometer as described by Bukovinszky et al. (2005). In short, filtered air was led through activated charcoal and split into two air streams (4 L min^{-1}) that were led through five-liter glass vessels containing the odor sources consisting on each experimental day of 4 plants each with the feeding caterpillars. The olfactometer was illuminated with 4 high-frequency fluorescent tubes (Philips 840, 36 W) from above at an intensity of 60±5 μmol m^{-2} sec^{-1} PPFD. All experiments were conducted in a climatized room (20±2°C).

Naïve, 3–7-d-old female *D. semiclausum* were transferred individually from the cage into the Y-tube olfactometer on a plant leaf; this was done by using alternately a Col-0 or *AtBSMT1-KO* leaf, that had been infested previously by *P. rapae* and from which the caterpillars and their products had been removed carefully. This procedure increases the general behavioral response of the parasitoids to plant cues but does not induce a shift of preference (Kaiser and Carde 1992; Bleeker et al. 2006; Smid 2006). Upon release in the olfactometer, parasitoid behavior and parasitoid choice for one of the two odor sources was observed and scored as described in detail by Bukovinszky et al. (2005). Parasitoids that did not explore one of the two arms of the olfactometer within 5 min or that did not make a final choice within 10 min after release were considered as non-responding individuals, and were excluded from preference analysis. After every 5 parasitoids tested, the odor sources were interchanged to compensate for any unforeseen asymmetry in the set-up.

Experiment 1. Effect of AtBSMT Knock-out on Parasitoid Attraction to Caterpillar-infested Plants To assess the role of MeSA as a cue for parasitoids *in planta*, we compared parasitoid behavior in response to an HIPV blend that lacked MeSA vs. a complete HIPV blend that included MeSA. Caterpillar-infested mutant *AtBSMT1-KO* and wild-type Col-0 plants were offered as odor sources in the olfactometer to *D. semiclausum*.

Experiment 2. Supplementing Headspace of Caterpillar-infested AtBSMT1-KO Plants with Synthetic MeSA To investigate further the role of MeSA, we supplemented the HIPV blend of *AtBSMT1-KO* plants by adding synthetic MeSA (Merck, 99% pure). The supplemented HIPV blend from these *AtBSMT1-KO* plants was tested against the emitted HIPV blend from *AtBSMT1-KO* or Col-0 plants. To determine if parasitoid behavior to MeSA was dose

dependent, different doses of MeSA (0.2 μg, 2 μg, 20 μg, 200 μg) were added downwind to the earlier infested *AtBSMT1-KO* plants. MeSA was diluted in n-hexane (Sigma-Aldrich, 95%). In all experiments, 0.1 ml of the MeSA solution was applied on filter paper (15 cm^2) and positioned in the last section of the olfactometer arm. A piece of filter paper with 0.1 ml hexane was placed at a similar position in the other arm as a control. The solvent was allowed to evaporate for 30 to 60 sec, after which a parasitoid was introduced into the olfactometer. New filter papers with MeSA or hexane were used for each parasitoid tested in the olfactometer.

Caterpillar-feeding Areas of consumed leaf-tissue were assessed for the caterpillar-infested Col-0 and *AtBSMT1-KO* plants that were used in experiment 1. Immediately after finishing an olfactometer bioassay, individual leaves were taped on paper and scanned with a Hewlett-Packard scan jet 3570c. Original leaf shapes were reconstructed by using Paint.NET v3.30, Microsoft Corporation. Quantification of consumed leaf-tissue area was performed using Winfolia pro 2006a, Regent instruments (Québec, Canada).

Headspace Collection And Volatile Analysis Dynamic headspace sampling was carried out in a climate room (20±2°C, 70% RH; 8:16 hr, L:D photoperiod and 90 to 110 μmol photons m^{-2} sec^{-1} PPFD). Twenty-four h before sampling, the pots were removed, roots and soil were carefully wrapped in aluminum foil, and 4 plants were placed together in a 2.5 L glass jar. The glass jars then were covered with insect-proof gauze. Just before trapping, the gauze was removed and the jars were closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through tubes filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, MI, USA). Air was sucked out of the jar with 100 ml min^{-1} by passing through a tube filled with 200 mg Tenax TA. Headspace volatiles from different treatments were collected for a period of 3.5 hr. Fresh weights of all rosettes were determined immediately after the experiments. On each experimental day, headspace samples for two or three replicates of each treatment were collected simultaneously.

Headspace samples were analyzed with a Thermo TraceGC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo TraceDSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with helium at 30 ml min^{-1} for 20 min at ambient temperature to remove moisture. Samples were desorbed from the cartridges by using a thermal desorption system at 250°C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow at 30 ml min^{-1}. Analytes were focused at 0°C on an electronically-cooled sorbent trap filled with Tenax and Carbograph (Unity™, Markes International LTD, Llantrisant, UK) and were then transferred in splitless mode to the analytical column (Rtx-5 ms, 30 m, 0.25 mm i.d., 1.0 μm film thickness, Restek, Bellefonte, PA, USA) by rapid heating of the cold trap to 250°C. The GC was held at an initial temperature of 40°C for 3.5 min, followed by a linear thermal gradient of 10°C min^{-1} to 280°C, and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45–400 m/z with a scan rate of 3 scans sec^{-1}.

Compounds were identified by using the deconvolution software AMDIS (version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from the literature (Adams 1995). For quantification, characteristic quantifier ions were selected for each compound of interest. MetAlign software (PRI-Rikilt, Wageningen, the Netherlands) was used to remove baseline noise, to align the peaks of all chromatograms of the samples, and to integrate peak areas of quantifier ions. The peak areas of all compounds were corrected for the fresh weight of the leaf rosettes.

Statistical Analysis

Y-tube Olfactometer Behavioral Assays Parasitoid preference for infested *AtBSMT1-KO* vs. infested Col-0 plants (i.e., experiment 1) were analyzed statistically by using a *Chi-square* test, with the null-hypothesis that parasitoids did not have a preference for any of the two odor sources. Second, we analyzed whether the parasitoids exhibited a MeSA-dose-dependent response when the HIPV blend from *AtBSMT1-KO* plants was supplemented with synthetic MeSA (i.e., experiment 2). We constructed an overall generalized linear model (GLM) including MeSA dose as a covariate, the tested genotype combination as a fixed factor, and the interaction of the terms. As the null-hypothesis, we defined that addition of MeSA did not affect attraction of parasitoids. Total number of parasitoids that preferred infested *AtBSMT1-KO* plants with the supplemented MeSA over the control (infested Col-0 or *AtBSMT1-KO*) plants was taken as response variate. Total numbers of parasitoids that made a choice per day were used as the binomial total, which were fixed on 10 except for a single case where 9 wasps responded, and we used a logit-link function. We used a *Chi-square* test, with the aforementioned null-hypothesis, to test for a significant preference of each tested MeSA dose per genotype combination. Parasitoids that did not make a choice were not included in the test.

Third, we investigated the effects of MeSA dose on the proportion of parasitoids that made a choice in the olfactometer experiments. We used the same overall GLM

model as described above, only now with number of non-responding parasitoids as the response variate (SPSS 15.0, Chicago, IL, USA).

A Mann-Whitney-*U* test was used to test whether the consumed leaf area of Col-0 and *AtBSMT1-KO* plants (experiment 1) was different (SPSS 15.0, Chicago, IL, USA).

Headspace Collection The fresh weight of the corrected peak area for a volatile compound quantifier ion was log10 transformed, and for each HIPV, the following mixed model was used to screen for HIPV compound differentiation per genotype: $\log 10(V_{ijk}) \sim G_i + T_j + G{:}T_{ij} + R_k + \varepsilon_{ijk}$, where V=area of quantifier ions per gram fresh weight; G=genotype; T=treatment; R=replicate; ε=residual; i=1,2; j=1,2; and k=1,…,5. Both G and T were used as fixed effects and R as a random effect. Subsequently, two-tailed *t*-tests followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction were conducted per compound for the genotypes [significance: $q<0.05$; Benjamini and Hochberg 1995].

Results

Headspace Volatile Analysis To evaluate the effects of the BSMT knock-out mutation, we analyzed the headspace of uninfested and *P. rapae*-infested plants of wild-type Col-0 and *AtBSMT1-KO* mutant plants. For the analysis, we selected compounds in the HIPV blend that are known to influence the behavior of carnivorous arthropods, e.g., methyl salicylate, hydrocarbon alcohols, and terpenoids, and compounds predicted to be affected by the KO mutation, i.e. methyl salicylate and methyl benzoate (MeBA) (Fig. 2) (Turlings and Fritzsche 1999; Dicke et al. 1990; Chen et al. 2003; De Boer et al. 2004; Shimoda et al. 2005).

Infested Col-0 and *AtBSMT1-KO* plants differed significantly in the emission of MeSA, ethyl salicylate (EtSA) and MeBA ($q<0.001$); these compounds were induced in Col-0 but not in *AtBSMT1-KO*. The Y-axis represents a ^{10}log scale. Thus, the headspace analysis for infested *AtBSMT1-KO* and infested Col-0 showed that the production of MeBA (No. 10), MeSA (No. 12), and ethyl salicylate (EtSA, No. 13) was hampered. These compounds are emitted from *AtBSMT1-KO* at 14, 59, and 17 times lower emission rates, respectively (Fig. 2).

Several other compounds—1-pentanol, linalool, indole, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12–trimethyltrideca-1,3,7,11–tetraene (TMTT)—similarly were induced in infested Col-0 and *AtBSMT1-KO* plants when compared to uninfested plants. The green leaf volatile (*Z*)-3-hexen-1-ol was induced significantly in only the mutant ($q=0.005$) but not in the wild-type. Uninfested Col-0 and *AtBSMT1-KO* plants did not differ in the emission of volatiles, except for TMTT, which was emitted in somewhat larger amounts by uninfested Col-0 plants than by uninfested *AtBSMT1-KO* plants ($q=0.011$). Finally, uninfested Col-0 plants emitted more 1-octen-3-ol and 1-nonanol than infested plants (Fig. 2).

Y-Tube Olfactometer Behavioral Assays

Experiment 1. Effect of AtBSMT Knock-out on Parasitoid Attraction to Caterpillar-infested Plants Diadegma semiclausum females are attracted to the headspace of *P. rapae*-infested Arabidopsis Col-0 plants (Loivamäki et al. 2008) in which MeSA is an induced volatile compound (Van Poecke et al. 2001). To investigate whether changes in this headspace, as a result of a knock-out mutation in the *AtBSMT1* gene, affected parasitoid attraction, we investigated the behavioral responses of *D. semiclausum* towards plant volatiles induced by *P. rapae* herbivory in a Y-tube olfactometer. Females of *D. semiclausum* preferred the volatiles emitted by herbivore-infested *AtBSMT1-KO* plants over those emitted by infested Col-0 plants ($P<0.05$; Fig. 3a).

The amount of leaf-tissue consumed by the caterpillars did not differ between Col-0 and *AtBSMT1-KO* plants (mean ± SE: Col-0 10.41±0.91 mm^2, *AtBSMT1-KO* 10.88±0.80 mm^2; Mann Whitney *U* test: $U=259.00$; $P=0.90$, $N=23$). Thus, the difference in attraction cannot be explained by a difference in the amount of feeding by the caterpillars.

Experiment 2. Supplementing Headspace of Caterpillar-infested AtBSMT1-KO Plants with Synthetic MeSA To assess whether the absence of MeSA in the HIPV blend can explain the effect on parasitoid behavior, the volatile blend of infested *AtBSMT1-KO* plants was supplemented by adding synthetic MeSA downwind of the plant. Different MeSA doses were used to test for dose-dependent effects of MeSA presence. The addition of MeSA eliminated the preference for the knock-out plants, as seen in experiment 1, and with increasing MeSA dose this effect was stronger. Parasitoid preference was not influenced by the different genotype combinations, but solely by the MeSA dose used (GLM: genotype combination $P=0.167$, MeSA dose $P<0.001$, $R^2=0.28$). Analysis of parasitoid choices for each tested MeSA dose within a tested genotype combination demonstrated that parasitoid preferences slightly varied among the MeSA doses used. Yet, for both genotype combinations tested, *AtBSMT1-KO* HIPV complementation with 0.2 µg MeSA did not result in discrimination between the two odor sources and a complementation with 200 µg MeSA resulted in significant preference for the odor without supplemented MeSA (Fig. 3b, c). When parasitoids were offered a choice between volatiles from infested *AtBSMT1-KO* plants supplemented with 2 or 20 µg MeSA vs. infested Col-0 HIPVs,

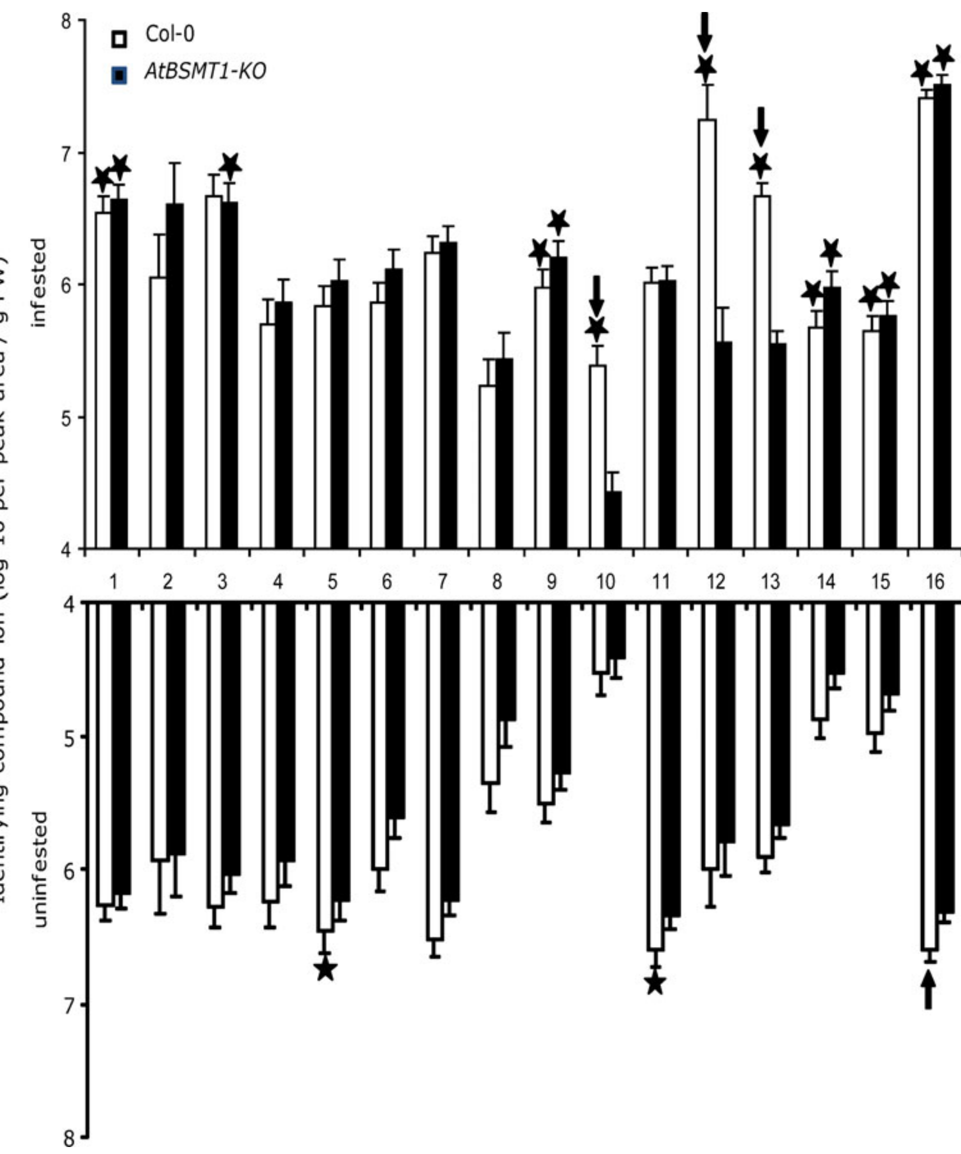

Fig. 2 Emission of volatile compounds of Col-0 wild-type and *AtBSMT-KO* Arabidopsis plants, either uninfested or infested with 20 *Pieris rapae* caterpillars, expressed as peak area (arbitrary units; mean ± SE; N=5) for the identifying ion per g FW. Compounds: 1=1-pentanol (*m/z* 70); 2= (*Z*)-2-penten-1-ol (*m/z* 57); 3= (*Z*)-3-hexen-1-ol (*m/z* 67); 4= α-pinene (*m/z* 93); 5= 1-octen-3-ol (*m/z* 57); 6= ß-myrcene (m/z 93); 7= (*Z*)-3-hexen-1-ol acetate (*m/z* 67); 8= (*E*)-ß-ocimene (*m/z* 93); 9= linalool (*m/z* 93); 10= methyl benzoate (*m/z* 136) 11= 1-nonanol (*m/z* 56); 12= methyl salicylate (*m/z* 120); 13= ethyl salicylate (*m/z* 120); 14= indole (*m/z* 117); 15= (*E*,*E*)-α-farnesene (*m/z* 93); 16= (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (*m/z* 69). Bars marked with * indicate a treatment for a genotype significantly emitting more volatiles than its opposite uninfested or infested genotype. Bars marked with arrows represent compounds emitted in significantly different amounts by the two genotypes

they preferred the latter. In contrast, no discrimination between odors from infested *AtBSMT1-KO* plants supplemented with 2 or 20 µg MeSA vs. infested *AtBSMT1-KO* plants was observed.

The addition of MeSA negatively influenced the proportion of parasitoids that made a choice for one of the two odor sources. Analysis of the number of wasps that did not make a choice for one of the two odor sources, showed no effect of the offered genotype combination, but only an effect of the MeSA dose used (GLM: genotype combination P=0.75, MeSA dose P=0.010, R^2=0.15).

Discussion

Variation in the attraction of carnivorous arthropods to HIPVs often is ascribed to the relative contribution of attractive compounds within the complex herbivore-induced blend (Van Den Boom et al. 2004; D'Alessandro et al. 2006, 2009). Plant species can differ in the relative emission rates of individual attractive compounds in response to feeding by distinct herbivores. The commonly induced attractive compounds comprise green leaf volatiles (GLV), terpenoids, phenyl propanoids, and benzenoids. Several studies already have shown *in planta* the importance of some GLV and terpenoids in attracting carnivorous arthropods (Kappers et al. 2005; Schnee et al. 2006; Shiojiri et al. 2006). Yet, empirical evidence for the quantitative importance of single compounds in the total HIPV blend of infested plants is still rare (but see De Boer and Dicke 2004). The parasitoid *D. semiclausum* is attracted to the headspace of *P. rapae*-infested Col-0 plants (Loivamäki et al. 2008), in which MeSA is an induced volatile (Van Poecke et al. 2001). Here, we studied the effect of MeSA on preference for volatile blends in the parasitoid *D. semiclausum*.

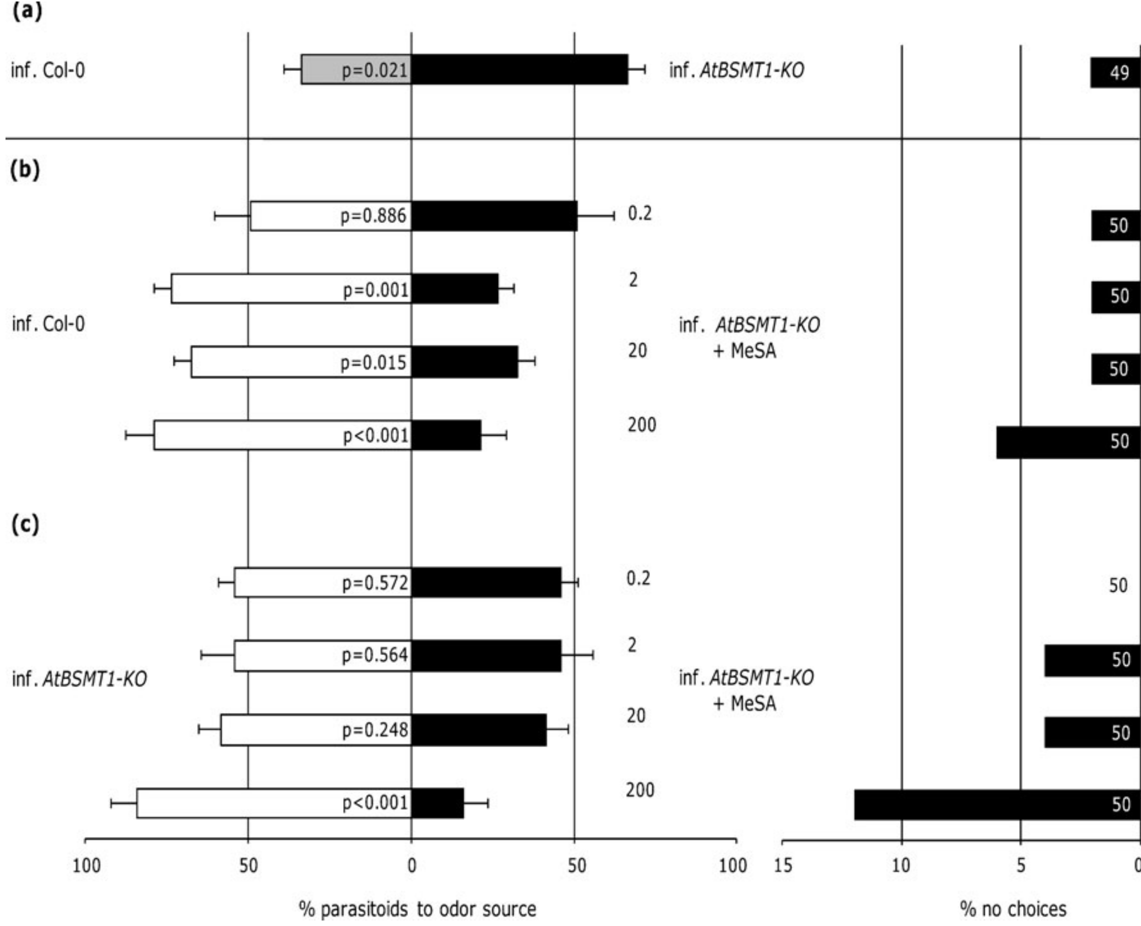

Fig. 3 Behavioral responses of naïve *Diadegma semiclausum* females to volatiles of two sets of *Arabidopsis thaliana* plants (Col-0 vs. *AtBSMT1-KO*), as assessed in the Y-tube olfactometer. All plants were infested (inf.) with 20 *Pieris rapae* caterpillars and in some cases the headspace was supplemented with synthetic methyl salicylate (MeSA), added downwind from the plants. Added MeSA-doses (µg) are indicated to the right of the bars in the left bar plot. Each bar represents the percentage of choices for each of the two odor sources as determined in five replicate experiments; on each replicate day 10 parasitoids were tested per odor source (X^2 test, *P* values). Error bars indicate SE. Generalized linear model analysis for experiments B and C, demonstrated that MeSA dosage ($P<0.001$) and not the offered genotype ($P=0.167$) explained parasitoid behavior. Parasitoid MeSA dosage responses between two tested genotype combinations did not differ ($P=0.270$). The right bar plots indicate the percentage of no choice in each experiment; total number of tested parasitoids are given in these bars

The newly-available Arabidopsis knock-out mutant for benzoic acid and salicylic acid carboxyl methyltransferase (*AtBSMT1-KO*) (Fig. 1, Fig. S1), allowed us to study *in planta* the ecological effects of an HIPV mixture lacking wild type levels of MeSA. Our data show clearly that caterpillar-infested *AtBSMT1-KO* plants attract more parasitoids than infested wild-type Col-0 plants (see Fig. 3a). Thus, although the total HIPV blend from wildtype plants strongly attracts *D. semiclausum* parasitoids (Loivamäki et al. 2008), a genotype that does not emit MeSA in response to caterpillar infestation is even more attractive to the parasitoids. These data indicate that MeSA does not contribute to the attraction of naïve *D. semiclausum* females but instead results in a reduced attractiveness.

This negative effect of MeSA on *D. semiclausum* attraction was not anticipated, as MeSA is commonly induced after herbivory in many plant species, e.g., in lima bean (Dicke et al. 1990), Brussels sprouts (Geervliet 1997; Bukovinszky et al. 2005), tomato (Ament et al. 2004), and Arabidopsis (Van Poecke et al. 2001; Chen et al. 2003). Moreover, the salicylic acid-deficient Arabidopsis mutant *NahG* is less attractive to *Cotesia rubecula* parasitoids upon infestation by *P. rapae,* which suggests an attractive role of MeSA for this parasitoid species (Van Poecke and Dicke 2002). Furthermore, synthetic MeSA has also been demonstrated to attract other carnivore species, such as predatory mites, lacewings, and mirid bugs (Dicke et al. 1990; De Boer and Dicke 2004; James and Price 2004).

However, in our approach we tested the role of MeSA within the total HIPV-blend, by using *AtBSMT1-KO* plants with a hampered MeSA production. By additionally testing the response of the wasps to an HIPV blend from *AtBSMT1-KO* plants that we had supplemented with synthetic MeSA against HIPVs from Col-0 plants and *AtBSMT1-KO* plants, we demonstrated that MeSA acts as a repellent to naïve *D. semiclausum*. Moreover, the repellent effect of MeSA was dose-dependent, and this was reflected in the response level of the parasitoids, as fewer wasps move upwind to make a choice between odor sources with higher MeSA-doses. Additionally, the KO-mutation did not affect the feeding rate of the caterpillars. Thus, the change in volatile emission and parasitoid attraction cannot be attributed to effects of the KO-mutation. The data show that this compound plays a role in the observed repellent effects.

A few other studies also have recorded repellent effects of HIPV compounds to carnivores. For example, an HIPV fraction of maize, containing (*Z*)-3-hexen-1-ol acetate, linalool, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was found attractive compared to other tested fractions of the HIPV blend of maize to the parasitoid *Cotesia marginiventris* (Turlings and Fritzsche 1999). For the same plant-herbivore system, naïve *Microplitis rufiventris* parasitoids preferred HIPV blends lacking the induced compound indole (D'Alessandro et al. 2006). Compounds that are repellent also may affect or override the attractiveness of other compounds in the headspace. Isoprenoids have, for example, been found to interfere with host-finding, as demonstrated for transgenic Arabidopsis plants emitting isoprene (Loivamäki et al. 2008). Therefore, to determine whether a hampered enzyme activity for SAMT and BAMT affected the headspace composition in other respects, we also analyzed the headspace of infested knock-out mutant and wild-type plants. We observed that the headspace of caterpillar-infested Col-0 and *AtBSMT1-KO* plants differed only in the emission of MeSA, MeBA, and EtSA. Yet, all other investigated HIPVs were emitted at similar rates by these infested plants (Fig. 2). The emission rates of the compounds whose rates were affected, were up to 60 times lower for *AtBSMT1-KO* plants compared to wild-type plants (Fig. 2). This agrees with the reported activity of the enzyme encoded by the *BSMT1* gene (Chen et al. 2003). The very low emission of MeSA that remains may be ascribed to activity of another SABATH enzyme that shows low levels of activity towards SA and BA (Chen et al. 2003). Eliminating a functional *BSMT1* gene also decreased the emission rate of EtSA. EtSA has been recorded in several plants (Hamilton-Kemp et al. 1988; Scutareanu et al. 1997; Deng et al. 2004) and is perceived by insect chemoreceptors (Ramachandran et al. 1990; Reinecke et al. 2002). Whether MeBA and EtSA also affect the attraction of *D. semiclausum* wasps remains to be investigated.

We investigated the role of MeSA by eliminating it from the total HIPV blend rather than by investigating its role as an isolated compound or as an addition to an artificial blend (see e.g., De Boer and Dicke 2004). We thus demonstrated that it has a repellent effect on the behavior of naïve *D. semiclausum* parasitoids. This finding provides another view on the fitness effects of individual components of herbivore-induced plant volatile blends that do not lure naïve carnivores but rather repel them. An alternative function of HIPV emitted compounds can be to repel herbivores (Dicke 1986; Bernasconi et al. 1998; Kessler and Baldwin 2001; Bruce et al. 2008; Piesik et al. 2008), which also has been demonstrated for MeSA (Hardie et al. 1994; James and Price 2004; Prinsloo et al. 2007; Ulland et al. 2008). MeSA may activate disease resistance and the expression of defense related genes in neighboring plants and in healthy tissue of infected plants (Shulaev et al. 1997). Therefore, the emission of MeSA also could benefit the plant through protecting it at herbivory-derived wounds against infectious pathogens, as has been reported for several GLVs (Brown et al. 1995; Shiojiri et al. 2006). In addition, evidence has accumulated for a plant–plant signaling role for MeSA (Ozawa et al. 2000; James and Price 2004), as well as indications for a priming effect of MeSA on plant defense (Turlings and Ton 2006).

A few studies indicate that parasitoids can learn to respond to individual compounds following a learning experience with an odor mixture (Meiners et al. 2003). This suggests that an innate response of *D. semiclausum* to avoid MeSA-emitting plants could potentially turn into attraction after a learning experience in the presence of a host.

In summary, our study provides evidence for an HIPV component that makes a plant less attractive to a carnivorous insect. This should be seen in the context of the different selection pressures on a plant's emission of volatiles in a multitrophic context. Understanding these selection pressures will provide insight into the role of induced volatiles in the biology of plants (Dicke and Baldwin 2010).

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Response of a Generalist Herbivore *Trichoplusia ni* to Jasmonate-Mediated Induced Defense in Tomato

Ian M. Scott · Jennifer S. Thaler · Jeffrey G. Scott

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Abstract The up-regulation of plant defense-related toxins or metabolic enzyme binding proteins often leads to a negative effect on herbivorous insects. These negative effects can manifest themselves at three points: changes in food ingestion, post-ingestive-changes, and post-digestive changes. Many studies have related the decrease in herbivore growth and/or survival with expression of chemicals that interfere with post-digestive effects such as the anti-nutritive effects of protease inhibitors. Nevertheless, it is unclear whether such compounds impact herbivores via earlier ingestive processes. We addressed this question by using a jasmonate-deficient mutant (Def-1), a jasmonate-overexpressor mutant (Prosystemin or Prosys), and wild-type tomato in three trials with 5th instar *Trichoplusia ni*. Decreases in relative growth rate (RGR) confirmed that *T. ni* fed on the Prosys plants developed poorly compared to those feeding on Def-1 plants (larvae on wild-types were intermediate). Preingestive and postingestive processes contributed to this effect. Total food ingested and the consumptive index were 25% lower on Prosys plants compared to Def-1 plants. Post-ingestive processes, measured by approximate digestibility, were 62% greater on Prosy plants**.** Post-digestive efficiency measured by conversion of ingested and digested food (ECI and ECD) decreased on Prosys plants two-fold compared to Def-1 plants. This post-digestive interference correlated well with the 2-fold decrease in activity of digestive enzymes, serine proteases, in Prosys-fed *T. ni* compared to those on Def-1 plants. No difference in detoxifying enzyme activity was detected.

Key Words Jasmonic acid · Induced plant response · Tomato · *Trichoplusia ni* · Prosystemin · Def-1 mutant · Nutritional index · Serine protease · Detoxification enzyme

Introduction

The induced responses in plants to herbivore and pathogen attack are well documented and often have strong negative consequences for herbivore survivorship, growth and reproduction (Green and Ryan 1972; Karban and Myers 1989; Farmer and Ryan 1992; Stout and Duffey 1996). The negative effects of plant responses can be based on lower nutritional value or more direct toxicity (Duffey and Stout 1996; Barbehenn et al. 2007). In the latter case, a toxic effect would be caused by direct interference with midgut processes or normal life processes (Duffey and Stout 1996). In the former case, many herbivores compensate for sub-optimal nutritional value by increasing food consumption or switching to an alternative food (pre-ingestive effects), altering utilization of the food (post-ingestive effects) or increasing the efficiency of conversion of digested food to solid body matter (post-digestive effects) (Hägele and Rowell-Rahier 1999). Reduced efficiency can be divided further into either: 1) an anti-digestive effect caused by a reduction in the enzymatic conversion of ingested food; or 2) an anti-nutritive effect that reduces the physical availability or chemical composition of the food. An example of an anti-digestive effect is when protease inhibitors (PIs) from the plant reduce the protease activity

I. M. Scott (✉)
Southern Crop Protection and Food Research Centre,
Agriculture and Agri-Food Canada,
1391 Sanford St.,
London N5V 4T3, ON, Canada
e-mail: Ian.Scott@agr.gc.ca

J. S. Thaler · J. G. Scott
Entomology Department, Cornell University,
Ithaca, 14853 NY, USA

in the insect gut, thus affecting the metabolism rate of proteins and the availability of amino acids (Chen et al. 2005). An example of an anti-nutritive effect occurs when polyphenol oxidases (PPOs) cause irreversible covalent modification of amino acids and plant proteins by quinines or quinine-generated reactive oxygen species (Mahanil et al. 2008).

The defense pathway regulated by jasmonic acid (JA) is known to be one of the plant's most important lines of defense against insect herbivores (Farmer and Ryan 1992; Lightner et al. 1993; McGurl et al. 1994; Howe et al. 1996). This pathway influences many different properties in Solanaceous plants, including the formation of defense proteins (PIs), oxidative enzymes (PPO), glandular trichome density, small compounds such as alkaloids (glycoalkaloids in potato and nicotine in tobacco), and volatiles (mono- and sesquiterpenes in tomato). These compounds are known to be inducible (Ryan 2000; Thaler et al. 2002; Boughton et al. 2005; Halitschke and Baldwin 2005; Wasternack 2007). Tomato leaves contain α-tomatine, chlorogenic acid, and rutin shown to be toxic to *Heliothis zea* at foliage concentrations (Elliger et al. 1981), but it has not been determined whether these are induced through the JA pathway. Because of this, plants with an activated jasmonate pathway may vary both in nutritive content as well as levels of toxins. Nutritional quality of the plant may be altered by either changes in the amount of nutrients present or in the presence of compounds that reduce the digestibility of nutrients by the insect. While it has been shown that herbivores feeding on plants with an induced jasmonate response often have severely reduced performance, it has not been investigated to what extent these negative effects on herbivores are due to decreased nutritional availability or increased toxicity.

Modeling the function of JA has been facilitated through the use of tomato mutants or genetic manipulation of wild-type plants to alter the JA biosynthetic pathway. Jasmonate-deficient (Def-1) tomato mutants have an altered octadecanoid pathway converting linolenic acid to JA resulting in a 3-fold lower induction of JA 90 min after wounding compared to wild-type (WT) plants (Howe et al. 1996). As a result, there is a 10-to >25-fold lower accumulation of wound-inducible proteinase inhibitors (PI) in the Def-1 wounded leaves compared to WT plants. The expression of a PI-II related gene, cathepsin D inhibitor (CDI), in Def-1 plants is also less than 10% of those in WT plants (Li et al. 2002). Mortality of *Spodoptera exigua* that fed on Def-1 plants was 30% less, and larval mass of survivors was double that of larvae that fed on WT tomatoes (Thaler et al. 2002). In a second transgenic tomato line (Prosys), prosystemin is over-expressed through the use of a promoter leading to the constitutive overproduction of JA, leading to an overproduction of the defense proteins, PPO (Howe and Ryan 1999), and PIs (McGurl et al. 1994), including arginase and threonine deaminase (Chen et al. 2005). In unwounded Prosys leaves, the typical levels of PI-I, PI-II, and PPO are 14.7x, 19.1x, and 69x greater than in the unwounded WT leaves, respectively (Howe and Ryan 1999). As a result, when thrips and spider mites fed on prosystemin-overexpressing tomatoes, there is reduced damage to the plant and decreased herbivore fecundity compared to WTs (Li et al. 2002). Compared to a wild-type tomato, a Prosys mutant that overexpresses arginase had dramatically higher leaf arginase activity, reduced *Manduca sexta* larval weight gain and leaf consumption, and the level of Arg in the *M. sexta* midgut was reduced significantly (Chen et al. 2005). Jasmonate-induced levels of PIs and PPO work in combination to either modify the dietary protein or bind and inhibit digestive enzymes in the caterpillar midgut, respectively (Chen et al. 2005).

In the present study, the cabbage looper *Trichoplusia ni* (Hbn.) (Noctuidae), an insect with a large range of host plants, was used to investigate the digestive and physiological response to variable JA expression in tomato. The tomato mutants Def-1 and Prosys provide excellent models for unraveling whether the JA-induced defenses act pre-ingestive, post-ingestive, or post-digestive. The objectives of this study were to examine the difference in digestive and physiological responses of *T. ni* that are indicative of: 1) consumption and digestibility of plant nutrients (e.g., leaf area consumed, mass gain, frass production, and nutritional indices); and 2) insensitivity to plant proteins via gut serine proteases, and detoxification by gut monooxygenases, esterases, and transferases. The expectations were that tomato plants expressing higher levels of JA will: 1) have increased levels of anti-digestive and anti-nutritive compounds that will lead to significant differences in the insect's post-digestive nutritional indices; and 2) have increased concentrations of toxic allelochemicals that will lead to significantly greater detoxifying enzymes in the insect.

Methods and Materials

Plant and Insect Material Jasmonic acid deficient (Def-1), wild-type (var. Castlemart) and prosystemin over-expressing 35S::Prosystemin (Prosys) mutant tomatoes were obtained from Gregg Howe, Michigan State University. Def-1 plants were backcrossed 5 times to the Castlemart variety and were homozygous for a mutation within the jasmonate pathway leading to lower JA production (Howe et al. 1996) and a reduced ability to induce proteinase inhibitor I and II (Lightner et al. 1993). The Prosys transgenic tomatoes were developed through an agrobacterium-mediated transformation of var. Better Boy (McGurl et al. 1994), and, as a

consequence, accumulate high levels of wound response proteins in the absence of wounding (Howe and Ryan 1999). Seeds for the Prosys plants were collected from a homozygous line backcrossed to var. Castlemart (Li et al. 2002). All tomato plants were grown in 4″ diam pots with Metromix potting soil in the greenhouse from seed. Plants were used when they were between 4 and 5 wk in age.

Cabbage looper *Trichoplusia ni* larvae were obtained from Benzon Research (Carlisle, PA, USA). Larvae were reared on multispecies diet (Southland Products, Lake Village AR, USA) until the 5th instar. All larvae used in trials were mid 5th instar. Larvae were reared at 26-28°C, 16:8 hr L:D.

Tomato—T. ni Greenhouse Trials Three greenhouse tomato trials were conducted between October 2006 and January 2007. The plants were grown in the greenhouse at 27°C under natural light conditions with supplemental lighting until 22:00 hr. Fertilizer (21:5:20 N:P:K, 150 ppm N) was added weekly to the pots. Fifty Def-1, WT, and Prosys plants without prior herbivore damage were selected for use in the trials. The third leaf from the top was selected for the insect feeding trial. Outlines of the first five leaflets from the leaf were obtained from each plant by using an underlying sheet of white paper and an overlying acetate sheet. A clear plastic clip cage (approx. 5 cm diam.) then was attached to the first or terminal leaflet of the leaf. When all leaflets were traced, a 6–8 hr-starved, pre-weighed *T. ni* larva was placed in each clip cage. After 5–6 hr, the clip cage was moved to an adjacent leaflet on the same leaf, and this was repeated up to three more times over a 24 hr period. The cage was moved depending on the degree of damage per leaflet. If the leaflet was largely or completely consumed by the larva, the cage was moved. If <50% of the leaf area was removed, the cage was not moved. At the end of the trial, the remaining leaf tissue was scanned (Canoscan, Canon, Burlington NJ, USA) and compared to a scan of the leaflet tracing in order to calculate the actual leaf area consumed. Each time a cage was moved the frass produced by the insect was collected.

After 24 hr, all larvae were removed from the cages and held in cups on ice. In the laboratory, the final fresh mass of each larva was measured. Ten of the 50 larvae were randomly selected for measurement of dry mass, the remainder were divided into three groups for enzyme assays. To determine dry mass, larvae were removed from the ice, starved at room temperature for 1–2 hr, weighed, and then frozen at −20°C. Later these larvae were dried at 65°C for 3 d and reweighed. Larvae used in the enzyme assays were placed on ice, and the midgut was removed by dissection as described below. Calculation of the dry mass of the tomato leaflet area consumed was based on dry mass of discs cut from 10–15 leaflets per variety with a #10 cork borer and dried for 3 d at 65°C. These leaf discs were cut from 10 plants, and the disc was cut from the base or the widest part of the leaflet. The surface area damaged on each leaflet was converted to dry mass based on the mean mass per disc for each tomato variety. Initial dry mass of the insects was calculated by multiplying the initial fresh mass by the ratio of the mean final dry mass : fresh mass. Frass collected from individual insects was dried at 65°C for 3 d and weighed.

Nutritional Analysis The nutritional indices calculated for the *T. ni*—fed tomato mutants and wild-type were as follows: relative growth rate (RGR), consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of ingested food (ECI). The indices were calculated according to the Waldbauer method as described in Slansky and Scriber (1985) and Rayapuram and Baldwin (2006). CI [(leaf ingested)/(larval mass gain x number of days)] provides a measure of the total amount of leaf consumed relative to the body mass gained during the 1 day feeding period. Increases in CI have been associated with compensation by nutritionally limited insects. AD [(leaf mass ingested—frass mass)/(leaf mass ingested)] is the difference between the amount of food consumed and the amount of frass produced per unit food consumed. This provides a measure of the efficiency of digestion of the ingested food. ECD [(larval mass gain)/(leaf mass ingested—frass mass)] and ECI [(larval mass gain)/(leaf mass ingested)] provide a measure of the efficiency of conversion of absorbed food and ingested food, respectively. The latter 2 indices measure the mass increment of the insect relative to the amount of food absorbed and eaten, respectively. Lower ECI and ECD indicate that insects are not getting essential macronutrients, whereas a change in AD reflects different mechanisms occurring after ingestion, but prior to digestion. RGR (growth attained per unit of body mass per unit of time) is the product of the relative consumption rate (RCR=mg consumed per mg gained per day) and nutritional indices (RGR=RCR x ECI) (Schoonhoven et al. 2005).

Total Serine Protease Activity Individual *T. ni* midguts were dissected on ice, and the whole midgut was placed in a preweighed cryovial with 100 µl of MilliQ water and reweighed. Samples then were frozen at−80°C until the protease assay was performed. The frozen whole midguts were thawed on ice, transferred to a 1.5 ml centrifuge tube, and additional distilled water was added, based on the midgut mass in order to have a 10% midgut solution. The midgut then was processed using a disposable pellet mixer (VWR) with pestle to fit the 1.5 ml microtube. The 10% homogenate was used to measure total serine protease

activity for 10 individual *T. ni* larvae per tomato variety per trial. Total midgut serine protease activity was quantified with azocasein (Sigma) substrate using a method adapted from Hegedus et al. (2003) (P. Wang, personal communication). The midgut homogenate (20 µl) was added to 150 µl of 1% azocasein solution in pH 11 glycine-sodium hydroxide (0.05 M). All samples were incubated at 28°C for 15 min. TCA (10%) was added (170 µl) and incubated for 1 h at room temperature. The sample was centrifuged at 13,000 RPM for 10 min, and the supernatant was collected. Sodium hydroxide (1 M) was added (340 µl), and the absorbance was read at 495 nm with a Beckman spectrophotometer (Li et al. 2004). Total midgut protease activity is calculated by subtracting the azocasein blank absorbance from the azocasein sample absorbance divided by the incubation time in min multiplied by 1000 ([(Abs $_{(sample)}$—Abs $_{(blank)}$]/min * 1000). The units are tryptic activity (mU) per min of incubation per mg insect body weight (mU/min/mg bw). Leaf homogenate from each of the tomato types also was assessed for protease activity using the above method to ensure that no background protease activity was present due to plant material present in the midguts.

Previous studies have shown that adaptation by insects to protease inhibitors (PIs) in their diet involves increases in the amount of PI-insensitive proteases (Broadway 1995; Jongsma and Bolter 1997). One method of examining the difference in the profile of proteases in the midgut is to apply a protease inhibitor with specific activity. Bowman-Birk soybean protease inhibitor (BBI) (Sigma) was selected as it targets trypsin and chymotrypsin proteases, the principal serine proteases in lepidopterans (Abdeen et al. 2005; Chougule et al. 2008). In order to test the hypothesis that *T. ni* larvae adjust to increasing levels of JA-induced defenses by up-regulating insensitive proteases, differences in the remaining amount of serine protease activity was measured between BBI-incubated midgut homogenates taken from *T. ni* that fed on the three tomato mutants in trial 1 relative to the homogenates incubated without BBI. The midgut homogenates of the treated *T. ni* were incubated for 15 min with BBI over a range of concentrations (0.1, 0.5, 1, 2, and 8 mg/ml) that showed a linear inhibition of protease activity with azocasein substrate with increasing inhibitor concentration until saturation was approached. Total serine protease activity was measured as described above, and the percent inhibition caused by the BBI incubation was determined.

Cytochrome P450/b$_5$ Assay Microsomes were prepared from the dissected midguts of 20 *T. ni* larvae fed on the wild-type and two tomato mutants. The midguts were dissected on ice and rinsed in Rinaldini's saline. The 20 midguts were combined and homogenized in 20 ml 0.1 M sodium phosphate homogenizing buffer (pH 7.5) (Lee and Scott 1989). Homogenates were centrifuged at 10,000 RPM for 20 min, the supernatant was transferred to a 30 ml centrifuge tube and centrifuged at 100,000 RPM for 1 h. The microsome pellet was homogenized in a resuspension buffer (pH 7.5) (Lee and Scott 1989) and frozen at−80°C until the assay was performed. Cytochrome P450 and b$_5$ were determined following the methods described for house fly (Lee and Scott 1989). Microsome protein was determined by the Bradford (1976) method.

Glutathione S-transferase Assay Individual *T. ni* midguts were dissected on ice, rinsed in Rinaldini's saline, and homogenized in 500 µl of sodium phosphate buffer (pH 7.5). Ten individuals per treatment from each of the three trials were analyzed. A disposable pellet mixer was used to grind the midgut-buffer mixture. After homogenizing the midgut, a further 500 µl of buffer were added so that the final preparation was one midgut per 1 ml buffer. Samples were centrifuged at 10,000 RPM, and the supernatant was transferred to 1.5 ml vials. Samples were kept frozen at−80°C until both the glutathione *S*-transferase (GST) and esterase assays were conducted. The substrates, 150 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) (1 µl), and 150 mM 1,2-dichloro-4-nitrobenzene (DCNB) (Sigma) (1 µl) were used with 15 mM reduced glutathione prepared in pH 7.0 potassium phosphate buffer (50 µl) and homogenate (25 µl). The methods were adapted from Baker et al. (1998) where three replicates were prepared for each midgut sample on a 96 well plate on ice. Readings at 340 nm were taken every 30 sec for 10 min at 25°C using a microplate reader (Thermo Fisher Scientific, Waltham MA, USA). The initial and final readings used for GST activity were at 3 and 8 min (5 min interval) after the initiation of the plate readings. Blank wells were prepared by replacing the homogenate with pH 7.0 buffer. Ten individuals per treatment from each of the three trials were analysed. Protein concentration was determined by the Bradford (1976) method.

Esterase Assay Midgut supernatants were used for the determination of esterase activity. The esterase substrates used were α-and β-naphthyl acetate (NA) and 4-methylumbelliferyl acetate (4-MUA), and a microplate assay was adapted from the methods described by Baker et al. (1998). For the α-and β-NA assays, supernatant was diluted 1:100 with pH 7.0 buffer. Into each plate well we added: 110 µl of buffer, 50 µl of either 4 mM α-or β-NA dissolved in acetone, and 40 µl of diluted sample. After 15 min at 25°C, a stop reagent, 25 µl of 0.8% Diazo Blue in 3.4% SDS was added, followed by a further 10 min wait before measuring the activity at 595 nm (α-NA) or 490 nm

(β-NA). Each plate was set up with triplicate wells of samples, standards with either α-naphthol or β-naphthol (4–40 μM) in buffer and blank wells (buffer only). Esterase activity with all three substrates was standardized to protein concentration.

Statistical Analyses ANCOVA was used to analyze each of the nutritional indices to avoid problems with the statistical analysis of ratios (Raubenheimer and Simpson 1992) (Proc GLM SAS ver 9.1). In all cases, the independent variables are plant type and trial. For RGR, final mass is the dependent variable and initial mass is the covariate. For CI, plant mass consumed is the dependent variable, and initial mass is the covariate. For AD, the plant mass consumed minus the frass mass produced is the dependent variable, and the mass consumed is the covariate. For ECD, the larval mass gain is the dependent variable, and amount consumed minus the frass produced is the covariate. For ECI, the larval mass gain is the dependent variable, and the amount consumed is the covariate. In the case of AD, ECI, and ECD, the interaction between the plant-type and the covariate was tested. Two-way ANOVA was used to analyze the biomarkers (protease, cytochrome P450, cytochrome b_5, esterase, and GST activity) for the three separate trials. Results were combined and analyzed, and the difference between the treatments was compared among and between the trials. Comparison of treatment means used Tukeys (SAS ver.9.1, SAS Institute, Cary NC, USA). Data were log + 1 transformed, and the normality and homogeneity of all data sets were confirmed (SAS ver.9.1). The effects of the sbBBI treatment on protease activity were ARC Sine (Squareroot) transformed and analyzed using one-way ANOVA (SAS ver. 9.1).

Results

Insect Relative Growth Rate, Leaf Area Consumption, Frass Production, and Nutritional Indices Plant-type had a large effect on caterpillar performance with a significantly lower relative growth rate (RGR) when comparing larvae on Prosys plants to those on Def-1 and WT plants (Fig. 1a). *Trichoplusia ni* caterpillars ate 27–31% less on Prosys plants compared to the Def-1 and WT plants (Table 1). The consumptive index (CI) decreased by 25% when larvae fed on Prosys plants and were compared to those on Def-1 plants; larvae feeding on WT plants showed a CI similar to those feeding on Prosys plants (Fig. 1a).

Post-ingestive efficiency, measured as approximate digestibility (AD), was 62% less on Def-1 plants and 34% less on wild type plants compared to Prosys plants. The caterpillars on Def-1 plants ate more, but also produced more frass (Table 1).

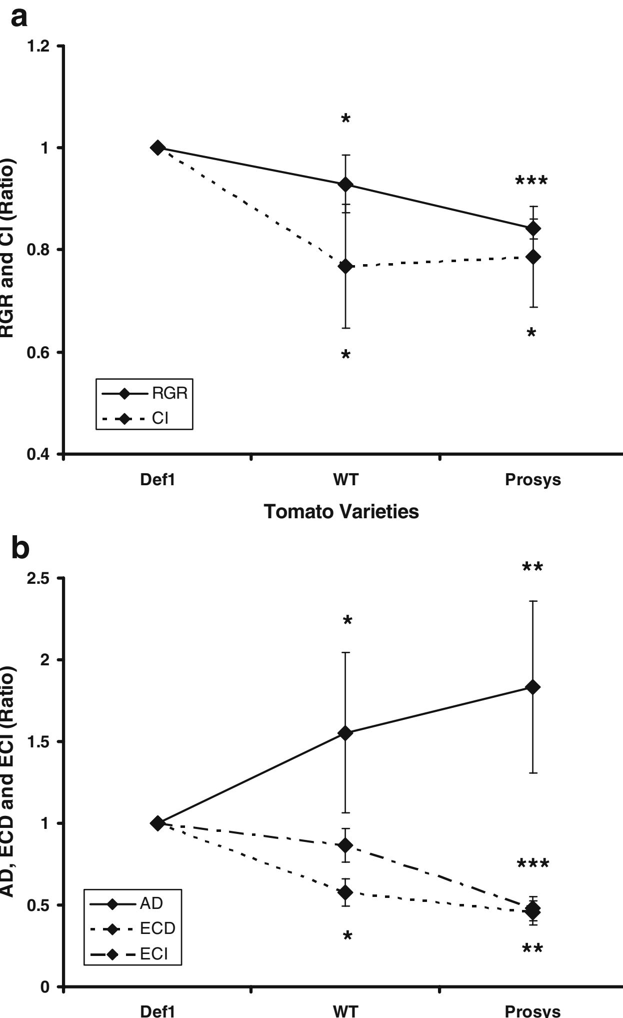

Fig. 1 Mean ratio (± S.E.) for Def-1:Def-1, WT:Def-1 and Prosys: Def-1 of the following: **a** relative growth rate (RGR) and consumption index (CI); and **b** approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of conversion of ingested food (ECI) for *Trichoplusia ni* feeding for 24 hr on three tomato mutants. RGR, CI, AD, ECD, and ECI ratios marked with either a *, ** or *** indicates that there was a significant difference (*LS Means*, *ANCOVA*, $P<0.05$) between the Def-1 and WT or Def-1 and Prosys mutants in 1, 2, or all of the 3 trials, respectively. $N=27$ (Def-1); 28 (WT) and 28 (Prosys)

In contrast, post-digestive measures of efficiency of food conversion (ECD and ECI) were 2-fold higher for caterpillars fed on Def-1 plants compared to those feeding on WT or Prosys plants (Fig. 1b). The nutritional indices of caterpillars feeding on WT plants were typically intermediate to those of larvae feeding on Prosys and Def-1 plants. (Table 2).

Total Serine Protease Activity Total serine proteases were highest in *T. ni* feeding on the Def-1 plants, intermediate on WT, and lowest on the Prosys plants (One way ANOVA:

Table 1 Mean total dry mass of tomato leaf consumed and dried frass produced by *Trichoplusia ni* feeding for 24 hr on Def-1, wild-type (WT) and Prosys tomato mutants

Plant	Leaf mass[a] mg (S.E.)	Ratio[b] (S.E.)	Frass mass mg (S.E.)	Ratio (S.E.)
Def-1[c]	15.23 (1.34)[A]	1	10.34 (0.49)[A]	1
WT[d]	9.7 (0.65)[A]	0.69 (0.16)	7.14 (0.46)[A]	0.74 (0.14)
Prosys[e]	10.36 (0.78)[A]	0.73 (0.14)	6.17 (0.49)[B]	0.62 (0.13)

[a] Mean (± S.E.) leaf and frass dry mass values having same upper case letter are not significantly different (*two way ANOVA*: $F_{8,69}$=5.57 and $F_{8,69}$=13.62, respectively; *Tukey's P*>0.05).

[b] Mean ratio (± S.E.) for Def-1:Def-1, WT:Def-1 and Prosys:Def-1 are provided for leaf and frass mass.

[c] *N*=27.

[d] *N*=28.

[e] *N*=28.

$F_{8,72}$=13.73; Tukey's P<0.05, Fig. 2). The serine protease activity in leaf samples of all tomato types was 5% or less of the midgut protease activity for larvae feeding on either plant type (data not shown), and therefore cannot be responsible for the observed results.

The estimated value of IC_{50} for Bowman-Birk Inhibitor (BBI) on the midgut total serine protease activity was 2 mg/ml for the insects feeding on WT plants. Saturation of the BBI inhibitor occurred at 8 mg/ml and before 50% inhibition of the Prosys-treated protease midgut activity was reached. At the WT-fed *T. ni* BBI IC_{50} concentration, the level of inhibition was 44% and 31% for the Def-1 and Prosys *T. ni*, respectively (Fig. 3). At this concentration there was greater inhibition of the total serine protease activity in the WT vs. the Prosys *T. ni* midguts (One way ANOVA: $F_{14,30}$=12.86; Tukey's P<0.05). Since BBI is a protease-specific inhibitor, this result indicates that *T. ni* larvae might modify BBI-insensitive proteases to adapt to increasing PI levels in the Prosys plants.

Detoxification Enzyme Activity The total P450 and cytochrome b_5 levels in *T. ni* larvae were unaffected by feeding on the different mutant tomato plants (One way ANOVA: $F_{8,15}$=7.45 and $F_{8,14}$=4.06, respectively; Tukey's P>0.05). No significant differences in the CDNB GST or DCNB GST activity were observed for *T. ni* feeding on the three tomato mutants (One way ANOVA: $F_{8,171}$=61.95 and $F_{8,183}$=5.88, respectively; Tukey's P>0.05).

In general, the *T. ni* 4-methylumbelliferyl acetate esterase, α-and β-naphthyl acetate activity were not affected by plant type (One way ANOVA: $F_{8,180}$= 274.06, $F_{8,195}$=124.49 and $F_{8,243}$=69.21, respectively; Tukey's P>0.05). However, activities of all three larval esterases were significantly higher (Tukey's P<0.05) on the Prosys plants compared to esterase activities of larvae on the other two tomato plants in one of the trials (a different trial for each esterase).

Discussion

Although effects of plant defenses were detected at all stages of caterpillar feeding, they appeared most strongly at the post-digestive stage. Overall caterpillar performance was greater on the Def-1 plants as seen in the significantly higher relative growth rate compared to larvae on WT and Prosys plants. This was due in part to an approximately 30% greater consumption on the Def-1 plants. The consumption index (CI) was lower on Prosys compared to the Def-1 plants, but no different from the wild type plants.

A post-ingestive measure of efficiency, approximate digestability (AD), was greater for *T. ni* that fed on Prosys compared to those on Def-1 plants. This may be a result of the reduced fecal output, likely due to a lowering of the food conversion rate in the midgut of the caterpillars feeding on Prosys plants. The increase in AD may compensate partially for the 2-to 3-fold decrease in efficiency of conversion of both digested (ECD) and ingested food (ECI). These results are comparable with a 3 d trial where *T. ni* larvae fed on Def-1, Prosys, and wild-type tomato plants (unpublished results, J. Thaler). The RGR and ECD for Prosys-fed *T. ni* were much less than the Def-1-fed *T. ni* after 3 d compared to 1 d of feeding, but the relative ECI between the two mutants was the same for the two feeding periods.

Insect larvae often compensate for detrimental digestive effects of their food plants by increasing the approximate digestibility (AD). However, over an 11 d trial, *M. sexta* larvae showed greater leaf consumption and ECD on the LOX3 silenced transgenic *Nicotiana attenuata* (Solanaceae) plants than on WT plants, but AD was no different (Rayapuram and Baldwin 2006). These results are similar in that both *M. sexta* and *T. ni* increase leaf consumption and increase digestibility on jasmonate-deficient plants. However, our study found lowered AD values in larvae feeding on Def-1 and WT plants compared to Prosys plants,

Table 2 Analysis of covariance for *Trichoplusia ni* feeding for 24 hr on Def-1, wild-type (WT) and Prosys tomato mutants

	df[a]	*F*	*P*
Relative Growth Rate (RGR)			
Initial dry weight (DW)	1	287.7	<0.001
Plant type	2	27.4	<0.001
Trial	2	39.2	<0.001
Trial x Plant type	4	5.3	0.001
Error	9,73	53.6	<0.001
Consumption Index (CI)			
Initial DW	1	12.1	0.001
Plant type	2	15.1	<0.001
Trial	2	9.3	<0.001
Trial x Plant type	4	6.4	<0.001
Error	9,73	9.5	<0.001
Approximate Digestability (AD)			
DW leaf consumed	1	231.7	<0.001
Plant type	2	14.1	<0.001
Trial	2	35.6	<0.001
Trial x Plant type	4	5.2	0.001
DW leaf consumed x Plant type[b]	2	7.9	0.001
Error	11,71	63.2	<0.001
Efficiency of Conversion of Digested Food (ECD)			
DW leaf consumed—frass produced	1	1.3	0.268
Plant type	2	10.1	<0.001
Trial	2	30.7	<0.001
Trial x Plant type	4	4.5	0.003
(DW leaf consumed—frass) x Plant type	2	1.9	0.161
Error	11,71	13.2	<0.001
Efficiency of Conversion of Ingested Food (ECI)			
DW leaf consumed	1	22.3	<0.001
Plant type	2	12.8	<0.001
Trial	2	40.5	<0.001
Trial x Plant type	4	2.6	0.039
DW leaf consumed x Plant type	2	8.2	0.001
Error	11,71	19.3	<0.001

[a] The degrees of freedom, *F* and *P* values are adjusted for the covariate.

[b] The covariate by Plant type interaction for AD and ECI was significant ($P<0.05$), but not for ECD ($P>0.05$)

whereas the *M. sexta* study did not detect any difference in AD in dependence of the food plant. This difference between *T. ni* and *M. sexta* responses to plant types could be due to different jasmonate-regulated traits in tobacco compared to tomato or to differences in the generalist feeder *T. ni* and the specialist feeder, *M. sexta*. In addition, trial length influences the effect of plant type on larval performance and digestion parameters. We observed also that lengthening the feeding period from 1 to 3 d for *T. ni* on tomato WT, Def-1 and Prosys mutants resulted in

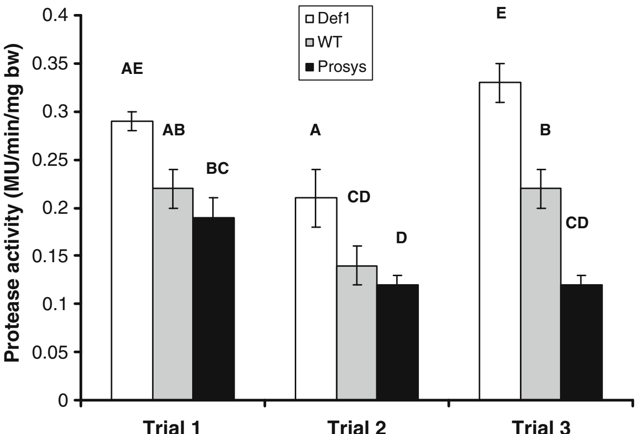

Fig. 2 Serine protease tryptic activity (mU) per min of incubation per mg insect body weight (mU/min/mg bw) in 5th instar *Trichoplusia ni* larvae 24 hr after feeding on three tomato JA mutants in three separate trials. Bars with different shading and the same upper case letter indicate no significant difference between mutants in each trial (two-*way ANOVA*, $P>0.05$). Bars with the same shading and the same upper case letter indicate no significant difference in protease activity for the same mutant between trials (two-*way ANOVA*, $P>0.05$). $N=28$ (Trial 1); 25 (Trial 2), and 28 (Trial 3)

significantly reduced growth and ECD on the Prosys plants, but no difference in AD was detected (unpublished results, J. Thaler).

Further support for the importance of post-digestive stage interference is the reduced serine protease activity in Prosys-fed *T. ni*, compared to those fed on Def-1 plants. There is an inverse relationship between the *T. ni* midgut serine protease levels after 24 hr feeding and the reported levels of defense proteins and enzymes in induced Def-1, WT, and Prosys plants (Howe et al. 1996; Howe and Ryan

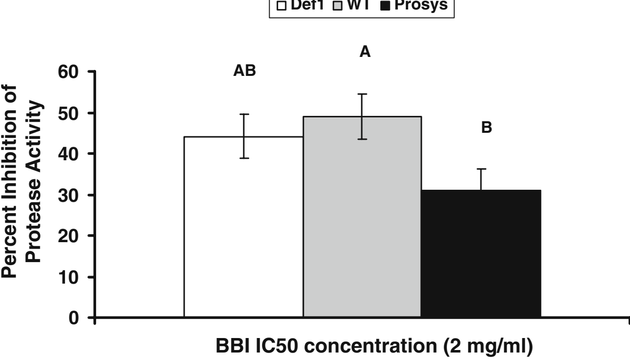

Fig. 3 Percent inhibition of serine protease activity in 5th instar *Trichoplusia ni* larvae after midgut homogenates were incubated with Soybean Bowman-Birk Inhibitor (BBI). Bars with different shading and the same upper case letter indicate no significant difference between mutants (*one way ANOVA*, $P>0.05$). $N=3$ midgut samples per plant type

1999; Li et al. 2002). An inverse correlation between the level of foliar PI activity and larval growth has been observed similarly with both *T. ni* fed on cabbage and *S. exigua* fed on tomato (Broadway et al. 1986; Broadway and Colvin 1992). Prosys plants have constitutively greater levels of PIs, PPO, and other compounds well-characterized by many researchers, so the reduction of serine protease levels in the *T. ni* midguts is just one measurable response that confirms a direct effect of plant nutrients on the insect metabolism. Insects other than the cabbage looper, such as the tomato hornworm, may be more successful herbivores on plants that express higher JA-inducible defenses through their greater ability to compensate for reduced protease levels.

A small compensation for post-digestive interference was detected through the use of the soybean Bowman-Birk inhibitor (BBI) in the present study. To our knowledge this is the first study to examine the effect of BBI on *T. ni* serine protease activity post-feeding on plants expressing a range of PI activity. According to Chougule et al. (2008), BBI has dual specificity towards tryptic and chymotryptic activities. Although the relative amount of trypsin-chymotrypsin activity inhibited by BBI was less in the midguts of the *T. ni* feeding on the Prosys tomatoes, the serine protease activity was reduced to a greater degree than in the midgut of *T. ni* fed on the other two tomato plants. Since we did not isolate the trypsin-like enzymes from *T. ni* that fed on the three tomato mutants, we cannot confirm an increase in the relative proportion of resistant trypsin-like enzymes in the midgut of *T. ni* that fed on the Prosys plants. However, the lower proportion of trypsin-chymotrypsin activity reduced by BBI in the Prosys-fed *T. ni* does agree with the results of a study by Broadway (1995) where *T. ni* fed a diet with cabbage proteinase inhibitors had increased levels of activity from enzymes that were less susceptible to inhibition.

Up-regulation of other proteases may require more than 24 hr, and thus may not be detectable in the WT-fed *T. ni* samples. An increase in the level of proteases with a lower affinity for BBI or the presence of other classes of proteolytic enzymes was thought to be the reason for the lower BBI-inhibitory activity in *Heliothis obsolete* feeding on hemizygote transgenic tomato plants compared to control plants (Abdeen et al. 2005). Those authors suggested that the 1.6-fold increase in BBI-insensitive digestive proteases in *H. obsolete* was a compensation for the partial loss of activity caused by the presence of the introduced serine protease inhibitors in the transgenic tomatoes. The differences between the two studies suggests that BBI may have an effect on a different range of trypsin proteases present in *T. ni* compared to *H. obsolete*, some of which might be up-regulated in response to induced tomato PI levels. Another possibility may be that the protein substrate casein does not reflect quantitatively the activity of the PI-insensitive proteases (Jongsma and Bolter 1997).

Among the many JA-pathway inducible compounds, PPO has a direct effect on digestive processes in insects by decreasing the availability of essential amino acids in the insect gut, considered to be an anti-nutritive effect (Barbehenn et al. 2007). This is different from the effect produced by PIs, since a reduction in protease activity is considered an anti-digestive effect. Despite these differences, tomato mutants that overexpress PPO had a similar negative effect on insect growth, consumption rate, ECD, and ECI (Mahanil et al. 2008) observed in the present study.

A toxic effect or combined mechanism of action that negatively impacts the post-digestive processes cannot be ruled out. However, there was no difference in the levels of cytochrome P450/b_5 and GST, and only small, inconsistent increases in esterase activity in the *T. ni* that fed on the Prosys tomatoes. Induction of insect monooxygenases by phenolic compounds such as indoles and flavones has been described by Yu (1984). However, for both a specialist and a generalist insect fed on transgenic tobacco lines, no correlation between foliar levels of phenolics (chlorgenic acid, rutin, and total flavonoids) and larval growth and survival was noted (Bi et al. 1997). Chlorogenic acid, rutin, and α-tomatine are phenolics found at toxic levels in tomato leaves (Elliger et al. 1981), but detoxification enzyme levels have, to our knowledge, never been measured after insect exposure to these compounds. In selected plant systems, induced monooxygenase or glutathione levels can play a role in the insect response to plant toxins, for example induced GST activity as a generalized detoxification response to plant allelochemicals (Yu 1982, 1984; Wadleigh and Yu 1987; Francis et al. 2005). In the present study, there was no affect on the levels of cytochrome P450/b_5 or glutathione transferases associated with feeding on the three tomato mutants. This confirms the importance of proteins as an induced defense in tomato, rather than alkaloids, in order to reduce herbivory.

We conclude that pre and post-ingestive processes work together to reduce insect growth on plants with high levels of jasmonate regulated defenses. JA-mediated plant responses directly impact midgut protein digestion at least in part through increased defense proteins (PIs) and oxidative enzymes (PPO). In this study, the reduction in serine proteases and the ability of the insect to absorb nutrients for growth was decreased significantly in defended plants, as exemplified by the lower efficiency of conversion of absorbed and ingested food on Prosys-fed versus Def-1-fed *T. ni*. Based upon what we know of the post-digestive stage, a reduction in ECI and ECD indicates that the insect is not able to obtain the necessary essential

macronutrients for growth. In the present case, we have empirical evidence that suggests an anti-digestive effect is occurring since there is a reduction in the protease activity in the insect gut. However, evidence from prior studies with the Prosys mutant details the significantly greater production of PPO relative to the wild-type, a factor that has an anti-nutritive effect. One of the two expectations of this study was met: post-digestive processes were significantly affected by JA-induced defenses due to both anti-digestive and anti-nutritive activity, whereas the toxic affect from tomato allelochemicals did not produce a measured response in the insect.

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Genetic Variation in Jasmonic Acid- and Spider Mite-Induced Plant Volatile Emission of Cucumber Accessions and Attraction of the Predator *Phytoseiulus persimilis*

Iris F. Kappers · Francel W. A. Verstappen · Ludo L. P. Luckerhoff · Harro J. Bouwmeester · Marcel Dicke

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Abstract Cucumber plants (*Cucumis sativus* L.) respond to spider–mite (*Tetranychus urticae*) damage with the release of specific volatiles that are exploited by predatory mites, the natural enemies of the spider mites, to locate their prey. The production of volatiles also can be induced by exposing plants to the plant hormone jasmonic acid. We analyzed volatile emissions from 15 cucumber accessions upon herbivory by spider mites and upon exposure to jasmonic acid using gas chromatography—mass spectrometry. Upon induction, cucumber plants emitted over 24 different compounds, and the blend of induced volatiles consisted predominantly of terpenoids. The total amount of volatiles was higher in plants treated with jasmonic acid than in those infested with spider mites, with (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E,E*)-α-farnesene, and (*E*)-β-ocimene as the most abundant compounds in all accessions in both treatments. Significant variation among the accessions was found for the 24 major volatile compounds. The accessions differed strongly in total amount of volatiles emitted, and displayed very different odor profiles. Principal component analysis performed on the relative quantities of particular compounds within the blend revealed clusters of highly correlated volatiles, which is suggestive of common metabolic pathways. A number of cucumber accessions also were tested for their attractiveness to *Phytoseiulus persimilis*, a specialist predator of spider mites. Differences in the attraction of predatory mites by the various accessions correlated to differences in the individual chemical profiles of these accessions. The presence of genetic variation in induced plant volatile emission in cucumber shows that it is possible to breed for cucumber varieties that are more attractive to predatory mites and other biological control agents.

Key Words *Cucumis sativus* L. · *Tetranychus urticae* · *Phytoseiulus persimilis* · Cucumber · Spider mite · Predatory mite · Herbivore-induced plant volatiles · Tritrophic interactions

I. F. Kappers · L. L. P. Luckerhoff · M. Dicke
Laboratory of Entomology,
Wageningen University and Research Centre Wageningen,
Wageningen, The Netherlands

I. F. Kappers (✉) · F. W. A. Verstappen · L. L. P. Luckerhoff ·
H. J. Bouwmeester
Plant Research International,
Wageningen University and Research Centre Wageningen,
Wageningen, The Netherlands
e-mail: i.f.kappers@wur.nl

F. W. A. Verstappen · H. J. Bouwmeester
Laboratory of Plant Physiology,
Wageningen University and Research Centre Wageningen,
Wageningen, The Netherlands

Introduction

Cucumis sativus L. is grown in nearly all temperate regions and is one of the ten most widely cultivated vegetable species, ranking fourth after tomato, onion, and cabbage (Tatlioglu 1993). Cucumber is indigenous to India, and its domestication started there approximately 3,000 years ago, followed by spreading into Western Asia and then southern Europe. In cucumber breeding programs, genotypes are selected for improved performance in specific environments (greenhouse, field, single, or multiple harvest), as well as for intended use (processing, fresh market), consumer preference (fruit shape and quality), pest and diseases

resistance, abiotic stress tolerance, and high fruit yield. It has been observed that the genetic base of commercial cucumber germplasm is not very broad (Staub and Meglic 1993). Despite the agricultural and biological importance of cucurbits, knowledge of their genetics and genome currently is limited (Huang et al. 2009). Moreover, the historical reservoirs of genetic variation in cucumber, such as India (primary center), Burma, and Southern China (secondary center), are subject to genetic erosion (Tatlioglu 1993). So far, plant breeders have hardly selected for crop characteristics that influence the success of biological control (but see Van Lenteren and De Ponti 1990) even though it is obvious that for successful biological control it is important that biological control agents also can find the herbivores, at low densities (Dicke 1999).

Plants respond to herbivore feeding with the production of volatiles that natural enemies use as cues to locate the herbivores. This phenomenon has been observed in various tritrophic systems and is an important mechanism by which plants defend themselves against herbivorous insects (e.g., Dicke and Van Loon 2000; Arimura et al. 2005; Dudareva et al. 2006; D'Alessandro and Turlings 2006; Mumm and Hilker 2006; Hilker and Meiners 2010). Induced defenses allow plants to be cost-effective and also to diminish the risk that herbivores will adapt to plant defenses (Heil 2008; Steppuhn and Baldwin 2008). Plants exhibit variation in these traits, which comprises both intra-individual phenotypic plasticity and genetic polymorphisms among individuals and populations. The plasticity of inducible defense allows individual plants to adapt to changing environments (Agrawal 2001). Polymorphism in traits for resistance against herbivory may result from varying selection pressures among populations.

Herbivory Induced Plant Volatile (HIPV) blends vary considerably both in quantity and quality with plant species or plant varieties (Krips et al. 2001; Hoballah et al. 2002; Degen et al. 2004; Van den Boom et al. 2004; Bukovinszky et al. 2005; Lou et al. 2006). This may result in differences in the degree of attraction of natural enemies of the herbivores (Krips et al. 2001; Hoballah et al. 2002; Bukovinszky et al. 2005; Lou et al. 2006). Knowledge of which of the induced volatiles have ecological significance may aid eventually the development of methods to enhance the attractiveness of crop varieties to beneficial arthropods. Typical plant volatiles that are induced by herbivory are green leaf volatiles: C6-alcohols, -aldehydes and -acetates; phenylpropanoids such as methyl salicylate; and various monoterpenes and sesquiterpenes, as well as two homoterpenes: (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Arimura et al. 2005; Dudareva et al. 2006). Green leaf volatiles are fatty acid derivatives that result from the lipoxygenase pathway (D'Auria et al. 2007). Terpenoids are synthesized either via the cytosolic mevalonate (MVA) pathway or via the methylerythritol 4-phosphate (MEP) pathway in the plastids (Aharoni et al. 2005; Rodríguez-Concepción 2006).

The objectives of this study were to explore the variation among cucumber accessions with respect to herbivory-induced volatile emission and to determine to what extent differences in volatile profiles affect the attraction of predatory mites. We compared the volatile emission of various cucumber accessions in response to spider-mite (*Tetranychus urticae*) infestation and jasmonic acid treatment, as well as the attractiveness of a number of these accessions to the predatory mite *Phytoseiulus persimilis*.

To take differences in direct defense into account, e.g., due to differences in cucurbitacin C content (Balkema-Boomstra et al. 2003), jasmonic acid was used to standardize the level of induction, as the production of many induced volatiles is mediated by the jasmonic acid signalling (Dicke et al. 1999; Gols et al. 1999; Degenhardt and Lincoln 2006; Ozawa et al. 2008). Previous work demonstrated that spider–mite infestation and jasmonic acid both induce similar components in the volatile blend of cucumber, albeit in different amounts and at a different time scale (Bouwmeester et al. 1999; Mercke et al. 2004).

Methods and Materials

Plants, Herbivores and Predators Cucumber (*Cucumis sativus*) seeds were obtained from the Centre for Genetic Resources, the Netherlands (CGN), which is part of Wageningen University and Research Centre. As there is little to no information about genetic variation in relation to induced volatiles in cucumber, seven accessions were chosen as representative of various places of origin. In addition, eight cucumber F1-hybrids from commercial breeders were chosen. Seeds were sown and grown at 22–23°C, 60–70% relative humidity, and 16 hr of light per day in a greenhouse at Wageningen University, The Netherlands. SON-T lamps were used to supply extra light when the natural light intensity decreased below 250 μmol m^{-2} sec^{-1}. Plants used for experiments were 3–4-wk-old and had four or five fully expanded leaves.

Two-spotted spider mites, *Tetranychus urticae* Koch, were reared on lima bean plants (*Phaseolus lunatus*) in a greenhouse at 24±4°C, 60±20% relative humidity and a 16:8 hr, L:D photoperiod. For spider mite-infestation, approximately 50 adult female spider mites were evenly distributed on the adaxial side of fully expanded leaves with a fine brush.

Predatory mites, *Phytoseiulus persimilis* Athias-Henriot, were reared on spider mite-infested lima bean leaves to exclude that predator behavior could be influenced by exposure to cues emitted from one specific cucumber accession above others (Dicke et al. 1990b; Takabayashi

and Dicke 1992). Predators were reared in a growth chamber at 23±1°C, 60±10% RH and continuous light. Before experiments, females were starved individually in 1.5 ml microcentrifuge tubes for 3±0.5 hr at 23±1°C.

Application of Jasmonic Acid Plants were sprayed with a 0.5 mM (±)-JA solution (Sigma–Aldrich, St. Louis, MO, USA) with 0.01% Tween-20 using an aerosol spray bottle until completely wet (ca 5 ml per plant). Mock treated plants were sprayed with water containing 0.01% Tween-20. JA-sprayed plants were separated from control and spider–mite infested plants in a different greenhouse compartment but under the same environmental conditions.

Collection and Analysis of Induced Cucumber Volatiles To allow valid comparison of the composition and quantities of the volatiles, headspace samples were collected from all accessions at the same time during the photoperiod. As there were several experiments in different periods of the year, the variety 'Hybrid C' was used as a reference in all experiments. Individual plants were enclosed in glass cuvettes (2.5 l) with a viton-lined lid equipped with an inlet and an outlet and placed in a climate room at 21±1°C and a light intensity of 120 µmol m^{-2} sec^{-1}. A vacuum pump was used to draw air through the glass cuvette at approximately 100 ml min^{-1} with the incoming air being purified through a cartridge containing 200 mg of Tenax TA (20/35-mesh, Alltech). At the outlet, the volatiles emitted by the plants were trapped on a similar cartridge. For quantification of the volatiles released, carvone and benzyl acetate in hexane were added as internal standards in a T-shaped glass liner placed between the cuvette and the Tenax outlet. For spider–mite infested plants, 600 ng of each compound were added to the cuvette, whereas in case of JA-treated plants, 6 ng were added. Volatiles were sampled for 3 hr. Volatiles from at most 11 accessions were collected simultaneously, and five replicate samples were collected for each accession over time (Hybrid C 12 times). To check for background volatiles, released by e.g., materials used, volatiles from empty jars were trapped as well. The amounts of the 24 dominating compounds were quantified in area units under the curve, and were corrected using the response factors of each compound towards the internal standard closest in retention time. For compounds for which we did not have a standard, we estimated the amounts emitted based on the average response factor of compounds between RI 850 and 1,000 (for compounds d, e, and f); on the response factor of (*E*)-β-ocimene (for compound k), or on the average response factor of homo- and sesquiterpenes q, t, w, and x (for compounds r, u, and v).

Gas Chromatography—Mass Spectrometry Headspace samples of cucumber plants were analyzed on a Hewlett–Packard GC-MS or on a Trace GC Ultra™ (Thermo Electron Corporation, Austin,TX, USA) equipped with a Trace DSQ quadrupole mass spectrometer (Thermo Electron Corporation, Austin,TX, USA). For this, Tenax cartridges either were eluted using 3×1 ml of re-distilled pentane : diethyl ether (4:1) or released from Tenax traps using a thermal desorption cold trap setup (TDS; UNITY™, Markes international LTD, UK). After elution, samples were concentrated to about 30 µl, of which 2 µl were analyzed by GC-MS using a gas chromatograph (5,890 series II, Hewlett–Packard) equipped with a 30-m× 0.25-mm i.d., 0.25-µm film thickness column (5MS, Hewlett–Packard) and a mass-selective detector (model 5972A, Hewlett–Packard). The GC was programmed at an initial temperature of 45°C for 1 min, with a ramp of 10°C min^{-1} to 220°C, and final time of 5 min. The injection port (splitless mode), interface, and MS source temperatures were 250°C, 290°C, and 180°C, respectively, and the He inlet pressure was controlled with an electronic pressure control to achieve a constant column flow of 1.0 ml min^{-1}. The ionization potential was set at 70 eV, and scanning was performed from 30 to 250 amu.

Tenax cartridges analyzed on the Trace GC were first dry-purged with helium at 100 ml min^{-1} for 10 min at ambient temperature to remove any water. Volatiles were released from Tenax traps using a thermal desorption cold trap setup by heating at 250°C for 3 min, with a He flow of 30 ml/min. Desorbed volatiles then were transferred to an electronically-cooled focusing trap at −10°C within the TDS. Volatiles were injected in splitless mode into the analytical column (Restek, RTX 5MS, 30 m 0.25 mm i.d., 1.0 µm *df*) by heating the cold trap to 300°C. The GC was held at the initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C/min to 280°C, and held for 2.5 min with a He flow of approximately 1 ml/min. The column was coupled directly to the ion source of the DSQ quadrupole mass spectrometer, which was operated in the 70 eV EI ionization mode and scanned from mass 45 to 400 amu. An auto sampler was used for the automatic desorption of the Tenax traps (UltrA™, Markes international Ltd, UK). The TC-20 Multi-tube conditioning unit (Markes International Ltd, UK) was used for cleaning the cartridges in between the measurements by heating them to 310°C for 40 min under a He flow. Compounds were identified by comparing the mass spectra with the Wiley and NIST mass spectral libraries and by comparing retention indices with Adams (1995). Headspace samples of plants exposed to spider–mite infestation in a time-series experiment were analyzed on the HP-GC-MS, whereas for the experiment where the headspace from JA-treated plants were compared to the headspace of spider–mite infestations was analyzed by thermodesorption GC-MS.

Olfactometer Set-up and Predatory Mite Bioassay To compare the responses of predatory mites to the induced volatiles of a selection of accessions we used a closed Y-tube olfactometer as previously described (Takabayashi and Dicke 1992; Bukovinszky et al. 2005). Odor sources consisted of a single plant with 4–5 leaves that were infested with 50 spider mites 1 wk prior to the experiment or induced with JA 24 hr prior to the experiment. Experiments compared plants of different accessions that both were infested with spider mites or that were both induced with JA. For each accession, induced plants also were tested against non-induced plants. Individual female predators were released on an iron wire in the basal tube, and their behavior was observed for a maximum of 5 min. The connections of the odor sources to the arms of the olfactometer were interchanged after testing a series of five predators. A choice was recorded when the finish line, halfway up one of the olfactometer arms, was reached within this period. Otherwise it was recorded as no-choice. Each predator was used only once. Per experimental day, 20 predators were tested for each odor combination, and each experiment was repeated on 3–5 different days.

Statistical Analysis Not all volatiles were detected in every replicate of every accession. To be able to determine ratios among accessions and allow log-transformation of data, we added 1,000 area units to all data points, representing the lowest recorded non-zero value. A number of volatiles were not included in the data analysis because they occurred in one or two accessions only, with an abundance of less than 0.1% of the total volatile production.

Peak areas of all compounds were corrected for fresh weight of the leaves. Per compound, the *Student's t-test* was used to determine whether the compound was induced significantly by herbivory. Experiments were conducted over a period of 2 yr. To correct for the inevitable variation in the absolute amounts of individual volatiles, emissions are given as least-squares means as calculated by two-factor ANOVA, with experimental period as the second factor.

Standard Pearson correlation coefficients were determined among individual accessions for each volatile and a *Student's t-test* was used to determine the significance of these correlations. Cluster and correlation analysis also were performed using Genemaths (Applied Maths, Sint-Martens-Latem, Belgium). Principal component analysis (PCA) involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. PCA gives complementary information to ranking lists based on correlation coefficients to specific volatiles. The significance level for correlations was set at $P<0.05$. To comply with ANOVA assumptions, all volatile emission data were log-transformed and normalized (mean of complete data-set set to zero) by dividing by the average volatile emission rate calculated from all experiments.

The choices of predatory mites between odor sources in the Y-tube olfatometer were analyzed with two-sided binominal tests to determine whether the distribution of the predators' choice was significantly different from 50:50. Predators that did not make a choice were excluded from the statistical analysis.

Results

The headspace of cucumber plants induced by spider–mite feeding or treatment with JA consisted of a maximum 24 volatile compounds that collectively represented more than 98% of the total emission of cucumber leaf volatiles (Table 1). Most of these compounds were not detected in the headspace of non-induced plants, except for nonanal, decanal, limonene, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, which were present also in trace amounts in non-induced plants. Methyl jasmonate and jasmone sometimes were detected in the headspace of JA-treated plants, but not in the headspace of mite-damaged plants. Because these two compounds likely are a direct consequence of the JA treatment, they were not included in the statistical analyses.

No visual damage was observed after JA-treatment, whereas spider–mite damage led to a decrease of about 8–14% of the chlorophyll content during a 7-d-period of feeding (data not shown). Feeding damage was sufficient to cause an increase in the total amount of volatiles from day 1 onwards (Fig. 1). Progressing infestation of the mites increased volatile release with time (Fig. 1). JA-application resulted in the release of volatiles from treated leaves within 2 hr and was maximal after 2–3 d (Table 2). The total emission of volatiles varied strongly among the various accessions, ranging from about 80 ng per plant to over 6 μg per plant (Table 2). After 3 d, the volatile emission rate diminished (data not shown).

Mite feeding and JA application both increased the emission of many compounds. By far, the most abundant compounds in plants induced either by *T. urticae* infestation or by JA treatment were DMNT, (*E*)-β-ocimene, (*E,E*)-α-farnesene, and (*Z*)-3-hexenyl acetate (Fig. 2). However, there is a large range of variation among accessions for all compounds (Table 1, Fig. 2). The majority of induced compounds were terpenoids: about 75% of the total volatile blend, consisting of six monoterpenoids [α-phellandrene, thujene, (*Z*)-β-ocimene, (*E*)-β-ocimene, limonene, linalool], two homoterpenes [DMNT, TMTT], and five sesquiterpenoids [(*E*)-β-caryophyllene, (*E,E*)-α-farnesene, and three unknown sesquiterpenoids]. The remaining compounds were an aldehyde, two alcohols, three acetate

Table 1 Variation in volatile compounds emitted by cucumber plants of different origin that were infested with spider mites during 7 days or were treated with jasmonic acid (JA) 2 days prior to headspace collection

	Compound[a]	Biochemical class	RI (RI STD)		Relative Quantities[2] of Compounds in Volatile Blend				
					Non-treated	JA treated (2 days)		Spider mite infested (7 days)	
					Mean±SE	Mean±SE	Range	Mean±SE	Range
a	(*E*)-2-Hexenal	lipid derivative	851 (849)	STD	43.0±7.9	0.45±0.2	0.8	0.41±0.2	0.7
b	(*Z*)-3-Hexen-1-ol	lipid derivative	857 (857)	STD	3.5±1.6	0.74±0.5	3.4	0.40±0.8	4.3
c	1-Hexanol	lipid derivative	868 (867)	STD	2.1±0.9	0.81±0.6	3.0	0.91±0.8	2.8
d	Oxime 1	N-aromatics	925	Rt/MS	–	0.57±0.2	1.0	0.50±0.2	0.7
e	3-Me-butanal-O-Me-oxime	N-aromatics	968	Rt/MS	5.8±1.7	4.22±1.6	9.4	0.13±0.1	0.1
f	Nitrogen-containing compound	N-aromatics	988	Rt/MS	3.6±2.7	2.05±0.9	4.8	0.64±0.3	0.9
g	(*Z*)-3-Hexen-1-yl butyrate	lipid derivative	1,159 (1,161)	STD	–	0.31±0.2	0.9	0.41±0.3	0.9
h	(*Z*)-3-Hexen-1-yl acetate	lipid derivative	1,013 (1,015)	STD	36.5±1.9	8.27±3.4	20.3	46.56±5.2	19.0
i	α-Phellandrene	monoterpenoid	999 (1,003)	STD	–	1.60±0.5	2.6	1.01±0.2	0.8
j	Thujene	monoterpenoid	928 (931)	STD	–	0.54±0.2	0.8	0.39±0.2	0.8
k	(*Z*)-β-Ocimene	monoterpenoid	1,037 (1,040)	Rt/MS	–	0.71±0.2	1.1	0.69±0.2	0.9
l	(*E*)-β-Ocimene	monoterpenoid	1,046 (1,050)	STD	2.6±0.9	26.78±6.9	42.3	7.11±2.3	9.2
m	Limonene	monoterpenoid	1,032 (1,031)	STD	–	2.05±0.7	4.2	1.04±0.4	1.7
n	Linalool	monoterpenoid	1,096 (1,098)	STD	–	0.15±0.1	0.6	0.23±0.2	0.6
o	Nonanal		1,097 (1,098)	Rt/MS	2.9±0.2	3.02±1.2	7.0	2.79±1.0	4.3
p	Decanal		1,201 (1,204)	RT/MS	–	3.17±1.1	6.4	3.14±1.5	5.9
q	DMNT	homoterpenoid	1,118	STD	–	16.28±8.5	55.5	12.74±4.7	21.9
r	Unknown sesquiterpenoid	terpenoid	1,121	MS	–	3.33±1.4	8.0	1.79±1.6	6.0
s	Methyl salicylate	phenylpropanoid	1,197 (1,194)	STD	–	0.73±0.6	3.2	1.07±0.9	2.3
t	(*E*)-β-Caryophyllene	sesquiterpenoid	1,434 (1,430)	STD	–	2.22±1.4	6.5	2.01±1.8	6.8
u	Unknown sesquiterpenoid	terpenoid	1,460	MS	–	0.17±0.2	0.8	0.27±0.2	0.8
v	Unknown sesquiterpenoid	terpenoid	1,487	MS	–	1.03±1.4	8.6	0.71±0.9	3.6
w	(*E,E*)-α-Farnesene	sesquiterpenoid	1,534 (1,534)	STD	–	13.05±3.1	18.2	13.30±3.8	12.6
x	(*E,E*)-TMTT	homoterpenoid	1,554 (1,555)	STD	–	6.31±1.9	10.9	3.3±0.8	3.1

[a] Identification of compounds is based on comparison with authentic (STD) or comparison with (KOVATS), retention index (RI) or on with mass spectra of various data bases (MS)

[b] Quantities are given as proportion of the total blend ±SE. The range of variation is an indication for genetic variation

esters, one nitrogen-containing aromatic compound, and two oximes. Most of these were released into the headspace within hours after the application of JA or within 1 d after spider–mite feeding started.

Experiments were performed during a 2-yr-period, and thus we have to deal with large absolute differences in volatile release. We, therefore, first analyzed volatile release of accessions for the relative contributions of individual compounds and found that these were rather constant over different experiments. Thus, the relative amount of e.g., (*E*)-β-ocimene varied between 35% and 40% in Hybrid T, 15–25% in CGN20890 and 9–16% in Hybrid C, and for DMNT, 25–32%, 4–8%, and 28–33%, respectively. Furthermore, (*E*)-β-caryophyllene never was detected in headspace samples of Hybrid C and CGN20890, but it was detected in all samples of Hybrid T (1.5–5%). We, therefore, decided to use the data on the relative amounts from the different experiments within one multivariate analysis.

PCA is used in statistics to extract the main relationships in data of high dimensionality. The first axis of the PCA accounted for 42% of the total variation recorded in JA-induced plants. It was positively correlated with the quantity of all compounds that was included as a factor in the analyses and hence separated the accessions according to the total volatile emission. Therefore, we focused rather on the proportions of the compounds in the blend, as this may lead to a better distinction between different accessions. The second axis of the PCA accounted for 17% of the total variation recorded in JA-induced plants (Fig. 3). This component was correlated positively to the proportion of DMNT in the induced blend and roughly separated the European hybrids from the Asian and African landraces (Fig. 3). Indeed, European hybrids ranged from 11% to

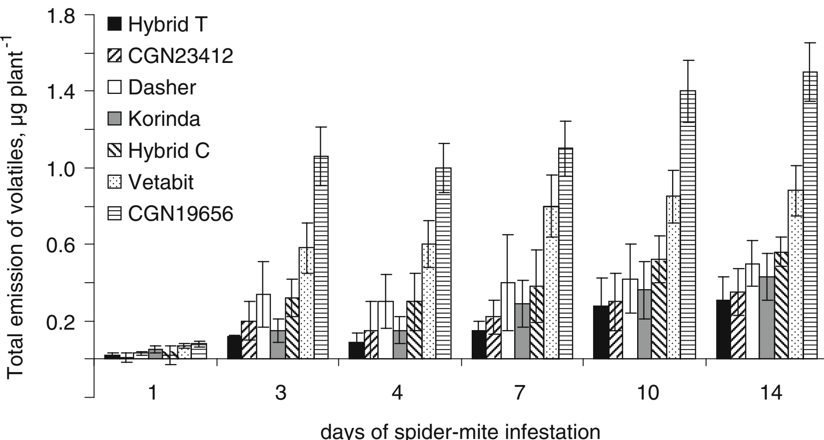


Fig. 1 Total amount of volatile compounds, i.e. the sum of 24 volatile compounds, Mean±SE emitted by 3-wk-old *Cucumis sativus* plants of seven different accessions in response to spider–mite feeding during 14 d. From *left* to *right*: accessions Hybrid T, CGN23412, Dasher, Korinda, Hybrid C, Vetabit and CGN19656. Headspaces were sampled on day 1, 3, 4, 7, 10 and 14 after infestation

56% DMNT in the volatile blend, whereas the accessions from Asia and Africa emitted only 15% to less than 1% of DMNT. The high proportion of DMNT in the European hybrids is correlated to a relatively low proportion of (*E*)-β-ocimene and (*E,E*)-α-farnesene in the volatile blends (Figs. 2, 3, Table 1).

A volatile-volatile matrix based on Pearsons correlation shows clusters of compounds whose proportions in the blend were correlated (positively): (*E*)-β-ocimene, (*E,E*)-α-farnesene, and an unknown sesquiterpenoid one (mutual Pearson correlations of >0.93) cluster strongly together (Fig. 4). A second cluster was formed by limonene, thujene, and α-phellandrene (mutual Pearson correlations >0.80) (Fig. 4). These three volatiles also clustered in the loading plot of the PCA (Fig. 3).

Responses of Predatory Mites We tested the preferences of the predatory mite *Phytoseiulus persimilis* for the volatile blends of a selection of accessions of spider-mite infested and JA-treated plants in a Y-tube olfactometer. Two hybrids were selected that had either a low (hybrid T) or a high volatile emission rate (hybrid C). Furthermore, three landraces were selected, among them the lowest (CGN19821) and the highest (CGN19656) emitter of all accessions used in this study. For all selected accessions, the predatory mites significantly preferred volatiles induced by spider mites or jasmonic acid to blends of non-induced plants (data not shown). Accessions CGN23412, Hybrid C, and Hybrid T were infested with 50 spider mites on each plant and tested against each other 7 d after the onset of infestation. Preferences of predatory mites could be ranked

Table 2 Total amount of volatiles [least-squares means ng plant ±SE], i.e. the sum of 24 compounds emitted by 3-wk-old plants of 15 cucumber accessions in response to (±)-JA (0.5 mM) spraying

Accession	Country of origin	Total amount [ng±SE] of volatiles emitted			
		Control plants	JA treated plants		N
			2d	3d	
CGN19821	Nepal	4.2±2.2	93.6±9.8	78.2±7.6	5
CPRO1	EU-1	9.5±2.5	127.7±19.2	364.2±25.3	5
Hybrid T	EU-2	14.3±3.6	168.0±33.5	334.2±51.7	5
CPRO2	EU-1	13.5±4.8	263.4±31.9	420.1±63.1	5
CGN23412	Egypt	23.7±5.6	349.7±31.4	614.5±67.5	5
CGN22310	Kazachstan	11.7±7.6	354.2±54.0	234.5±35.7	5
Dasher II	EU-3	19.7±2.5	393.4±37.9	889.5±61.7	5
Korinda	EU-4	6.9±1.8	480.9±64.8	753.6±98.3	5
Sharp1	JP-1	12.3±7.3	616.7±80.1	648.5±100.2	5
Hybrid C	EU-2	17.6±2.6	836.3±59.4	807.4±100.3	12
CGN21623	Libanon	39.0±9.4	952.1±74.6	2,090.4±232.7	5
Vetabit	unkown	22.7±3.8	1,086.9±131.4	4,449.4±395.6	5
CGN20890	India	14.6±2.6	1,095.1±171.6	3,526.0±387.2	5
CGN19568	Pakistan	29.5±4.2	1,740.9±168.0	2,451.2±368.3	5
CGN19656	Iran	12.8±6.8	2,642.4±355.2	6,051.4±573.7	5

EU-1, F1 hybrid developed by PRI formerly CPRO-DLO, The Netherlands

EU-2, F1 hybrid developed by Dutch Seed company

EU-3, F1 hybrid developed by Seminis, India

EU-4, F1 hybrid developed by Nunhems, The Netherlands

JP-1, Japanese F1 hybrid

Fig. 2 Relative contribution of individual volatile compounds to the total volatile blend of different *Cucumis sativus* accessions 2 d after treatment with JA (0.5 mM; 15 accessions) or after 7 d of spider–mite infestation (Sp, seven accessions)

from CGN23412 > Hybrid C > Hybrid T ($P<0.01$; Fig. 5). Although Hybrid C had the highest total release of volatiles (Fig. 1), this did not result in the strongest attraction of predatory mites. The individual volatiles of the mite-infested plants could be ranked according to their attractiveness to predatory mites. This ranking was correlated positively with the relative amounts of (*E*)-2-hexenal, (*E*)-β-ocimene, (*E*)-β-caryophyllene, and (*E,E*)-α-farnesene in the volatile blend (Fig. 5b).

In a parallel experiment, a selection of accessions were treated with JA and used in the Y-tube olfactometer at different times after the onset of the treatment (Fig. 6). Predatory mites preferred the volatiles released by JA-treated CGN19656 above those of Hybrid C, Hybrid T, or CGN19821. The preference was maximal 2–3 d after JA application and diminished afterwards (Fig. 6). As CGN19656 was by far the highest emitter of volatiles (Table 1), this preference is correlated strongly with the

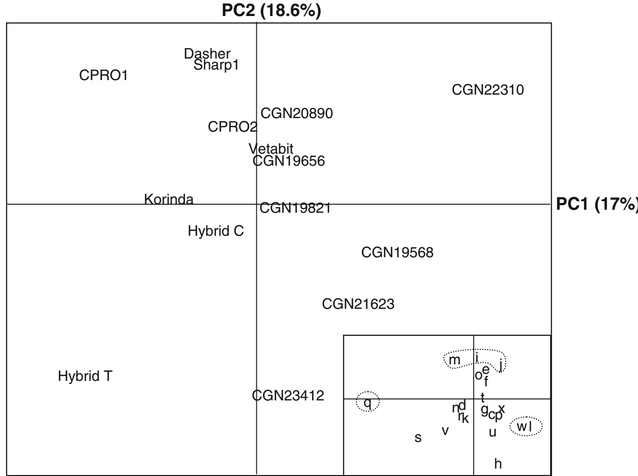


Fig. 3 Principal component analysis based on Pearson's correlations: the second principal component PC2 versus the third principal component PC3 of JA-induced volatiles in cucumber. *Inset* shows the loading plot, *letters* indicate individual volatile compounds, as explained in Table 2. *Dotted circles* indicate those volatiles that are discussed in the text

absolute amount of induced volatiles. Therefore, it is valuable to study the difference between e.g., Hybrid T and CGN19821, that both emit low amounts of volatiles (Table 2). In this case, volatiles released by CGN19821 were significantly more attractive to predatory mites 2 and 3 d after JA application (Fig. 6). CGN19821 had significantly higher proportions of 3-methyl-butanal-*O*-methyloxime, (*E*)-β-ocimene, and (*E*)-β-caryophyllene in its blend, whereas the blend of Hybrid T was relatively rich in (*Z*)-3-hexenyl acetate and DMNT (Fig. 2). The blend of Hybrid C also was more attractive than that of Hybrid T on day 3 (Fig. 6). Hybrid C is relatively rich in (*E,E*)-α-farnesene and TMTT, and low in (*Z*)-3-hexenyl acetate as compared to the blend of Hybrid T (Fig. 2).

Discussion

Varietal or genotypic differences in herbivore-induced plant volatiles have been studied primarily in cultivars of agricultural crops, including cotton (Loughrin et al. 1995),

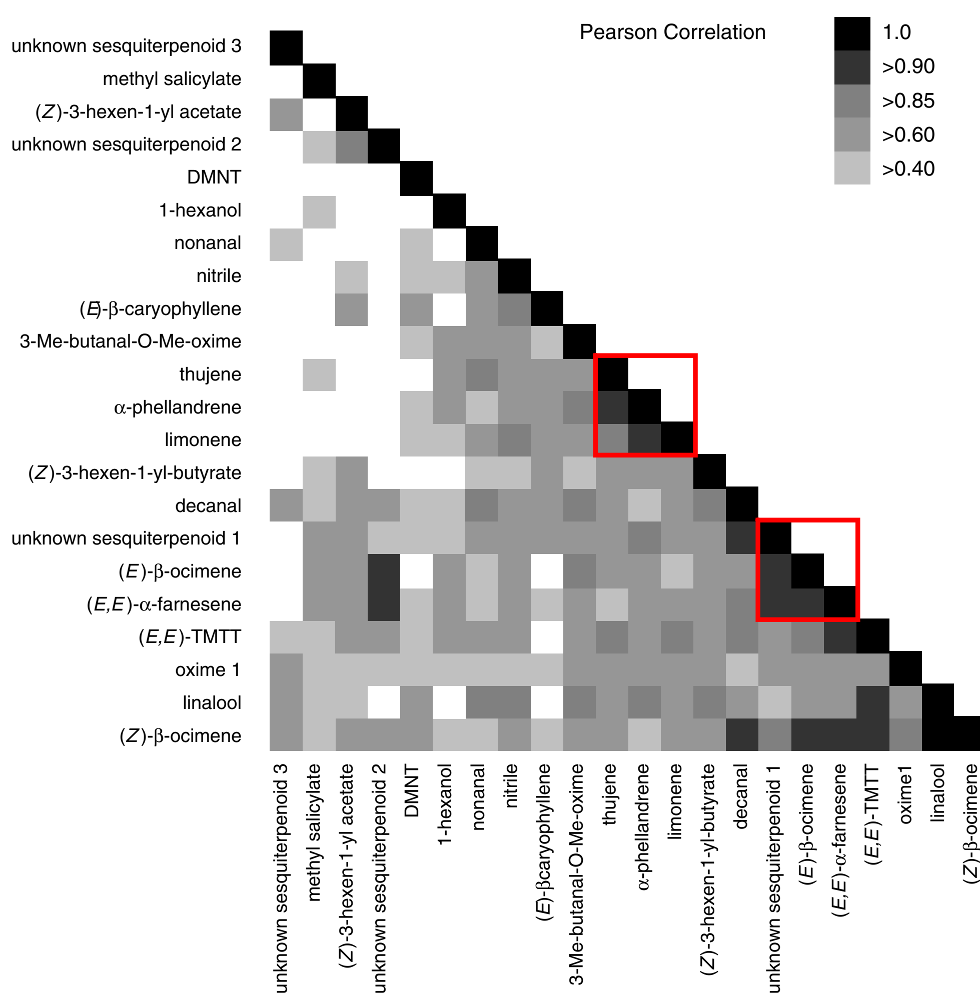
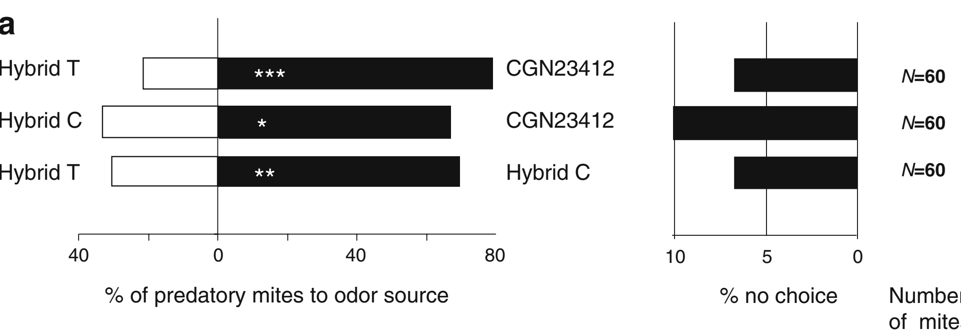


Fig. 4 Volatile—volatile correlation matrix of the 24 compounds emitted by JA-treated cucumber plants. Correlations between volatiles are shown in *gray scale*: the darker the gray color, the higher the percentage of similarity between metabolite expression patterns. *Lined boxes* show those clusters that are discussed in the text



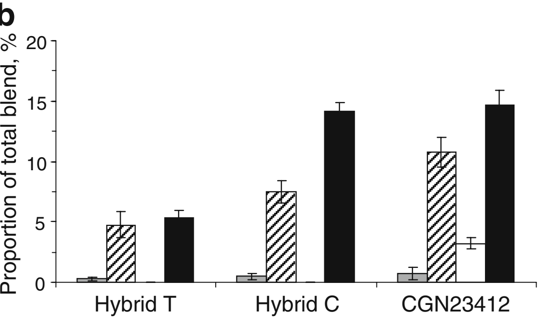


Fig. 5 Relative preference of *Phytoseiulus persimilis* for headspace from three different *Cucumis sativus* accessions infested with *Tetranychus urticae* for 7 d. **a** % of predatory mites to each odor source, *asterisks* indicate level of significance, as tested with a two-sided binomial test: *ns*, not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$; and % of predatory mites that did not make a choice within the given time period. **b** Relative contribution to the total blend (means ±SE) of (*E*)-2-hexenal (*grey bars*), (*E*)-β-ocimene (*dashed bars*), (*E*)-β-caryophyllene (*white bars*), and (*E,E*)-α-farnesene (*black bars*) in accessions Hybrid C, Hybrid T and CGN23412

gerbera (Krips et al. 2001), pear (Scutareanu et al. 2003), maize (Hoballah et al. 2004), carrot (Nissinen et al. 2005), rice (Lou et al. 2006), and cruciferous crops (Bukovinszky et al. 2005). In a study that used 31 maize hybrid lines, for example, about 70-fold variation in the quantity of volatiles emitted was found (Degen et al. 2004). The significance of such variation in volatile production among different accessions with respect to the attraction of natural enemies can be evaluated only by behavioral bioassays. For some natural enemies, differential attraction to the odors of

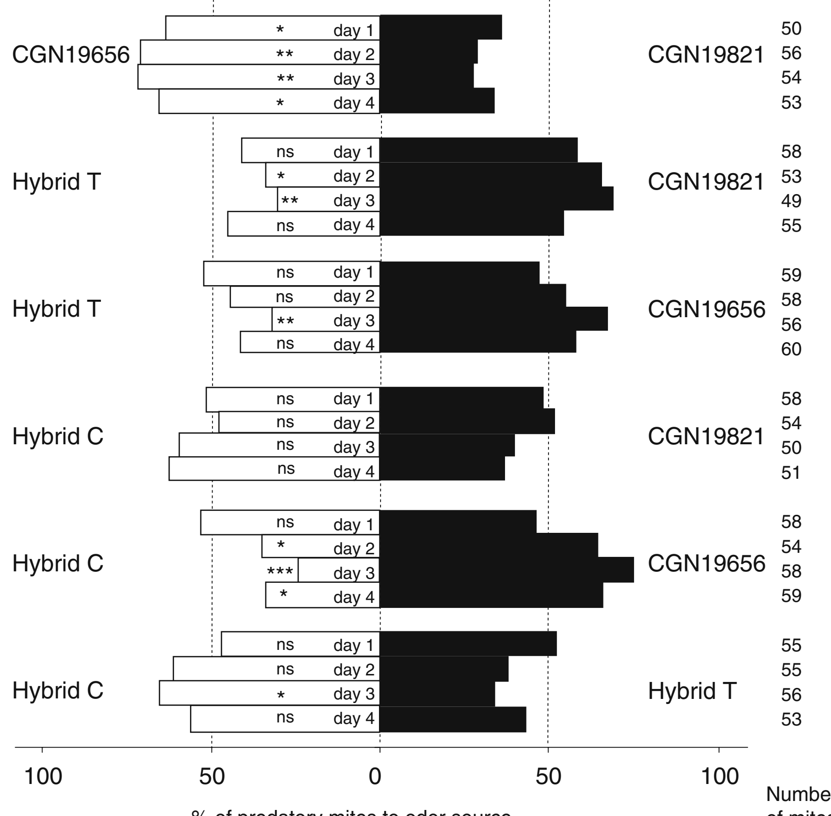


Fig. 6 Relative preferences of *Phytoseiulus persimilis* for headspace of three different *Cucumis sativus* accessions sprayed with 0.5 mM jasmonic acid. Bioassays were conducted three times during 4 d following JA treatment (day 1, 2, 3, 4 since JA treatment, as indicated in the *bars*). *Asterisks* indicate level of significance, as tested with a two-sided binomial test: *ns*, not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$. Numbers of mites are those mites that made a choice for one of either odor sources

particular accessions has been demonstrated (Krips et al. 2001; Hoballah et al. 2002; Bukovinszky et al. 2005). Here, we showed that differences in induced volatile profiles also exist among cucumber accessions and have consequences for predator attraction.

Uninduced cucumber plants released less than 3% of the volatiles emitted by induced plants with (*E*)-2-hexenal and (Z)-3-hexenyl acetate as predominant compounds (Table 2). There were no pronounced differences in the composition of the volatiles collected from undamaged leaves of the European F1-hybrids and landraces from Asia and Africa. The release of these compounds is caused probably by minor damage due to handling of the plants.

The release of induced volatiles varied from 80 ng to over 6 µg per plant after JA application. *Tetranychus urticae* infestation did not cause a similar increase in volatile production, likely because feeding damage is progressing relatively slowly with the development of the mite population, and the plant can adapt to the changing situation, whereas JA application quickly activates the whole signalling cascade to a maximum within 2–3 d. JA application was used to standardize the level of induction and, therefore, is a more accurate induction treatment to investigate potential differences in induced volatile production among various accessions.

A total of 24 compounds, representing approximately 98% of the trapped volatiles, were identified. The majority of the volatile blend consisted of terpene hydrocarbons, with the most abundant components being the monoterpene (*E*)-β-ocimene and the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) Also, the sesquiterpene (*E,E*)-α-farnesene and another homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were among the most abundant volatiles in the released blend. The homoterpenes DMNT and TMTT result from oxidative degradation of the sesquiterpenoid nerolidol and the diterpenoid geranyllinalool, respectively (Gäbler et al. 1991). Although both nerolidol and geranyllinalool are volatile, we detected only nerolidol occasionally in the volatile blend of some of the accessions used, indicating an efficient conversion of the alcohols into the homoterpenes. In other species, like e.g., maize and rice, nerolidol has been found in the herbivory-induced blend, but always together with the degradation product DMNT (Degen et al. 2004; Lou et al. 2006). Besides nerolidol, the only other terpene alcohol detected was linalool, which was found in six accessions. In induced volatile blends of many plant species, linalool is the only terpene alcohol, although in Norway spruce α-terpineol, camphenilol, and borneol also were found (Martin et al. 2003).

Compared to previous studies (Dicke et al. 1990a; Bouwmeester et al. 1999; Mercke et al. 2004), we detected a number of new herbivory-induced volatiles: 2-hexanal, linalool, methyl salicylate, β-caryophyllene, α-phellandrene, thujene, and three unknown sesquiterpenoids in some of the accessions. These differences may be due to treatment differences. Herbivore- or elicitor-induced volatile release varies with time and treatment, applied amount of elicitor (Halitschke et al. 2000), and the herbivore damage level (Gouinguene et al. 2003). In our study, spider–mite infestation and JA treatment led to qualitative as well as quantitative differences in the volatile profile (Fig. 2). The volatile components released by the accessions used due to spider–mite infestation also were detected in the blend of JA-treated plants, although in most of the accessions JA-treatment led to the emission of a larger number of compounds than were emitted in response to spider–mite feeding (Fig. 2). Exceptions are (*Z*)-3-hexen-1-yl butyrate and an unknown sequiterpenoid, which were found in the headspace of spider–mite infested plants of accessions Dasher and Korinda, respectively, but not in the blends of JA-treated plants of these accessions. Apparently, spider mites are not capable of triggering the whole induced volatile potential that cucumber plants have, and JA application resulted in a more 'JA-specific volatile response' that does not mimic exactly the herbivore-induced volatile blend. Herbivore damage likely influences alternative defense pathways that control volatile emission (De Boer and Dicke 2004). Herbivores with piercing-sucking mouthparts may induce more salicylic acid (SA)-mediated defense genes compared to chewing herbivores (Leitner et al. 2005; Zhang et al. 2009). The SA and JA signal-transduction pathways may interact (Pieterse et al. 2009) and lead to different induced volatiles blends

By using PCA based on Pearson correlations on least square means of absolute amounts, the accessions investigated were separated principally on the basis of total volatile emission. Therefore, we focused on the proportions of the compounds in the blend, as this may lead to a better distinction between accessions. The accessions used are not genetically characterized, but quite randomly selected. Nonetheless, our PCA data show a clear difference between European F1-hybrids and the landraces from Asia and Africa, mostly distinguished by the proportions of DMNT, (*E*)-β-ocimene, and (*E,E*)-α-farnesene in the odor blend (Fig. 3). Our results show that, at least for this limited selection of accessions, the European F1 hybrids have lost the capacity to produce large amounts of (*E*)-β-ocimene and (*E,E*)-α-farnesene. As a 'chemical signature' is determined by relative amounts, the consequence is that the induced blends of European hybrids are characterized by a relatively higher proportion of DMNT.

Several compounds identified in cucumber volatile blends could be clustered by using a Pearson correlation matrix. With the exception of (*E*)-β-ocimene, the principal volatile emitted in ten accessions, the emission rates of the

non-alcohol monoterpenes, limonene, α-phellandrene, and thujene were mutually correlated, suggesting that these three monoterpenes may be formed by a single enzyme that is induced upon herbivory. Indeed, several multiproduct synthases have been cloned, e.g., from maize (Köllner et al. 2004), Arabidopsis (Tholl et al. 2005), and *Medicago truncatula* (Arimura et al. 2008). Multi-product terpene synthases significantly contribute to the plasticity of blends and are increasingly found in plants, especially in the context of herbivory (Köllner et al. 2004; Tholl et al. 2005; Arimura et al. 2008). Interestingly, the monoterpene (*E*)-β-ocimene does not correlate with the other monoterpenes, but it is correlated positively with the sesquiterpene (*E,E*) α-farnesene, and another unknown sesquiterpene (Pearson coefficient=0.97). This matches well with previously published data that show that the (*E,E*)-α-farnesene synthase cloned from spider mite-induced cucumber plants also catalyzes the formation of (*E*)-β-ocimene from geranyl diphosphate (Mercke et al. 2004). Furthermore, the (*E,E*)-α-farnesene synthase also produced sesquiterpene in enzymatic assays with a similar RI as 'unknown sesquiterpenoid one' detected in this study. This gene possesses a plastid targeting peptide and hence is likely to produce (*E*)-β-ocimene when activated/induced by herbivory. Indeed, in time course studies, (*E*)-β-ocimene is among the first volatiles released by cucumber (Bouwmeester et al. 1999; Mercke et al. 2004). When herbivory progresses, other volatile compounds are released, amongst them (*E,E*)-α-farnesene. These results suggest then farnesyl diphosphate becomes available in the plastids. To date, increasing evidence is accumulating that the plastidial MEP pathway also may significantly contribute to cytosolic sesquiterpene biosynthesis by allowing IPP to be shuttled between cell compartments (Bick and Lange 2003). Aharoni et al. (2003) suggest that FPP is available in the plastids, based on the observation that transgenic *Arabidopsis* plants harboring a heterologous sesquiterpene synthase from strawberry emit low amounts of the sesquiterpene (*E*)-nerolidol. The exact mechanism remains to be elucidated.

Jasmonic-acid-induced volatiles of accessions CGN19656 and CGN19821 were significantly preferred by *P. persimilis* predatory mites over those of Hybrid T and Hybrid C. As CGN19656 is by far the highest emitter of volatiles, it is not unexpected that this landrace is highly attractive. In contrast, CGN19821 is a landrace that releases low amounts of volatiles, but its blend attracts more predators than the JA-induced blend of Hybrid T, and is equally attractive to the blend of Hybrid C, both of which had an almost 2-fold (Hybrid T) to 9-fold (Hybrid C) higher release of volatiles (Table 1). The relative contributions of 3-methyl-butanal-*O*-methyloxime, β-caryophyllene, and (*E*)-β-ocimene to the 'chemical signature' of the induced blend were significantly higher in CGN19821 than in Hybrid C and Hybrid T (Fig. 2), and this could correlate with its higher degree of predator attraction. In contrast, the proportions of the green leaf volatiles (*Z*)-3-hexen-1-ol, 1-hexanol, and (*Z*)-3-hexen-1-yl butyrate were lower in CGN19821 than in the other two accessions.

Preferences of predatory mites also were studied after spider–mite infestation in cucumber accessions CGN23412, Hybrid T, and Hybrid C. The ranking order of attractiveness in accessions infested with spider mites correlated with the relative proportion of (*E*)-2-hexenal, (*E*)-β-ocimene, (*E*)-β-caryophellene, and (*E,E*)-α-farnesene. Similarly, Loughrin et al. (1995) found that differences in volatile production of cotton cultivars were based mainly on differences in the production of terpenes. Moreover, Krips et al. (2001) concluded that the lower attractiveness of *P. persimilis* to certain gerbera cultivars correlated with the relatively lower emission rates of (*E*)-β-ocimene and linalool in the total volatile blend of these less attractive cultivars. Our study indicates that in cucumber (*E*)-β-ocimene and (*E*)-β-caryophyllene play an important role in the attraction of predatory mites. Previously, it has been shown that *P. persimilis* and other predatory mites are attracted to linalool, methyl salicylate, (*E*)-β-ocimene, DMNT, and (*E*)-nerolidol, when offered individually (Dicke et al. 1990b; De Boer et al. 2004; Kappers et al. 2005). From our data, it seems that the proportion of DMNT within the total blend is not very important for the attraction of *P. persimilis* to induced cucumber volatiles: accessions Hybrid T and Hybrid C, both with relatively high proportions of DMNT, were less attractive in comparison to CGN19656 and CGN19821. However, transgenic *Arabidopsis* plants expressing a nerolidol synthase gene from strawberry (Kappers et al. 2005) emit more DMNT and were significantly more attractive to predatory mites. Additionally, Mumm et al. (2008) found less attraction of parasitoid wasps *Diadegma semiclausum* when *Arabidopsis* plants were treated with fosmidomycin, which specifically inhibits 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXPR), a key enzyme in the methylerythritol 4-phosphate (MEP) pathway, located in the plastids. However, other terpenoid compounds also were reduced in this study, which limits the conclusion concerning DMNT. Another example of an individual compound that affects the attraction of natural enemies is (*E*)-α-bergamotene that increased *Manduca sexta* egg predation rates on *Nicotiana attenuata* by the generalist predator *Geocoris pallens* (Kessler and Baldwin 2001). Most of these studies used single compounds or mixtures, ignoring the fact that background odors of plants may have a large impact on the 'chemical signature' (Schroeder and Hilker 2008).

The relative production of (*Z*)-3-hexen-1-yl-acetate was higher in Hybrid T than in other accessions. (*Z*)-3-Hexen-1-yl-acetate was found to be neither attractive nor

repellent for *P. persimilis* (Dicke et al. 1990b). Van Wijk et al. (2008) also did not find a response of predatory mites towards this compound. Takabayashi et al. (1994) found indications that two oximes might be repellent to *P. persimilis*. In our study, the relatively high proportion of 3-methyl-butanal-*O*-methyloxime in accession CGN19821 was not found to prevent predatory mites from choosing this accession when tested against accessions with lower production of oxime. Interestingly, 3-methyl-butanal-*O*-methyloxime has been reported to be induced by *Pythium aphanidermatum* infestation of cucumber plants as well (Jansen et al. 2007), thus suggesting a common defense signalling pathway for these two different types of biotic stress.

In summary, this study demonstrates a large range of quantitative as well as qualitative variation for herbivory induced volatile emission by different cucumber accessions. Behavioral studies with predatory mites *P. persimilis* show that these differences have a profound effect on the attraction of a natural enemy of spider mites. We currently are addressing the effects of differentially attractive accessions on predatory mites under greenhouse conditions to exploit the genetic variation for the realization of sustainable horticulture with a reduced use of pesticides.

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Modifications of the Chemical Profile of Hosts after Parasitism Allow Parasitoid Females to Assess the Time Elapsed Since the First Attack

Sebastien Lebreton · Jean-Philippe Christidès · Anne-Geneviève Bagnères · Claude Chevrier · Eric Darrouzet

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Abstract In solitary parasitoids, only one adult can emerge from a given host. In some of these species, when several eggs are laid on the same host, supernumerary individuals are eliminated by lethal larval fights. In the solitary parasitoid *Anisopteromalus calandrae*, the probability of a second larva winning the fight depends on the time elapsed since the first oviposition. The older the first egg is at the moment a second egg is laid, the less chance the second egg has of winning the competition. As a consequence, females of this species lay their eggs preferentially on recently parasitized hosts rather than on hosts parasitized by an egg about to hatch. *Anisopteromalus calandrae* females parasitize bruchid larvae located in cowpea seeds. In a series of choice test experiments using an artificial seed system, we demonstrated that the cue that allows parasitoid females to differentiate between hosts parasitized for different lengths of time comes from the host and not from the artificial seed or the previously laid egg. This cue is perceived at short range, indicating that the chemicals involved are probably partly volatile. Interestingly, although parasitism stops host development, cuticular profiles continue to evolve, but in a different way from those of unparasitized hosts. This difference in the host's cuticular profile after parasitism, therefore, probably underlies the parasitoid female's discrimination.

Key Words Cuticular hydrocarbons · Semio-chemicals · Kairomone · Oviposition strategies · Solitary parasitoids · *Anisopteromalus calandrae*

S. Lebreton · J.-P. Christidès · A.-G. Bagnères · C. Chevrier · E. Darrouzet (✉)
Institut de Recherche sur la Biologie de l'Insecte, UMR 6035 CNRS, Université François Rabelais,
37200 Tours, France
e-mail: eric.darrouzet@univ-tours.fr

Introduction

Parasitoid females have to localize hosts to lay their eggs. This localization often is mediated by semiochemicals that can have different origins, such as volatiles released by plants when attacked by herbivores, chemical markers left by host females when they oviposit, or host sex or aggregative pheromones (for a review see Hilker and McNeil 2008). For example, the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) is attracted by volatiles emitted by the blackcurrant when attacked by aphids (Birkett et al. 2000). Similarly, the Pteromalid wasps *Dinarmus basalis* and *Anisopteromalus calandrae* can localize their bruchid larvae hosts in cowpea seeds due to chemical markers deposited by the bruchid females after oviposition (Kumazaki et al. 2000; Onodera et al. 2002).

After locating their hosts, females have to select which host to parasitize. Indeed, all hosts are not of equal quality, and one of the important parameters influencing this quality is the host's parasitized status. Hosts that have been parasitized already have a lower quality than those that are unparasitized because of the competition among larvae resulting from several clutches laid on the same host (Godfray 1994). In some solitary parasitoids, when several eggs are laid in or on the same host, larvae fight until only one remains. Only one adult thus can emerge from a given host in these species (Hubbard et al. 1987). Parasitoid females generally are able to discriminate between these two categories of hosts (Gauthier et al. 1996; Weber et al. 1996; Darrouzet et al. 2007; Lebreton et al. 2009a). These discrimination capacities may be based on perception of different chemical markers deposited by the first parasitoid during parasitization (Völkl and Mackauer 1990; Jaloux et al. 2005; Stelinski et al. 2007) as well as on alarm

pheromones produced by the host itself after having been stung the first time (Outreman et al. 2001). In other species, host discrimination can be based on the interaction of several protagonists. In the ectoparasitoid *D. basalis* for example, the cue is located on the host, but a transfer of molecules from the parasitoid egg to the host is needed over an extended period (Gauthier and Monge 1999).

It would appear disadvantageous for a parasitoid female to lay several eggs on the same host or to lay eggs on already parasitized hosts. This behavior, called superparasitism, has, however, been demonstrated to be adaptive under certain circumstances, particularly when unparasitized hosts are scarce or distances between oviposition patches are long (van Alphen and Visser 1990). When females are confronted with several parasitized hosts, the survival probability of their offspring can vary from one host to another depending on different factors. Among these, the time elapsed between the two ovipositions is one of the more important (Visser et al. 1992; van Baaren and Nénon 1996; Goubault et al. 2003; Lebreton et al. 2009a); the older the first egg, the less chance the second egg has of surviving, because the previously laid egg hatches first and the larva kills the second egg before it can hatch (Godfray 1994). In a previous study with the solitary ectoparasitoid *Anisopteromalus calandrae*, we demonstrated that survival probability of a second egg decreases strongly when the time interval between two ovipositions increases (Lebreton et al. 2009a). On the other hand, two eggs laid at the same time have about the same probability of winning the competition, while the survival probability of the second egg decreases to 15% when it is laid shortly before the hatching of the first egg. As a consequence, females lay their eggs preferentially on recently parasitized hosts rather than on those parasitized by an egg about to hatch (Lebreton et al. 2009a).

The present study aimed to identify the cue responsible for this discrimination capacity. *Anisopteromalus calandrae* females parasitize bruchid larvae contained in cowpea seeds. When a female encounters a seed containing a host, she taps the seed surface with her antennae to locate the exact position of the host, and then she inserts her ovipositor into the seed in order to probe the host. The cue perceived by females thus could come from a quantitative or qualitative change in an external marker deposited by the first female after oviposition. It also could be due to an internal marker that originates from the host in response to the first sting, which could evolve with time, or from modifications to the egg itself that probably take place during its development. In some species, consecutive cues of different origins inform females about the parasitized status of a host (Islam and Copland 2000; Outreman et al. 2001). Perception of one or more of these cues can inform parasitoid females of the time elapsed since the first parasitism. However, to our knowledge, the few studies that have dealt with this discrimination capacity have never focused on the chemical signals involved.

In the present study, we aimed to investigate whether the cue perceived by females and involved in discrimination between hosts parasitized for different lengths of time originates from the seed, the host, or the egg. For this purpose, by using an artificial seed system, we performed a series of choice tests, in which we presented females with either parasitized seeds, previously laid eggs, or parasitized hosts. After locating the cue, we investigated which compounds could be involved by using gas chromatography and mass spectrometry.

Methods and Materials

Insect Rearing Anisopteromalus calandrae were reared in the laboratory on larvae and pupae of the bruchid *Callosobruchus maculatus* (Coleoptera: Bruchidae). Both *C. maculatus* and *A. calandrae* originated from Ivory Coast (collected in 2000) and were mass-reared under conditions close to that of their area of origin: 12 hr light at 29°C, 12 hr dark at 22°C, and 65% r.h.

Behavioral Analysis To obtain parasitized hosts, we used an artificial seed system consisting of gelatine capsules (1.5 cm long x 0.6 cm diam) containing a bruchid larva (Gauthier and Monge 1999; Darrouzet et al. 2003). The bruchid L4 larvae were placed inside the capsules after removal from seeds by dissection and selection by size. Five capsules were proposed to a group of 5 virgin females during a 2 hr period in a climatic chamber (29°C, 65% r.h.). Because Hymenopteran parasitoids reproduce by arrhenotokous parthenogenesis, virgin females were able to lay only male eggs, which avoids a bias due to the sex of the eggs during the choice test. At the end of the exposure period, eggs on parasitized hosts were located with a dissecting microscope, and parasitized artificial seeds were selected for choice tests.

Choice tests consisted of two artificial seeds each containing one host, one which had been parasitized for 2 hr (recently parasitized capsule, P 2 hr) and the other for 28 hr (parasitized by an egg about to hatch, P 28 hr), placed in a Petri dish (3.5 cm diam x 1 cm high) (Lebreton et al. 2009a). The two capsules were separated by 0.5 cm. To determine whether the cue perceived by females was located on the capsule, on the egg, or on the host, three choice tests were performed, each offering a different part of the artificial seed system to the females. In test 1, capsules were presented that had contained hosts previously parasitized for 2 hr and 28 hr, but now contained an unparasitized host. In test 2, 2- and 28-h-old eggs were

offered in a clean capsule, on an unparasitized host. Finally, in test 3, hosts parasitized for 2 and 28 hr, from which eggs had been removed, were offered in clean capsules.

In a previous study, we demonstrated that mated females distinguish between 2 and 28 hr parasitized artificial seeds (Lebreton et al. 2009a). In the present study, to avoid a bias due to the mating status of females, mated females also carried out these choice tests. The whole oviposition sequence was observed (first capsule visited, capsule with oviposition). The tests ended as soon as females laid an egg but lasted no more than 2 hr to avoid the first egg hatching during the test. After the test, females were removed from the Petri dish. The first capsules visited and the selected capsules were noted. Statistical analyses were performed with a χ^2*-test*.

Chemical Analysis of the Hosts' Cuticular Profile We quantified and identified the chemical compounds present on the host cuticle. For this purpose, samples from one bruchid larva were extracted in 10 µl of pentane, to which 2 µl of an internal standard (C20) were added for 1 min. Samples were analyzed with a Perkin-Elmer Autosystem XL GC (Perkin-Elmer, Wellesley, MA, USA) equipped with a flame ionization detector (FID) and interfaced with Turbochrom workstation software. Each sample was dried to 2 µl. These 2 µl then were injected into the GC-FID injector heated to 220°C in splitless mode, and analyzed by using a BP1 capillary column, which was temperature programmed from 50°C (2 min hold) to 310°C at 7°C/min with a final hold of 10 min. Five categories of host were analyzed: larvae parasitized for 2 hr (P 2 hr, N=6) or 28 hr (P 28 hr, N=14) and unparasitized larvae stored in the same conditions as 2 hr (UnP 2 hr, N=9) or 28 hr parasitized hosts (UnP 28 hr, N=9). As some unparasitized larvae develop to a pre-pupa stage after 28 hr, unparasitized pre-pupae (UnP Pre-pupae, N=6) also were analyzed. The components were identified by GC-MS analysis, performed using a Hewlett-Packard 5890 GC system coupled to a 5989A MS, controlled by HP chemstation software. Hydrocarbons were identified tentatively by their mass spectra (Lockey 1988; Blomquist 2010) and corroborated by their ECL indices. Individual samples and pools of 2 hr and 28 hr parasitized hosts were injected into the GC/MS injector following the same procedure as described above. To analyze whether there was a difference in the cuticular profile of the different categories of host, we performed a discriminant analysis based on the relative amount of the 30 major peaks.

Bioassays with Chemical Extracts from the Host To confirm that compounds observed in GC-MS were responsible for the parasitoid females' capacity to perceive the time elapsed since the first oviposition, we performed another series of choice tests. In these, mated females (N=42) were presented with two capsules, each containing an unparasitized host on which an extract of parasitized hosts had been deposited. These extracts were obtained by immersing 100 hosts parasitized for 2 hr or 28 hr in 200 µl of pentane for one min. Then, 2 µl of each extract (2 or 28 hr; amount corresponding to one host) were deposited on an unparasitized host previously rinsed in pentane to remove its own chemical compounds. After rinsing, we checked that hosts were still alive. One host of each category (with the 2 hr or 28 hr parasitized extract) was used for the choice tests, which took place in a climatic chamber as described above. The first capsule visited and the one selected for oviposition were noted. The proportion of each category of capsules visited first and those on which females oviposited then was calculated. Statistical analyses were performed with a χ^2*-test*. All statistical analyses were performed using Statistica 6.0 software (Statsoft, Inc.).

Results

Behavioral Analysis In tests 1 and 2, parasitoid females first visited each category of capsules equally (test 1, χ^2 = 0.03, df = 1, P=0.87; test 2, χ^2 = 0.61, df = 1, P=0.43) and laid the same number of eggs in each category of capsules (Fig. 1; test 1, χ^2 = 1.58, df = 1, P=0.21; test 2, χ^2 = 0.03, df = 1, P=0.85). In test 3, most females (69.2%) first visited the capsules containing the 2 hr parasitized host rather than the 28 hr parasitized host (χ^2 = 7.69, df = 1, P=0.006). Moreover, most (72.7%) selected the 2 hr parasitized hosts to lay their eggs (Fig. 1; χ^2 = 6.82, df = 1, P=0.009).

Chemical Analysis of the Hosts' Cuticular Profile The GC-MS analysis showed 44 major peaks (Fig. 2) and 71 identified components (Table 1) on hosts. These included a series of n-alkanes and methyl-branched alkanes (C_{25}-C_{35}; Table 1). The same compounds were found on each category of host but in different proportions (Table 1).

The discriminant analysis revealed a difference between UnP Pre-pupae and UnP Larvae (UnP 2 hr: F=3.53, df = 30.1, P=0.020; UnP 28 hr: F=3.35, df = 30.1, P=0.023), but no statistical differences were found between the two categories of UnP Larvae (F=1.21, df = 30.1, P=0.39; Fig. 3). P 2 hr were not different from UnP 2 hr (F=1.28, df = 30.1, P=0.30), whereas P 28 hr were different from P 2 hr (F=3.64, df = 30.1, P=0.018) and had a tendency to diverge from UnP 28 hr (F=2.39, df = 30.1, P=0.073; Fig. 3). P 28 hr also were different from UnP Pre-pupae (F=3.12, df = 30.1, P=0.031; Fig. 3).

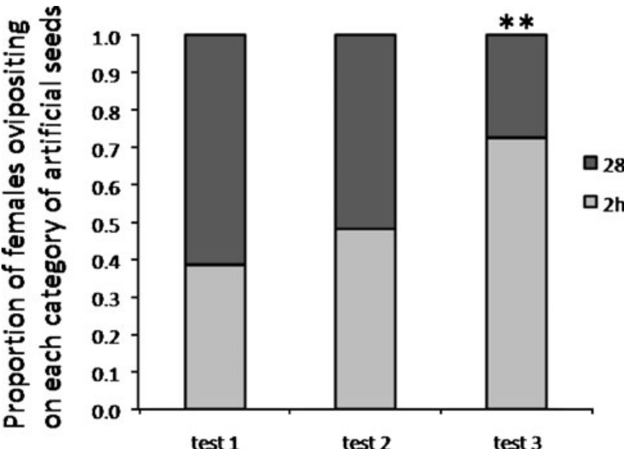


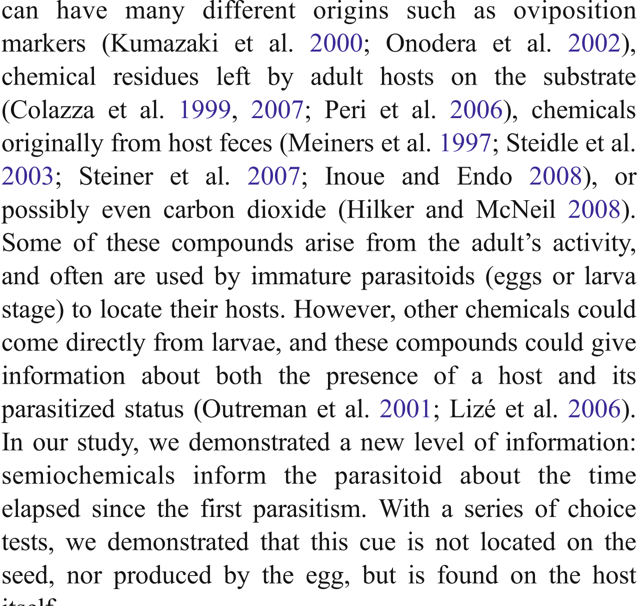
Fig. 1 Proportion of *Anisopteromalus calandrae* females selecting each category of artificial seed on which to lay their eggs (2 hr or 28 hr parasitized) when confronted with each test condition: capsules that previously contained parasitized hosts, but which were presented with an unparasitized host inside for the test (test 1), eggs on an unparasitized host in a clean capsule (test 2), and parasitized hosts on which eggs had previously been removed, in a clean capsule (test 3). Marks show significant differences between proportions observed and a random distribution of 50:50 ** $P<0.01$

UnP Larvae and P 2 hr could be distinguished from P 28 hr and UnP Pre-pupae on the basis of the first discriminant variable and were characterized by the occurrence of n-C_{28} (peak 15) and n-C_{29} (peak 22). In contrast, P 28 hr and UnP Pre-pupae were characterized by a higher proportion of 3-MeC_{25} (peak 2), n-C_{26} (peak 3), MeC_{27} (peaks 10, 11, 12, 14), DiMeC_{27} (peaks 13, 14), MeC_{28} (peaks 17, 19, 21), and MeC_{29} (peaks 23, 24). The P 28 hr and UnP Pre-pupae differed in the second discriminant variable. P 28 hr were characterized by the occurrence of 3-MeC_{28} (peak 21), MeC_{29} (peak 23), 9,13-DiMeC_{29} (peak 25), and MeC_{31} (peak 32), whereas UnP Pre-pupae were more particularly characterized by the presence of n-C_{26} (peak 3), an unidentified compound (peak 35), and 3,9,13-TriMe-C_{31}.

Bioassays with Cchemical Extracts from Hosts Most females (70.7%) visited the capsule containing the 2 hr parasitized extract first ($\chi^2 = 7.04$, $df = 1$, $P=0.008$). Ovipositing females tended to lay more eggs on these hosts (64.3% vs. 35.7% on hosts with the 28 hr parasitized extract), but the difference was not significant ($\chi^2 = 2.29$, $df = 1$, $P=0.13$).

Discussion

Semio-chemicals are known to play a crucial role in host localization in parasitoids (Afsheen et al. 2008). Host cues


can have many different origins such as oviposition markers (Kumazaki et al. 2000; Onodera et al. 2002), chemical residues left by adult hosts on the substrate (Colazza et al. 1999, 2007; Peri et al. 2006), chemicals originally from host feces (Meiners et al. 1997; Steidle et al. 2003; Steiner et al. 2007; Inoue and Endo 2008), or possibly even carbon dioxide (Hilker and McNeil 2008). Some of these compounds arise from the adult's activity, and often are used by immature parasitoids (eggs or larva stage) to locate their hosts. However, other chemicals could come directly from larvae, and these compounds could give information about both the presence of a host and its parasitized status (Outreman et al. 2001; Lizé et al. 2006). In our study, we demonstrated a new level of information: semiochemicals inform the parasitoid about the time elapsed since the first parasitism. With a series of choice tests, we demonstrated that this cue is not located on the seed, nor produced by the egg, but is found on the host itself.

Our behavioral results show that most of the tested females visited the capsule containing the 2 hr parasitized host first (test 3, 70%), thus indicating that compounds involved in this discrimination capacity are detected not by direct contact but from a short distance. In fact, when females were introduced in the choice test, they came within a few millimetres of the capsules with their antennae but without touching them before finally making their choice (Lebreton, personal observation).

When females were confronted with only the chemical compounds (experimentally deposited on an unparasitized host), they also more often visited the host exhibiting the "most recently parasitized" profile first. However, although they tended to lay their eggs on these hosts, this choice was not as marked as for actually parasitized hosts (Lebreton et al. 2009a). This result suggests that several cues could in fact be necessary for a female to assess host quality accurately. During the first parasitism, *A. calandrae* females also paralyze their hosts by injecting venom (Lebreton et al. 2009b). In the present experiment (bioassays of chemical extracts from hosts), hosts were unparasitized and therefore not paralyzed. It is possible that initially females perceive hosts as "recently parasitized", but on coming closer detect that they are not in fact parasitized because they are not paralyzed.

In a previous study, Outreman et al. (2001) showed that an alarm pheromone produced by aphids after being parasitized allows parasitoid females to determine whether a host is already parasitized or not. They correlated an antennal rejection of the parasitized host with the presence of the pheromone in the hours following the first parasitism. As the pheromone disappeared, the antennal rejection was replaced by a sting rejection. This result indicates that several cues can give the same information.

Fig. 2 Gas chromatogram of the cuticular pentane extract of hosts parasitized for 2 hr (**a**) and 28 hr (**b**). IS = internal standard. Peak numbers correspond to those listed in Table 1

In our study, the difference in the cuticular profile of hosts was visible mainly after 28 hr. It is thus possible that this cue does not inform parasitoids about the parasitized status of hosts just after parasitism, whereas other cues, such as oviposition markers deposited by parasitoids, give this information for shorter periods. When *A. calandrae* females oviposit on unparasitized hosts, generally they deposit a substance with their ovipositors after laying (Lebreton, personal observation). Moreover, when confronted with parasitized capsules (test 1), females tend to select 28 hr parasitized capsules slightly more, even if this difference is not statistically significant from a random choice. This observation may support the hypothesis of an oviposition marker that is more detectable shortly after its deposit. Oviposition markers generally are produced in the Dufour gland. In *A. calandrae*, the Dufour gland contains a mixture of *n*-alkanes (C_{30} to C_{39})(Howard and Baker 2003). Such marking-pheromones commonly are used by parasitoids to inform competitors about previous ovipositions and thus avoid competition (Hoffmeister 2000; Rosi et al. 2001; Stelinski et al. 2007). They also seem to be used by females to discriminate between hosts parasitized by themselves and those parasitized by other parasitoids (van Dijken et al. 1992). More recently, it also has been shown

Table 1 Tentatively identified cuticular hydrocarbons present on the host cuticle, with their relative amount for each host category

Peak	Hydrocarbons	ECL[a]	CN[b]	Relative amount (% of total)				
				UnP 2hr	UnP 28hr	P 2hr	P 28hr	UnP pre-pupae
1	*n*-C25	25.00	25	0.23	0.25	0.21	0.55	0.59
2	3-MeC25	25.68	26	0.07	0.10	0.14	0.40	0.44
3	*n*-C26	26.00	26	0.10	0.22	0.17	0.30	0.46
4	9MeC26	26.30	27	0.00	0.01	0.03	0.06	0.04
5	6-MeC26	26.47	27	0.00	0.00	0.00	0.03	0.00
6	5-MeC26	26.49	27	0.03	0.01	0.01	0.02	0.05
7	4-MeC26	26.51	27	0.01	0.03	0.03	0.10	0.10
8	3-MeC26	26.67	27	0.02	0.03	0.04	0.13	0.13
9	*n*-C27	27.00	27	4.88	9.99	6.29	8.58	10.19
10	9-MeC27	27.30	28	0.75	0.83	1.45	2.63	2.18
11	7-MeC27	27.32	28					
12	5-MeC27	27.43	28	0.11	0.23	0.35	0.65	0.57
13	9,15-DiMeC27	27.59	29	0.11	0.19	0.18	0.32	0.25
14	3-MeC27 +	27.69	28	0.78	1.54	1.62	3.54	3.36
	5,9-DiMeC27	27.72	29					
15	*n*-C28	28.00	28	4.00	4.77	4.05	3.46	3.70
16	3,9-DiMeC27	28.05	29	0.21	0.20	0.15	0.09	0.24
17	10-MeC28	28.26	29	0.27	0.32	0.46	0.77	0.65
18	6-MeC28	28.30	29	0.01	0.04	0.03	0.14	0.06
19	4-MeC28	28.50	29	0.11	0.20	0.16	0.32	0.28
20	2-MeC28	28.66	29	0.03	0.07	0.09	0.10	0.04
21	3-MeC28	28.67	29	0.07	0.26	0.29	0.51	0.41
22	*n*-C29	29.00	29	56.95	48.43	47.55	34.47	36.66
23	15-MeC29 +	29.28	30	5.25	4.79	7.32	10.44	7.52
	13-MeC29 +	29.28	30					
	11-MeC29 +	29.28	30					
	9-MeC29 +	29.30	30					
	7-MeC29	29.32	30					
24	5-MeC29	29.39	30	0.14	0.15	0.26	0.33	0.27
25	9,13-DiMeC29	29.49	31	5.26	5.45	5.72	7.06	6.05
26	7,11-DiMeC29 +	29.60	31	0.80	1.45	1.11	1.45	1.93
	7,13-DiMeC29	29.60	31					
27	3-MeC29	29.68	30	2.44	4.14	3.75	4.08	4.08
28	*n*-C30 +	30.00	30	4.02	3.32	3.44	3.20	3.67
	3,9-DiMeC29 +	30.05	31					
	3,11-DiMeC29 +	30.05	31					
	3,13-DiMeC29	30.05	31					
29	14-MeC30 +	30.28	31	0.80	0.82	1.05	1.23	1.11
	12-MeC30	30.28	31					
30	10,14-DiMeC30 +	30.48	32	0.62	0.79	0.84	0.92	0.95
	9,13-DiMeC30 +	30.48	32					
	8,12-iMeC30 +	30.48	32					
	8,18-DiMeC30	30.48	32					
31	*n*-C31	31.00	31	4.96	4.03	3.86	3.65	3.76
32	15-MeC31 +	31.28	32	2.27	1.98	2.97	3.34	2.47
	13-MeC31 +	31.28	32					
	11-MeC31 +	31.28	32					
	9-MeC31 +	31.30	32					
	7-MeC31	31.30	32					
33	9,13-DiMeC31	31.46	33	2.43	2.81	3.23	3.48	3.75
34	7,11-DiMeC31 +	31.59	33	0.44	0.24	0.39	0.49	0.52

Table 1 (continued)

Peak	Hydrocarbons	ECL[a]	CN[b]	Relative amount (% of total)				
				UnP 2hr	UnP 28hr	P 2hr	P 28hr	UnP pre-pupae
	7,13-DiMeC31 +	31.59	33					
	3-MeC31 +	31.66	32					
	5,13DiMe-C31	31.64	33					
35	*unknown*			0.30	0.38	0.38	0.33	0.51
36	*n*-C32	32.00	32	0.04	0.08	0.06	0.06	0.15
37	3,9,13-TriMeC31	32.11	34	0.25	0.29	0.30	0.34	0.46
38	14-MeC32 + 12-MeC32	32.28 32.28	33 33	0.18	0.20	0.26	0.28	0.30
39	10,14-DiMeC32	32.46	34	0.11	0.15	0.21	0.20	0.16
40	*n*-C33	33.00	33	0.13	0.13	0.10	0.15	0.29
41	15-MeC33 + 13-MeC33	33.28 33.28	34 34	0.31	0.30	0.48	0.59	0.50
42	11,15-DiMeC33 + 9,13-DiMeC33 +	33.48 33.48	35 35	0.48	0.64	0.75	0.89	0.94
	7,11-DiMeC33	33.60	35					
43	*n*-C35 + 13-MeC35	35.00 35.29	35 36	0.04	0.09	0.13	0.15	0.09
44	13,17-DiMeC35 + 11,15-DiMeC35 +	35.46 35.48	37 37	0.02	0.06	0.12	0.17	0.09
	9,13-DiMeC35	35.48	37					

[a] ECL = equivalent chain length

[b] CN = carbon number

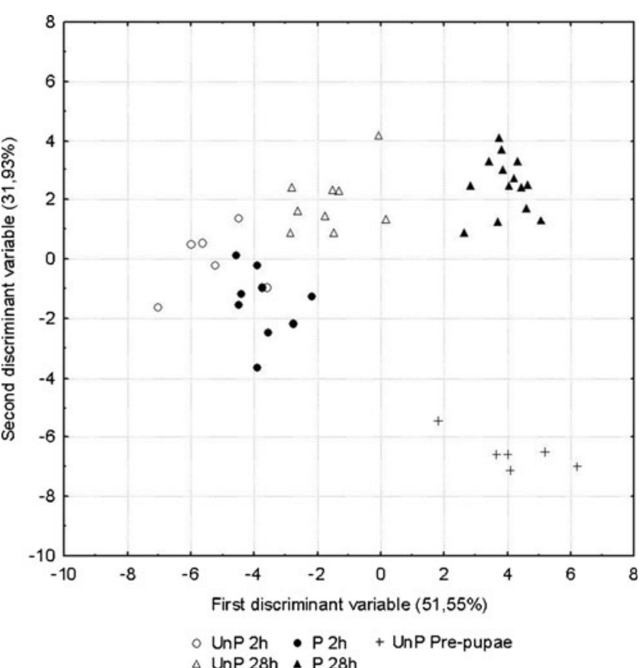


Fig. 3 Discriminant analysis based on the relative amount of the 30 major peaks of the cuticular profile of the different host categories (L4 larvae parasitized for 2 hr (P 2 hr) or 28 hr (P 28 hr), unparasitized L4 larvae stored under the same conditions as 2 hr (UnP 2 hr) and 28 hr (UnP 28 hr) parasitized hosts, and unparasitized pre-pupae (UnP Pre-pupae))

that oviposition markers deposited by larval parasitoids can be perceived by adult hosts, and thus avoid laying their eggs on unsuitable substrate (Stelinski et al. 2009).

Our results show differences in the cuticular profiles of the hosts analyzed. First, the profiles of the unparasitized hosts depend on their developmental stage, with larvae and pre-pupae exhibiting different profiles. The profile of parasitized hosts also changes with time, with hosts parasitized for 2 hr and 28 hr showing different profiles. However, it evolves differently in unparasitized hosts. Although unparasitized pre-pupae and 28 hr parasitized host profiles were similar in terms of the first discriminant variable, they diverged from each other in regard to the second discriminant variable. This result suggests that parasitism affects the evolution of the host profile. The compounds identified (a series of linear and methyl alkanes) were in accordance with those previously identified by Howard (2001). Although these consist of heavy long-chain hydrocarbons (C_{25} to C_{35}), and generally are considered as being detected through direct contact, in our choice test experiments, parasitoid females were able to detect the time elapsed since the first oviposition at a short distance (a few millimetres), i.e., outside the capsule. Nevertheless, considering the high temperature in our experimental procedure (29°C, the mean temperature of the area of origin of these species), some of these

compounds may be semi-volatile. A previous study demonstrated that cuticular hydrocarbons from C_{25} to C_{27} can be volatile and perceived at a distance at 25°C (Saïd et al. 2005). It is possible that only the lighter components play a role in this discrimination. This hypothesis is in accordance with our results, which show that the major difference between 2 hr and 28 hr parasitized hosts is related to these compounds (more particularly from C_{25} to C_{29}). This remains to be tested. Another possibility is the spontaneous oxidation of heavy cuticular lipids to volatile aldehydes or alcohols, as previously observed in other insect species (Bartlelt and Jones 1983; Bartlelt et al. 2002). However, the compounds involved in the discrimination observed in our experiment were pentane-soluble (obtained by immersing bruchids in a pentane solution), and only alkanes were found in these pentane extracts. Further studies are necessary to determine precisely which compounds are involved in this discrimination. The use of selected fractions of the hosts cuticular profiles or the use of blends of synthetic compounds would give us some indications. Moreover, by using olfactometry experiments, we would be able to differentiate between an attractant and an arrestment effect of these compounds. It also would be possible to know whether the profile of 2 hr parasitized hosts could be attractive for parasitoid females or whether that of hosts parasitized for 28 hr could be repellent.

Our results also raise an interesting question about the action of parasitoid venom on host physiology. When females inject their venom into hosts, the latter are paralyzed and their development is stopped. Their cuticular profile, however, continues to evolve. This indicates that some elements of host physiology still operate after venom injection. However, the evolution of the cuticular profile of a parasitized host is perceptibly different from that of an unparasitized host. The parasitoid venom thus appears to disturb the metabolism of hydrocarbons, interfering either with their production or with their dynamic release at the cuticle level. In a previous study, we observed that parasitism disturbs lipid metabolism by decreasing the amount of total lipids present in the host haemolymph (Lebreton et al. 2009b). Studies with other species also have demonstrated that modifications in the composition of the host haemolymph occur after venom injection (Rivers and Denlinger 1995; Nakamatsu and Tanaka 2003). However, in most of these species, the amount of lipids increases after parasitism, probably due to the lysis of fat body cells by the components of the venom (Nakamatsu and Tanaka 2004). The opposite result found in our study suggests other mechanisms. This result raises new questions about the affect of parasitism on a host's lipid metabolism. Identifying the components of venom could shed light on the way parasitoid venom acts on the host physiology.

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RAPID COMMUNICATION

Nosema spp. Infection Alters Pheromone Production in Honey Bees (*Apis mellifera*)

Claudia Dussaubat · Alban Maisonnasse · Cedric Alaux · Sylvie Tchamitchan · Jean-Luc Brunet · Erika Plettner · Luc P. Belzunces · Yves Le Conte

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Abstract Pheromones in social insects play a key role in the regulation of group homoeostasis. It is well-established that parasites can modify hormone signaling of their host, but less is known about the effect of parasites on pheromone signaling in insect societies. We, thus, tested in honey bees (*Apis mellifera*) the effect of the widespread parasite *Nosema* spp. on the production of ethyl oleate (EO), the only identified primer pheromone in honey bee workers. Since environmental stressors like pesticides also can weaken honey bees, we also analyzed the effect of imidacloprid, a neonicotinoid widely used in agriculture, on EO production. We show that, contrary to imidacloprid, *Nosema* spp. significantly altered EO production. In addition, the level of *Nosema* infection was correlated positively with the level of EO production. Since EO is involved in the regulation of division of labor among workers, our result suggests that the changes in EO signaling induced by parasitism have the potential to disturb the colony homoeostasis.

Claudia Dussaubat and Alban Maisonnasse contributed equally.

C. Dussaubat (✉) · A. Maisonnasse · C. Alaux · Y. Le Conte
UMR 406 Abeilles et Environnement, Laboratoire Biologie et Protection de l'abeille, INRA,
Site Agroparc,
84914 Avignon, France
e-mail: cdussaubat@avignon.inra.fr

S. Tchamitchan · J.-L. Brunet · L. P. Belzunces
UMR 406 Abeilles et Environnement, Laboratoire Toxicologie Environnementale, INRA,
Site Agroparc,
84914 Avignon, France

E. Plettner
Department of Chemistry, Simon Fraser University,
Burnaby V5A 1S6, BC, Canada,

Key Words Primer pheromone · Honey bee · Ethyl oleate · *Nosema* spp. · Imidacloprid

Introduction

Analogous to the hormones that control the organism homoeostasis, pheromones in social insects play a key role in the regulation of group homoeostasis. However, homoeostasis of both organisms and insect societies can be threatened by parasite infection. For example, in mammals there is evidence that parasites can modify the endocrine system of the host to favor their development and reproduction (Escobedo et al. 2005). In honey bees, the cuticular hydrocarbon profile involved in social recognition can be altered by an activation of the immune system (Richard et al. 2008) or parasitization by the mite *Varroa destructor* (Salvy et al. 2001). However, it is not known whether, analogous to the modification of hormone signaling in the organism, parasites can affect pheromone signaling in insect societies.

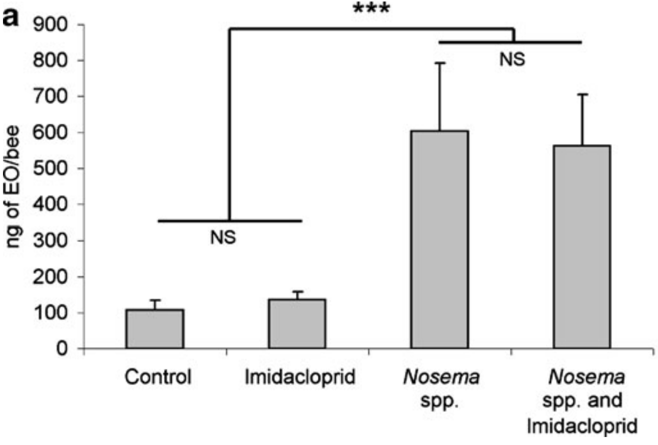
To answer this question, we asked whether the microsporidia *Nosema* spp., potentially involved in worldwide honey bee losses (Higes et al. 2008), could affect the production of pheromone in workers. We analyzed the production of the only indentified primer pheromone in workers: ethyl oleate (EO), which regulates worker behavioral maturation (i.e., inhibits the transition from inside-nest tasks performed by young bees (nurse) to foraging tasks performed by old bees (forager)) (Leoncini et al. 2004). The focus was on primer pheromones because they are essential to the regulation of social behaviors and colony homeostasis. Therefore, a modification in their production could affect the whole colony organization and endanger its survival. Since survival of honey bees can be

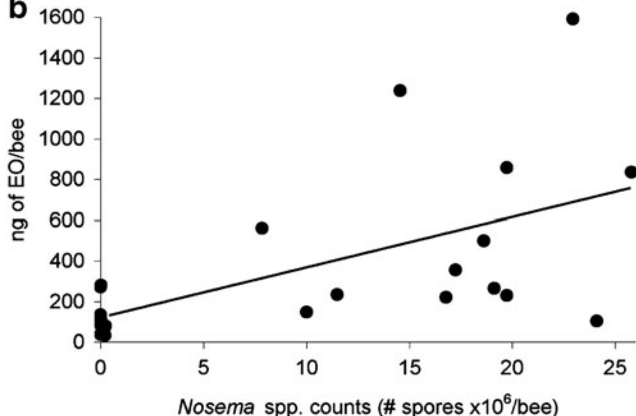
threatened by other stressors, such as pesticides, we also tested the effects of a neonicotinoid (imidacloprid) widely used in agriculture on EO production. Pesticides are known to disrupt pheromone perception, but they also can affect their production (Desneux et al. 2007).

Methods and Materials

Nosema infection and imidacloprid exposure This experiment was part of a larger study described in detail by Alaux et al. (2010). Briefly, in order to test the effect of *Nosema* infection and/or imidacloprid exposure, 1-d-old bees were reared in cages and split into four experimental groups: control groups, groups infected with *Nosema*, groups exposed to imidacloprid, and groups both infected with *Nosema* and exposed to imidacloprid. For each experimental group, 3 colonies were used, with 2 cage replicates for each colony (N=120 bees per cage). For the *Nosema* infection, bees were fed individually at the beginning of the experiment with a sugar solution containing 200,000 spores (Alaux et al. 2010). Spores were isolated previously from infected colonies as in Higes et al. (2007), and genetic analysis showed that our bees were infected with both *N. apis* and *N. ceranae* (see Alaux et al. 2010). For the pesticide exposure, caged bees were chronically exposed 10 h per day to imidacloprid by ingesting a sugar solution containing 7 μg/kg of imidacloprid (a concentration encountered in the environment) (see Alaux et al. 2010). The solution was replaced each day. After 10 d, bees were collected and stored at−20°C in order to measure the level of EO and *Nosema* infection.

EO Quantification Pools of 5 bees were analyzed. Whole-body extracts were prepared in 1.9 ml of iso-hexane with the addition of 100 μl of two internal standard solutions at 10 ng/μl (arachidic acid methyl ester, and methyl heptadecanoate, Sigma-Aldrich, France). Samples were crushed with a glass rod for 2 min at 0°C and centrifuged for 20 min at 4°C (2,500 g). The supernatant was collected and applied to a silica column (silica gel 60, particle size 40–63 m, 230–400 mesh). The first fraction was eluted in 3 ml of a solvent mixture (98.5% iso-hexane, 1.5% diethyl ether). The second fraction containing the EO was eluted in 3 ml of a second solvent mixture (94% iso-hexane, 6% diethyl ether). One ml of this fraction was concentrated to 10 μl under a nitrogen stream, and 1 μl was injected into a fast gas chromatograph (2014, Shimadzu, Japan) equipped with a split-splitless inlet, a flame ionization detector, and a capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 μm film thickness). Samples were injected in split mode. Hydrogen was used as carrier gas with a column flow of 0.52 ml/min. Oven temperature was set at 90°C for 1 min, raised to 195°C at 40°C min^{-1}, stabilized for





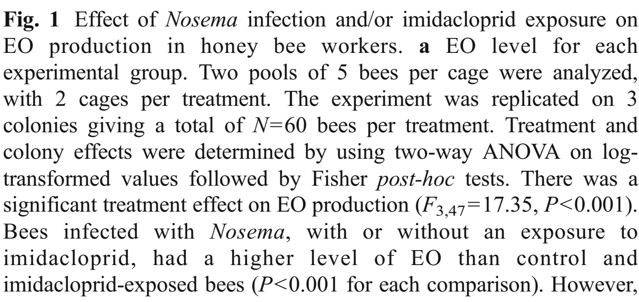


Fig. 1 Effect of *Nosema* infection and/or imidacloprid exposure on EO production in honey bee workers. **a** EO level for each experimental group. Two pools of 5 bees per cage were analyzed, with 2 cages per treatment. The experiment was replicated on 3 colonies giving a total of N=60 bees per treatment. Treatment and colony effects were determined by using two-way ANOVA on log-transformed values followed by Fisher *post-hoc* tests. There was a significant treatment effect on EO production ($F_{3,47}$=17.35, P<0.001). Bees infected with *Nosema*, with or without an exposure to imidacloprid, had a higher level of EO than control and imidacloprid-exposed bees (P<0.001 for each comparison). However, imidacloprid did not affect EO production (control vs. imidacloprid-exposed bees: P=0.81; or *Nosema*-infected bees vs. *Nosema*-infected and imidacloprid-exposed bees: P=0.14). There also was a significant effect of colony origin ($F_{2,47}$=4.59, P=0.017), but no significant interaction with the treatments was found ($F_{6,95}$=0.88, P=0.52) thus demonstrating a consistent effect of the treatments. Data show mean ± SE. *** and NS denote significant (P<0.001) and non-significant differences between treatments, respectively. **b** Relation between EO production and the level of *Nosema* infection. There was a significant positive correlation between the quantity of EO produced and the number of *Nosema* spores infecting bees (r=0.58, P<0.005, N=24)

3 min, then augmented to 210°C at 1°C min^{-1}, stabilized again for 2 min, then increased to 270°C at 40°C min^{-1} and held at 270°C for 3 min. Identification and quantification of EO was based on retention times of EO synthetic compound (Sigma-Aldrich, France) and by comparison of internal standard area, respectively, using a gas chromatography solution program (Shimadzu, Japan). The EO confirmation was done by a mass spectrometer (CP2010, Shimadzu, Japan) operated in the electron impact mode at 70 eV with continuous scans (every 0.2 sec) from a mass to charge ratio (m/z) of 70–400.

Nosema spore counting Since *Nosema* is an intestinal parasite, the honey bee intestinal tract was dissected and macerated in distilled water as in Higes et al. (2007). The spore concentration from the suspension then was determined by using a haemocytometer.

Results and Discussion

Nosema infection caused a significant increase in EO production compared to non-infected groups (Fig. 1a), thus demonstrating that pheromone production can be modified by environmental stressors. However, EO production in imidacloprid-exposed bees did not differ significantly from non-exposed bees (Fig. 1a). Neonicotinoids target the nicotinic acetylcholine receptors and thus can affect neural function (Decourtye et al. 2004), but here no effect was found on pheromone production. Studies with different pesticides are needed to determine whether this absence of modification that we observed is a general phenomenon.

One would expect that *Nosema* infection induces a cost to pheromone production. Contrary to this expectation, parasitized bees produced more EO than healthy ones. Since, EO is present at higher levels in foragers compared to nurses (Leoncini et al. 2004), and because *Nosema* causes a precocious onset of foraging (Wang and Moeller 1970), the EO increase might reflect a forager profile of infected bees compared to control bees. However, further investigation tended to show that the EO increase is not just a consequence of a forager profile. First, the level of EO in parasitized bees was 6 times higher than healthy bees, which is greater than the difference naturally found between nurses (young bees) and foragers (old bees) (100±19 ng EO/nurse, N=60 and 213±25 ng EO/forager, N=120, unpublished data from N=3 colonies, A. Maisonnasse). Second, there was a positive and significant relationship between EO level and the number of *Nosema* spores per bee (Fig. 1b), showing that the EO increase is not an all-or-nothing response but is linked to the level of *Nosema* infection.

Even if the earlier onset of foraging could be a bee response that decreases the *Nosema* load within the hive, the higher EO level in infected bees has the potential to disturb colony organization. The abnormally high level of EO could mislead the colony on the actual number of foragers and delay the onset of foraging in non-infected nurses. It is not known, however, how infected bees who accelerate their behavioral maturation would react to the high inhibitory effects of EO. On the other hand, since *Nosema* infection decreases worker lifespan (Higes et al. 2007), a loss of EO in the colony also can be expected and to accelerate nurse maturation. Field studies are needed to determine the actual response of the colony and whether a failure in pheromone communication induced by parasitism or disease could lead to the colony collapse.

To our knowledge, this is the first demonstration that parasites can modify pheromone production in insect societies. Therefore, our finding indicates that pathogens, besides their effect at the individual level, also can cause damage at the social level.

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Changes of Sex Pheromone Communication Systems Associated with Tebufenozide and Abamectin Resistance in Diamondback Moth, *Plutella xylostella* (L.)

Zhen Xu · Guang-Chun Cao · Shuang-Lin Dong

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Abstract Many insect pests have evolved resistance to insecticides. Along with this evolution, the sex pheromone communication system of insects also may change, and subsequently reproductive isolation may occur between resistant and susceptible populations. In this study of the diamondback moth, we found that resistant females (especially Abamectin resistant females) produced less sex pheromone and displayed a lower level of calling behavior. Resistant males showed higher EAG responsiveness to the sex pheromone mixture of females, and responded to a broader range of ratios between the two major components compared to the responses of susceptible moths. In addition, wind tunnel experiments indicated that changes associated with insecticide resistance in the Abamectin resistant strain (Aba-R) significantly reduced female attractiveness to susceptible males. Furthermore, mating choice experiments confirmed that non-random mating occurred between the two different strains. Aba-R females with an abnormal pheromone production and blend ratio exhibited significantly lower mating percentages with males from either their own strain or other strains, which corroborates the results obtained by the wind tunnel experiments. The implications of this non-random mating for insect speciation and insecticide resistance management are discussed.

Z. Xu · S.-L. Dong (✉)
Entomology Department/Key Laboratory of Monitoring and Management of Plant Diseases and Insects,
Ministry of Agriculture, Nanjing Agricultural University,
Nanjing 210095, China
e-mail: sldong@njau.edu.cn

G.-C. Cao
State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection,
Chinese Academy of Agricultural Sciences,
Beijing 100094, China

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Introduction

The sex pheromone communication system plays a vital role in insect mating and reproduction. This system, particularly in moths, is highly complex and species-specific (Roelofs and Comeau 1969; Lanier and Wood 1975; Costa et al. 1997). Therefore, it often is considered as a powerful force for reproductive isolation and, thus, speciation (Cardé and Baker 1984). Some studies with closely related species have shown that reproductive isolation under natural conditions is the result of differences in sex pheromone systems, as these species can hybridize when confined in the laboratory (Gadenne et al. 1997; Huang et al. 2002; Tabata and Ishikawa 2005; Laurent et al. 2007). Traditionally, sex pheromone communication systems in moths have been assumed to involve strong stabilizing selection on emitters and receivers, and such stabilizing pressure would allow only small, incremental changes in pheromone systems over time (Paterson 1980). However, more recent investigations have revealed that many environmental factors can cause notable changes in these systems (Krokos et al. 2002; Svensson et al. 2002; Bashir et al. 2003; Jun et al. 2003; Yang and Du 2003; Pelozuelo et al. 2004; Nosil et al. 2007; Robbins et al. 2008), and assortative mating can occur between different strains of the same insect species with different pheromone components or different ratios among components (Zhu et al. 1997; Pelozuelo et al. 2004).

Human activity now is a great evolutionary force, and has greatly facilitated the evolution of pesticide resistance in insects (Palumbi 2008). Many studies have focused on

the monitoring, management, and mechanism of pesticide resistance. However, few have addressed changes in sex pheromone communication associated with insecticide resistance (Delpuech et al. 1998, 1999, 2001; Delisle and Vincent 2002; Wei and Du 2004). Considering the importance of insect sex pheromone communication systems in reproductive isolation, studies of changes between pesticide resistant and susceptible insects should provide insight into the process of speciation. In addition, sex pheromones, as selective and environmentally friendly pest means of control, have been used successfully to control some important insect pests including diamondback moth (DBM), *Plutella xylostella* (L.) by trapping-and-killing or mating disruption (Chisholm et al. 1983; Schroeder et al. 2000). If there are changes in an insect sex pheromone communication system associated with insecticide resistance, sex pheromone formulations may require modification for the best control efficiency, and strategies for insecticide resistance management based on gene dilution between susceptible and resistant individuals also may require a re-evaluation.

The diamondback moth (DBM) is a worldwide pest, mainly of cruciferous vegetable and rapeseed crops. It also is the first crop pest in the world to develop resistance to DDT and *Bacillus thuringiensis* (Lingappa and Kulkarni 2004), and has developed resistance to most classes of pesticides. Tebufenozide and Abamectin are two insecticides widely used to control DBM. Tebufenozide was first developed by Rohm and Haas Co. as an agonist of 20-hydroxyecdysone, and kills insects by initiating premature molting (Wing 1988; Wing et al. 1988). Abamectin is a bacterial (*Streptomyces avermitilis*) based toxin that acts on the GABA receptor/chloride ionophore complex and glutamate-gated chloride channel (Clark et al. 1995; Rugg et al. 2005). Resistance of DBM to Tebufenozide has been reported in laboratory selected strains, and to Abamectin in field populations (Cao and Han 2006; Qian et al. 2008). In this study, we report the differences in the DBM sex pheromone communication system associated with Tebufenozide and Abamectin resistance, and a pronounced non-random mating between resistant and susceptible strains.

Methods and Materials

Insects Three strains of *P. xylostella* were used. The susceptible strain (S) was a laboratory strain provided by the Wuhan Insect Virus Research Institute. Previously, a Tebufenozide resistant strain (74-fold) was developed in our laboratory by selection with Tebufenozide for 17 generations during a 37 generation period from the S strain, which carried a cross resistance to Abamectin of 36-fold (Cao and Han 2006). From this strain, Tebufenozide and Abamectin resistances were selected further, respectively, with one of the two insecticides for another 20 generations to obtain the two resistant strains used in this study. The resistance level to Tebufenozide (Teb-R strain) was 141-fold, and to Abamectin (Aba-R strain) was 304-fold, calculated as the LC_{50} ratios between the resistant and susceptible strains. The two resistant strains were maintained in the laboratory by continuous exposure to 3 ppm Abamectin and 2,000 ppm Tebufenozide, respectively. Moth larvae of all strains were reared routinely with vermiculite-cultured radish (*Raphanus sativus*) seedlings under conditions of 25°C, 70–80% RH and a 14:10 hL:D photoperiod. Males and females were separated at the fourth-instar stage based on morphological differences. Pupae were transferred to a dark room, and eclosed moths were fed with 10% honey solution.

Insecticides and Chemicals Tebufenozide (20% suspension concentrate) and Abamectin (0.05% wettable powder) were provided by Dow AgroSciences and Nanjing Baofeng Chemical Co., Ltd., respectively. Two pheromone components Z11-16:Ald and Z11-16:Ac were provided by Professor Du at the Institute of Plant Physiology & Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The purity of these two chemicals was determined to be >95% by gas chromatograph (GC) analysis.

Calling Behavior Observation A 2-d-old virgin female moth was introduced into a transparent glass cylindrical container (3.5 cm diam×10 cm height), and placed in an environmentally controlled room (25±1°C, 70–80% RH, and a 14:10 hL:D photoperiod). Calling behaviors were observed by eye under dim incandescent red backlighting at 0.5 hr intervals throughout the day (24 hr). Each female was noted as either calling or not calling. There were three replications for each strain and 10 females per replication.

Electroantennogram Recordings EAG values were recorded by using a previous method (Yang et al. 2009). The binary mixture of Z11-16:Ald and Z11-16:Ac (1:1) were prepared in hexane at different concentrations (1, 10, 100, and 1,000 ng/µl) to test for a dose-response relationship, and in different ratios between Z11-16:Ald and Z11-16:Ac (9:1, 3:7, 5:5, 7:3, 1:9) to test the ratio-response relationship of male moths. One µl of test solution was deposited on a filter paper strip (2.5×1 cm), the solvent was allowed to evaporate for 1 min, then the paper strip was inserted into a Pasteur pipette placed perpendicularly through a hole in a matalline tube with an airflow of 4 ml/s. Stimulations were achieved by directing a puff of air (4 ml/s) through the pipette with a timer-controlled solenoid valve. Control stimulations were performed by

using a pipette with a paper strip treated with pure hexane. Measurements were repeated on 10 individuals of each strain using newly prepared paper strips for each individual. Male moths from the three strains were tested in random order.

Gas Chromatography Analysis Ovipositors and the associated sex pheromone glands were dissected from 2-d-old virgin female moths during the peak calling period, i.e., 1–3 hr after the initiation of scotophase (D1-D3 h). Each gland was soaked for 2 hr in 5 µl redistilled hexane containing an internal standard (11:Ac; 11.3 ng/µl) to extract pheromone components. Twenty replicates (glands) were made for each of the three strains.

To determinate the amounts of Z11-16:Ald and Z11-16:Ac, 2 µl of the 5 µl extract were injected into a GC (GC-14B, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID), a split/splitless injector, and a fused silica capillary column (SPB-50, 30 m× 0.25 mm ID, 0.25 µm film thinckness, Supelco, USA) in splitless mode. Nitrogen was used as carrier gas. The oven temperature was maintained at 100°C for 2 min and then programmed at 10°C/min to 250°C. The injector and FID temperatures were set at 200°C and 270°C, respectively. The quantity of each component was calculated based on the peak area, and calibrated by comparing it with that of the internal standard 11:Ac.

Wind Tunnel Bioassay The methods used in the wind tunnel experiments were similar to those of Lelito et al. (2008). Behavioral responses of males to calling females were observed in a Plexiglas wind tunnel (60 cm high, 90 cm wide, 230 cm long). For all tests, wind tunnel conditions were 3 lux red light, 25±2°C, 60–80% RH with 0.3 m/sec wind speed.

Just before the onset of the scotophase, 2-d-old virgin male moths were transferred individually into test tubes (2.5 cm diam×10 cm height), which were labeled with the strain name. All test tubes then were placed in the darkened room that housed the wind tunnel. At the same time, females of each strain were placed into seperate metal screen cages (4 cm diam×6 cm height) in an adjacent fume hood to prevent the males from being exposed to pheromone prior to testing. One calling female that served as the pheromone source was transferred into a metal screen cage (4 cm diam×10 cm height) and placed in the middle of the wind tunnel (equidistant from the two lateral sides of the tunnel) on a steel screen platform 30 cm high and 150 cm from the release point of male moths. The female was allowed to acclimate for up to 10 min for calling behavior to resume. After that, males were introduced individually into the wind tunnel by using a cylindrical one-end-opened screen cage (release cage, 4 cm diam×8 cm height). The release cage was placed into the tunnel with the open end facing the calling female on a steel screen platform 28 cm high. Each male was given up to 2 min to respond, and was scored for the following behavioral categories: no flight, taking flight, locked on plume, half the distance to source, 10 cm from source, and source contact. Each male was tested once.

To measure the females' attractiveness, 20 male moths were tested for each strain in each replication, with 3 replications for the experiment (total of 60 males for each strain). In each replication, the male individuals from 3 strains were tested randomly (in no particular order) to avoid bias due to female attractiveness and male responsiveness. The female (lure source) was replaced when she was no longer visibly calling. Tests were conducted between D1 to D3 h.

Mating Choice Measurement To elucidate further the effects of insecticide resistance-associated changes in the sex pheromone communication system, mating choice experiments were conducted. Six inter-strain combinations of the 3 strains were tested in a mating cage (35×35× 35 cm). Two-d-old virgin moths were used in this experiment. Ten males and 20 females of each strain were introduced into a cage to provide enough females among which to choose. Males and females of different strains were marked on their wings with different colored inks. The ink marking was tested prior to the study and found not to influence moth mating behaviors. Observations were made at 30 min intervals throughout the 10-h scotophase. Mated moths were removed after recording to avoid repeat mating. Five replicates (cages) were performed for each inter-strain combination.

Statistics Data were analyzed with a one-way analysis of variance, $P<0.05$. Fisher's least significant difference test was used for multiple comparisons among treatments in all experiments. All values were mean±SE unless otherwise designated.

Results

Calling Behavior of Females The periodicities of female calling behavior of the two resistant strains were similar to that of the S strain (Fig. 1). For all three strains, high percentages of individuals started calling as soon as the photophase ended. The highest calling percentages were displayed at 0.5 hr into scotophase (D0.5 hr) for S and Aba-R, and at D1.0 hr for Teb-R. Females of all 3 strains maintained the highest calling percentages for about one hour, and then the calling percentages gradually declined. However, the calling percentages at each time-point were

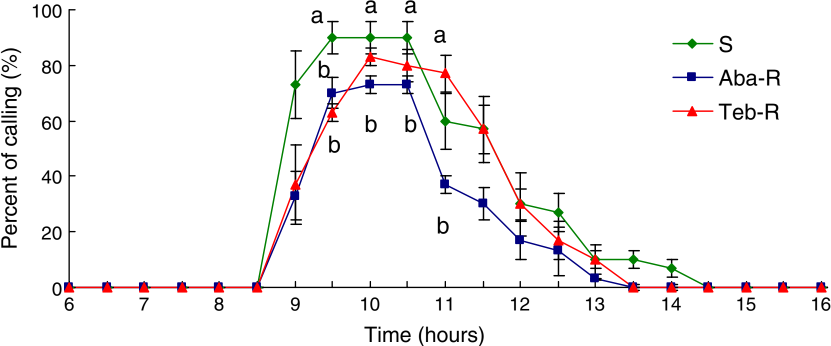


Fig. 1 Calling percentages by S, Teb-R and Aba-R strain females during a 24 hr period. Scotophase was from the 9th to 19th hour. The data series in each time point marked with different letters are significantly different at the 5% level using Fisher's least significant difference test

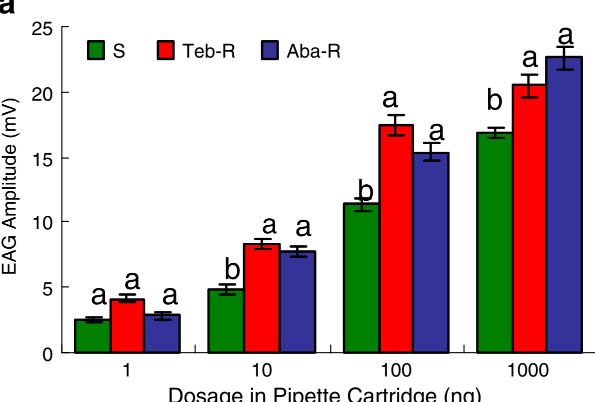
much different among the 3 strains. Compared with the S strain, calling percentages of Aba-R females were consistently lower at each point in time, and significant differences were observed during calling peak time (D0.5h-D2h). This was different for Teb-R females, where the calling percentages were lower than those of S females before D1.5h, but similar to that of S females afterwards. Both Aba-R and Teb-R resistant strains stopped calling at D5.0h, one hour earlier than the S strain, which stopped at D6.0h. For all three strains, no calling behavior was observed in photophase.

EAG Response of Males The dose-response bioassay showed that the 1:1 binary pheromone mixture elicited significantly higher EAG responses in male moths of both resistant strains compared with those of the S strain at all, except the lowest (1 ng), dosages (Fig. 2a). However, no significant difference in EAG responses was observed between the two resistant strains at all dosages tested. Similar results were obtained for pheromone mixtures (100 ng) of differing Z11-16:Ald/Z11-16:Ac ratios. Both resistant strains displayed higher EAG responses compared to the S strain at all five ratios tested (Fig. 2b).

Among different ratios of the binary mixtures, the 5:5 mixture elicited the highest EAG responses in males of all three strains. In addition, the EAG differences between resistant and susceptible males were much greater for ratios of 3:7 and 1:9 than for those of 9:1 and 7:3 (Fig. 2b).

GC Analysis of Female Sex Pheromone Titers The S strain females produced higher amounts of Z11-16:Ald than Aba-R and Teb-R females, and S strain females also produced significantly more Z11-16:Ac than Teb-R females (Table 1). In addition, significantly more Z11-16:Ald was found in the Teb-R strain than in the Aba-R strain females. The ratio of Z11-16:Ald/Z11-16:Ac in S females was 4.57:5.43, similar to that of Teb-R females (4.31:5.69), but much different from that of Aba-R females (1.59:8.41).

Wind Tunnel Bioassay The S strain males exhibited similar behavioral responses to the S and Teb-R strain females with respect to response percentages of all behavioral categories (Table 2). However, these response percentages exhibited by S strain males were reduced significantly when Aba-R strain females were used as the lure source. In other words, Aba-R females, in comparison with S and Teb-R females, were significantly less attractive to S males. For males of Teb-R or Aba-R strains, there were generally no significant



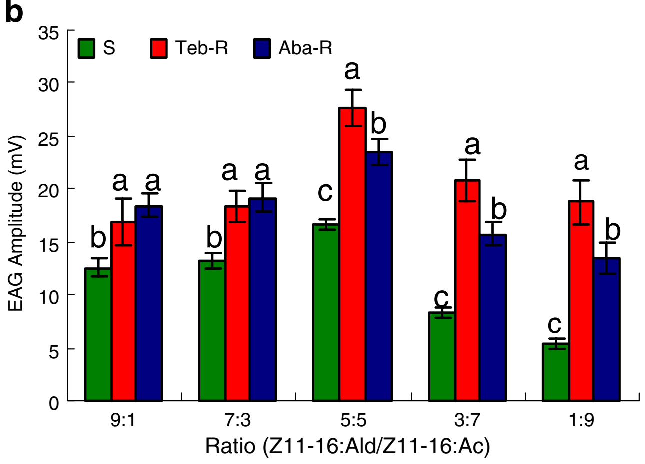


Fig. 2 EAG responses of S, Teb-R and Aba-R strain males to a 1:1 binary female sex pheromone mixture (Z11-16:Ald/Z11-16:Ac) at different doses (**a**), and at the same dose (100 ng) but different ratios (**b**), N=10. The data series at the same dose in panel A or of the same ratio in panel B marked with different letters are significantly different at the 5% level using Fisher's least significant difference test

Table 1 Mean titers (ng±SE) and blend ratios of Z11-16:Ald/Z11-16:Ac in females of three DBM strains (N=20)

Insect strain	Z11-16:Ald	Z11-16:Ac	Sum	Z11-16:Ald/Z11-16:Ac
Aba-R	2.17±1.14 c	11.60±0.85 ab	13.69±0.86 c	1.59:8.41
Teb-R	8.65±0.51 b	11.16±0.75 b	20.09±1.17 b	4.31:5.69
S	11.29±0.65 a	13.43±0.68 a	24.72±1.16 a	4.57:5.43

The data within each column followed by the same letters are not significantly different at the level of 5% using Fisher's least significant difference test

differences in behavioral response to calling females of the different strains. So, females of the 3 strains were of the same attractiveness to males of the 2 resistant strains. There was no significant difference in male responsiveness among different males when an S strain female or a Teb-R strain female was used as the lure. However, when an Aba-R train female was used as lure, the responses of the S strain males were significantly different from that of resistant males. Males of the two resistant strains showed significantly higher response percentages than S males in all five behavioral categories (Fig. 3). There was no difference between Teb-R and Aba-R strain males in their response percentages.

Mating Choice Experiment Observations showed that two types of non-random mating existed in the three two-strain combinations (Fig.4). One was assortative mating, and the other was designated as preferential mating. In the S+Teb-R combination, assortative mating was found, with males of the two strains mating preferentially with the females from the same strain. The number of mated pairs in which both females and males were from the S strain (S♂×S♀) was the highest, significantly higher than those of the other 3 types of mated pairs (S♂+Teb-R♀, Teb-R♂+S♀, and Teb-R♂+Teb-R♀). The number of mated pairs for Teb-R♂+Teb-R♀ was the second highest, and the number for S♂+Teb-R♀ was the lowest. In contrast, in the other two combinations, preferential mating was found. In the S+Aba-R combination, both the S and Aba-R strain males displayed a significant mating preference for S strain females (Fig. 4b). In the Aba-R+Teb-R combination, Teb-R females were mated more than Aba-R females, regardless of the strain origin of the male (Fig. 4c).

Discussion

Sex pheromone communication systems may be a major force that drives moth speciation by causing behavioral reproductive isolation via assortative meeting and mating of conspecific individuals (Linn et al. 2007). In this study, we found differences in sex pheromone communication between susceptible and two insecticide resistant strains of DBM, and we showed that the differences led to an assortative or preferential mating between different strains. The results are important, both for understanding speciation mechanisms potentially caused by changes in sex pheromone communication, and for effective management of insecticide resistance in insect pests.

We found that the titers of sex pheromones in females of the two resistant strains were reduced significantly compared to females of the susceptible strain. This is consistent with the results obtained with Azinphosmethyl susceptible and resistant *Choristoneura rosaceana* (Harris) (Delisle and Vincent 2002; Trimble et al. 2004), and Pyrethroid susceptible and resistant *Heliothis virescence* females (Campanhola et al. 1991). More importantly, the ratio of the two main components of the sex pheromone produced by Aba-R strain females was, surprisingly, shifted to 1.59 :

Table 2 Behavioral responses of males to females in the wind tunnel study (%±SE)

Male		Female S	Female Teb-R	Female Aba-R
S	TF	91.7±1.7a	86.7±1.7ab	81.7±1.7b
	LP	83.3±3.3a	81.7±1.7a	71.7±1.7b
	HD	61.7±6.0a	53.3±3.3a	38.3±1.7b
	10 cm	45.0±5.8a	41.7±3.3a	21.7±4.4b
	CS	38.3±1.7a	36.7±4.4a	20.0±2.9b
Teb-R	TF	93.3±4.4a	98.3±1.7a	90.0±2.9a
	LP	81.7±3.3a	88.3±4.4a	83.3±4.4a
	HD	58.3±4.4a	51.7±4.4a	51.7±3.3a
	10 cm	48.3±1.7a	41.7±4.4ab	35.0±2.9b
	CS	38.3±3.3a	38.3±3.3a	30.0±2.9a
Aba-R	TF	95.0±2.9a	95.0±2.9a	91.7±1.7a
	LP	91.7±1.7a	93.3±1.7a	86.7±3.3a
	HD	68.3±4.4a	53.3±6.0a	56.7±4.4a
	10 cm	41.7±4.4a	43.3±3.3a	36.7±1.7a
	CS	38.3±3.3a	40.0±2.9a	35.0±2.9a

TF take flight, *LP* locked on the plum, *HD* half the distance, *10 cm* 10 cm from the pheromone source, *CS* contact the source. The data within each row followed by the same letters are not significantly different at the level of 5% using Fisher's least significant difference test

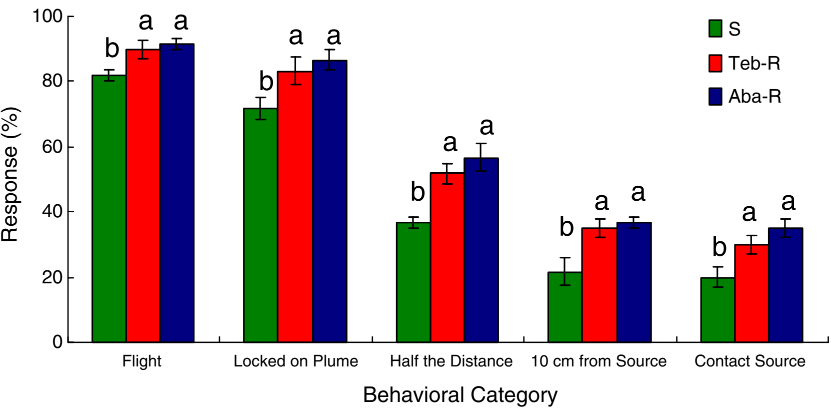


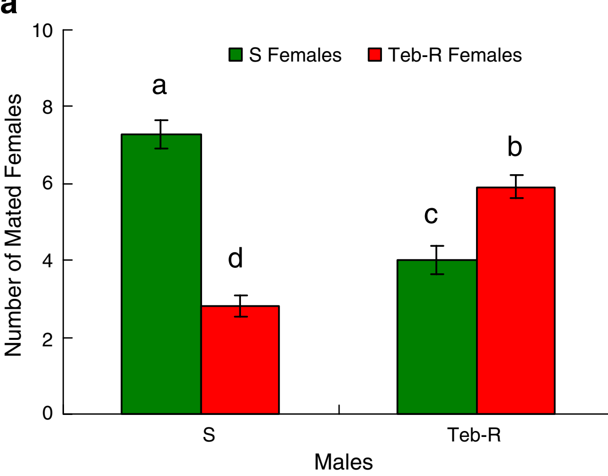
Fig. 3 Behavioral responses by males of the different strains to Aba-R strain females. Within each behavioral category, *error bars* marked with different letters are significantly different from each other at the 5% level using Fisher's least significant difference test

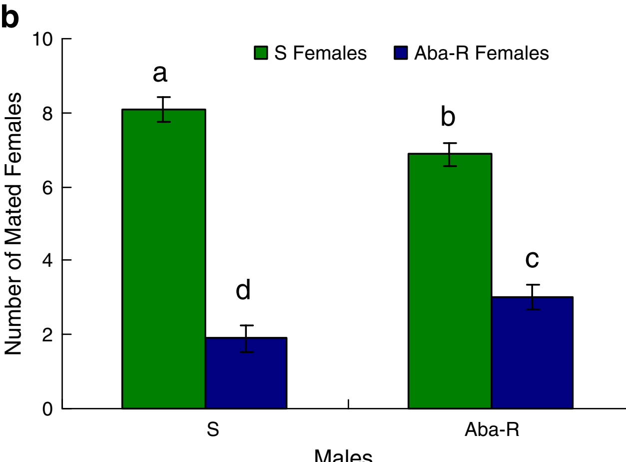
8.41 from 4.57:5.43 in susceptible females. This shift of blend ratio, together with the reduction of pheromone production, led to a lower competitiveness of Aba-R females in luring males. Aba-R females elicited significantly lower behavioral responses of S strain males when compared with S strain females in the wind tunnel experiment. Similarly, Aba-R females were less preferred for mating by S males, and even by males of the same strain, in the mating choice experiment. In contrast, S females and Teb-R males mated significantly less than Teb-R females and Teb-R males, although S females and S males mated significantly more than Teb-R females and S males. These results suggest that blend ratios are more important for mating success than are absolute titers of each pheromone component.

Another interesting result of our study is that insecticide-resistant males showed increased EAG responses to the sex pheromone. To our knowledge, this is the first report of resistant males exhibiting hypersensitivity to sex pheromones, although similar hypersensitivity effects have been reported in male *Grapholita molesta* treated with sublethal Octopamine (Linn and Roelofs 1986), male *Trichogramma brassicae* treated with Chlorpyrifos (Delpuech et al. 1998), and male *Spodoptera litura* treated with Endosulfan (Wei and Du 2004). Males of the two resistant strains also showed a wider response range in the ratios between the two major pheromone components; resistant males displayed higher EAG response to the ratios of 3:7 and 1:9. In the wind tunnel tests, resistant males showed similar behavioral responses to both Aba-R females with the pheromone ratio of 1.59:8.41 and to S females with a ratio of 4.57:5.43. It is reasonable to believe that Aba-R strain males were selected to respond to a wider range of blend ratios as the female pheromone ratio changed. However, the males of Teb-R strain also responded to a wider range of blend ratios with no change in pheromone ratio of the females. Such similar changes in male response (both in blend ratio and sensitivity) of the two resistant strains may be due to similar insecticide resistant mechanisms, although the two insecticides used for selection have different modes of action. For example, enhancement of cytochrome P450 monooxygenase activity was reported recently as one mechanism of resistance against Abamectin and Tebufenozide in DBM (Qian et al. 2008).

Notably, there was a discrepancy between the results of the wind tunnel and the mating choice experiments. Neither Aba-R nor Teb-R strain males displayed significantly different responsiveness to Aba-R and Teb-R strain females in wind tunnel tests, but, in mating choice experiments, Aba-R females were less frequently mated than were Teb-R females to Teb-R males or even to Aba-R males. This possibly is due to two factors. One is that in the mating choice experiment, there may be interactions between sexes and strains, while in wind tunnel tests just one factor (strain) may be operational. Another possible explanation is that the two experiments focused to some extent on different aspects of mating behavior. Wind tunnel tests of female attractiveness focused on a relatively long distance effect, while the mating choice tests were more focused on recognition between the two sexes and female attractiveness at closer range.

In the two DBM resistant strains, there may be a partial compensation of the sex pheromone communication between the two sexes, i.e., females released less amounts and varied ratios of sex pheromone while males responded to female sex pheromone with higher sensitivity and at a wider ratio range. This phenomenon also has been found in *Trichogramma brassicae* treated with $LD_{0.1}$ Deltamethrin (Delpuech et al. 2001). Such compensation in resistant strains is consistent with the "asymmetric tracking" model for the evolution of sex pheromone communication systems (Phelan 1992, 1997), in which the sex pheromone of females has been modified, and some males are able to track the changes. The model of asymmetric tracking involves two stages. In the first, males can be attracted to unusual females while still retaining their responsiveness to the ancestral pheromone blend. The second stage is characterized by the occurrence of assortative mating





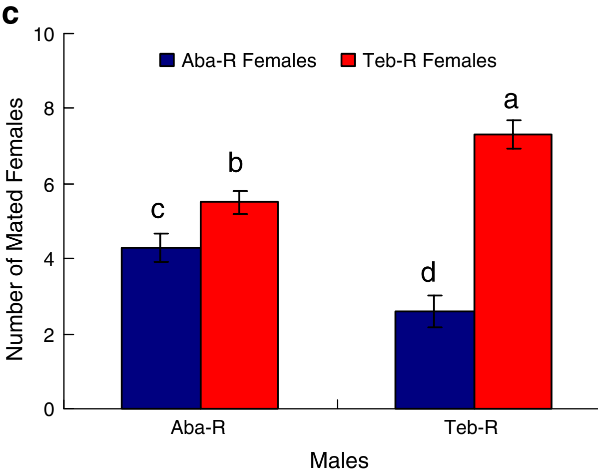
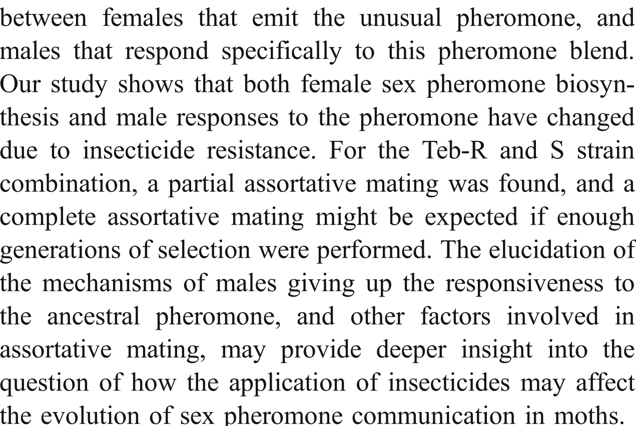


Fig. 4 Number (mean±SE) of females that mated with males of different strains. **a** S+Teb-R combination; **b** S+Aba-R combination; **c** Aba-R+Teb-R combination. *Error bars* with different letters are significantly different from each other at the 5% level by the Fisher's least significant difference test



between females that emit the unusual pheromone, and males that respond specifically to this pheromone blend. Our study shows that both female sex pheromone biosynthesis and male responses to the pheromone have changed due to insecticide resistance. For the Teb-R and S strain combination, a partial assortative mating was found, and a complete assortative mating might be expected if enough generations of selection were performed. The elucidation of the mechanisms of males giving up the responsiveness to the ancestral pheromone, and other factors involved in assortative mating, may provide deeper insight into the question of how the application of insecticides may affect the evolution of sex pheromone communication in moths.

Changes in sex pheromone communication associated with insecticide resistance are of significance to the gene dilution-based insecticide resistance prevention and management strategy. Given that the insecticide resistance is recessive (Liang et al. 2003), our present study suggests that the change of the sex pheromone system in the Aba-R strain would have a positive effect on resistance management, as the mating frequency of S females was much higher than that of R females, and the mated pairs of R male and R female were less than one fourth (the proportion when R and S individuals mate randomly). Thus, the changes in the sex pheromone system would be favorable for the management of Abamectin resistance. However, in the case of the Teb-R strain, the effect would be neutral, because the number of mated pairs of R female and male was approximately one fourth, although the total number of mated S females (either with S male or R male) was higher than that of R females. Another implication of our study for pest resistance management is the possibility of using a pheromone lure that is more attractive to resistant males than is the lure for non-resistant individuals. As our wind tunnel experiment revealed, Aba-R resistant females displayed a significantly higher attractiveness to both Aba-R and Teb-R males than to S males. Therefore, use of lures with the blend ratio of the Aba-R female could trap more resistant males in the fields.

Although the two resistant strains with considerably different blend ratios (1.59:8.41 in the Aba-R strain and 4.57:5.43 in the Teb-R strain) were selected from the same S strain, the possibility that the change in sex pheromone is a result of random effects due to genetic bottlenecks can not be excluded. To confirm that the changes truly are due to insecticide resistance, one method might be to develop several new resistant strains from susceptible strains with considerable genetic difference, and check the reproducibility of this effect. Alternatively, insight could be obtained by exploring the resistance mechanisms to the insecticides. For example, Abamectin acts on the GABA receptor/ chloride ionophore complex and glutamate-gated chloride channel (Clark et al. 1995; Rugg et al. 2005). Any variation

associated with insecticide resistance that involves these targets should affect the neurophysiological activity of olfactory receptor neurons, and thus affect a male's response to sex pheromone and a female's pheromone biosynthesis in direct and/or indirect ways. Currently, the resistant mechanisms of the two resistant strains are not completely understood. Enhancement of cytochrome P450 monooxygenase activity was reported recently as one mechanism of resistance against both insecticides in DBM (Qian et al. 2008). This may contribute to the reduction of sex pheromone titers in resistant females, as a metabolic resistance mechanism often is thought to reduce the overall fitness of insects (Roush and McKenzie 1987). A complete understanding of insecticide resistant mechanisms will be helpful in the exploration of how insecticide resistance-associated changes affect the DBM sex pheromone system.

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Biosynthesis of Unusual Moth Pheromone Components Involves Two Different Pathways in the Navel Orangeworm, *Amyelois transitella*

Hong-Lei Wang · Cheng-Hua Zhao · Jocelyn G. Millar · Ring T. Cardé · Christer Löfstedt

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Abstract The sex pheromone of the navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), consists of two different types of components, one type including (11*Z*,13*Z*)-11,13-hexadecadienal (11*Z*,13*Z*-16:Ald) with a terminal functional group containing oxygen, similar to the majority of moth pheromones reported, and another type including the unusual long-chain pentaenes, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-tricosapentaene (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H) and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-pentacosapentaene (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H). After decapitation of females, the titer of 11*Z*,13*Z*-16:Ald in the pheromone gland decreased significantly, whereas the titer of the pentaenes remained unchanged. Injection of a pheromone biosynthesis activating peptide (PBAN) into the abdomens of decapitated females restored the titer of 11*Z*,13*Z*-16:Ald and even increased it above that in intact females, whereas the titer of the pentaenes in the pheromone gland was not affected by PBAN injection. In addition to common fatty acids, two likely precursors of 11*Z*,13*Z*-16:Ald, i.e., (*Z*)-11-hexadecenoic and (11Z,13Z)-11,13-hexadecadienoic acid, as well as traces of (*Z*)-6-hexadecenoic acid, were found in gland extracts. In addition, pheromone gland lipids contained (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaenoic acid, which also was found in extracts of the rest of the abdomen. Deuterium-labeled fatty acids, (16,16,16-D_3)-hexadecanoic acid and (*Z*)-[13,13,14,14,15,15,16,16,16-D_9]-11-hexadecenoic acid, were incorporated into 11*Z*,13*Z*-16:Ald after topical application to the sex pheromone gland coupled with abdominal injection of PBAN. Deuterium label was incorporated into the C_{23} and C_{25} pentaenes after injection of (9*Z*,12*Z*,15*Z*)-[17,17,18,18,18-D_5]-9,12,15-octadecatrienoic acid into 1–2 d old female pupae. These labeling results, in conjunction with the composition of fatty acid intermediates found in pheromone gland extracts, support different pathways leading to the two pheromone components. 11*Z*,13*Z*-16:Ald is probably produced in the pheromone gland by Δ11 desaturation of palmitic acid to 11*Z*-16:Acid followed by a second desaturation to form 11*Z*,13*Z*-16:Acid and subsequent reduction and oxidation. The production of 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H may take place outside the pheromone gland, and appears to start from linolenic acid, which is elongated and desaturated to form (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaenoic acid, followed by two or three further elongation steps and finally reductive decarboxylation.

Key Words Sex pheromone · Biosynthesis · *Amyelois transitella* · Linolenic acid · (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaenoic acid · (3*Z*, 6*Z*, 9*Z*, 12*Z*, 15*Z*)-3,6,9,12,15-tricosapentaene · (11*Z*,13*Z*)-11,13-hexadecadienal · Bifunctional Δ11 desaturase · PBAN · Pyralidae

H.-L. Wang · C. Löfstedt (✉)
Department of Ecology, Lund University,
223 62 Lund, Sweden
e-mail: christer.lofstedt@ekol.lu.se

H.-L. Wang · C.-H. Zhao
State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology,
The Chinese Academy of Sciences,
Beijing 100101, China

J. G. Millar · R. T. Cardé
Department of Entomology, University of California,
Riverside, CA 92521, USA

Introduction

Unsaturated C_{10}–C_{18} straight chain alcohols, aldehydes, and acetates have been designated as "Type I" sex

pheromones of lepidopteran insects, and the majority of the known lepidopteran pheromones fall into this class (Ando et al. 2004). In contrast, a number of species in the families Geometridae, Arctiidae, Lymantriidae, and Noctuidae use polyunsaturated C_{17}–C_{23} straight chain hydrocarbons and the corresponding mono- and diepoxide derivatives as pheromone components, classed as "Type II" sex pheromones because of their distinct differences from the Type I group (Millar 2000; Ando et al. 2004).

The Type I alcohol, aldehyde, and acetate components are biosynthesized *de novo* from fatty acid precursors by a series of desaturation, chain elongation, and chain shortening steps that produce pheromone components with specific chain lengths, double bond positions, and double bond geometries. The structures are completed by the adjustment of the terminal functional group by reduction, oxidation, or tranesterification to provide alcohols, aldehydes, or acetates, respectively (Jurenka and Roelofs 1993; reviewed in Tillman et al. 1999). The evidence to date suggests that all of these transformations are carried out in the pheromone gland.

The biosynthesis of Type II pheromones also has been studied, and from reports of pheromone biosynthesis in the Lymantriidae (Kasang et al. 1979), Arctiidae (Rule and Roelofs 1989), and Geometridae (reviewed in Ando et al. 2008), it is clear that there are significant differences between the biosynthetic pathways leading to the two types of pheromones. These include differences in the biosynthetic sites, the substrates, the enzymes involved, and the endocrine regulation of biosynthesis. In distinct contrast to the site of production of Type I pheromones, it is likely that the polyunsaturated hydrocarbons that constitute Type II pheromones are produced in oenocyte cells by chain extension of diet-derived linoleic or linolenic acids, culminating in reductive decarboxylation to remove the terminal functional group (Rule and Roelofs 1989; reviewed in Ando et al. 2008). The final steps are slightly different for odd- vs. even-numbered carbon skeletons. The former probably result from 0–3 cycles of 2-carbon unit chain extension of linoleic or linolenic precursors and subsequent removal of one carbon by reductive decarboxylation to give an odd-numbered chain. In contrast, recent evidence suggests that even-numbered chains probably are produced by the same series of 2-carbon chain extension steps, then α-oxidation and loss of one carbon, followed by loss of a second carbon as CO or CO_2 by an oxidative mechanism (Goller et al. 2007). In some cases, further desaturation also occurs somewhere in the pathway to produce compounds such as the tetraene hydrocarbon pheromones of the arctiid moth *Utetheisa ornatrix* (Linnaeus) (Choi et al. 2007) and the geometrid moth *Operophtera brumata* (L.) (Zhao and Löfstedt, unpublished data). The newly formed polyene skeletons then are transported through the hemolymph by lipophorin carriers from the oenocytes to the pheromone gland (Wei et al. 2004; Matsuoka et al. 2006). The polyene hydrocarbons are emitted from the pheromone gland unchanged, or are further transformed by one or more epoxidation steps to produce unsaturated epoxide pheromone components for release (Miyamoto et al. 1999; Jurenka et al. 2003; Wei et al. 2003; Fan et al. 2004; Wei et al. 2004; Matsuoka et al. 2006).

The endocrine regulation of Type II pheromone biosynthesis also is markedly different from that of Type I pheromones. According to studies published to date, pheromone biosynthesis activating neuropeptides (PBANs) are not involved in the production of unsaturated hydrocarbon pheromone components in the arctiid moth *U. ornatrix* (Choi et al. 2007) or the geometrid moth *Ascotis selenaria cretacea* (Butler) (Wei et al. 2004), but they are involved in the epoxidation of these hydrocarbons in the pheromone gland, for those species that use epoxide pheromones alone or in combination with unsaturated hydrocarbons (e.g., the lymantriid moth *Lymantria dispar* L. (Jurenka et al. 2003) and the geometrid moth *A. selenaria cretacea* B. (Ando et al. 1997; Wei et al. 2004)).

For many years, it was presumed that a particular lepidopteran species produced either Type I or Type II pheromones, but not both. However, examples of species whose pheromone blends contain compounds of both types recently have been found in two moth families. For example, the pheromone blend of the crambid moth *Neoleucinodes elegantalis* consists of (*E*)-11-hexadecen-1-ol and (3Z,6Z,9Z)-3,6,9-tricosatriene (3Z,6Z,9Z-23:H) (Cabrera et al. 2001), whereas another crambid species, *Deanolis sublimbalis*, uses a blend consisting of (*Z*)-11-hexadecenal and 3Z,6Z,9Z-23:H (Gibb et al. 2007). Within the pyralid moth family, several examples are known, including the meal moth, *Pyralis farinalis* (L.) [(11*Z*,13*Z*)-11,13-hexadecadienal (11*Z*,13*Z*-16:Ald) (Landolt and Curtis 1982) + 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H; Leal et al. 2005; Kuenen et al. 2010], the navel orangeworm, *Amyelois transitella* (Walker) [11*Z*,13*Z*-16:Ald (Coffelt et al. 1979) + 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H; Leal et al. 2005; Millar et al. 2005; Kuenen et al. 2010], and the fir coneworm moth, *Dioryctria abietivorella* (Grote) [(9*Z*,11*E*)-9,11-tetradecadienyl acetate (9*Z*,11*E*-14:OAc) + 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H; Millar et al. 2005]. Several other *Dioryctria* species also appear to have analogous pheromone blends containing both Type I and Type II components (Miller et al., 2010a, b; Löfstedt et al. unpub. data).

The biosynthesis of the blends of Type I and Type II pheromone components in such species has not yet been studied. On the basis of previous pheromone biosynthesis studies, the aldehyde and pentaene pheromone components found in *A. transitella* pheromone glands probably arise from two independent biosynthetic pathways. The conjugated diene aldehyde, 11*Z*,13*Z*-16:Ald, may be produced

from palmitic acid in the pheromone gland, by pathways analogous to those for other conjugated diene Type I pheromones. For example, production of 11*Z*,13*Z*-16:Acid, a precursor to the conjugated pheromone in the pine processionary moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller), involves a bifunctional Δ11 desaturase (Quero et al. 1997; Abad et al. 2007). By analogy to other systems, it seems likely that the two long-chain pentaenes, 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, are synthesized in oenocyte cells via chain elongation, desaturation, and decarboxylation of diet-derived linolenic acid or other unsaturated fatty acid precursors, and transported to the gland through the hemolymph (Schal et al. 1998a, b; Jurenka and Subchev 2000; Matsuoka et al. 2006). It also is noteworthy that the above polyenes differ in structure by only one two-carbon unit, suggesting that the polyene motif that is common to both components may be synthesized first, followed by chain length adjustment in the later steps of the biosynthesis.

Thus, the objectives of the work reported here were to study the biosynthesis of the polyene hydrocarbon components and the diene aldehyde pheromone component present in the navel orangeworm pheromone gland, and to gain a better understanding of the mechanisms by which the biosynthesis of these two distinct types of pheromone components are regulated in *A. transitella*, including examining the possible role of PBAN.

Methods and Materials

Insects The *A. transitella* moths used in this study were obtained from the Department of Entomology, University of California, Riverside, CA, USA. The larvae were fed on a wheat germ diet as previously described (Coffelt et al. 1978; Girling and Cardé 2006), and kept in 4 liter glass jars in controlled environment chambers with 17:7 L:D, 25± 1°C and 65% relative humidity. Newly emerged adult females were collected daily and held in single-sex cohorts fed with a 10% honey solution. One- to 2-d old female pupae and adults were used throughout this study. Pupae were sexed by the morphological differences in their external genitalia.

Chemicals PBAN (*Helicoverpa zea* pheromone biosynthesis-activating neuropeptide) was purchased from Bachem (Weil am Rhein, Germany). Methyl (9*Z*,12*Z*,15*Z*,18*Z*,21*Z*)-9,12,15,18,21-tetracosapentaenoate and (16,16,16-D_3)-hexadecanoic acid (D_3-16:Acid) were purchased from Larodan Fine Chemicals, Malmö, Sweden. The synthesis of (*Z*)-[13,13,14,14,15,15,16,16,16-D_9]-11-hexadecenoic acid (D_9-11*Z*-16:Acid) was described in Löfstedt et al. (1994), and (9*Z*,12*Z*,15*Z*)-[9,10,12,13,15,16-D_6]-9,12,15-octadecatrienoic acid (D_6-linolenic acid), (9*Z*,12*Z*,15*Z*)-[17,17,18,18,18-D_5]-9,12,15-octadecatrienoic acid (D_5-linolenic acid), and (11*Z*,14*Z*,17*Z*)-[8,8,9,9-D_4]-icosatrienoic acid (D_4-icosatrienoic acid) were obtained from R. Adlof as gifts. 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H were synthesized as described by Millar et al. (2005), and 11Z,13*Z*-16:Ald and 11Z,13*Z*-16:OH were synthesized as described in Kuenen et al. (2010).

(*Z*)- and (*E*)-6-Hexadecenol (6*Z*-16:OH and 6*E*-16:OH) from our laboratory collection of pheromone components were used to prepare the reference standards needed to identify the double bond configuration of methyl 6-hexadecenoate in the insect extracts. Thus, 2 mg of 6*Z*-16:OH or 6*E*-16:OH were dissolved in 1 ml of dimethylformamide (DMF) in a 4 ml screw-cap vial, 100 mg of pyridinium dichromate were added, and the mixture was stirred overnight at room temperature. Then 1 ml of diethyl ether was added, and the mixture was vortexed briefly to precipitate the chromium salts. Distilled water was added, and the ether layer was then collected, washed twice with distilled water to remove traces of DMF, dried over anhydrous sodium sulfate, and evaporated to dryness. Methyl esters were produced by acid-catalyzed esterification (MeOH/HCl at 80°C for 1 h).

A reference standard of methyl (11*Z*,13*Z*)-11,13-hexadecadienoate (11*Z*,13*Z*-16:Me) also was prepared from 11*Z*,13*Z*-16:Acid by acid-catalyzed esterification.

Extraction of Insect Tissues The pheromone glands of female *A. transitella*, a broad, chevron-shaped structure located on the ventrolateral surface of the intersegmental membrane between abdominal scleromata VIII and IX (Srinivasan et al. 1986), were dissected from cohorts of individual 1- to 2-d-old intact virgin female moths in both the photophase (1 h before lights off) and scotophase (6 h after lights off). Each gland was extracted for 30 min in 10 μl of hexane containing 1 ng of (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene (3*Z*,6*Z*,9*Z*-21:H) as internal standard. After removing the pheromone glands, the hemolymph, the abdominal cuticle, and the whole abdomen (i.e., cuticle, hemolymph, and abdominal tissues) were extracted separately. For extraction of the hemolymph, ca. 10 μl of modified Weever's saline (21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 170 mM glucose, 5 mM PIPES, 9 mM KOH, adjusted to pH 6.6; Carrow et al. 1981) were injected first into the abdomen, and then the mixture of hemolymph and saline was transferred into a glass vial insert by syringe. Methanol (ca. 30–40 μl per 3–4 female equivalents of hemolymph) was added, and the mixture was extracted × 3 with 10 μl hexane containing 0.1 ng/μl of internal standard (3*Z*,6*Z*,9*Z*-21:H).

The abdominal cuticle was extracted as previously described (Jurenka et al. 2003), first removing the majority of the scales on the abdomen by application of a gentle

vacuum and then removing the abdomen at the junction of the thorax. The abdomen was cut laterally and pinned open. The ovaries were removed along with the majority of the remaining fat body and organs, leaving mostly epidermal tissue attached to the cuticle. The cuticle was rinsed first with 30 µl methanol and then extracted by vortexing 3× with 100 µl hexane containing 0.1 ng/µl 3*Z*,6*Z*,9*Z*-21:H internal standard, 10 min each time. After extraction, the hexane layers were combined into a new glass tube. The external abdominal cuticle also was extracted in this study by successively dipping five intact female abdomens into 100 µl hexane, with 5 sec dipping per individual. The hexane extract then was concentrated to approximately 10 µl for GC-MS analysis.

To extract the hydrocarbons from the whole abdomen with only the pheromone gland removed, the abdominal tissues were immersed in 100 µl methanol in a 2 ml vial and disrupted with a probe sonicator for a few seconds. The resulting homogenate was extracted by sequential vortexing 3× with 100 µl hexane containing 0.1 ng/µl 3*Z*,6*Z*,9*Z*-21:H internal standard. The hemolymph, cuticle, and abdomen tissue homogenate extracts were dried with anhydrous sodium sulfate and purified by passage through a 7× 0.2 cm column of 100-200 mesh Florisil. Approximately 30-40 µl of a crude hexane extract were added to the column, and hydrocarbons were eluted with 1 ml hexane.

Base Methanolysis and Methylthiolation After the first extraction with hexane, the remaining glandular and abdominal tissues were extracted further for fatty acyl moieties. Thus, the glandular tissue was extracted with 20 µl chloroform:methanol (2:1 *v*:*v*) in a glass vial insert for 24 h at room temperature, then the tissue was removed and the extract was concentrated under a stream of nitrogen. The residue was subjected to base methanolysis to convert fatty acyl moieties to the corresponding methyl esters as described by Bjostad and Roelofs (1984). First, 20 µl of 0.5 M KOH in methanol were added, and the vial insert was placed inside a 4 ml screw-cap vial that was heated for 1 h at 40°C. After cooling, 20 µl of 0.5 M HCl in methanol were added to neutralize the alkali, and the mixture was extracted 3× with 20 µl hexane. The combined hexane layers were washed twice with 30 µl distilled water, then dried over anhydrous sodium sulfate.

The residue from extraction of the abdominal tissue with hexane, in 100 µl methanol, was re-extracted with 200 µl chloroform for 24 h at room temperature. The chloroform/methanol extract was transferred to a new 2 ml vial and concentrated under a stream of nitrogen, then subjected to methanolysis by addition of 200 µl of 0.5 M KOH in methanol and warming to 40°C for 1 h. After cooling, 200 µl of 0.5 M HCl in methanol were added, and the mixture was extracted 3× with 200 µl hexane, vortexing for 10 min each time. The hexane layers were combined and washed 2× with 200 µl distilled water, and dried over anhydrous sodium sulphate. The resulting solutions of fatty acid methyl esters were analyzed by GC-MS as described below. The double bond positions of monounsaturated methyl esters were determined by GC-MS analysis of their dimethyldisulfide (DMDS) adducts (Buser et al. 1983; Dunkelblum et al. 1985). DMDS derivatizations were carried out by adding 50 µl DMDS and 10 µl of 5% (by weight) I_2 in diethyl ether to 20 µl of the solution of methylated fatty acids extracted from the pheromone glands, or 50 µl of the methylated fatty acids extracted from the abdomen tissues. Each reaction was stirred in a 2 ml screw-cap vial overnight at 40°C, then 200 µl of hexane were added, and the mixture was washed with 50 µl 5% aqueous sodium thiosulfate. The organic layer then was dried over anhydrous sodium sulfate and evaporated to dryness. The product was taken up in hexane for analysis.

In addition, an extract of artificial diet (1 g diet in 1 ml chloroform/methanol for 24 h) was used to analyze fatty acids from the diet.

Effect of PBAN on Pheromone Production Unmated female moths (0–24-h-old) were decapitated at the end of the photophase and kept individually in small plastic containers. A piece of wet filter paper was placed inside each container to maintain high humidity so that the decapitated animals would not desiccate. After 24 h, 10 pmol PBAN in 4 µl of Weever's saline were injected into the abdomen of each decapitated female. Equal volumes of Weever's saline were injected into decapitated animals as controls. The pheromone glands of treated and control insects were dissected 2 h later, and each gland was extracted with 10 µl hexane containing 0.1 ng (*Z*)-12-tetradecenyl acetate and 0.2 ng (1,3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatetraene as internal standards.

Labeling Experiment The proposed pheromone precursors, D_3-16:Acid, D_9-11*Z*-16:Acid, D_6-linolenic acid, D_5-linolenic acid, and D_4-11*Z*,14*Z*,17*Z*-20:Acid were used to probe the aldehyde and pentaenes biosynthetic pathways. D_3-16:Acid, D_9-11*Z*-16:Acid, and D_6-linolenic acid were dissolved in dimethylsulfoxide (DMSO) at a concentration of 12.5 µg/µl and topically applied to the extruded pheromone gland (0.2 µl of DMSO solution; 2.5 µg/gland) of PBAN-injected decapitated female adults. D_5-linolenic acid and D_4-11*Z*,14*Z*,17*Z*-20:Acid dissolved in DMSO also were topically applied to pheromone glands of the control group of intact female adults that had not been injected with PBAN. After a 2 h incubation, pheromone glands were dissected and extracted as described above.

For injection of the possible pheromone precursors, D_5-linolenic acid and D_4-11*Z*,14*Z*,17*Z*-20:Acid were dissolved

in vegetable oil (75% rapeseed and 25% sunflower oil, ICA, Sweden.) and injected into the abdomens of 1- to 2-d-old female pupae (1 µl of 100 µg/µl solution per female). After adult emergence, at the 6th h of the scotophase, the pheromone gland was removed from 2-d-old females and extracted with 10 µl hexane containing 0.1 ng/µl 3*Z*,6*Z*,9*Z*-21:H as internal standard. After removal of the pheromone gland, the remaining abdominal tissue including the scales was extracted 3× with 100 µl hexane containing 0.1 ng/µl 3*Z*,6*Z*,9*Z*-21:H as internal standard as described above. D_4-11*Z*,14*Z*,17*Z*-20:Acid dissolved in vegetable oil also was injected into 2-d-old female adults at the 2nd h in the scotophase, and the pheromone gland and remaining abdominal tissue were subsequently extracted at the 6th h in the scotophase, followed by the same protocol.

Coupled Gas Chromatography-Mass Spectrometry The aldehyde and pentaene components in pheromone gland extracts, the corresponding deuterium labeled compounds and precursors, and the fatty acid methyl esters derived from methanolysis of tissue and diet extracts were analyzed with a Hewlett-Packard (Palo Alto CA, USA) 5972 mass selective detector coupled to an HP 5890 series II GC equipped with a capillary column (HP-1MS or InnoWax (SGE, Austin, TX, USA), both 30 m×0.25 mm i.d.). The HP-1MS column was programmed from 80°C at 10°C/min to 220°C, hold for 3 min, then to 250°C at 4°C/min, hold for 5 min, and finally to 280°C at 20°C/min, hold for 5 min. The InnoWax column was programmed from 80°C at 10°C/min to 200°C, then to 230°C at 1°C/min, with a final hold for 20 min at 230°C.

Selected ion monitoring (SIM) was used to monitor the pheromone compounds and the incorporation of labeled precursors. The following characteristic ions were chosen for the aldehyde and the hydrocarbons: when each of D_3-16:Acid and D_9-11*Z*-16:Acid was applied, the molecular ion at *m/z* 236 was chosen for 11*Z*,13*Z*-16:Ald, with *m/z* 239 and 243 being used to monitor the corresponding D_3-11*Z*,13*Z*-16:Ald and D_7-11*Z*,13*Z*-16:Ald, respectively. When D_6-linolenic acid was applied to probe the pentaenes biosynthetic pathway, the diagnostic ions at *m/z* 108 and 178 were used to monitor unlabeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H, and *m/z* 108 and 206 were used to monitor unlabeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, whereas *m/z* 112 (108 + 4) and *m/z* 178 or 206 were used to monitor labeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H or 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, respectively. Specifically, the ions at *m/z* 178 and 206 remain unchanged in the labeled pentaenes because they arise from cleavages from the nondeuterated end of the chains (Millar et al. 2005). When D_5-linolenic acid was applied, *m/z* 113 (108 + 5) and *m/z* 178 or 206 were used to monitor labeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, respectively; and when D_4-*Z*11,*Z*14,*Z*17-20:Acid was used, *m/z* 108 and *m/z* 180 (178+2) were chosen for monitoring labeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H.

Results

Regulation of Pheromone Production The amount of 11*Z*,13*Z*-16:Ald in pheromone gland extracts from decapitated females 24 h after decapitation and 2 h after injection of saline was barely detectable (0.02±0.02 ng) and was significantly lower than the titer in intact control females (0.25±0.27 ng, P=0.016, N=10) (Fig. 1). However, 2 h after injection of PBAN into the abdomens of decapitated females, the pheromone gland titer of 11*Z*,13*Z*-16:Ald increased to 1.6±1.2 ng (N=9), significantly higher than the titer in either decapitated, saline-injected control females (P<0.001) or intact females (P=0.001). By contrast, the titers of 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H were not affected by PBAN; the levels of each compound found in glands from normal, decapitated PBAN-injected, or decapitated saline-injected control females were not significantly different (Fig. 1).

The two long-chain pentaenes were found in hexane extracts from various tissues including the pheromone gland, the abdominal cuticle, the abdominal tissue, and the hemolymph of 1- to 2-d-old virgin females (Table 1). The titers of each of the two pentaenes in the pheromone gland and abdominal tissue extracts did not differ significantly between the photophase and scotophase (Table 1). There was no evidence for either of the pentaenes on external cuticular surfaces; extracts prepared by quickly dipping intact adult insects in solvent contained no detectable amounts of the pentaenes.

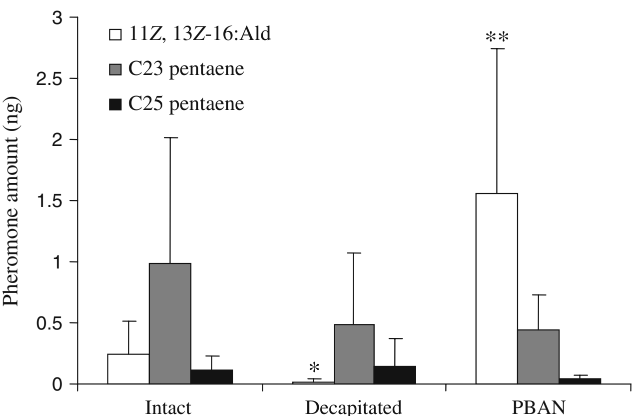

Fig. 1 Titers of 11*Z*,13*Z*-16:Ald, 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H, and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H in pheromone gland extracts from *Amyelois transitella* intact females, decapitated females injected with saline, and decapitated females injected with 10 pmol PBAN in saline (PBAN). For each column color, significant differences between means were determined by Student's *t*-tests ("*": P<0.05; "**": P<0.01)

Table 1 Titer (ng) of C_{23} and C_{25} pentaenes in various tissues of 1–2 day old virgin female navel orangeworm moths[a]

Compound	Pheromone Gland		Abdomen[b]		Abdominal Cuticle[c]	Hemolymph[d]
	(*N*=5)		(*N*=5)		(*N*=7)	(*N*=2)
	photophase	scotophase	photophase	scotophase	scotophase	scotophase
3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H	0.40±0.47	0.45±0.47	135±70	111±39	17±8	2.9±0.2
3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H	0.03±0.01	0.07±0.05	10±6	18±11	2.0±1.7	0.71±0.12

[a] Individual 1- to 2-d-old intact virgin female moths (or in the case of hemolymph, samples of four females) were extracted during the last hour of photophase or the 6th hour of scotophase, respectively. The titer of each polyene (mean ± SD) was determined by GC-MS in selected ion monitoring mode using the base peak (*m/z* 79) for quantification versus 3*Z*,6*Z*,9*Z*-21:H as internal standard. Student's *t*-tests showed no significant differences at the 5% level between photophase and scotophase extracted polyenes in the pheromone gland or whole abdominal tissue

[b] Abdomen = the whole abdomen with the pheromone gland removed, homogenized and extracted

[c] Abdominal cuticle = the abdominal cuticle with the pheromone gland, hemolymph, fat body, and other tissues removed

[d] Hemolymph = the mixture of hemolymph and saline extracted from the abdomen, after injection of 10 µl Weever's saline per female

Fatty Acid Composition in Abdominal Tissue and Pheromone Gland In addition to saturated and unsaturated fatty acid methyl esters such as methyl tetradecanoate (14:Me, related abbreviations are used hereafter for analogous fatty acid methyl esters), 15:Me, 16:Me, 9*Z*-16:Me, 17:Me, 18:Me, 9*Z*-18:Me, 9*Z*,12*Z*-18:Me, and 9*Z*,12*Z*,15*Z*-18:Me, the methanolized abdominal tissue and pheromone gland extracts contained the methyl esters of some longer chain saturated and unsaturated fatty acids, including 20:Me, 22:Me, 24:Me, 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-20:Me, as well as traces of

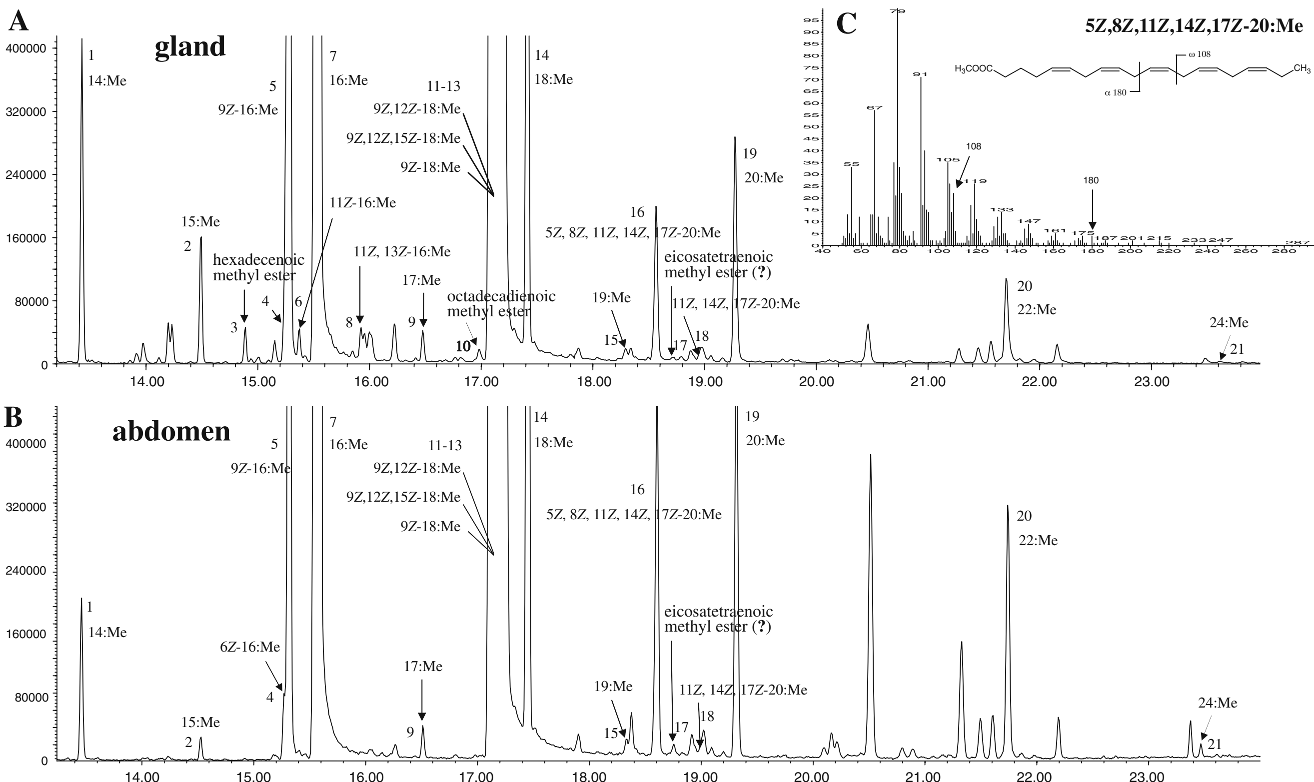

Fig. 2 Total ion chromatograms of the fatty acid compositions (as the methyl esters) of representative pheromone gland (**a**) and abdominal tissue (**b**) extracts (HP-1MS capillary column). Fatty acid methyl esters are coded with a number indicating chain length, followed by a number indicating the number of double bonds, with the positions and geometries given if known. Peak numbers correspond to those listed in Table 2. The omega ion at *m/z* 108, diagnostic for a 3,6,9-triene motif, and the alpha ion at *m/z* 180, diagnostic for a 5,8,11-triene motif counting from the other end of the chain are indicated in the mass spectrum of the C20:5 acid methyl ester (**c**). The weak molecular ion at *m/z* 316 was not detected in full scan mode, but it was detected in the more sensitive selected ion monitoring mode, at a same retention time as the two diagnostic ions at *m/z* 79 and 108

two compounds tentatively identified as 11*Z*,14*Z*,17*Z*-20: Me and a C20:4 methyl ester (Fig. 2, Table 2). In particular, the mass spectrum of 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-20:Me contained diagnostic ions at *m/z* 108 and *m/z* 180 from the characteristic cleavages and rearrangements of a fatty acid methyl ester with a 5,8,11,14,17 pentaene system (Fellenberg et al. 1987), as well as *m/z* 74 from McLafferty rearrangement of the methyl ester. The molecular ion (*m/z* 316) was not detected in full scan, but it was found in the more sensitive selected ion monitoring mode. Furthermore, this acid must be produced by the insect because it was not found in extracts of the diet used to rear the insects. 11*Z*,14*Z*,17*Z*-20: Acid also was tentatively identified on the basis of its matching retention time with that of an authentic standard, the characteristic base peak at *m/z* 79, and the diagnostic fragment at *m/z* 108 from the same cleavage and rearrangement as shown in Fig. 2c for 5*Z*,8*Z*,11*Z*,14*Z*, 17*Z*-20:Acid.

In extracts of the pheromone gland, the aldehyde pheromone intermediates 11*Z*-16:Me and 11*Z*,13*Z*-16:Me were found, along with traces of a nonconjugated doubly unsaturated C_{18} fatty acid that was not completely identified (peak 6, peak 8, peak10, Fig. 2a). These three compounds were not found in the abdominal tissue extracts.

Table 2 Fatty acid methyl esters detected in the pheromone gland and abdomen of female navel orangeworm

Compound	Peak No.[a]	Pheromone gland	Abdomen[c]	Criteria used in identifications		
		Relative amount[b] (*N*=7)	Relative amount (*N*=6)	MS[d]	Retention time[e]	DMDS[f] derivative
14:Me	1	12.8±8.8	2.0±0.9	√	√	
15:Me	2	8.2±5.7	0.20±0.04	√	√	
Hexadecenoic methyl ester[g]	3	2.2±1.4	–			
6*Z*-16:Me[h]	4	–	0.4±0.2	√	√	√
9*Z*-16:Me	5	31.8±12.0	20.1±7.3	√	√	√
11*Z*-16:Me	6	1.4±0.8	–	√	√	√
16:Me	7	100	100	√	√	
11*Z*,13*Z*-16:Me	8	1.9±1.0	–	√	√	
17:Me	9	1.9±1.1	0.26±0.03	√	√	
Octadecadienoic methyl ester[g]	10	1.3±0.8	–			
9*Z*,12*Z*-18:Me	11	33.3±4.5	38.3±6.2	√	√	
9*Z*,12*Z*,15*Z*-18:Me	12	18.5±4.7	10.7±2.8	√	√	
9*Z*-18:Me	13	90.7±8.4	76.3±10.2	√	√	√
18:Me	14	21.2±5.2	10.1±1.4	√	√	
19:Me	15	0.8±0.5	0.14±0.07	√	√	
5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-20:Me	16	2.5±0.8	2.0±0.4	√	√	
eicosatetraenoic methyl ester(?)[g, i]	17	<0.1	0.10±0.04			
11*Z*,14*Z*,17*Z*-20:Me[i]	18	<0.1	<0.1	√	√	
20:Me	19	3.6±1.1	2.2±0.4	√	√	
22:Me	20	3.1±2.2	1.6±0.3	√	√	
24:Me	21	0.8±0.5	0.17±0.14	√	√	

[a] Numbers refer to gas chromatographic peaks in Fig. 2

[b] Amounts are reported relative to that of methyl palmitate, the most abundant component

[c] Abdomen = the whole abdomen with the pheromone gland removed, homogenized and extracted

[d] Full scan mass spectrum

[e] Retention time match on both the HP1-MS and InnoWax columns

[f] DMDS derivative confirmation of double bond location and configuration

[g] The double bond position was not determined

[h] 6*Z*-16:Me in pheromone gland extract was well separated from 9*Z*-16:Me on the InnoWax column, but the two compounds were not resolved on the HP-1MS column. However, the same compounds in the abdomen extracts were separated on both columns. The double bond position of 6*Z*-16:Me was confirmed by DMDS analysis and the configuration was confirmed by comparing the mass spectrum and retention time of both the methyl ester and the corresponding DMDS adduct with those of the reference 6*Z*-16 and 6*E*-16 isomers

[i] Present in trace amount

Another unusual monoenoic C16 fatty acid methyl ester was found in the gland and abdomen extracts after DMDS derivatization of the methyl esters fraction. Specifically, a C16 DMDS adduct was found with a molecular ion at *m/z* 362 (6%) and diagnostic fragment ions at *m/z* 175 (42%) and 187 (81%), corresponding to the DMDS derivative of Δ6-16:Me. By comparing the mass spectrum and gas chromatographic retention times of the methyl ester and its DMDS adduct with those of authentic 6*Z*-16: Me and 6*E*-16:Me and their DMDS adducts, the natural Δ6-16 component was shown to be the 6*Z*-16:Me isomer (Fig. 3).

Labeling Experiment Results Topical application of D_3-16: Acid and D_9-11*Z*-16:Acid to the pheromone gland of PBAN-injected decapitated females resulted in incorporation of the labeled precursors into 11*Z*,13*Z*-16:Ald, with a labeled to unlabelled compounds ratio of 2.7±2.0 % (N=4) and 44±23% (N=6), respectively (Fig. 4a and b). These results demonstrated that both of these acids likely are biosynthetic precursors to 11*Z*,13*Z*-16:Ald, and that 11*Z*, 13*Z*-16:Ald synthesis occurs in the pheromone gland.

When D_5-linolenic acid was injected into 1-to 2-d-old female pupae, the labeled precursor was incorporated into both the C_{23} and C_{25} pentaenes (Fig. 5), with a labeled to

Fig. 3 Representative sections of the total ion chromatograms (TIC) of fatty acid methyl ester DMDS adducts, analyzed on an InnoWax capillary column. **a** Section of TIC from pheromone gland fatty acid methyl esters. **b** Selected ion monitoring GC-MS analyses of monounsaturated C16 fatty acid methyl ester DMDS adducts. Double bond positions were identified from the diagnostic ions from cleavage of the C-C bond between the two carbons bearing methylthiol groups: Δ6-16, 175/187/362; Δ9-16, 217/145/362; Δ11-16, 245/117/362. **c** Confirmation of the "*cis*" configuration in the insect-produced Δ6-16 monounsaturated fatty acid methyl ester by comparison of the chromatographic retention times of the DMDS adducts, using the diagnostic ions at *m/z* 175 and 187. *a*: Δ6-16:Me DMDS adduct in insect abdomen extract; *b*: authentic 6*Z*-16:Me DMDS adduct; c: authentic 6*E*-16:Me DMDS adduct. **d** Full scan mass spectrum of the Δ6-16 monounsaturated fatty acid methyl ester DMDS adduct from insect abdomen extract (*a*) and authentic 6*Z*-16:Me DMDS adduct (*b*)

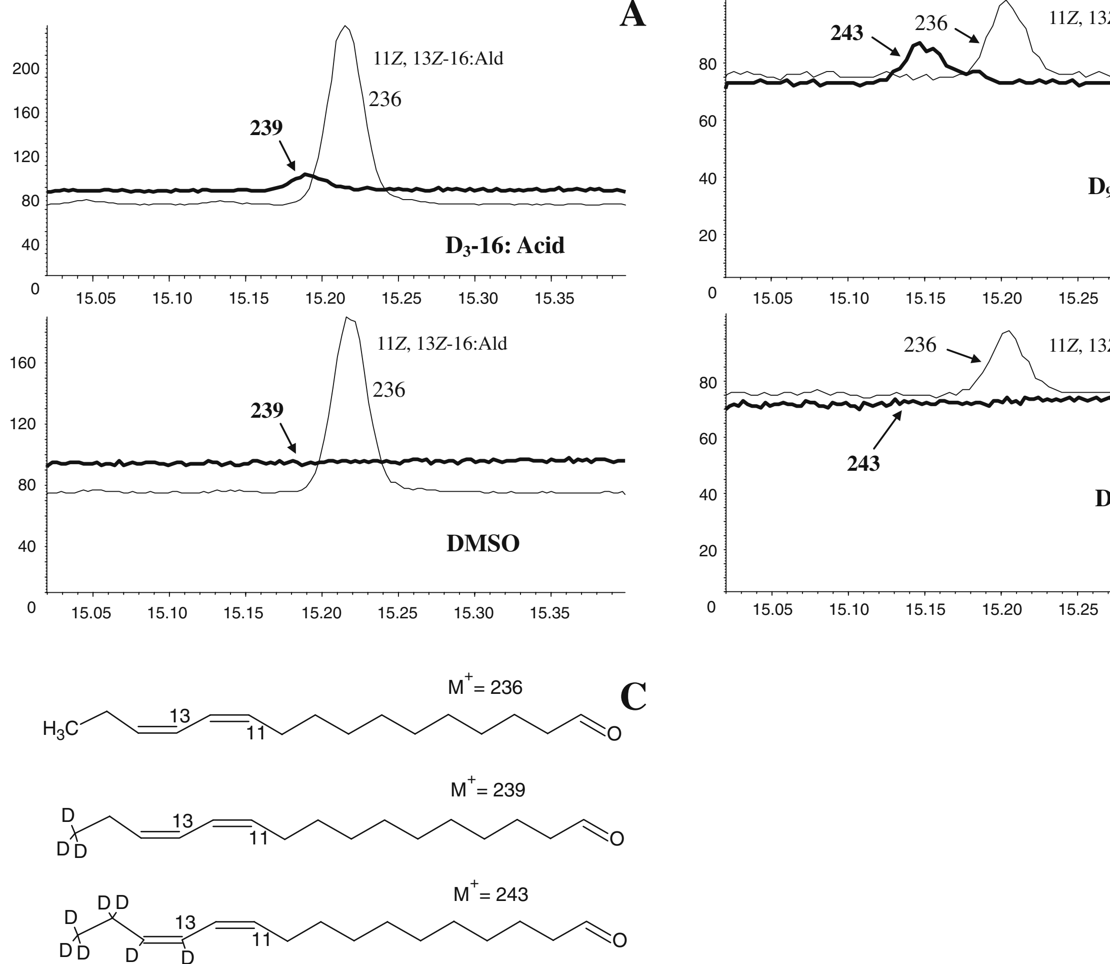

Fig. 4 Selected ion monitoring chromatograms showing incorporation of labeled fatty acid precursors into 11*Z*,13*Z*-16:Ald, using ions 239 and 243 to monitor D_3-11*Z*,13*Z*-16:Ald and D_7-11*Z*,13*Z*-16:Ald, respectively. **a** Deuterium label incorporation into D_3-11*Z*,13*Z*-16:Ald after topical application of D_3-16:Acid in DMSO onto the pheromone gland; **b** Deuterium label incorporation into D_7-11*Z*,13*Z*-16:Ald after topical application of D_9-11*Z*-16:Acid in DMSO onto the pheromone gland; **c** diagnostic ions for unlabeled 11*Z*,13*Z*-16:Ald, D_3-labeled 11*Z*,13*Z*-16:Ald and D_7-labeled 11*Z*,13*Z*-16:Ald

unlabeled compounds ratio of 4.9±0.6% for C_{23} pentaene in the pheromone gland extracts, a 4.0±1.2% ratio in C_{23} pentaene extracted from the abdominal tissues (N=3), and a 3.6±0.2% ratio (N=3) in C_{25} pentaene extracted from abdominal tissues. Because of the small amount of native C_{25} pentaene in the pheromone gland, among the labeled samples only one provided clear evidence for incorporation of label (6.0%). These results demonstrated that linolenic acid is a precursor for both of the pentaene components, but they do not reveal where the biosynthesis of the pentaenes occurs. However, topical application of D_6-linolenic acid to the pheromone gland coupled with PBAN injection, or topical application of D_5-linolenic acid onto the pheromone gland without PBAN injection resulted in no detectable incorporation of label into either of the pentaenes. This suggests that the pentaenes are not synthesized in the pheromone gland, and consequently, that they must be synthesized elsewhere in the abdomen and transported to the pheromone gland for release.

Topical application of D_4-11*Z*,14*Z*,17*Z*-20:Acid onto the pheromone gland of intact females without PBAN injection or injection of D_4-11*Z*,14*Z*,17*Z*-20:Acid into the abdomens of either pupae or adults also did not result in detectable label incorporation into the pentaenes, indicating that this acid probably is not a precursor to the pheromone.

Discussion

This is the first example of a lepidopteran insect in which both of the biosynthetic pathways leading to Type I and Type II moth pheromone components, respectively, have been demonstrated. The general steps of the pathways are parallel to those found in moths which use only one or the

Fig. 5 Selected ion monitoring chromatograms showing incorporation of labeled fatty acid precursors into 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H after abdominal injection of D_5-linolenic acid into 1–2 d old pupae. **a** Deuterium label incorporation into D_5-3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H; **b** Deuterium label incorporation into D_5-3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H; **c** Ions 108 and 178, and ions 108 and 206 were used to monitor unlabeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H and 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, respectively. Ions 113 and ion 178 were used to monitor D_5-3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-23:H, and ions 113 and 206 were used to monitor D_5-3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H. Because of the small amounts of both native and labeled 3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H, the 206 ion was not detected in labeled D_5-3*Z*,6*Z*,9*Z*,12*Z*,15*Z*-25:H

other pathway, with our results suggesting that navel orangeworm females synthesize 11*Z*,13*Z*-16:Ald from palmitic acid in the pheromone gland. In contrast, the two pentaenes apparently originate from linolenic acid. The fact that the two pentaenes were found in extracts of the pheromone gland, the abdominal tissue, the abdominal cuticle, and the hemolymph of female navel orangeworm moths (Table 1) are consistent with the hypothesis that the pentaenes are synthesized outside the pheromone gland, possibly in oenocyte cells, and then transferred to the gland through the hemolymph by carrier proteins, as has been shown in other lepidopteran species that produce Type II pheromone components (Jurenka et al. 2003; Wei et al. 2003; Matsuoka et al. 2006; Choi et al. 2007).

As expected, the production of 11*Z*,13*Z*-16:Ald in pheromone glands of decapitated females was increased significantly by PBAN injection, whereas the titer of the pentaenes in the gland extracts was not affected by PBAN injection. In addition, the titers of both pentaenes in the gland extracts were the same regardless of whether the glands were extracted during the photophase or the scotophase (Table 1). Thus, PBAN does not appear to be involved in the production of the pentaenes, similar to what was found with the production of polyenes in the geometrid moth *A. selenaria cretacea* (Wei et al. 2004) and the arctiid moth *U. ornatrix* (Choi et al. 2007), and the titer of the pentaenes does not fluctuate with the light-dark cycle.

The fatty acid composition of extracts of pheromone glands and abdominal tissues, coupled with the results showing incorporation of labeled precursors, suggest two distinctly different biosynthetic pathways for the aldehyde and the pentaenes (Figs. 6 and 7). In the pheromone gland, the fatty acid compositions found in the extracts suggested that saturated C_{16} palmitic acid may be a key substrate, being first desaturated at the 11 position to form 11*Z*-16: Acid. A second desaturation, possibly mediated by a bifunctional desaturase such as the one known from *T. pityocampa* (Quero et al. 1997; Abad et al. 2007) then may

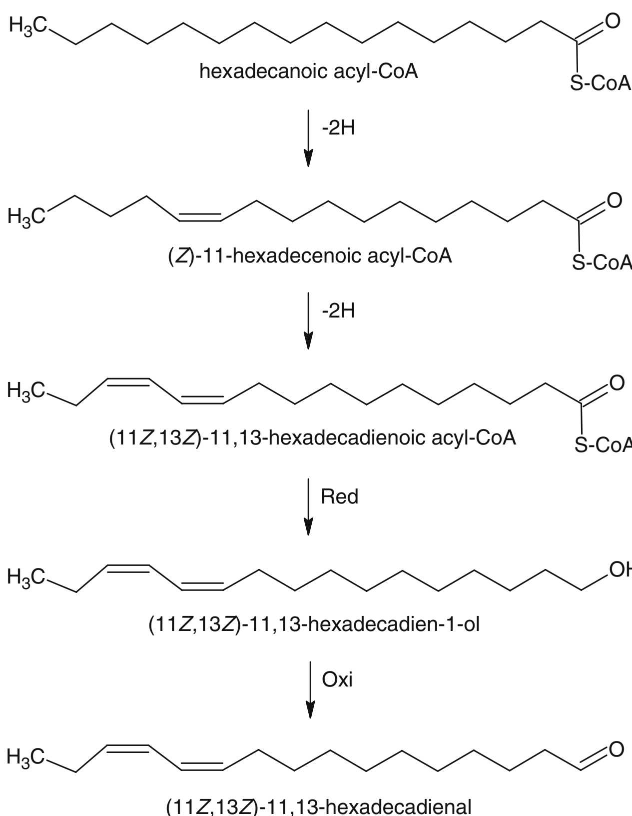

Fig. 6 Proposed biosynthetic pathway for the Type I 11*Z*,13*Z*-16:Ald sex pheromone component produced by female *Amyelois transitella*. "–2H" represents desaturation to form a double bond, "*Red*" indicates functional group reduction and "*Oxi*" indicates terminal oxidation

form 11*Z*,13*Z*-16:Acid, which is subsequently reduced to the corresponding alcohol and finally oxidized to the aldehyde (Fig. 6). The incorporation of deuterium label from saturated D_3-16:Acid and monoenoic D_9-11*Z*-16:Acid precursors into 11*Z*,13*Z*-16:Ald is in agreement with this proposed biosynthetic pathway.

In contrast, our results suggest that the biosynthesis of the two long-chain pentaenes must take place in a markedly different fashion, beginning with different precursors, and likely occurring at a site outside the pheromone gland. The incorporation of deuterium label from labeled linolenic acid into the two pentaenes confirms that linolenic acid can serve as a precursor to these compounds. Furthermore, (5Z,8Z,11Z,14Z,17Z)-icosapentaenoic acid, a likely later-stage intermediate in the biosyntheses of the pentaene hydrocarbons, was found in both abdominal tissue and pheromone gland extracts. Two or three 2-carbon chain extensions of this icosapentaenoic acid would provide the homologous tetracosapentaenoic and hexacosapentaenoic acids, respectively, which would yield the C_{23} and C_{25} pentaene hydrocarbons after decarboxylation. These data suggest that the icosapentaenoic acid with all the double bonds in place is a key intermediate in the biosynthesis of the pentaene hydrocarbons. The fact that we could detect no trace of the corresponding C_{22}, C_{24}, or C_{26} homologs suggests that this intermediate then undergoes a series of chain extensions and the final decarboxylation with all of the intermediates being continuously bound to the relevant enzymes, so that none of them are available as free entities that would have shown up in the analysis (Fig. 7).

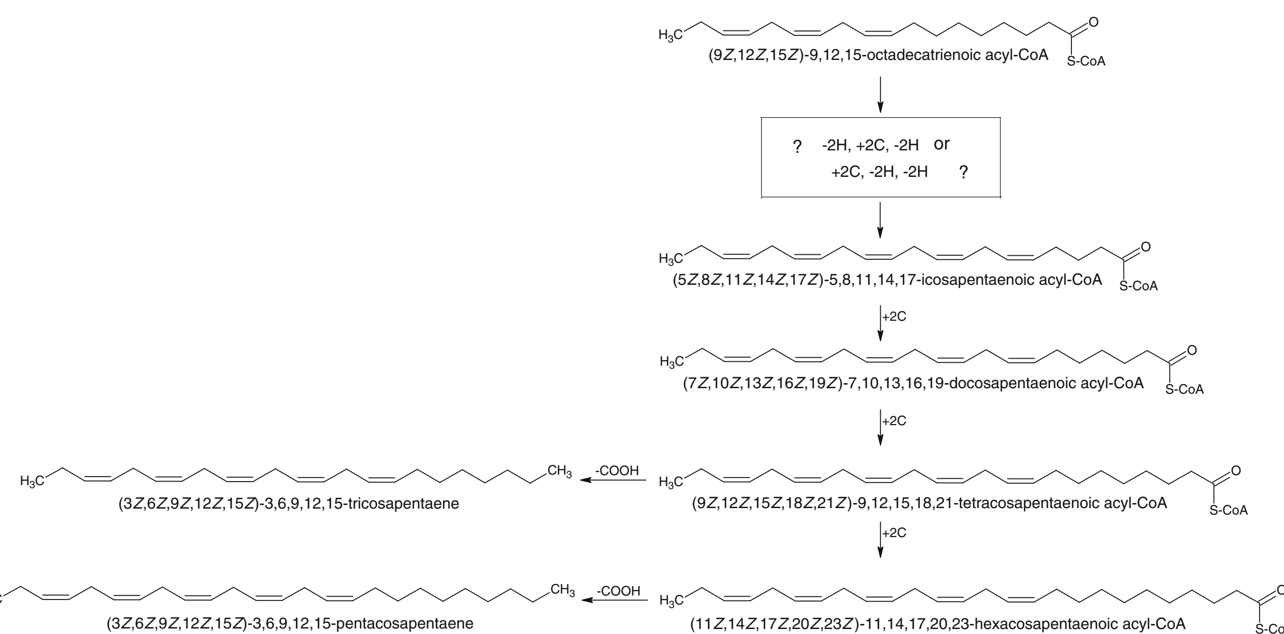

Fig. 7 Proposed biosynthetic pathway for the Type II pentaene sex pheromone components produced by female *Amyelois transitella*. "–2H" represents desaturation to form a double bond, "+2C" represents one cycle of chain elongation by two carbons, "–COOH" means decarboxylation

The presence of (5Z,8Z,11Z,14Z,17Z)-icosapentaenoic acid in the pheromone gland tissue was unexpected, given that most of the total pentaene titer was found in the abdominal tissues (Table 1). However, this acid actually may have been present in the gland extracts only as the result of contamination of the pheromone gland preparations with abdominal tissues, i.e., it is difficult to dissect out pheromone glands that are completely free of surrounding tissues.

Prior studies in other organisms from a broad array of taxa, such as plants, algae, and mammals, suggested a conserved "Δ6 pathway", i.e., the Δ6 desaturation of linolenic acid followed by elongation to produce 8*Z*,11*Z*,14*Z*,17*Z*-icosatetraenoic acid, which is further desaturated at the Δ5 position to produce the icosapentaenoic acid (Napier 2007). In the zebrafish, a bifunctional Δ6/Δ5 desaturase has been identified (Hastings et al. 2001), providing a precedent for this proposed pathway. In the present study, small amounts of the unusual (*Z*)-6-hexadecenoic acid were found in the pheromone gland and the abdomen extracts, which implies that the navel orangeworm may have a corresponding Δ6 desaturase. The fact that labeled 11*Z*,14*Z*,17*Z*-icosatrienoic acid was not incorporated into the pentaenes also suggests that at least the fourth double bond is introduced before elongation of the 18-carbon chain.

The shift from Type I to Type II pheromone components represents a major evolutionary transition that involves a new site of production, a mechanism for transport and selective uptake of polyunsaturated hydrocarbons into the pheromone gland before release, as well as for the evolution of corresponding receptors in male moths, with matching specificity for the detection of the novel pheromone components. It is difficult to envisage all these changes taking place simultaneously, and it is surprising that there are not more reports of species with mixed/intermediate pheromone systems in, for instance, Geometroidea and Noctuoidea, because these two moth superfamilies have many species that possess either Type I or Type II pheromones. The majority of the species for which mixed pheromones have been reported instead belong to the Pyraloidea, from which no cases of pure Type II (hydrocarbon-type) pheromones have been described (Witzgall et al. 2004; El-Sayed 2008). This suggests that the detailed mechanisms and sites of hydrocarbon biosynthesis may differ between the Geometroidea and Noctuoidea on one hand and the Pyraloidea on the other.

Once the hydrocarbon compounds started being exported to and taken up by the pheromone gland, this opened up the possibility for the modification of these compounds by the gland before release. The production of the epoxide derivatives of polyunsaturated hydrocarbons used by some species in the Geometroidea and Noctuoidea should, thus, represent a more advanced character state. Continued radiation then resulted in epoxidation at different positions, yielding one or the other, or in some cases both of the two possible enantiomers of each epoxide, thus allowing a variety of different semiochemicals to be made from a common precursor.

In vitro molecular cloning and functional analysis of the genes for the specific desaturase, elongase, and decarboxylase enzymes postulated in the biosynthesis of the hydrocarbon pheromone components would be helpful in explaining the entire pathway. Likewise, studies of tissue specific expression and *in situ* hybridization could clarify where the different reactions actually take place.

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Effects of Age and Mating on Female Sex Attractant Pheromone Levels in the Sorghum Plant Bug, *Stenotus rubrovittatus* (Matsumura)

Keiko Oku · Tetsuya Yasuda

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Abstract Previous work has shown that male sorghum plant bugs, *Stenotus rubrovittatus* (Matsumura) (Heteroptera: Miridae), are more attracted to young virgin females than to old virgin females and are not attracted to mated females. Therefore, we examined the effects of age and mating status on female sex pheromone levels. The pheromone components, hexyl butyrate, (*E*)-hex-2-en-1-yl butyrate, and (*E*)-4-oxohex-2-enal, were collected from females in two different ways. First, the compounds were extracted from whole bodies of each female and quantified by gas chromatography-mass spectrometry (GC-MS). Pheromone levels extracted from virgin female bodies decreased with age. In females that had just mated, pheromone levels did not differ from those of virgin females, and pheromone levels in mated females remained relatively constant up to 15 d after mating. This absence of change in pheromone levels extracted from whole bodies of mated females is not congruent with previous reports of lack of male attraction to these females. In a second method for pheromone recovery (adsorption on a PDMS-coated stir bar), GC-MS analysis showed that mated females released lower amounts of pheromone components compared to those emitted by virgin females of the same age (4 d). As was the case with whole body extracts, young virgin females (3 d) released higher amounts of pheromone components than did old virgin females (8 d). The results suggest that male response in *S. rubrovittatus* is dictated more by the quantities of pheromone components released into the volatile headspace by females than by the quantities present in the body.

Key Words Adsorbent · Age · Gas chromatography-mass spectrometry · Mating · Miridae · Polydimethylsiloxane (PDMS)-coated stir bar · Sex pheromone · *Stenotus rubrovittatus*, Heteroptera

Introduction

Females of many insect species produce sex pheromones that attract conspecific males. However, female sex pheromone levels vary with age (Raina et al. 1986; Babilis and Mazomenos 1992; Tang et al. 1992; Delisle and Royer 1994; del Mazo-Cancino et al. 2004) and decline after mating (Raina et al. 1986; Giebultowicz et al. 1991; Ramaswamy and Cohen 1992; Babilis and Mazomenos 1992; Tang et al. 1992; del Mazo-Cancino et al. 2004). Accordingly, male responses to females should change with female age and mating status (Shu et al. 1998; Fukuyama et al. 2007; McClure et al. 2007).

In the sorghum plant bug, *Stenotus rubrovittatus* Matsumura (Heteroptera: Miridae), males are attracted to females (Okutani-Akamatsu et al. 2007). Males of *S. rubrovittatus* are caught in traps baited with young virgin females more frequently than in traps baited with old virgin females, and are rarely caught in traps baited with mated females (Okutani-Akamatsu et al. 2007). Therefore, we hypothesized that young virgin females have higher levels of sex pheromones than old virgin females and that virgin females have higher levels than mated females of the same age. In

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K. Oku (✉) · T. Yasuda
National Agricultural Research Center,
National Agriculture and Food Research Organization,
Kannondai 3-1-1,
Tsukuba, Ibaraki 305-8666, Japan
e-mail: okeiko@affrc.go.jp

S. rubrovittatus, hexyl butyrate, (*E*)-hex-2-en-1-yl butyrate, and (*E*)-4-oxohex-2-enal have been identified as sex pheromone components (Yasuda et al. 2008). In this study, we examined the effects of age and mating status on female sex pheromone levels and discuss relationships between female sex pheromones and male responses to females in *S. rubrovittatus*.

Methods and Materials

Insects Adult *S. rubrovittatus* were collected from gramineous fields in the National Agricultural Research Center, Tsukuba, Japan (36°01′ N, 140°06′ E), on May 26, and June 2, 2008 and were permitted to lay eggs on millet, *Setaria italica*, seedlings at 25°C under conditions of 16:8h, L:D (light phase, 6:00–22:00; hereafter called "laboratory conditions"). Newly hatched nymphs were transferred onto wheat, *Triticum aestivum*, seedlings and kept under laboratory conditions. In this way, *S. rubrovittatus* was reared for several generations under laboratory conditions. Fifth-instar nymphs were isolated in vials (50 ml), each containing five wheat seedlings. Each vial was covered with a sheet of gauze to prevent the nymphs from escaping. The cultures of *S. rubrovittatus* were checked for adults every morning. Emerged adults were used for the following experiments.

Effects of Age and Mating on Female Sex Pheromone Levels—Whole Body Samples To determine whether age and mating affect female sex pheromone levels, 297 virgin females and 158 unmated males (3 d after emergence) were collected from the culture. Each female was placed in a glass vial (50 ml) with five wheat seedlings. Each vial was covered with a sheet of gauze. Then, one male was introduced at 10:00 a.m. into each of 158 vials to mate with the female; the rest of the vials (N=139) contained females that were not allowed to mate (= virgin females). We observed each male-female pair for 1 hr to determine whether they mated. A total of 124 females mated with the male during the 1-hr observation period (mated females, N=124). After mating, males were removed from vials. The 34 females that did not mate within 1 hr were excluded from this experiment. The females were kept for 0, 1, 3, 5, 10, or 15 d (i.e., 3, 4, 6, 8, 13, or 18 d after emergence) under laboratory conditions. The wheat seedlings were replaced every 5 d.

To extract sex pheromone components from female bodies, one female was dipped in 1 ml of hexane with heptadecane (10 μg) for 5 min at room temperature. Heptadecane was used as an internal standard to calculate relative values of each component. Then, the extract was transferred into a glass vial (2 ml) and stored at −20°C until gas chromatography-mass spectrometry (GC-MS) analyses were performed. All extractions were conducted between 11:00 a.m. and 12:00 p.m.

Do Females Release Sex Pheromones? The experimental setup (Fig. 1) for trapping female sex pheromone components from the volatile headspace consisted of a glass tube with a side arm and black screw caps with Teflon®-faced rubber liners. A stir bar coated with polydimethylsiloxane (Twister™, GERSTEL GmbH & Co. KG, Germany, film thickness 1 mm, length 10 mm) was used as an adsorbent (Bicchi et al. 2005) to collect the compounds released from females. To compare relative levels of sex pheromone released by virgin females with those released by mated females, 24 virgin females and 20 mated females (4 d after emergence) were introduced separately into the experimental setup between 2:00 p.m. and 3:00 p.m. and were kept for 1 d under laboratory conditions. Then, the stir bars were removed from each of the experimental chambers. Relative levels of sex pheromone released were also compared between young (3-d-old, N=23) and old (8-d-old, N=23) virgin females. Their sex pheromone components also were collected by using the procedure described above.

To extract sex pheromone components from the stir bars, each stir bar was soaked in 1 ml of hexane with heptadecane (5 μg) as an internal standard. The bars were stirred while soaking on a magnetic stirrer for 30 min at room temperature. The extract was transferred into a glass vial (12 ml). Then, the stir bar was soaked again in 1 ml of hexane and stirred for another 30 min. The second extract was added to the glass vial containing the first extract. The extract was evaporated at 30°C until the volume was reduced to approximately 1 ml. The concentrate was

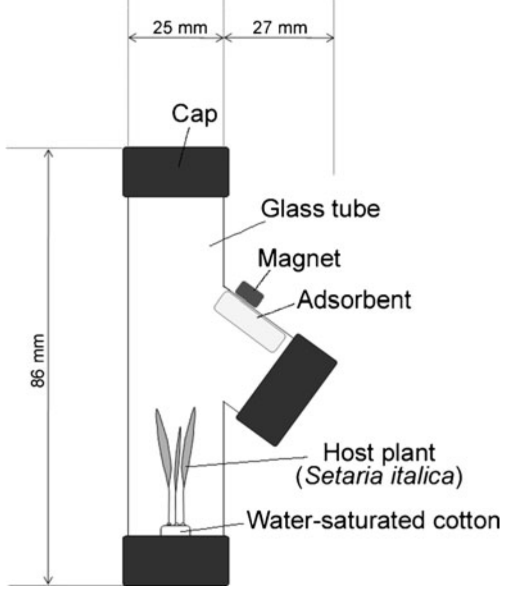

Fig. 1 Experimental setup to collect sex pheromone components released from female *Stenotus rubrovittatus*

transferred into a glass vial (2 ml) and stored at −20°C until GC-MS analyses were performed.

Chemicals Hexyl butyrate (>98.0% chemical purity) and (*E*)-hex-2-en-1-yl butyrate (>95.0%) were purchased from Tokyo Chemical Industry Co., Ltd., Japan. (*E*)-4-Oxohex-2-enal (96.9%) was obtained from the Shin-Etsu Chemical Co. Ltd, Japan.

GC-MS Analysis To determine the quantity of sex pheromone components in each female, GC-MS analysis was performed on an Agilent 6890N GC with an HP-INNOWax column (30 m length×0.25 mm internal diam×0.25 μm film thickness) by splitless injection combined with an Agilent 5975 Network Mass Selective Detector. Mass spectrometric data were acquired by continually alternating between full scanning (range: *m/z* 35–350) and selected ion monitoring (SIM) modes. The quantitative and reference ions for SIM were *m/z* 240 and 99 for heptadecane, *m/z* 129 and 89 for hexyl butyrate, *m/z* 170 and 100 for (*E*)-hex-2-en-1-yl butyrate, and *m/z* 112 and 83 for (*E*)-4-oxohex-2-enal, respectively. Injection temperature was 230°C. Helium was used as carrier gas, and the flow rate was constant at 1.0 ml/min. The initial GC oven temperature was 50°C (2 min hold), increased to 240°C at 10°C/min, and then held for 5 min. The quantity of each sex pheromone component was estimated by using standard linear calibration curves obtained from authentic samples of hexyl butyrate, (*E*)-hex-2-en-1-yl butyrate, and (*E*)-4-oxohex-2-enal, analyzed together with the internal standard heptadecane (Supplemental Data Fig. 1). The absence of co-eluting compounds that might confound the analysis of each analyte and the standard was confirmed by examining mass spectra of each component in the analysis (Supplemental Data Fig. 2). This method has also been used to quantify pheromone components from another plant bug (Byers 2006).

Statistical Analysis To test for effects of age and mating status on total female sex pheromone levels, the quantities of the three sex pheromone components were summed. For the first experiment (whole body extraction), the data were analyzed by two-way analysis of variance (ANOVA) using age and mating as independent variables (Sokal and Rohlf 1995). When an interaction effect between effects of mating and age was significant at the 0.05 level, the data were reanalyzed within each group of females by one-way ANOVA with a Bonferroni correction to test for effects of age on female sex pheromone content (Bonferroni-corrected significance level, 0.025). Then, when the effect of age was significant at the 0.025 level, a Tukey-Kramer honestly significant difference (HSD) test with a Bonferroni correction was used to compare treatments within each group of females (Bonferroni-corrected significance level, 0.025). For the second experiment (headspace analysis), amounts of the female sex pheromone components adsorbed on the stir bars were compared between female groups by one-way ANOVA. JMP version 7.0.1 (SAS Institute 2007) was used for these analyses.

Table 1 Two-way ANOVA table from the test of the effects of age and mating on sex pheromone levels in female *Stenotus rubrovittatus*

Factor	*df*	Mean squares	*F*	*P*
Mating	1	1180.924	97.055	<0.001
Age	5	43.752	3.596	0.004
Mating × age	5	76.978	6.327	<0.001
Residual	224	12.168		

Table 2 Secondary data analysis of the effect of age on sex pheromone levels of *Stenotus rubrovittatus*, separated by mating status (one-way ANOVA)

Factor	*df*	Mean squares	*F*	*P*
Virgin female				
Age	1	72.881	9.209	<0.001
Residual	113	7.914		
Mated female				
Age	1	47.528	2.881	0.018
Residual	111	16.498		

Results

Effects of Age and Mating on Female Sex Pheromone Levels—Whole Body Samples Both age and mating affected sex pheromone levels extracted from female bodies (Table 1). Furthermore, a significant interaction effect was detected, indicating that the effects of age differed between virgin and mated female treatment groups. Hence, the effect of age on sex pheromone levels was tested within each group of females separately. As a consequence, age affected female sex pheromone levels within virgin as well as mated female treatment groups (Table 2). In virgin females (Fig. 2a), female sex pheromone level decreased with age. In contrast, sex pheromone levels in mated females (Fig. 2b) (mated 3 d after emergence), did not differ from those of virgin females at the 3-d time point ($F_{1,38}$=0.08, P=0.722; Fig. 2a, b). Subsequently, however, mated females (Fig. 2b) maintained sex pheromone components at a constant level.

Do Females Release Sex Pheromone? Although (*E*)-4-oxohex-2-enal was not detected from any of the females,

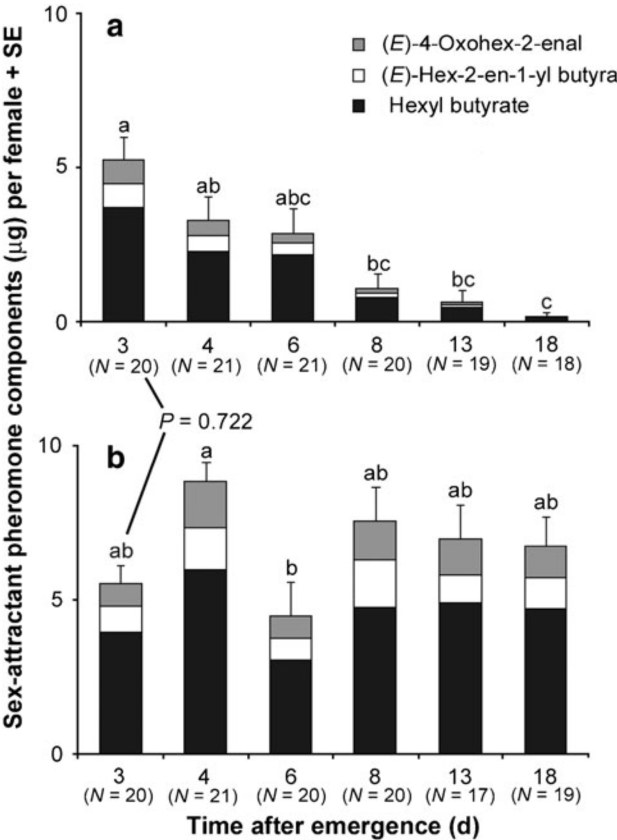

Fig. 2 Changes in the sex pheromone levels extracted from whole bodies of (**a**) virgin female *Stenotus rubrovittatus* and (**b**) mated females. *Letters* indicate significant differences between differently-aged females in each group (Tukey-Kramer HSD test with Bonferroni correction, α=0.025)

other sex pheromone components were detected from both virgin and mated females (Fig. 3). Adsorbed sex pheromone levels were higher in virgin females than in mated females ($F_{1,42}$=12.39, P=0.001; Fig. 3a). On the other hand, adsorbed amounts of sex pheromone components were higher in young virgin females than in old virgin females ($F_{1,44}$=7.313, P<0.01; Fig. 3b).

Discussion

Sex pheromone levels of *S. rubrovittatus* females were altered by both aging and mating. However, the results differed from our predictions in that virgin females have higher levels than mated females of the same age. We first determined the quantity of sex pheromone components of *S. rubrovittatus* females and found that sex pheromone levels decreased with age in virgin females, whereas mated females maintained higher levels. Males of *S. rubrovittatus* are more frequently attracted to young virgin females than to old virgin females and are rarely attracted to mated females (Okutani-Akamatsu et al. 2007). However, the changes in sex pheromone levels in mated female were not congruent with the reported changes in the male response to females. Hence, we determined the quantities of sex pheromone components released from *S. rubrovittatus* females using adsorbents. Adsorbed sex component levels were higher in virgin females than in mated females, although (*E*)-4-oxohex-2-enal was not detected from any of the females. Adsorbents do not always adsorb all of the compounds present in the air. The adsorbed amount of (*E*)-4-oxohex-2-enal might have been too low to detect. However, the amounts of the other compounds adsorbed within a given time should be comparable. Therefore, the result suggests that virgin females release greater amounts of sex pheromones than do mated females. Furthermore, adsorbed sex pheromone levels were higher for young virgin females than for old virgin females, suggesting that young virgins release more amounts of sex pheromones than do old virgins. From these results, it is likely that the levels of sex pheromones released from females affect the response of *S. rubrovittatus* males. Males of *S. rubrovittatus* would be attracted to females that release large amounts of sex pheromone components.

Generally, virgin females have sex pheromones, whereas mated females no longer produce them (Raina et al. 1986; Giebultowicz et al. 1991; Ramaswamy and Cohen 1992;

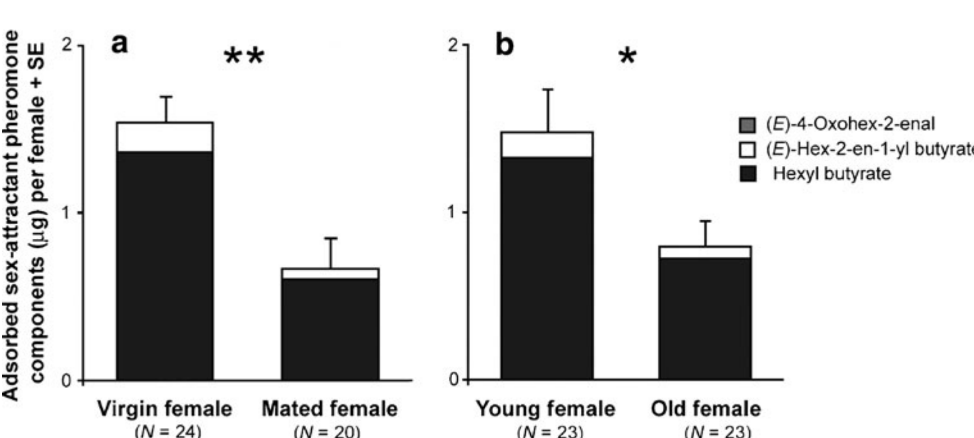

Fig. 3 Comparison of adsorbed sex pheromone levels between (**a**) virgin female *Stenotus rubrovittatus* and mated females of the same age and (**b**) young (3 d) virgin females and old (8 d) virgin females. * P<0.01, **P<0.005 (ANOVA)

Babilis and Mazomenos 1992; Tang et al. 1992; del Mazo-Cancino et al. 2004). In *S. rubrovittatus*, however, the amount of sex pheromone components did not decline after mating. Although it is possible for mated females to have increases in sex pheromone components when remating (Shu et al. 1998), mated *S. rubrovittatus* females normally do not re-mate (Okutani-Akamatsu et al. 2009; Kitsunezuka et al. unpublished data). The sex pheromone components of *S. rubrovittatus* might have other functions in mated females, but this remains to be studied.

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Regulation of Cell Division and Growth in Roots of *Lactuca sativa* L. Seedlings by the *Ent*-Kaurene Diterpenoid Rabdosin B

Lan Ding · Hongwei Jing · Bo Qin · Linlin Qi · Jing Li · Tao Wang · Guoan Liu

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Abstract Rabdosin B, an *ent*-kaurene diterpenoid purified from the air-dried aerial parts of *Isodon japonica* (Burm.f) Hara var. *galaucocalyx* (maxin) Hara, showed a biphasic, dose-dependent effect on root growth and a strong inhibitory effect on root hair development in lettuce seedlings (*Lactuca sativa* L.). Lower concentrations of rabdosin B (20–80 μM) significantly promoted root growth, but its higher levels at 120–200 μM, by contrast, had inhibitory effects. Additionally, all tested concentrations (10–40 μM) inhibited root hair development of seedlings in a dose-dependent manner. Further investigations on the underlying mechanism revealed that the promotion effect of rabdosin B at the lower concentrations resulted from increasing the cell length in the mature region and enhancing the mitotic activity of meristematic cells in seedlings' root tips. In contrast, rabdosin B at higher concentrations inhibited root growth by affecting both cell length in the mature region and division of meristematic cells. Comet assay and cell cycle analysis demonstrated that the decrease of mitotic activity of root meristematic cells was due to DNA damage induced cell cycle retardation of the G_2 phase and S phase at different times.

Lan Ding and Hongwei Jing—co-first-authors.

L. Ding (✉) · H. Jing · L. Qi · J. Li · T. Wang · G. Liu
College of Life Sciences, Northwest Normal University,
No. 967 Anning East Road,
Lanzhou 730070, People's Republic of China
e-mail: nwnudinglan@gmail.com

B. Qin
Key Laboratory for Natural Medicine of Gansu Province,
Lanzhou Institute of Chemical Physics,
Chinese Academy of Sciences,
Lanzhou 730000, People's Republic of China

Key Words *Ent*-kaurene diterpenoid · *Lactuca sativa* L. · Root growth · Cell cycle arrest · DNA damage · Root hair

Introduction

As the largest group of secondary metabolites in plants, terpenes show a wide spectrum of biological activities including allelopathic potential (Calera et al. 1995; Skaltsa et al. 2000; Cangiano et al. 2002; Macías et al. 2002). Numerous mono- and sesquiterpenes have been identified as allelochemicals and their modes of action have been investigated (Muller 1966; Rice 1984; Duke and Oliva 2002; Macías et al. 2004, 2007; Morimoto and Komai 2005; Nishida et al. 2005; Cantrell et al. 2007). However, there are fewer reports concerning the phytotoxic properties of diterpenoids (Macías et al. 1999, 2000). A clerodane diterpene, isolated from *Viguiera tucumanensis*, inhibited both germination and root growth of *Sorghum halepense* and *Chenopodium album* (Vaccarini et al. 1999). A series of diterpenes, primarily *ent*-labdane derivatives from *Potomageton natans* and *Ruppia maritime*, have been found to have toxicity to a number of animals and the alga *Selenastrum capricornornutum* (DellaGreca et al. 2004). The natural diterpenes from *Croton ciliatoglanduliferus* Ort. inhibit photophosphorylation, electron transport, and the partial reactions of both photosystems in spinach thylakoids (Morales-Flores et al. 2007). Macías et al. (2008) reported that helikauranoside A, a new *ent*-kaurane glycosylated diterpenoid isolated from the aerial parts of *Helianthus annuus* L., might be related to the allelopathic behavior shown by this species.

Isodon (formally *Rabdosia*) is a cosmopolitan and important genus of the Labiatae (Lamiaceae) family. *Ent*-kaurene diterpenoids are the main constituent in this genus

and are especially abundant in fresh branches and leaves (Sun et al. 2001). To date, over 400 *ent*-kaurene diterpenoids have been isolated from these plants and been reported to have antibacterial, anti-inflammatory, and antitumor activities (Sun et al. 2001). However, whether these chemical components mediate interactions in natural surroundings is still unknown. Rabdosin B (Fig. 1), a diterpenoid belonging to 6, 7-seco-*ent*-kaurene spirolactone type, was isolated as the most abundant compound from the aerial parts of *Isodon japonica* (Burm.f) Hara var. *galaucocalyx* (maxin) Hara obtained from Gansu, China (Liu et al. 2006). The phytotoxic effect and action modes of this diterpenoid have not yet been reported.

Seeding growth bioassays are a primary tool for determining phytotoxic activity (Inderjit and Dakshini 1995; Szabo 2000), and they may detect potential phytotoxic effects under controlled laboratory conditions (Reigosa and Pazos-Malvido 2007). The growth-limiting effects of various kinds of stress have been related to their effects on the regulation of elongation and cell division (Burssens et al. 2000; West et al. 2004; Peres et al. 2007). Hence, in our study, the elongation of cells in the mature region and the cell division of meristematic cells were both used to evaluate the effects of rabdosin B on root growth. We also used flow cytometry with cell synchronization (Blanco Fernández et al. 2001), together with the conventional determinations of the mitotic index to study the effects of rabdosin B on the cell cycle of root meristematic cells. Additionally, to elucidate further the mechanism of its phytotoxic effects, the extent of DNA damage induced by rabdosin B in roots was checked by using the comet assay or single cell gel electrophoresis (SCGE) assay (Gichner and Plewa 1998; Angelis et al. 1999; Tice et al. 2000), since it has been reported that cell cycle progression can be delayed in response to DNA stress (Cools and De Veylder 2008). The root hair is an extension of the root epidermis and is particularly important due to its many roles within the soil rhizosphere (Bertin et al. 2003). Hence, inhibition of root hair development also was used to evaluate the phytotoxicity of rabdosin B.

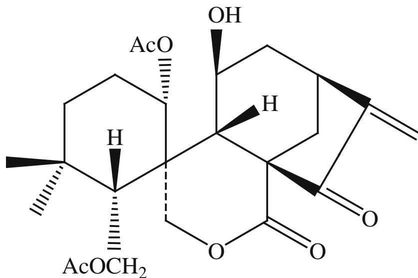


Fig. 1 Chemical structure of rabdosin B, an *ent*-kaurene diterpenoid isolated from the species of *Isodon*

The specific objectives of this study were: (1) to test the effects of rabdosin B on root growth and on root hair development of lettuce seedlings; (2) to establish a link among radicle growth, altered cell cycle, and the extent of DNA damage to reveal the action modes of rabdosin B.

Methods and Materials

Plant Materials and Chemicals Lettuce seeds (*Lactuca sativa* L. cv. Taiyuan) were obtained from Dadi Seed Service (Lanzhou, China). Rabdosin B was isolated previously in our laboratory from *Isodon japonica* (Burm. f) Hara var. *galaucocalyx* (maxin) Hara (Liu et al. 2006). Hydroxyurea (HU) and RNase A were purchased from BBI, USA. Propidium iodide, ethidium bromide (EtBr), normal melting point (NMP) and low melting point (LMP) agarose, were purchased from AMRESCO Inc. (Solon, OH, USA). All other general laboratory reagents were obtained from Sango Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Culture Conditions and Treatments Lettuce seeds were rinsed in tap water for 5 min, followed by rinsing × three in distilled water. About 500 seeds were placed into Petri dishes (15 cm diam) that contained two sheets of qualitative filter paper soaked with 13 ml distilled water. They were allowed to germinate for 48 h at 22°C and a photoperiod of 16:8 h L:D. Twelve uniform 2-d-old seedlings were transferred onto 6 cm Petri dishes containing filter paper moistened with different concentrations of treatment solutions (4 ml) and incubated under the same conditions.

Rabdosin B was dissolved in methanol and diluted to the desired concentrations with distilled water. The amount of methanol was uniform for each treatment and never exceeded 1%—i.e., below a level where root growth and root hair development were affected (data not shown).

Measurement of Net Growth Rates (NGR) Seedlings were cultivated as described above. After germination, 12 seedlings per 6 cm Petri dishes were incubated for 48 h. Net growth rates (NGR) were measured to evaluate the influence of rabdosin B on root growth according to the method of Pan et al. (2001). Seminal root lengths of the seedlings in each treatment were measured before and after treatment. NGR was calculated as follows: (final length—initial length)/initial length. Three parallel dishes were examined per treatment, and the experiment was repeated three times.

Measurement of Cell Sizes For determination of cell size, root tips (8 mm long) were excised from 2-d-old seedlings after treatment, fixed in an ethanol/acetic acid (9:1) solution overnight, followed by washing in 90%, 70%, 50%, and

30% ethanol, sequentially. Root tips were cleared with a chloral hydrate/glycerol/water solution (8:1:2, w/v/v) as described by Yadegari et al. (1994). Cells were observed under an optical microscope (Nikon Eclipse E 400) equipped with an ocular micrometer. Cells at the root-hair-forming region of the roots were chosen to determine the mature cell size. The length and width of 20 mature cells were measured from each root, with six roots used per treatment. Three independent experiments were carried out, giving the same statistically significant results.

Measurement of Mitotic Index Squash techniques were modified from Akinboro and Bakare (2007). Briefly, primary roots of lettuce seedlings after treated with rabdosin B were fixed in freshly prepared ethanol-acetic acid (3:1, v/v) for 24 h, then transferred into 70% ethanol and stored at 4°C until use. Roots were hydrolyzed for 8–10 min with 1 M HCl at 60°C followed by rinsing in distilled water (three to four times). Root tips (1 mm) were excised, stained with carbol fuchsin for 10–15 min, and the meristems were squashed under a coverslip to separate the cells. Three slides were prepared for each treatment, and at least 1,000 cells were examined randomly under a Nikon Eclipse E 400 optical microscope to determine the mitotic index, which was calculated as the number of dividing cells per 1,000 observed cells. Three replicate slides were examined per treatment, and the experiment was repeated three times.

Cell Cycle Analysis The cell cycle was analyzed according to the method of Sánchez-Moreiras et al. (2008) with a few modifications. In the present work, cell cycle of lettuce root meristems was synchronized by using the inhibitor hydroxyurea (HU), which reversibly halted the cell cycle in the G_1 and early S phase by inhibiting ribonucleotide reductase, and hence the production of deoxyribonucleotides (Doležěl et al. 1999).

Seedlings with roots 1 mm to 2 mm long were obtained by germinating seeds in the dark for 28 h on moistened filter paper at 22°C. Then, these seedlings were transferred, 50 per dish, to Petri dishes 7.5 cm diam containing filter paper moistened with 5 ml of 2.5 mM HU (pH 6.0). After 6 h incubation in dark at 25°C, seedlings were cleansed of HU by washing × three in distilled water (pH 6.0) to remove the inhibitor (Coba de la Peña and Sánchez-Moreiras 2001). Seedlings then were transferred to 7.5 cm Petri dishes containing filter papers moistened with 5 ml of either distilled water supplemented with methanol (pH 6.0, controls) or rabdosin B solution at a concentration of 200 μM (pH 6.0), and then incubated in the dark at 25°C. Samples were taken every 3 h and were processed as described below.

The apical root meristem (2±0.5 mm long) of each seedling was removed and chopped with a fresh razor blade on clean Petri dishes containing 600 μl Galbraith's nuclear buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% Triton X-100; pH 7.0), supplemented with 100% Tween-20 (8 μl in 3 ml buffer), 100% β-mercaptoethanol (7 μl in 1 ml buffer), and RNase A (100 μg/ml). The obtained suspension was filtered twice through 30 μm mesh stainless-steel cell filters, and the filtrate (450 μl) was collected in Eppendorf tubes. Immediately, the nuclei suspension was centrifuged at 100×*g* for 5 min, and the supernatant was decanted and the nuclei pellet resuspended in 200 μl chopping buffer. After resuspension, the solution was stained with propidium iodide (5 μg/ml) for 5–8 min at room temperature before cell DNA contents were evaluated by Epics XL-4 flow cytometry (BECKMAN-Coulter, USA) using a Coulter Elite apparatus with a 488 nm excitation laser. For each sample, at least 10,000 nuclei were analyzed. Histogram profiles were calculated from DNA content histograms using Cellquest software (version 2).

Comet Assay Isolation of nuclei from root tips and comet assay were performed following the method described by Gichner et al. (2004, 2008) with slight modifications. After incubated for 24 h and 48 h, 80-seedlings of each treatment were used for the nuclei isolation and comet assay. The apical 2±0.5 mm long root tip of each seedling was removed and placed in a Petri dish kept on ice and spread with 250 μl of cold 400 mM Tris buffer (pH 7.5). Using a fresh razor blade, the root tips were gently sliced. The plate was kept tilted in the ice so that the isolated root nuclei would be collected in the buffer. Immediately, the obtained suspension was filtered twice through 30 μm mesh stainless-steel cell filters, and the filtrate (200 μl) was collected in Eppendorf tubes. All operations were conducted under dim or yellow light.

The frosted microscope slides were coated with 0.5% normal melting point (NMP) agarose prepared with water at 50°C, dried overnight at room temperature, and kept dry in slide boxes until use. Nuclear suspension (50 μl) and 1% low melting point (LMP) agarose (50 μl) prepared with phosphate-buffered saline were added at 40°C onto each slide. The nuclei and the LMP agarose were mixed gently by repeated pipetting with a cut micropipet tip, and a coverslip was placed on the mixture. The slides were kept at 4°C for 15 min for the gel agarose to solidify. After removing the cover glass, slides were placed in a horizontal gel-electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH>13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.74 V/cm (25 V, 300 mA) for 25 min at 4°C. After electrophoresis, slides were rinsed × three with 400 mM Tris buffer, pH 7.5, stained with 80 μl EtBr (20 μg/ml) for

5 min, dipped in ice-cold water to remove the excessive stain, and covered with a coverslip. Nuclei were analyzed under a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm.

Digital images were captured and analyzed using CASP image-analysis program (Końca et al. 2003). Twenty-five randomly chosen nuclei were analyzed for each slide. Three slides were evaluated per treatment, and each treatment was repeated three times. From the repeated experiments, the averaged median percentage of tail DNA (% tail DNA) and Olive tail moment [OTM, the product of the distance between the gravity centers of the DNA head and the DNA tail ($LX_{Gravity}$) and percent tail DNA ($Tail_{DNA}$), OTM= $LX_{Gravity} \times Tail_{DNA}$, Liu et al. 2004] were used to measure DNA damage for each treatment group.

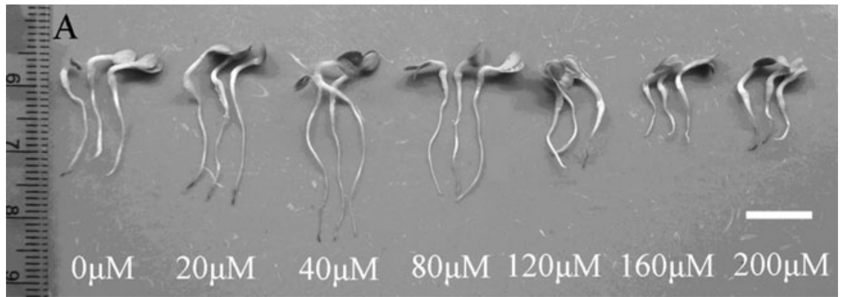
Measurements of Root Hairs Root hairs were evaluated with slight modifications of the method of Yang et al. (2004). Seedlings (ten) were randomly selected from each treatment. A 1 cm root segment was excised from 0.5 cm behind the root tip, fixed in 70% ethanol, placed onto a microscope slide, and covered by a glass cover for microscopic observation. The length and number of root hairs present in certain regions (424 μm root segment) were measured (×3) from the ends and the middle of root segments with an ocular micrometer. Measurements for each treatment were determined from the ten root segments, and mean values were calculated. The experiment was repeated three times.

Statistical Analysis All data are presented as means±SD. Data were subjected to analysis of variance (ANOVA) with significant differences among means identified by LSD multiple range tests using SPSS 11.5. Differences were considered significant at $P<0.05$. To verify the relationship between pairs of data (root NGR/cells length; root NGR/mitotic index; mitotic index/prophase index) at the same rabdosin B concentrations, linear regression analysis was performed, and the coefficient of regression (r) and its level of significance (P) was determined.

Results

Effects of Rabdosin B on Seedling Growth The roots of lettuce seedlings responded to different concentrations of rabdosin B in a dose-dependent, biphasic manner (Fig. 2a). Lower levels of rabdosin B (20–80 μM) significantly promoted the root growth, and the root NGR reached a maximum at 40 μM. Inhibitory effects were observed at high concentrations of rabdosin B (120–200 μM). At all concentrations above 120 μM, the NGR of seedlings decreased, but the NGR of 160 μM and 200 μM rabdosin B treated seedlings remained, respectively, at about 42% and 43% of the control, which displayed a similar level of inhibition (Fig. 2b).

Effects of Rabdosin B on Elongation of Root Cells To evaluate the impact of rabdosin B on cell elongation, we measured the size of mature cells at the root-hair-forming regions of lettuce roots. Rabdosin B affected the cell length in a pattern that was similar to the NGR (Fig. 3). Regression analysis revealed a positive correlation between NGR and cell length ($r=0.992$, $P<0.001$). Lower levels of rabdosin B (20–80 μM) significantly increased the cell length, but



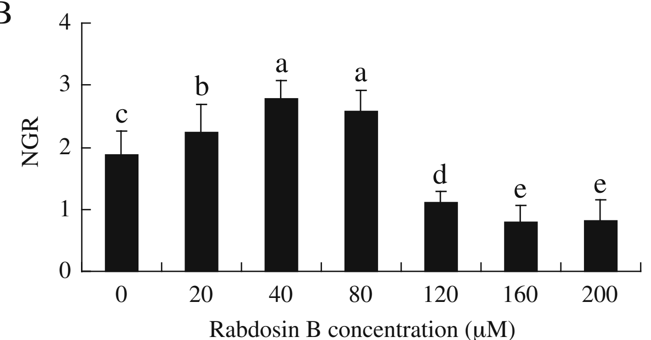


Fig. 2 Effect of rabdosin B on *Lactuca sativa* seedling growth. **a** Effects of increasing concentrations of rabdosin B on the root growth of lettuce seedlings 48 h after treatment. **b** Root NGR in lettuce seedlings calculated after 48 h of treatment. Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD. *Bar*=1 cm

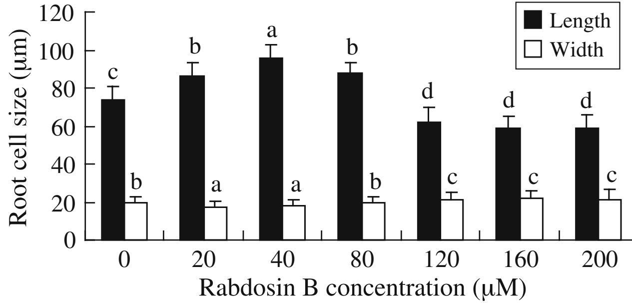


Fig. 3 Effect of rabdosin B on the mature cell sizes (length and width) in lettuce roots 48 h after treatment. Each datum represents average value from 120 cells. Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD

decreased the cell width except at 80 μM. In contrast, after treatment with higher levels of rabdosin B (120–200 μM), the cell length was reduced significantly, but the cell width was increased (Fig. 3).

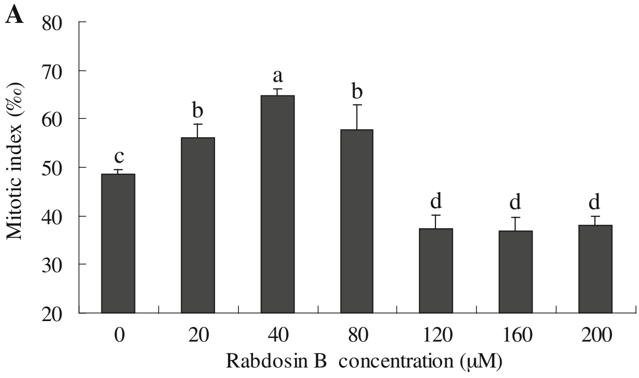
Effects of Rabdosin B on Mitotic Activity Different concentrations of rabdosin B affected the mitotic index in a pattern that was similar to the NGR ($r=0.989$, $P<0.001$) (Fig. 4a). The mitotic index for root tip cells treated with lower levels of rabdosin B (20–80 μM) significantly increased compared with the control, but at the higher concentrations 120–200 μM, by contrast, the mitotic index decreased. In addition, rabdosin B affected the relative proportion of the various stages of mitosis in meristematic cells with different stages showing different sensitivity to rabdosin B (Fig. 4b). The number of meristematic cells in prophase was analogous to the mitotic index ($r=0.994$, $P<0.001$). However, the percentage of the other phases did not change significantly compared to the control.

Cell Cycle Analysis To determine whether the reduction of prophase treated with 200 μM rabdosin B involved cell cycle changes at some earlier stages, we examined cell cycle phase distribution of the root tip cells by flow cytometry. The inhibitor hydroxyurea (HU) was used to increase the number of mitotic cells and to prove a clear effect of rabdosin B on the cell cycle.

Figure 5a shows DNA content histograms of non-rabdosin B and rabdosin B-treated root tips. The proportion of cells in the S phase of rabdosin B-treated root tips was similar to that of control 3 h after removal of HU, but cells in the G_2 phase were double that of the control. However, at 6 h, the proportion of control cells in phase S was 14.7%, and that of rabdosin B-treated cells was 23.2% (Fig. 5a), while G_2 phase of the treatment group was triple that of the control. Over the next 6 h, the S population of control cells fell steadily to a much lower level between *5.2%* and 6.5%, but the fall in the rabdosin B-treated S phase population was much less pronounced. Instead, the G_2 phase population was similar to that of the control. Cell cycle progression was so slow in rabdosin B treated meristems that the number of cells in S phase was triple that of control meristems after 9 h and 12 h treatments. Figure 5b shows that the S-phase proportion of control cells significantly decreased from 3 h to 12 h, concurrent with a gradually increased G_2-phase proportion. However, after treatment with 200 μM rabdosin B, S-phase and G_2-phase proportions remained at a relative stable level. These results indicate that the root tip cells treated with 200 μM rabdosin B were first arrested in G_2 phase from 3 h to 6 h, and then the cells were arrested in S phase from 9 h to 12 h.

Since flow cytometry could not measure mitotic events, we measured the mitotic index at the same time that the cell cycle was analyzed (3–12 h after removal of HU). Figure 5c showed that the mitotic index of control root tips was only 13‰ 3 h after removal of HU, peaked sharply at 99‰ at 6 h, and by 9 h returned again to 58‰. The mitotic index peak of rabdosin B-treated root tips was lower (75‰), later (at 9 h) and broader (6–12 h).

DNA Damage in Root Nuclei Using Comet Assay To elucidate whether the cell cycle arrest of root tips involved DNA damage, DNA strand breaks were assessed by comet



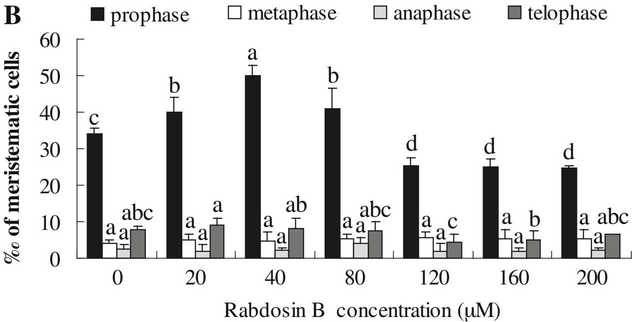


Fig. 4 Effect of rabdosin B on the mitotic activity of lettuce seedling root apical meristem cells after treatment (48 h). **a** The mitotic index of root apical cells; **b** percentage of different mitotic phases calculated relative to the total number of meristematic cells. Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD

Fig. 5 Effect of rabdosin B on cell cycle of lettuce root tips. **a** Flow-cytometric DNA content histograms of Hydroxyurea (HU)-synchronized lettuce root tips 3 h, 6 h, 9 h, and 12 h after removal of HU and transfer to distilled water supplemented with methanol (controls) or 200 μM rabdosin B solution. **b** Time course of the cell-cycle distribution in root tip cells after the treatment. **c** Mitotic index of HU-synchronized lettuce root tips at the same time that cell cycle was measured (from 3 h to 12 h) after removal of HU. Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD

A

G1: 76.5 %
S: 19.0 %
G2: 4.5 %

Control (3 h after HU release)

G1: 68.8 %
S: 21.6 %
G2: 9.5 %

Rabdosin B (3 h after HU release)

G1: 82.0 %
S: 14.7 %
G2: 3.3 %

Control (6 h after HU release)

G1: 66.0 %
S: 23.2 %
G2: 10.8 %

Rabdosin B (6 h after HU release)

G1: 85.1 %
S: 5.2 %
G2: 9.7 %

Control (9 h after HU release)

G1: 70.1 %
S: 17.1 %
G2: 12.8 %

Rabdosin B (9 h after HU release)

G1: 81.3 %
S: 6.5 %
G2: 12.2 %

Control (12 h after HU release)

G1: 69.9 %
S: 18.6 %
G2: 11.5 %

Rabdosin B (12 h after HU release)

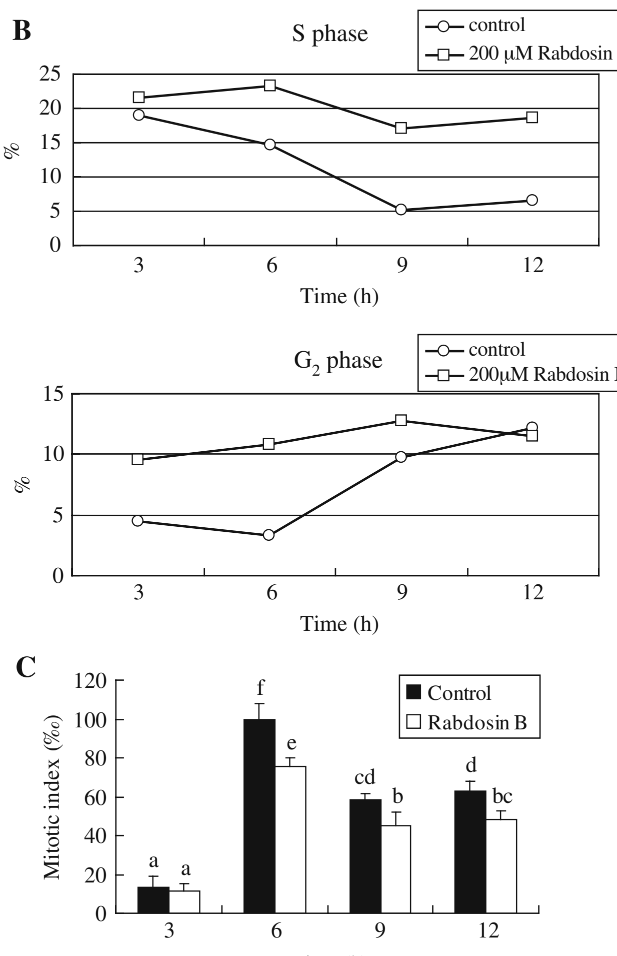
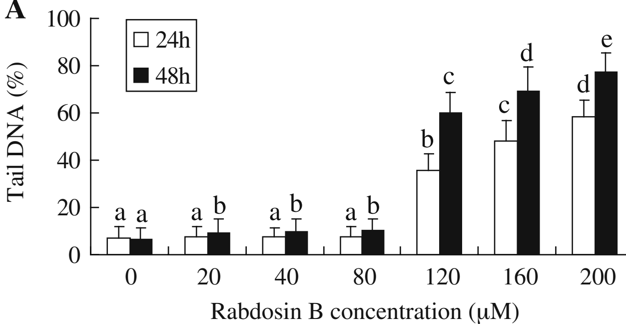


Fig. 5 (continued)

assay (Fig. 6). Figure 7 shows a time- and dose-dependent increase in DNA damage in the form of % tail DNA values and averaged median OTM values by 120–200 μM rabdosin B.

Just after the treatment with rabdosin B (120–200 μM) for 24 h, the data of comet assay show a significant increase in averaged median % tail DNA values from 35.4±7.5% to 58.6±6.7%, and also in averaged median OTM values from 12.0±3.2 to 24.8±4.8 pixels. There was no significant



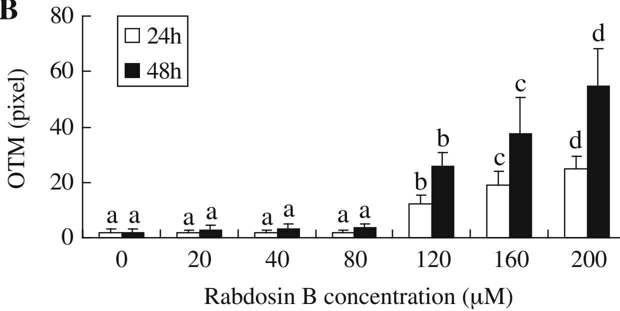


Fig. 7 Effect of rabdosin B on the DNA damage of lettuce roots after treatment for 24 h and 48 h. **a** The average median % tail DNA; **b** the average median Olive tail moment (OTM). Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD

difference in either % tail DNA or OTM at the lower levels of rabdosin B (20–80 μM) (Fig. 7).

When the cells were treated with rabdosin B (120–200 μM) for 48 h, both % tail DNA and OTM increased in a dose-dependent manner, and they nearly reached 77.5±8.0% and 54.7±13.7 pixels, respectively, as shown in Fig. 7. After treatment with lower levels of rabdosin B (20–80 μM) for 48 h, although the % tail DNA were significantly higher compared with the control, the values were very low, and raised only from 9.1±6.1% to 10.2±5.2%. Moreover, there was no significant difference in OTM values.

Effect of Rabdosin B on Root Hair Development Root hair development in lettuce seedlings (length and density) was significantly inhibited by rabdosin B after treatment for

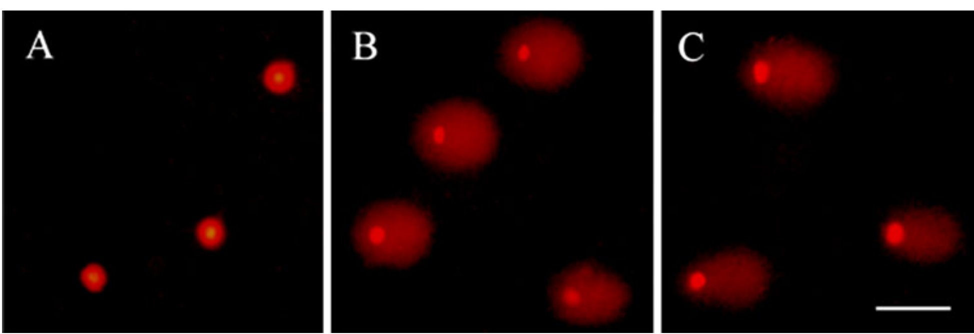


Fig. 6 Comet character showing tails of different length induced by rabdosin B in nuclei of lettuce root cells for a period of 24 h in order to determine the dose response for DNA damage: control (**a**), treated with rabdosin B 120 μM (**b**) and 200 μM (**c**). *Bar*=50 μm

48 h (Fig. 8). The diterpenoid decreased the average root hair length and root hair density in a dose-dependent manner at the concentrations between 10–40 μM, with a larger effect on root hair length than on root hair density (Fig. 9). Only a few bulges were observed on root surfaces when rabdosin B concentrations were raised to 40 μM (Fig. 8).

Discussion

In this study, both significant promotion and inhibition of root growth in lettuce seedlings were observed at the different concentrations of rabdosin B (20–200 μM). Root hair development also was inhibited significantly by rabdosin B in a dose-dependent manner at all of the tested concentrations (10–40 μM). Plant roots, as the first plant organ in contact with the soil, serve a multitude of functions in the plant including anchorage, provision of nutrients and water, and production of exudates with growth regulatory properties (Bertin et al. 2003). Root hairs are tubular outgrowths on the root surface that play an important role in increasing the root's ability to capture nutrients (Forde and Lorenzo 2001). Based on our study, we hypothesize that the abundant *ent*-kaurene diterpenoid might play a critical role in mediating ecological interactions between *Isodon* and other plant species in nature.

Root growth arises from the proliferation of meristematic cells followed by cell expansion that results in root elongation. Lettuce seedlings treated with higher levels of rabdosin B experienced a reduction in root NGR that is strongly correlated with both the changes of cell sizes in the mature region and the decrease of the mitotic index of root meristematic cells. Additionally, the number of meristematic cells in prophase was correlated with the mitotic index, while the percentages of other phases were less affected. Hence, we hypothesized that it depends on the general mechanism that arrests the cell cycle at earlier stages. To test this directly, we performed flow cytometry with nuclei derived from the root tips of seedlings. Flow cytometry analyses showed a significant retardation of the cell cycle in rabdosin B-treated meristems with selective activity at G_2/M and S/G_2 checkpoints. These data are consistent with the supposition that 200 μM

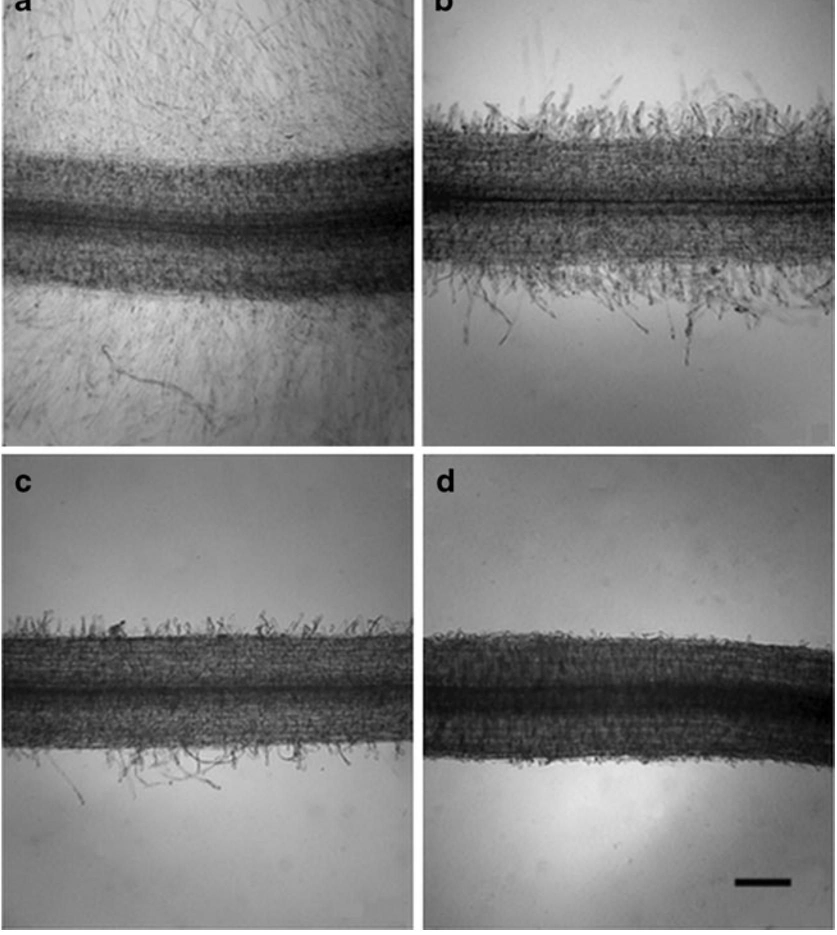
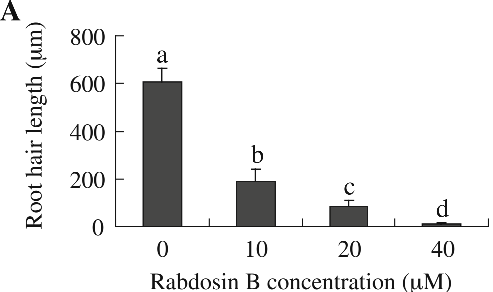


Fig. 8 Light micrographs of lettuce roots showing the inhibitory effects of rabdosin B on root hair development 48 h after treatment. **a** Control; **b**–**d** treatments with 10 μM, 20 μM, and 40 μM rabdosin B, respectively. *Bar*=200 μm



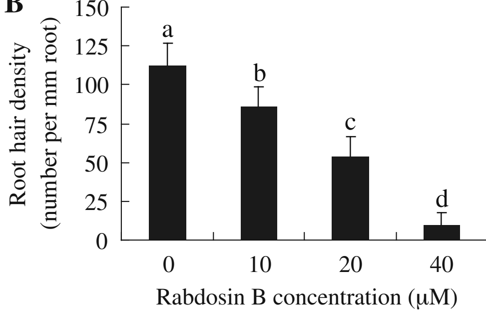


Fig. 9 Influence of rabdosin B on root hair development in lettuce seedlings 48 h after treatment. Root hair development was evaluated by average root hair length (**a**) and root hair density (**b**). Different letters indicate significant differences at $P<0.05$. *Bars* indicate SD

rabdosin B treated root tips experience a cell cycle arrest in the measure G_2 and S phase at different times. The mitotic index results, measured at the same time that the cell cycle was measured (3–12 h after removal of HU),confirmed the flow cytometry results.

Upon induction of DNA damage or after alteration of intracellular redox homeostasis, the cell cycle slows down at the G_1/S and G_2/M transition points (Den Boer and Murray 2000). Hefner et al. (2006) reported that DNA damage-induced cell cycle checkpoints could be initiated in cells of the meristematic organs (root and shoot tip) of irradiated *Arabidopsis* seedlings. In our study, we used the comet assay to determine whether rabdosin B could induce DNA damage in the root tips of lettuce seedlings, and to elucidate the mechanism of the cell cycle arrest. It was shown clearly that higher concentrations of rabdosin B induced a time- and dose-dependent increase in DNA damage in the form of % tail DNA and OTM when compared with control. We confirmed that the DNA damage of root tips could lead to cell cycle retardation. When DNA damage takes place in meristems, plants apparently have evolved DNA stress checkpoint mechanisms that arrest the cell cycle and activate the DNA repair machinery to preserve the genome (Cools and De Veylder. 2008). Cell cycle arrest as a consequence of the retardation of DNA replication and the delay of the start of mitosis also has been associated with an inhibition of the activity of cyclin-dependent kinases, cell cycle gene expression, and a concomitant activation of stress genes (Reichheld et al. 1999; De Veylder et al. 2007; Peres et al. 2007). When DNA stress checkpoints are activated, the induction of DNA-repair genes and the inactivation of genes required for mitosis switch on. This signaling cascade after biotic or abiotic stress affects plant metabolism and "stimulates cell cycle checkpoints, resulting impaired G_1 to S transition, a slowing down of DNA replication, and a delayed entry into mitosis" (Peres et al. 2007).

Our study confirmed that DNA damage-induced cell cycle arrest was initiated in lettuce root tips after treatment with higher concentrations of rabdosin B (200 μM). Interestingly, the roots responded to rabdosin B concentrations in a biphasic way. Concentrations of rabdosin B lower than 80 μM showed a stimulatory effect on root growth, and rabdosin B (20–80 μM) increased the cell length but reduced the cell width, while the mitotic index for root tip cells treated with rabdosin B (20–80 μM) increased compared to the control. An increase in the number of cells in prophase followed a pattern that was similar to the mitotic index. Thus, we conclude that 20–80 μM rabdosin B promotes the root growth in lettuce seedlings by affecting both cell size and cell division.

Examples of hormesis (the stimulation effect of a toxin at low doses) with natural phytotoxins have been reported with single compounds (e.g., gramine and hordenine, benzoxazolin- 2(3*H*)-one, and allyl isothiocyanate), plant extracts (e.g., *Triticum aestivum* L.), and root exudates (e.g., *T. aestivum* and *Triticum spelta* L.) (An et al. 1993; Belz et al. 2005, 2007). Belz (2008) reported that hormetic effects might occur in a natural setting if doses released are low, and they should be regarded as a potential low dose component of plant/plant interference. Research will be needed in order to understand the ecological conditions necessary for hormesis and the potential impacts of low dose stimulation by the diterpenoid.

Parker et al. (2000) reported that root hairs contribute as much as 77% to the total root surface area in cultivated crops, being the major point of contact between plants and the rhizosphere. Our results indicate that rabdosin B strongly inhibits root hair development in a dose-dependent manner. Ethylene plays a role in root hair initiation and elongation (Dolan 2001; Yang et al. 2004; Dugardeyn and Van Der Straeten 2008). Our previous study proposed that leukamenin E (a C-20-non-oxygenated-*ent*-kaurene diterpenoid) might act as a potential ethylene action antagonist in the inhibition of lettuce root hair development (Ding et al. 2008). Therefore, we hypothesize that rabdosin B inhibits root hair development similar to that of leukamenin E through this mechanism of action, as a potential ethylene action antagonist. More studies are needed to confirm this hypothesis.

In summary, this study demonstrated the dual stimulatory and inhibitory effects of rabdosin B on root growth and inhibitory effects on root hair development in lettuce

seedlings, and has provided some possible explanatory mechanisms for this action.

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Workers Make the Queens in *Melipona* Bees: Identification of Geraniol as a Caste Determining Compound from Labial Glands of Nurse Bees

Stefan Jarau · Johan W. van Veen · Robert Twele · Christian Reichle · Eduardo Herrera Gonzales · Ingrid Aguilar · Wittko Francke · Manfred Ayasse

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Abstract Reproductive division of labor in advanced eusocial honey bees and stingless bees is based on the ability of totipotent female larvae to develop into either workers or queens. In nearly all species, caste is determined by larval nutrition. However, the mechanism that triggers queen development in *Melipona* bees is still unresolved. Several hypotheses have been proposed, ranging from the proximate (a genetic determination of caste development) to the ultimate (a model in which larvae have complete control over their own caste fate). Here, we showed that the addition of geraniol, the main compound in labial gland secretions of nurse workers, to the larval food significantly increases the number of larvae that develop into queens. Interestingly, the proportion of queens in treated brood exactly matched the value (25%) predicted by the two-locus, two-allele model of genetic queen determination, in which only females that are heterozygous at both loci are capable of developing into queens. We conclude that labial gland secretions, added to the food of some cells by nurse bees, trigger queen development, provided that the larvae are genetically predisposed towards this developmental pathway. In *Melipona beecheii,* geraniol acts as a primer pheromone representing the first caste determination substance identified to date.

Key Words Social insects · Stingless bees · *Melipona* · Caste determination · Larval provision · Labial gland secretions · Primer pheromone · Geraniol · Apidae · Hymenoptera

S. Jarau (✉) · C. Reichle · M. Ayasse
Institute for Experimental Ecology, Ulm University,
Albert-Einstein-Allee 11,
89069 Ulm, Germany
e-mail: stefan.jarau@uni-ulm.de

J. W. van Veen · E. H. Gonzales · I. Aguilar
Center for Tropical Bee Research,
National University of Costa Rica,
PO Box 475-3000, Heredia, Costa Rica

R. Twele · W. Francke
Institute for Organic Chemistry, Hamburg University,
Martin-Luther-King Platz 6,
20146 Hamburg, Germany

Introduction

Female larvae of the eusocial honey bees (Hymenoptera: Apidae: Apini) and stingless bees (Hymenoptera: Apidae: Meliponini) may develop into either an adult queen or a worker. In the vast majority of species, queens are reared in special cells, which are larger than worker cells, and receive more food, which is sometimes of special quality (de Wilde and Beetsma 1982; Wheeler 1986; Hartfelder et al. 2006). Thus, the caste fate of the larvae in these species is determined trophically, which means that an individual's nutritional history triggers endocrine signals, which are caste-specific modulations of juvenile hormone and ecdysteroid titers (Hartfelder and Emlen 2005; Hartfelder et al. 2006) that mediate the subsequent patterns of developmental differentiation (Wheeler 1986). However, queen production in bees of the genus *Melipona* is unique among social bees because brood cells that produce queens and workers are indistinguishable, and all larvae feed on similar amounts of food (Sakagami 1982). Furthermore, *Melipona* queens are reared in significant numbers all year round (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al.

2006). To explain the observation that up to 25% of female larvae in *Melipona* nests develop into queens, Kerr proposed a mechanistic, proximate model of genetic caste determination, based on two unlinked loci, each with two alleles, in which only double heterozygous females could develop into queens (Kerr 1948, 1950a, b) (the original assumption of 3 loci for some species was later dismissed; Kerr 1969). Since male bees are haploid, their gametes must have an equal set of alleles at each locus. Therefore, the female progeny of a single mated queen, which are produced from fertilized diploid eggs, separate into 25% queens that are heterozygous at both loci, and 75% workers that are homozygous at one or both loci (Kerr 1948, 1950a, b). However, usually the proportion of queens in *Melipona* broods is lower than 25% (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al. 2006). Thus, it was proposed that female larvae that are genetically queens also can develop into workers under sub-optimal food conditions (Kerr et al. 1966; Kerr 1969; Maciel-Silva and Kerr 1991; Velthuis and Sommeijer 1991). A different set of studies, based on theoretical considerations on caste conflict and colony kin structure, viewed the large numbers of queens in *Melipona* as support for the model of self determination of caste fate in social insects (Bourke and Ratnieks 1999; Ratnieks 2001; Wenseleers et al. 2003, 2004; Wenseleers and Ratnieks 2004). According to this evolutionary or ultimate model, a female larva that develops in a normal sized, mass provisioned brood cell has the ability to control her own caste fate (Bourke and Ratnieks 1999; Ratnieks and Helanterä 2009). Thus, when resources are equally available, she could become a queen instead of a worker, thus becoming the reproductive dominant rather than an altruistic worker rearing sisters. One of the assumptions of this model is that all larvae not only feed on the same amount of food, but also on food of the same quality. However, this assumption overlooks existing experimental evidence, which demonstrates that caste fate can be influenced by quantitative, and possibly qualitative, differences in nutrition (Kerr et al. 1966; Kerr 1969; Darchen and Delage-Darchen 1975; Maciel-Silva and Kerr 1991). Nevertheless, specific nutrients that influence the developmental pathway of a female *Melipona* larva have not been described.

In recent chemical analyses involving the species *Melipona beecheii*, we found striking similarities between the pattern of volatiles from labial gland secretions of nurse workers and that of the rectal waste of emerging queens (Jarau, unpublished data). This suggests, that nurse workers may specifically add labial gland secretions to the food provision of certain brood cells, thus inducing the development of queens. In the present study, we tested this hypothesis through structure elucidation of volatiles of labial gland secretions, as well as bioassays, in which synthetic samples of the identified compound were added to the food provisions of developing larvae.

Methods and Material

Study Site and Bee Colonies We used two colonies of *Melipona beecheii* BENNETT, 1831 (Hymenoptera: Apidae: Meliponini) that were kept in a dark room at the Center for Tropical Bee Research (CINAT) of the National University of Costa Rica in Heredia, Costa Rica (9°58′22″N, 84°07′45″W). The nests were connected to the outside via plastic tubes leading through the wall of the building. Colony 1 was used for the collection of bees for chemical analyses (March and April 2007), whereas colony 2 was used for the food manipulation bioassays (March to May 2009). All manipulations in the nests were done under red light without disturbance of the bees.

Chemical Analyses We extracted labial glands of workers that were engaged in the construction and provisioning of brood cells (nurse bees) as well as the hind gut (rectum) of both newly emerged queens and workers. To prepare the extracts, an individual was killed by freezing, the respective body part dissected under a stereo microscope and left in 200 μl pentane for 24 hr at room temperature. The volume of each extract was reduced to 30 μl, and an internal standard (n-undecane) was added to allow quantification of the compounds.

For quantitative analyses, we used a Hewlett Packard HP 5890 gas chromatograph (Series II, Palo Alto, CA, USA) equipped with a DB-5MS capillary column (30 m× 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA) and a flame ionization detector (FID), with hydrogen as carrier gas (2 ml/min constant linear flow rate). Injection of 1 μl extract (per sample) was done in the splitless mode with an initial temperature of 50°C. After 1 min, the splitter was opened and the temperature increased by 10°C/min until the oven reached 310°C. The final temperature was held for another 23 min.

For qualitative analyses, the samples were analyzed by using a combined Fisons Instruments GC 8000 series / MD 800 mass spectrometer (carrier gas: helium; column: DB-5MS, 30 m×0.25 mm i.d., 0.25 μm film thickness, J & W Scientific; electron impact: 70 eV). The temperature was initially 60°C for 5 min, then increased by 10°C/min to 300°C and held at this temperature for 30 min. Identification of compounds was based on comparisons of mass spectra with literature data (McLafferty and Stauffer 1989), and with mass spectra and retention times of authentic reference substances. Non-commercial esters were synthesized from the corresponding acid chlorides and alcohols

according to standard procedures. Mass spectra and NMR-data were in accordance with expected data (Francke et al. 2000).

Bioassays To test the effect of geraniol on larval development, we added 10 μg of geraniol (Sigma-Aldrich®) to the larval food provision of recently sealed brood cells by puncturing each treatment cell with a fine needle and injecting 0.01 μl (=10 μg; equivalent to the labial gland contents of 3–4 nurse bees) of geraniol using a 0.5 μl gastight® syringe (Hamilton, Switzerland). The larvae in the treated brood cells were left to develop in their normal environment within the nest for 7–8 wk. We then opened the treatment cells (N=158) and untreated control cells (N=212) from the same colony in order to determine the caste of each pupa. We compared the observed distribution of queens and workers in treated and untreated cells using a 2×2 *chi-square* analysis of contingency table. A Yates correction was applied to adjust the *chi-square* value.

Results

Chemical Analyses Chemical analyses revealed that the main component in labial gland secretions of nurse workers of *Melipona beecheii* was (E)-3,7-dimethyl-2,6-octadien-1-ol, also known as geraniol (2.7±0.9 μg, N=6). Geraniol also was found in rectal extracts collected from newly emerged queens (3.8±1.2 μg, N=6), but was absent from extracts collected from newly emerged workers (N=12; Fig. 1).

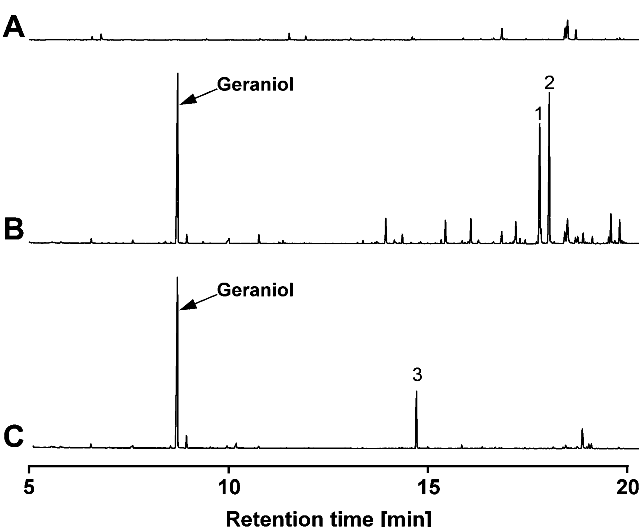


Fig. 1 Gas chromatograms of extracts from the rectum of a newly emerged *Melipona* worker (**A**) or a newly emerged virgin queen (**B**) and from the labial glands of a nurse bee (**C**). Peak height is a measure of abundance (flame ionization detector, A–C same scale). Additional compounds: *1*, (Z)-9-octadecenol; *2*, 1-octadecanol; *3*, geranyl hexanoate

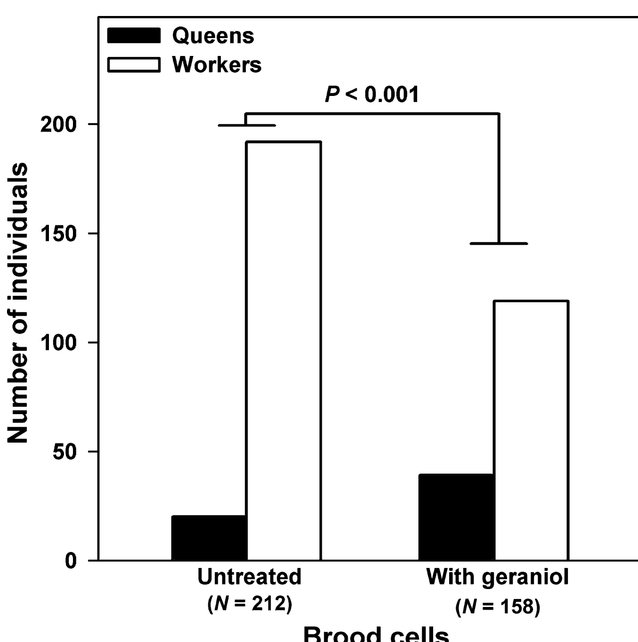


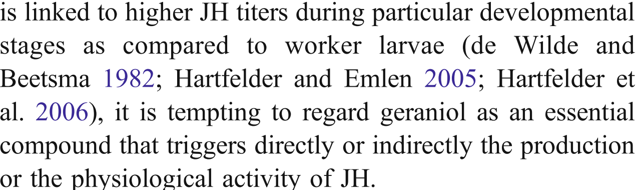
Fig. 2 Distribution of *Melipona* queens and workers among untreated brood and among brood that developed in cells to which 10 μg geraniol were added to the larval provision. 2×2 *Chi-square* analysis of contingency table, Yates correction, df=1, χ^2=14.590

Bioassays The holes in the cells made by injection of geraniol were closed by workers after the treatment, which demonstrates that they were accepted as normal brood cells. Naturally provisioned brood cells contained 116.6±9.4 mg (mean ± SD; N=14) of food. Thus, the addition of 10 μg of geraniol is far below the naturally occurring variation in food amount. We, therefore, assume that any observed effect of geraniol on larval development was caused by the qualitative rather than the quantitative alteration of the food. To test the effect of geraniol on larval development we compared the numbers of queens and workers in brood cells in which 10 μg of synthetic geraniol were added to the larval food provision, with the respective numbers in untreated brood cells from the same colony. The addition of geraniol to the larval food caused significantly more larvae to develop into queens as compared to untreated brood (geraniol: 39 queens, 119 workers; untreated: 20 queens, 192 workers; 2×2 *chi-square* analysis of contingency table, Yates corrected χ^2=14.590, df=1, P<0.001; Fig. 2). Furthermore, the proportion of larvae that developed into queens in geraniol treated brood cells perfectly matched the value (25%) predicted by Kerr's two-locus, two-allele model of genetic caste determination, whereas in untreated cells only 9% of the larvae developed into queens.

Discussion

The mechanism that triggers queen development in bees of the genus *Melipona* has remained unresolved to this day despite more than half a century of research and debate. In the present study, we identified geraniol, the main compound in labial gland secretions of nurse workers, as an exogenous caste determination factor in *M. beecheii*. The finding that in our bioassays 25% of the female brood in geraniol treated cells developed into queens corroborates the two-locus, two-allele model of genetic caste determination in *Melipona* proposed by Kerr (see Introduction). We, therefore, conclude that caste fate in *Melipona* is controlled both genetically and by a factor associated with larval provision: Female larvae that are genetically predisposed towards being queens only will follow this developmental pathway if they received sufficient amounts of a caste determining compound, which, in the case of *M. beecheii*, is geraniol. From the observation that the proportion of queens among larvae in *Melipona* nests is typically less than 25% (Kerr 1948; Darchen and Delage-Darchen 1975; Moo-Valle et al. 2001; Sommeijer et al. 2003; Wenseleers et al. 2004; Morais et al. 2006), we conclude that the concentration of geraniol usually is below this critical threshold in the majority of cells in nature. This indicates that nurse bees only add labial gland secretions to the normal provision (pollen, carbohydrates, hypopharyngeal gland secretions; Velthuis and Sommeijer 1991) in selected brood cells (we are currently investigating the distribution of geraniol among naturally provisioned brood cells). Thus, adult *Melipona* workers, which have little power to control the caste fate of developing larvae by limiting the *quantity* of their food, may exert suppression of queen development by limiting the access to a specific *qualitative* food factor—the primer pheromone geraniol. As a consequence, our results refute the assumption that *Melipona* larvae completely control their own caste fate (Bourke and Ratnieks 1999; Ratnieks 2001; Wenseleers et al. 2003; Wenseleers and Ratnieks 2004; Ratnieks and Helanterä 2009).

The high abundance of young queens in *Melipona* and, thus, the colony-level costs that are associated with the production of excess queens (Wenseleers and Ratnieks 2004), may well be a constraint imposed by its unique mechanism of caste determination. To answer this, detailed knowledge about the exact proximate mechanism by which geraniol acts at the molecular level is needed. It may function directly on gene regulation by triggering the expression of new genes or the repression of others (e.g., Schlichting and Pigliucci 1995; Nijhout 1999; West-Eberhard 2003). Geraniol may represent an essential precursor of a terpenoid compound exhibiting hormonal activity, such as juvenile hormone (JH), or, possibly, geranyl esters. Since queen development in eusocial bees



is linked to higher JH titers during particular developmental stages as compared to worker larvae (de Wilde and Beetsma 1982; Hartfelder and Emlen 2005; Hartfelder et al. 2006), it is tempting to regard geraniol as an essential compound that triggers directly or indirectly the production or the physiological activity of JH.

The impact of exogenous agents, including nutrition, on the development of an organism is well documented for a large variety of species (Gilbert and Epel 2009). However, to the best of our knowledge, geraniol is the first caste determination substance identified as a physiologically active signal for queen development from the larval food of a social insect. Moreover, geraniol is the only primer pheromone, as defined by Nordlund (1981), identified from an insect other than the honey bee (Le Conte and Hefetz 2008)—almost 50 years after the identification of 9-oxo-(*E*)-2-decenoic acid, the queen substance primer pheromone of honey bees (Butler et al. 1962). The impact of geraniol on an individual's physiology sheds new light on the versatility of this compound and its derivatives, which are generally regarded as typical releaser pheromones. It should be noted that a second major compound in the labial gland secretions of nurse bees of *Melipona beecheii* is geranyl hexanoate (Fig. 1), which also is widespread among solitary and other eusocial bees (Bergström 2008). Whether geranyl hexanoate represents a lipophilic storage form of geraniol, or shows physiological activities on its own, awaits further investigation.

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Identification and Field Activity of a Male-Produced Aggregation Pheromone in the Pine Sawyer Beetle, *Monochamus galloprovincialis*

Juan A. Pajares · Gonzalo Álvarez · Fernando Ibeas · Diego Gallego · David R. Hall · Dudley I. Farman

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Abstract The pine sawyer beetle, *Monochamus galloprovincialis*, is a pest of pine trees in Europe and North Africa. Previously considered a secondary pest of stressed and dying trees, it is now receiving considerable attention as a vector of the pine wood nematode, *Bursaphelenchus xylophilus*, the causal agent of a lethal wilting disease in susceptible species of pines. Adult beetles are attracted to traps baited with a kairomone blend consisting of a host volatile, α-pinene, and two bark beetle pheromone components, ipsenol and 2-methyl-3-buten-2-ol. More recently it has been shown that mature male *M. galloprovincialis* produce a pheromone that attracts mature females in a laboratory bioassay. Here, volatiles were collected from mature male and female *M. galloprovincialis*, and a compound produced specifically by mature males was identified as 2-undecyloxy-1-ethanol from its gas chromatographic retention times, its mass spectrum, and by comparison with synthetic standards. The naturally-derived and synthetic compounds elicited electroantennographic responses from both females and males. Sealed polyethylene vials and polyethylene sachets were shown to be effective dispensers with zero-order release, the latter giving a higher release rate than the former. In two field tests, multiple-funnel traps baited with synthetic 2-undecyloxy-1-ethanol caught both female and male *M. galloprovincialis*, with higher catches at the higher release rate. This compound also synergized the attractiveness of the kairomone blend, the combined mixture catching 80–140% more beetles than the sum of the catches to each bait separately and luring up to two beetles/trap/d in a moderate-density population. We conclude that 2-undecyloxy-1-ethanol is a male-produced aggregation pheromone of *M. galloprovincialis*. This is the first example of a sex-specific compound in the cerambycid subfamily Lamiinae with significant behavioral activity in the field at a range sufficient to make it a useful trap bait. The possible roles of this pheromone in the chemical ecology of *M. galloprovincialis* and its potential use in pine wilt disease management are discussed.

Key Words Aggregation pheromone · *Bursaphelenchus xylophilus* · Cerambycidae · Ethers · Gas chromatography-electroantennographic recording · Kairomone · Lamiinae · Monochaminae · *Monochamus galloprovincialis* · Pine sawyer · Pine wilt disease · Pine wood nematode · Trapping · 2-Undecyloxy-1-ethanol

J. A. Pajares · G. Álvarez · F. Ibeas
Institute of Sustainable Forestry, University of Valladolid,
34004 Palencia, Spain

J. A. Pajares
e-mail: jpajares@pvs.uva.es

D. Gallego
Departamento de Zoología y Antropología Física,
University of Murcia,
Murcia, Spain

D. R. Hall (✉) · D. I. Farman
Natural Resources Institute, University of Greenwich,
Chatham Maritime,
Kent ME4 4TB, UK
e-mail: d.r.hall@gre.ac.uk

Introduction

Woodboring beetles of the genus *Monochamus* Dejean (Coleoptera: Cerambycidae) comprise a group of species in which the larvae usually colonize woody plants that are dead, dying, or severely stressed. As such they can be considered secondary pests included in the stressed or dead

host species categories described by Hanks (1999). Economic damage produced by these species is due to timber degradation caused by larvae boring into the sapwood and heartwood (McIntosh et al. 2001; Evans et al. 2004). In rare cases, species of *Monochamus* are known to oviposit in and kill standing coniferous trees following forest disturbances (Gandhi et al. 2007). However, woodborers in the genus *Monochamus* are of particular importance since the adults are vectors of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, the causal agent of pine wilt disease. The pine wood nematode is native to North America and causes a lethal wilting in susceptible species of pines (Wingfield et al. 1982). Nematodes typically are introduced into healthy trees during maturation feeding of adults on the pine shoots (Mamiya and Enda 1972), but nematode transmission also occurs when females oviposit into dying or dead trees. *Bursaphelenchus xylophilus* was introduced into Japan around 1905, later spreading to southeastern China in 1982, Taiwan in 1983, and Korea in 1988, causing widespread mortality and severe losses in pine forests of these Asian countries (Shing 2008; Zhao 2008).

The pine sawyer, *Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae), is a woodboring insect distributed in Europe and North Africa. It mainly colonizes *Pinus* species, but also will colonize several species of *Abies* and *Picea*. Eggs are deposited in niches chewed within the bark. Larvae bore under the bark, feed in the phloem tissue, later mine into the sapwood, and finally into the heartwood to complete their development and pupate in characteristic pupal cells (Bense 1995). Young adults feed on the bark of pine shoots in the crown of healthy trees for sexual maturation. In southern Europe, it completes its life cycle in 1 year (Vives 2000; Naves et al. 2008), but it may require 2 years in northern latitudes (Tomminen 1993). This longhorn beetle previously was considered a secondary forest pest, but it is now receiving increased attention following the recent introduction of pine wood nematode into Portugal. This disease is killing *Pinus pinaster* Aiton (Mota et al. 1999), and *M. galloprovincialis* has been confirmed as the vector of the disease in this area (Sousa et al. 2001).

Among the Cerambycidae, the genus *Monochamus* has been one of the more well-studied groups from the perspective of its chemical ecology; kairomonal attractants and repellents and oviposition stimulants have been reported for several species, but no pheromones have been isolated and identified (Allison et al. 2004). Both males and females of *M. galloprovincialis* are attracted by blends of pine volatiles and compounds used as pheromone components by *Ips* species bark beetles (Pajares et al. 2004; Francardi et al. 2009), and a kairomonal lure composed of α-pinene, ipsenol, and 2-methyl-3-buten-2-ol has been developed for use in monitoring operations (Ibeas et al. 2007). Presumably, both sexes are brought together on the host plant by these kairomones, and this is followed by copulation and oviposition. Ibeas et al. (2008) described the mating behavior of *M. galloprovincialis* in the laboratory and proposed a chemically-mediated sex recognition by males through the contact of the female elytra with the labial and/or maxillary palpi of the mouthparts. Later, the role of female cuticular compounds in mate recognition was demonstrated, and significant differences between the global hydrocarbon profiles of males and females were found, indicating that females have a contact pheromone on their cuticle that stimulates male copulatory behavior (Ibeas et al. 2009).

Ibeas et al. (2008) also showed that mature female *M. galloprovincialis* were strongly attracted to mature males feeding on pine shoots in an olfactometer. Mature females also were attracted to mature males in the absence of pine shoots, but less strongly. Mature females were not attracted to immature males, and immature females were not attracted to mature or immature males. Males were not significantly attracted by males or females.

The olfactometer data suggested that mature male *M. galloprovincialis* produce a pheromone that attracts mature females. The work reported here was undertaken to isolate and identify this pheromone and to test its attractiveness in the field to male and female *M. galloprovincialis* alone and with the kairomone lures described earlier. Traps baited with the latter are already in use for monitoring the pest, and if the pheromone increases their attractiveness, then the sensitivity of the traps would be enhanced and control by mass trapping might be feasible.

Methods and Materials

Insect Material Insects were allowed to emerge from infested logs of *Pinus halepensis* Miller, *P. pinaster*, and *P. sylvestris* L. collected in the field and brought into the laboratory in Spain. They then were sexed and sent by courier to the UK in individual tubes. They arrived within 2 d and were maintained on shoots of *P. halepensis* in an insectary with 12L:12D cycle (0700–1900 h L: 1900–0700 h D), at temperatures of 25°C and 20°C, respectively, and at ambient humidity. Initially, freshly-emerged beetles were sent from Spain and used immediately. Subsequently, beetles were allowed to feed on pine shoots for 2 wk in Spain before shipment to the UK. These correspond to immature and mature beetles, respectively (Ibeas et al. 2008).

Collection of Volatiles For collection of volatiles, beetles and/or pine shoots were held in a silanized flange flask

(1 l). Air was drawn in through a charcoal filter (10 cm× 2 cm; 10–18 mesh; Fisher Scientific, Loughborough, Leicestershire, UK) at 2 l/min, and volatiles were trapped on Porapak Q (50–80 mesh; 200 mg; Supelco, Gillingham, Dorset, UK) packed between glass wool plugs in a Pasteur pipette (i.d. 4 mm). The Porapak Q was cleaned previously by Soxhlet extraction with chloroform for 8 h, and filters were washed well with dichloromethane before use. Trapped volatiles were eluted from the filter with dichloromethane (3×0.5 ml) and used directly for analyses. Separate flasks were used for male and female beetles, and each flask was fitted with two collection filters attached to pumps with electronic timers arranged to collect volatiles emitted during the light and dark periods on separate filters.

Gas Chromatography (GC) and GC Coupled to Mass Spectrometry (GC-MS) Collections of volatiles were analyzed by GC with flame ionization detection (FID) on an HP6850 instrument (Agilent Technologies, Stockport, Cheshire, UK) fitted with a split/splitless injector and fused silica capillary column (30 m×0.32 mm i.d.×0.25 μm film thickness) coated with polar SupelcoWax10 (Supelco, Gillingham, Dorset, UK). Carrier gas was helium (2.4 ml/min), and the oven temperature was held at 60°C for 2 min then programmed at 10°C/min to 250°C. Injection was splitless at 200°C. Peaks were quantified by comparing peak areas with an internal standard of 1-undecanol (1 μg).

Collections of volatiles also were analyzed by GC-MS on an HP6890 GC coupled directly to an HP5973 MS (Agilent Technologies) operated in EI mode. The capillary column (30 m×0.25 mm i.d.×0.25 μm film thickness) was coated with SupelcoWax10 or non-polar SPB5 (Supelco). Injection was splitless (220°C); the carrier gas was helium (1 ml/min); and the oven temperature was held at 50°C for 2 min then programmed at 6°C/min to 250°C.

GC retention times were converted to Retention Indices (RI) by comparison with the retention times for *n*-alkanes (Zellner et al. 2008). Compounds were identified by comparison of their mass spectra with those in the spectral library (NIST 05 Mass Spectral Library; Agilent Technologies) and subsequent comparison of mass spectra and GC retention times with those of synthetic standards.

GC Coupled to Electroantennographic (EAG) Recording For GC-EAG analyses, an HP6890 instrument was fitted with fused silica capillary columns coated with both polar and non-polar phases as above except that column i.d. was 0.32 mm. The ends of the two columns were connected to a short piece of deactivated fused silica tubing with a glass, push-fit Y-piece. The outlet from this then was split by means of a similar Y-piece connected to the flame ionization detector and to a silanized, glass T-piece (arms 5 cm, i.d. 4 mm), with similar lengths of deactivated fused silica tubing. One arm of the T-piece was connected to a device delivering nitrogen (200 ml/min) in a 3-sec pulse at 17-sec intervals. The third arm of the T-piece passed through the GC oven wall to the insect EAG preparation (Cork et al. 1990).

EAG recording was carried out with a portable device (INR-02; Syntech, Hilversum, The Netherlands) consisting of integrated electrode holders, micromanipulators, and an amplifier. Electrodes were silver wires fitted with glass electrodes pulled to a fine point with an electrode puller and containing saline solution (0.1 M potassium chloride with 1% polyvinylpyrrolidine to reduce evaporation). An antenna was excised at the base and suspended between the glass electrodes, which were cut so that they just accommodated the ends of the antenna. The signal was amplified × 500 and the amplifier was connected to the GC as a detector device. Data were processed with EZChrom Elite v3.0 (Agilent Technologies).

Microacetylation An aliquot (200 μl) of a collection of volatiles from mature male *M. galloprovincialis* in dichloromethane was blown down carefully just to dryness with charcoal-filtered nitrogen. Pyridine (10 μl) and acetic anhydride (10 μl) were added, and the mixture stood at room temperature overnight. The mixture was dissolved in hexane (200 μl) and extracted × 3 with water (100 μl) before drying with a little magnesium sulfate and analyzing by GC-MS.

Synthetic Chemicals Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a JEOL EX270 machine at 270 MHz for ^1H and 67.8 MHz for ^{13}C. Infrared (IR) spectra were recorded as thin films with a Perkin Elmer 298 grating spectrophotometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK).

2-Undecyloxy-1-ethanol (I, Fig.1) was synthesized by adding an oil dispersion of sodium hydride (60%; 2.8 g, 70 mM) portionwise to a stirred solution of ethanediol (6.2 g, 100 mM) in dry dimethylformamide (70 ml) at room temperature over 30 min. The suspension then was heated at 60°C for 30 min until effervescence had ceased. After cooling to room temperature, 1-bromoundecane (11.8 g, 50 mM) was added dropwise. The mixture was stirred for 2 h at room temperature, then for 2 h at 60°C after which time GC analysis showed all the 1-bromoundecane had reacted. The mixture was cooled and quenched carefully with half-saturated brine (100 ml). After adding 20% diethylether in petroleum ether (150 ml), the mixture was extracted × 3 with half-saturated brine (100 ml). The aqueous extracts were re-extracted with 20% diethylether in petroleum ether (150 ml), and the combined organic extracts were dried with magnesium sulfate and filtered through silica gel (10 g). Analysis by GC showed the product and 1,2-diundecyloxyethane in 57:43 ratio. After removal of solvents

(I) H_3C ... O ... OH

(II) H_3C ... O ... OH

(III) H_3C ... O ... CHO

(IV) H_3C ... O ... O ... OH

Fig. 1 Structures of male-specific compounds from *Monochamus galloprovincialis* (I), *Anoplophora glabripennis* (II) and (III) (Zhang et al. 2002), and *M. leuconotus* (IV) (Hall et al. 2006b)

on a rotary evaporator, the residue (13.5 g) was distilled to give the product b.p. 125°C/0.04 mm Hg (5.8 g, 54%). IR (film): ν 3440 cm^{-1}; ^{1}H NMR: δ 0.88 (t, J=6.2 Hz, 3H), 1.26 (br, 16H), 1.59 (quin, J=6.8, 2H), 2.21 (s, 1H), 3.47 (t, J=6.6 Hz, 2H), 3.53 (t, J=4.6 Hz, 2H), 3.73 (t, J=4.6 Hz, 2H); ^{13}C NMR: δ 14.13, 22.71, 26.14, 29.35, 29.51, 29.63 (m), 29.69, 31.93, 45.55, 61.90, 71.46, 71.69 MS: m/z (%) 41 (40), 43 (65), 45 (41), 55 (41), 57 (100), 63 (28), 69 (20), 71 (43), 75 (10), 82 (10), 83 (21), 85 (34), 97 (24), 99 (10), 111 (8), 126 (8), 154 (4), 185 (2).

4-Heptyloxy-1-butanol (II, Fig. 1) and 4-heptyloxy-butyraldehyde (III, Fig. 1), male-specific compounds from the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky), were synthesized as described by Zhang et al. (2002).

2-(4-Heptyloxy-1-butyloxy)-1-ethanol (IV, Fig. 1), a male-specific compound from the coffee stemborer, *Monochamus leuconotus* (Pascoe), was synthesized from II (Fig. 1) after conversion to the corresponding bromide with phosphorus tribromide in diethylether and reaction with ethanediol as for I (Fig. 1) (Hall et al. 2006b).

Release Rates of Pheromone from Dispensers Release rates of 2-undecyloxy-1-ethanol (I, Fig. 1) from closed polyethylene vials and polyethylene sachets were determined in the laboratory. The compound (41 mg) was impregnated onto a cellulose cigarette filter (15×6 mm; Swan, High Wycombe, Buckinghamshire, UK) inserted into the vial (22×8×1.5 mm thick; Just Plastics Ltd., London, UK) or polyethylene sachet (2.5 cm×2.5 cm×120 μm thick) made from heat-sealed, layflat tubing (International Pheromone Systems Ltd., Wirral, UK). Dispensers were maintained in a laboratory windtunnel (27°C, 8 km/h windspeed), and release rates were determined gravimetrically over 41 d. Data were recorded from only two replicates for each release device as release from these dispensers is known to be uniform (Hall et al. 2006a).

Field Tests Two trapping experiments (Table 1) were carried out in a mature forest of *P. halepensis* at Sierra Espuña (Murcia) National Park, southeastern Spain (37º 57′ N, 1º 24′W). Pine trees were healthy, and breeding material for the pine sawyer was not particularly abundant. Thus, the population of *M. galloprovincialis* in the area was assumed to be at a moderate level. In Experiment 1, we tested the responses of the beetles to the synthetic pheromone alone, to the standard kairomone lure of α-pinene, ipsenol (2-methyl-6-methylene-7-octen-4-ol), and 2-methyl-3-buten-2-ol (Pajares et al. 2004; Ibeas et al. 2007) and to combinations of the two. We also investigated the flight response elicited by verbenone to determine whether this compound could replace α-pinene in the kairomonal mixture, as suggested by Ibeas et al. (2007). In Experiment 2, we replaced the bark beetle pheromone component ipsenol in the kairomone mixtures with ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol).

Dispensers for bark beetle semiochemicals and host compounds were as described previously by Pajares et al. (2004) and Ibeas et al. (2007). Thus, racemic ipsenol, racemic ipsdienol, 2-methyl-3-buten-2-ol, and ~80%-(–)-

Table 1 Treatments in field experiments testing flight response by *Monochamus galloprovincialis* to pheromone and/or kairomones

Treatments[a]	
Kairomone	Pheromone
Experiment 1	
Is + Mb + αP	–
Is + Mb + Vb	–
Is + Mb + αP + Vb	–
Is + Mb	–
Vb	–
–	PhL
–	PhH
Is + Mb + αP	PhL
Is + Mb + αP	PhH
Experiment 2	
Id + Mb + αP	–
Id + Mb + Vb	–
Id + Mb + αP + Vb	–
Id + Mb	–
Vb	–
–	PhL
–	PhH
Id + Mb	PhL
Id + Mb	PhH

[a] Is = ipsenol; Id = ipsdienol; Mb = 2-methyl-3-buten-2-ol; αP = α-pinene; Vb = verbenone; PhL = Pheromone low release rate; and PhH = Pheromone high release rate

verbenone were dispensed from separate bubble-cap or pouch dispensers (Phero Tech Inc. Delta, BC, Canada; now Contech Enterprises) with release rates (and active ingredient loads) quoted by the manufacturer as 0.40 mg/d (40 mg, bubble cap, at 25°C), 0.20 mg/d (40 mg, bubble cap, at 25°C), 11–65 mg/d (3.3 mg, pouch, at 20°C and 30°C), and 2–15 mg/d (790 mg, bubble cap, at 20°C and 30°C), respectively. Racemic α-pinene was dispensed from polyethylene tubing (300 cm long × 11 mm diam; Esencias Catalá, Gandia, Valencia, Spain) at 2.1 g/d as measured gravimetrically at 27°–30°C. The pheromone was dispensed from polyethylene vials (low release rate) or polyethylene sachets (high release rate) as described above except that the loading was 82 mg.

Traps were 12-unit, multiple funnel traps (115 cm long; Phero Tech Inc.) suspended from ropes between trees with the top funnel 1.8 m above ground. The collecting cups were provided with a small piece of DDVP (dimethyl 2,2-dichlorovinyl phosphate) insecticide strip (Econex S. L., Murcia, Spain) to kill trapped beetles. Both experiments were deployed in seven randomized complete blocks. Traps were at least 100 m apart and nearest blocks were 700 m apart. Experiments 1 and 2 were conducted from 3 July to 7 August 2008 and from 7 August to 26 September 2008, respectively. Traps were emptied every 7–10 d and *M. galloprovincialis* in the trap catches were identified and separated by sex (Vives 2000).

Previous experience has shown that *M. galloprovincialis* are not caught in unbaited traps (Pajares, unpublished; Francardi et al. 2009). Thus, no unbaited controls were included in the experiments in order to avoid having treatments with zero mean and variance (Reeve and Strom 2004). Even so, in both experiments, no beetles were caught in traps baited with verbenone. These were excluded from analysis, and the remaining data were square-root transformed to meet the assumptions of normality and homoscedasticity, as confirmed by quantile-quantile plots, and subjected to analysis of variance (ANOVA) by using a generalized linear model for randomized complete blocks (SAS System software, SAS Institute Inc. 1999–2000). Treatment mean catches were compared by Least Significant Difference (LSD) Fisher tests (α=0.05). In addition, normalized data from treatments with lowest catches were subjected to one sample *t*-tests to test for departure of treatment true means from zero.

Results

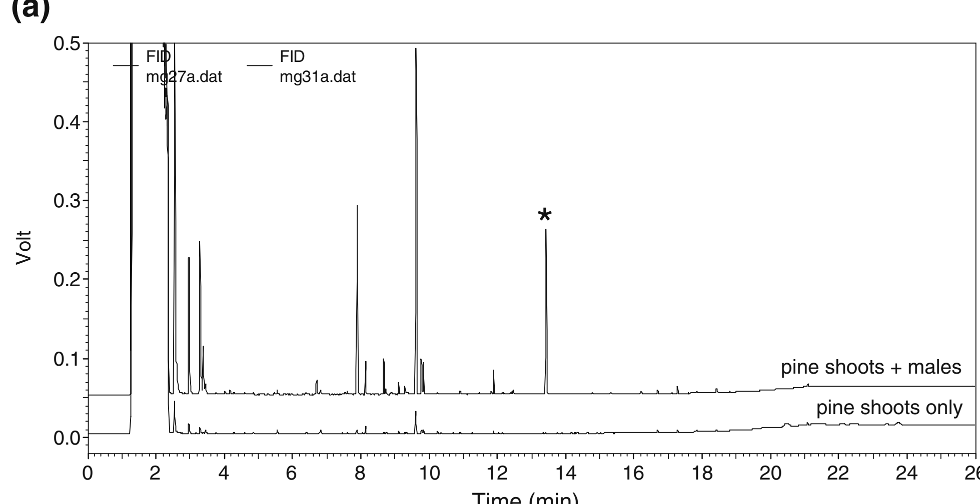
Pheromone Identification Initially, volatiles were collected from freshly-emerged, immature adult *M. galloprovincialis*. Collections were made from two to three males or females separately, with and without shoots of *P. halepensis*, and from shoots of *P. halepensis* only. Collections were made from 0–24 h and 24–72 h after introduction of beetles, and volatiles emitted during the light and dark periods were collected separately. All samples were replicated twice. Analysis of these collections by GC-FID showed no obvious differences between collections from males and females.

Subsequent collections were made from mature beetles that had fed for at least 2 wk on pine shoots. Volatiles were collected from pine shoots only for 24 h, and then one to three beetles added. After addition of beetles much larger amounts of volatiles were collected from the pine shoots, presumably due to the feeding of the beetles (Fig. 2). The main compounds in the extracts were identified (GC-MS) as α- and β-pinene, myrcene, limonene, methyl benzoate, methyl salicylate, α- and β-caryophyllene, and α-farnesene.

An additional compound was observed only in collections from the pine shoots with mature male beetles (Fig. 2). GC retention data for this compound are shown in Table 2 and the EI mass spectrum in Fig. 3a). A library search on the mass spectrum gave 2-hexyloxy-1-ethanol as one of the best fits, albeit with a fit of only 38%. However, the mass spectrum showed several of the ions as doublets two mass units apart, as reported by Zhang et al. (2002) in the mass spectrum of the two *A. glabripennis* male-specific compounds (II and III, Fig. 1). We also observed this phenomenon in the mass spectrum of a male-specific compound (IV, Fig. 1) from *M. leuconotus* (Hall et al. 2006b).

The difference between the RI's on polar and non-polar GC phases (Table 2) showed that the compound from male *M. galloprovincialis* was much less polar than the diether alcohol from *M. leuconotus* (IV, Fig. 1) and closer in polarity to the monoether alcohol (II, Fig. 1). The presence of an alcohol function was confirmed by acetylation (Table 2, Fig. 3b). The difference in RI on the non-polar column between that for the compound from *M. galloprovincialis* and the alcohol from *A. glabripennis* (II, Fig. 1) suggested the former had two carbons more than the latter.

The mass spectrum of the compound from male *M. galloprovincialis* (Fig. 3a) showed an unusual ion at *m/z* 63 corresponding to protonated ethane diol ($HOCH_2$-$CH_2OH_2^+$). The ion at *m/z* 154 could be due to (C_9H_{19}-$CH=CH_2)^+$ formed by elimination of an ether group from a $C_{11}H_{23}$- moiety. A McLafferty-type arrangement of the same moiety would give $C_7H_{15}CH=CH_2^+$ at *m/z* 126. The ion at *m/z* 185 then would correspond to loss of -CH_2-OH from a molecular weight of 216 by cleavage α-β to the ether. Thus, 2-undecyloxy-1-ethanol (I, Fig. 1) was considered a possible structure. This was synthesized from 1-bromoundecane and ethanediol and found to have identical GC retention times (Table 2) and mass spectrum (Fig. 3c) to those for the compound from mature male *M. galloprovincialis*.



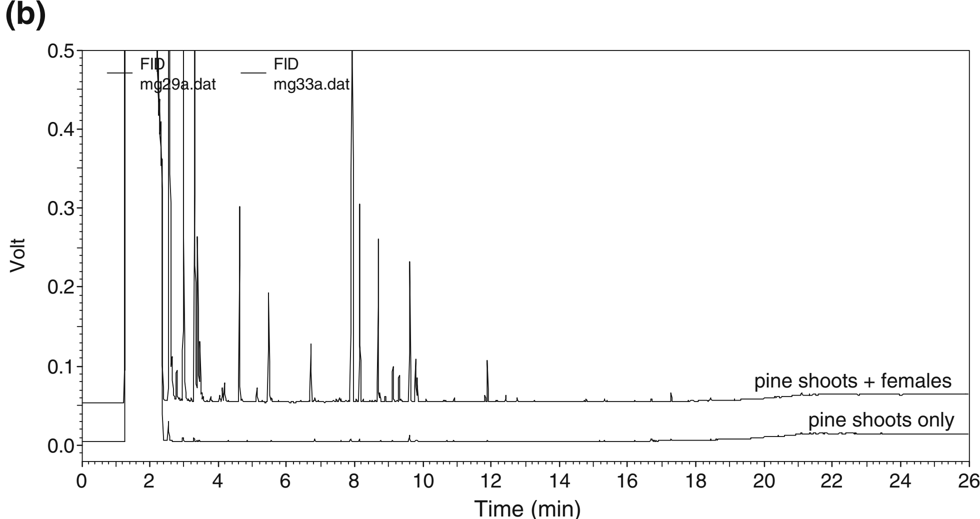


Fig. 2 GC-FID analyses of volatile collections from *Monochamus galloprovincialis* during light period: (**a**) analyses of collection from pine shoots only (24 h; lower trace) and after adding 1 mature male *M. galloprovincialis* (48 h; upper trace); (**b**) analyses of collection from pine shoots only (24 h; lower trace) and after adding two mature female *M. galloprovincialis* (48 h; upper trace). Male-specific compound marked with *

Compound I (Fig. 1) was detected in collections made from mature males during both light and dark periods, although the amount collected during the dark period was approximately half that collected during the light period. The two collections with the most compound I contained approximately 10 µg from two males during a single 12 h light period and approximately 30 µg from one male over two successive light periods. If production were uniform throughout the light period, these figures are equivalent to approximately 0.4 µg/h/male and 1.3 µg/h/male, respectively.

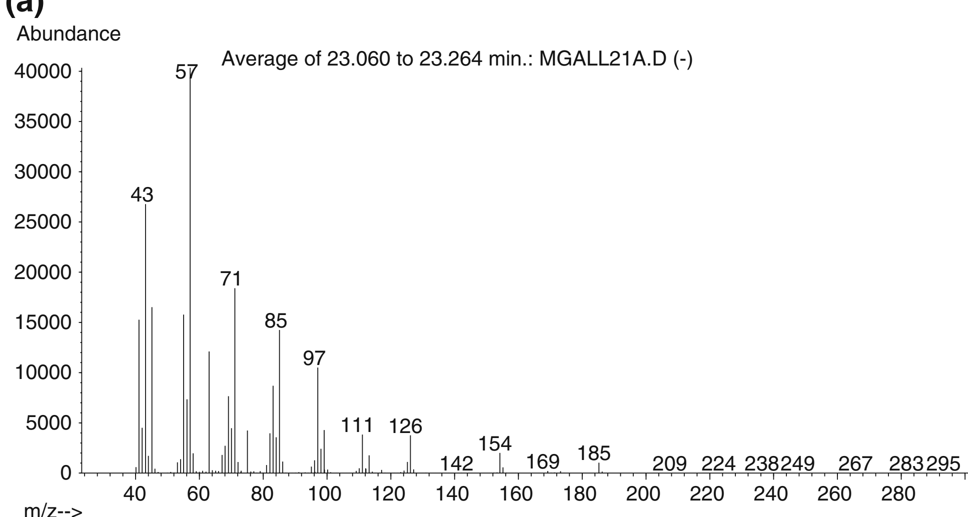
GC-EAG Recording Analyses of collections of volatiles from male and female *M. galloprovincialis* showed a significant and consistent EAG response from antennae of both female (Fig. 4a) and male beetles (Fig. 5a) to the male-specific compound (I, Fig. 1) in volatiles from mature males. No responses to volatiles from the pine were

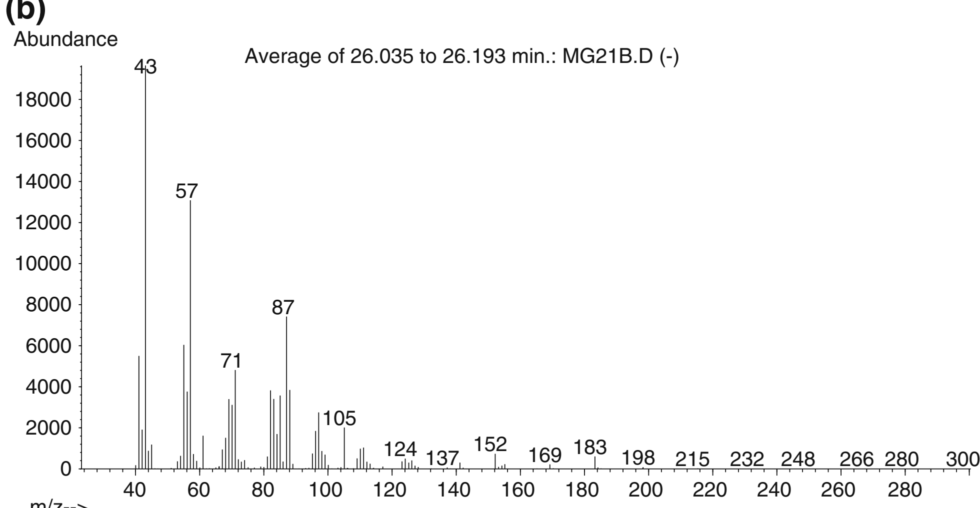
Table 2 Gas chromatography retention data for male-specific compounds from *Monochamus galloprovincialis* and other cerambycid species

Species (compound)	Retention index (RI)[a]		
	Wax	SPB5	Δ[b]
M. galloprovincialis (male-specific compound)	2126	1617	509
M. galloprovincialis (after acetylation)	2152	1778	374
2-undecyloxy-1-ethanol (I, Fig. 1)	2126	1617	509
Anoplophora glabripennis alcohol (II, Fig. 1)	1990	1438	552
Anoplophora glabripennis aldehyde (III, Fig. 1)	1753	1374	379
M. leuconotus (IV, Fig. 1)	2293	1685	608

[a] Retention index relative to retention times of *n*-alkanes

[b] Difference between RI on polar column and RI on non-polar column





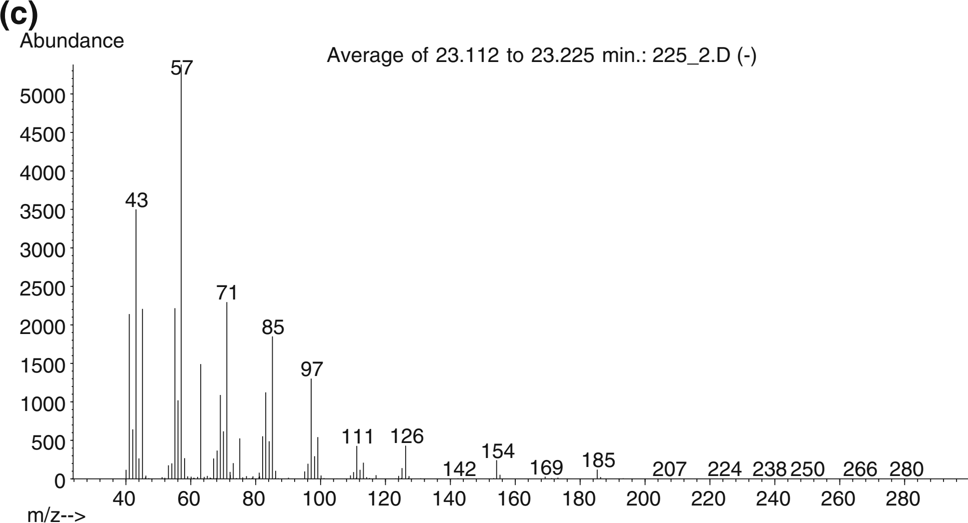


Fig. 3 EI mass spectra of **a** male-specific compound from *Monochamus galloprovincialis*, **b** this compound after acetylation, and **c** synthetic 2-undecyloxy-1-ethanol (I, Fig. 1)

recorded in these analyses even though α-pinene at least is a known kairomone (Pajares et al. 2004; Ibeas et al. 2007) and has been shown to elicit an EAG response from *M. galloprovincialis* (Álvarez, unpublished). Presumably, the antennal receptors are less sensitive to the kairomones than to the pheromones, and the concentrations of the former were too low in these analyses. Synthetic 2-undecyloxy-1-ethanol (I, Fig. 1) also elicited an EAG response from the antennae of female (Fig. 4b) and, to a lesser extent, male (Fig. 5b) beetles. In these analyses, the EAG responses from the antennae of female beetles seemed to be generally higher than those from the antennae of male beetles. Although the sensitivities of individual preparations were not normalized with a reference standard, the absolute responses above blank to 14 ng injected (7 ng to the antenna) of the natural male-specific compound were 1.85± 0.15 mV (± S.E., N=3) and 0.83±0.32 mV (± S.E., N=3) from females and males, respectively. The responses to 10 ng injected of synthetic I were 1.51±0.15 mV (± S.E., N=9) and 0.66±0.09 mV (± S.E., N=5), respectively. These differences were significantly different by a simple t test (P=0.001 for the natural compound; P=0.023 for the synthetic).

In unreplicated tests, cutting the tip of the antenna before inserting into the recording electrode did not seem to affect the magnitude of the response recorded. The antenna also seemed to be uniformly responsive along its length.

Release Rates of Pheromone from Dispensers Release of synthetic 2-undecyloxy-1-ethanol (I, Fig. 1) from polyethylene sachet dispensers was uniform over the period of measurement of 41 d (Fig. 6). Release from the polythene vials did not start for approximately 5 d, while the compound diffused through the walls but was uniform after about 10 d (Fig. 6). Rates at 27°C and 8 km/h windspeed were 0.16 mg/d (± 0.005 mg/d for the two replicates) for the vial and 0.74 mg/d (± 0.013 mg/d for the two replicates) for the sachet, equivalent to 67 μg/h and 330 μg/h, respectively. Thus, with initial loadings of 82 mg, even the sachets would be expected to last for at least 3 mo under these conditions.

Field Tests In Experiment 1, carried out during 5 wk in the first half of the summer, we tested the responses by both sexes of *M. galloprovincialis* to the synthetic pheromone at two release rates alone or in combination with the standard kairomone lure and to four variations of the kairomone lure designed to investigate the effect of α-pinene and verbenone (Fig. 7). This experiment confirmed that the kairomone mixture of ipsenol, 2-methyl-3-buten-2-ol, and α-pinene is attractive to *M. galloprovincialis*, catching up to one beetle/trap/d. Responses of females were 17–34% lower when α-pinene was either removed or replaced by verbenone in the kairomone mixture, although the differences were not significant. Catches of males were approximately half those of females, but the males responded similarly to the kairomone mixtures with the response significantly reduced by 40% if α-pinene was excluded. Traps baited with verbenone alone captured no beetles during the experiment, and this compound had no significant effect on attractiveness to females or males when added to the kairomone standard.

The male-produced compound, 2-undecyloxy-1-ethanol, attracted both female and male *M. galloprovincialis* (Fig. 7). The high-release dispensers trapped three times more females than the low-release baits, and this difference was significant. Catches of female *M. galloprovincialis* in traps baited with the low and high-release dispensers were significantly different from zero (t-test, P=0.009; P<0.001, respectively), whereas only the high-release dispensers resulted in catches of males significantly different from zero (t-test, P=0.004).

Although traps baited with the high-release dispensers caught two to three times fewer female *M. galloprovincialis* and three to six times fewer males than were caught in traps baited with the kairomone standard, there was a synergism between the pheromone and the kairomones (Fig. 7). The numbers of female *M. galloprovincialis* attracted by the combinations were more than 80% higher than the sum of the catches obtained by the separate components; the numbers of males attracted were 65–75% higher. As a result, the proportion of the sexes trapped by the pheromone-plus-kairomone mixture was less biased to the females (1:2 male:female) than with the pheromone alone (1:3.5). Also, the dose-response effect found to the pheromone alone was not so apparent when it was combined with the kairomone. Catches with the high-release dispensers were only 13% more than catches from the low-release dispensers, and this difference was not significant. The combinations of the pheromone and the standard kairomone bait trapped means of 64.8 and 73.6 beetles/trap over 35 d for low- and high-release pheromone dispensers, respectively, i.e., approximately two *M. galloprovincialis* beetles per trap each day.

Experiment 2 was carried out in the same site for 7 wk during the second half of the beetle flight period and evaluated similar blends to those used in the first experiment except that ipsenol was replaced by ipsdienol (Fig. 8). The four kairomone blends were significantly attractive relative to a zero trap catch, but numbers of beetles trapped were 50–75% lower than those trapped by the analogous blends in the first experiment, and removal of α-pinene did not reduce catches. As in Experiment 1, traps baited with verbenone alone caught no beetles.

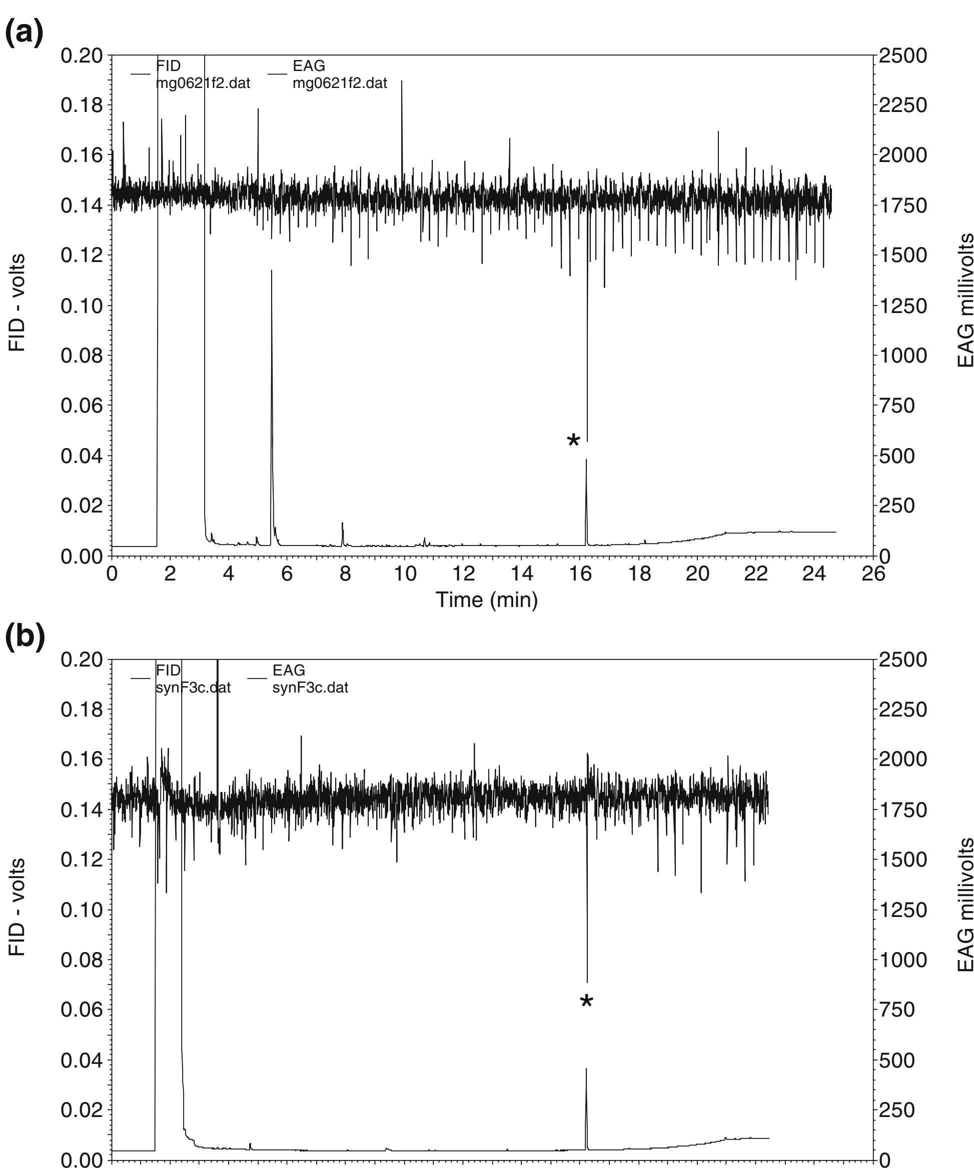


Fig. 4 Linked GC-EAG analyses with EAG recording from female *Monochamus galloprovincialis* antenna: **a** volatiles from mature males and **b** synthetic 2-undecyloxy-1-ethanol (I) (10 ng injected; EAG responses marked with *)

As observed in the first half of the season, *M. galloprovincialis* females were significantly attracted to traps baited with 2-undecyloxy-1-ethanol alone in a dose-response pattern (Fig. 8). Catches with the high-release lures were higher than those with the four kairomonal lures containing ipsdienol, significantly so for two of them. Catches of males were approximately 63% of the catches of females, a proportion much higher than during the first part of flight, when male catches were 30% of female catches.

The synergistic effect of the kairomones and the pheromone on attraction of *M. galloprovincialis* was even greater than in the first experiment. Catches of females were 175% higher and of males 125% higher than the sum of the catches with the pheromone and kairomones separately when the low-release pheromone dispensers were added to the kairomone mixture of ipsdienol and 2-methyl-3-buten-2-ol. Catches of both sexes were 155% higher when the high-release pheromone lures were added. Overall, the kairomone-pheromone mixtures trapped means of 31 and 58 beetles/trap during the 49 d of the experiment for low- and high-release pheromone lures, respectively, i.e., approximately one *M. galloprovincialis* beetle per trap each day.

Discussion

2-Undecyloxy-1-ethanol has been shown to be produced by mature, adult male *M.galloprovincialis* feeding on pine.

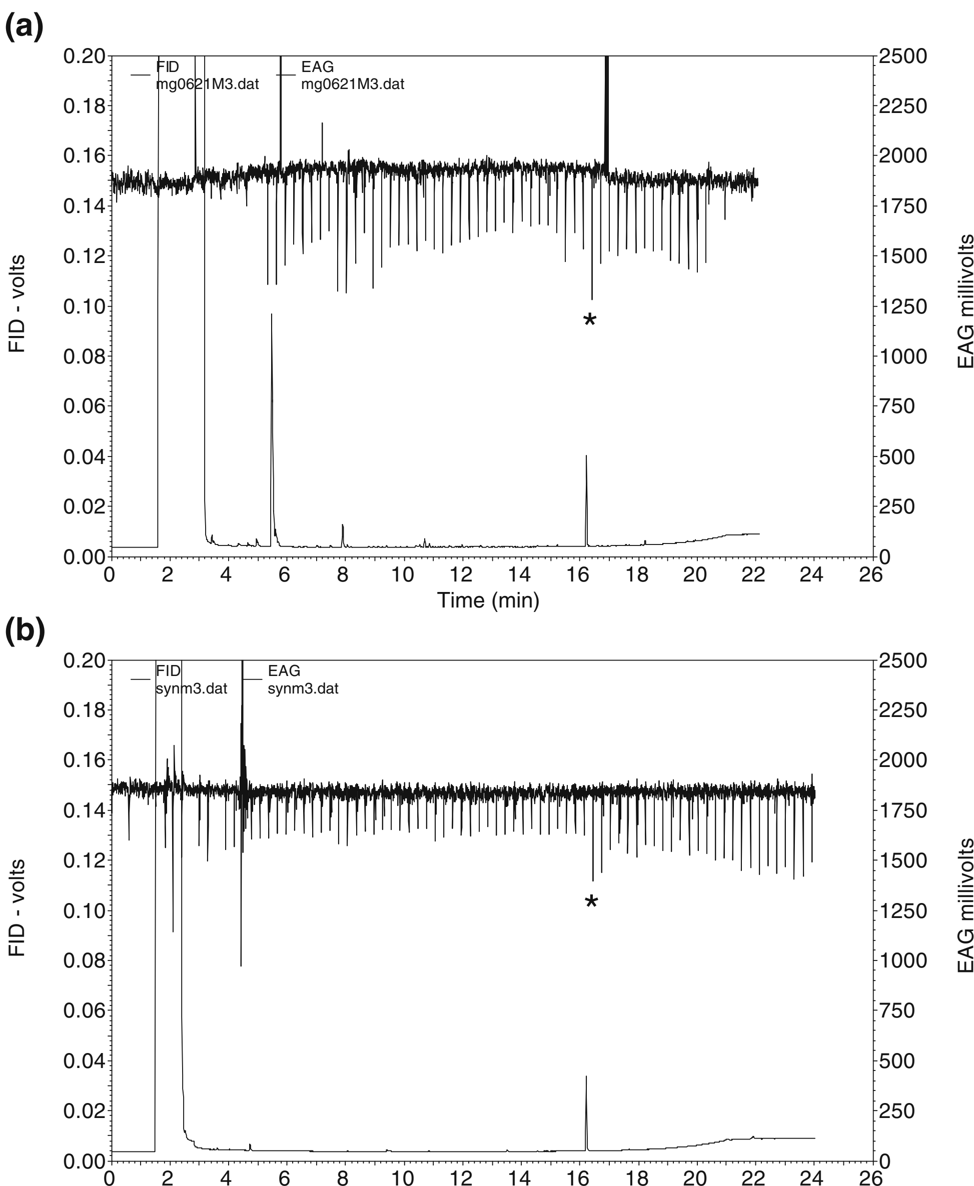


Fig. 5 Linked GC-EAG analyses with EAG recording from male *Monochamus galloprovincialis* antenna: **a** volatiles from mature males and **b** synthetic 2-undecyloxy-1-ethanol (I) (10 ng injected; EAG responses marked with *)

This compound elicits EAG responses from the antennae of both males and females, is attractive to females and males in the field, and enhances the attractiveness of traps baited with blends of pine volatiles and bark beetle pheromone components. We conclude that 2-undecyloxy-1-ethanol is a male-produced aggregation pheromone of *M. galloprovincialis*. These results are partially in agreement with those of Ibeas et al. (2008), who reported that reproductively mature females were consistently attracted to plumes containing odors from mature males in a Y-tube olfactometer. However, these authors did not observe significant attraction of males to the odors from males. This may have been due to the overall lower levels of response by males or to the differences in experimental procedure between the laboratory and field bioassays.

Ethers have been identified as male-specific volatiles in two other species of cerambycids of the subfamily Lamiinae: compounds II and III (Fig. 1) by *A. glabripennis* (Zhang et al. 2002) and IV (Fig. 1) by *M. leuconotus* (Hall et al. 2006b). The structures of these clearly are related (Fig. 1) and quite different from the hydroxyketones and dihydroxy compounds that have been found frequently as male-specific compounds in species from other subfamilies, particularly the Cerambycinae (e.g., Hanks et al. 2007). More recently, other structural types have been identified as pheromones in cerambycids, including the Lamiinae. Males of *Megacyllene caryae* (Cerambycinae) produce several monoterpenes as well as 2,3-hexanediols (Lacey et al. 2008); males of *Rosalia funebris* (Cerambycinae) produce (*Z*)-3-decenyl (*E*)-2-hexenoate (Ray et al. 2009); males of

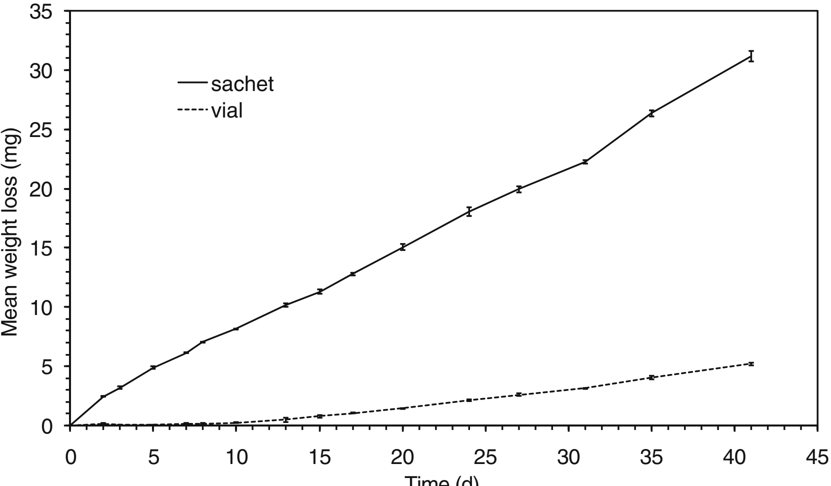


Fig. 6 Release of 2-undecyloxy-1-ethanol from polyethylene vials and sachets maintained in a wind tunnel at 27°C and 8 km/h wind-speed as measured gravimetrically (N=2 for each release device; bars show the range between the two measurements)

Tetropium fuscum (Spondylidinae) produce two homomonoterpenoids (Silk et al. 2007); females of *Prionus californicus* (Prioninae) produce 3,5-dimethyldodecanoic acid, which attracts males (Rodstein et al. 2009); and males of *Hedypathes betulinus* (Lamiinae) produce three homomonoterpenoids (Vidal et al. 2008; Fonseca and Zarbin 2009).

Long-range sex pheromones have not been found in the Lamiinae so far. Hanks (1999) suggested that long-range pheromones might be expected in species where the adults do not feed. Species in the Lamiinae generally feed as adults, and it is thought the adults are brought together on the host plant that is often weakened or stressed. The sexes then locate each other by visual stimuli as well as short-range and contact pheromones. Observations on the reproductive behavior of *M. alternatus* showed evidence of short-range attraction of females by male-emitted volatiles (Fauziah et al. 1987; Kim et al. 1992). Also,

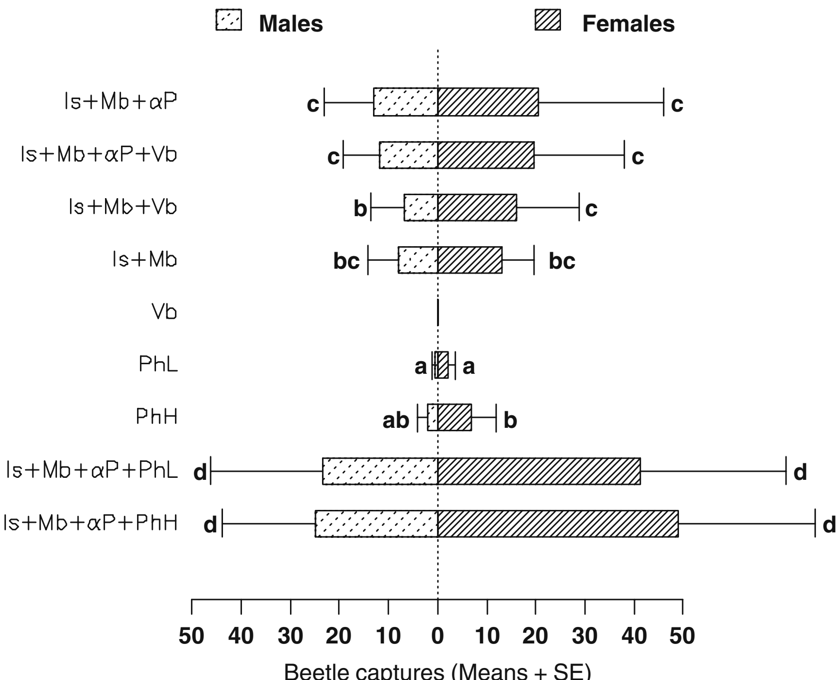


Fig. 7 Catches of *Monochamus galloprovincialis* during Experiment 1 in Sierra Espuña, Murcia, Spain, from 11 July to 7 August 2008 in semiochemical-baited multiple-funnel traps. Compounds are: αP = α-pinene; Is = ipsenol; Mb = 2-methyl-3-buten-2-ol; Vb = Verbenone; PhH and PhL = pheromone at high and low release rates, respectively. For each sex, bars followed by the same letter are not significantly different after ANOVA on data omitting zero catches with verbenone and square-root transformed followed by LSD test (α=0.05); for males F=15.20, df=7,42, P<0.001, N=7; females F=17.98, df=7,42, P<0.001, N=7. Actual means and standard errors are shown in the histogram. Differences among treatment means were assessed after ANOVA on transformed data, which removed variability among replicates

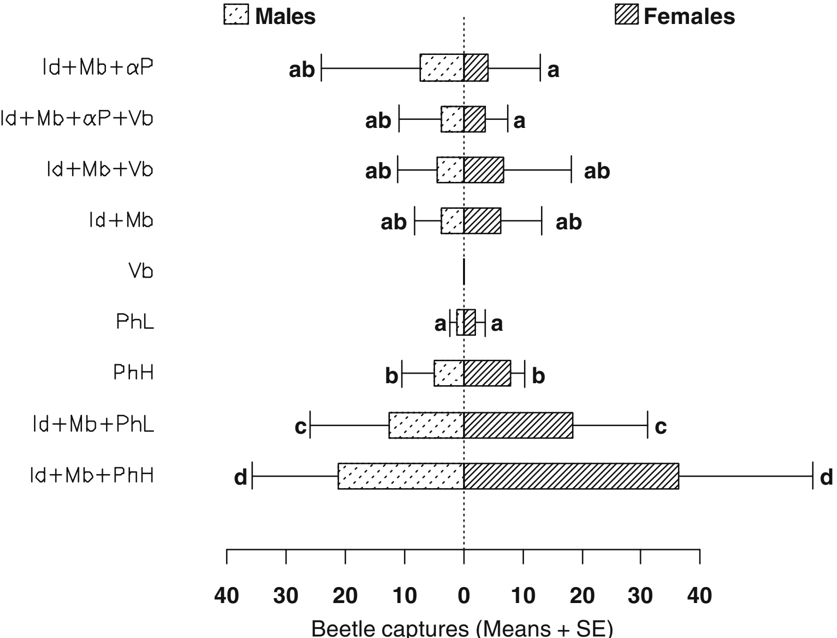


Fig. 8 Catches of *Monochamus galloprovincialis* during Experiment 2 in Sierra Espuña, Murcia, Spain, from 7 August to 26 September 2008 in semiochemical-baited multiple-funnel traps. Compounds are: αP = α-pinene; Id = ipsdienol; Mb = 2-methyl-3-buten-2-ol; Vb = Verbenone; PhH and PhL = pheromone at high and low release rates, respectively. For each sex, bars followed by the same letter are not significantly different after ANOVA on data omitting zero catches with verbenone and square-root transformed followed by LSD test (α=0.05); for males F=9.6, df=7,42, P<0.001, N=7; females F=17.92, df=7,42, P<0.001, N=7. Actual means and standard errors are shown in the histogram. Differences among treatment means were assessed after ANOVA on transformed data, which removed variability among replicates

Fukaya et al. (2004, 2005) reported that olfactory and visual cues mediated short-distance orientation in *A. malasiaca*, presenting clear evidence that males were able to recognize females at close range without direct physical contact, and that olfactory cues were essential and visual cues synergistic for mate location by males. In *Steirastoma breve*, the odors emitted by males elicited strong EAG responses in the females and also attracted females in a two-choice olfactometer (Liendo et al. 2005). Lu et al. (2007) stated explicitly that long-range pheromones are not involved in mate location by *Glenea cantor*.

Among the Lamiinae, the two male-specific compounds (II and III, Fig. 1) identified by Zhang et al. (2002) in volatiles from male *A. glabripennis* elicited EAG responses from the antennae of both sexes of the beetle. A blend of these compounds was significantly attractive to *A. glabripennis* adult females in laboratory olfactometer tests at one critical loading, but attempts to use these compounds as lures for traps in the field have been less successful (Nehme et al. 2009). The male-specific compound detected in volatiles from *M. leuconotus* (IV, Fig. 1) by Hall et al. (2006b) also elicited EAG responses from the antennae of both sexes, but no behavioral function for this compound alone or in combination with host plant volatiles has yet been established despite extensive olfactometer and field trapping studies (Hall and Kutywayo, unpublished).

The range of action of the pheromone in *M. galloprovincialis* is unknown, but the field trapping results indicate that it is attractive over more than the few centimeters reported for the pheromones of other species of the Lamiinae described above and suggest that it might well be active over much more than within-tree distances. Thus, 2-undecyloxy-1-ethanol is the first example in the Lamiinae of a sex-specific compound having a significant behavioral activity in the field at a range sufficient to make it a useful lure for trapping beetles. Several chemical signals appear to be involved in the reproductive behavior of *M. galloprovincialis*. Long-range host selection is driven by a kairomonal response to host volatiles and bark beetle semiochemicals that brings together both sexes to larval host trees (Pajares et al. 2004, Ibeas et al. 2007), and this now seems to be synergized by the male-produced aggregation pheromone. The latter also may play a role in attracting females at relatively short range, as in the Y-tube bioassays reported by Ibeas et al. (2008), and even in recognition of males by females. Finally, the chemically-mediated reproductive behavior of *M. galloprovincialis* is completed when males recognize mates by contact chemoreception of female cuticular compounds (Ibeas et al. 2009).

The field trapping experiments described here confirmed the kairomonal response by *M. galloprovincialis* to lures containing ipsenol, 2-methyl-3-buten-2-ol, and α-

pinene (Pajares et al. 2004; Ibeas et al. 2007; Francardi et al. 2009). Attempts to simplify or improve the response to this mixture by removing the host volatile or replacing it with verbenone showed that the host monoterpene should be included in any operative kairomonal lure to maximize attraction. In field Experiment 2, when ipsenol was replaced by ipsdienol, catches with the four kairomone blends were lower than in the first experiment. It is unlikely that this was due to lower populations later in the season, as catches with the pheromone and mixtures of kairomones and pheromone generally were higher than in the first experiment. The results are consistent with a lower attractiveness of the blends with ipsdienol than those with ipsenol, as reported by Pajares et al. (2004) and Ibeas et al. (2007), but changes in relative responses to kairomones and pheromone during the season cannot be excluded and need to be investigated. The responses to the kairomones ended somewhat earlier than to the pheromone, as only the baits containing the pheromone attracted beetles in mid-late September in Experiment 2 (data not shown). The difference in release rate of the two bark beetle pheromone components (ipsenol was 2× greater than ipsdienol) also may have contributed to the difference in trap catch.

The synergy between the kairomones and pheromone resulted in trap catches of both sexes to the combined mixture increasing by almost 80% in Experiment 1 and by 140% in Experiment 2, compared to the sum of the catches to each bait separately. The kairomone-pheromone combination thus provides a more powerful attractant for *M. galloprovincialis*, able to lure as many as two beetles/trap/d in a moderate population. Only two release rates of the pheromone were evaluated here (67 μg/h and 330 μg/h). Although these were significantly higher than the rate of release of the pheromone by male *M. galloprovincialis* (up to 1.3 μg/h), there was some evidence for a positive dose-response effect of the pheromone, and higher release rates might increase catches even further.

This result allows us to revise the potential of trapping in the management of *M. galloprovincialis*. Traps baited with host attractants have been useful for monitoring *Monochamus* species in Asia and Portugal, and also have been evaluated for control of beetle populations, although the latter trials were reported to be unsuccessful in Japan (Nakamura 2008; Rodrigues 2008). Previous experiments have demonstrated that the response by *M. galloprovincialis* to host monoterpenes alone is very low (Pajares et al. 2004; Ibeas et al. 2007), but we have shown that it is increased by almost two orders of magnitude when bark beetle semiochemicals and the male pheromone also are released. Ipsenol, 2-methyl-3-buten-2-ol, and α-pinene are well-established compounds, currently used for operational monitoring of bark beetles and other xylophagous insects (e.g., Francardi et al. 2009). 2-Undecyloxy-1-ethanol is relatively easy to synthesize and can be conveniently dispensed from polyethylene vials or sachets for long periods in the field. This bait should be useful in management of pine wilt disease, not only for monitoring of the insect vectors and of the nematodes they carry, but also possibly for effective control of the beetle by mass trapping in outbreak situations.

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Attractiveness of a Four-component Pheromone Blend to Male Navel Orangeworm Moths

Hiroo Kanno · L. P. S. Kuenen · Kimberly A. Klingler · Jocelyn G. Millar · Ring T. Cardé

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Abstract The attractiveness to male navel orangeworm moth, *Amyelois transitella*, of various combinations of a four-component pheromone blend was measured in wind-tunnel bioassays. Upwind flight along the pheromone plume and landing on the odor source required the simultaneous presence of two components, (11*Z*,13*Z*)-hexadecadienal and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene, and the addition of either (11*Z*,13*Z*)-hexadecadien-1-ol or (11*Z*,13*E*)-hexadecadien-1-ol. A mixture of all four components produced the highest levels of rapid source location and source contact. In wind-tunnel assays, males did not seem to distinguish among a wide range of ratios of any of the three components added to (11*Z*,13*Z*)-hexadecadienal. Dosages of 10 and 100 ng of the 4-component blend produced higher levels of source location than dosages of 1 and 1,000 ng.

Key Words Lepidoptera · Pyralidae · (11*Z*,13*Z*)-hexadecadienal · (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene · (11*Z*,13*Z*)-hexadecadien-1-ol · (11*Z*,13*E*)-hexadecadien-1-ol

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H. Kanno · K. A. Klingler · J. G. Millar · R. T. Cardé (✉)
Department of Entomology, University of California,
Riverside, CA 92521, USA
e-mail: ring.carde@ucr.edu

L. P. S. Kuenen
USDA-Agricultural Research Service,
9611 South Riverbend Avenue,
Parlier, CA 93648, USA

Introduction

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is a major pest of the multibillion dollar almond, pistachio, and walnut crops in California. The sex pheromone produced by the female navel orangeworm moth was characterized originally as a single component, (11*Z*,13*Z*)-hexadecadienal (Z11,Z13-16:Ald) (Coffelt et al. 1979a). Although this compound stimulated male activation, wing fanning, and upwind movement in laboratory bioassays (Coffelt et al. 1979b), its activity as an attractant for field traps was inconsistent, and it was only weakly attractive when compared to virgin female moths (e.g., Kuenen et al. 2001, 2010). In recent studies, a number of additional compounds have been identified from pheromone gland extracts (Leal et al. 2005; Kuenen et al. 2010), including analogs of Z11,Z13-16:Ald, such as (11*Z*,13*E*)-hexadecadienal (Z11,E13-16:Ald), (11*E*,13*Z*)-hexadecadienal (E11,Z13-16:Ald), (11*Z*,13*Z*)-hexadecadien-1-ol (Z11,Z13-16:OH), (11*Z*,13*Z*)-hexadecadien-1-yl acetate (Z11,Z13-16:Ac), hexadecanal (16:Ald), (*Z*)-11-hexadecenal (Z11-16:Ald), (*Z*)-13-hexadecenal (Z13-16:Ald), ethyl hexadecanoate (16:COOEt, ethyl palmitate), and ethyl (11*Z*,13*Z*)-hexadecadienoate (Z11,Z13-16:COOEt). Two unusual polyunsaturated hydrocarbons, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene (C_{23} pentaene) and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene (C_{25} pentaene), also were found in gland extracts. The first group of compounds is typical of the so-called Type I lepidopteran pheromones, that is, compounds with C_{10} to C_{18} straight chains terminating in alcohol, aldehyde, or ester functions. In contrast, the two pentaenes belong to the Type II lepidopteran pheromones, characterized by polyunsaturated hydrocarbons and related epoxides and ketones, with C_{17} to ~C_{25} carbon chain lengths (Ando et al. 2004). Thus, this insect is one of the first examples of a

moth species that might use a combination of both Type I and Type II compounds in its pheromone blend. In field trials, Leal et al. (2005) demonstrated that blends of five of these components, in combination with Z11,Z13-16:Ald, were attractive to male moths, but they did not report further attempts to refine the blend. Thus, it remained unclear as to which specific compounds were necessary and sufficient to constitute an attractive blend. More rigorous attempts to delineate the true blend by using a combination of wind tunnel and field bioassays spanning several field seasons, provided evidence that the pheromone blend is composed of at least four components, Z11,Z13-16:Ald, Z11,Z13-16:OH, (11*Z*,13*E*)-hexadecadien-1-ol (Z11,E13-16:OH), and C_{23} pentaene (Kuenen et al. 2010). Furthermore, Kuenen et al. (2010) found no evidence that other components found in gland extracts, Z11,E13-16:Ald, E11,Z13-16:Ald, Z11,Z13-16:Ac, Z11-16:Ald, 16:Ald, hexadecan-1-ol, and C_{25} pentaene, were components of the pheromone blend.

The aim of the work reported here was to delineate and define the optimal attractive pheromone blend of the navel orangeworm. Our specific objectives were to conduct wind tunnel bioassays to assess: 1. Attraction of male moths to the major component, Z11,Z13-16:Ald, plus one, two, or three of the additional minor components that had been implicated in previous studies; 2. attraction to various blends in which the proportion of a single component was varied systematically while holding the ratio of the other components fixed; 3. the effect of adding other compounds, identified in pheromone gland extracts, to an optimized blend of 4 components; 4. the effect of dose of the pheromone blend on responses of male *A. transitella*.

Methods and Materials

Insects Our colony of *A. transitella* was established with eggs obtained from the USDA, Agricultural Research Service, Parlier, CA, USA, from a colony founded from individuals collected in 2001 from walnuts in Fresno County, CA, USA. Larvae were reared in 3.8-l jars on a red flaky wheat diet (Coffelt et al. 1979a). Cultures were maintained at 26°C, with a photoperiod of 16:8L:D and 50–60% relative humidity. Approximately 80 last instar males were removed daily from the colony and placed in 180-ml plastic cups (20 per cup). These larvae were held in a controlled temperature cabinet separated from the rearing colony, under a reversed photoperiod, 14:10L:D regime at 25°C. Males that emerged each day were transferred to a 30×30×30 cm plastic screen cage, where they were held with access to 10% sucrose solution. All individuals used in bioassays were 2–4-d-old.

Wind-tunnel Bioassays A wind tunnel (1 m wide×1 m high×3 m long), described in Justus et al. (2002), was used for all experiments. Because male *A. transitella* often ascend 15 cm or more after take-off in a pheromone plume, the 1 m height of this tunnel was essential for the bioassay. The wind speed was set to 50–60 cm s^{-1} by regulating two variable-speed fans, one on the tunnel input and the other on the exhaust. The wind speed was verified with a Barnant Tri-Sense (Cole-Parmer 37000-61) anemometer. All air flowing through the tunnel was exhausted from the building. The tunnel's floor was covered with white fabric, and red paper circles, 5.4 cm diam., set randomly (30 per m^2) on the floor in a non-overlapping pattern to provide non-directional visual cues. All experiments were conducted at 24–26°C with a relative humidity of 50–60%. Two lateral banks of 25-W, clear red (Philips Colortone R) and 25-W tungsten lights, on each side of the room, illuminated the ceiling and were set with a rheostat to provide a diffuse light of 7–10 lux in the wind tunnel. A Sanyo VCB-3512T monochrome CCD camera (shutter speed, 1/100 sec), with a 75 mm lens, was mounted at the side of the wind tunnel to record male behavior at the pheromone source (see below).

All experiments were conducted during the last 3 hr of scotophase, the period when female *A. transitella* call and mate (Coffelt et al. 1979b). Test males were brought into the assay room and transferred individually into a small wire mesh cylindrical (3 cm diam.×5 cm high) cage, with one end open and the other closed. Males were acclimated to the conditions of the assay room for 60–180 min. before being tested. To test male responsiveness to a pheromone/odor source, a cage was placed, closed-end facing upwind, on a wire-mesh stand set 21 cm above the tunnel floor and 160 cm downwind of the pheromone source. Once a male began wing fanning, the cage was rotated 180°, thereby allowing him to initiate upwind flight. Test males were used once and discarded.

The following were noted:

Percentage taking flight: proportion of males initiating wing fanning that take flight within 1 min. of the cage being placed on the stand in the pheromone plume.

Latency of taking flight: time from cage being placed on stand in pheromone plume until the initiation of flight.

Percentage of source contact: proportion of males locking onto the plume that landed on the pheromone source.

Latency of source contact: time from initiation of flight to landing on the pheromone source.

Duration of source contact: time of continuous contact with the source after landing. All males walked and wing fanned while in contact with the source. If a male

flew off the source and then re-contacted it (a relatively rare occurrence), we did not consider time spent in a subsequent landing in our measurement of duration of source contact. Observations were terminated after 2 min. of continuous source contact.

Odor Sources All compounds used were synthesized as described in detail in Kuenen et al. (2010). Stock solutions (1 mg/ml in heptane) were formulated from compounds that were re-purified (see Kuenen et al. 2010) immediately before making up the solutions, and had the following purities (known impurities): Z11,Z13-16:Ald, >98% pure (~0.6% Z11,E13-16:Ald and ~0.6% E11,Z13-16:Ald); Z11, Z13-16:OH, >98% chemically and isomerically pure; C_{23} pentaene, >99% pure; Z11,E13-16:OH, >96% pure. Working solutions were prepared by dilution of stock solutions in hexane. Point sources of odor were created by applying 20 µl of a hexane solution of a particular blend to a 1-cm diam. filter paper disk (Whatman #1) mounted on a #3 insect pin. The solvent was allowed to evaporate for 3 min. before the paper disks were placed in glass vials until used (within 2 hr). The filter paper disk was pinned vertically into the eraser of a pencil, 22 cm above the tunnel floor. Each source was placed 100 cm from the upwind end of the tunnel and used for no longer than 20 min.

Statistical Analyses In each experiment, a treatment was blocked daily as a group of five males; within the block, five males were tested consecutively. The order in which treatments were tested was randomized. All treatments were tested on each test day. The proportions of individuals initiating flight or contacting the lure were analyzed for differences by treatment using a *G*-test in SAS 9.1 (SAS Institute 2004). Results from asymptotic and exact tests were the same in all instances. Results of the exact test were reported because effective sample sizes for moths making contact with the lure in some instances were small. Time to initiation of flight, time to lure contact, and the duration of contact, were compared by treatment using Minitab's Regression with Life Data in Minitab 14 (Minitab Statistical Software 2004). The data for each of the above variables were determined to fit a lognormal distribution adequately ($P>0.94$). Treatments with $N<10$ were excluded from analyses, but their means and standard deviations are reported.

Results

Our first experiment tested the effects of addition of one, two, or all three, of these compounds added to Z11,Z13-16:Ald (Fig. 1) in the 100:100:5:15 ratio used by Kuenen et al. (2010). Z11,Z13-16:Ald, as a single component, elicited some activation and flight initiation (59%), but no oriented upwind flight along the plume; no moths reached the

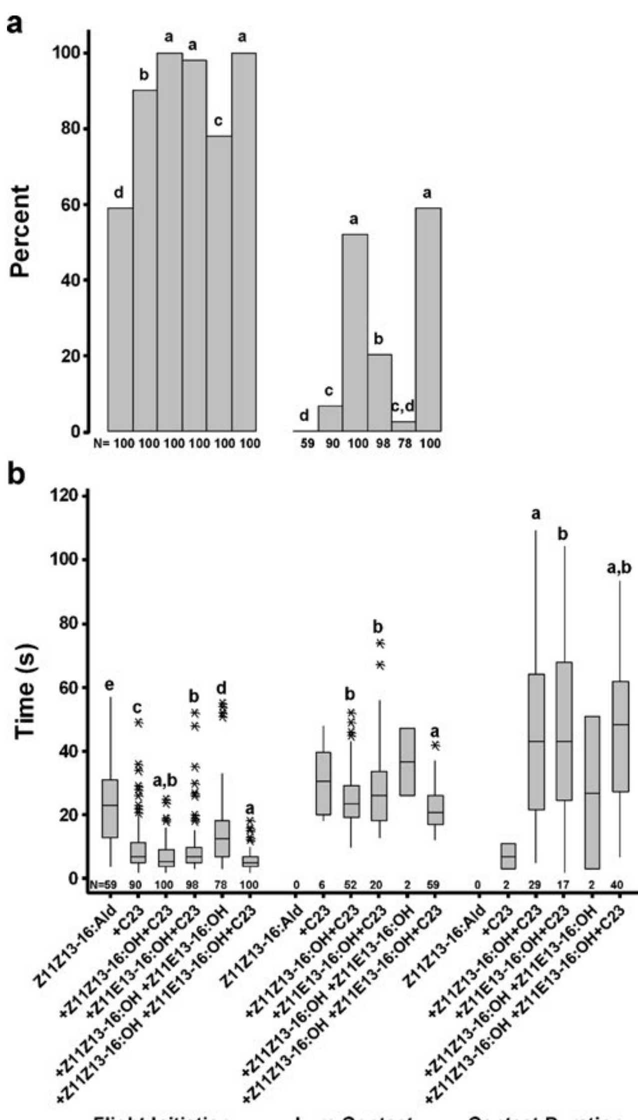

Fig. 1 Flight responses of male *Amyelois transitella* to various pheromone blends containing (11*Z*,13*Z*)-hexadecadienal (Z11Z13-16:Ald; in all blends), (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene (C23), (11*Z*,13*Z*)-hexadecadien-1-ol (Z11Z13-16:OH), or (11*Z*,13*E*)-hexadecadien-1-ol (Z11E13-16:OH). **a** Percentage of males that initiated flight within 60 sec of exposure to lure, and percentage of males that contacted a lure. N indicates the number of males tested for each response. **b** Time to flight initiation (censored at 60 sec), time to lure contact, and contact duration (censored at 120 sec). Boxes indicate 25th and 75th percentiles, bars inside boxes are medians, whiskers denote the range, and asterisks represent outliers. Z11Z13-16:Ald , Z11Z13-16:Ald + C23 and Z11Z13-16:Ald + Z11Z13-16:OH + Z11E13-16:OH were not included in the analysis of time to contact and contact duration because of inadequate sample sizes. N indicates the number of males tested for each response. Different letters above columns or boxes [in (a) or (b)] indicate significant ($P<0.05$) differences

source. Addition of C_{23} pentaene to Z11,Z13-16:Ald increased the proportion of moths initiating flight, and reduced the mean latency to flight, but the percentage of moths contacting the source was still low (6.7%). Similarly, the blend of Z11,Z13-16:Ald + Z11,Z13-16:OH + Z11,E13-16:OH resulted in greater flight initiation and a shorter mean latency to flight than recorded to Z11,Z13-16:Ald alone but, again, there were few source contacts (2.6%). Addition of either Z11,Z13-16:OH or Z11,E13-16:OH, or both, to the blend of Z11,Z13-16:Ald and C_{23} pentaene, resulted in high levels of flight initiation (98–100%), and short mean latencies (7.3–9.6 sec).

The 4-component mixture evoked the most rapid initiation of flight, followed by the two 3-component mixtures containing C_{23} pentaene, the mixture containing Z11,Z13-16:Ald and C_{23} pentaene, and the remainder of the treatments (Fig. 1). The time of source location followed the same trends seen in percentages of source location. There were 3 treatments that had sufficient numbers of males locating the source that permitted an analysis of latencies of source location (Fig. 1). The 4-component mixture evoked the most rapid source location, followed by the two 3-component mixtures containing C_{23} pentaene. The duration of source contact was longest for the 4-component blend and the Z11,Z13-16:Ald, Z11,Z13-16:OH, and C_{23} pentaene blend, and somewhat shorter for the mixture of Z11,Z13-16:Ald, Z11,E13-16:OH, and C_{23} pentaene.

Taken together, these comparisons demonstrate that a high proportion of source location required Z11,Z13-16:Ald, C_{23} pentaene, and either Z11,Z13-16:OH or Z11,E13-16:OH, or both. Although the 3-component blend of Z11,Z13-16:Ald, Z11,Z13-16:OH, and C_{23} pentaene was statistically indistinguishable from the full 4-component blend for percentage of moths locating the pheromone source, the 4-component blend evoked significantly more rapid flight to the source, supporting the role of Z11,E13-16:OH as a component of the optimal blend. In a follow-up wind tunnel test, aimed at verifying the role of Z11,E13-16:OH and comparing the same series of blend components, the proportion of moths contacting the lure was highest (all 3 treatments differed at $P<0.05$, Hantel-Haenszel-Cochran test) for the 4-component mixture (80.0%), over the Z11,Z13-16:Ald, Z11,Z13-16:OH and C_{23} pentaene blend (56.7%), and the Z11,Z13-16:Ald, Z11,E13-16:OH and C23 pentaene mixture (21.7%), confirming that all 4 compounds are involved in eliciting the full behavioral response.

The second experiment tested the effects of adding different proportions of C_{23} pentaene to a base blend, consisting of Z11,Z13-16:Ald, Z11,Z13-16:OH, and Z11,E13-16:OH (100:100:5). Although the C_{23} pentaene was required for source location, males were remarkably insensitive to the percentage (from 1.5 to 150% of Z11,Z13-16:Ald) of this compound in the 4-component blend (Fig. 2). Based on these results, in further tests we used a 100:15 ratio of Z11,Z13-16:Ald to C_{23} pentaene, equivalent to the optimum ratio used in bioassays of Kuenen et al. (2010).

In the third experiment, the proportion of Z11,Z13-16:OH was varied while holding the ratio of Z11,Z13-16:Ald, C_{23} pentaene, and Z11,E13-16:OH constant (100:15:5). Four-component blends, that varied greatly in the proportion of Z11,Z13-16:OH, elicited similar levels of attraction

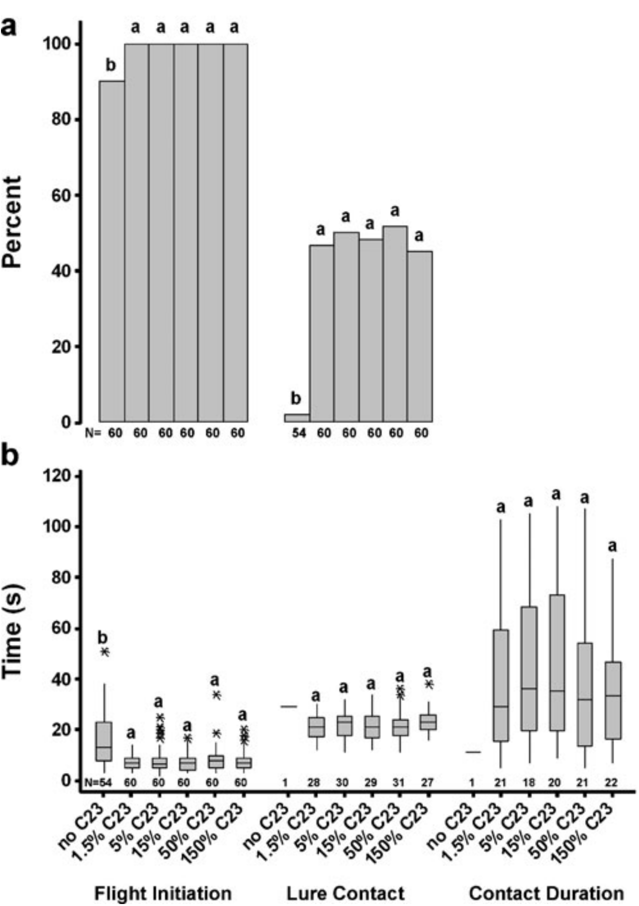

Fig. 2 Flight responses of male *Amyelois transitella* to pheromone blends with different amounts of (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene (C23). All treatments contain (11*Z*,13*Z*)-hexadecadienal (10 ng), (11*Z*,13*Z*)-hexadecadien-1-ol, and (11*Z*,13*E*)-hexadecadien-1-ol in a 100:100:5 ratio. The percentage of C23 is relative to the amount of (11*Z*,13*Z*)-hexadecadienal. **a** Percentage of males that initiated flight within 60 sec of exposure to lure, and percentage of males that contacted a lure. N indicates the number of males tested for each response. **b** Time to flight initiation (censored at 60 sec), time to lure contact, and contact duration (censored at 120 sec). Boxes indicate 25th and 75th percentiles, bars inside boxes are medians, whiskers denote the range, and asterisks represent outliers. The treatment lacking C23 was not included in the analysis of time to contact and contact duration due to inadequate sample size. N indicates the number of males exhibiting a response. Different letters above columns or boxes [in (a) or (b)] indicate significant ($P<0.05$) differences

and source contact by males; ratios of Z11,Z13-16:OH to Z11,Z13-16:Ald of 30:100 to 300:100 were statistically indistinguishable for all metrics (Fig. 3). Thus, for subsequent experiments, we used the intermediate ratio of 100:100 Z11,Z13-16:Ald to Z11,Z13-16:OH.

Varying the proportion of Z11,E13-16:OH, while holding the ratio of the three other components constant (Z11,Z13-16:Ald to Z11,Z13-16:OH to C_{23} pentaene, 100:100:15), also had little effect on male responses (Fig. 4). Percent source contact increased with inclusion of Z11,E13-16:OH in the blend, but all other metrics were not affected significantly by the presence or absence of this component.

A dose-response comparison using 1, 10, 100, and 1,000 ng, all with the 4-component blend at the optimized ratio, showed that the 10-ng dose elicited the best overall response when comparing across all metrics (Fig. 5). The 100-ng dose resulted in a similar percentage of source contacts, but moths took longer to reach the source and

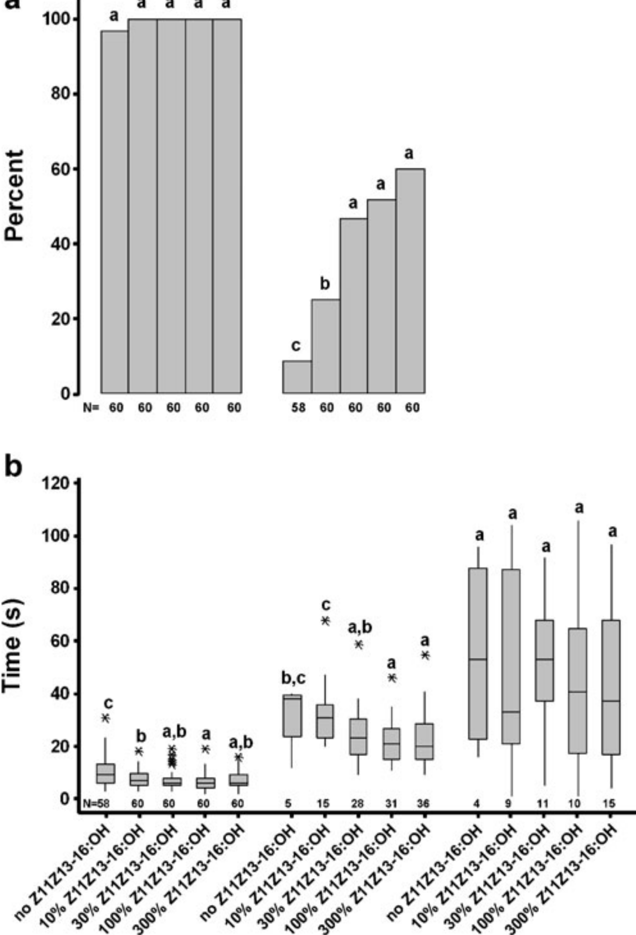

Fig. 3 Flight responses of male *Amyelois transitella* to pheromone blends with different amounts of (11*Z*,13*Z*)-hexadecadien-1-ol (Z11Z13-16:OH). All treatments contain (11*Z*,13*Z*)-hexadecadienal (10 ng), (11*Z*,13*E*)-hexadecadien-1-ol and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene in a 100:5:15 ratio. The percentage of Z11Z13-16:OH is relative to the amount of (11*Z*,13*Z*)-hexadecadienal. **a** Percentage of males that initiated flight within 60 sec of exposure to lure, and percentage of males that contacted a lure. N indicates the number of males tested for each response. **b** Time to flight initiation (censored at 60 sec), time to lure contact, and contact duration (censored at 120 sec). Boxes indicate 25th and 75th percentiles, bars inside boxes are medians, whiskers denote the range, and asterisks represent outliers. N indicates the number of males exhibiting a response. Different letters above columns or boxes [in (a) or (b)] indicate significant (P<0.05) differences

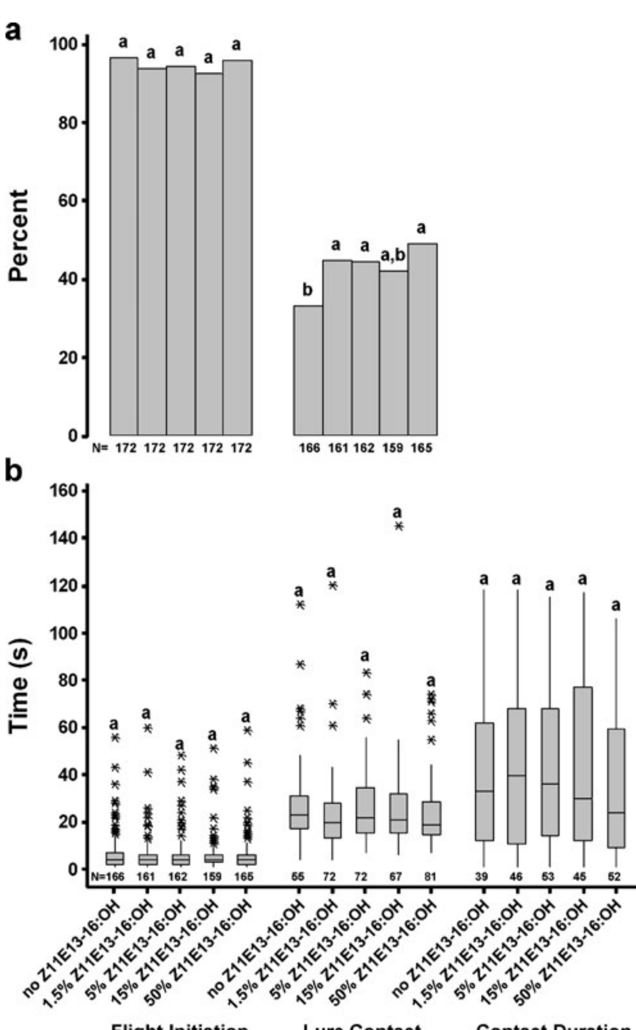

Fig. 4 Flight responses of male *Amyelois transitella* to pheromone blends with different amounts of (11*Z*,13*E*)-hexadecadien-1-ol (Z11E13-16:OH). All treatments contain (11*Z*,13*Z*)-hexadecadienal (10 ng), (11*Z*,13*Z*)-hexadecadien-1-ol and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene in a 100:100:15 ratio. The percentage of Z11E13-16:OH is relative to the amount of (11*Z*,13*Z*)-hexadecadienal. **a** Percentage of males that initiated flight within 60 sec of exposure to lure, and percentage of males that contacted a lure. N indicates the number of males tested for each response. **b** Time to flight initiation (censored at 60 sec), time to lure contact, and contact duration (censored at 120 sec). Boxes indicate 25th and 75th percentiles, bars inside boxes are medians, whiskers denote the range, and asterisks represent outliers. N indicates the number of males exhibiting a response. Different letters above columns or boxes [in (a) or (b)] indicate significant (P<0.05) differences

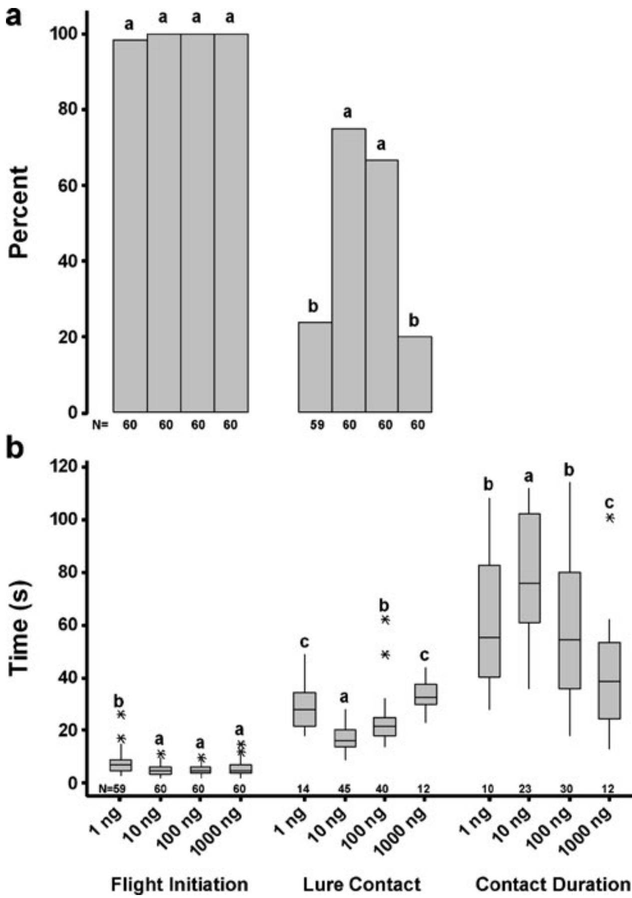

Fig. 5 Flight responses of male *Amyelois transitella* to 4 doses of a 100:100:5:15 ratio of the optimized 4-component pheromone blend of (11*Z*,13*Z*)-hexadecadienal, (11*Z*,13*Z*)-hexadecadien-1-ol, (11*Z*,13*E*)-hexadecadien-1-ol, and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene. Dose = amount of (11*Z*,13*Z*)-hexadecadienal. **a** Percentage of males that initiated flight within 60 sec of exposure to lure, and percentage of males that contacted a lure. N indicates the number of males tested for each response. **b** Time to flight initiation (censored at 60 sec), time to lure contact, and contact duration (censored at 120 sec). Boxes indicate 25th and 75th percentiles, bars inside boxes are medians, whiskers denote the range, and asterisks represent outliers. N indicates the number of males exhibiting a response. Different letters above columns or boxes [in (a) or (b)] indicate significant ($P<0.05$) differences

remained in contact with the source for less time than for the 10 ng dose. Both the 1-ng and the 1,000-ng doses resulted in markedly less source contact, longer latencies for source location, and shorter times in contact with the source, than were observed with the 10-ng dose.

The final experiment tested the effects of adding four other compounds that had been identified in pheromone gland extracts [(3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene, ethyl hexadecanoate, Z11,Z13-16:COOEt, and Z11,Z13-16:Ac (Leal et al. 2005; Kuenen et al. 2010), to the optimized 4-component blend. None of these compounds, added to the 4-component blend in the proportions reported by Leal et al. (2005), significantly affected any measure of attraction (flight latency, proportion contacting the source, time to lure contact, or duration of lure contact) compared to the responses to the 4-component blend (data shown in supplementary material).

Discussion

The *A. transitella* pheromone was characterized initially as Z11,Z13-16:Ald by Coffelt et al. (1979a), but this component alone has proven to be minimally attractive in field tests over the last two decades (J.G.M. unpublished data). A number of possible additional components of the *A. transitella* pheromone were described by Leal et al. (2005), but this study did not define the subset of compounds (and their optimal ratio) that was both necessary and sufficient to obtain high attraction. Iterative refinement of the active blend did not prove trivial, requiring several years of wind tunnel and field trials (Kuenen et al. 2010). The present study has expanded on these previous reports by examining the roles of the various compounds in mediating the sequential steps in attraction to the source.

The wind-tunnel assay allowed assessment of the effects of a wide range of component combinations and ratios on sequential stages of the male moths' flight responses, from activation of quiescent moths, to initiation of flight, flight upwind along the pheromone plume, landing on the pheromone source, and time spent in contact with the odor source. As in past work with wind tunnel assays (e.g., Linn et al. 1988; Zhu et al. 1999; Trimble and Marshall 2008), the most diagnostic measure of attraction was the proportion of males reaching and contacting a point source of odor. However, in the present study, several other metrics, including latencies of response and lure contact, were consistent with the proportion of lure contact. The duration of lure contact was less discriminatory among treatments: once males landed on an odor source, they spent many seconds wing fanning and walking on the source, even if the blend was not as attractive as other blends.

The data from our study reinforced the work of Kuenen et al. (2010), by verifying that C_{23} pentaene and Z11,Z13-16:OH are components of the pheromone blend. Z11,E13-16:OH had a less marked effect on the attraction of male moths to blends than C_{23} pentaene or Z11,Z13-16:OH, but still demonstrably affected the overall suite of behaviors.

One of the unexpected results from our studies was the general lack of sensitivity to component ratios by male *A. transitella*, especially given the importance of a precise component ratio in attraction of other moth species (reviewed in Cardé and Haynes 2004). This is likely because production of consistent blend ratios by females is more difficult when components have major structural

differences (Roelofs and Cardé 1977). Furthermore, probably because C_{23} pentaene is biosynthesized in oenocytes and then transported to the pheromone gland (Wang et al. 2010), whereas the other fatty acid-derived components are biosynthesized *de novo* in the gland, it is possible that the blend emitted by females may change during a calling bout as the available C_{23} pentaene becomes depleted.

Male *A. transitella* were sensitive to the dose of pheromone, with attraction being maximal to 10 ng and dropping to higher doses. This peak of response might be indicative of male response being linked to an optimum dose of pheromone, as has been noted with *Grapholita molesta* (Baker and Cardé 1979) and other moth species. Alternatively, the drop in attraction with increasing dose may be the result of small percentages of antagonistic impurities in the synthetic pheromone, which reached the threshold value required to affect the behavior of males, as the overall pheromone dose increased.

Our results, in combination with the data reported by Kuenen et al. (2010), suggest that other minor components found in gland extracts, including (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene, ethyl hexadecanoate, ethyl (11*Z*,13*Z*)-hexadecadienoate, and (11*Z*,13*Z*)-hexadecadienyl acetate (Leal et al. 2005; Kuenen et al. 2010), play no role in attraction of male *A. transitella*. However, the latter compound antagonizes attraction of a sympatric species, the meal moth *Pyralis farinalis* L. (Leal et al. 2005; Kuenen et al. 2010) that also uses both Z11,Z13-16:Ald and C_{23} pentaene as components of its pheromone blend. Thus, this compound, and possibly some of the other minor components, may function as behavioral antagonists mediating interspecific interactions.

This characterization of attractive blends for *A. transitella* will help advance the development of field lures for surveillance and population monitoring of this important pest. However, the optimal blends described in this study are not yet ready for operational use, because there are lingering problems with longevity of lures in the field. In particular, Kuenen et al. (2010) reported that pheromone lures decreased dramatically in activity within a day of being deployed, for a variety of lure types that incorporated both an antioxidant and an ultraviolet light stabilizer in their formulations. This suggests that male *A. transitella* are extremely sensitive to degradation of one or more of the components of the blend under field conditions. We have not noted analogous decreases in activity of test solutions in our wind tunnel trials carried out under low-light conditions. This suggests that photooxidation processes occurring in sunlight may be responsible for the rapid loss of activity of field lures.

Past work on mating disruption of *A. transitella* has centered mainly on the use of widely spaced (5 per ha^{-1}) "puffers", which dispensed metered doses of technical grade Z11,Z13-16:Ald in aerosol puffs at set intervals (Shorey and Gerber 1996). This disruptant system has been reported to be effective in suppressing male attraction to traps baited with virgin females and in reducing damage levels in almonds and pistachios (Higbee and Burks 2008), likely acting via the mechanism of "sensory impairment." Use of the complete blend might increase efficacy of disruption, however, especially if the pheromone were formulated with point sources that were attractive (Minks and Cardé 1988; Cardé and Minks 1995; Cardé 2007).

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Male and Female Noctuid Moths Attracted to Synthetic Lures in Europe

Miklós Tóth · István Szarukán · Béla Dorogi · Attila Gulyás · Pál Nagy · Zoltán Rozgonyi

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Abstract In field tests in Europe, traps baited with a blend of isoamyl alcohol, acetic acid, and isobutanol (compounds previously found attractive to a number of noctuids in North America) caught the following noctuid moths: *Agrotis segetum* Schiff., *Agrotis crassa* Hbn., *Agrotis exclamationis* L., *Amathes (Xestia) c-nigrum* L., *Apatele rumicis* L., *Amphipyra pyramidea* L., *Dipterygia scabriuscula* L., *Discestra trifolii* Hfn., *Euxoa aquilina* Schiff., *Euclidia glyphica* L., *Mamestra brassicae* L., *Mamestra oleracea* L., *Mamestra suasa* Schiff., *Mythimna albipuncta* Den. & Schiff., *Mythimna l-album* L., *Noctua pronuba* L., and *Trachea atriplicis* L. A substantial percentage of the catch of each species of moths was females. The presence of isobutanol in the mixture was important for catching *A. rumicis, D. trifolii*, and *E. glyphica*. The addition of 3-methyl-1-pentanol to the ternary mixture did not increase trap captures of any of the moths. Traps baited with the floral attractant phenylacetaldehyde alone caught several species of noctuid moths. However, when phenylacetaldehyde was added to the isoamyl-alcohol ternary blend, no increases in catches of any of the species, relative to the ternary blend or phenyacetaldehyde alone, were observed, with catches of most species being depressed. Comparing the noctuid species attracted to the phenylacetaldehyde- and isoamyl alcohol-based lures showed that phenylacetaldehyde attracted predominantly Plusiinae and Melicleptriinae spp., while isoamyl alcohol-based lures attracted species mostly from the Noctuinae or Hadeninae subfamilies.

Keywords Female attractant · Isoamyl alcohol · Isobutanol · Acetic acid · Phenylacetaldehyde · Lepidoptera · Noctuidae

M. Tóth (✉)
Plant Protection Institute, HAS,
Budapest Pf 102, H-1525, Hungary
e-mail: h2371tot@ella.hu

I. Szarukán · B. Dorogi · A. Gulyás · P. Nagy · Z. Rozgonyi
Faculty of Agricultural Sciences, Department of Plant Protection,
University of Debrecen,
Debrecen, Hungary

Present Address:
P. Nagy
Gárdonyi Géza Mg. Zrt,
Agárd, Hungary

Introduction

The Noctuidae is one of the largest and most important families of Lepidoptera, both by total number of species and by economic importance of agricultural pests. The detection and monitoring of flight activity of adult males of many pest noctuid species is performed routinely by using traps baited with sex pheromone. Such sex pheromone-baited traps have proven to be highly sensitive and selective, but they have the inherent weakness for pest control of attracting only male moths. Traps catching female moths would potentially provide more valuable information for pest control decisions. Isoamyl alcohol (3-methyl-1-butanol) or 3-methyl-1-pentanol (naturally occurring in fermenting molasses), in combination with acetic acid, has been found to attract females of several noctuid pests in North America (Landolt 2000; Landolt and Alfaro 2001; Landolt and Highbee 2002). The related isobutanol (2-methyl-1-propanol), in combination with acetic acid, also is known to attract yellowjacket spp. (Hymenoptera, Vespidae) (Landolt 1998; Landolt et al. 1999, 2000). Furthermore, phenylacetaldehyde is a well-known floral

compound that attracts both sexes of several noctuid species, as well as other Lepidoptera (e.g., Creighton et al. 1973; Cantelo and Jacobson 1979).

The principal objective of this study was to test the above compounds for ability to attract European noctuid species, with a particular application as female attractants in pest control programs. Also, we aimed to determine whether there were any synergistic interactions, in attraction to noctuid species, between the various alcohols/acid, produced by fermenting molasses and the floral attractant phenylacetaldehyde.

Methods and Materials

Traps The standard CSALOMON® VARL + funnel traps, produced by the Plant Protection Institute, HAS (Budapest, Hungary) (Tóth et al. 2000, 2002; Subchev et al. 2003) were used. This trap consists of an opaque plastic funnel (top opening, 13 cm o.d., funnel-hole diam., 3 cm, height of funnel, 16 cm), with a 20 × 20 cm flat, plastic roof, and a transparent plastic round container (ca 1 l, held in place by a rubber band). Photos of the trap can be viewed at <www.julia-nki.hu/traps>. The lure was suspended from the middle of the roof and positioned slightly above the level of the upper edge of the large funnel opening. A small piece (1 × 1 cm) of household anti-moth strip (Chemotox®, Sara Lee, Temana Intl. Ltd, Slough, UK; active ingredient 15% dichlorvos) was placed in the container to kill captured insects.

Baits Isoamyl alcohol, isobutanol (2-methyl-1-propanol), 3-methyl-1-pentanol, phenylacetaldehyde, and acetic acid were obtained from Sigma-Aldrich Kft. (Budapest, Hungary) and were stated by the suppliers to be >95% pure. The compounds were loaded onto a 1-cm piece of dental roll (Celluron®, Paul Hartmann Ag. Heidenheim, Germany), and placed inside a polyethylene bag (ca 1.0 × 1.5 cm; 0.02 mm-thick polyethylene foil). The lure dispensers were heat sealed and attached to 8 × 1 cm plastic strips for easy handling when assembling the traps. Lures were wrapped singly in pieces of aluminum foil and stored at −18°C until used. One or three lures were used in the traps to test for effect of lure concentration. Lures in traps were changed at 2–3 wk intervals, as previous experience with similar lures showed that they may lose activity after this period (Tóth et al. 2002).

Trapping Tests were conducted at several sites in Hungary. Traps were suspended at a height of ca 1.0–1.5 m. One replicate of each treatment was incorporated into a block, so that treatments were 8–10 m apart, and blocks were 30–50 m apart.

Experiment 1 was a preliminary, unreplicated test, run at Debrecen, Hajdú-Bihar County, May 3–October 18, 2001. Treatments included binary blends of isoamyl alcohol with acetic acid, isobutanol with acetic acid, and the ternary mixture of all three compounds. Each compound was loaded at 200 mg.

Experiment 2 studied the addition of 10, 30, and 100 mg of isobutanol to the binary isoamyl alcohol: acetic acid mixture (1:1, 100 mg each). Unbaited traps were included as a control. Site: Debrecen, July 1–October 10, 2003, with 4 blocks of traps.

Experiment 3 checked the addition of 3-methyl-1-pentanol (200 mg) to the ternary isoamyl alcohol + acetic acid mixture + isobutanol (1:1:1, 200 mg each) mixture. Unbaited traps were used as a control. Site: Debrecen, July 1–October 10, 2002, with 4 blocks of traps.

Experiment 4 compared the activity of the ternary isoamyl alcohol + acetic acid + isobutanol mixture (1:1:1, 200 mg each) against phenylacetaldehyde (200 mg). Unbaited traps were used as a control. Site: Túrkeve, Jász-Nagykun-Szolnok county, May 2–September 22, 2003, with 5 blocks of traps.

Experiments 5–6 compared the activities of the ternary isoamyl alcohol + acetic acid + isobutanol mixture (1:1:1, 200 mg each), phenylacetaldehyde (200 mg), or the combination of the two baits inside one trap. Unbaited traps were included in the tests as a control. Both tests were run with 5 blocks of traps, Experiment 5: Csenger, Szabolcs-Szatmár County, May 14–September 20, 2004; Experiment 6: Debrecen, May 11–October 11, 2004.

Experiments 7–8 compared the activity of the ternary isoamyl alcohol + acetic acid + isobutanol female-targeted lure (1:1:1, 200 mg each) with that of the respective sex pheromone baits for *Agrotis segetum* Schiff., *Mamestra brassicae* L. and *M. oleracea* L. Pheromone baits used were commercially available lures (Plant Protection Institute, HAS, Budapest, Hungary) Both tests were run with 3 blocks of traps. Experiment 7: Tiszavasvári, Hajdú-Bihar County, May 13–September 25, 2002; Experiment 8: Hajdúnánás, Hajdú-Bihar County, May 14–June 11, 2002.

Statistics The catches (number of insects caught / trap / inspection) from field trapping tests were transformed by $(x+0.5)^{1/2}$ (Tukey 1949, 1955) and analyzed by ANOVA. When the ANOVA showed a significant effect, treatment means were separated by a Games-Howell Test (Games and Howell 1976; Jaccard et al. 1984). In experiments in which one of the treatments caught no insects, the Bonferroni-Dunn test (Dunn 1961) was used to check whether mean catches in other treatments were significantly different from zero or not. All statistical procedures were conducted using the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

In a preliminary test (Experiment 1), substantial numbers of nine noctuid spp. (data not shown, but see Tables 1 and 2 for typical species caught) were caught in traps containing isoamyl alcohol plus acetic acid or the ternary combination with isobutanol. The combination of isobutanol + acetic acid caught few moths. Thus, further experiments concentrated on the isoamyl alcohol/acetic acid mixture. Percentages of females in the traps ranged from 10 to 100%.

Addition of increasing amounts of isobutanol to the isoamyl alcohol + acetic acid blend (Experiment 2), had little effect on catches of *Agrotis exclamationis* L., *Agrotis segetum* Schiff., *Agrotis crassa* Hbn., *Amathes (Xestia) c-nigrum* L., *Euxoa aquilina* Schiff, *Mamestra brassicae* L., *Mamestra oleracea* L., *Mamestra suasa* Schiff., and *Noctua pronuba* L., relative to traps baited with the binary mixture of isoamyl alcohol + acetic acid (Table 1). However, for *Apatele rumicis* L., *Discestra trifolii* Hufn., and *Euclidia glyphica* L., increasing amounts of isobutanol resulted in increased catches (Table 1). Traps baited with the binary mixture of isoamyl alcohol + acetic acid caught significantly more moths for the majority of species than did unbaited traps (Table 1). Again, sizeable percentages of the catches were females, ranging from 18% (for *A. exclamationis*) to 58% (for *M. brassicae*).

The addition of 3-methyl-1-pentanol to the ternary isoamyl alcohol + acetic acid + isobutanol mixture (Experiment 3), had no effect on trap catches of *A. exclamationis, A. segetum, Am. c-nigrum, D. trifolii, M. brassicae, M. oleracea, Mythimna albipuncta* (Denis & Schiff) *Mythimna. l-album* L., *N. pronuba*, or *Trachea atriplicis* L., but resulted in a decreased catch of *Dipterygia scabriuscula* L, relative to catches in traps baited with the ternary blend (Table 2). Traps with the ternary mixture caught significantly more moths of all species than did unbaited traps. The percentages of female moths caught in these traps ranged from 39% (*A. segetum*) to 75% (*M. oleracea*).

Traps baited with the ternary isoamyl alcohol + acetic acid + isobutanol mixture caught significantly more *A. exclamationis, A. segetum, Amphipyra pyramidea* L., *Apatele rumicis* L., *Am. c-nigrum, M. brassicae, M. oleracea*, and *M. suasa* moths than did traps baited with phenylactaldehyde or unbaited controls (Experiment 4**;** Fig. 1). In contrast, traps baited with phenylacetaldehyde caught more moths of *Autographa gamma* L., *Helicoverpa*

Table 1 Mean catches of noctuid moths in field tests in traps baited with mixtures of isoamyl alcohol, acetic acid, and differing amounts of isobutanol, and unbaited (**Experiment 2**). Means with same letter within a given species are not significantly different (*P*=0.05; ANOVA, followed by Games-Howell or Bonferroni-Dunn *post-hoc*)

Species caught	Mean catch/trap/inspection by blends (isoamyl alcohol: acetic acid: isobutanol (amounts in mg)					Total No. caught	% of females
	0 : 0 : 0	100 : 100 : 0	100 : 100 : 10	100 : 100 : 30	100 : 100 : 100		
Agrotis exclamationis	0.00a	0.28b	0.38b	0.32b	0.45b	114	18
Agrotis segetum	0.07a	1.57b	2.02b	2.46b	2.12b	1360	40
Agrotis crassa	0.03a	0.60b	0.40b	0.80b	0.77b	78	50
Amathes c-nigrum	0.01a	0.55b	0.54b	0.78b	0.85b	328	34
Apatele rumicis	0.00a	0.07ab	0.03a	0.21bc	0.36c	67	34
Discestra trifolii	0.04a	0.21b	0.21b	0.41bc	0.58c	195	33
Euclidia glyphica	0.01a	0.11ab	0.28bc	0.34bc	0.43c	93	20
Euxoa aquilina	0.06a	0.37ab	0.29ab	0.40b	0.29ab	92	29
Mamestra brassicae	0.00a	0.13b	0.12b	0.20b	0.21b	66	58
Mamestra olearcea	0.00a	0.25b	0.19b	0.40b	0.38b	160	53
Mamestra suasa	0.00a	0.35b	0.28b	0.34b	0.42b	194	54
Noctua pronuba	0.02a	0.25b	0.32b	0.23ab	0.29b	72	50

Table 2 Mean catches of noctuids in field tests in traps baited with a ternary isoamyl alcohol + acetic acid + isobutanol lure, a quaternary lure (with 3-methyl-1-pentanol added), or unbaited (**Experiment 3**). Means with same letter within a given species are not significantly different (P=0.05; ANOVA, followed by Games-Howell or Bonferroni-Dunn *post-hoc*)

Species caught	Mean catch/trap/inspection			Total No. caught	% of females
	unbaited	ternary blend	quaternary blend		
Agrotis exclamationis	0.00a	0.25b	0.38b	70	39
Agrotis segetum	0.00a	1.79b	1.62b	382	39
Amathes c-nigrum	0.00a	0.21b	0.13b	38	58
Dypterygia scabriuscula	0.00a	1.20c	0.49b	189	49
Discestra trifolii	0.00a	0.14b	0.13b	31	71
Mamestra brassicae	0.00a	0.23b	0.15b	43	49
Mamestra olearcea	0.00a	0.12b	0.17b	32	75
Mythimna albipuncta	0.00a	0.41b	0.33b	83	47
Mythimna l-album	0.00a	0.13b	0.14b	31	61
Noctua pronuba	0.00a	0.25b	0.12b	41	46
Trachea atriplicis	0.00a	0.97b	0.77b	195	49

armigera Hn., *Macdunnoughia confusa* Steph., and *Meristris trigrammica* Hufn. than did traps baited with the ternary mixture or the unbaited controls. *E. glyphica* moths were caught in similar numbers in traps baited with either phenylacetaldehyde or the ternary blend, but catches were significantly higher than those in unbaited traps only for traps baited with 3 dispensers of the ternary mixture. Traps with 3 dispensers of the ternary mixture showed a general tendency of catching more than those with a single dispenser for all species, but the difference was significant only for *A. exclamationis* (Fig. 1). The effect of increased catches with increased number of dispensers was similar for traps baited with phenylacetaldehyde, with only a significant difference for *M. confusa* catches. Female percentages in traps with the ternary mixture ranged from 45% (*E. exclamationis*) to 65% (*M. brassicae*), while in those with phenylacetaldehyde from 11% (*Me. trigrammica*) to 57% (*H. armigera*) (Fig. 1).

In Experiments 5 and 6, traps with both the ternary mixture and phenylacetaldehyde caught significantly fewer moths of *A. exclamationis, A. segetum, Am. c-nigrum, M. brassicae, M. oleracea*, and *M. suasa* than did those baited with only the ternary mixture (Fig. 2). Traps with both phenylacetaldehyde and the ternary mixture also tended to

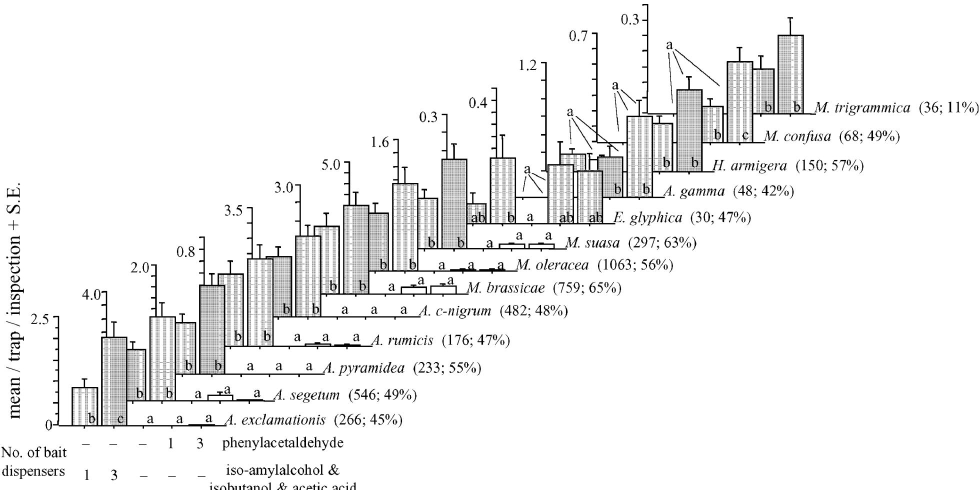

Fig. 1 Mean catches of various noctuid species in field tests in traps baited with one or three dispensers of, a ternary isoamyl alcohol + acetic acid + isobutanol lure, a phenylacetaldehyde lure, or unbaited (Experiment 4). Columns with the same letter within a given species are not significantly different (P<0.05; ANOVA, followed by Games-Howell or Bonferroni-Dunn *post-hoc* tests). Numbers in parentheses after the species show total number of moths caught and % of females in the catch. Species caught were: *Agrotis exclamationis, A. segetum, Amathes c-nigrum, Amphipyra pyramidea, Apatele rumicis, Autographa gamma, Euclidia glyphica, Heliothis armigera, Mamestra brassicae, M. oleracea, M. suasa, Meristris trigrammica*, and *Macdunnoughia confusa*. L

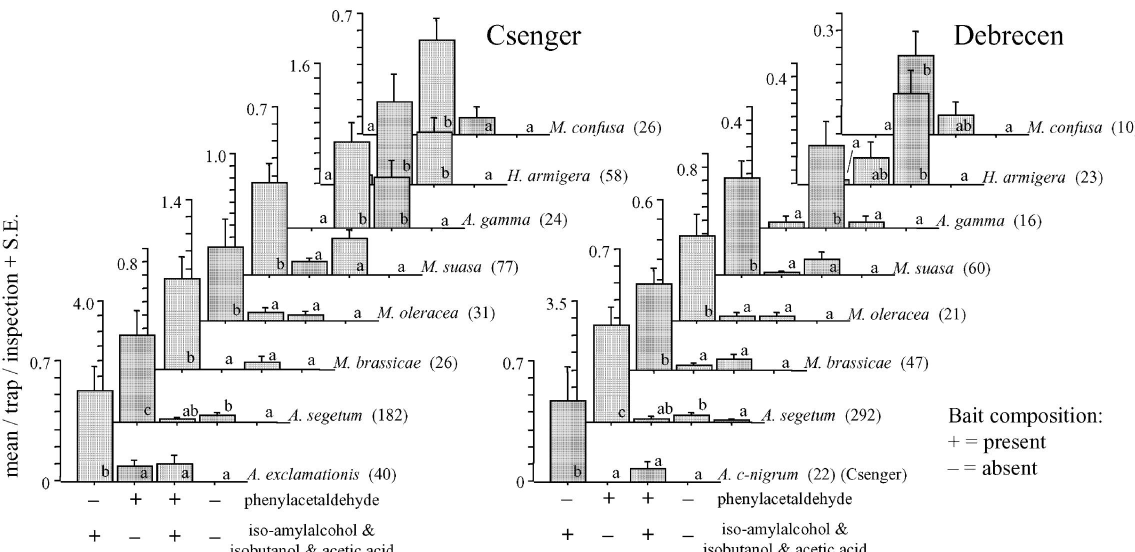

Fig. 2 Mean catches of noctuids in field tests in traps baited with a ternary isoamyl alcohol + acetic acid + isobutanol lure, a phenylacetaldehyde lure, the two types of lures together, or unbaited, at Csenger (Experiment 5) and Debrecen (Experiment 6). Columns with same letter within a given species are not not significantly different ($P<0.05$; ANOVA, followed by Games-Howell or Bonferroni-Dunn *post-hoc* tests). Numbers in parentheses after the species show total number of moths caught. Species caught were: *Agrotis exclamationis, A. segetum*, *Amathes c-nigrum, Autographa gamma*, *Heliothis armigera*, *Mamestra brassicae, M. oleracea*, *M. suasa,* and *Macdunnoughia confusa*

catch fewer moths of *Au. gamma* and *M. confusa* than did traps with phenylacetaldehyde alone, but the difference was significant in only one out of two tests for each species (respectively, Debrecen and Csenger). Traps with phenylacetaldehyde alone or with both types of baits caught similar numbers of *H. armigera*.

In Experiments 7 and 8, the respective sex pheromone-baited traps caught significantly more males of *A. segetum, M. brassicae*, and *M. oleracea* (although the pheromone data were lost for this species in Experiment 7) than did traps baited with the ternary blend of isoamyl-alcohol + acetic acid + isobutanol (which caught ca 5-7%, 25–26%, and 20% of the total amount in the pheromone traps for the respective species). Traps baited with the ternary mixture generally caught more moths of both sexes than did unbaited traps (Table 3).

Discussion

In the present experiments, the blend of isoamyl alcohol, acetic acid, and isobutanol was attractive to male and female moths of 16 species of European noctuids. Previously, isoamyl alcohol, or 3-methyl-1-pentanol, with acetic acid had been shown to attract a North American population of *Am. c-nigrum* (Landolt 2000; Landolt and Alfaro 2001). In this study, we confirmed the attraction of a European population of *Am. c-nigrum* to isoamyl alcohol plus acetic acid. Isoamyl alcohol and acetic acid in a binary mixture was attractive to 13 of the noctuid species, while the presence of isobutanol was important for catching the three additional spp. The addition of 3-methyl-1-pentanol to this ternary mixture did not increase captures of any of these moths. Importantly, the ternary blend attracted a significant proportion of female moths of all the species.

In a study of the species of North American Lepidoptera captured in traps baited with isoamyl alcohol plus acetic acid, Landolt and Hammond (2001) recorded specimens belonging to ca 60 species, 90% of which were Noctuidae. Combined with our European results, this shows that isoamyl alcohol plus acetic acid are probably widely exploited as attractants to food in Noctuidae. The presence of acetic acid and short-chain alcohols generally is attributed to a fermentation process, and may indicate the presence of a carbohydrate source for feeding (Landolt et al. 1999, 2007; Landolt 2000).

We also demonstrated attraction of four noctuid species to phenylacetaldehyde. Phenylacetaldehyde is a floral scent constituent of many flowers (Knudsen et al. 1993), and is known as an attractant of several species of Lepidoptera (e.g., Creighton et al. 1973; Cantelo and Jacobson 1979). Our study confirmed the previously reported attraction of *H. armigera* to this compound (Pawar et al. 1983) or blends containing this compound (Bruce and Cork 2001; Bruce et al. 2002). To our knowledge, the attraction of the other species (*Me. trigrammica*, *M. confusa*, *Au. gamma*) to phenylacetaldehyde has not been reported previously, although the closely related plusiines, *Autographa californica* Speyer and *Trichoplusia ni* Hbn., respond to lures containing this compound (Landolt et al. 2001, 2006).

Table 3 Mean catches of *Agrotis segetum*, *Mamestra brassicae,* and *Mamestra oleracea* in field tests in traps baited with a ternary isoamyl alcohol + acetic acid + isobutanol lure, or the respective pheromone lures (**Experiments 5–6**). Means with same letter within a given species are not significantly different (P=0.05; ANOVA, followed by Games-Howell or Bonferroni-Dunn *post-hoc*)

Species caught	Mean catch/trap/inspection								Total No. caught (both sexes)
	ternary lure		*A. segetum* pheromone		*M. brassicae* pheromone		*M. oleracea* pheromone		
	males	females	males	females	males	females	males	females	
Exp. 7									
A. segetum	0.51b	1.50B	9.59c	0.00A	0.00a	0.00A	not av.	not av.	750
M. brassicae	0.17b	0.57B	0.00a	0.00A	1.83c	0.00A	not av.	not av.	101
M. olearcea	3.57b	3.20B	0.00a	0.00A	0.00a	0.00A	not av.	not av.	465
Exp. 8									
A. segetum	1.46b	1.79B	23.17c	0.00A	0.00a	0.00A	0.00a	0.00A	634
M. brassicae	0.25a	1.46B	0.00a	0.00A	6.29b	0.00A	0.00a	0.00A	192
M. olearcea	14.96b	18.83B	0.00a	0.00A	0.00a	0.00A	160.50c	0.00A	4663

not av. not available, as several of the traps from this treatment were missing

The species caught in greatest numbers in the study of Landolt and Hammond (2001) in North America belonged to the subfamilies Amphipyrinae, Hadeninae, and Noctuinae. Similarly, the majority of species caught in our study in traps baited with isoamyl alcohol-based lures also belonged to these subfamilies. In contrast, the moths caught in traps baited with phenylacetaldehyde belonged to the subfamilies Plusiinae (2 spp.), Melicleptriinae (1 sp.) and Amphipyrinae (1 sp.). The different subfamily distribution of species that respond to isoamyl alcohol- and phenylacetaldehyde-based lures may reflect general differences in feeding habits of the respective noctuids. It has long been known to moth collectors that *Autographa* (Plusiinae) and *Helicoverpa* (Melicleptriinae) spp. frequent and feed on flowers, while *Agrotis* (Noctuinae) or *Mamestra* (Hadeninae) spp. do not (e.g., Abafi-Aigner 1907; Balachowsky 1972). Interestingly, we found traps containing both isoamyl alcohol- and phenylacetaldehyde-based baits tended to catch fewer moths than traps with the respective single baits. The reason for this negative interactive effect of the two types of compounds is unknown.

Among the species captured in the present study, *Au. gamma*, *A. segetum, A. crassa, A. exclamationis, Am. c-nigrum, D. trifolii, E. aquilina, H. armigera, M. brassicae, M. oleracea, M. suasa,* and *N. pronuba* are regarded as agricultural pests, with, *Au. gamma, A. segetum, H. armigera, M. brassicae,* and *M. oleracea* among the most important economic noctuid pests in Europe (e.g., Balachowsky 1972; Mészáros 1993). In experiments (7 and 8) that compared the isoamyl alcohol-based lures against pheromone lures, relatively high percentages (20–25% of catches in the respective pheromone traps) of *M. brassicae* and *M. oleracea* moths were caught in traps baited with isoamyl alcohol-based lures. Therefore, for these species, especially with further optimization of lures, these isoamyl alcohol-based attractants could become a useful tool. For instance, they could be used to attract both females and males in a lure and kill strategy, thus lowering both the adult moth population as well as the potential offspring of the female. A previous study that used phenylacetaldehyde and a sucrose-methomyl toxicant yielded promising results with mortality of 61% (females) and 44% (males) of released *T. ni* moths (Landolt et al. 1991). However, for *A. segetum* trapped in Experiments 7 and 8, very low percentages of moths (only 5–7% of the numbers in pheromone traps) were caught in traps baited with the isoamyl alcohol-based lures, suggesting that these lures may not be suitable for practical usage for this species.

In summary, isoamyl alcohol-based lures show promise as male and female noctuid moth attractants. A characteristic of the lures tested in this present study is that they attract a number of species, and thus are more general attractants than species-specific sex pheromones. Use of such a single lure would allow several important pests to be trapped in an agricultural system, thereby minimizing monitoring efforts. However, this lack of specificity also can be a disadvantage in that non-target, or even rare and endangered species of moths could be caught. The advantages and disadvantages of the use of such a method should, therefore, be evaluated according to the actual scenario.

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Flying the Fly: Long-range Flight Behavior of *Drosophila melanogaster* to Attractive Odors

Paul G. Becher · Marie Bengtsson · Bill S. Hansson · Peter Witzgall

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Abstract The fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), is a model for how animals sense, discriminate, and respond to chemical signals. However, with *D. melanogaster* our knowledge of the behavioral activity of olfactory receptor ligands has relied largely on close-range attraction, rather than on long-range orientation behavior. We developed a flight assay to relate chemosensory perception to behavior. Headspace volatiles from vinegar attracted 62% of assayed flies during a 15-min experimental period. Flies responded irrespective of age, sex, and mating state, provided they had been starved. To identify behaviorally relevant chemicals from vinegar, we compared the responses to vinegar and synthetic chemicals. Stimuli were applied by a piezoelectric sprayer at known and constant release rates. Re-vaporized methanol extracts of Super Q-trapped vinegar volatiles attracted as many flies as vinegar. The main volatile component of vinegar, acetic acid, elicited significant attraction as a single compound. Two other vinegar volatiles, 2-phenyl ethanol and acetoin, produced a synergistic effect when added to acetic acid. Geosmin, a microbiological off-flavor, diminished attraction to vinegar. This wind tunnel assay based on a conspicuous and unambiguous behavioral response provides the necessary resolution for the investigation of physiologically and ecologically relevant odors and will become an essential tool for the functional analysis of the *D. melanogaster* olfactory system.

Key Words Acetic acid · *Drosophila melanogaster* · Flight behavior · Fruit fly · Geosmin · Long-range attraction · Olfactory system · Vinegar fly · Wind tunnel

P. G. Becher (✉) · M. Bengtsson · P. Witzgall
Chemical Ecology Group, SLU,
23053,
Alnarp, Sweden
e-mail: paul.becher@ltj.slu.se

B. S. Hansson
Department of Evolutionary Neuroethology,
Max Planck Institute for Chemical Ecology,
Jena, Germany

Introduction

What triggers take-off and upwind flight behavior in an insect? Two types of flight initiation are distinguished in the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae): A fast escape in response to threatening visual stimuli and a slow voluntary take-off based on the assessment of a stimulus that reflects the internal physiological state of the animal (Trimarchi and Schneiderman 1995; Allen et al. 2006; Card and Dickinson 2008). Olfactory cues elicit premeditated flights. Following take-off, the odor signal mediates upwind oriented flight and guides the insect towards the source (Budick and Dickinson 2006; Chow and Frye 2008).

The molecular, genetic, and neurosensory knowledge available from *D. melanogaster* provides the most complete picture of an olfactory system, from odorant receptor genes to neuroanatomy and central processing of the olfactory input (Couto et al. 2005; Benton et al. 2007; Jefferis et al. 2007; Dickson 2008). A current challenge is to expand further our view to include also odor-mediated behavior, which would complete the entire integration pathway from ligand-binding to motor output. A distinct behavior, such as the voluntary initiation of odor-mediated flight, is an opportunity to investigate how *D. melanogaster* processes

chemical signals that evoke the behavior of flying into the scented wind.

Towards this goal, we need to improve our knowledge of the behavioral effect of olfactory receptor ligands in *D. melanogaster*. This relies largely on screening of compounds by extracellular recordings and imaging of the input of olfactory receptor neurons into the antennal lobe. The response of *D. melanogaster* to odors is usually studied in trapping or oviposition assays, using small containers, in spite of common long-range orientation to odor sources in nature. Small dimensions, the lack of an air-flow, and long test duration have led to an elevated response to suboptimal stimuli and have limited the discriminative power and sensitivity of such experiments (Vosshall and Stocker 2007). In addition, the chemicals used as olfactory stimuli in current molecular and neurophysiological studies of the *D. melanogaster* olfactory system do not necessarily match the stimuli used by flies for location of food sources and oviposition sites.

Wind tunnels have been employed to investigate odor-mediated upwind flight behavior in *D. melanogaster* (Budick and Dickinson 2006), but have, with the exception of the pheromone compound *cis*-vaccenyl acetate (Bartelt et al. 1985), not been used to identify behavior-modifying chemicals. We report the use of a wind tunnel to study long-range attraction in relation to odor quality and the internal physiological state of the flies. After measuring fly attraction to vinegar and some of its key chemical components, we investigated the effect of fly age, sex, mating state, and starvation on flight response. The results demonstrate that a flight tunnel is a sensitive instrument for the analysis of odor-mediated behavior in *D. melanogaster*.

Methods and Materials

Insects An Oregon R strain of the fruit fly, *D. melanogaster*, was reared on a standard sugar-yeast-cornmeal diet at room temperature (19–22°C) and under a 8:16 hr L:D photoperiod. Newly eclosed flies were removed from the larval diet daily. Adult flies either were transferred and kept in 30-ml plexiglass vials on fresh diet, or were starved up to 3 d on a humidified piece of cotton wool. Thus, the adults were exposed to the larval diet for a limited period (several hours) after eclosion. For tests with mated males or females, newly emerged test flies were kept together with an excess ratio (ca. 1.5:1) of older virgin flies (ca. 1 wk old) of the opposite sex. Flies were anesthetized with CO_2, and the sexes were separated 24 hr before the experiment. For tests with unmated insects, the sexes of newly emerged flies were separated up to 3 hr after eclosion. Exposure to CO_2 did not exceed 5 min, and preparatory control experiments (data not shown) as well as our subsequent flight assays (e.g., Fig. 2b) did not indicate an adverse effect of anesthetization on fly behavior. The flies were tested during the last 3 hr of the photophase.

Headspace Collection, Chemical Analysis, and Chemicals Volatiles were collected from ca. 70 ml balsamic vinegar (Aceto balsamico di Modena, Urtekram, Denmark, aged at least 3 yr) by blowing charcoal-filtered air (0.9 l/min) with an aquarium pump through the vinegar in a 1-l gas wash bottle, exiting through a Super Q trap at the gas outlet. The volatile trap was a 4×40-mm glass tube containing 35 mg Super Q (80/100 mesh; Alltech, Deerfield, IL, USA) held between glass wool plugs (Tasin et al. 2006). Before use, the trap was rinsed with 3 ml of methanol (redistilled >99.9% purity, Merck, Darmstadt, Germany) and *n*-hexane (redistilled >99.9% purity; Labscan, Malmö, Sweden). After 30 min of collecting vinegar headspace volaitles, compounds were eluted with 0.3 ml of redistilled methanol. These samples were analyzed on a gas chromatograph coupled to a mass spectrometer (GC-MS; 6890 GC and 5,975 MS, Agilent Technologies Inc., Santa Clara, CA, USA). The gas chromatograph was equipped with a DB-Wax or an HP-5MS fused silica capillary column (30 m×0.25 mm× 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA or Agilent Technologies). The amounts of acetic acid, 2-phenyl ethanol, acetoin, and ethyl acetate in the samples were quantified in comparison with synthetic standards (Sigma-Aldrich, Stockholm, Sweden). Ethyl acetate and ethanol were quantified in a headspace collection eluted with pentane. The methanol Super Q extracts were re-vaporized in the wind tunnel by the piezoelectric sprayer (see below).

Wind Tunnel Most of our flight assays were conducted in a wind tunnel, which was built of glass and had a flight section of 30×30×100 cm. The airstream in the tunnel (0.25 m/s) was produced by a fan (Fischbach GmbH, Neunkirchen, Germany), which blew air into the tunnel through an array of four activated charcoal cylinders (14.5 cm diam.×32.5 cm long; Camfil, Trosa, Sweden). A 30×30×30 cm compartment at the upwind end of the tunnel held the piezoelectric sprayer (see below) and was separated from the flight section by a polyamide mesh (pore size 0.5×0.5 mm; Sintab, Oxie, Sweden). The downwind end of the tunnel was closed by the same mesh. The tunnel was lit diffusely from above, and light intensity inside the wind tunnel was 13 lux. Temperature ranged from 19°C to 22°C; relative humidity from 35% to 50%.

The speed of the airstream was selected based on preliminary tests of the response of the flies. Significantly more flies (2-d-old, starved for 2 d) flew to the standard stimulus vinegar at 0.25 m/s (62±10% attraction) and 0.15 m/s (54±9% attraction) than at a lower speed of 0.05 m/s (40±11%; ANOVA, $F=6.20$, $df=14$, $P<0.05$).

Odor Delivery Charcoal-filtered air was blown through the wash bottle with balsamic vinegar (similar as described above but without an air filter) or distilled water (MilliQ, Millipore, Bedford, MA, USA), into a teflon tube (0.5 cm diam.), leading to an attached Pasteur pipette. The pipette was facing downward into a wide-mouth 225-ml glass jar (38 mm diam.). The air stream containing the stimulus (e.g., volatiles of authentic vinegar or distilled water) emanated as a wide plume from the opening of the jar, in the center of the upwind end of the tunnel. The shape of the odor plume was verified with titanium oxide. Flies were scored when landing at the tip of the pipette, at the top edge of the jar, or inside the jar. All glassware was heated to 375°C for 8 hr before use.

Headspace samples and solutions of synthetic compounds were released from a piezoelectric sprayer. A microinjection pump (CMA Microdialysis AB, Solna, Sweden) delivered the solutions at a rate of 10 µl/min from a 1-ml syringe, through teflon tubing, to a 25-µl glass capillary tube with an elongated tip. A piezo-ceramic disc (Valvo, Hamburg, Germany) vibrated the glass capillary at ca. 200 kHz, and thus produced an aerosol that evaporated from the tip of the capillary (El-Sayed et al. 1999). The capillary was placed at the vertical midline, 20 cm from the floor, at the upwind end of the tunnel. The dimension of a hexane plume was measured at the downwind end of the tunnel with a photoionization detector (ppbRAE Plus, Scantec, Sävedalen, Sweden). The plume was approximately in the center of the tunnel, and its cross-section was oval, with a vertical axis of ca. 12 cm and a horizontal axis of ca. 7 cm. The piezoelectric sprayer ensured application of odor solutions at a constant rate and known chemical purity. A glass cylinder (60×95 mm diam.) covered with a metal mesh (pore size 2 mm) shielded the vibrating capillary from flies and mechanical damage. When the sprayer was used to deliver the stimuli for the flight assay, flies were scored when landing at the metal mesh.

Initially, four solvents (Table 1) were assayed in the wind tunnel to help to choose an appropriate solvent that would be efficient to elute and dissolve volatile organic compounds without attracting fruit flies by itself. Distilled water (MilliQ) and the redistilled organic solvents methanol, hexane, and heptane (redistilled >99.9% purity, Labscan) were delivered at 10 µl/min (Table 1). Flies were not attracted to the non-polar solvents hexane and heptane, but these were not suitable for dissolving acetic acid, which is the major headspace component of vinegar. Methanol was a suitable solvent for acetic acid and other vinegar compounds, and was used in subsequent tests.

Table 1 Upwind flight and landing of *Drosophila melanogaster* in a wind tunnel in response to solvents delivered from a piezoelectric sprayer

Solvent (10µl/min)	Flies landing at source[a] (% ±SD)[b]
Water	6±9 a
Methanol	4±2 a
Hexane	0 a
Heptane	0 a

[a] Flies were 2-d-old and starved

[b] Percentages (N=5 batches of 20 flies) followed by different letters indicate significant differences (Tukey's test following ANOVA; F=2.12, df=19, P=0.138)

Trapping Study of Diel Flight Period in the Laboratory A trap was made of a 225-ml capped glass jar filled with 25 ml of a 0.1% Triton X-100 (Tamro Medlab AB, Mölndal, Sweden) solution in distilled water. It contained a 30-ml plastic vial that was filled with the bait, balsamic vinegar (the same as used in the wind tunnel assay and chemical analyses) or a macerated banana-water mixture. Distilled water was the control. A plastic lid holding a cut pipette tip allowed the odor to emanate and provided a trap entry for the flies. Traps were placed in plastic trays filled with water. Five replicates of three different trap lures were randomly distributed in the laboratory. Seventy 1- to 3-d-old flies were released into the wind tunnel room (N=3), 4 hr after onset of the photophase, under a 8:16 hr L:D photoperiod. The traps were checked every 2 hr during the photoperiod.

Test Protocol and Statistical Analyses For wind tunnel assays, batches of 18–24 flies were allowed to walk or fly into a 225-ml glass jar (12 cm high) that was then closed with a screw cap and covered with tin foil. The jar was exposed to the odor plume at the downwind end of the tunnel. A plug closing a 10-mm hole in the middle of the screw cap was removed to allow the flies to exit. The test period was 15 min. Variances of the mean attraction (N=5) were checked for homogeneity (F test or Bartlett's test) and analyzed statistically by a t-test or Tukey's test following an analysis of variance (ANOVA). For the trapping study in the laboratory differences between the two baits and differences between sexes were analyzed by a t-test.

Results

Attraction to Vinegar and Synthetic Vinegar Compounds in the Wind Tunnel Starved, 2-d-old flies were strongly attracted to volatiles of authentic vinegar emanating from the wash bottle (50% of the test flies reached the source after 9.0±2.8 min, and overall attraction was 62±10% within the 15-min experimental period, Fig. 1c). The

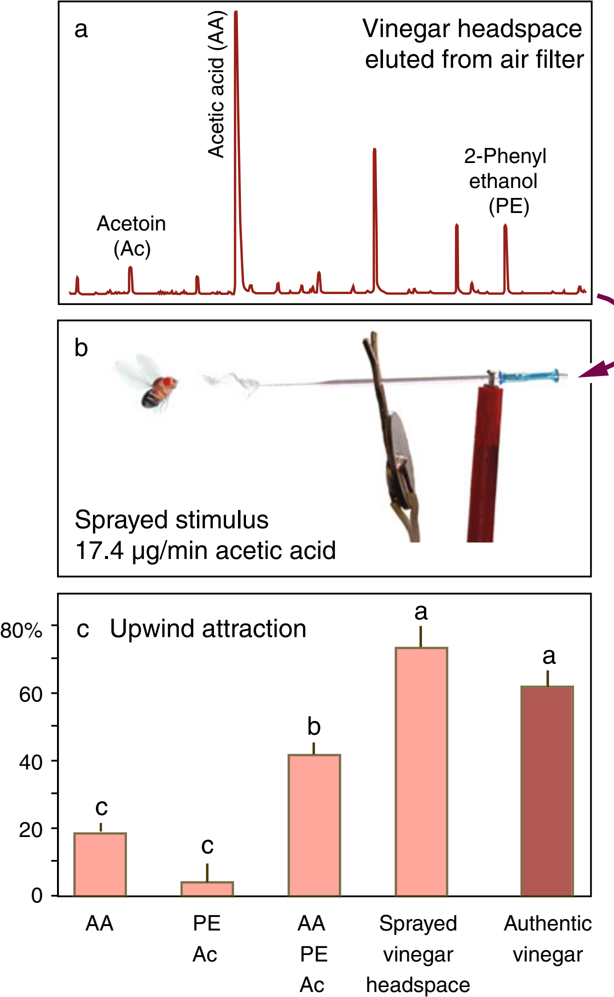
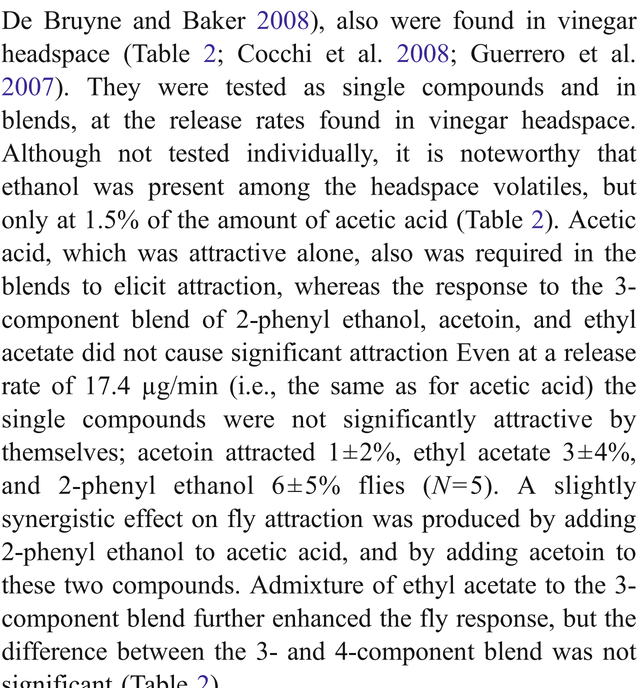


Fig. 1 Attraction of 2-d-old starved *Drosophila melanogaster* adults in a wind tunnel to GC-MS analyzed (**a**) and defined odor samples that were released from a glass capillary attached to a piezo-ceramic disk (piezoelectric sprayer) (**b**). The release rates of acetic acid (AA), the main vinegar compound (17.4 µg/min), ethyl acetate (0.103 µg/min), acetoin (Ac; 0.58 µg/min), and 2-phenyl ethanol (PE; 1.22 µg/min) in the sprayed treatment and in the synthetic blends mimicked the release rates from authentic vinegar. Flies were attracted to sprayed synthetic vinegar compounds, sprayed methanol extract of Super Q-trapped vinegar volatiles, and to authentic vinegar from a wash bottle (**c**). *Different lowercase letters* in (**c**) indicate significant differences according to ANOVA followed by Tukey's test (N=5 batches of 20 flies; F=50.61; df=24, P<0.001; *error bars* show standard deviation of the mean)

quantitative assessment of vinegar headspace volatiles revealed that acetic acid was the most abundant compound (Fig. 1a). It was released from the wash bottle at 17.4 µg/min (GC-MS). Re-vaporized (sprayed) methanol extract of vinegar volatiles collected on Super Q, releasing acetic acid at the same rate, attracted 74 ±14% flies (Fig. 1c).

Three known ligands of *D. melanogaster* olfactory receptor neurons, acetoin, ethyl acetate, and 2-phenyl ethanol (Stensmyr et al. 2003; Hallem and Carlson 2006; De Bruyne and Baker 2008), also were found in vinegar headspace (Table 2; Cocchi et al. 2008; Guerrero et al. 2007). They were tested as single compounds and in blends, at the release rates found in vinegar headspace. Although not tested individually, it is noteworthy that ethanol was present among the headspace volatiles, but only at 1.5% of the amount of acetic acid (Table 2). Acetic acid, which was attractive alone, also was required in the blends to elicit attraction, whereas the response to the 3-component blend of 2-phenyl ethanol, acetoin, and ethyl acetate did not cause significant attraction Even at a release rate of 17.4 µg/min (i.e., the same as for acetic acid) the single compounds were not significantly attractive by themselves; acetoin attracted 1±2%, ethyl acetate 3±4%, and 2-phenyl ethanol 6±5% flies (N=5). A slightly synergistic effect on fly attraction was produced by adding 2-phenyl ethanol to acetic acid, and by adding acetoin to these two compounds. Admixture of ethyl acetate to the 3-component blend further enhanced the fly response, but the difference between the 3- and 4-component blend was not significant (Table 2).



Threshold Concentration of Orientation to Vinegar in the Wind Tunnel A dose-response test showed that re-vaporized methanol extracts of Super Q-trapped vinegar volatiles elicited significant attraction at release rates ranging over three orders of magnitude (Table 3). The release rate of acetic acid from vinegar was 17.4 µg/min as measured from Super Q traps, but flies were still attracted to a 1,000-fold diluted headspace collection that released as little as 17.4 ng/min acetic acid. This agrees with the significant attraction to a 1,000-fold dilution of vinegar in water. In contrast, the response to a 1:10 dilution of acetic acid alone was not different from the response to the blank, thus corroborating the synergistic effect of the other vinegar compounds (Tables 2 and 3).

Antagonistic Effect of an Off-flavor on Attraction to Vinegar in the Wind Tunnel During preliminary trapping tests with the banana–water mixture (see below), we regularly observed a decrease in attraction when the bait was growing moldy. Geosmin, an off-flavor produced by mold fungi, had an antagonistic effect on attraction to vinegar. Methanol extracts of trapped vinegar volatiles containing geosmin in a ratio of 1:10 (w/w) relative to acetic acid, sprayed at release rates of 0.174 and 0.0174 µg/min acetic acid, attracted fewer flies (18±12% and 17±10%, respectively) than trapped vinegar volatiles without geosmin (51 and 42% attraction, respectively; Table 3) (t-test, t=3.30 and 3.28, df=8, P<0.05).

Effect of Age, Starvation, Sex, and Mating Status on Response of D. melanogaster in the Wind Tunnel The

Table 2 Attraction of *Drosophila melanogaster* to synthetic vinegar compounds and to vinegar headspace volatiles delivered by a piezoelectric sprayer

Compound (µg/min)[a]																	
Acetic acid	17.4	•				•	•	•				•	•	•		•	•
2-Phenyl ethanol	1.22		•			•			•	•		•	•		•	•	•
Acetoin	0.58			•			•		•		•	•		•	•	•	•
Ethyl acetate	1.03				•			•		•	•		•	•	•	•	•
Other compounds[b]	6.80																•
Attraction (%)[c]		19 de	8 ef	2 f	0 f	31 cd	10 ef	2 f	4 f	0 f	3 f	42 bc	17 e	16 e	9 ef	52 b	74 a

[a] Release rates mimic a headspace collection of vinegar (last column)

[b] Other compounds (>1% relative to acetic acid) found in vinegar headspace, according to GC-MS analysis and in agreement with literature (Zeppa et al. 2002; Guerrero et al. 2007; Cocchi et al. 2008) included: ethanol (1.5%), 3-methyl-1-butanol (2.1%), 2,3-butanediol diacetate (1.7%), acetyl furan (1.0%), 2-methyl-propanoic acid (1.0%), 5-methyl-2-furancarboxyaldehyde (2.6%), isovaleric acid (17.9%), 2-phenylethyl acetate (6.8%), hexanoic acid (2.3%), and octanoic acid (2.1%)

[c] Percentages (N=5 batches of 20 flies) followed by different letters are significantly different (ANOVA followed by Tukey's test, F=57.920; df=15,64; P<0.001)

Table 3 Attraction of *Drosophila melanogaster* to different release rates of acetic acid and vinegar

Release rates of acetic acid (µg/min)	Attraction and SD (%) to		
	Acetic acid	Sprayed methanol extract of vinegar headspace volatiles[a]	Headspace of bubbled vinegar[a]
blank	2±1	2±1	1±2
0.00174	nt[b]	2±3	4±5
0.0174	nt	**42**±14	**21**±18
0.174	nt	**51**±19	**21**±8
1.74	6±7	**69**±15	**70**±7
17.4	**19**±5	**74**±14	**62**±10
174	13±6	nt	nt

[a] Percentages in bold-faced font (N=5 batches of 20 flies) are significantly different from the blank (t-test; P<0.005). Sprayed methanol extract of Super Q-trapped vinegar volatiles were diluted further with methanol to obtain known amounts of acetic acid. Bubbled vinegar from a wash bottle was diluted in water; standard vinegar headspace contained acetic acid at 17.4 µg/min

[b] *nt* not tested

response of fed flies to authentic vinegar, disregarding age, was not significantly different from a blank test with water (Fig. 2a). Starvation for one day significantly increased the rate of upwind attraction of 1-d-old flies to the vinegar. Attraction of older, 2- to 6-d-old flies peaked when they were deprived of food for 2 d. During the 24 hr period after eclosion, few flies were attracted (1.6%, data not shown). With flies of 1 d and older, age did not have a significant effect on upwind attraction (Fig. 2a). These tests were done with mixed batches of females and males, of unknown mating status. In order to verify a possible effect of sex and mating, virgin or mated flies were separated by sex either immediately after eclosion (virgin test flies) or 1 d before the experiment (mated test flies). Neither sex nor mating status had a significant effect on attraction (Tukey's test following ANOVA, F=1.54, df=24, P=0.23) (Fig. 2b).

Trapping Study of Diel Flight Period in the Laboratory After *D. melanogaster* were released from their food vials, traps baited with vinegar or banana–water mixture captured 15% of the flies during the remaining 4 hr of the photoperiod. On the following day, 39% and 62% of the remaining flies were captured, during the first and second half of the photophase, respectively. Significantly more flies were attracted to vinegar (76% and 72% of the trapped males and females) than to the banana–water mixture (t-test, t=3.72, df=10, P<0.005). The ratio between trapped males and females was not different (t-test, t=1.71, df=4, P=0.16). Most flies were trapped during the photophase, only 6% of the flies were caught during the scotophase.

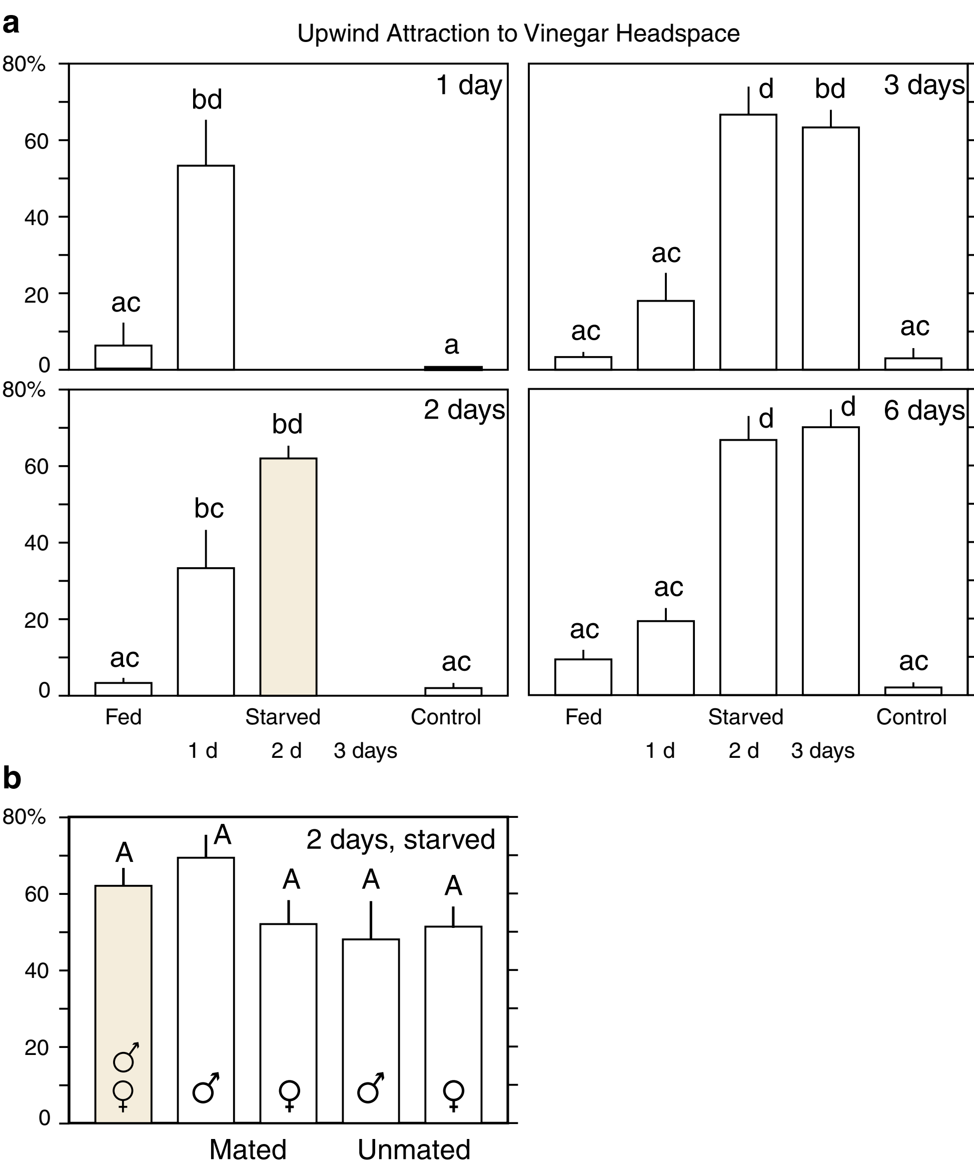


Fig. 2 Attraction of *Drosophila melanogaster* adults in a wind tunnel to vinegar headspace volatiles. **a** Mean percentage of 18–24 flies (N=5) flying upwind and landing at the outlet of an air stream passing a wash bottle with vinegar. Flies, which were tested between 1 d and 6 d after eclosion, were fed, or starved for 1–3 d. For each age, water was tested as control on flies of similar starvation time as those flies showing the highest attraction to vinegar. Tests were done with a mixture of males and females of unknown mating status. The shaded bar indicates conditions chosen as standard for the other wind tunnel experiments of the study. Bars with different letters are significantly different (ANOVA followed by Tukey's test; F=20.62; df=84, P<0.05; error bars show standard deviation of the mean). **b** Attraction of 2-d-old, 2-d-starved males and females, sexes unseparated (shaded as shown in panel **a**), and sexes separated before and after mating (ANOVA followed by Tukey's test; F=1.54, df=24, P=0.23)

Discussion

Flies and other insects rely on odors to detect food and mates. Perception of odors has been studied thoroughly in *D. melanogaster*, particularly by extracellular recordings from olfactory neurons (De Bruyne et al. 2001; Stensmyr et al. 2003; Hallem and Carlson 2006). These neurons generate excitation patterns that are transmitted to higher brain centers where a behavioral response is created (Jefferis et al. 2007; Schlief and Wilson 2007; Shang et al. 2007; Root et al. 2008). A current challenge with *D. melanogaster* is to relate chemosensory perception and coding to the natural behavior of long-range attraction to odors.

Airborne odor typically is encountered in intermittent bursts (Baker et al. 1998), and the generation of an appropriate behavioral response requires instantaneous assessment of its quality. Since long-range displacement costs energy and involves risks, it is vital to evaluate odor quality downwind from the source. Wind tunnel assays monitor the conspicuous outcome of this sensory evaluation, i.e., upwind flight orientation behavior, which is elicited within short time intervals after perception of relevant signals. We have established a sensitive and discriminative flight tunnel assay that facilitates the identification of behaviorally relevant odors, thus providing a link between the neurophysiology, behavioral physiology, and chemical ecology of *D. melanogaster.*

Balsamic vinegar is a robust stimulus for studying the fly's odor-mediated upwind flight attraction. Following chemical analysis, we applied the wind tunnel assay to reduce the chemical complexity of the vinegar headspace (Zeppa et al. 2002; Guerrero et al. 2007; Cocchi et al. 2008) to a simple blend of volatiles that produced nearly the same

attractive response as vinegar. Acetic acid, the main component of vinegar headspace, attracted flies as a single compound. Adding 2-phenyl ethanol and acetoin had a synergistic effect (Fig. 1, Table 2). This agrees with recent findings that the complex odor of apple cider vinegar activates several olfactory glomeruli in the antennal lobe; only two glomeruli (and consequently few odor components) are required for close-range attraction in *D. melanogaster* (Semmelhack and Wang 2009).

Overripe mango, which is another powerful attractant for *D. melanogaster*, also releases acetic acid and 2-phenyl ethanol. A blend of these two compounds with ethanol is an attractive trap lure for *D. melanogaster* (Zhu et al. 2003), and addition of ethanol to the 3- and 4-component synthetic blends that we tested in our study might complement the attraction found for the complete vinegar bouquet. Thus, the role of ethanol in the long-range attractant for *D. melanogaster* merits additional study.

Attraction to acetic acid is not unique to *D. melanogaster*. Noctuid moths attracted to fermenting sweet baits also respond to acetic acid (Landolt 2000). 2-Phenyl ethanol is, like acetic acid, a yeast product; it is also found in insect-pollinated plants and known to attract many insect species from different taxa (Andersson et al. 2002; El-Sayed 2009).

Sensitivity to acetic acid is expressed in *D. melanogaster* adults and larvae (Hoffmann and Parsons 1984; Cobb 1999; Ruebenbauer et al. 2008; Joseph et al. 2009), supporting the idea that this compound is of ecological relevance. Acetic acid probably serves as a cue for the presence of fermenting fruit and other substrates used as food or oviposition sites. Interestingly, Joseph et al. (2009) recently demonstrated gustatory-mediated attraction vs. olfactory-mediated positional repulsion in response to egg-laying substrates containing acetic acid, under close-range conditions. Under long-range conditions, acetic acid plays an essential role in upwind flight attraction (Table 2), which we assume is relayed through olfactory neurons. Despite its strong behavioral effect, it is yet unclear how *D. melanogaster* perceives and processes acetic acid. In comparison, olfactory neurons expressing receptors for 2-phenyl ethanol, acetoin, and ethyl acetate, and their associated glomeruli in the antennal lobe are already known (Fishilevich and Vosshall 2005; Hallem and Carlson 2006; De Bruyne and Baker 2008; Asahina et al. 2009).

Semmelhack and Wang (2009) showed that activity in the glomeruli DM1 and VA2 is associated with close-range attraction of *D. melanogaster* to vinegar. The vinegar compounds ethyl acetate and acetoin are the strongest known stimuli for these two glomeruli (De Bruyne and Baker 2008). Ethyl acetate and acetoin may be sufficient for close range attraction and oviposition (Ruebenbauer et al. 2008; Semmelhack and Wang 2009), but a blend of these compounds did not induce upwind flight attraction in the absence of other vinegar volatiles (Table 2). It will be rewarding to study the representation of defined blends of synthetic compounds in comparison with complex authentic odors such as vinegar.

The threshold concentration for initiation of upwind flight reflected odor quality. *Drosophila melanogaster* responded to the odor of vinegar even at a 1,000-fold dilution, or a release rate of 17.4 ng/min of the main compound acetic acid. In comparison, attraction to a 10-fold dilution of acetic acid alone was not significant (Table 3). Admixture of further vinegar volatiles to acetic acid had a synergistic effect on attraction (Table 2), while admixture of geosmin, an off-flavor produced by micro-organisms such as mold fungi (La Guerche et al. 2006), produced an opposite, antagonistic effect on attraction to vinegar.

The stimulus application method is a keystone element of wind tunnel bioassays. The piezoelectric sprayer (El-Sayed et al. 1999) enables the release of chemicals at a known constant rate and purity. It enables the parallel chemical and behavioral analysis of headspace collections of natural odour sources, as a starting point for tests with synthetic chemicals. The comparison of fly attraction to authentic and re-vaporized headspace corroborates the validity of the spray application procedure (Fig. 1).

The piezoelectric sprayer disperses solutions of synthetic and authentic chemicals in solvent. Methanol, which did not elicit a significant response from *D. melanogaster* (Table 1), is a good choice for a solvent in this spray system. It is easily vaporized and dissolves polar- and to some extent, non-polar compounds. Our results on the response to methanol agree with a study by Hoffmann and Parsons (1984) that showed no attraction of *D. melanogaster* and three other species of *Drosophila* to methanol. Comparison between authentic vinegar- and sprayed methanol extracts of Super Q-trapped volatiles did not show an impact by methanol (Fig. 1c). Interestingly, tests on higher dilutions (1:100 and 1:1,000) indicated a stronger attraction to the methanol extracts (diluted in methanol) than to the authentic vinegar (diluted in distilled water) (Table 3). This suggests a possible synergistic activity of methanol to stimuli tested at threshold concentrations, and the effect of methanol in the context of other components of the attractant bears further investigation. It is, however, unclear if this effect was due to the solvent or due to the different odor application methods.

Water is a straightforward solvent for vinegar, however dilutions in water cannot be analyzed by gas chromatography. It is noteworthy that a few flies responded to distilled water, although attraction was not significantly different from blank (Table 1). Water has been reported earlier to be critically important for attraction to traps baited with synthetic fruit odors (Zhu et al. 2003). *Drosophila*

melanogaster sense water with gustatory receptor neurons on the proboscis that projects into a specific region of the suboesophagal ganglion, and with hygrosensory neurons located on the antennae that projects into the antennal mechanosensory centre (Fischler et al. 2007; Liu et al. 2007; Inoshita and Tanimura 2008). It is remarkable that stimuli from outside the antennal lobe, i.e., the olfactory center, generate or contribute to an upwind flight response in *D. melanogaster*.

The response of *D. melanogaster* to vinegar was modulated by hunger, whereas sex and age did not have an effect (Fig. 2). The olfactory system is under circadian clock control (Tanoue et al. 2008). Sexual activity peaks during the night (Fujii et al. 2007), but flies respond to food during the day. Starvation had a decisive effect on attraction to vinegar, in both sexes, irrespective of mating status. Upwind flight of hungry flies to the odor of vinegar demonstrates that odor cues are processed with respect to the physiological state of the fly to generate an appropriate behavioral response. In the case of a hungry fly, this response is expressed as a voluntary take off and the initiation of flight towards the odor source.

We conclude that our wind tunnel assay and the piezoelectric delivery system enables the measurement of a conspicuous and unambiguous behavioral response. The measurement of this response provides the necessary resolution for the investigation of physiologically and ecologically relevant odors and will become an essential tool for the functional analysis of the *D. melanogaster* olfactory system.

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Carotenoid Composition of Invertebrates Consumed by Two Insectivorous Bird Species

Tapio Eeva · Samuli Helle · Juha-Pekka Salminen · Harri Hakkarainen

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Abstract Dietary carotenoids are important pigments, antioxidants, and immune-stimulants for birds. Despite recent interest in carotenoids in bird ecology, we know surprisingly little about the carotenoid content of invertebrates consumed by birds. We compared carotenoid (lutein, β-carotene, and total) concentrations in invertebrates brought to nestlings by two insectivorous passerines, the great tit, *Parus major* and the pied flycatcher, *Ficedula hypoleuca*. We also compared carotenoid levels between environments that were either polluted by heavy metals or were not polluted, because the carotenoid-based plumage color of *P. major* nestlings is affected by environmental pollution. Lepidopterans were the most carotenoid-rich food items and contained the largest proportion of lutein. There were no differences in carotenoid concentrations in the food items of the two bird species but *P. major* nestlings obtained more carotenoids from their invertebrate diet than *F. hypoleuca* nestlings because the *P. major* diet had a higher proportion of lepidopteran larvae. In polluted areas, *P. major* nestlings consumed lower levels of dietary carotenoids than in unpolluted areas because of temporal differences in caterpillar abundance between polluted and unpolluted sites. Our study suggests that pollution-related difference in nestling plumage color in *P. major* is related to varying dietary proportion of lutein-rich food items rather than pollution-related variation in insect carotenoid levels.

T. Eeva (✉) · S. Helle · H. Hakkarainen
Section of Ecology, University of Turku,
FI-20014 Turku, Finland
e-mail: tapio.eeva@utu.fi

J.-P. Salminen
Laboratory of Organic Chemistry and Chemical Biology,
University of Turku,
20014 Turku, Finland

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Introduction

Carotenoid pigments have been studied intensively in avian ecology during the last decade (Hill and McGraw 2006). Besides their important role in visual signaling, plant-derived carotenoids have essential physiological functions as antioxidants, immunostimulants, and pro-vitamins in birds (Surai et al. 2001). Birds acquire all of their carotenoids through their diet (Goodwin 1986; Brush 1990). For example, some herbivorous invertebrates that are rich in plant-derived carotenoids are important sources of carotenoids for insectivorous birds, such as the great tit, *Parus major* (Partali et al. 1985; Slagsvold and Lifjeld 1985; Tummeleht et al. 2006; Isaksson and Andersson 2007; Eeva et al. 2008). While carotenoid concentrations have been documented for one important food item, herbivorous caterpillars (Partali et al. 1985; Isaksson and Andersson 2007; Sillanpää et al. 2008), there is little information on other important invertebrate groups. A better understand of dietary carotenoids will elucidate the sources of variation in carotenoid levels in wild birds.

We compared carotenoid (lutein, β-carotene, unidentified carotenoids, and total) concentrations in dietary invertebrates for two insectivorous passerines, the great tit and the pied flycatcher, *Ficedula hypoleuca*. To get the most relevant information about the bird's diet, we collected the food items that birds brought to their nestlings. Sampling invertebrates collected by birds ensures that we are sampling just those species that are important for birds during breeding. Furthermore, with this sampling procedure we could estimate the mass proportions of the

main invertebrate groups in the diets of birds, which enabled us to calculate species-specific dietary carotenoid profiles. Because plasma carotenoid levels and plumage color of *P. major* are related to environmental pollution (Eeva et al. 1998, 2009), we collected data at two separate sites, one polluted by heavy metals and the other a relatively unpolluted reference site.

Methods and Materials

Study Site The data were collected from 12 study sites in the summers 2000 and 2002, in the pollution gradient of a copper smelter in the town Harjavalta (61°20′ N, 22°10′ E), SW Finland. Elevated heavy metal concentrations occur in soil, vegetation, insects, and birds in the polluted area (Kiikkilä 2003), and metal contents decrease exponentially with increasing distance to the smelter, approaching background levels at sites further than 5 km from the smelter (Jussila and Jormalainen 1991). A more detailed description of the study area and the pollution gradient has been given in Eeva et al. (1997). Six study sites in the proximity (<2 km) of the pollution source and six more distant (>5 km) sites were established with 40–50 nest boxes at each site.

Food Samples We collected food samples (N=338 individual food items) from *F. hypoleuca* and *P. major* parents feeding their nestlings at the age of 6–11 or 8–13 d, respectively. Since *P. major* nestlings hatched, on average, 16 d earlier than *F. hypoleuca*, the sampling of invertebrates also took place about 16 d later in the latter species. Birds were captured with a shutter trap at the entrance hole of a nest-box. A piece of plastic transparency above the begging nestlings prevented parents from giving the food items to nestlings. When an adult bird entered the nest-box we took the food item. Maximum duration of disturbance per brood was 20 min. Invertebrates were identified and classified into eight groups as follows: spiders (Aranae), beetles (Coleoptera), ants (Formicidae), adult moths, and butterflies (Lepidoptera), lepidopteran caterpillars, sawfly caterpillars (Symphyta), cockroaches (Ectobiidae), and other invertebrates (including Diptera, adult Hymenoptera, Isopoda, Lithobiidae, Myriapoda, and Gastropoda). Food items were weighed (mg) and preserved at −20°C in the dark until analysis. Sampling was conducted following the Finnish guidelines for ethical treatment of vertebrate experimental subjects.

Carotenoid Analyses For carotenoid analyses, 1–9 individuals from the same taxonomic group were put together, freeze-dried, and ground into fine powder. The total number of samples was 63 from *F. hypoleuca* diets and 23 from *P. major* diets. The powder (15 mg) was extracted three times in dark at 10°C with 400 µl of acetone, and the residue was then washed with 100 µl of acetone. The combined acetone extract (1300 µl) was concentrated for 50 min with an Eppendorf concentrator and was freeze-dried. The freeze-dried residue was dissolved in 300 µl of acetone, and 60 µl were injected into the HPLC system. The carotenoid composition was analyzed at 450 nm using a YMC C-30 column (250× 4 mm, i.d., 5 µm). β-carotene was quantified as β-carotene equivalents and the other carotenoids as lutein equivalents, using commercial lutein and β-carotene as standards (Extrasynthese, France). In some of the samples, the concentrations were below the detection limit (0.5 µg/g) and were replaced with a value of $0.5/\sqrt{2}$ in the statistical analyses (Hornung and Reed 1990).

Although the degradation of carotenoids is relatively slow at −20°, it is likely that some degradation occurred during the relatively long sample storage (7–9 yr). The first-order degradation kinetics of total carotenoids in wheat flour at −20° is described by the rate equation $\ln C = \ln C_0 - kt$; C = concentration, k = reaction rate constant, 0.15×10^{-3} day^{-1}, t = time in days (Hidalgo and Brandolini 2008). From that rate equation, we estimate that when we analyzed our samples they contained 61–68% of the original carotenoid concentration. The exact degradation rate is unknown since it may depend on the matrix (Hidalgo and Brandolini 2008). Therefore, absolute concentrations found in this study cannot be compared directly with levels found in fresh invertebrates. The main interest in our study, however, was to compare relative concentrations among different invertebrate groups.

Statistical Analyses We analyzed carotenoid concentrations (lutein, β-carotene, unidentified carotenoids, and total carotenoids) with separate generalized linear models with a negative binomial error distribution and log link function by using the GLIMMIX procedure of SAS 9.1 (SAS Institute 2003). The independent factors in the models were: bird species, year, area (polluted vs. unpolluted), and invertebrate group. Due to the small number of samples in most invertebrate groups, we did not consider the interactions between the main effects. Statistical interpretation of the group-differences was based on the 95% confidence intervals of the means. For example, if the 95% confidence intervals of means overlap half the length of one arm, this corresponds approximately to statistical significance at p= 0.05 (Cumming 2009).

Results

Carotenoid concentrations in different invertebrate groups are shown in Table 1. There were no statistically significant

Table 1 Mean concentrations (μg/g, d.w.) of lutein, β-carotenoid, unidentified carotenoids, and total carotenoids in invertebrate groups normally consumed by *Parus major* and *Ficedula hypoleuca* nestlings in polluted and unpolluted areas

Group	n	Lutein mean (±SE)	β-carotenoid mean (±SE)	Unidentified mean (±SE)	Total mean (±SE)
Aranae	9	1.7 (±0.27)	1.2 (±0.17)	3.3 (±0.82)	6.1 (±1.3)
Coleoptera	16	1.5 (±0.51)	2.4 (±0.79)	8.6 (±4.6)	12.4 (±5.9)
Formicidae	7	<0.5[b]	0.69 (±0.12)	0.86 (±0.20)	1.8 (±0.35)
Lepidoptera adults	13	6.4 (±1.8)	7.9 (±3.1)	13.6 (±4.9)	27.8 (±9.5)
Lepidoptera larvae	22	14.0 (±2.0)	4.7 (±0.81)	10.1 (±3.2)	28.5 (±5.2)
Symphyta larvae	5	3.0 (±1.6)	1.9 (±0.68)	6.1 (±2.5)	10.7 (±4.4)
Blattaria	4	<0.5[b]	1.0 (±0.20)	1.3 (±0.67)	2.4 (±0.78)
Others	10	0.70 (±0.17)	0.77 (±0.39)	0.79 (±0.44)	1.8 (±1.0)
Among group effect[a]		$F_{7,75}$=15.7 P<0.001	$F_{7,75}$=5.6 P<0.001	$F_{7,75}$=4.4 P<0.001	$F_{7,75}$=8.5 P<0.001

[a] GLM with negative binomial error distribution. Independent factors were: species, year, pollution zone and invertebrate group

[b] All values below the detection limit (0.5 μg/mg)

differences in mean concentrations of carotenoids in dietary invertebrates consumed by the two bird species, nor between years or study areas (for lutein, β-carotene, unidentified carotenoids, and total: P>0.05 in all cases). Invertebrate groups, however, differed significantly in their mean carotenoid concentrations (Table 1). The highest lutein, β-carotene, and total carotenoid concentrations were found in lepidopterans (Fig. 1). Moderate total concentrations were found in Coleoptera, Symphyta, and Aranae (Fig. 1). Ants, cockroaches, and the group "other" invertebrates showed the lowest total concentrations (Fig. 1). The proportion of lutein from total carotenoids was highest in lepidopterans (mean±SE proportions calculated over individual samples: 56±4.9% for caterpillars; 40±6.5% for adults). The proportion of β-carotene was highest in cockroaches (44±18.9%), and the proportion of unidentified carotenoids was highest in beetles (45±7.4%) and spiders (44±6.7%).

Although there were no differences in the concentration of carotenoids in food items used by the two bird species, the birds used different invertebrate groups in different proportions (Table 2). For example, in the unpolluted area, the biomass-based proportion of carotenoid-rich lepidopteran larvae in diet was 3.4 times higher in *P. major* than in *F. hypoleuca* (Table 2). We calculated the carotenoid profile for the nestling diet of each species, taking the varying dietary proportions of different invertebrate classes into account (Fig. 2). *Parus major* nestlings received 1.4 and 5.7 times more carotenoids than *F. hypoleuca* nestlings in the polluted and unpolluted areas, respectively (Fig. 2). This

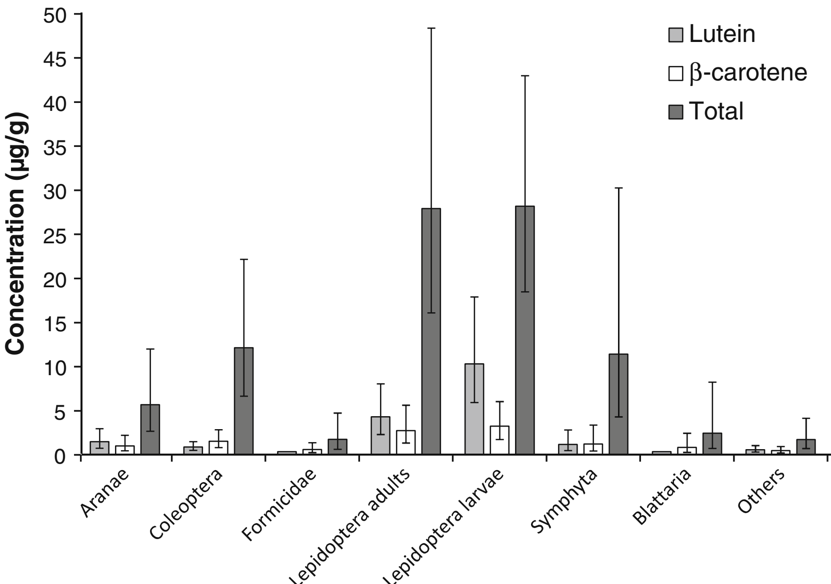

Fig. 1 Mean concentrations (μg/g, d.w.) of lutein, β-carotenoid, and total amount of carotenoids in invertebrate groups consumed by *Parus major* and *Ficedula hypoleuca* nestlings. Values are model based expected marginal means backtransformed from a log-scale. Error bars indicate the 95% confidence interval. N=86

Table 2 Proportions (% of total fresh mass) of invertebrate groups in the diet of *Parus major* and *Ficedula hypoleuca* nestlings in polluted and unpolluted areas

Group	*P. major*		*F. hypoleuca*	
	Polluted	Unpolluted	Polluted	Unpolluted
Aranae	8.1	3.9	2.5	10.8
Coleoptera	12.4	1.4	29.0	10.1
Formicidae	3.5	1.5	2.5	13.5
Lepidoptera adults	14.4	9.0	15.3	21.5
Lepidoptera larvae	50.8	77.1	37.9	22.8
Symphyta larvae	3.6	0.0	3.9	2.0
Blattaria	0.0	0.3	1.2	5.8
Others	7.2	6.9	7.8	13.7

Calculated from total biomass of collected food items (*P. major*: *N*=121; *F. hypoleuca*: *N*=217)

was due primarily to the much higher proportion of lepidopteran larvae in diet of *P. major* (Table 2). In *P. major*, dietary carotenoid content was lower in the polluted area, while in *F. hypoleuca* it was higher in the polluted area (Fig. 2). For both polluted and unpolluted areas the proportion of dietary lutein was somewhat higher in *P. major* (48%) than in *F. hypoleuca* (37%).

Discussion

Carotenoid concentrations differed among the main invertebrate food groups in the nestling diets of *P. major* and *F. hypoleuca*. Lepidopterans were the most carotenoid-rich food items, with lepidopteran caterpillars (e.g., *Operophtera brumata*, *Epirrita autumnata*) containing the largest amount of lutein on a mass and proportional basis. Relatively high carotenoid concentrations also were found in adult Lepidoptera (e.g., *Bupalus piniarius, Thera obeliscata*), which are an important source of carotenoids especially for *F. hypoleuca*. Beetles, sawfly larvae, and spiders contained moderate total carotenoid concentrations, but with a much lower proportion of lutein than in lepidopterans. Some invertebrate groups that were important in the diet of *F. hypoleuca*, such as ants and cockroaches, had relatively low carotenoid concentrations.

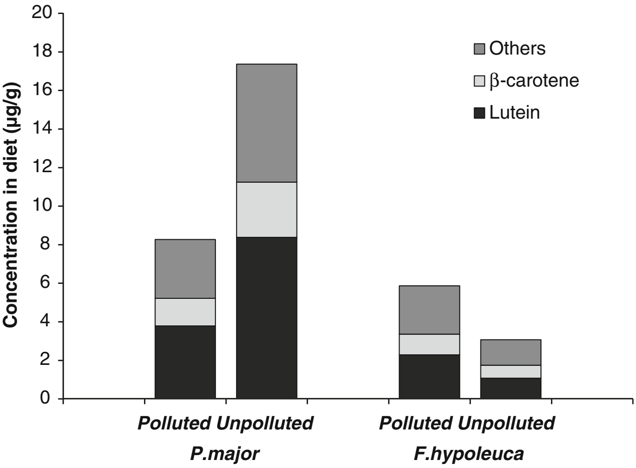

Fig. 2 Concentrations of lutein, β-carotenoid and other carotenoids in the invertebrate diet of *Parus major* and *Ficedula hypoleuca* nestlings. The values take account the concentration in each invertebrate group (Table 1) and the proportion of each group in the diet (Table 2)

The levels of carotenoids in the insects in our study, were lower than previously published values. Sillanpää et al. (2008) found lutein concentrations of 80–90 µg/g (d.w.) in a sample of autumnal moth (*Epirrita autumnata*) larvae, while in our sample the average caterpillar lutein concentrations was only 14 µg/g (d.w.). Isaksson and Andersson (2007) reported lutein concentrations of 16–26 µg/g (w.w.) in lepidopteran caterpillars, equivalent to 123–200 µg/g (d.w.) assuming that caterpillar water content is 87% (Sillanpää et al. 2009). Lutein concentration of sawfly larvae was 15–18 µg/g (d.w.) in a study of Sillanpää et al. (2008), while it was 3 µg/g (d.w.) in our sample. The relatively large differences most likely are due to degradation of carotenoids during the relatively long storage of our samples (see Methods). However, the rather diverse species composition in our samples also may partly explain these differences. For example, lepidopteran caterpillars include species of Geometridae, Noctuidae, Arctiidae, Lasiocapidae, and Nymphalidae, and there is likely variation in carotenoid concentrations among the species within this group (Isaksson and Andersson 2007).

The carotenoid profiles of the invertebrates normally consumed by *P. major* and *F. hypoleuca* nestlings were diverse. In all, 34% of total carotenoids was lutein, 21% β-carotene, and 46% unidentified carotenoids. The large number of species in our samples suggest that the unidentified carotenoids may include numerous compounds. For example, astaxanthin and lycopene are important carotenoids in insects, and likely are represented in our samples (Goodwin 1986). Carotenoid profiles of our samples also varied within the same insect group, possibly due to highly variable species composition. For example,

within Coleoptera, the highest carotenoid concentrations (up to 98 μg/g) occurred in samples containing only ladybirds (Coccinellidae). *Coccinella septempunctata*, which was the primary ladybird species in our sample, has a very diverse carotenoid profile (Britton et al. 1977). These unpalatable beetles, which are especially numerous in the polluted area, frequently were observed in the nestling diet of *F. hypoleuca,* while *P. major* seems to avoid them (Eeva et al. 2005).

Invertebrate carotenoid profiles differ from plasma carotenoid profiles of *P. major* and *F. hypoleuca* nestlings. In plasma, lutein is the dominant carotenoid, comprising up to 57% (*F. hypoleuca*) and 75% (*P. major*) of the total carotenoids (Biard et al. 2006; T. Eeva, unpublished data). Birds absorb different carotenoids disproportionally, and xanthophylls (like lutein) generally are absorbed relatively efficiently (Goodwin 1986; Surai et al. 2001; McGraw 2005). Xanthophylls also are the primary carotenoids in the integument coloration of many bird species, including *P. major* (McGraw 2006; Eeva et al. 2008). Many bird species can modify dietary carotenoids chemically, but in *P. major* the main feather pigments (lutein and zeaxanthin) are transported from food to feathers without modification (Brush 1990; McGraw 2006).

We found no significant differences in carotenoid concentrations of food items collected by the two bird species. This is not surprising since the diets of *P. major* and *F. hypoleuca* include some of the same species (Cramp and Perrins 1993; Eeva et al. 2005). Furthermore, due to a large variation in concentrations among samples and relatively small sample size (especially in *P. major*), our analysis could not have detected small differences in carotenoid concentrations. The normal diet of *P. major* nestlings, however, contains considerably more carotenoids than the diet of *F. hypoleuca* because *P. major* consumes a much larger proportion of carotenoid-rich lepidopterans (Eeva et al. 2005). In agreement with our study, Sillanpää et al. (2008) showed that there was no pollution-related difference in concentrations of lutein and β-carotene in caterpillars of one important dietary lepidopteran species, the autumnal moth (*Epirrita autumnata*).

Temporal differences in caterpillar abundance between polluted and unpolluted sites are an important determinant of dietary carotenoid concentration and carotenoid intake in our study species (Eeva et al. 2005; Sillanpää et al. 2009). Heavy metal rich food is known to retard growth and increase mortality of herbivorous insect larvae (Heliövaara and Väisänen 1990; Martens and Boyd 1994; Ruohomäki et al. 1996). At least in some years, the peak abundance of lepidopteran caterpillars occurs later in the polluted area (Sillanpää et al. 2009). As a consequence, the diet of *P. major* nestlings in the polluted sites contains lesser amounts of carotenoids than the diet in the unpolluted area. In contrast, the nestlings of later-breeding *F. hypoleuca* may consume more carotenoids in the polluted area than their conspecifics in the unpolluted area.

Carotenoid concentrations differ markedly among the main invertebrate taxa in the diet of *P. major* nestlings. Lepidopterans are the most carotenoid-rich food items, and contain a high proportion of lutein. Dietary variation in carotenoid levels may have important consequences for bird species that use carotenoid pigments for their feather coloration. Lutein is the most important feather color pigment in *P. major*, the pale yellow coloration of *P. major* nestlings in a polluted area is a manifestation of low dietary carotenoid levels (Eeva et al. 1998). Our study suggests that this difference in nestling plumage color is due to varying dietary proportion of lutein-rich food items rather than due to pollution-related variation in insect carotenoid levels.

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Predator Avoidance by Phytophagous Mites is Affected by the Presence of Herbivores in a Neighboring Patch

Yasuyuki Choh · Junji Takabayashi

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Abstract When predators invade a leaf patch inhabited by herbivores, the herbivores disperse to a neighboring predator-free leaf patch, thus escaping from the predators. However, the neighboring patch might already be used by con- or heterospecific herbivores. We used laboratory bioassays to examine whether perception of odor from con- or heterospecific competitors on a neighbored lima bean leaf patch influences dispersal behavior of the herbivorous mite *Tetranychus urticae* when attacked by predatory mites *Phytoseiulus persimilis*. The dispersal rates of *T. urticae* that perceived odors from leaf patches infested by conspecifics or cowpea aphids (*Aphis craccivora*) did not differ from the control (the dispersal rate of *T. urticae* that perceived odor from uninfested leaf patches). By contrast, the dispersal rate of *T. urticae* was reduced when they perceived odors from leaf patches that were currently or had previously been infested by larvae of the common cutworm (*Spodoptera litura*). Previous herbivory by *S. litura* larvae induced resistance in leaf patches to *T. urticae* as indicated by the reduced number of eggs laid by *T. urticae*. Our results are discussed with respect to the feeding behavior of the tested competitors of *T. urticae* and the impact of the plant and arthropod community on the dispersal behavior of these mites.

Key Words Antipredator behavior · Competitors · Induced resistance · Dispersal · Herbivore-induced plant volatiles · *Phytoseiulus persimilis* · *Tetranychus urticae* · *Spodoptera litura* · *Aphis craccivora* · Tritrophic

Y. Choh · J. Takabayashi (✉)
Center for Ecological Research, Kyoto University,
Otsuka 509-3, Hirano,
Kamitanakami, Otsu 520-2113, Japan
e-mail: junji@ecology.kyoto-u.ac.jp

Introduction

Herbivorous arthropods are known to leave the host plant that they are currently infesting in the presence of their predators (Loose and Dawidowicz 1994; Tikkanen et al. 1996; Nilsson and Bengtsson 2004; Kunert et al. 2005; Choh and Takabayashi 2007). The leaving prey may evaluate a new host plant nearby before and/or after leaving the host plant. Our previous study showed that the suitability of new host plants affects the dispersal behavior of an herbivorous mite (*Tetranychus urticae*) (Choh and Takabayashi 2007). However, even if the neighboring new host plant is acceptable to them, the plant may already be inhabited by conspecific and/or heterospecific herbivore species. Since herbivore-infested plants can change their defensive levels against herbivores depending on the herbivore species (Karban and Baldwin 1997; Ohgushi 2005; Schaller 2008), differences in whether a plant is infested by con- or heterospecific herbivores will affect the suitability of the plant. Thus, the presence of other herbivore individuals on a neighboring host plant would also affect the dispersal behavior of herbivores in response to the invasion of predators.

Many herbivore species are reported to use plant odors as a cue for host plant selection (Visser 1986; Bernays and Chapman 1994; Dicke 2000). Furthermore, herbivores may respond to odors from plants infested by conspecifcs and heterospecifics as cues to the quality of host plants (Dicke 1986; Loughrin et al. 1995: Bolter et al. 1997; Pallini et al. 1997; De Moraes et al. 2001; Horiuchi et al. 2003; Meiners et al. 2005). For example, *T. urticae* mites avoid odors from cucumber plants infested with the western flower thrips (*Frankliniella occidentalis*), which is not only a competitor, but also an intraguild predator (Pallini et al. 1997). We showed previously that *T. urticae* respond to odors from

neighboring patches that result in leaving or staying on the current predator-invaded patch or not (Choh and Takabayashi 2007).

Based on our previous study, we hypothesized that herbivores change their dispersal behavior for predator avoidance in response to odors from host plants infested by other herbivores. To test this hypothesis, we used lima beans, a herbivorous mite (*T. urticae*), and a predatory mite (*Phytoseiulus persimilis*). As heterospecific competitors, common cutworm (*Spodoptera litura*) and cowpea aphid (*Aphis craccivora*) were used. We examined the dispersal of *T. urticae* from the current leaf patch invaded by predators to a clean leaf patch when they perceived odors from a neighboring leaf patch with conspecific or heterospecific competitors. Furthermore, we tested whether herbivore-induced leaf volatiles were involved in the dispersal changes. The oviposition of *T. urticae* on a leaf patch that had been infested previously by heterospecific competitors was measured also to evaluate plant resistance induced by competitors.

Methods and Materials

Plants and Arthropods Lima bean plants (*Phaseolus lunatus* cv. Sieva) were grown in soil in a greenhouse at 25±2°C, 60–70% relative humidity (RH), and a photoperiod of 16 L-8hD. We used plants at 10–15 d post-germination.

Herbivorous spider mites (*T. urticae*) were obtained from the Laboratory of Ecological Information (Graduate School of Agriculture, Kyoto University) in 2002, and reared on lima beans in an incubator (25±2°C, 60–70% RH, 16 L-8hD).

Cowpea aphids (*Aphis craccivora*) were collected from a crop field at the Center for Ecological Research in Otsu, Shiga, Japan in May 2007. The insects were reared on broad bean plants (*Vicia faba*) in an incubator (20±2°C, 60–70% RH, and a photoperiod of 16 L-8hD).

Common cutworms (*Spodoptera litura*) were obtained from a culture maintained at the National Institute of Agrobiological Sciences in Tsukuba, Ibaraki, Japan in 2006. The insects were reared on an artificial diet (Insecta LF; Nihon Nousan Kogyo Ltd., Yokohama, Japan) in an incubator (25±2°C, 50–70% RH, and a photoperiod of 16 L-8hD).

Predatory mites (*P. persimilis*) were purchased from Koppert BV (Berkel and Rodenrijs, The Netherlands). They were reared on detached lima bean leaves, heavily infested with *T. urticae*, in an incubator (25±2°C, 60–70% RH, 16 L-8hD). Fresh *T. urticae*-infested leaves were added every other day.

All experiments were conducted in a climate controlled room (25±2°C, 60–70% RH, 16 L-8hD).

Wind Tunnel Experiments: Dispersal Behavior of T. urticae General A 3.5×2.5 cm intact leaf patch was cut from a primary leaf of a lima bean plant (hereafter, current patch), and placed on water-saturated cotton wool in a Petri dish (15 cm diam., 1 cm height). Then, a 3.5×2.0 cm intact leaf patch (hereafter, alternative patch) also was cut from another primary leaf of the same plant, and was placed in the same Petri dish, 5 cm away from the current patch. Thirty randomly selected adult female *T. urticae* were placed on the current patch and were allowed to lay eggs for 24 hr. We then connected the current patch and the alternative patch with a Parafilm bridge (5 cm length, 0.5 cm width) and placed ten adult female *P. persimilis* on the current patch. As *P. persimilis* prefer the eggs of *T. urticae* to the adults (Blackwood et al. 2001), we assumed that the introduced predatory mites were arrested on the current patch by the presence of prey eggs that had been laid 24 hr before the introduction.

We studied the question whether odors from patches with conspecific and heterospecific competitors affect dispersal behavior of the herbivorous mites from the current patch to an uninfested alternative patch. A patch with competitors was prepared of a 3.5×3.5 cm leaf from a primary leaf of a lima bean plant; the patch was placed on water-saturated cotton wool in another Petri dish (15 cm diam.). We placed either ten female adult *A. craccivora*, or 3 2nd–3rd instars of *S. litura*, or 30 female adult *T. urticae* on a leaf patch, and kept it for 24 hr. We used such leaf patches as the odor source patches.

Dispersal Movement Upwind of Odor Source (= Leaf Patch with Con- and Heterospecific Competitors) The set-up was placed in a wind tunnel (air flow: 10 cm/s, size: 40×40×80 cm), with the three leaf discs being positioned in the following order: the odor source patch with the competitors was placed upwind, the current patch was placed downwind, and the alternative clean patch was placed between the current patch and the odor source patch. In this way, *T. urticae* perceived odors from the odor source patch with competitors when dispersing upwind from the current patch to the alternative patch. The number of *T. urticae* that dispersed to an alternative patch and the number of *T. urticae* killed by predators on each current patch were counted 24 hr after the introduction of the three patches into the wind tunnel. We repeated the above experiments 40 times per treatment (Fig. 1a).

Dispersal Movement Downwind of Odor Source (= Leaf Patch with Heterospecific Competitors) For control, the experiment described above was modified by changing the position of patches in the wind tunnel. The odor source

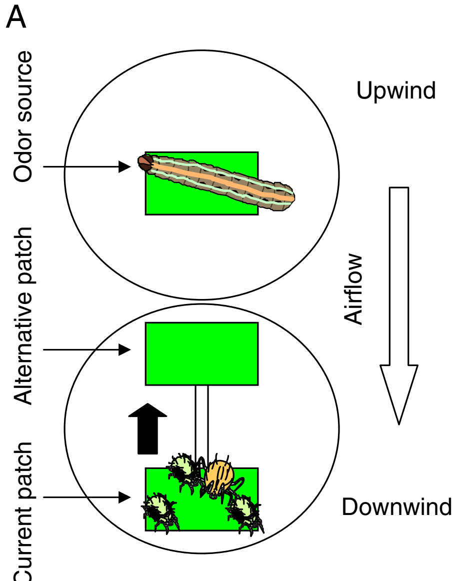
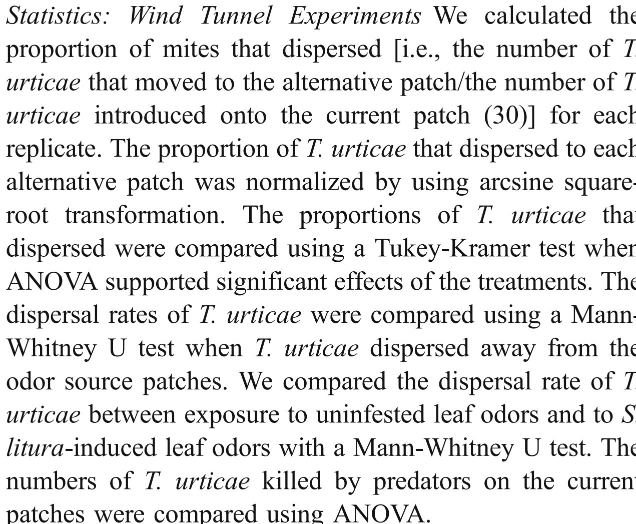


Fig. 1 Set-up for wind tunnel experiments. The current patch and alternative leaf patch were connected by a "bridge" made of Parafilm. The black arrow indicates the direction in which dispersal from a current patch to an alternative patch was possible. *White arrow*: direction of airflow. *Small mites*: symbol for herbivorous *Tetranychus urticae*; *large mite*: symbol for predatory *Phytoseiulus persimilis*; larva on odor source patch: symbol for competitors located on odor source patch: tested competitors for set-up A where dispersal was possible upwind of the odor source: *T. urticae*, *Spodoptera litura*, and *Aphis craccivora*; for set-up B where dispersal was possible downwind of the odor source: only *S. litura*

patch with competitors was placed upwind, the alternative (uninfested) patch was placed downwind, and the current patch was placed between the alternative patch and the odor source patch. Thus, mites moved downwind of the odor source patch when dispersing to the alternative patch. We used as odor source patch only those infested with *S. litura*. The number of *T. urticae* on each alternative patch was counted 24 hr after the introduction of the three patches into the wind tunnel. We repeated the above experiments 18 times per treatment (Fig. 1b).

Dispersal Movement Upwind of Odor Source (= Leaf Patch that Has Previously Been Infested with Heterospecific Competitors) To test whether leaf odors induced by herbivory affect the dispersal of *T. urticae*, we conducted experiments similar to those described above, but using an odor source leaf patch that had been infested previously by three 2nd–3rd instars of *S. litura* for 24 hr and, for control, an uninfested odor source leaf patch. Prior to the experiments, the associated products of herbivores (i.e., feces and exuviae of larvae) were removed. These patches were placed into the wind tunnel in the following order: the odor source patch upwind, the current patch downwind, and the alternative clean patch between the odor source and the current patch. The number of *T. urticae* on each alternative patch was counted 24 hr after the introduction of the three patches into the wind tunnel. We repeated the above experiments 18 times per treatment (Fig. 1a).



Statistics: Wind Tunnel Experiments We calculated the proportion of mites that dispersed [i.e., the number of *T. urticae* that moved to the alternative patch/the number of *T. urticae* introduced onto the current patch (30)] for each replicate. The proportion of *T. urticae* that dispersed to each alternative patch was normalized by using arcsine square-root transformation. The proportions of *T. urticae* that dispersed were compared using a Tukey-Kramer test when ANOVA supported significant effects of the treatments. The dispersal rates of *T. urticae* were compared using a Mann-Whitney U test when *T. urticae* dispersed away from the odor source patches. We compared the dispersal rate of *T. urticae* between exposure to uninfested leaf odors and to *S. litura*-induced leaf odors with a Mann-Whitney U test. The numbers of *T. urticae* killed by predators on the current patches were compared using ANOVA.

Competitor-Induced Plant Resistance to T. Urticae When competitors have fed previously on a new leaf patch, the leaf may show induced resistance against *T. urticae*. To test this possibility, we made a 5×5 cm leaf patch from a primary leaf of a lima bean plant, and put it on water-saturated cotton wool in a Petri dish (9 cm diam, 1.4 cm high). Ten female adult *A. craccivora*, or three 2nd–3rd instars of *S. litura*, or 30 female adult *T. urticae* were placed on the leaf patch. To keep a part of a leaf uninfested, the herbivores were enclosed in a cage (4 cm diam., 0.5 cm high) which was covered with nylon gauze. All herbivore

individuals and eggs of *T. urticae* were removed from the leaf disc 24 hr after the introduction. As a control, no herbivorous arthropods were kept in the cage on the leaf patch. Subsequently, a female adult *T. urticae* that had been randomly selected from the culture was placed on the uninfested part of the leaf patch, and the number of eggs laid by the mite during the following 3 d was measured. We repeated the experiment 24 times. The numbers of *T. urticae*-eggs were compared with a Tukey-Kramer test among treatments when ANOVA supported significant effects of the treatments.

Results

Wind Tunnel Experiments Dispersal Movement Upwind of Odor Source (= Leaf Patch with Con- and Heterospecific Competitors) Dispersal behavior of *T. urticae* towards a clean, uninfested patch differed when mites were exposed to odor source patches with different herbivore species ($F_{3, 156}$=3.635, P<0.014, ANOVA). When *T. urticae* perceived odors from the odor source patch with *S. litura*, the dispersal rate was lower than when they perceived odors from the odor source patches with no herbivores or with *A. craccivora* (P<0.05, Tukey-Kramer test; Fig. 2). Although the dispersal rate of mites exposed to odors from the patch with *S. litura* tended to be lower than that the one of mites exposed to odors from the patch with *T. urticae*, the difference was not significant (P>0.05, Tukey-Kramer test; Fig. 2). There were no significant differences in the number of *T. urticae* killed by predators on the current patch among the four treatments ($F_{3, 156}$=1.377, P=0.252, ANOVA; Fig. 3) (Fig. 1a).

Dispersal Movement Downwind of Odor Source (= Leaf Patch with Heterospecific Competitors) When *T. urticae* dispersed to an uninfested patch away from the odor source patch infested with *S. litura*, the dispersal rate did not differ from the one of mites dispersing to an uninfested patch away from a control patch without competitors (leaf patches with *S. litura*; 0.24±0.051, uninfested control leaf patches; 0.25±0.042, Mann-Whitney *U* test, U=76, P=0.66) (Fig. 1b).

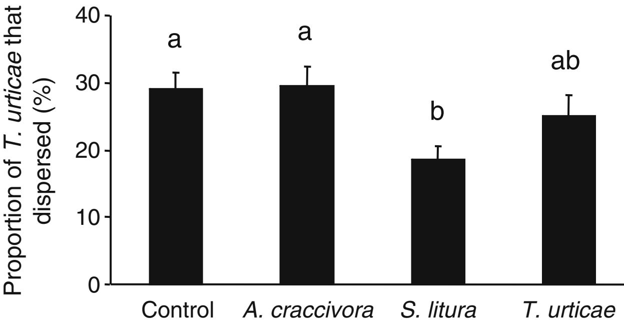


Fig. 2 The proportion (mean+SE) of *Tetranychus urticae* female adults that dispersed from the current patch to the alternative patch when competitors were on the odor source patches. The *letters* above each bar indicate significant differences among treatments by the Tukey-Kramer test (P<0.05)

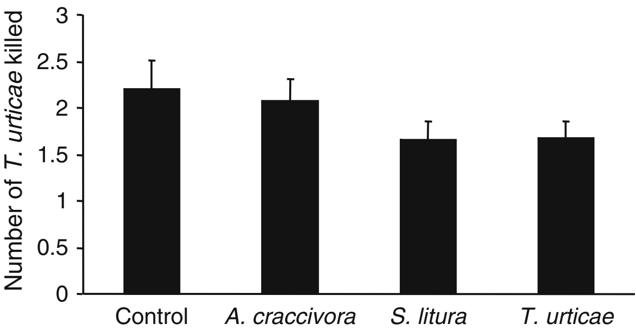


Fig. 3 The number (mean+SE) of *Tetranychus urticae* female adults killed by *Phytoseiulus persimilis* on the current patch when they dispersed from the current patch to an alternative patch next to an odor source patch with competitors

Dispersal Movement Upwind of Odor Source (= Leaf Patch that Has Previously Been Infested with Heterospecific Competitors) When *T. urticae* was exposed to odors from a patch that had been infested previously by *S. litura*, the dispersal rate to a clean patch was lower compared to the rate when exposed to odors from an uninfested leaf patch (*S. litura*-induced leaf odors; 0.12 ± 0.023, uninfested leaf odors; 0.26±0.041, Mann-Whitney *U* test, U=76, P=0.011, (Fig. 1a).

Competitor-Induced Plant Resistance to T. Urticae Oviposition activity of *T. urticae* differed on the leaf patches with previous herbivory by *S. litura, A. craccivora, T. Urtica*, and an untreated control ($F_{3, 92}$=2.896, P<0.039, ANOVA). When *T. urticae* was placed on a leaf patch with previous herbivory by *S. litura*, the oviposition rate was lower than on an uninfested leaf patch (P<0.05, Tukey-Kramer test; Fig. 4). Although the oviposition rate on a leaf patch with previous herbivory by *S. litura* tended to be lower than that on leaf patches with previous herbivory by conspecifics and by *A. craccivora*, there were no significant differences between them (P>0.05, Tukey-Kramer test; Fig. 4).

Discussion

Tetranychus urticae disperses from a patch with predators to an uninfested patch for predator avoidance (Choh and

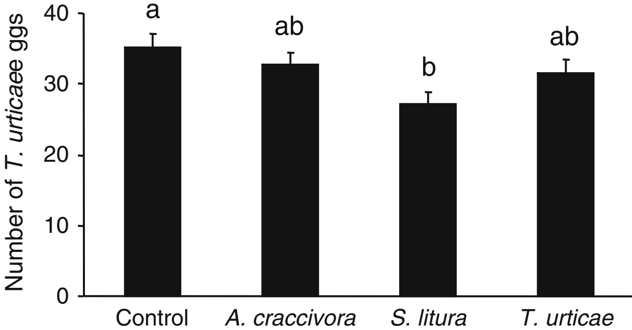


Fig. 4 The number (mean+SE) of eggs laid by *Tetranychus urticae* female adults on the leaf patches that had previously been infested by competitors. The *letters* above each bar indicate significant differences among treatments by the Tukey-Kramer test ($P<0.05$)

Takabayashi 2007). When *T. urticae* perceived odors from an odor source patch with *S. litura*, it showed reduced dispersal compared to when exposed to odors from the control patch (no herbivores). As the mortality of *T. urticae* killed by predators on the current leaf patch was the same irrespective of herbivore species on the odor source leaf patches, the reduced dispersal was not due to a difference in the mortality of adult *T. urticae* killed by the predators.

In contrast to the dispersal of mites exposed to odors from patches with *S. litura*, their dispersal did not change when exposed to odors from patches with conspecifics or *A. craccivora*. These results suggest that *T. urticae* distinguishes herbivore species on the odor source patches by using volatile infochemicals. Odors from *S. litura* larvae themselves, from feces of the larvae, and from the leaf patch are potential cues of the volatile infochemicals that affect the dispersal of *T. urticae*. When lima bean plants are infested by herbivores of different species, they emit different blends of volatile compounds, which can be specific to the herbivore species (Ozawa et al. 2000). In fact, *T. urticae* also showed reduced dispersal when exposed to leaf volatiles from an odor source patch that had been infested previously by *S. litura*. *T. urticae* might change its dispersal in response to such differences in the volatiles. The leaf area consumed by *S. litura* was larger than that consumed by the other two herbivore species. In addition, the feeding mode of *S. litura* (chewing) differs from that of *T. urticae* and *A. craccivora* (sucking). Such differences might partly explain the volatile emission from herbivore-infested lima bean leaves, and the different responses of spider mites to these volatiles. Turlings et al. (1998) reported that herbivores with different feeding modes (caterpillar: *Spodoptera littoralis*, stemborer; *Ostrinia nubilalis*, and aphid *Rhopalosiphum maidis*) could trigger distinctly different quantities of volatiles in plants. It also has been reported that volatiles from lima bean leaves infested by *S. exigua* differ from those from *T. urticae*-infested lima bean leaves (Ozawa et al. 2000). Further study is needed to test how volatile emission from herbivore-infested leaves differs among the three herbivore species that we used in this study.

The intensity and effects of herbivore-induced plant resistance depend on the herbivore species (Agrawal 2000; Agrawal and Karban 2000; van Zandt and Agrawal 2004). We found reduced oviposition of *T. urticae* on the leaf patches that had been infested previously by *S. litura*, suggesting that feeding of *S. litura* induced resistance to *T. urticae* in lima bean leaves. It remains unanswered which defensive traits (e.g., physical or chemical traits) are involved in the *S. litura*-induced resistance to *T. urticae*. It also has been reported that some herbivores induce susceptibility to other herbivores after previous feeding on plants (Underwood 1998; Agrawal 2000; Karban and Agrawal 2002; Rotem and Agrawal 2003). Thus, when prey disperses to a patch with competitors, the effects of the previous feeding of the competitors on the dispersed prey would depend on the combination of herbivore and plant species.

Herbivorous arthropods are known to select and/or distribute on host plants according to herbivore-induced changes in plants (Gilbert et al. 2001; Rostás and Hilker 2002; Kessler et al. 2004; Carroll et al. 2006; Delphia et al. 2007; Viswanathan et al. 2008). For example, several studies have shown that herbivores use volatiles from herbivore-infested plants to find new host plants (Rostás and Hilker 2002; Carroll et al. 2006; Delphia et al. 2007). In this study, dispersing *T. urticae* avoided the leaf patches with *S. litura* with induced resistance against *T. urticae* by using leaf odors. When *T. urticae* leaves the current patch invaded by predators, the resistance of neighboring leaves infested by competitors might be a factor that affects the behavior.

Predators might induce not only antipredator behavior in prey but also nonconsumptive effects on prey that would not result in death (Peacor and Werner 2000; Schmitz et al. 2004; Pangle and Peacor 2006; Werner and Peacor 2006). There have been many demonstrations that nonconsumptive predator effects on prey affect communities through a trait-mediated indirect interaction (see Werner and Peacor 2003; Schmitz et al. 2004; Peckarsky et al. 2008). However, it still is not well understood how communities affect predator-prey interactions. We showed here that the dispersal behavior of prey to escape from predators was affected by the presence of competitors on neighboring patches. In addition, other organisms (e.g., plants, predators, parasites, symbionts) in the neighboring patches might also affect the antipredator responses of prey. Predator avoidance by prey is a key component that determines predator-prey dynamics. We suggest that predator-prey dynamics are not independent of the community, and that theory and experiments should focus on the effects of community on predator-prey interactions.

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Present or Past Herbivory: A Screening of Volatiles Released from *Brassica rapa* Under Caterpillar Attacks as Attractants for the Solitary Parasitoid, *Cotesia vestalis*

Soichi Kugimiya · Takeshi Shimoda · Jun Tabata · Junji Takabayashi

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Abstract Females of the solitary endoparasitoid *Cotesia vestalis* respond to a blend of volatile organic compounds (VOCs) released from plants infested with larvae of their host, the diamondback moth (*Plutella xylostella*), which is an important pest insect of cruciferous plants. We investigated the flight response of female parasitoids to the cruciferous plant *Brassica rapa*, using two-choice tests under laboratory conditions. The parasitoids were more attracted to plants that had been infested for at least 6 hr by the host larvae compared to intact plants, but they did not distinguish between plants infested for only 3 hr and intact plants. Although parasitoids preferred plants 1 and 2 days after herbivory (formerly infested plants) over intact plants they also preferred plants that had been infested for 24 hr over formerly infested plants. This suggests that parasitoids can distinguish between the VOC profiles of currently and formerly infested plants. We screened for differences in VOC emissions among the treatments and found that levels of benzyl cyanide and dimethyl trisulfide significantly decreased after removal of the host larvae, whereas terpenoids and their related compounds continued to be released at high levels. Benzyl cyanide and dimethyl trisulfide attracted parasitoids in a dose-dependent manner, whereas the other compounds were not attractive. These results suggest that nitrile and sulfide compounds temporarily released from plants under attack by host larvae are potentially more effective attractants for this parasitoid than other VOCs that are continuously released by host-damaged plants.

Key Words Herbivore-induced plant volatiles · Indirect defense · Tritrophic interaction · *Brassica rapa* · *Plutella xylostella* · *Cotesia vestalis*

S. Kugimiya (✉) · J. Tabata
National Institute for Agro-Environmental Sciences,
Kannondai 3-1-3,
Tsukuba, Ibaraki 305-8604, Japan
e-mail: kugimiya@affrc.go.jp

T. Shimoda
National Agricultural Research Center,
Kannondai 3-1-1,
Tsukuba, Ibaraki 305-8666, Japan

J. Takabayashi
Center for Ecological Research, Kyoto University,
Hirano 2-509-3,
Otsu, Shiga 520-2113, Japan

Introduction

Many plant species release specific blends of volatile organic compounds (VOCs) in response to attack by herbivores. The release of VOCs induced by herbivory can attract natural enemies of herbivores and may guide parasitoids or predators to their hosts or prey (Turlings et al. 1990; Takabayashi and Dicke 1996). Chemical information of this nature has attracted considerable attention as an indirect defense of plants against herbivorous insects mediated by their natural enemies at a higher level in a tritrophic interaction system (Karban and Baldwin 1997; Sabelis et al. 2007). However, the plant defense may not be constant, since the composition of plant VOCs can vary both in quality and in quantity, depending on various biotic and abiotic factors, and these changes can affect the attractiveness of the plants to natural enemies of the herbivores (Takabayashi et al. 1994; Maeda et al. 2000; Gouinguené and Turlings 2002).

The profile of VOCs also can change during the course of herbivory (Loughrin et al. 1994; Turlings et al. 1998; Scascighini et al. 2005). Diurnal cycles in the release of VOCs are related to the attraction of natural enemies of herbivores or to deterrence of herbivores (Loughrin et al. 1994; De Moraes et al. 2001; Shiojiri et al. 2006a), and the importance of the effects of light in a diurnal cycle have been reported (Gouinguené and Turlings 2002). However, there is a little information on changes in VOCs of infested plants during and after herbivory, particularly from the perspective of potential differences in plant-insect interactions between the two phases (Mattiacci et al. 2001; Hoballah and Turlings 2005). In natural ecosystems, herbivores may not stay on a single plant, but may move among plants, be eliminated by other predators (Shiojiri and Takabayashi 2005), or pupate and become unsuitable targets for parasitoids to oviposit. Thus, parasitoids may be able to distinguish between plants currently being attacked (hereafter, “infested plants”) and plants that were formerly attacked by herbivores (hereafter, “formerly infested plants”). By focusing on the difference in compositions of VOCs released from infested and formerly infested plants, we may be able to identify effective attractants for parasitoids.

In Japan, *Brassica* plants (Capparales: Brassicaceae) are grown during the spring, close enough together for adjacent plants to touch. A tritrophic system forms among the plant species, herbivorous insects, and their parasitoids. Caterpillars of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), are oligophagous on cruciferous plants. The specialist parasitoid wasp *Cotesia vestalis* (Haliday) [= *C. plutellae* (Kurdjumov)] (Hymenoptera: Braconidae) oviposits on *P. xylostella* larvae. The female parasitoids are attracted to crucifers infested by *P. xylostella* larvae through a specific blend of VOCs (Shiojiri et al. 2000) and lay a single egg per host.

Under laboratory conditions, infested cruciferous plants attract various parasitoids by emitting specific blends of VOCs (Mattiacci et al. 1994; Shiojiri et al. 2000; Van Poecke et al. 2001), and the mechanisms for the induction, regulation, and biosynthesis of those VOCs have been characterized (Mattiacci et al. 1995; Van Poecke and Dicke 2002; Shiojiri et al. 2006b; D’Auria et al. 2007; Herde et al. 2008). However, it is unclear which VOC components attract *C. vestalis*. In the present study, we screened for potential parasitoid attractants, by investigating the flight response of female parasitoids to plants that had been infested by their host larvae, *P. xylostella*, for different durations (infested plants), and their response to plants at various times after herbivory (formerly infested plants). We then analyzed the headspace volatiles released from infested and formerly infested plants to establish the time course of changes in the relative amounts of plant VOCs after the start of herbivory and after removal of the larvae, and tested synthetic versions of VOCs to evaluate their effectiveness as attractants for parasitoids.

Methods and Materials

Plants and Insects Japanese mustard spinach, *Brassica rapa* L. var. *perviridis* (Capparales: Brassicaceae), was cultivated in a greenhouse (25±3°C, 60±10% relative humidity (RH), 16L:8D photoperiod). Five plants were reared in a plastic pot (90 mm diam, 70 mm depth) for 4–5 wk, and were used for both insect rearing and flight preference tests. Diamondback moths, *P. xylostella*, originally collected from fields in Ayabe, Kyoto Prefecture, Japan, in 2001, were mass-reared on potted plants in a climate-controlled room (25±3°C, 60±10% RH, 16L:8D photoperiod). Eggs were collected every day, and hatched larvae were reared on cut plants in small cages (width 25 cm, depth 15 cm, height 10 cm). The solitary parasitoids *C. vestalis*, which parasitize mainly *P. xylostella* larvae, were obtained from their hosts collected in the same fields and were reared on *P. xylostella*-infested plants under the same conditions as their hosts. For use in the two-choice test, wasp cocoons were collected from the stock culture, and newly emerged adults were maintained with 50% aqueous honey in acryl cages (width 35 cm, depth 25 cm, height 30 cm) separately from the host-infested plants until the experiments.

Flight Response of Parasitoids to Host-infested Plants and to Synthetic Compounds The flight response of *C. vestalis* females was assessed by using a two-choice test in an acrylic chamber (width 35 cm, depth 25 cm, height 30 cm, 3,000 lux) in a climate-controlled room (25±3°C, 60±10% RH) (Shiojiri et al. 2000). Infested plants were prepared by allowing 15 third-instar *P. xylostella* to feed on potted plants of *B. rapa* for 3, 6, and 24 hr (hereafter, 3, 6, and 24 hr-infested plants, respectively; Fig. 1). The infestation began at 12:00 am, during the middle of the light phase of the photoperiod. Formerly infested plants were prepared by removing the host larvae from 24 hr-infested plants and then by using the plants for experiments 48 and 72 hr after the start of the infestation (1 and 2d-after plants, respectively; Fig. 1). In every treatment, we used almost the same size larvae, showing similar feeding activity, and the areas of damage caused by the larvae were identical for a given 24 hr-infested plant and the 1d-after and 2d-after versions of that plant. Intact plants were prepared as controls, without any treatment. To produce a two-choice test design, one potted plant from a given treatment was placed ca. 10 cm from a plant that had been subjected to a different treatment inside the acrylic chamber, with the leaves not overlapping.

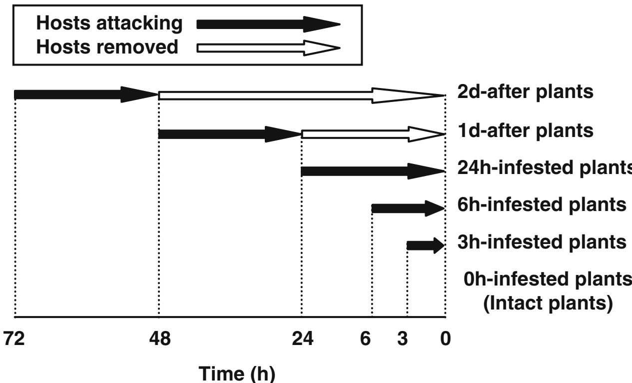
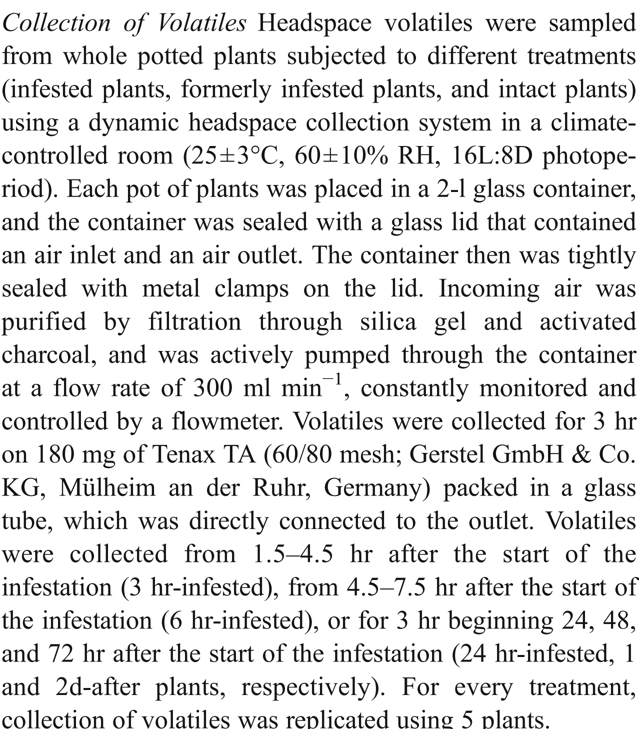


Fig. 1 Experimental time line for infestation of *Plutella xylostella.* Data were collected at various times following infestation (0, 3, 6, and 24 hr-infested plants) and following removal of larvae (1 and 2d-after plants)

At the start of each trial, a group of 10 female parasitoids was released from glass tubes (25 mm diam, 120 mm height) at a position centered between the two pots. After hovering between the two potted plants inside the chamber, the females landed on one of the plants. The total numbers of first landings on each potted plant by the parasitoids were counted for 30 min. After landing, parasitoids were carefully removed from the chamber with an insect aspirator. A few females that did not land on any potted plants within 30 min were recorded as no-choice subjects. At the end of each test, the test chamber was wiped with 70% ethanol aq., and the inside air was flushed out with a fan to avoid potential contamination by volatiles. Another group of 10 parasitoids was tested on the same day, with the positions of the plants subjected to different treatments switched to counteract any potential positional effects. These data were pooled as a single replicate. Three replications were carried out on different days. Individual insects (60 parasitoids in all) were used only once.

To test the flight response of parasitoids to compounds that were identified from infested plants and formerly infested plants (as described in the next section), we used triethyl citrate (Wako Pure Chemical industries, Ltd., Osaka, Japan) as a solvent and prepared 1–100 mg/l solutions of the test compounds. Each solution (200 μl) was applied to two square pieces of filter paper (20× 20 mm, Advantec No. 1, Japan) that were placed together on a cover glass (24×24 mm, Matsunami No. 1, Japan), which was then placed in the pot beside the intact plants. As a control, solvent only (200 μl) was applied to filter papers on the cover glass and placed beside intact plants. The preference of the parasitoids for intact plants presented together with the synthetic compounds vs. control plants was tested in the acrylic chamber. The amount of each compound in the headspace of the plants was checked and compared with the amount in the headspace of infested plants.


Collection of Volatiles Headspace volatiles were sampled from whole potted plants subjected to different treatments (infested plants, formerly infested plants, and intact plants) using a dynamic headspace collection system in a climate-controlled room (25±3°C, 60±10% RH, 16L:8D photoperiod). Each pot of plants was placed in a 2-l glass container, and the container was sealed with a glass lid that contained an air inlet and an air outlet. The container then was tightly sealed with metal clamps on the lid. Incoming air was purified by filtration through silica gel and activated charcoal, and was actively pumped through the container at a flow rate of 300 ml min^{-1}, constantly monitored and controlled by a flowmeter. Volatiles were collected for 3 hr on 180 mg of Tenax TA (60/80 mesh; Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) packed in a glass tube, which was directly connected to the outlet. Volatiles were collected from 1.5–4.5 hr after the start of the infestation (3 hr-infested), from 4.5–7.5 hr after the start of the infestation (6 hr-infested), or for 3 hr beginning 24, 48, and 72 hr after the start of the infestation (24 hr-infested, 1 and 2d-after plants, respectively). For every treatment, collection of volatiles was replicated using 5 plants.

Chemical Analysis of Headspace Volatiles The headspace volatiles collected in the Tenax tubes were analyzed using a gas chromatography-mass spectrometry (GC-MS) system consisting of an Agilent 6890N gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA, USA) coupled with an Agilent 5973N quadrupole mass selective detector. The collected volatiles were desorbed from the Tenax in a Thermodesorption System (Gerster, Inc.) by heating the tube from 20°C (1 min hold) to 200°C (4 min hold) at a rate of 60°C min^{-1}. The released volatile compounds were carried through a transfer line (250°C) to a Cold Injection System (Gerster, Inc.) and cryofocused at −100°C (30 sec hold). They then were heated at a rate of 12°C min^{-1} to 260°C (5 min hold) and injected by a splitless mode into an analytical capillary column (HP-5MS, 30 m×0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as carrier gas at a flow rate of 1.2 ml min^{-1}. The GC oven was programmed to start at 40°C (9 min hold) and then to rise at a rate of 10°C min^{-1} to 280°C (5 min hold). The column effluents were ionized by electron impact ionization (70 eV) with an ion source temperature of 250°C and monitored in the mass selective mode with a scan range from 35 to 350 m/z. Volatile compounds were identified tentatively by comparing their mass spectra with those in the Wiley Library database and further confirmed by comparing retention times and mass spectra to commercially available authentic standards (Wako Pure Chemical Industries, Ltd., Osaka, Japan and Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). (*E*)-*β*-Ocimene was synthesized from (*Z*)-*β*-ocimene (available as mixture of ocimene

isomers; SAFC Supply Solutions, St. Louis, MO, USA) by isomerizing the *cis* to the *trans* configuration (Sgoutas and Kummerow 1967).

Statistics In the flight response test, significant differences between the numbers of wasps that landed on either of the two potted plants and the null hypothesis of an expected ratio of 0.5:0.5 were analyzed by using a replicated *G*-test to account for suspected heterogeneities among the replications (Sokal and Rohlf 1995). To compare the relative amounts of each volatile compound released from the plants after different treatments, the total ion peak area of each compound detected by GC-MS analysis was calculated by integration. We applied one-way analysis of variance (ANOVA), followed by Tukey's HSD test, to the results of the chemical analysis. When necessary, data were normalized using logarithmic transformation [ln $(10x + 1)$] to meet the assumptions for ANOVA. These analyses were performed using JMP software (version 7.0.1, SAS Institute Inc., Cary, NC, USA).

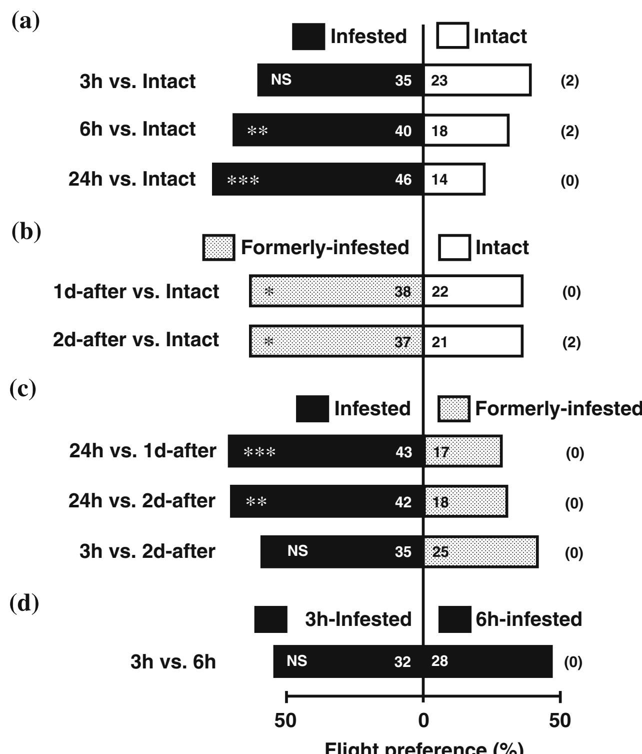


Fig. 2 Flight preferences (%) of female parasitoids *Cotesia vestalis* (N=60) among intact plants (open bars), *Plutella xylostella* larvae-infested plants (3, 6, and 24 hr: filled bars) and formerly infested plants (1 and 2d-after: dotted bars). Numbers in bars indicate the number of parasitoids that landed on each plant. Numbers in parentheses indicate parasitoids that did not choose any plants. Asterisks mean significant differences within each preference test set (replicated *G*-test; *: P<0.05, **: P<0.01, ***: P<0.001, NS = no significance)

Results

Flight Response of Parasitoids to Infested and Formerly Infested Plants Female parasitoids preferred 6 and 24 hr-infested plants over intact plants in the two-choice test (Fig. 2a) but 3 hr -infested plants were not distinguished from intact plants (Fig. 2a) or from 6 hr-infested plants (Fig. 2d). Female parasitoids also preferred 1 and 2d-after plants over intact plants, respectively (Fig. 2b). When offered both 24 hr-infested plants and formerly infested plants (1 or 2d-after) at the same time, parasitoids preferred the 24 hr-infested plants (Fig. 2c). When offered 3 hr-infested plants and 2d-after plants in the two-choice test, parasitoids showed no significant preference (Fig. 2c). There was no significant heterogeneity among replicates in any tested sets (replicated *G*-test, df=2, P>0.05 for each G_h), suggesting good reproducibility of the two-choice test.

Volatiles Released from Infested and Formerly Infested Plants Profiles of VOCs released by infested plants, formerly infested plants, and intact plants were quantitatively but not qualitatively different. In the headspace of these plants, 11 major compounds were identified by GC-MS analysis, and other unidentified minor compounds were ignored. Various alkanes and aldehydes with lengths of C9 to C24 were ignored as impurities, since they were also detected from the blank headspace. Ten compounds were identified by using authentic standards, and junipene was tentatively identified although an authentic standard was not available. The compounds included the nitrile, benzyl cyanide (Fig. 3); the sulfur-containing compounds, aryl isothiocyanate and dimethyl trisulfide (Fig. 3); the monoterpenes, myrcene, limonene, (E)-β-ocimene, and α-pinene (Fig. 4); the sesquiterpenes, (E,E)-α-farnesene and junipene (Fig. 4); and the terpenoid related ketones, 6-methyl-5-hepten-2-one and 6,10-dimethyl-5,9-undecadien-2-one (Fig. 4).

The relative amounts of benzyl cyanide (ANOVA, MS=1.885, df=5, F=27.415, P<0.001) and dimethyl trisulfide (MS=0.923, df=5, F=22.998, P<0.001) in the headspace increased after the start of the infestation (Fig. 3). The relative amounts of the four monoterpenes (myrcene, MS=0.148, df=5, F=4.662, P=0.004; limonene, MS=0.314, df=5, F=8.494, P<0.001; (E)-β-ocimene, MS=0.369, df=5, F=9.901, P<0.001; α-pinene, MS=1.144 df=5, F=9.385, P<0.001; Fig. 4), and one sesquiterpene [(E,E)-α-farnesene, MS=0.372, df=5, F=5.134, P=0.0024; Fig. 4] increased following infestation. However, no significant differences were observed in the relative amounts of the remaining compounds, aryl isothiocyanate (MS=0.401, df=5, F=2.189, P=0.089; Fig. 3), junipene (MS=0.069, df=5,

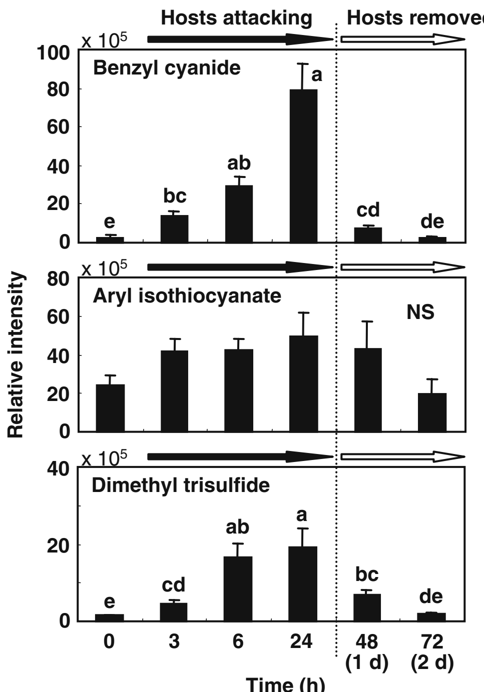
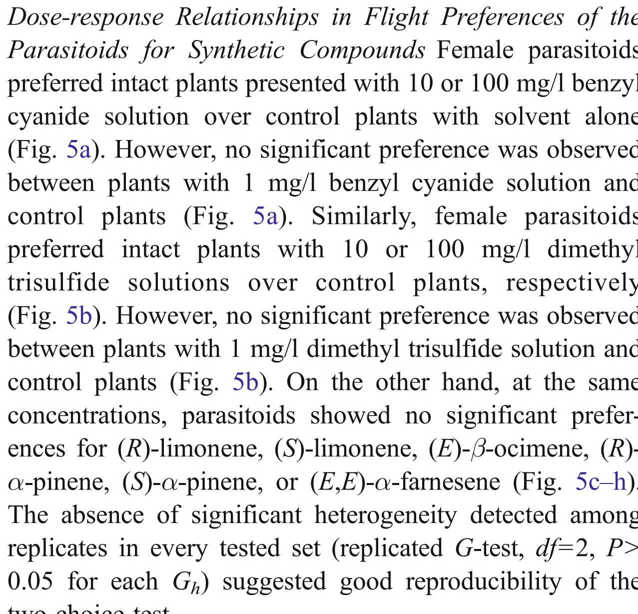


Fig. 3 Time-course indicating relative amounts of a nitrile and sulfur-containing compounds detected by GC-MS analysis of the headspace volatiles released from intact plants (0 hr), *Plutella xylostella* larvae-infested plants (3, 6, and 24 hr) and formerly infested plants (1 and 2d-after) (N=5). Different small letters mean significant differences in relative amounts of each compound (ANOVA followed by Tukey's HSD test; P<0.05, NS = no significance)

F=1.968, P=0.120; Fig. 4), 6-methyl-5-hepten-2-one (MS=0.281, df=5, F=1.354, P=0.276; Fig. 4), and 6,10-dimethyl-5,9-undecadien-2-one (MS=0.133, df=5, F= 1.118, P=0.377; Fig. 4).

The compounds with significant differences during the time-course measurements were analyzed further by using a pairwise test. In particular, benzyl cyanide, dimethyl trisulfide, (E)-β-ocimene, and α-pinene were induced as soon as 3 hr after the start of the infestation (Figs. 3 and 4). Limonene and (E,E)-α-farnesene were not induced until 24 hr after infestation (Fig. 4). After removal of the larvae, benzyl cyanide and dimethyl trisulfide decreased rapidly, and the amounts of both compounds released from 1 and 2d-after plants were smaller than those released from 24 hr-infested plants (Fig. 3). The relative amounts of the two compounds from 1d-after plants were larger than those from intact plants (Fig. 3), but the amounts in 2d-after plants did not differ significantly from intact plants (Fig. 3). In contrast, most terpenoids were released from the plants at significantly higher levels than from intact plants after the larvae had been removed (Fig. 4), and the amounts of limonene, (E)-β-ocimene, α-pinene, and (E,E)-α-farnesene released from 1 and 2d-after plants were not significantly different from those of 24 hr-infested plants (Fig. 4).



Dose-response Relationships in Flight Preferences of the Parasitoids for Synthetic Compounds Female parasitoids preferred intact plants presented with 10 or 100 mg/l benzyl cyanide solution over control plants with solvent alone (Fig. 5a). However, no significant preference was observed between plants with 1 mg/l benzyl cyanide solution and control plants (Fig. 5a). Similarly, female parasitoids preferred intact plants with 10 or 100 mg/l dimethyl trisulfide solutions over control plants, respectively (Fig. 5b). However, no significant preference was observed between plants with 1 mg/l dimethyl trisulfide solution and control plants (Fig. 5b). On the other hand, at the same concentrations, parasitoids showed no significant preferences for (R)-limonene, (S)-limonene, (E)-β-ocimene, (R)-α-pinene, (S)-α-pinene, or (E,E)-α-farnesene (Fig. 5c–h). The absence of significant heterogeneity detected among replicates in every tested set (replicated G-test, df=2, P> 0.05 for each G_h) suggested good reproducibility of the two-choice test.

The highest levels of benzyl cyanide (3.68±0.59 ng) and dimethyl trisulfide (0.50±0.16 ng) were released by 24 hr-infested plants. When intact plants were treated with 10 mg/l benzyl cyanide, levels in the headspace were approximately three-fold higher than the levels from 24 hr-infested plants. Even higher levels were detected with benzyl cyanide applied at 100 mg/l. When dimethyl trisulfide was applied at concentrations of 10 and 100 mg/l, 200–500 times the amount released from 24 hr-infested plants were detected in the headspace. The tested concentrations of other synthetic VOCs were similar to the amounts released by the infested plants detected in the headspace.

Discussion

Female *C. vestalis* parasitoids showed a significant preference for *B. rapa* plants that had been infested for 24 hr with larvae of their host herbivores, *P. xylostella*, over formerly infested plants from which the larvae had been removed (Fig. 2c). GC-MS analysis revealed that benzyl cyanide and dimethyl trisulfide were released at significantly higher levels from 24 hr-infested plants than from formerly infested plants (Fig. 3). These results suggest that the two compounds released from 24 hr-infested plants are potentially better attractants for parasitoids than the other VOCs released from formerly infested plants.

We further tested synthetic versions of benzyl cyanide and dimethyl trisulfide as strong candidates for parasitoid attraction, and found that intact plants presented together with the single compounds attracted parasitoids in a dose-dependent manner (Fig. 5a,b), in contrast to the other compounds found in the headspace (Fig. 5c–h). When

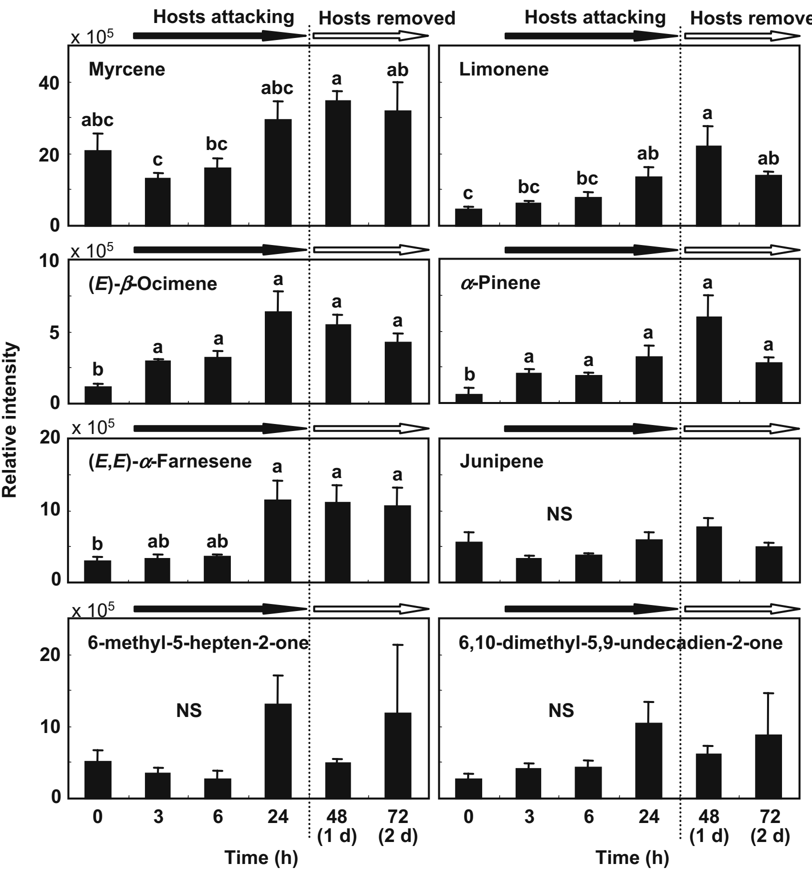


Fig. 4 Time-course indicating relative amounts of terpenoids detected by GC-MS analysis of the headspace volatiles released from intact plants (0 hr), *Plutella. xylostella* larvae-infested plants (3, 6, and 24 hr) and formerly infested plants (1 and 2d-after) (N=5). Different small letters mean significant differences in relative amounts of each compound (ANOVA followed by Tukey's HSD test; $P<$ 0.05, NS = no significance)

benzyl cyanide was applied to intact plants at the most attractive concentration (10 mg/l; Fig. 5a), its concentration in the headspace was only a few-fold higher than the concentration detected in the headspace of 24 hr-infested plants. On the other hand, when dimethyl trisulfide was applied at the attractive concentrations (10 and 100 mg/l; Fig. 5b), its concentration in the headspace was far larger than the concentration released from 24 hr-infested plants. These results suggest that although either compound can act as a parasitoid attractant, benzyl cyanide is most likely to be responsible for parasitoid discrimination between 24 hr-infested plants and formerly infested plants. Dimethyl trisulfide may have little effect at the levels normally released by these plants.

Significant differences in the amounts of VOCs detected by chemical analysis may not always result in significant preferences by parasitoids. For example, significantly larger amounts of benzyl cyanide were analytically detected from 3 hr-infested plants than from 2d-after plants (Fig. 3), but no significant preference was shown by parasitoids between these plants (Fig. 2c). Differences in the amounts of VOCs are necessary for parasitoids to discriminate between plants, but small differences sometimes may not be enough to be perceived by parasitoids with their limited sensitivity to VOCs.

Female parasitoids preferred 6 or 24 hr-infested plants over intact plants (Fig. 2a). There was also a slight, but not significant, trend in the preference for 3 hr-infested plants over intact plants. This trend is supported by the observation that parasitoids did not discriminate between 3 and 6 hr-infested plants (Fig. 2d). GC-MS analysis revealed that the emission of some VOCs was induced within a few hours of the initial damage by larvae. Together with benzyl cyanide and dimethyl trisulfide, (E)-β-ocimene and α-pinene were significantly increased in 3, 6, and 24 hr-infested plants (Fig. 4). (E)-β-Ocimene and α-pinene were not attractive to parasitoids at the tested concentrations (Fig. 5e–g), which are similar to the levels these compounds released by the infested plants. Benzyl cyanide may be the main compound that attracts parasitoids to infested plants, and dimethyl trisulfide may be of less importance. However, the relative attractiveness for parasitoids of mixtures of terpenoids with benzyl cyanide and dimethyl trisulfide has not been tested, but may be important in light of the potential ability of the parasitoids to use associative learning about VOCs (Turlings et al. 1993; Vet et al. 1995).

Formerly infested plants, which no longer had larvae, were still preferred by the parasitoids over intact plants (Fig. 2b). It appears that the plants kept releasing VOCs that attracted the parasitoids for 2 d after herbivory had

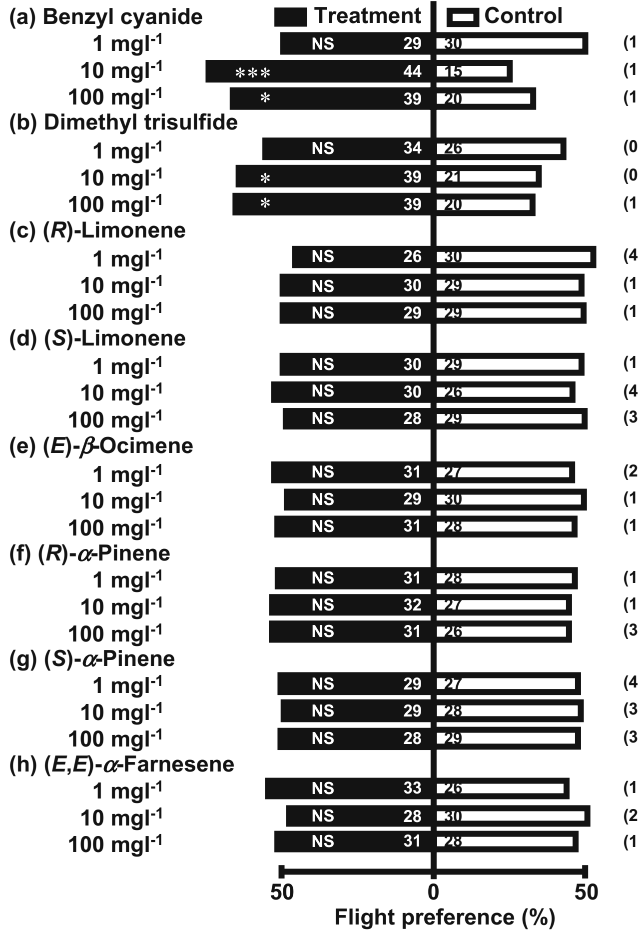
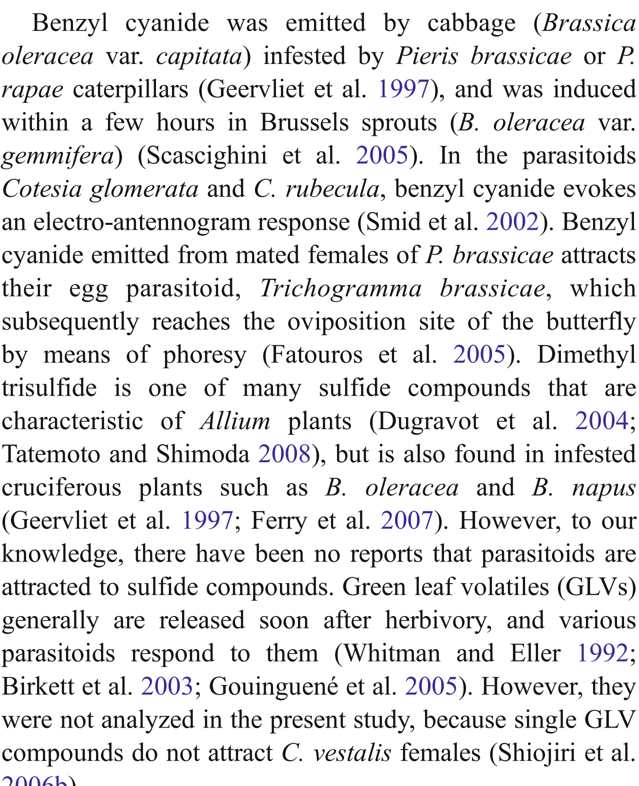


Fig. 5 Flight preferences (%) of female parasitoids *Cotesia vestalis* (N=60) between intact plants presented together with different concentrations of synthetic compounds (filled bars) and control plants (open bars). Numbers in bars indicate the number of parasitoids that landed on each plant. Numbers in parentheses indicate parasitoids that did not choose any plants. Asterisks mean significant differences within each preference test set (replicated G-test; *: $P<0.05$, ***: $P<0.001$, NS = no significance)

stopped. Benzyl cyanide and dimethyl trisulfide cannot explain the observed preferences, since the two compounds decreased rapidly after removal of the larvae (Fig. 3). On the other hand, terpenoids were emitted from formerly infested plants at significantly higher levels than from intact plants (Fig. 4). Ibrahim et al. (2005) reported that *C. vestalis* preferred intact cabbages plus synthetic (*R*)-(+)-limonene to control cabbages, depending on the cabbage subspecies. In this study, intact plants plus individual synthetic terpenoids, including (*R*)-(+)-limonene, did not attract parasitoids significantly (Fig. 5c–h). These results imply that a certain blend of VOCs, especially of terpenoid compounds, was responsible for the preference for formerly infested plants over intact ones. There was also another possibility that the hosts themselves and visual cues, such as damage holes, might have affected the preference for infested and formerly infested plants over intact plants.


Benzyl cyanide was emitted by cabbage (*Brassica oleracea* var. *capitata*) infested by *Pieris brassicae* or *P. rapae* caterpillars (Geervliet et al. 1997), and was induced within a few hours in Brussels sprouts (*B. oleracea* var. *gemmifera*) (Scascighini et al. 2005). In the parasitoids *Cotesia glomerata* and *C. rubecula*, benzyl cyanide evokes an electro-antennogram response (Smid et al. 2002). Benzyl cyanide emitted from mated females of *P. brassicae* attracts their egg parasitoid, *Trichogramma brassicae*, which subsequently reaches the oviposition site of the butterfly by means of phoresy (Fatouros et al. 2005). Dimethyl trisulfide is one of many sulfide compounds that are characteristic of *Allium* plants (Dugravot et al. 2004; Tatemoto and Shimoda 2008), but is also found in infested cruciferous plants such as *B. oleracea* and *B. napus* (Geervliet et al. 1997; Ferry et al. 2007). However, to our knowledge, there have been no reports that parasitoids are attracted to sulfide compounds. Green leaf volatiles (GLVs) generally are released soon after herbivory, and various parasitoids respond to them (Whitman and Eller 1992; Birkett et al. 2003; Gouinguené et al. 2005). However, they were not analyzed in the present study, because single GLV compounds do not attract *C. vestalis* females (Shiojiri et al. 2006b).

It is reasonable for parasitoids to use benzyl cyanide as a host-searching cue, since it is common in cruciferous plants as a breakdown product of glucosinolates through a rapid reaction catalyzed by myrosinase at damaged sites (Wittstock and Halkier 2002). Benzyl cyanide was not detected in feces of *P. xylostella* larvae, which can disarm consumed glucosinolates by means of glucosinolate sulfatase (Ratzka et al. 2002). Nonetheless, female parasitoids could detect benzyl cyanide released from damaged plant tissue. In addition, benzyl cyanide could be systemically inducible, like terpenoids and GLVs, through the jasmonic acid (JA) pathway as suggested for other *Brassica* species (Bruinsma et al. 2009). Further physiological studies are needed to investigate JA-dependent induction of benzyl cyanide and dimethyl trisulfide in *B. rapa*.

In the present study, we clarified the attractants for parasitoids by screening for candidates based on the relative amounts of VOCs released from plants currently under attack and from plants that were attacked in the past. Further comparative studies to identify the VOCs that attract foraging parasitoids to various plant species are necessary, to effectively use VOCs for biological control. Further studies could also reveal details of the chemical basis for the tritrophic systems that are formed in ecological processes.

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Influence of Feeding and Oviposition by Phytophagous Pentatomids on Photosynthesis of Herbaceous Plants

Violeta Velikova · Gianandrea Salerno · Francesca Frati · Ezio Peri · Eric Conti · Stefano Colazza · Francesco Loreto

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Abstract Feeding by herbivorous insects may change photosynthetic activity of host plants. We studied how feeding and oviposition by herbivorous stink bugs, *Murgantia histrionica* and *Nezara viridula* (Heteroptera: Pentatomidae), affect photosynthetic parameters of *Brassica oleracea* (savoy cabbage) and *Phaseolus vulgaris* (French bean). First, we measured photosynthetic gas exchange, chlorophyll fluorescence imaging, and emission of induced volatile organic compounds (VOC) immediately after feeding and during a post-feeding period. Photosynthesis decreased rapidly and substantially in *B. oleracea* and *P. vulgaris* infested by feeding bugs. Stomatal conductance did not decrease proportionally with photosynthesis; instead, inhibition of photosynthesis likely was due to a reduced diffusion of CO_2 in the mesophyll. We also measured the impact of oviposition *per se* and oviposition associated with feeding on photosynthetic parameters. A surprisingly large inhibition of photosynthesis was detected in cabbage leaves in response to oviposition by *M. histrionica*, even when oviposition was not associated with feeding activity. High resolution chlorophyll fluorescence imaging revealed that the damage to photochemistry caused by feeding and oviposition was restricted to the attacked areas. By contrast, the photochemical yield increased temporarily in the unaffected areas of the attacked leaves, indicating the onset of a compensatory response. Measurement of volatile organic compounds (VOC) revealed that feeding-damaged plants did not emit detectable amounts of VOC, indicating cellular damage (methanol and green leaf volatiles). However, feeding by *M. histrionica* induced emission of mono- and sesquiterpenes in savoy cabbage leaves. The different time-course of the induction of these two classes of terpenes may reflect the induction of two different biosynthetic pathways and indicate different roles of these terpenoids in tritrophic interactions.

Key Words Heteroptera · Pentatomidae · *Murgantia histrionica* · *Nezara viridula* · Feeding · Oviposition · Photosynthesis · Tritrophic interactions

V. Velikova
Bulgarian Academy of Sciences, Institute of Plant Physiology,
Acad. G. Bonchev Str., Bl. 21,
Sofia 1113, Bulgaria

G. Salerno · F. Frati · E. Conti
Dipartimento di Scienze Agrarie e Ambientali,
Università di Perugia,
Borgo XX Giugno 74,
06121 Perugia, Italy

E. Peri · S. Colazza
Dipartimento di S.En.Fi.Mi.Zo., sezione di Entomologia,
Zoologia ed Acarologia, Università di Palermo,
Viale delle Scienze 13,
90128 Palermo, Italy

F. Loreto (✉)
Consiglio Nazionale delle Ricerche,
Istituto per la Protezione delle Piante (CNR-IPP),
50019 Sesto Fiorentino,
Firenze, Italy
e-mail: francesco.loreto@ipp.cnr.it

Present Address:
V. Velikova
Consiglio Nazionale delle Ricerche, Istituto di Biologia
Agroambientale e Forestale (CNR-IBAF),
Via Salaria Km. 29,300, 00015 Monterotondo,
Roma, Italy

Introduction

Infestation by insects can trigger complex physiological responses in plants (Nabity *et al.* 2009). In particular, the

impact of herbivorous insects on photosynthesis is variable. Different insect feeding habits (*e.g.*, chewing, piercing-sucking) contribute to this variability (Welter 1989; Peterson and Higley 2001). Where photosynthetic tissue is lost during insect feeding, both an increase (Welter 1989) and a decrease (Zangerl *et al*. 2002) of photosynthesis of the remaining leaf tissue may be observed. Water balance in the remaining tissue also may be altered after tissue loss by caterpillar feeding (Zangerl *et al*. 2002; Aldea *et al*. 2006). When no loss of photosynthetic tissues occurs (*e.g*., when piercing-sucking insects attack a plant), photosynthetic damage often is less apparent. In these cases, measurements of gas exchange (CO_2 uptake and H_2O release) have been used to show a reduction in photosynthetic rates (Burd and Elliott 1996; Haile *et al*. 1999; Macedo *et al*. 2003).

The effects of insect oviposition have received less attention than the direct impact of insect feeding activity on photosynthesis. Schröder *et al*. (2005) reported that net photosynthetic rate, electron transport rate (J_{max}), and maximum rate of Rubisco activity (V_{cmax}) of pine needles laden with eggs of an herbivorous sawfly were lower than in egg-free control plants that were not attacked. Egg deposition by herbivorous sawflies is associated with wounding of a plant since sawflies "saw" with their ovipositor into plant tissue and lay eggs into this ovipositional wound. To date, it is still unknown how insect oviposition that is not associated with ovipositional plant wounding affects photosynthesis. Furthermore, it is unknown how the combination of oviposition and feeding damage affects photosynthesis (Hilker *et al*. 2002).

In addition to gas exchange, chlorophyll fluorescence provides a powerful, non-invasive tool for investigating the effects of various stresses on the component processes of photosynthesis (Baker *et al*. 2001). One of the key parameters provided by chlorophyll fluorescence is the quantum yield of photosystem II in light-adapted leaves (Φ_{PSII}). This parameter is related to the rate of carbon fixation (Genty *et al*. 1989), and it may be used to calculate the photosynthetic electron transport rate driving photosynthesis and photorespiration (Di Marco *et al*. 1990). Damage to the photochemistry of photosynthesis also is revealed by measurements of the quantum yield in dark-adapted leaves (the ratio between variable and maximal fluorescence, F_v/F_m) and by the amount of photochemical energy lost as heat (the non-photochemical quenching of fluorescence, NPQ) (Genty and Harbinson 1996). The development of imaging fluorescence has further improved the suitability of this technique to assess damage to the photosynthetic apparatus (Baker *et al.* 2001). Imaging fluorescence indicates the topography of damage and may detect systemic damage, *i.e*., damage to parts that are not affected directly by the stressor (Chaerle *et al*. 2007). Herbivore damage to foliage frequently initiates changes in the primary photosynthetic reactions. For instance, fluorescence imaging showed that feeding by larvae of *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) led to reduction of Φ_{PSII} and the rate of CO_2 uptake in host plants (Zangerl *et al*. 2002; Tang *et al*. 2006).

Another indicator of damage in plants is the emission of volatile organic compounds (VOC). Several studies have shown that insects can induce or change the spectrum of volatiles emitted by plants (Karban and Baldwin 1997; Dicke and Hilker 2003). Some VOC are emitted upon damage to membranes (*e.g*., six-carbon (C6) VOC produced by lipoxygenation of membranes, often referred to as LOX compounds) or cell walls (methanol) (Loreto *et al*. 2006). Other VOC are emitted as part of sophisticated defensive strategies that have evolved and defend plants against herbivores, either directly (deterring the attack) or indirectly (*e.g*., allowing communication with predators or parasitoids of the herbivores) (Dicke and Hilker 2003). Induction of volatiles occurs locally at the site of oviposition and systemically at leaf lamina adjacent to the oviposition site (Meiners and Hilker 2000; Colazza *et al*. 2004a; Conti *et al*. 2006; Hilker and Meiners 2006).

In this study we examined how feeding, oviposition, and both feeding and oviposition by the stink bugs *Murgantia histrionica* (Hahn) and *Nezara viridula* (L.) (Heteroptera Pentatomidae) affect photosynthetic processes in two herbaceous plants, savoy cabbage, *Brassica oleracea* L., and French bean, *Phaseolus vulgaris* L., respectively. We chose these stink bugs because of their different feeding habits on host plant leaves. Preliminary observations showed that *M. histrionica* adults adopt a lacerate-and-flush feeding mode (*sensu* Miles 1972). This involves heavy damage to the mesophyll tissue due to mechanical cell laceration and extra-oral digestion by salivary enzymes (Miles 1972; Hory 2000). Conversely, *N. viridula* adults prefer to feed on leaf veins with a stylet-sheath feeding mode (*sensu* Miles 1972), destroying only a few cells and causing minimal mechanical damage (Miles 1972; Hory 2000). In contrast to their feeding modes, oviposition behavior of the two pentatomids is similar. Neither species cuts or otherwise physically damages the host substrate during oviposition; the eggs are laid in clusters on the leaf surface. Eggs are glued together and adhere to the leaf surface by a sticky oviduct secretion (Bin *et al*. 1993; Colazza *et al*. 2004b). Since oviposition habits do not differ between the pentatomid species, experiments on the effects of oviposition alone or in combination with feeding were conducted only with *M. histrionica* ovipositing on *B. oleracea.*

The influences of feeding and oviposition activities on plant leaves were investigated by measuring leaf gas exchange and by chlorophyll fluorescence imaging. The latter approach enabled us to visualize and quantify spatial variation in the components of the photosynthetic process. The emission of VOC also was measured to determine

whether VOC can provide an early indicator of damage to the photosynthetic apparatus. Furthermore, our measurements elucidated the time-course of induction of volatile molecules that may have antioxidant action (Vickers *et al*. 2009) and that may be involved in indirect defense against biotic stressors (Dicke *et al*. 2009).

Methods and Materials

Plants and Insects

Two plant—herbivore systems were used: savoy cabbage (*Brassica oleracea*), a host plant of *Murgantia histrionica*; and French bean (*Phaseolus vulgaris*), a host plant of *Nezara viridula*.

Seeds of *B. oleracea* var. sabauda (cv. Salto, kindly provided by Royal Sluis Brand) were placed individually in pots filled with peat. Seven days later, seedlings were transplanted into plastic pots filled with a mixture of inert substrates, agriperlite (BPB Vic; Agrimport, Italy) and vermiculite (Sopram, Italy). Plants were kept in a greenhouse under controlled conditions (25±3°C, 50–60% RH, 12:12 L:D), watered daily, and fertilized with a solution of Flory 9 Hydro (N-P-K 15-7-22) (Planta Regenstauf), sequestrene (NK 3–15 containing Fe EDDHA, Syngenta) and urea (Hydro Agri Italia Milano) (1 l solution: 1 g Flory 9, 0.04 g sequestrene and 0.1 g urea). All experiments were conduced with 4–5-wk-old plants with 5–6 fully expanded leaves.

Seeds of French bean plants (*P. vulgaris*, cv Lingua di Fuoco) were placed individually in plastic pots filled with fertilized commercial soil (Trflor - HOCHMOOR) and grown in a greenhouse under controlled conditions (25±3°C, 50–60% RH, 12:12 L:D). Plants were watered regularly to pot capacity and fertilized once per week with full-strength Hoagland's solution. Ten-d-old plants with 3–4 fully expanded leaves of the central plant portion of savoy cabbage and French bean were used for the experiments.

Murgantia histrionica originally was collected from cabbage in the area of Beltsville, MD, USA, in the year 2000 and maintained under quarantine conditions in a growth chamber (25±1°C, 60±5% RH, 16:8 L:D) at the entomology laboratory of the University of Perugia, Italy. The colony of *M. histrionica* was reared in plastic cages and fed on cabbage as described by Conti *et al*. (2004).

Nezara viridula adults were collected in Central Italy, and the colony was reared under laboratory conditions (25±1°C, 60±5% RH, 16:8 L:D) in a plastic cage where it was fed on sunflower seeds, seasonal fresh vegetables, and water as described by Colazza *et al*. (1999).

Mated *M. histrionica* and *N. viridula* females of the same age (10-14-d-old) were used for plant treatments.

Plant Treatment

Plants were treated by exposing single leaves to single insect females, placed on the lower surface inside a suitable "clip cage" (3.8 cm diam; 1 cm high; modified Petri dish with the rim covered by a small sponge ring). One insect per cage and two cages per plant were kept under controlled environmental conditions (25±1°C, 60±5% RH). After 8 h, the cages with insects were removed, and measurements were started.

Savoy cabbage plants were subjected to the following treatments with *M. histrionica*: a) feeding punctures, b) combination of feeding punctures and oviposition, and c) oviposition only. Gravid *M. histrionica* with excised stylets were used to obtain plants with egg masses uninjured by adult feeding activity. To excise the stylets, mated females in the pre-ovipositional state were immobilized at −4°C for ~4 min, and their stylets were drawn from the labium with an entomological pin (no. 000) and half their length amputated with precision micro-scissors under a stereomicroscope (Zeiss® Stemi SV8) illuminated by optical fiber illumination (Intralux® 5000). French bean plants were exposed to *N. viridula* to assess the impact of feeding activity only.

Measurements of gas exchange, chlorophyll fluorescence, and VOC emissions were carried out before insects started to feed (control), immediately after insect removal (time 0), and 24, 48, and 72 h after insect removal. The effect of oviposition and of the combination of feeding and oviposition was assessed 24 h after removing the insect, *i.e*., 24–32 h after oviposition. All experiments were replicated on five different plants.

Visible Injury

Evidence of insect feeding was recorded 0 and 48 h after insect removal. For observation and documentation, we used a stereomicroscope (Wild® M420) equipped with a high resolution digital camera (SIS® ColorView 12).

Gas Exchange Analyses

Gas exchange was measured in the upper lamina, using the LI-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). A portion (6 cm^2) of the leaf area on which the insect was allowed to feed and/or oviposit was clamped in a gas-exchange cuvette and exposed to a 0.44 L min^{-1} flow of synthetic air, contaminant-free and made by mixing N_2 (80%), O_2 (20%) and 400 ppm CO_2. The relative humidity within the chamber was controlled at 45–55%. Measurements were done at a photosynthetically active radiation (PAR) of 400 μmol $m^{-2}s^{-1}$ and a cuvette temperature of 25°C. Photosynthesis (A), transpiration (T), and stomatal

conductance (g_s) were calculated using the LI-6400 software from the difference between CO_2 and H_2O concentration at the cuvette inlet and outlet. Water use efficiency (WUE) was calculated as WUE = A/T. To measure VOC emissions the outlet of the cuvette was disconnected from the LI-6400 system, and the flow was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Agilent, Cernusco sul Naviglio, Italy). A volume of 30 L of air was pumped through the trap at a rate of 150 mL min^{-1}. The cartridge was analyzed immediately by GC-MS (see below).

Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence imaging was used as an effective indicator of invisible injury to the leaf caused by the treatments, in particular, damage to the photochemical leaf apparatus. Fluorescence images generally were taken from upper leaf part; however, in the oviposition treatment, images also were taken from the lower leaf part to better visualize the effect of eggs deposited on that leaf side (see *e.g.* Fig. 5). Images of chlorophyll fluorescence were obtained using the MINI-Imaging-PAM Chlorophyll Fluorometer (Walz, Effelrich, Germany). The MINI-Imaging-PAM employs blue LEDs, with a wavelength peak at 450 nm, for pulse modulated measuring light, continuous actinic illumination, and saturation pulse. The fluorescence signal emitted by leaves was collected by a charge-coupled device (CCD) camera with a resolution of 640×480 pixels (exposed leaf area 3×1.6 cm). Pixel value images of the fluorescence parameters were displayed with the help of a false color code ranging from black (0.000) through red, yellow, green, blue, to pink (1.000 = end scale). Leaves were dark adapted for 15 min prior to determination of minimal (F_0) and maximal (F_m) chlorophyll fluorescence. These measurements were used to calculate the maximum quantum yield of PSII photochemistry ($F_v/F_m = (F_m–F_0)/F_m$). Leaves then were exposed to actinic, moderate light intensity (371 µmol $m^{-2}s^{-1}$) and, after a steady-state fluorescence (F_s) was reached, a saturating pulse was applied to determine the maximum fluorescence (F'_m). Chlorophyll fluorescence images taken from illuminated leaves were used to calculate the operating photochemical efficiency of PSII ($\Phi_{PSII} = (F'_m–F_s)/F'_m$; Genty *et al.* 1989). Non-photochemical quenching (NPQ), which reflects thermal dissipation of excess light energy, then was calculated from $NPQ = (F_m–F'_m)/F_m$ (Bilger and Björkman 1991).

Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

GC-MS analyses were performed with an Agilent 6850 gas chromatograph coupled to an Agilent 5975C Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA). The GC was supplied with a thermal desorber UNITY (Markes International Limited). The GC was equipped with a splitless injector and an HP-5MS capillary column (30 m in length, 250 µm i.d. and 0.25 µm film thickness). The column oven temperature was kept at 40°C for the first 5 min, then increased by 5°C min^{-1} to 250°C, and maintained at 250°C for 2 min. Helium was used as carrier gas. The concentration of each volatile was calculated by comparison with the peak area of a gaseous standard. The GC-MS was calibrated weekly using cylinders with standard mixtures of the main isoprenoids emitted by plants at an average concentration of 60 ppb (Rivoira, Milan, Italy). Compound identification was made by using the NIST library provided with the GC/MS ChemStation software (Agilent). GC peak retention time was substantiated by analysis of parent ions and main fragments of the spectra.

Statistical Analyses

Data shown represent the means ± s.e. of measurements of five different plants. Means were analyzed by ANOVA and statistically separated by Tukey's test. Means significantly different at the 95% confidence level ($P<0.05$) are shown by different letters. The package GraphPad InStat version 3.10 for Windows was used (GraphPad Software, San Diego, CA, USA, www.graphpad.com). ANOVA *P* and *F* values, and degrees of freedom are given in figure legends. Chlorophyll fluorescence images are shown as single measurements that are representative of the mean effect of the treatment.

Results

Visible Injury

Feeding by *M. histrionica* caused visible injury to cabbage leaves. This damage was evident soon after insect removal (Fig. 1a) and did not develop further after 48 h (Fig. 1b). When examined with the stereomicroscope, the injury caused appeared as sub-circular discolored lesions that were more evident in the upper than in the lower leaf lamina. These lesions were formed by conglomerates of radial stripes starting from the central stylet puncture sites (Fig. 1c). In the lower leaf lamina, the stylet punctures were visible as black spots (Fig. 1d) with a protruding salivary sheath (flange) (Fig. 1d insert). No damage was dectectable by stereomicroscopic studies of French bean leaf lamina 0–48 h after feeding by *N. viridula*.

Gas Exchange Analyses

The effect of *M. histrionica* feeding and egg deposition on photosynthesis, stomatal conductance, transpiration, and

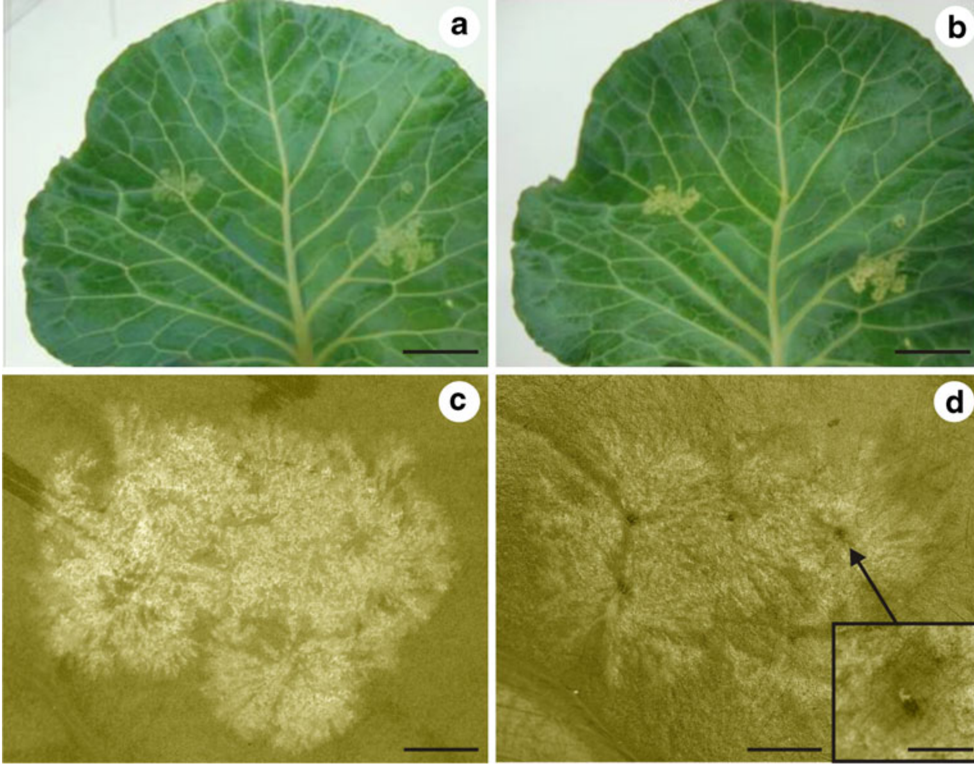


Fig. 1 Damage caused by feeding *Murgantia histrionica* adults on savoy cabbage leaves. **a** Feeding damage immediately after insect removal (time = 0 h) (scale bar = 1 cm); **b** Feeding damage 48 h after insect removal (scale bar = 1 cm); **c** Magnification of feeding damage on upper leaf lamina (time = 0 h) (scale bar = 1 mm); **d** Magnification of feeding damage on lower leaf lamina at time = 0 h (scale bar = 1 mm), inset: external salivary sheath material (flange) (scale bar = 0.5 mm) (time = 0 h)

WUE of cabbage leaves is shown in Fig. 2. Insect feeding reduced photosynthesis activity to 50% of the control leaf immediately after feeding (0 h, Fig. 2a), whereas stomatal conductance and transpiration rate were not significantly affected at this stage (Fig. 2b, c). During the post-feeding period, photosynthesis remained considerably lower than in controls, and stomatal conductance and transpiration decreased significantly reaching the lowest value 72 h after feeding. The WUE reflected the large inhibition of photosynthesis after insect feeding, decreasing dramatically immediately after feeding and slowly but incompletely recovering during the following 72 h (Fig. 2d). Photosynthesis also was measured in leaves that were offered to the insects but on which the insects did not feed. In this case, no inhibition of photosynthesis was observed (data not shown).

Oviposition by *M. histrionica* on cabbage leaves significantly reduced photosynthesis, stomatal conductance, and WUE with respect to controls (Fig. 2, hatched bars). The combination of oviposition and feeding damage resulted in the strongest negative effect in all gas exchange parameters (Fig. 2a-c, cross-hatched bars). However, as a consequence of the simultaneous and sharp decrease of photosynthesis and transpiration, WUE in these leaves remained unchanged (Fig. 2d).

Photosynthesis of bean leaves was affected strongly by *N. viridula* feeding (Fig. 3a). During the post-feeding period, photosynthesis of treated leaves recovered to some extent but remained significantly lower than in controls. Stomatal conductance and transpiration rate were slightly but not significantly reduced immediately after feeding (Fig. 3b, c). However, a statistically significant inhibition of these two parameters was found 24 and 48 h after feeding. The WUE was strongly reduced immediately after feeding, and slowly recovered during the post-feeding period (Fig. 3d). After 72 h, the WUE value was slightly lower than in control leaves but not statistically different.

Chlorophyll Fluorescence Imaging

Leaf damage inflicted by stink bug feeding was separated into light and severe symptoms by using chlorophyll fluorescence images (Fig. 4a), and in particular the F_v/F_m parameter. This is a conserved parameter in non-stressed leaves in which F_v/F_m values between 0.70–0.80 are recorded (Bjorkman and Demmig 1987). Decreases in F_v/F_m are a clear indicator of damage to the photosynthetic apparatus, which is often unrecoverable when the F_v/F_m drops to very low values (Maxwell and Johnson 2000). In our experiments, an F_v/F_m of 0.73 ± 0.005 (N=5) was recorded in control leaves and in areas of the test leaves that were distant from areas attacked by the insects of both plant species. Areas in which the F_v/F_m ratio was in the range of 0.50 to 0.70 were defined as 'light damage', and areas where the ratio F_v/F_m was less than 0.50 were defined as 'severe damage'. Immediately after feeding, the severe damage caused by *M. histrionica* was about 15% of the total damage, but this fraction increased slightly within 48 h after feeding (Fig. 4b). This increase was due not to a

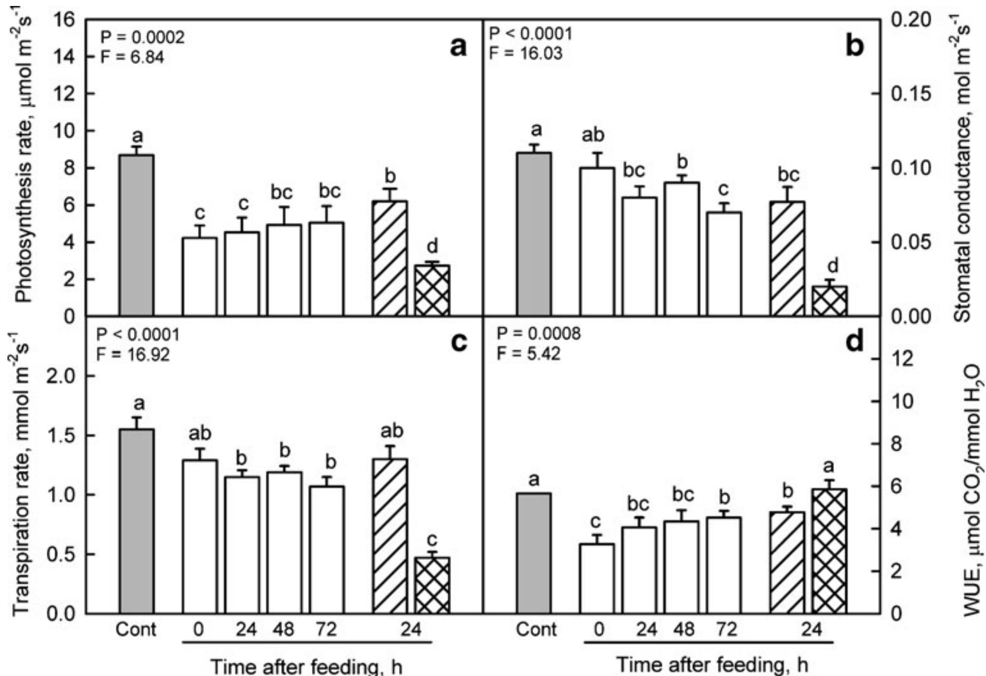


Fig. 2 Photosynthesis rate **a**, stomatal conductance **b**, transpiration rate **c**, and water use efficiency (WUE) **d** of savoy cabbage leaves after an 8 h long exposure to *Murgantia histrionica* adults. Bars represent control leaves (Cont, grey bars), leaves after feeding (white bars), leaves after oviposition (hatched bar), and leaves after oviposition and feeding (cross- hatched bar). Post-feeding measurements were carried out after 0, 24, 48, and 72 h, whereas in the cases of oviposition, and feeding and oviposition, measurements were carried out after 24 h only. Mean values ± s.e. (N=5) are given. Different letters indicate significant (P<0.05) differences as evaluated by ANOVA and Tukey's test. ANOVA degrees of freedom are the same for each plot: treatments (between columns) = 6, residuals (within columns) = 28

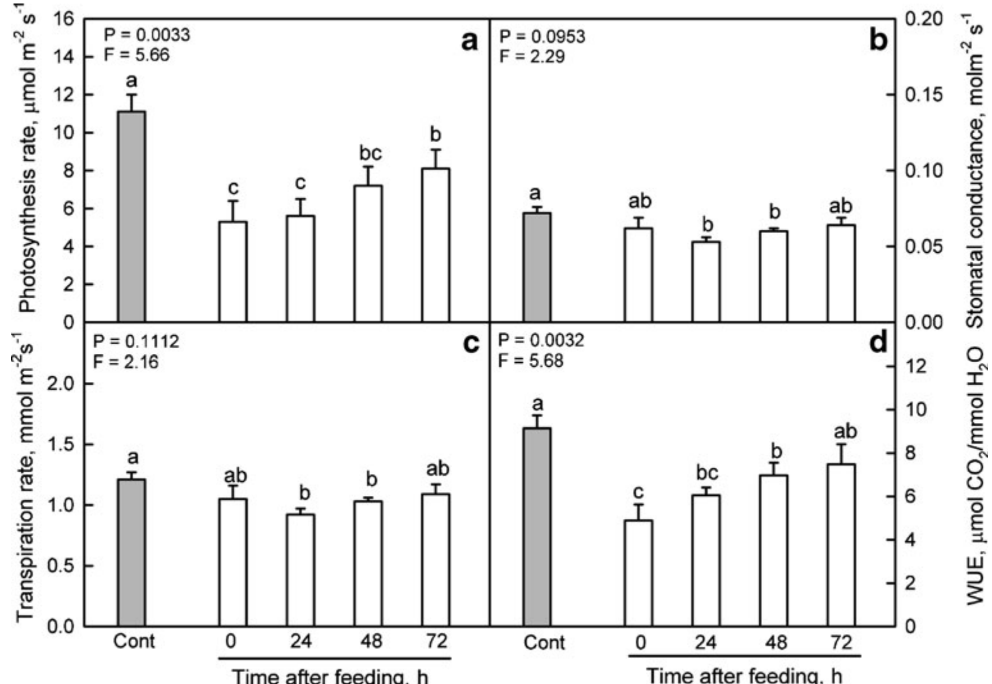


Fig. 3 Photosynthesis rate **a**, stomatal conductance **b**, transpiration rate **c** and water use efficiency (WUE) **d** of French bean leaves after an 8 h long exposure to *Nezara viridula* adults. Bars represent control leaves (Cont, grey bars) and of leaves after feeding (white bars). Post-feeding measurements were carried out after 0, 24, 48, and 72 h. Mean values ± s.e. (N=5) are given. Different letters indicate significant (P< 0.05) differences as evaluated by ANOVA and Tukey's test. ANOVA degrees of freedom are the same for each plot: treatments (between columns) = 4, residuals (within columns) = 20

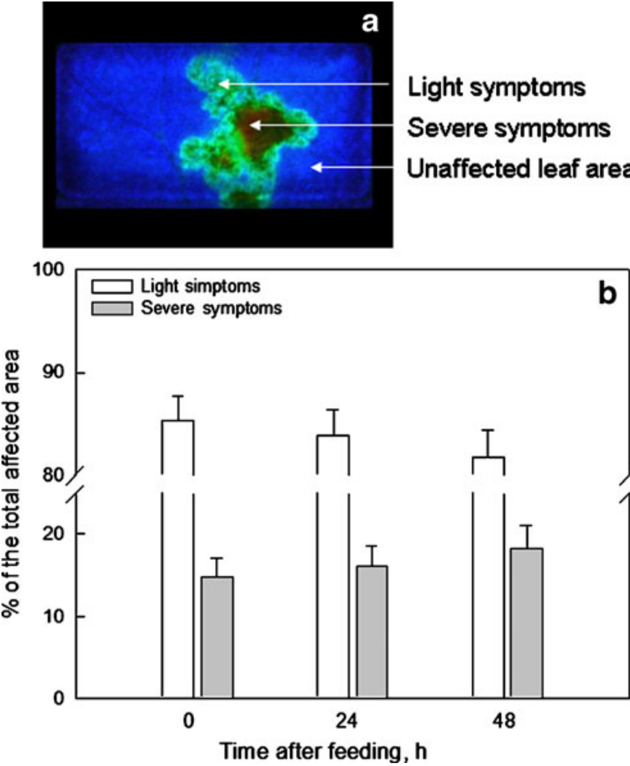


Fig. 4 Savoy cabbage leaves injured by feeding activity of *Murgantia histrionica* adults. **a** Chlorophyll fluorescence image of an attacked area. Light symptoms are defined those of areas in which the ratio F_v/F_m was in the range of 0.70 to 0.50 (green areas in our false color scale), whereas severe symptoms identify the areas in which the ratio F_v/F_m was less than 0.50, compared to the values in unaffected leaf area (0.73±0.005). **b** percentage of total feeding damage with light and severe symptoms 0, 24, or 48 h after feeding. Mean values ± s.e. (N=5) are given. Separation of means over time by ANOVA did not indicate significant differences

progressive enlargement of the damaged area but to the reduction of F_v/F_m in areas that showed only light symptoms immediately after the feeding.

Feeding damage on cabbage leaves caused by *M. histrionica* also was clearly visible by fluorescence emission measurements (Fig. 5c–h). In this case, fluorescence was used to investigate whether the damage spread beyond the areas that were visibly injured over time. There were no negative effects of herbivores on fluorescence parameters in the areas of leaves classified as unaffected (Fig. 6); by contrast, photochemical efficiency of PSII (Φ_{PSII}) of these areas slightly increased after feeding (Fig. 6c, white bars). In the leaf areas with symptoms of injury, F_v/F_m and Φ_{PSII} were reduced, particularly when the symptoms were severe (Fig. 6a, c, black bars). Interestingly, the expected increase of NPQ that generally accompanies the reduction of photochemical efficiency and the consequent dissipation of the photochemical energy (see *e.g.*, Fig. 6b, black bars) was not observed; NPQ even was reduced in the areas affected by severe symptoms.

Chlorophyll fluorescence also was measured in the cabbage leaves on which *M. histrionica* laid eggs but did not feed (Fig. 5i, j) and in leaves with both oviposition and feeding damage (Fig. 5k, l). The values of F_v/F_m of the areas surrounding the eggs were not affected significantly (Fig. 6a, hatched bar), but NPQ and Φ_{PSII} were reduced in these leaves (Fig. 6b, c, hatched bars). Again, as for gas-exchange measurements, the most negative effect was found in the leaves with co-occurring feeding damage and egg deposition, where the chlorophyll fluorescence parameters were reduced significantly (Fig. 6, cross-hatched bars).

Chlorophyll fluorescence images of French bean leaves attacked by feeding *N. viridula* are shown in Fig. 7, and quantitative fluorescence data are shown in Fig. 8. Despite the absence of visible damage induced by the feeding of this insect, the values of F_v/F_m and Φ_{PSII} were reduced

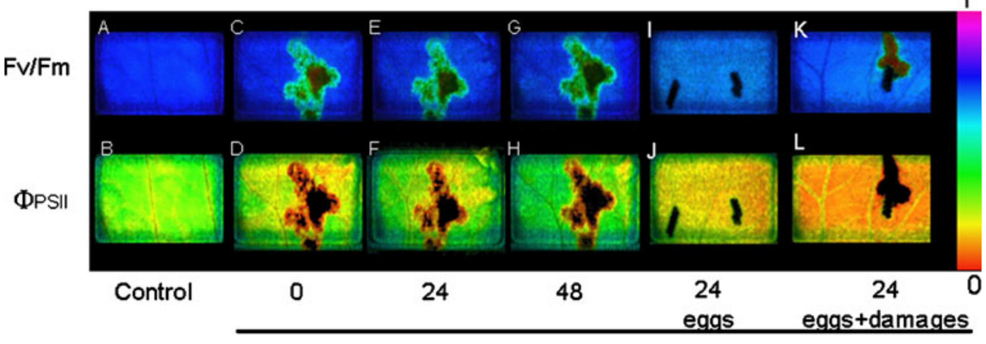
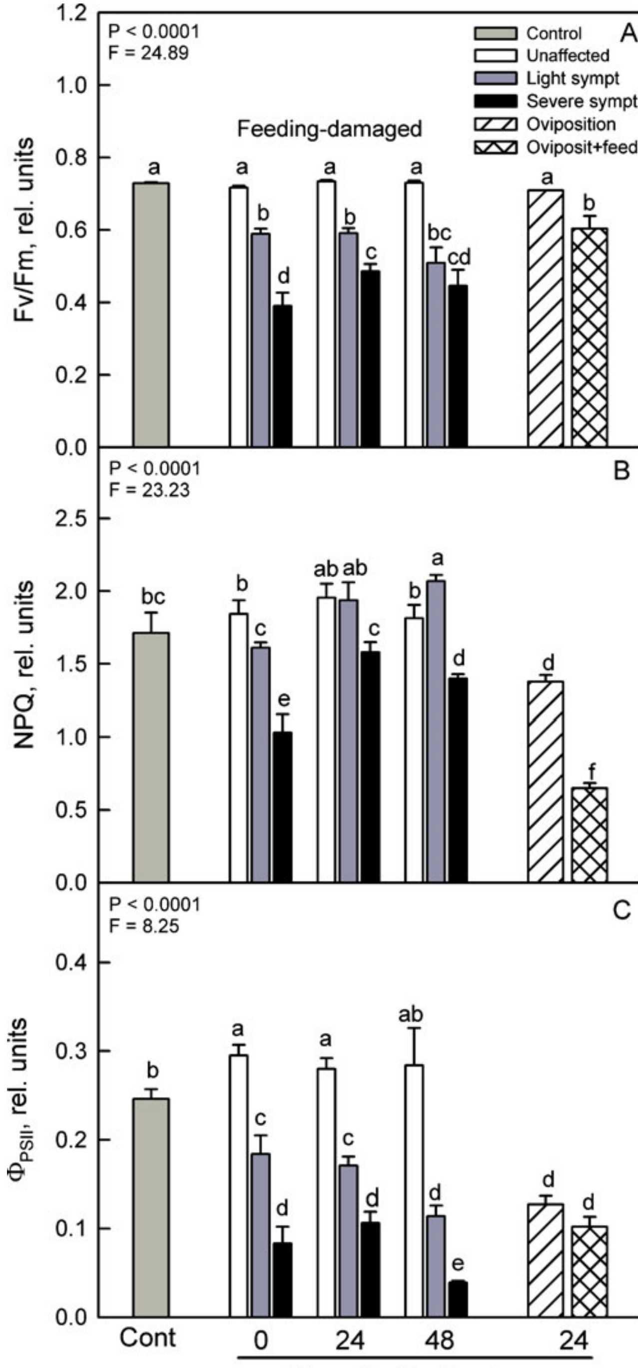


Fig. 5 Fluorescence images of savoy cabbage leaves after feeding (C–H), oviposition (I–J) and feeding and oviposition (K, L) by *Murgantia histrionica* adults. The panels show false color images of maximum photosynthetic efficiency (F_v/F_m, top) and PSII operating efficiency (Φ_{PSII}, bottom) of a control leaf (A, B) and a leaf exposed to insect (C–L). The images were taken 0 h (C–D), 24 h (E–F; I–J; K–L) and 48 h (G–H) after an 8 h long exposure to a single insect

Fig. 6 Effect of *Murgantia histrionica* on chlorophyll fluorescence parameters of savoy cabbage leaves: maximum quantum yield of PSII in dark adapted leaves **a**, non-photochemical quenching **b**, and quantum efficiency of PSII in light adapted leaves **c**, in cabbage leaves. Measurements were performed 0, 24, and 48 h after feeding, and 24 h after oviposition or oviposition and feeding. Bars represent control leaves (Cont, grey bar), and leaves after feeding (color-coded bars), oviposition (hatched bar), and oviposition and feeding (cross-hatched bar). Chlorophyll fluorescence parameters were measured in leaf parts characterized with different types of injury after feeding: white—unaffected area, dark grey—light symptoms, black—severe symptoms. Mean values ± s.e. (N=5) are given. Different letters indicate significant (P<0.05) differences as evaluated by ANOVA and Tukey's test. ANOVA degrees of freedom are the same for each plot: treatments (between columns) = 10, residuals (within columns) = 48



significantly (Fig. 8a, c), and NPQ was enhanced significantly (Fig. 8b) 24 h after feeding, with respect to control leaves. The damage revealed by the fluorescence parameters was transient, as all indicators returned to pre-stress levels within 72 h after feeding.

Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

VOC emissions were undetectable in control plants of cabbage and bean (Fig. 9). VOC emissions that indicate cellular damage (methanol and green leaf volatiles generated by membrane lipoxygenation) also were undetectable in both plant-insect associations. However, feeding by *M. histrionica* induced emission of monoterpenes and sesquiterpenes, among which β-phellandrene, sabinene, eucalyptol, and β-caryophyllene were the most abundant. The induction of jasmonates (methyl jasmonate, jasmonoyl-serin, jasmonoyl-leucine) also was observed, but only immediately after *M. histrionica* feeding. The time course analysis also identified significant differences among the emitted VOC. In particular, β-caryophyllene only appeared 24 h after feeding, whereas the emission of the other terpenes was observed immediately after feeding and remained high for at least 72 h after feeding. These induced terpenes were not detected in French bean leaves after feeding by *N. viridula*.

Discussion

Plant injury caused by insects with piercing-sucking mouthparts often is less evident than damage caused by chewing insects. The latter directly reduce the photosynthetic leaf lamina and induce emission of LOX that indicate damage to cell membranes (Hatanaka 1993). As a compensatory response, the remaining part of the lamina that is not attacked may present higher photosynthesis (Welter 1989). However, chlorophyll fluorescence imaging reveals that the injury caused by chewing herbivores often extends beyond the removal of the lamina, affecting adjacent tissues (Zangerl *et al*. 2002).

Piercing-sucking herbivores may feed on sap of xylem, phloem, or other plant cells. Their feeding site and the amount of tissue damage may vary considerably (Walling 2000). Visible damage often is absent when feeding is limited to a few hours (Guerrieri and Digilio 2008). Damage to photosynthesis also is expected to be lower in piercing-sucking than in chewing herbivores (Hare and Elle 2002). Photosynthesis injuries do not develop when cotton

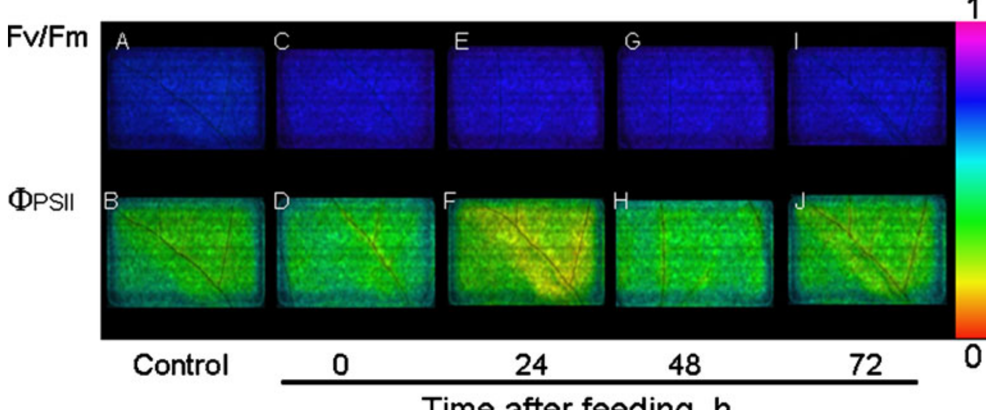


Fig. 7 Fluorescence images of French bean leaves after *Nezara viridula* feeding. The panels show false color images of maximum photosynthetic efficiency (F_v/F_m, top) and PSII operating efficiency (Φ_{PSII}, bottom) of control leaves (A, B) and of leaves exposed to insect feeding (C–J). The images were taken immediately (= 0 h) after removal of the insect (C–D), and 24 h (E–F), 48 h (G–H) and 72 h (I–J) after insect feeding

leaves are attacked by aphids for few hours (Gomez *et al*. 2006), but occur after herbivores attack a plant for several days (Reddall *et al*. 2004).

The damage caused on cabbage leaves by *M. histrionica* adults was visible immediately, whereas that provoked by *N. viridula* was not. However, both gas exchange and chlorophyll fluorescence imaging revealed substantial damage to the photosynthetic apparatus caused by both insect species. The reduction of photosynthesis following an 8-h feeding-period by a single insect was more rapid and greater than the reduction of transpiration and stomatal conductance. An increase of transpiration was observed in sugar maple seedlings attacked by thrips (Kolb *et al*. 1991). This increase was attributed to the larger conductance of gases both at stomatal and cuticular level caused by the insect punctures, and explained an unexpected increase of photosynthesis in damaged leaves (Kolb *et al*. 1991). In our experiments, however, the negative impact of insect feeding damage on photosynthesis seems to be independent of the effect on water relations. Consequently, a strong reduction of the WUE was observed immediately after feeding. WUE recovered slowly over 72 h post feeding.

Chlorophyll fluorescence imaging was used to clarify i) whether *N. viridula* feeding induced damage to the photochemistry of photosynthesis of French bean leaves, which might explain the rapid reduction of CO_2 gas exchange; and ii) whether injuries induced by both *N. viridula* and *M. histrionica* extended beyond the areas on which they actually fed, as observed for chewing insects (Zangerl *et al*. 2002). The large transient inhibition of photochemical efficiency of PSII (Fig. 8c) confirmed the substantial reduction of photosynthesis caused by *N. viridula* feeding. The limited and transient damage to the maximal quantum yield of PSII caused by *N. viridula* (Fig. 8a) indicated no permanent damage of the photochemistry. This is similar to the situation observed during photoinhibition (Powles and Björkman 1982). In *Nicotiana attenuata* feeding-damaged by *Tupiocoris notatus*, expression of genes that encode proteins involved in photosynthetic processes is up-regulated, potentially mitigating damage to photosynthesis caused by the piercing-sucking insect (Voelckel and Baldwin 2003). We observed a more complete recovery of fluorescence than of photosynthetic gas exchange after a 72-h recovery (Figs. 8c and 3a). Thus, photosynthesis is not permanently impaired by *N. viridula*, and the electron transport driving photorespiration compensates for the lower electron transport driving photosynthesis. This, in turn, suggests a lower CO_2 concentration in the chloroplasts after *N. viridula* feeding. Thus, the transient impairment of photosynthesis in *N. viridula*-fed leaves likely is caused by larger resistances to CO_2 diffusion in the mesophyll (Evans and Loreto 2000).

Murgantia histrionica caused visible and permanent damage to the leaf lamina in cabbage. As for *N. viridula*, the photosynthetic damage occurred rapidly, whereas transpiration and stomatal conductance declined more slowly; consequently, a fast reduction of WUE was observed. Because areas of *M. histrionica*-fed leaves were injured permanently, recovery of photosynthesis was incomplete. Chlorophyll fluorescence imaging allowed us to distinguish between damage with severe and light symptoms, and to follow the development of damage over 72 h after feeding. A permanent impairment of photosynthetic photochemistry was recorded in all damaged areas (see Fig. 6a, dark grey and black bars). Non-photochemical quenching of fluorescence increases when the light energy is dissipated non-radiatively and is not used for photochemistry (Bilger and Bjorkman 1991). However, in areas that suffered severe injuries, NPQ actually was reduced (Fig. 6b). This indicated permanent damage to the photochemical apparatus and inefficient dissipation of energy. Interestingly, the Φ_{PSII} of lightly and severely damaged areas did not recover during the post-feeding period, and in fact decreased over the 72 h after feeding (Fig. 6c). The recovery of photosynthesis (Fig. 2a) therefore was driven by an improved performance of the unaffected areas (Fig. 6c, white bars). This finding supports the observation that the attack of a plant by herbivores may

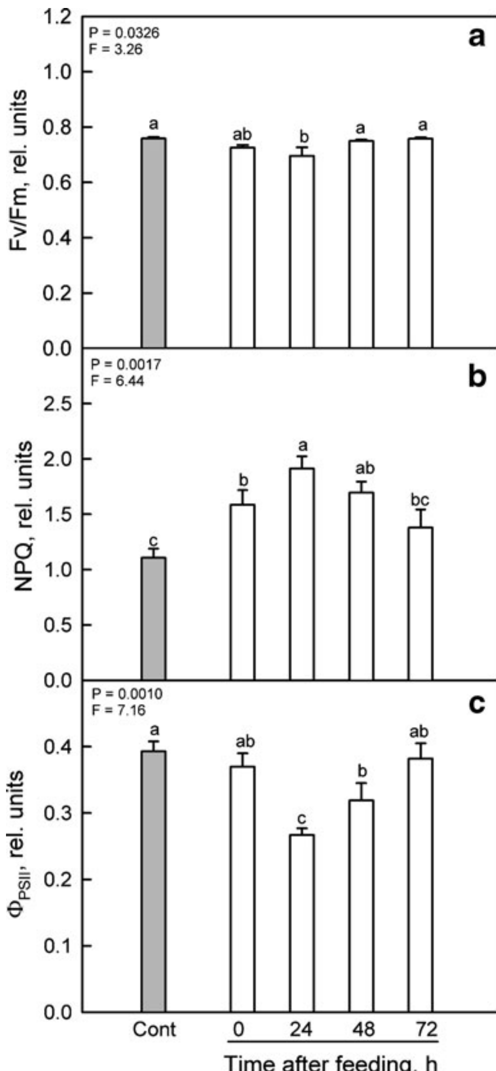
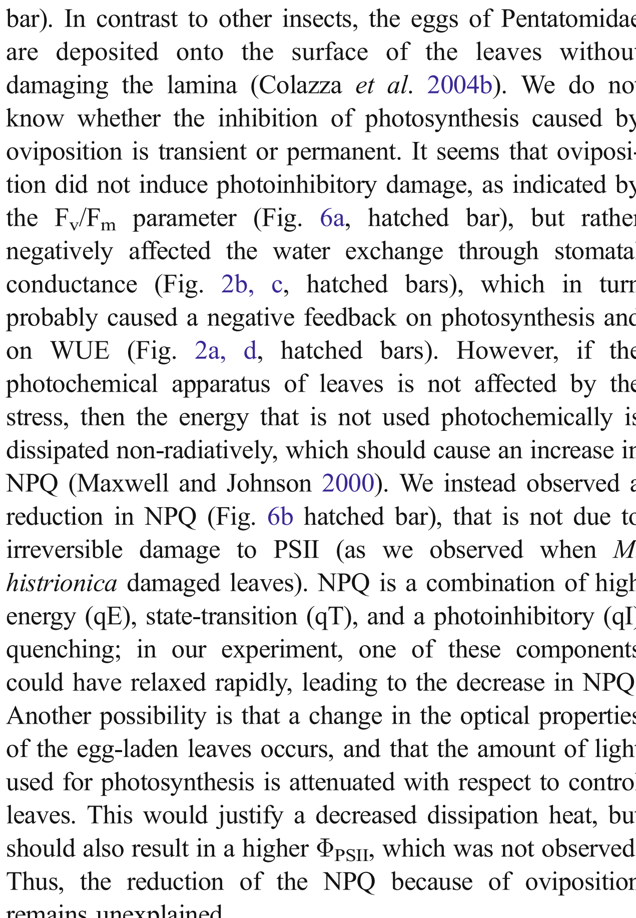


Fig. 8 Effect of feeding by *Nezara viridula* on chlorophyll fluorescence parameters of French bean leaves: maximum quantum yield of PSII in dark adapted leaves **a**, non-photochemical quenching **b**, and quantum efficiency of PSII in light adapted leaves **c**. Measurements were performed in control leaves (Cont, gray bars) and 0, 24, 48, and 72 h after feeding (white bars). Mean values ± s.e. (N=5) are given. Different letters indicate significant (P<0.05) differences as evaluated by ANOVA and Tukey's test. ANOVA degrees of freedom are the same for each plot: treatments (between columns) = 4, residuals (within columns) = 20

stimulate compensatory responses in parts of the leaf that are not directly affected (Voelckel and Baldwin 2003). This is not always observed. For instance, in cotton leaves infested by spider mites, stomatal conductance was reduced but photosynthetic performance was not affected (Schmidt *et al*. 2009).

Oviposition by *M. histrionica* inhibited photosynthesis. This is expected when oviposition is associated with nearby mechanical damage (Fig. 2a, cross-hatched bar). Surprisingly, however, photosynthesis also was inhibited when oviposition only (without feeding damage) occurred (Fig. 2a, hatched


bar). In contrast to other insects, the eggs of Pentatomidae are deposited onto the surface of the leaves without damaging the lamina (Colazza *et al*. 2004b). We do not know whether the inhibition of photosynthesis caused by oviposition is transient or permanent. It seems that oviposition did not induce photoinhibitory damage, as indicated by the F_v/F_m parameter (Fig. 6a, hatched bar), but rather negatively affected the water exchange through stomatal conductance (Fig. 2b, c, hatched bars), which in turn probably caused a negative feedback on photosynthesis and on WUE (Fig. 2a, d, hatched bars). However, if the photochemical apparatus of leaves is not affected by the stress, then the energy that is not used photochemically is dissipated non-radiatively, which should cause an increase in NPQ (Maxwell and Johnson 2000). We instead observed a reduction in NPQ (Fig. 6b hatched bar), that is not due to irreversible damage to PSII (as we observed when *M. histrionica* damaged leaves). NPQ is a combination of high energy (qE), state-transition (qT), and a photoinhibitory (qI) quenching; in our experiment, one of these components could have relaxed rapidly, leading to the decrease in NPQ. Another possibility is that a change in the optical properties of the egg-laden leaves occurs, and that the amount of light used for photosynthesis is attenuated with respect to control leaves. This would justify a decreased dissipation heat, but should also result in a higher Φ_{PSII}, which was not observed. Thus, the reduction of the NPQ because of oviposition remains unexplained.

Emission of induced VOC is a well-known response to biotic and abiotic stresses (Dicke and Baldwin 2010; Loreto and Schnitzler 2010). Some VOC are induced by physical damage. In particular, methanol emission results from cell

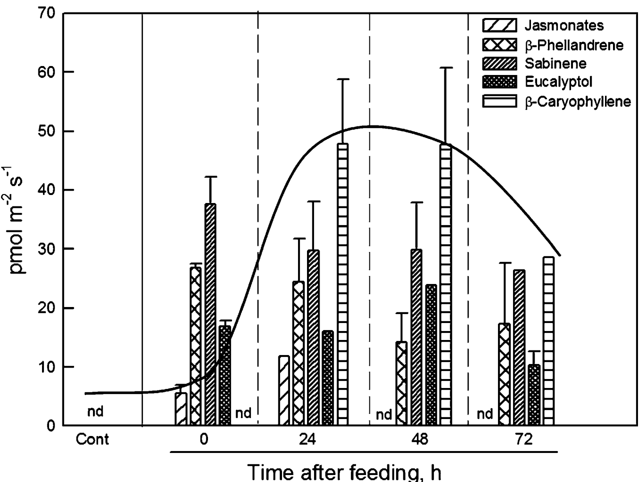


Fig. 9 Effect of feeding by *Murgantia histrionica* on the composition of induced volatile organic compounds (VOC) released from savoy cabbage plants. Measurements were carried out in control plants (Cont), and 0, 24, 48, and 72 h after feeding. Means ± s.e. (N=5) are given. The third-order best fit of β-caryophyllene emission was generated by Sigmastat

wall damage, and a family of C6 compounds results from degradation of cellular membranes (Loreto *et al*. 2006). Colazza *et al*. (2004a) and Williams *et al*. (2005) observed minimal emissions of these stress-induced compounds in leaves attacked by *N. viridula,* and they concluded that the damage caused by feeding was minor. Similarly, we also did not find detectable emissions of these compounds in leaves of *P. vulgaris* damaged by feeding *N. viridula*. Other classes of compounds are induced by biotic stresses and may serve to allow plant communication with predaceous or parasitic insects that feed on herbivores or their eggs (Dicke and Hilker 2003). For instance, *N. viridula* feeding on French beans induced an increase of the emissions of the monoterpene linalool, of the C16 homosesquiterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and of the C11 homomonoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene (Colazza *et al*. 2004a). Feeding injury of maize by *N. viridula* females also induced emissions of linalool; the sesquiterpenes (*E*)-β-caryophyllene, α-trans-bergamotene, and (*E,E*)-β-farnesene; and the homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Williams *et al*. 2005). Induced sesquiterpenes seem to act as olfactory cues that attract enemies of herbivores during tritrophic interactions (Schnee *et al*. 2006). In this study, we report the induction of several monoterpenes and sesquiterpenes, but only when cabbage leaves were attacked by feeding *M. histrionica*; in contrast to previous studies, we did not observe induction of volatiles when *N. viridula* fed upon *P. vulgaris.* It is likely that the feeding damage caused by *N. viridula* in our study was too small to induce biosynthesis and emission of defensive VOC. Alternatively, it is possible that VOC emission is absent or very low in some cultivars of French bean. This may reflect artificial or natural selection that limits carbon loss to improve productivity.

The profile of emitted compounds following the attack of *M. histrionica* confirms the observation that the lacerate-and-flush feeding mode induces classes of compounds that are more similar to those induced by chewing insects, rather than those induced by piercing-sucking insects that feed on phloem (Williams *et al*. 2005). In our study, the compound with the highest emission rate was the sesquiterpene (*E*)-β-caryophyllene. It was not observed until 24 h after feeding, as previously reported by Conti *et al*. (2008). We suggest that this delay, relative to other volatiles that are emitted immediately after feeding, reflects delayed induction, and that the maximal putative ecological activity of (*E*)-β-caryophyllene occurs 24–48 h after insect feeding.

Monoterpenes were induced more rapidly than sesquiterpenes, and their emission was sustained over the 72 h of our investigation (Fig. 9). This is more consistent with the very rapid time-course of induction that generally has been observed for insect-induced volatiles (Paré and Tumlinson 1997). Monoterpenes and sesquiterpenes are formed from two different biochemical pathways (Loreto and Schnitzler 2010). Flux through the monoterpene pathway is closely linked with photosynthesis, whereas the sesquiterpene pathway is not. Thus, the time course of the appearance of these compounds in our experiments may indicate a different rate of induction of the two pathways. This may be related to different functions for the two classes of volatiles. Unknown VOC, produced by plants attacked by *M. histrionica* (Conti *et al*. 2008), and terpenoids induced by *N. viridula* (Colazza *et al*. 2004a) act as attractants for the parasitoids *Trissolcus brochymenae* Ashmead (Conti *et al.* 2006) and *T. basalis* Wollaston (Hymenoptera: Scelionidae), respectively, which parasitize the eggs of the attacking pentatomids (Colazza *et al*. 2004b).

Finally, we note that jasmonate and its derivatives also were detected, but only immediately after the end of feeding. Jasmonates are important signaling molecules involved in a range of physiological responses; in particular, they are produced in response to plant wounding during insect attack (Browse and Howe 2008) and may trigger a range of physiological responses such as the induction of proteinase inhibitors and pathogen defense genes (Glazebrook 2005). In line with this physiological function, we observed very rapid induction of jasmonates in response to the attack of piercing-sucking insects.

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Nitrogen Deficiency Affects Bottom-Up Cascade Without Disrupting Indirect Plant Defense

Thorsten R. Winter · Michael Rostás

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Abstract Nitrogen (N) is an important macronutrient for plants and insects alike, and the availability of this critical element may considerably modify bottom-up effects in tritrophic systems. By using hydroponically cultured *Glycine max*, we investigated the impact of N deficiency on plant growth, photosynthetic efficiency, primary metabolism, and herbivore-induced volatile (VOC) emission. Cascading effects of N deficiency on higher trophic levels were assessed by measuring the performances of the herbivore *Spodoptera frugiperda* and its parasitoid *Cotesia marginiventris*. In addition, we studied the volatile-guided foraging behavior of *C. marginiventris* to explore whether nutrient stress affects the plant's indirect defense. Our results show that photosynthetic efficiency, leaf N, and soluble protein content were significantly reduced in N deficient plants whereas root biomass was increased. Nitrogen starved plants emitted the same range of herbivore-induced VOCs as control plants, but quantitative changes occurred in the release of the main compound and two other volatiles. Herbivore growth and the performance of parasitoids developing inside the affected hosts were attenuated when caterpillars fed on N deficient plants. The behavioral response of *C. marginiventris* to induced VOCs from N deficient hosts, however, remained unaffected. In summary, N stress had strong bottom-up effects over three trophic levels, but the plant's indirect defense remained intact.

T. R. Winter · M. Rostás (✉)
Department of Botany II, Julius-von-Sachs Institute
for Biosciences, University of Würzburg,
Julius-von-Sachs-Platz 3,
97082 Würzburg, Germany
e-mail: rostas@botanik.uni-wuerzburg.de

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Introduction

In terrestrial ecosystems, nitrogen (N) is an important macronutrient for plants. Due to soil properties, N availability can be patchy and may vary even on a small scale (Keddy 2007). Plants show plastic responses to N deficiency by profoundly reprogramming N and carbon (C) metabolism (Lou and Baldwin 2004; Scheible et al. 2004). In an effort to acquire the missing nutrients more efficiently, plants resort to altered biomass allocation between shoot and root and enhanced root branching. Deficiency also leads to sugar and starch accumulation in leaves, and exerts negative feedback on photosynthesis. Eventually, impeded uptake will lead to reduced leaf N content and a higher C/N ratio (Hermans et al. 2006).

Ecologically, a shift towards more C and less N can cause significant changes in bottom-up interactions between primary producers and subsequent trophic levels. Plant N content may affect either herbivore development directly (Scriber 1977; Fischer and Fiedler 2000; Berner et al. 2005; Coley et al. 2006), or deficiency effects can cascade up to higher trophic levels, thus altering top-down influences. Soil conditions may indirectly alter the abundance or performance of parasitoids and predators. For instance, ladybird beetles (*Aiolocaria hexaspilota*) feeding on willow leaf beetles (*Plagiodera versicolora*) had a higher adult mass and shorter developmental time when their prey was reared on leaves with high N content (Kagata et al. 2005).

A plant's nutritional quality is determined not only by the amounts of primary compounds such as proteins or carbohy-

drates but also by the levels of secondary metabolites. Both factors are intertwined as N availability may affect the synthesis of constitutive and induced defensive secondary compounds. Depending on the metabolites and plant species in question, N availability may lead to changing levels of secondary compounds (e.g., Dudt and Shure 1994; Stout et al. 1998; Hemming and Lindroth 1999; Cipollini et al. 2002; Lou and Baldwin 2004; Chen et al. 2008a).

As a response to feeding or egg deposition by herbivores, plants release volatile organic compounds (VOC), which comprise mainly fatty acid derivatives, terpenoids, phenyl propanoids, and benzenoids. Within the ecosystem, these metabolites can have multiple functions, but primarily they are known as signals that guide natural enemies to their herbivorous prey or host (Holopainen 2004; Heil 2008; Dicke 2009). The production and release of VOCs may vary considerably depending on the plant's nutritional status. Maize seedlings, for example, show decreased emissions of induced plant VOCs when N-P-K fertilization was reduced (Gouinguené and Turlings 2002). Manipulating N availability alone, however, resulted in enhanced levels of induced VOCs in maize (Schmelz et al. 2003) and cotton (Chen et al. 2008a) but not in *Nicotiana attenuata* at low levels of N (Lou and Baldwin 2004). Concentrations of the phytohormone jasmonic acid (JA) correlated negatively with N availability and positively with VOC induction, thus, it was suggested that changes in JA provide a mechanism to regulate the magnitude of plant defense responses (Chen et al. 2008a).

A blend of VOCs that varies in the composition or quantity of its components due to abiotic factors may constitute a signal with altered information content and may potentially modify the host finding behavior of natural enemies (Turlings and Wäckers 2004; Rostás and Turlings 2008). Several studies have explored the effects of abiotic factors, such as light (Maeda et al. 2000; Gouinguené and Turlings 2002), humidity (Gouinguené and Turlings 2002), carbon dioxide (Vuorinen et al. 2004b), UV radiation (Winter and Rostás 2008; Blande et al. 2009), ozone (Vuorinen et al. 2004a), or nutrient supply (Gouinguené and Turlings 2002) on VOC induction. Only some of these also have tested whether altered VOC blends affect parasitoid or predator attraction (Maeda et al. 2000; Vuorinen et al. 2004a, b; Winter and Rostás 2008; Blande et al. 2009). However, behavioral experiments are necessary to understand whether a given abiotic factor has the potential to disrupt the facultative mutualism between plants and natural enemies. Changes in the release rate of certain compounds do not automatically translate into differential host searching behavior (Rostás et al. 2006), while stronger or weaker attraction may not always be reflected by detectable changes in the measured VOCs of an induced plant (Gouinguené et al. 2005; Rostás and Turlings 2008; D'Alessandro et al. 2009).

Soybean plants (*Glycine max*) demand high amounts of N, and a large proportion of it is acquired from N-fixing rhizobacteria. This makes soybean generally less dependent on soil N, but nevertheless, deficiency may occur in patches where appropriate symbionts are lacking or whenever plants and bacteria fail to establish good root nodulation. Unfavorable environmental conditions or fungicide application has been shown to reduce strongly root nodulation and thus nitrogen acquisition (Roth 2009; Zilli et al. 2009).

Here, we addressed the question, whether N deficiency would result in significant bottom-up effects in a tritrophic system consisting of soybean, the herbivore *Spodoptera frugiperda,* and its larval parasitoid *Cotesia marginiventris.* In addition to direct effects on the growth, development, survival, and longevity of plants and insects, we focused on the impact of low N availability on the quantity and quality of herbivore induced plant VOC and the attractiveness of the blends for host searching parasitoids.

Methods and Materials

Plant and Insect Material Soybean seeds (*Glycine max* (L) Merr. cv. London) were obtained from Saatbau Linz (Leonding, Austria). Seedlings were grown in plastic trays (30×20×4.5 cm, Wiesauplast, Wiesau, Germany) containing silica sand for 14–16 d and then subjected to N treatments. Further rearing conditions of plants and insects are described in Winter and Rostás (2008).

Nitrogen Treatments After 14–16 d, plants (V 1 stage, McWilliams et al. 1999) were removed carefully from the sand, and roots were rinsed with deionized water. All seedlings then were transferred to black plastic containers (30.5×20.3×13.3 cm, Rotilabo® Drehstapelwanne, Carl Roth, Karlsruhe, Germany) containing 5.6 l of hydroponic solution, aerated with a membrane pump. Fifteen plants were grown in each container with a distance of 7 cm between each individual. The hydroponic solution was exchanged every 3–4 d. To maintain appropriate salt concentrations and pH of the solution, electric conductivity (EC) and pH were regularly controlled with a combined pH/EC tester (Combo 2, Carl Roth, Karlsruhe, Germany). EC was adjusted to 2.2 mS, pH was kept at 5.9–6.1.

Plants were exposed for 5 d to a modified Hoagland solution (Hoagland and Arnon 1938). For plants growing in nitrogen deficient solution (–N treatment), KNO_3 was replaced with K_2SO_4 (Carl Roth, Karlsruhe, Germany), and $Ca(NO_3)_2$ with $CaCl_2$ (AppliChem, Darmstadt, Germany) in equivalent concentrations.

Effects of Nitrogen Deficiency on Plant Growth and Physiology The effect of N deficiency on growth and

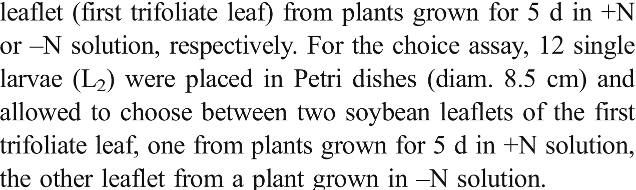
physiology of soybean was assessed by exposing plants to the respective N treatments for 5 d. Then, shoots and roots of six plants per treatment were freeze-dried for 48 h and weighed to calculate shoot-to-root-ratios. Eight plants per treatment grown in the same cohort were used to determine C/N-ratios and concentrations of soluble proteins in the leaves. For this, leaf discs were cut out with a cork borer (diam 17.8 mm) from unifoliate leaves, freeze-dried, weighed, and ground. Half of the material was used to analyze total C and N content by quantitative decomposition of substances by oxidative combustion (CHN-O-Rapid, Heraeus, Hanau, Germany). The other half was extracted 3 × with 500 µl deionized water. Extracts were combined, and soluble protein content was determined with Bradford reagent (Sigma-Aldrich, Seelze, Germany) using bovine serum albumin (1.4 mg ml^{-1} in water) as standard. Samples were arranged in a 96 well plate, and absorbance was measured with a photometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) at 595 nm.

To assess the effects of N limitation on the photosynthetic efficiency of soybean, the adaxial leaf side of the first trifoliate leaf of 5 plants per treatment was examined with a PAM-2000 fluorometer (Walz Mess–u. Regeltechnik, Effeltrich, Germany). Maximum photochemical yield of photosystem II (PSII) was measured in dark-adapted leaves as the ratio of variable (Fv) to maximal (F_M) chlorophyll fluorescence at room temperature with Fv/F_M = (F_M - F_0)/F_M (Schreiber et al. 1986). Minimum fluorescence (F_0) was excited at 655 nm and 600 Hz modulation frequency, and maximum fluorescence (F_M) was measured with 100 kHz modulation frequency. The F_M was elicited by saturating pulses of 0.8 s duration from a built-in halogen lamp.

Plant-Mediated Effects of Nitrogen Deficiency on Herbivore Growth, Developmental Time, Survival and Feeding Behavior An herbivore performance test was conducted to assess the effects of plant N limitation. Fifty neonate larvae of *Spodoptera frugiperda* were weighed and kept individually in Petri dishes (diam. 8.5 cm) with moistened filter paper in a climate chamber as described above. They were fed *ad libitum* with fresh cut leaflets of the first trifoliate soybean leaves from plants kept for 5 d in +N or –N solution. Each plant was harvested only once. Weight increases of larvae were measured between day 5 and 6 (L_2) and day 10 and 11 (L_3). In addition, pupal and adult weights, as well as developmental times were recorded.

Two feeding trials were performed to see whether larvae (L_2) compensated for potentially lower food quality due to N limitation by ingesting larger quantities of leaf tissue (no-choice assay), or by choosing food with potentially higher quality (choice assay).

For the no-choice assay, 15 single larvae (L_2) were placed in Petri dishes (diam. 8.5 cm) and fed with one soybean


leaflet (first trifoliate leaf) from plants grown for 5 d in +N or –N solution, respectively. For the choice assay, 12 single larvae (L_2) were placed in Petri dishes (diam. 8.5 cm) and allowed to choose between two soybean leaflets of the first trifoliate leaf, one from plants grown for 5 d in +N solution, the other leaflet from a plant grown in –N solution.

After 24 h, leaf consumption was measured by scanning the leaves and calculating the removed areas as described in Rostás et al. (2006).

For C/N analyses, another cohort of 8 neonate larvae was treated as described for the performance test. After 6 and 15 d, respectively, the larvae were starved for 1 hr, frozen, freeze dried, and ground. Total C and N content was analysed as described above.

Herbivore-Mediated Effects of Plant Nitrogen Deficiency on Parasitoid Growth, Developmental Time and Longevity The developmental time, growth, and longevity of the parasitoid *Cotesia marginiventris* developing inside *S. frugiperda* was measured to assess host-mediated effects of plant N limitation. Three-day old *S. frugiperda* larvae were fed with freshly cut soybean leaves of the respective N treatment for 2 d. The larvae then were offered in six groups of 5 to a 4–6 d old mated *C. marginiventris* female in a Petri dish (diam. 5.5 cm). After parasitism of 15 larvae per treatment was observed, caterpillars were kept separately in Petri dishes (diam. 8.5 cm) with moist filter paper and fed *ad libitum* with freshly cut leaves from +N or –N plants. Food was exchanged at least every 2nd day. Emerging parasitoid cocoons were transferred to individual Petri dishes (diam. 5.5 cm) with dry filter paper. Eclosed parasitoids were provided with water only. Developmental time, pupal weight at 24 hr post emergence, and longevity of the adult parasitoids were recorded.

Plant-Mediated Nitrogen Effects on VOC Emission and Parasitoid Behavior The effects of N limitation on the emission of herbivore-induced volatiles and consequently on the behavior of the parasitoid was investigated in a six-arm-olfactometer (for details see Turlings et al. 2004). Soybean plants exposed for five days were placed individually into the cup of an odor source vessel of the six-arm-olfactometer and provided with approximately 50 ml of the accordant hydroponic solution. The cup was covered with two semicircular polycarbonate plugs that had an opening in the center to hold the plant in an upright position and to prevent larvae from falling into the solution. Twenty-five *S. frugiperda* larvae (L_2) were placed on each plant and were allowed to feed overnight (approx. 16 hr). On the following day, volatile collections and behavioral assays were carried out simultaneously from 9:00 AM till 12:00 AM. Ten fluorescent lamps (PAR inside odor source vessels: 130 µmol photons m^{-2} sec^{-1} at 30 cm distance from

lamps) were switched on 3 hr before testing the wasps. Odor source vessels containing a plant that had received either +N or –N treatment were placed vis-à-vis in the olfactometer. The other four vessels remained empty as controls. After connecting the vessels to the air delivery and the olfactometer, the air stream was allowed to stabilize for 10 min. The flow rate was 1.2 l min^{-1} for incoming air and 0.6 l min^{-1} for air going out to the behavioral arena or the volatile traps, respectively.

Mated 3–5 d old females of *C. marginiventris* were used in the behavioral assays. Wasps had no oviposition experience prior to the experiment. All wasps were tested in groups of 6, as they do not interfere with each other's choices (Turlings et al. 2004). After 30 min, the choices made by the parasitoids were recorded, and the group was replaced by a new one. Five groups of wasps were tested on the same day. One day with 5 releases was considered as one replicate. Six replicate days were carried out with a new pair of plants and new wasps each day. Volatiles emanating from soybeans were collected with SuperQ traps as described previously (Rostás and Eggert 2008). After each experimental day, the glass and teflon parts of the olfactometer were cleaned with deionized water and rinsed with ethanol (v/v 70%), acetone, and hexane. After evaporation of the solvents, all glass parts were placed in an oven at 200°C for 1 hr.

Trapped volatiles were eluted with 150 µl methylene chloride. Two internal standards (*n*-octane and nonyl acetate, Sigma-Aldrich, Taufkirchen, Germany, 400 ng each in 20 µl methylene chloride), were added, and the samples were stored at −80°C. The qualitative and quantitative volatile composition of each sample was analyzed on an Agilent Technologies 6890N Network GC System coupled with a 5973 Network Mass Selective Detector. Three µl of each sample were injected with an automated injection system in pulsed splitless mode. The column was an Agilent 19091-s933 HP-1 capillary column (length 30 m, diam 0.25 mm, film thickness 0.25 µm). The oven was held at 35°C for 3 min and then increased with 8°C min^{-1} to a final temperature of 230°C that was held for 10 min. Helium (1.5 ml min^{-1}) was used as carrier gas. Compounds were identified using MSD ChemStation (Agilent Technologies) software with the Wiley 275 mass spectrum library and by using the software MassFinder3/Terpenoids library (Hochmuth Scientific Software, Hamburg, Germany). Identities were confirmed further by co-injection of authentic standards (Sigma-Aldrich, Taufkirchen, Germany). Quantification was obtained by comparing the area of the compounds to the areas of the internal standards.

Statistical Analyses Plant parameters were analyzed with a Mann-Whitney-*U*-test. Values of plant C/N ratios and soluble protein contents were Bonferroni corrected prior to the analyses. Performance parameters of *S. frugiperda* and *C. marginiventris* were compared with ANCOVA using initial weight of *S. frugiperda* as covariable and measured performance parameters as variables. Herbivore mortality was analyzed with a χ^2-test.

Plant photosynthetic efficiency, larval feeding in the no-choice assay, larval C/N ratios, and differences in VOC compositions were analyzed with Student's *t*-tests for independent samples. Only VOCs occurring in at least 50% of all samples were analyzed statistically. Larval feeding in the choice assay was assessed with a *t*-test for dependent samples. The analyses were conducted using the STATISTICA 7.1 software package (StatSoft, Tulsa, OK, USA).

For the six-arm-olfactometer, the entity computing a repetition in the statistical analysis corresponds to the response of a group of 6 wasps released, which was shown to follow a multinomial distribution (Ricard and Davison 2007). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasilikelihood functions to compensate for the overdispersion of parasitoids within the olfactometer. The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings et al. 2004).

Results

Effects of Nitrogen Limitation on Plant Morphology and Physiology Plants exposed to –N conditions for 5 d showed a significantly decreased shoot-to-root ratio due to increased root biomass (dry weight) compared to +N exposed plants (Table 1). In leaves of –N plants, the amounts of soluble proteins were reduced significantly whereas the C/N ratio was increased due to a lower N content (Table 1). Maximum photochemical efficiency of PS II, measured as F_V/F_M, was reduced significantly in –N compared to +N treated plants (Table 1).

Plant-Mediated Nitrogen Effects on Herbivore Growth, Developmental Time, Survival, and Behavior Herbivore larvae fed with leaves from N deficient plants gained significantly less biomass during their development, which resulted in significantly lower pupal and adult weights (Table 2). The C/N ratios in these larvae were reduced compared to larvae fed with +N treated plants. However, developmental time (Table 2), as well as larval mortality (χ^2 test, χ^2=0.08, *df*=1, *P*>0.5) and successful adult emergence (χ^2 test, χ^2=0.35, *df*=1, *P*>0.5), were not influenced by plant N limitation. In choice and no-choice tests, the

Table 1 Morphological and physiological parameters of soybean exposed to different N treatments

Parameter	+N[a]	–N[b]	Statistics[c]
Shoot/root ratio	9.84±1.74	3.73±0.44	N=6, Z=2.88, ***P*<0.005**
Soluble leaf protein content (mg g^{-1} DW)	57.74±6.02	37.36±2.78	N=8, Z=2.42, ***P*<0.05**
Leaf C/N ratio	10.19±0.40	16.26±1.16	N=8, Z=−3.05, ***P*<0.005**
Maximum photochemical efficiency of PS II (F_v/F_m)	0.806±0.003	0.788±0.006	N=5, t=2.87, ***P*<0.05**

P-values in bold indicate statistically significant differences

[a] Mean values ± SE for plants exposed to full Hoagland solution for 5 days

[b] Mean values ± SE for plants exposed to N deficient Hoagland solution for 5 days

[c] Statistical parameters are indicated for Mann-Whitney-*U*-tests and Student's *t*-test (F_V/F_M)

larvae did not consume significantly more leaf tissue from –N treated than from +N treated plants (Fig. 1).

Plant-Mediated Nitrogen Effects on Parasitoid Growth, Developmental Time, and Longevity Individuals of *C. marginiventris* reared in caterpillars of *S. frugiperda*, which in turn were fed with leaves from –N treated soybeans, had significantly lower pupal weights. Developmental time and longevity did not differ between wasps reared in herbivore larvae fed with +N or –N treated leaves (Table 3).

Plant-Mediated Nitrogen Effects on VOC Emission and Parasitoid Behavior In both N treatments, only trace amounts of (*E,E*)-α-farnesene could be detected from undamaged soybean plants. In contrast, plants damaged by *S. frugiperda* larvae released 22 different compounds of which 18 were identified (Fig. 2). The main constituents were (*E,E*)-α-farnesene (20% and 41% of total emission for +N and –N plants, respectively) and indole (24% and 12% of total emission for +N and –N plants, respectively) when emission was corrected for plant fresh weight. While the same compounds were emitted by +N and –N treated soybean plants, three substances were released in significantly different amounts. The release rates of the sesquiterpenes β-bergamotene and (*E,E*)-α-farnesene were approx. five times (t=−2.51. df=10, P<0.05) and three times (t=−2.64. df=18, P<0.05) higher in –N plants than in +N plants. In contrast, a 50% decrease was observed in the emission of (*Z*)-3-hexenyl-α-methylbutyrate (t=2.30. df= 10, P<0.05). All other volatiles were released in similar amounts. The statistical values given here refer to emission rates corrected for plant fresh weight, but the same pattern was obtained for uncorrected values (Student's-*t*-test). Differences in the quantities of the three volatiles did not cause a significant shift in overall emission.

Naïve females of *C. marginiventris* were highly attracted by the herbivore-induced volatiles of both +N and –N-treated plants when compared to clean air. But wasps had no preference for either one of the offered plant odors (log-linear- model, P>0.05, N=6, Fig. 3).

Discussion

Deficiency in N severely affected the morphology and physiology of soybeans. In our study, plants lacking N increased their root biomass while shoot biomass remained

Table 2 Performance parameters of *Spodoptera frugiperda* reared on soybean exposed to different N treatments

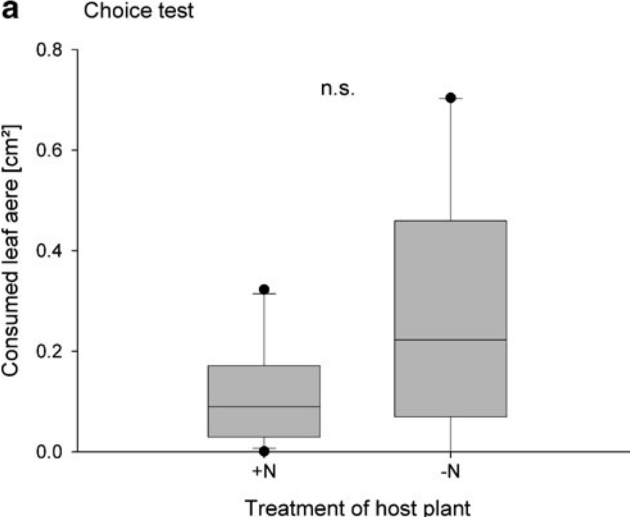
Parameter	+N[a]	–N[b]	Statistics[c]
Weight increase day 5/6 [mg]	3.04±0.29	2.12±0.25	N=21/24, F=5.28, ***P*<0.01**
Weight increase day 10/11 [mg]	34.17±4.52	21.63±2.84	N=19/22, F=5.19, ***P*<0.05**
Pupal weight [mg]	138.88±5.43	113.86±5.20	N=12/11, F=9.2, ***P*<0.01**
Adult weight [mg]	63.76±2.08	47.84±5.15	N=7/9, F=5.67, ***P*<0.05**
Larval C/N (day 15)	3.52±0.39	4.16±0.25	N=6/8, t=−0.369, ***P*<0.005**
Larval time [d]	21.08±0.75	22.36±0.81	N=12/11, F=0.79, P>0.1
Pupal time [d]	8.88±0.23	8.30±0.21	N=8/10, F=2.27, P>0.1

P-values in bold indicate statistically significant differences

[a] Mean values ± SE for plants exposed to full Hoagland solution for 5 days

[b] Mean values ± SE for plants exposed to N deficient Hoagland solution for 5 days

[c] Statistical parameters are indicated for ANCOVA and Student's *t*-test (Larval C/N). N = $\text{individuals}_{+N}/\text{individuals}_{-N}$



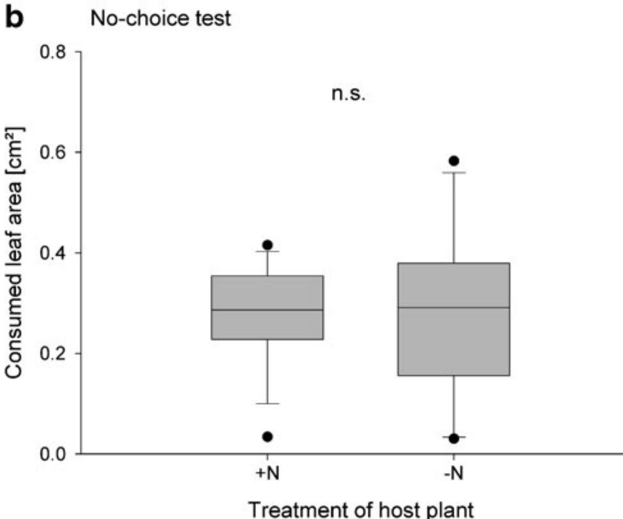


Fig. 1 Leaf consumption by second instar *Spodoptera frugiperda* larvae measured as leaf area removed in 24 hr. Soybean plants were exposed to either full (+N) or N deficient (–N) Hoagland solution for 5 d. Box-plots show median (*line*), 25–75% percentiles (*box*), 10–90% percentiles (*whisker*) and outliers (*dots*). **a** Choice test: Students *t*-test for dependent samples ($t=-2.14$ $P>0.05$, $N=12$). **b** No-choice test: Students *t*-test for independent samples ($t=0.00$, $P>0.5$, $N_{+N}=15$, $N_{-N}=14$)

unchanged (Table 1). Shifting the root-shoot-ratio is a well known response to low N availability and is considered to be an adaptation to suboptimal substrates which allows plants to more efficiently absorb nitrate from the soil (Schopfer and Brennicke 2005; Hill et al. 2006). It has been proposed that changes in phytohormonal balances in conjunction with sugar signals orchestrate cell division and differentiation, thus leading to optimized root morphology (Hermans et al. 2006).

We found that shortage in N supply negatively affected photosynthesis as indicated by the reduced maximum photochemical efficiency of PSII (Table 1). These results corroborate several studies that have used chlorophyll fluorescence to monitor the responses to N deprivation (Lu and Zhang 2000; Kumagai et al. 2007). Deficiency downregulates open PS II reaction centers and leads to increased light induced non-photochemical quenching and enhanced susceptibility to photoinhibition (Lu and Zhang 2000). A lack in chlorophyll content and ribulose-1, 5-bisphosphate-carboxylase/-oxygenase (RuBisCo), the most abundant protein in plants, is likely to also have contributed to a decline in photosynthesis. Chlorophyll and RuBisCo were not measured specifically, but leaves of soybeans exposed to N stress had lower leaf N levels, contained less protein (Table 1), and were lighter in color.

Negative effects on the leaf's N and protein content correlated with poor growth of *S. frugiperda* larvae. Caterpillars fed with –N leaves gained less weight during their development and had reduced pupal and adult weights compared to larvae reared on +N leaves (Table 2). Similar results were reported from *Spodoptera exigua* developing on N deficient cotton (Chen et al. 2008b) or larvae of other Lepidoptera feeding on several host plants (Coley et al. 2006). However, studies exist where the correlation between food N content and herbivore growth is less consistent, implying that N is a limiting nutrient for larval development, but not the only one (Tabashnik 1982). Clancy (1992) hypothesized that host plant N determines the amount of food ingested and thus affects the amount of other nutrients incorporated, resulting in an altered growth

Table 3 Performance parameters of *Cotesia marginiventris* reared inside *Spodoptera frugiperda*

Parameter	+N[a]	–N[b]	Statistics[c]
Pupal weight [mg]	2.18±0.05	1.73±0.09	$N=14/12$, $F=19.52$, $\mathbf{P<0.001}$
Larval time [d]	8.1±0.1	8.4±0.09	$N=15/12$, $F=1.02$, $P>0.1$
Pupal time [d]	5.2±0.2	5.0±0.0	$N=9/8$, $F=1.01$, $P>0.1$
Longevity [d]	14.6±0.3	14.6±0.6	$N=7/7$, $F=0.08$, $P>0.5$

P-values in bold indicate statistically significant differences

[a] Mean values ± SE for caterpillars fed with plants exposed to full Hoagland solution for 5 days

[b] Mean values ± SE for caterpillars fed with plants exposed to N deficient Hoagland solution for 5 days

[c] Statistical parameters are indicated for an ANCOVA. N = individuals$_{+N}$/individuals$_{-N}$

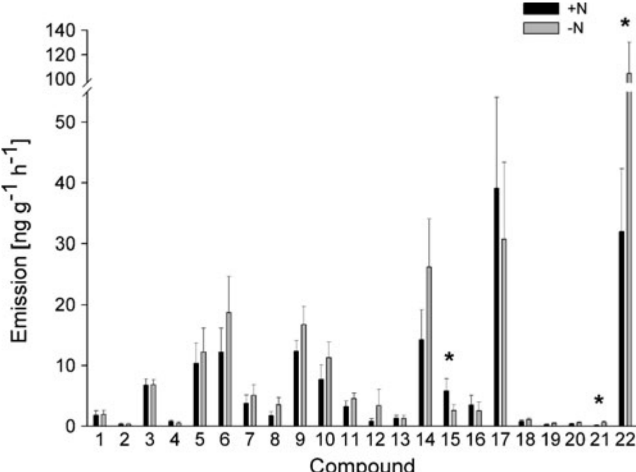


Fig. 2 Plant VOC emission in response to herbivory and full (+N) or deficient (–N) supply of N for 5 days. Bars indicate mean values, whiskers are ± SE. Asterisks indicate statistically significant differences (*$P<0.05$, Student's *t*-test, $N=10$). Compounds were identified according to their retention times, library mass spectra and by coelution with commercial standards and are arranged by retention times in the Figure. 1) n. i. 2) (*Z*)-3-Hexenal 3) n. i. 4) n. i. 5) (*Z*)-3-Hexenol 6) n. i. 7) α-Pinene 9) (*Z*)-3-Hexenyl acetate 10) (*E*)-β-Ocimene 11) (*Z*)-3-Hexenyl propionate 12) Benzeneacetonitrile 13) (*Z*)-3-Hexenylisobutyrate 14) Methyl salicylate 15) (*Z*)-3-Hexenyl-α-methylbutyrate 16) n. i. 17) Indole 18) (*E*)-Caryophyllene 19) α-Humulene 20) Germacrene D 21) β-Bergamotene 22) (*E,E*)-α-Farnesene. n.i. = Compound not identified

rate and survival rates of the herbivore caused by a deficiency of other nutrients than N.

Interestingly, despite poorer growth on –N leaves, *S. frugiperda* larvae did not prefer the superior food when both types of leaves were offered in a dual-choice assay, nor did they show any compensational feeding in a no-choice setup (Fig. 1). This contrasts with findings reported by Chen et al.(2008b) and Merkx-Jacques et al.(2008) who found that *S. exigua* larvae opted for leaves of high N content or a protein-biased diet, respectively. The incongruent feeding behavior of the two species may reflect their different feeding preferences. Larvae of *S. exigua*, for instance, are known to prefer leaves while *S. frugiperda* may often change to fruiting structures when available.

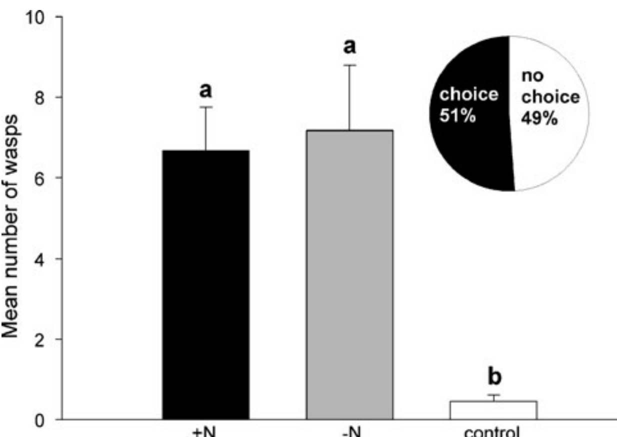


Fig. 3 Response of naïve *Cotesia marginiventris* to herbivore induced volatiles of soybean. Plants were reared in either full (+N) or nitrogen deficient (–N) Hoagland solution. Control was clean air; total numbers of wasps choosing one of the four control arms were divided by four. Bars represent mean (+SE) numbers of wasps making a choice in the olfactometer. Different letters indicate statistically significant differences (Log-linear model fitted to quasipoisson distribution; $N=6$ independent experiments with 30 wasps each). Pie chart shows mean percentage of wasps making a choice

The impact of N deficiency on the first trophic level was transmitted indirectly to the third trophic level as the growth of *C. marginiventris* also was affected. Parasitoids had significantly lower pupal and adult weights when their hosts had fed on N-deprived soybean leaves (Table 3). Pupal weight often is correlated with fecundity and consequently with fitness (Bourchier 1991). Thus, females of *C. marginiventris* that hatch from poor hosts may be expected to produce fewer eggs and have fewer offspring. Plant quality is crucial for the performance and fertility of parasitoids in many cases (Campan and Benrey 2004; Setamou et al. 2005; Caron et al. 2008; Sarfraz et al. 2008). *Cotesia flavipes* had a higher mean progeny size on its host *Chilo partellus,* when this was reared on cultivated compared to wild gramineous plants (Setamou et al. 2005). Eventually, the effect of poor plant quality on parasitoid fitness depends on the species' life history. Gregarious, koinobiont parasitoids, for instance, should be affected more strongly than solitary parasitoids when choosing poor hosts. They spend a considerable amount of time in examining their hosts and will lay their eggs in one or relatively few individuals (Brodeur and Boivin 2004). In contrast, females of the generalist, solitary endoparasitoid *C. marginiventris* allocate their offspring to many caterpillars by feeding on different host plants and thus reducing the fitness costs imposed by parasitizing an inferior host. Nevertheless, fitness costs could be substantial if a larger patch is affected by N deficiency and if wasps forage exclusively within this patch.

The host foraging behavior of parasitoid wasps is influenced by herbivore-induced plant VOCs, which are used as signals to locate those particular plants that are infested by herbivores. Soybeans grown in N deficient hydroponic solution emitted the same spectrum of herbivore-induced VOCs as fertilized plants. However, quantitative changes were found for some VOCs. Low N availability led to more than three times higher emission rates of the main compound (*E,E*)-α-farnesene, and significantly increased the release of the sesquiterpene β-bergamotene. Emission of the green leaf volatile (*Z*)-3-hexenyl-α-methylbutyrate, however, was significantly reduced (Fig. 2). Despite these changes, total amounts of VOCs were not affected by N deficiency.

Increased levels of constitutive mono- and sesquiterpenes have been found in N limited *Heterotheca subaxillaris* (Mihaliak and Lincoln 1985). Looking at induced VOCs, higher levels were found also in N deficient, hydroponically cultivated maize, and in soil-grown cotton. In both plants, the accumulation of the phytohormone jasmonic acid (JA), an important factor in the signaling cascade leading to volatile biosynthesis, correlated negatively with N (Schmelz et al. 2003; Chen et al. 2008a). It has been argued that N starved plants produce greater induced defense responses because N deficiency commonly leads to higher levels of leaf sugars and starch. This larger pool of nonstructural carbohydrates may be used for enhanced VOC biosynthesis (Schmelz et al. 2003).

Females of *Cotesia* spp. have been reported to respond in a dose-dependent manner to the total blend of herbivore-induced VOCs (NgiSong et al. 1996; Turlings et al. 2004). With the main compound (*E,E*)-α-farnesene being emitted in higher amounts by N deficient soybeans, a preference for this odor could have been expected. Naïve *C. marginiventris* females, however, did not differentiate between the blends of stressed and fertilized plants (Fig. 3). Our results thus confirm observations by Chen et al. (2008a) from cage experiments with cotton plants. The authors found that *C. marginiventris* parasitized the same numbers of caterpillars irrespective of the plants' N levels and concluded that the parasitoids did not differentiate between the herbivore induced VOC blends.

Several reasons for this behavior are conceivable. First, the VOCs that were emitted in higher quantities were not the relevant key compounds that induce wasp attraction. This notion is supported by an elegant experiment using transgenic *Arabidopsis thaliana* that overexpressed the maize terpene synthase gene TPS10. In this study, Schnee et al. (2006) demonstrated that several sesquiterpenes, among them (*E*)-β-farnesene or (*E*)-α-bergamotene, were not attractive to naïve *C. marginiventris*. Moreover, the wasps responded only if they had experienced these compounds during oviposition. Further evidence comes from studies that tested specific fractions of the induced odor blend. D'Alessandro and Turlings (2005) confirmed that a blend lacking most sesquiterpenes was as attractive to naïve and experienced *C. marginiventris* as the full mix. The same holds true for a blend that lacked VOCs from the shikimic acid pathway, while on the other hand, wasps responded strongly to unknown compounds in quantities that were too low to be detected (D'Alessandro et al. 2006, 2009; Rostás and Turlings 2008). In fact, our knowledge on which VOCs innately trigger attraction in parasitoids is fairly rudimentary and needs further testing. Moreover, in nature, such cues are rarely isolated but always occur within the context of background odor, that may mask or enhance the odors of the target plant (Mumm and Hilker 2005; Schroeder and Hilker 2008).

In summary, N deprivation had a strong negative impact on the whole tritrophic system, attenuating the performance of soybean plants, herbivorous caterpillars, and the parasitoid larvae that developed inside *S. frugiperda*. Despite such adverse effects on the plant's physiology and some alterations in VOC emission, the signal that indicates the presence of potential hosts obviously remained unchanged as the wasps' host searching behavior was not affected. These results suggest that the induced indirect defense against herbivores remains stable and effective even under low N conditions. On the parasitoid's side, reduced fitness due to low N availability cannot be ruled out, but might be negligible if only few plants within a patch are affected. From the plant's perspective and from a biocontrol point of view, this should be beneficial as abiotic stress in this case does not promote higher susceptibility to another biotic stress factor.

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A Biochemical Interpretation of Terpene Chemotypes in *Melaleuca alternifolia*

Andras Keszei · Yasmin Hassan · William J. Foley

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Abstract The variation of foliar monoterpenes in the Australian Tea Tree (*Melaleuca alternifolia*) has been of significant interest both to the essential oil industry as well as to ecologists. The majority of studies on leaf chemistry have been aimed directly towards obtaining oil of higher quality or quantity. In the current study, we aimed to understand how molecular mechanisms contribute to the chemical variability of this species, based on chemical analysis of the leaf oils from a biochemical perspective. Correlations between monoterpenes across the species as well as within chemotypes show strong, persistent patterns, which enable us to establish groups based on possible common biosynthetic origins. We found that three distinct enzymes corresponding to these groups: a sabinene-hydrate synthase, a 1,8-cineole synthase, and a terpinolene synthase may be sufficient to explain all six chemotypes in *M. alternifolia*.

Key Words *Melaleuca alternifolia* · Australian Tea Tree · Chemotypes · Terpene synthase (TPS) · Terpinen-4-ol · Cineole · Terpinolene · Barycentric plot · Viridiflorene · Aromadendrene · Calamenene

Introduction

Identification of the genes that underlie phenotypic traits provides a crucial link to understanding evolutionary processes, such as adaptive evolution and genetic drift, since genetic variation forms the basis for selection and evolution. Understanding how genetic information is related to phenotypic variation of ecologically important traits presents a major challenge in evolutionary biology. It requires first the identification of those traits, their mode of expression, and finally, information of the key genes involved. Since it is hard to meet all these criteria, the relationship between genes, phenotypes and ecological function has only been achieved in a few cases in natural populations.

Distinct intra-specific chemical variation in foliar terpenes can occur in natural populations (Vernet et al. 1986) and is widespread in many Australian plants of the family Myrtaceae (Keszei et al. 2008). *Melaleuca alternifolia* is a Myrtaceous tree that shows chemotypic variation of foliar terpenes throughout its range (Butcher et al. 1994). The chemotype rich in terpinen-4-ol is widely used medicinally (Cox et al. 2001; Hammer et al. 2006), and therefore, a lot of research in the species has been restricted to the one chemotype. The presence of chemotypes in Tea-Tree indicates that the foliar leaf oil traits may be the result of adaptation to complex biotic interactions, as those found to influence chemical variability in thyme (Linhart and Thompson 1999; Linhart et al. 2005). To gain a similar understanding of the well-studied system that Australian Tea-Tree represents, we need to focus on leaf oil in light of its variability and biosynthetic origin, and not as a raw product.

Melaleuca alternifolia contains foliar oils that are a complex mixture of mono- and sesquiterpenes (Brophy et al. 1989). Terpene synthases are responsible for the direct production of the majority of the terpenes found in essential oils. They are capable of producing single or multiple compounds from the same prenyl-diphosphate substrate (Degenhardt et al. 2009). Examining correlations between groups of terpenes and working under the assumption that

A. Keszei (✉) · Y. Hassan · W. J. Foley
Research School of Biology, Australian National University,
Canberra 0200, Australia
e-mail: andras.keszei@anu.edu.au

strong positive correlation indicates a common biogenetic origin, Zavarin (1970) predicted the presence of a single terpene synthase in *Picea* that catalyzes the formation of sabinene, terpinolene, and γ-terpinene in a set ratio. An enzyme was isolated later that encodes a terpene synthase that showed the predicted catalytic profile (Gijzen et al. 1991). Qualitative variation in plant terpenes likely is due to variation in terpene synthase genes, and may be deduced from identifying the correlations among compounds that potentially have the same biosynthetic origin.

In *M. alternifolia*, previous work has established chemotypes based on variation in the concentration of 1,8-cineole, terpinen-4-ol, and terpinolene (Butcher et al. 1992). *Melaleuca alternifolia* is restricted to coastal flood plains of north-eastern New South Wales (NSW) with neighboring populations in the 'granite belt' in southeast Queensland, Australia. The geographic distribution of the three major monoterpenes reveals terpinen-4-ol in the majority in populations in the center of the natural range, while terpinolene dominates in the northwest, and 1,8-cineole in the south. Butcher et al. (1994) have shown that this corresponds to co-occurrence with the sister species *M. linariifolia* in the south and *M. trichostachya* in the north of NSW. *Melaleuca linariifolia* also is characterized by having both terpinen-4-ol and 1,8-cineole rich chemotypes, while *M. trichostachya* has terpinolene and 1,8-cineole rich chemotypes. It has been proposed that the respective *M. alternifolia* chemotypes may be a result of past hybridization. However, it also is possible that the convergence of chemical traits is due to similar patterns of herbivore pressure where the species geographically co-occur.

The purpose of the current work was to identify correlations and chemical patterns that allow us to predict the biosynthetic origin of chemotypic variation in *M. alternifolia* terpenes. This in turn will facilitate a search for the molecular components involved, and ultimately an understanding of the forces and mechanisms that have brought about and maintain chemical variation in Australian Tea-Tree.

Methods and Materials

Population Sampling Mature leaf material (ca. 20 g wet mass) was collected from 20 mature trees at eight sites and 15 from a ninth site across the species' natural geographic range (Table 1). However, populations from the far south of the range (Port Macquarie) were excluded, because they have been proposed to be hybrids with southern lineages of *M. linariifolia* (Butcher et al. 1995). We chose trees that were at least 100 m apart to avoid collecting from related trees (Rossetto et al. 1999), and the location of each tree was recorded. Samples were refrigerated at 4°C within 2 hr of collection. The extraction of leaf oils was completed on the day of collection in order to minimize the possibility of evaporation and oxidative degradation.

Table 1 Frequency of chemotypes sampled at each site across the natural geographic range of *Melaleuca alternifolia*

Population[a]	Chemotype						
	1	2	3	4	5	6	7
Flaggy Creek	–	–	–	2	18	–	–
Wooli Road	1	–	–	–	19	–	–
Chaffin Swamp	1	1	–	3	7	8	–
Dilkoon Creek	12	–	–	7	1	–	–
Devil's Pulpit	20	–	–	–	–	–	–
Casino Racecourse	15	–	–	–	–	–	–
Yellow Creek	19	–	–	1	–	–	–
Cannon Creek	–	14	5	–	–	1	–
Bald Rock Creek	–	14	2	–	1	2	1

[a] Sites in New South Wales (NSW), Australia; for further information and a map of the sites, see Rossetto et al. (1999)

Extraction and Analysis of Terpenes Mature leaf material was separated from stems, weighed, and placed in 10 ml of ethanol (Baker et al. 2000) containing 0.25 $g \cdot l^{-1}$ of *n*-tridecane as an internal standard. The samples were left in ethanol for 7 d to complete extraction. A further 4 g leaf material from each tree were oven-dried at 60°C and weighed to calculate oil concentration on a dry weight basis.

Gas chromatography was carried out on an Agilent 6890 GC using an Alltech AT-35 (35% phenyl, 65% dimethylpolyoxylane) column (Alltech, Wilmington, DE, USA). The column was 60 m long (internal diam. 0.25 mm) with a stationary phase film thickness of 0.25 μm. Helium was used as carrier gas. The ethanol extract was filtered through a 0.45 μm filter, and 1 μl was injected at 250°C at a 1:25 split ratio. The temperature program was as follows: 100°C for 5 min, ramping to 200°C at 20°C·min^{-1}, followed by a ramp to 250°C at 5°C·min^{-1}, and held at 250°C for 4 min. The total elution time was 25 min. The components of the solvent extract were detected by using an FID and an Agilent 5973 Mass Spectrometer dual setup through an SGE MS/FID splitter. Several peaks were identified tentatively by comparisons to authentic standards (α-pinene, β-pinene, β-myrcene, β-phellandrene, terpinen-4-ol, 1,8-cineole, α-terpineol, β-caryophyllene, kindly made available by Mike Lacey and Thomas Wallenius, CSIRO Canberra). The remaining peaks were identified by comparison to mass spectra and retention times of compounds published using the same AT35 chromatographic column and run parameters (Southwell and Lowe

1999; Russell and Southwell 2002). Quantities of compounds were calculated by recording peak areas of the FID trace in MSD Chemstation Data Analysis (Agilent Technologies, Deerfield, IL, USA) and relating them to the internal standard (tridecane) and leaf dry weight.

Statistical Analysis We separated monoterpene and sesquiterpene data, as the two chemical groups are biosynthetically distinct, and are influenced by separate genetic factors (Keszei et al. 2008). Initially, we used principal components analysis to identify chemotypes as had been done previously (Butcher et al. 1994). However, this method did not unambiguously separate some of the published chemotypes that were based on polymodal and disjunct distributions of major oil components (data not shown). Consequently, we calculated Pearson's correlation coefficients on the entire sample set based on the absolute concentrations of terpenes to eliminate false negative correlations potentially introduced when data on terpene proportions are used. The individual components were divided into groups based on the correlations, and the proportion of each of these groups was plotted on a ternary barymetric plot. To predict the effect of individual biosynthetic genes, we re-calculated Pearson's correlation coefficients within each of the resulting clusters to obtain chemical groups characteristic of individual chemotypes.

Results

GC-MS analyses of ethanolic extracts of mature leaf material of *M. alternifolia* revealed the presence of 47 compounds comprising 20 monoterpenes and 27 sesquiterpenes. Significant variation in leaf oil concentration was found both between sites and among chemotypes. The lowest foliar oil concentration recorded was of a tree of chemotype 3 from Bald Rock Creek, Queensland (10 $mg \cdot g^{-1}$ dry matter (DM)), whereas the highest yielding was of an individual of chemotype 5 at Wooli Road, NSW, with a foliar oil concentration of 141 $mg \cdot g^{-1}$ DM. The distribution of oil yield shows a normal distribution throughout the species, and this pattern persists when examined at the level of individual chemotypes (data not shown).

Monoterpenes Monoterpenes are the dominant components of *M. alternifolia* leaf oil, and comprised between 9 and 128 $mg \cdot g^{-1}$ of dry leaf weight. Sesquiterpene content ranges between 0.8 $mg \cdot g^{-1}$ and 14 $mg \cdot g^{-1}$ or between 5 and 21% of the total leaf oils. Concentrations of mono- and sesquiterpene concentrations are positively correlated with each other. The ratio of monoterpenes to sesquiterpenes is continuous and polymodal, with a major mode at 62%, one at 80%, and one at 86%. The three major monoterpenes: terpinen-4-ol, 1,8-cineole, and terpinolene show well defined, discontinuous concentration ranges from which it is possible to assign all but one of the sampled individuals to known chemotypes.

Pearson's correlation values among concentrations of all of the monoterpenes within the full sample set established three groups showing strong intercorrelations ($r>0.8$): **Group A**: α-thujene, α-terpinene, γ-terpinene, terpinen-4-ol, **Group B**: α-pinene, β-pinene, myrcene, limonene, 1,8-cineole, α-terpineol, and **Group C**: α-phellandrene, terpinolene, linalool.

Common biosynthetic intermediates (Fig. 1) suggest that these three main groups may be the products of three distinct terpene synthases. Concentrations of *(Z)*-piperitol, *p*-cymene, β-phellandrene, sabinene, and *(Z)*-sabinene hydrate did not show strong ($r<0.8$) correlations to any other group, however, they showed the strongest correlation to group A. As each of these compounds readily undergoes post-biosynthetic conversion or is the product of such a process (Bohlmann et al. 1999; Keszei et al. 2008), their concentration may not directly reflect enzymatic activity. As such, the lack of a strong positive correlation need not rule out biosynthetic relatedness. Provided that there was either a strong statistical correlation or evidence of a common biosynthetic origin, monoterpenes were assigned to one of the three groups. The variation in the proportions of these three groups relative to the total monoterpene pool is discontinuous, and clearly resolves all chemotypes (Fig. 2).

The leaf oil of one individual from Bald Rock Creek, Queensland, showed 1,8-cineole and terpinolene as the major components. However, the ratio between the two compounds did not fit any of the established chemotypes. Furthermore, the ternary plot resolved the individual as a distinct separate group.

Looking at the contributions of the three independent biochemical groups in Fig. 2, it is evident that out of the seven chemotypes, chemotypes 1, 2, and 5 represent cardinal chemotypes dominated by a single compound, and chemotypes 3, 4, and 6 fall between these as intermediates. In the ternary plot, only chemotype 5 is plotted on an apex, while chemotype 1 shows significant contributions from both Groups B and C, and chemotype 2 shows significant contributions from Groups A and B. This can be explained by two processes: the major monoterpene synthases in chemotypes 1 and 2 may also synthesize compounds characteristic of the other major biosynthetic groups. In this case, compounds belonging to separate groups will show strong correlations in the cardinal chemotypes. It also is possible that the monoterpene profile of these chemotypes is determined by more than one active terpene synthase. If this were the case, we would expect the

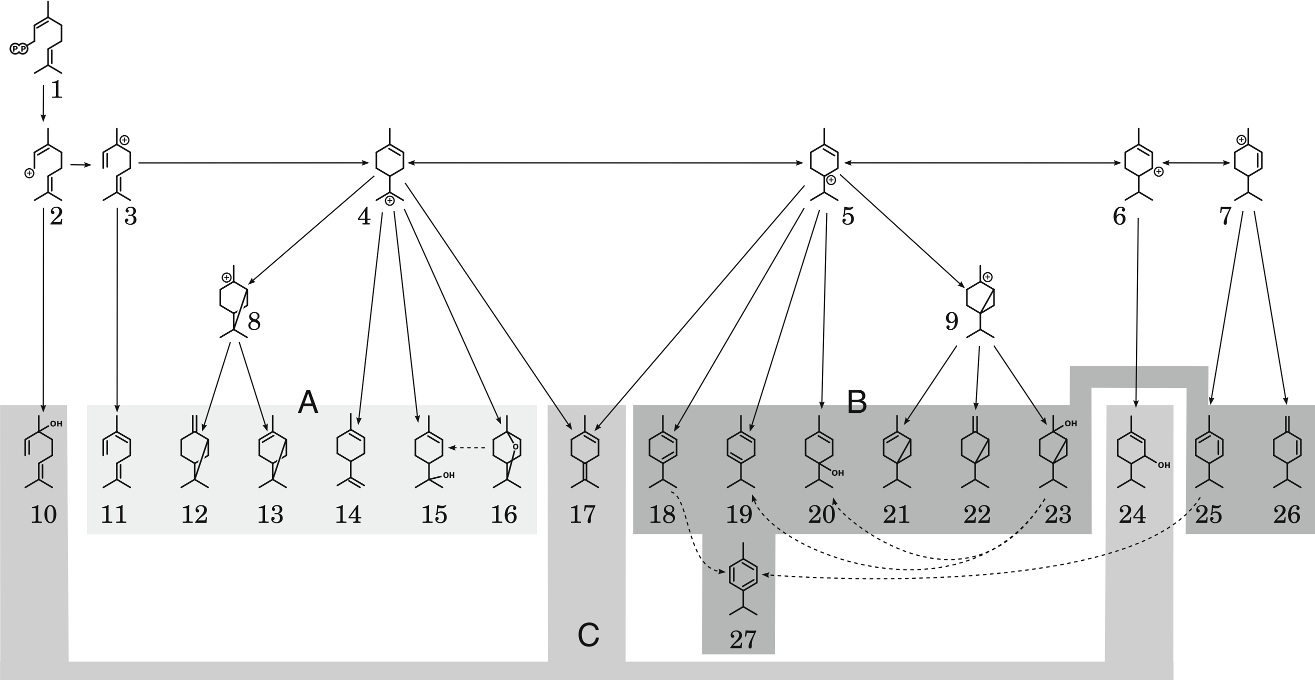


Fig. 1 Biosynthetic relationships between monoterpenes found in *Melaleuca alternifolia*. The products of the three proposed terpene synthases are indicated by shaded boxes. 1: geranyl diphosphate, 2: linalyl carbocation, 3: geranyl carbocation, 4: α-terpinyl carbocation, 5: terpinenyl carbocation, 6: piperitenyl carbocation, 7: phellandryl carbocation, 8: pinyl carbocation, 9: sabinyl carbocation, 10: linalool, 11: myrcene, 12: β-pinene, 13: α-pinene, 14: limonene, 15: α-terpineol, 16: 1,8-cineole, 17: terpinolene, 18: α-terpinene, 19: γ-terpinene, 20: terpinen-4-ol, 21: α-thujene, 22: sabinene, 23: sabinene hydrate, 24: piperitol, 25: α-phellandrene, 26: β-phellandrene, 27: *p*-cymene

correlation matrices to separate monoterpenes of different biosynthetic origins into separate groups.

To test these possibilities, Pearson's correlation coefficients were calculated between concentrations of each of the monoterpenes in the cardinal chemotypes, and compared to the overall pattern. Table 2 shows the resulting correlation matrices. The correlations between monoterpenes in the individual chemotypes illustrate the likely products of the distinct enzymes (Table 3).

In chemotype 1, dominated by terpinen-4-ol, two groups emerge: Group 1A: α-thujene, a-terpinene, γ-terpinene, terpinolene, terpinen-4-ol, *(Z)*-piperitol; and Group 1B: limonene, 1,8-cineole. Concentrations of myrcene, α-pinene, β-pinene, α-terpineol, α-phellandrene, and β-phellandrene show significant correlation to compounds in both groups, but they show somewhat stronger correlation to Group 1A. Concentration of linalool, *p*-cymene, sabinene and sabinene hydrate show no significant correlations to other compounds.

In the terpinolene dominated chemotype 2, two strong groups can be distinguished: Group 2C: α-thujene, α-phellandrene, α-pinene, α-terpinene, γ-terpinene, terpinolene, linalool; Group 2B: α-pinene, myrcene, limonene, β-phellandrene, 1,8-cineole, α-terpineol, γ-terpinene, terpinen-4-ol. Concentrations of β-pinene, *p*-cymene, sabinene, and sabinene hydrate show no significant correlation to other compounds.

In chemotype 5, dominated by 1,8-cineole, only a single distinct group could be identified: Group 5B: α-pinene, β-pinene, myrcene, a-phellandrene, limonene, β-phellandrene, 1,8-cineole, α-terpineol, terpinen-4-ol.

Sesquiterpenes Sesquiterpenes have always been considered minor and therefore insignificant components of the leaf oils of all *M. alternifolia* samples. However, we found that their contribution to some of the oils was as high as 20% of the total oil, which is higher than that of the main monoterpene groups in some chemotypes.

Calculation of the Pearson correlation coefficients among the concentrations of the sesquiterpenes (Table 3) showed strong correlations. The sesquiterpenes β-gurjunene, unknown peaks at 12.85 min, 12.95 min, and 13.59 min, α-elemene, unknown peak at 13.76 min, calamenene, unknown peak at 15.11 min, β-eudesmol showed discontinuous distributions. Interestingly, none of the sesquiterpenes with discontinuous distributions correlated with each other. Based on Pearson's correlation coefficients of $r>0.8$, we established the following groups; Group D: elixene, bicyclogermacrene; Group E: guaiene, α-gurjunene, aromadendrene, unknown peak at 13.59 min,

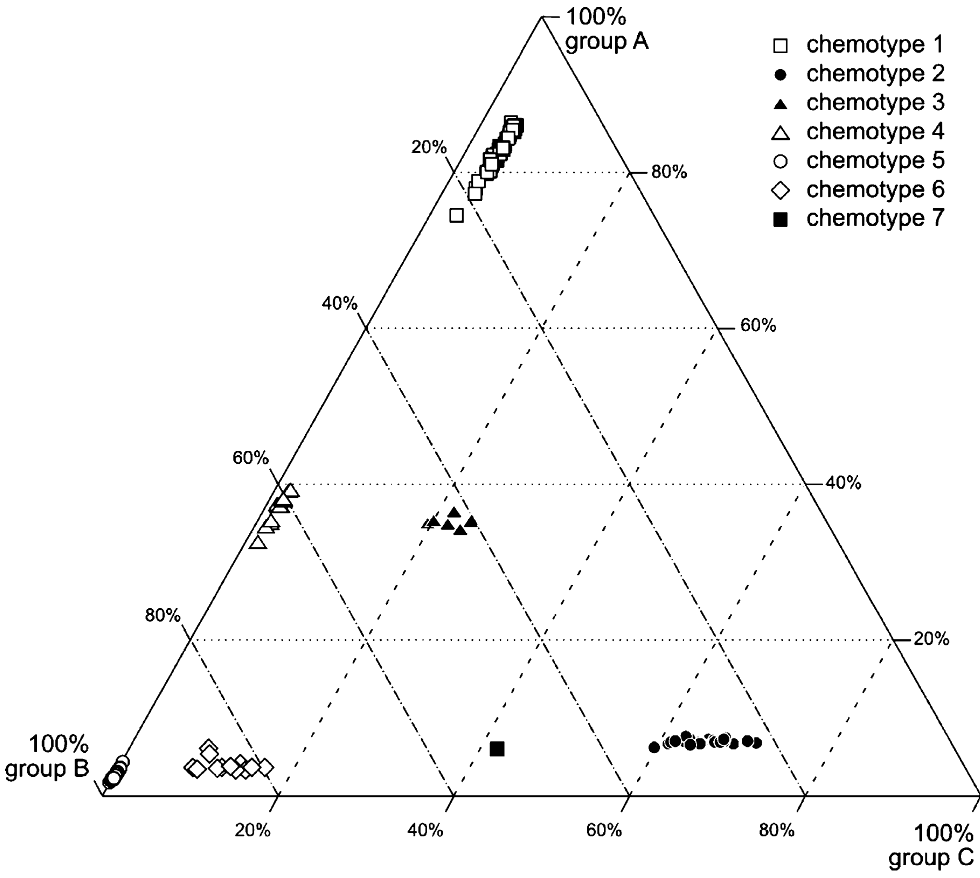
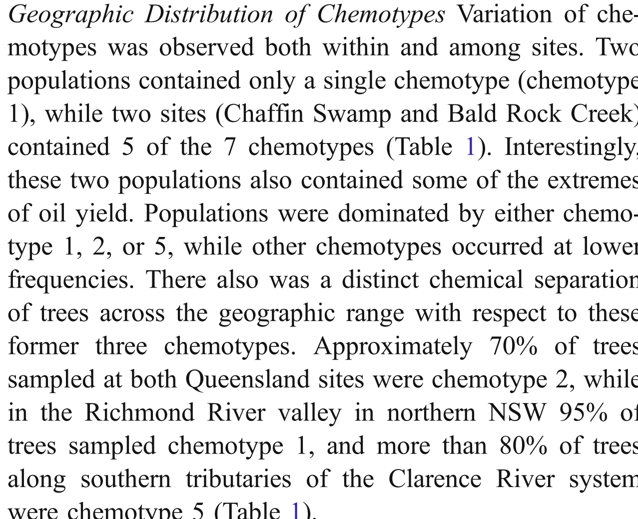


Fig. 2 A ternary barycentric plot showing the contributions of the three biochemical groups of compounds (as shown in Fig. 1) to the monoterpene fraction of the leaf oil profile of *Melaleuca alternifolia*. All six chemotypes are resolved and show discontinuous distributions. The intermediate chemotypes (3,4 and 6) are positioned between the cardinal chemotypes (1,2 and 5), which confirms a codominant relationship between the genetic components involved

allo-aromadendrene, viridiflorene, globulol, viridiflorol, isoaromadendrene epoxid e, unknown peak at 15.11 min, β-gurjunene; Group F: α-elemene, γ-muurolene; Group G: 13.79 min, δ-cadinene, β-cadinene, γ-cadinene, cubenol, (also showed strong correlation to α-gurjunene and γ-muurolene). None of the other sesquiterpenes showed significant correlation to any other compounds.

The two strongest sesquiterpene patterns that reflect the monoterpene chemotype were the ratio between aromadendrene and viridiflorene (Fig. 3), and the presence/absence of calamenene, shown together with another sesquiterpene that showed discontinuous but independent distribution: β-eudesmol. Chemotype 2 shows a viridiflorene to aromadendrene ratio different from all other chemotypes, although correlation between the two compounds is high ($r>0.8$) in all chemotypes. Calamenene content also is indicative of chemotype 2. Of the cardinal chemotypes, chemotype 2 is the only one that showed no calamenene content in any of the individuals sampled (Fig. 3). None of the other chemotypes was correlated with any of the sesquiterpene patterns, and no more sesquiterpene patterns were found to be correlated with monoterpene chemotypes. The correlations among sesquiterpene concentrations generally were lower than among monoterpenes. However, this may be a result of their lower concentrations leading to lower measurement accuracy.



Geographic Distribution of Chemotypes Variation of chemotypes was observed both within and among sites. Two populations contained only a single chemotype (chemotype 1), while two sites (Chaffin Swamp and Bald Rock Creek) contained 5 of the 7 chemotypes (Table 1). Interestingly, these two populations also contained some of the extremes of oil yield. Populations were dominated by either chemotype 1, 2, or 5, while other chemotypes occurred at lower frequencies. There also was a distinct chemical separation of trees across the geographic range with respect to these former three chemotypes. Approximately 70% of trees sampled at both Queensland sites were chemotype 2, while in the Richmond River valley in northern NSW 95% of trees sampled chemotype 1, and more than 80% of trees along southern tributaries of the Clarence River system were chemotype 5 (Table 1).

Discussion

Monoterpene Chemotypes We identified six chemotypes in this study, all of which had been described previously (Butcher et al. 1994), and an individual tree that did not group with any of the recognized chemotypes. We grouped the monoterpenes on the basis of their likely biosynthetic

Table 2 Pearson's pairwise correlation coefficients between individual terpene concentrations (mg·g^{-1} dry leaf weight) from all individuals of *Melaleuca alternifolia*

	β-gurjunene	guaiene	12.85	12.92	α-gurjunene	β-caryophyllene	aromadendrene	13.59	α-elemene	13.78	alloaromadendrene	γ-muurolene	viridiflorene	bicyclogermacrene	δ-cadinene	β-cadinene	γ-cadinene	calamenene	15.11	globulol	isoaromadendrene epoxide	viridiflorol	α-eudesmol	cubenol	β-eudesmol
elixene	0.19	0.41	0.42	0.01	0.59	0.61	0.17	0.17	0.76	0.67	0.73	0.66	0.54	0.91	0.73	0.61	0.71	-0.06	0.06	0.33	0.33	0.53	0.33	0.48	-0.05
β-gurjunene		0.74	-0.11	0.62	0.64	0.37	0.75	0.77	0.37	0.40	0.62	0.38	0.75	0.14	0.47	0.36	0.47	0.49	0.70	0.70	0.62	0.57	0.34	0.52	0.18
guaiene			0.09	0.60	0.92	0.66	0.87	0.82	0.65	0.63	0.79	0.69	0.90	0.34	0.77	0.73	0.80	0.61	0.77	0.84	0.73	0.65	0.41	0.73	0.18
12.85				-0.07	0.15	0.26	-0.08	-0.04	0.33	0.37	0.21	0.31	0.08	0.55	0.24	0.23	0.24	-0.16	-0.08	0.00	-0.03	0.09	0.09	0.07	-0.01
12.92					0.42	0.18	0.66	0.67	0.16	0.22	0.32	0.15	0.52	-0.05	0.26	0.09	0.26	0.54	0.68	0.41	0.28	0.24	0.22	0.29	0.23
α-gurjunene						0.78	0.82	0.77	0.83	0.73	0.87	0.83	0.93	0.54	0.85	0.82	0.88	0.41	0.66	0.82	0.69	0.70	0.43	0.77	0.16
β-caryophyllene							0.49	0.46	0.81	0.79	0.67	0.77	0.66	0.59	0.79	0.77	0.80	0.18	0.38	0.54	0.48	0.54	0.32	0.63	0.03
aromadendrene								0.94	0.46	0.40	0.69	0.47	0.86	0.11	0.48	0.46	0.53	0.60	0.86	0.89	0.71	0.57	0.38	0.57	0.31
13.59									0.44	0.42	0.64	0.46	0.83	0.11	0.46	0.42	0.49	0.56	0.86	0.81	0.63	0.53	0.32	0.54	0.30
α-elemene										0.79	0.73	0.93	0.71	0.77	0.85	0.82	0.87	0.02	0.30	0.51	0.40	0.57	0.34	0.68	-0.05
13.78											0.69	0.75	0.66	0.68	0.82	0.72	0.81	0.19	0.32	0.44	0.37	0.51	0.27	0.60	0.04
alloaromadendrene												0.68	0.91	0.69	0.79	0.70	0.79	0.36	0.52	0.83	0.80	0.85	0.46	0.71	0.10
γ-muurolene													0.70	0.65	0.84	0.86	0.88	0.04	0.31	0.52	0.43	0.53	0.29	0.69	-0.02
viridiflorene														0.50	0.77	0.66	0.78	0.49	0.71	0.87	0.77	0.76	0.47	0.75	0.23
bcg															0.68	0.53	0.64	-0.17	0.00	0.27	0.27	0.52	0.28	0.43	-0.12
δ-cadinene																0.89	0.98	0.33	0.37	0.56	0.54	0.65	0.37	0.78	0.03
β-cadinene																	0.93	0.27	0.32	0.59	0.55	0.59	0.31	0.72	-0.02
γ-cadinene																		0.34	0.41	0.60	0.55	0.64	0.39	0.79	0.06
calamenene																			0.63	0.52	0.51	0.35	0.30	0.41	0.28
15.11																				0.72	0.54	0.44	0.31	0.49	0.35
globulol																					0.92	0.79	0.39	0.63	0.17
isoaromadendrene epoxide																						0.85	0.34	0.56	0.03
viridiflorol																							0.44	0.63	-0.03
α-eudesmol																								0.53	0.42
cubenol																									0.30

	α-thujene	α-pinene	sabinene	b-pinene	myrcene	α-phellandrene	α-terpinene	limonene	β-phellandrene	p-cymene	1,8-cineole	g-terpinene	terpinolene	linalool	(Z)-sabinene hydrate	terpinen-4-ol	α-terpineol
α-pinene	-0.51																
sabinene	0.26	0.08															
β-pinene	-0.71	0.82	0.03														
myrcene	-0.63	0.94	0.12	0.93													
α-phellandrene	0.35	-0.53	-0.14	-0.21	-0.48												
α-terpinene	0.77	-0.16	0.30	-0.60	-0.35	-0.30											
limonene	-0.80	0.80	-0.06	0.97	0.91	-0.24	-0.67										
β-phellandrene	0.03	0.65	0.28	0.57	0.66	-0.13	0.11	0.45									
p-cymene	0.36	-0.12	-0.04	-0.46	-0.33	-0.24	0.63	-0.48	-0.12								
1,8-cineole	-0.82	0.77	-0.08	0.96	0.90	-0.22	-0.71	1.00	0.41	-0.51							
γ-terpinene	0.74	-0.14	0.26	-0.58	-0.34	-0.31	0.99	-0.66	0.11	0.71	-0.69						
terpinolene	0.32	-0.58	-0.16	-0.24	-0.52	0.99	-0.32	-0.27	-0.21	-0.24	-0.25	-0.33					
linalool	0.26	-0.54	-0.18	-0.19	-0.48	0.98	-0.38	-0.22	-0.21	-0.27	-0.19	-0.39	0.99				
(Z)-sabinene hydrate	0.25	-0.01	0.75	-0.08	-0.03	-0.06	0.25	-0.14	0.05	0.02	-0.15	0.22	-0.07	-0.08			
terpinen-4-ol	0.71	-0.12	0.26	-0.58	-0.32	-0.35	0.99	-0.65	0.11	0.71	-0.68	1.00	-0.37	-0.43	0.21		
α-terpineol	-0.70	0.90	0.04	0.94	0.97	-0.41	-0.46	0.96	0.55	-0.34	0.94	-0.44	-0.45	-0.41	-0.08	-0.43	
(Z)-piperitol	0.79	-0.20	0.45	-0.55	-0.36	-0.12	0.89	-0.65	0.13	0.53	-0.68	0.88	-0.13	-0.18	0.35	0.87	-0.46

Monoterpenes (bottom) and sesquiterpenes (top). Values above 0.7, 0.8, and 0.9 are emphasized using incremental shading. Strong positive correlations (>0.7) between monoterpenes correspond to grouping based on common carbocation intermediates as shown in Fig. 1

origins (Fig. 1) and calculated intercorrelations between them. Here we discuss the possible underlying biochemical factors that lead to these distinct patterns.

The two likely processes are based on different types of catalytic action. First, each terpene may be produced by a single, specific enzyme, and certain terpenes may co-occur as a result of genetic linkage (Wilderman et al. 2004). Alternatively, terpenes in each group may be products of the same reaction by a single enzyme (Wise et al. 1998). It is more common for a single terpene synthase to produce multiple products than a single product exclusively, and therefore, we expect that the latter explanation is the most likely. Support for this proposal comes from functional assays of terpene synthase enzymes in other species. Heterologous expression and functional characterisation of 1,8-cineole synthase from *Arabidopsis thaliana* (Chen et al. 2004) and *Salvia officinalis* (Wise et al. 1998) show that those enzymes catalyze not only the formation of 1,8-cineole, but also of limonene, myrcene, α-and β-pinene, and α-terpineol as by-products, and that the ratios of products are constant characteristics of these enzymes. These compounds also are the major contributors to the Group B terpenes of *M. alternifolia*.

A single enzyme can also synthesise one product, which can subsequently be modified into multiple products by other processes. In particular, the Group A terpenes in *M. alternifolia* may result from re-arrangement of unstable *cis*-sabinene hydrate. In *Origanum majorana*, sabinene synthase also produces sabinene hydrate, which is rearranged after steam distillation to give terpinen-4-ol, α- and γ-terpinene, and terpinolene (Fischer et al. 1987). Young leaves of chemotype 1 in *M. alternifolia* are rich in *cis*-sabinene hydrate (41%) (Russell and Southwell 2002), and these also are known to undergo temperature-catalysed transformation to the aforementioned products during hydrodistillation (Brophy et al. 1989). It also has been shown that *cis*-sabinene hydrate can undergo a non-enzymatic acid solvolysis *in vivo* as the leaf ages (Southwell and Stiff 1990; Cornwell et al. 1995), resulting in Group A monoterpenes in the mature leaf of *M. alternifolia*. This reaction would be expected to contain terpinolene as a by-product, and in chemotype 1 individuals, there is indeed a strong positive correlation between concentrations of terpinen-4-ol and terpinolene.

In chemotypes 2, 3, 6, and 7, however, terpinolene most likely is synthesized by a separate terpinolene synthase. In *M. alternifolia*, the concentration of terpinolene is positively

Table 3 Pearson's pairwise correlation coefficients between individual monoterpene concentrations shown for only the three cardinal chemotypes of *Melaleuca alternifolia*

Chemotype 1

	α-pinene	sabinene	b-pinene	myrcene	α-phellandrene	α-terpinene	limonene	β-phellandrene	p-cymene	1,8-cineole	g-terpinene	terpinolene	linalool	(Z)-sabinene hydrate	terpinen-4-ol	α-terpineol	(Z)-piperitol
α-thujene	0.64	0.56	0.68	0.93	0.70	0.89	0.00	0.77	-0.55	-0.23	0.67	0.82	-0.09	0.33	0.65	0.58	0.77
α-pinene		0.35	0.84	0.79	0.76	0.85	0.43	0.75	0.22	0.01	0.98	0.93	0.04	0.21	0.96	0.87	0.68
sabinene			0.62	0.60	0.23	0.43	0.04	0.30	-0.31	-0.06	0.26	0.36	-0.08	0.87	0.24	0.37	0.66
β-pinene				0.86	0.70	0.76	0.62	0.73	-0.02	0.33	0.79	0.80	0.05	0.48	0.76	0.83	0.68
myrcene					0.80	0.92	0.25	0.86	-0.39	-0.03	0.80	0.90	-0.08	0.38	0.79	0.73	0.77
α-phellandrene						0.82	0.30	0.96	-0.16	-0.02	0.81	0.86	-0.07	0.05	0.81	0.70	0.54
α-terpinene							0.16	0.85	-0.27	-0.19	0.88	0.97	-0.07	0.22	0.86	0.73	0.72
limonene								0.29	0.39	0.89	0.36	0.25	0.21	0.07	0.30	0.52	0.04
β-phellandrene									-0.27	-0.01	0.80	0.88	-0.07	0.11	0.81	0.72	0.61
p-cymene										0.24	0.15	-0.10	0.25	-0.17	0.13	0.17	-0.16
1,8-cineole											-0.06	-0.14	0.16	0.02	-0.10	0.17	-0.22
γ-terpinene												0.96	0.03	0.09	0.98	0.86	0.68
terpinolene													-0.03	0.16	0.95	0.81	0.72
linalool														-0.05	0.00	0.17	-0.06
(Z)-sabinene hydrate															0.07	0.21	0.38
terpinen-4-ol																0.86	0.68
α-terpineol																	0.68

Chemotype 2

	α-pinene	sabinene	b-pinene	myrcene	α-phellandrene	α-terpinene	limonene	β-phellandrene	p-cymene	1,8-cineole	g-terpinene	terpinolene	linalool	(Z)-sabinene hydrate	terpinen-4-ol	α-terpineol	(Z)-piperitol
α-thujene	0.86	0.32	0.21	0.84	0.96	0.83	0.76	0.96	-0.27	0.74	0.80	0.88	0.84	-0.16	0.72	0.80	-0.17
α-pinene		0.24	0.51	0.82	0.93	0.96	0.90	0.92	0.16	0.84	0.99	0.98	0.93	0.04	0.95	0.85	0.27
sabinene			0.12	0.39	0.30	0.21	0.23	0.32	-0.21	0.23	0.18	0.22	0.24	-0.04	0.22	0.31	-0.05
β-pinene				0.40	0.37	0.50	0.47	0.33	0.41	0.41	0.57	0.49	0.48	0.39	0.60	0.32	0.56
myrcene					0.78	0.68	0.93	0.90	-0.19	0.95	0.76	0.74	0.81	-0.13	0.78	0.94	0.13
α-phellandrene						0.95	0.77	0.96	-0.08	0.71	0.91	0.97	0.90	0.02	0.83	0.78	-0.05
α-terpinene							0.76	0.87	0.19	0.69	0.97	0.99	0.90	0.12	0.91	0.73	0.15
limonene								0.86	0.09	0.99	0.87	0.81	0.85	-0.09	0.89	0.95	0.35
β-phellandrene									-0.16	0.84	0.87	0.91	0.89	-0.05	0.83	0.89	-0.03
p-cymene										0.02	0.25	0.14	0.07	0.20	0.26	-0.03	0.71
1,8-cineole											0.80	0.74	0.79	-0.13	0.84	0.95	0.30
γ-terpinene												0.98	0.93	0.09	0.97	0.80	0.33
terpinolene													0.93	0.05	0.92	0.77	0.16
linalool														0.08	0.91	0.84	0.24
(Z)-sabinene hydrate															0.13	-0.13	0.15
terpinen-4-ol																0.84	0.42
α-terpineol																	0.20

Chemotype 5

	α-pinene	sabinene	b-pinene	myrcene	α-phellandrene	α-terpinene	limonene	β-phellandrene	p-cymene	1,8-cineole	g-terpinene	terpinolene	linalool	(Z)-sabinene hydrate	terpinen-4-ol	α-terpineol	(Z)-piperitol
α-thujene	0.20	0.01	0.22	0.23	0.24	0.01	0.26	0.07	-0.05	0.27	0.37	0.62	0.62	-	0.31	0.34	-
α-pinene		0.23	0.88	0.92	0.78	0.70	0.90	0.74	-0.04	0.89	0.71	0.21	0.21	-	0.83	0.86	-
sabinene			0.38	0.41	0.29	0.30	0.27	0.48	-0.09	0.27	0.27	0.12	0.12	-	0.08	0.31	-
β-pinene				0.99	0.87	0.69	0.96	0.89	-0.18	0.95	0.69	0.19	0.19	-	0.83	0.92	-
myrcene					0.86	0.71	0.95	0.89	-0.14	0.94	0.72	0.23	0.23	-	0.82	0.91	-
α-phellandrene						0.61	0.89	0.76	-0.02	0.88	0.77	0.18	0.18	-	0.83	0.88	-
α-terpinene							0.61	0.72	-0.09	0.61	0.60	0.25	0.25	-	0.52	0.54	-
limonene								0.76	0.02	1.00	0.78	0.14	0.14	-	0.92	0.97	-
β-phellandrene									-0.26	0.75	0.52	0.15	0.15	-	0.59	0.69	-
p-cymene										0.02	0.24	-0.04	-0.04	-	0.05	0.01	-
1,8-cineole											0.77	0.14	0.14	-	0.92	0.97	-
γ-terpinene												0.33	0.33	-	0.79	0.78	-
terpinolene													1.00	-	0.10	0.19	-
linalool														-	0.10	0.19	-
(Z)-sabinene hydrate															-	-	-
terpinen-4-ol																0.92	-
α-terpineol																	-

Values above 0.8 and 0.9 are emphasized using incremental shading. The compounds with the highest levels of correlation in these groups are expected to be the products of a common terpene synthase

correlated with those of linalool and α-phellandrene, and together they comprise Group C terpenes. However, when just focusing on chemotype 2 individuals, it can be seen that α-terpinene, γ-terpinene, α-phellandrene, and β-phellandrene also show strong correlation with terpinolene. These compounds, therefore, may be synthesized by more than one of the enzymes that define the terpene composition of Tea Tree oil.

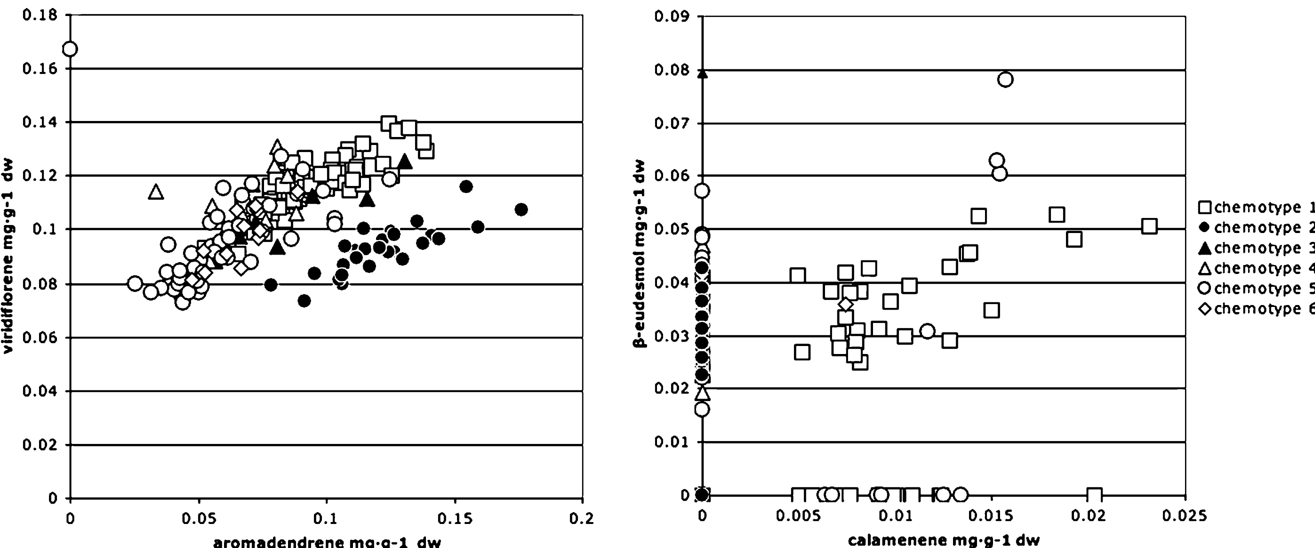


Fig. 3 Correlations of concentrations of the sesquiterpenes viridiflorene and aromadendrende, as well as of β-eudesmol and calamenene with respect to chemotypes

Three studies that examined the expression of terpinolene synthases show different products (Wise et al. 1998; Bohlmann et al. 1999; Huber et al. 2005). This suggests that terpinolene can be produced from different carbocation intermediates, and that it is not unusual that the monoterpene components positively correlated to terpinolene in *M. alternifolia* do not match the profile of any currently known terpinolene synthase.

Three groups of monoterpenes describe the majority of variation in Australian Tea Tree. Pairwise correlation of concentrations of individual monoterpenes as a proportion of total monoterpenes shows that almost all monoterpenes show strong correlation to one of the major groups. The lowest correlation to any single group is shown by *p*-cymene. However, it has been shown that this compound may be an oxidation product that can be derived from multiple monoterpenes (Brophy et al. 1989), and its concentration may depend more on extrinsic factors that affect *in vivo* oxidation, rather than on individual enzymatic processes.

Sesquiterpene Chemotypes This is the first study to examine sesquiterpene patterns in *M. alternifolia*, and it is clear from the analyses that there are strong correlations among concentrations of individual compounds. Viridiflorene and aromadendrene showed two distinctly different ratios, and the higher viridiflorene ratio was exclusive to chemotype 2. As both share the same viridiflorane structure, it leads us to expect two aromadendrene synthase variants, both responsible for synthesizing the same suite of compounds in slightly different, but nevertheless characteristic ratios. The only other pattern linked to a monoterpene chemotype was the concentration of calamenene, which was consistently absent from all chemotype 2 individuals. As calamenene has two less hydrogens ($C_{15}H_{22}$) than would result from the cyclization and dephosphorylation of FPP ($C_{15}H_{24}(P_2O_7H_3)$), it is likely that its synthesis is linked to the presence of a reductase from the P450 superfamily of enzymes, as in mint monoterpene biosynthesis (Bertea et al. 2001). Compared to monoterpenes, there were many more compounds that were not correlated to other constituents, and our sampling probably missed some combinations. It is interesting to note, however, that the only variation linked to monoterpene variation was found in chemotype 2. None of the other sesquiterpenes showing discontinuous distributions was correlated to other monoterpene chemotypes. This suggests that formation of sesquiterpenes is independent of the monoterpene chemotype in Tea Tree, and that the correlation in chemotype 2 may be at a molecular level.

Biochemical Origin of the Chemotypes We propose that three major enzymes are responsible for the three major chemotypes. These are (i) sabinene hydrate synthase that produces the Group A compounds, (ii) 1,8-cineole synthase that yields Group B compounds, and (iii) a terpinolene synthase responsible for group C products. The barycentric plot showing the individual contributions suggests that in chemotypes 1 and 2, a separate 1,8-cineole synthase also may be contributing to the oil profile. Chemotype 3 demonstrates that all three enzymes may be present at once; therefore, in a diploid organism such as *M. alternifolia*, at least two loci need to be involved in coding for the foliar monoterpene synthases that determine chemotype.

The effect of the proposed individual terpene synthases appears to be codominant. This is supported by the intermediate position of chemotypes 3, and 4, and 6

compared to 1, 2, and 5 as shown in Fig. 2. While chemotypes 3 and 4 fall exactly halfway between the contributing cardinal chemotypes, indicating equal contribution from all sides, chemotypes 6 and 7 are not as straightforward. In chemotype 6, the contribution of the group B and group C compounds is 3:1 and 2:1. This may indicate that: (i) our hypothetical 1,8-cineole synthase utilizes GDP as a substrate more efficiently than does terpinolene synthase, or that (ii) the two genes have a cumulative effect, where product proportions are determined by the number of alleles present of each synthase. In the case of multiple copy gene families such as terpene synthases, duplication often is the mechanism behind the increase in the number of genes contributing to the same process. Among duplicated genes, the process of gene conversion can occur whereby similar sequences recombine with each other regardless of actual chromosomal topology, whereby in effect a sharing of alleles will occur across loci. In such a scenario, the 3:1 contribution may be explained by two loci sharing alleles.

The distribution of chemotypes across the natural range further supports the idea that chemotypes 3, 4, 6, and 7 are likely intermediate chemotypes, particularly because, unlike chemotypes 1, 2, and 5, neither our nor previous studies (Homer et al. 2000) have found them to occur in monochemotypic populations. Furthermore, if they are intermediates, then they should be found in populations that contain the contributing cardinal chemotypes. In one population, Dilkoon Creek, which occurs between the two chemically distinct regions of NSW, 35% of trees recorded were chemotype 4. Chemotypes 1 and 5 also are present in this population. These are the two chemotypes that were used in a controlled cross experiment that reinforced the intermediate nature of chemotype 4 (Shelton et al. 2002). Finally, chemotypes 3, 6, and 7 that contain group C compounds occur mostly in Queensland populations, where chemotype 2 is dominant.

Butcher et al. (1994) have raised the possibility of chemotypes 2 and 5 being the results of introgression from sister species *M. linariifolia* and *M. trichostachya*. In the case of *M. linariifolia*, chemical analyses have been devised that are able to separate the two species based on the ratio of *cis*- and *trans*-sabinene hydrate (Southwell and Stiff 1990). This shows that even though the two species apparently share similar chemotypes, the final chemistries are obtained via enzymatically different routes. This separation does, however, depend only on differences between Group A compounds, and without at least a thorough analysis of the corresponding group B dominated chemotypes, it is hard to reach any conclusive answer. On the other hand, chemotype 2 of *M. alternifolia* is very similar to a corresponding chemotype of *M. trichostachya* (Brophy and Doran 1996). Furthermore, not only does chemotype 2 differ from all others in its monoterpene composition, but it also displays further differences in two independent sesquiterpene traits. In other plants, genes for terpene biosynthesis often co-locate on the chromosomes, and if this is also true in *Melaleuca*, the consistent co-variation of these traits indicate that chemotype 2 is not just the result of variation of a single gene, but of multiple linked loci.

In summary, the examination of purely chemical data from *M. alternifolia* from a biochemical point of view has enabled us to make much deeper conclusions about the origins of the different chemotypes. By using biochemical knowledge with the power of statistically analyzing a large number of samples, it has been possible to develop a set of hypotheses about the nature of terpene synthases that are contributing to natural variation. Ultimately, this provides a framework for uncovering the individual molecular processes and identifying the actual genes that contribute to the maintenance of such remarkable chemical variation of an industrially, medicinally, and ecologically important species.

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High Molecular Size Humic Substances Enhance Phenylpropanoid Metabolism in Maize (*Zea mays* L.)

Michela Schiavon · Diego Pizzeghello · Adele Muscolo · Silvia Vaccaro · Ornella Francioso · Serenella Nardi

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Abstract A high molecular weight humic fraction (>3,500 Da) was characterized chemically by DRIFT and ^{1}H NMR spectroscopy, and was applied to *Zea mays* L. plants to evaluate its effect on phenylpropanoid metabolism. The activity and gene expression of phenylalanine (tyrosine) ammonia-lyase (PAL/TAL), and the concentrations of phenolics and their amino acid precursors phenylalanine and tyrosine were assayed. Maximum induction of PAL/TAL activity and expression was obtained when the concentration of added humic substance was 1 mg C/l hydroponic solution. Phenylalanine and tyrosine significantly decreased (−16% and −22%, respectively), and phenolic compounds increased in treated plants. The effects of the humic substance could be ascribed partly to indoleacetic acid (27 nmol/mg C) in the humic fraction. Our results suggest that this humic fraction induces changes in phenylpropanoid metabolism. This is the first study that shows a relationship between humic substances and the phenylpropanoid pathway.

M. Schiavon · D. Pizzeghello (✉) · S. Vaccaro · S. Nardi
Department of Agricultural Biotechnologies,
University of Padova,
Agripolis, Viale dell'Università 16,
35020 Legnaro, Padova, Italy
e-mail: diego.pizzeghello@unipd.it

A. Muscolo
Department of Agriculture and Forest Systems Management,
Mediterranean University of Reggio Calabria,
89100 Reggio di Calabria, Italy

O. Francioso
Department of Agroenvironmental Science and Technology,
University of Bologna,
Viale Fanin 44,
40127 Bologna, Italy

Key Words High molecular weight humic substances · *Zea mays* L. · DRIFT (Diffuse Reflectance Infrared Fourier Transform spectroscopy) · ^{1}H NMR (Proton Nuclear Magnetic Resonance spectroscopy) · Phenylalanine (tyrosine) ammonia-lyase · Phenolics

Introduction

Humic substances (HS) are ubiquitous constituents of environmental organic matter that are responsible for many complex chemical reactions in soil (Stevenson 1994). The general term "humic substances" includes fractions that are identified on the basis of their molecular size and solubility in alkali and acids, and that build up in soil during the microbial degradation of organic matter (Stevenson 1994). These degradation products may be modified further through reactions such as polymerization, condensation, and oxidation to produce complex substances that differ in molecular weight from a few hundred up to several million Daltons (Stevenson 1994).

It is believed that humic substances in solution are a set of relatively small molecules, which are loosely bound by intermolecular hydrophobic interactions (Piccolo 2002). HS have a high apparent molecular weight, but can be reversibly disrupted by treatment with low concentrations of mono-, di-, and tricarboxylic acids. The amphiphilic properties of organic acids in root exudates can dissociate HS into low and high molecular weight fractions (Piccolo 2002; Nardi et al. 2005; Canellas et al. 2008). Bioactive molecules such as indoleacetic acid may be released from the HS upon dissociation of the parent material (Nardi et al. 2009).

Humified organic matter stimulates plant growth and improves the yields of agricultural crops (Canellas et al. 2002; Eyheraguibel et al. 2008). High molecular weight HS

also can act as root growth promoters via auxin-mediated pathways (Trevisan et al. 2009). HS may have practical applications in agriculture as a source of new organic-mineral fertilizers and as inhibitors of plant stress (Cavani et al. 2006; Kaufmann et al. 2007; Schiavon et al. 2008).

HS induce carbon and nitrogen metabolism by increasing the activity of the enzymes of glycolysis, the Krebs cycle, and N assimilation (Quaggiotti et al. 2004; Muscolo et al. 2007). However, there is no information on the interactions between HS and secondary metabolism. Among secondary metabolites, phenolic compounds (e.g., flavonoids and phenylpropanoids) provide protection to plants against a range of biotic and abiotic stresses (Dixon and Paiva 1995). Phenylalanine (tyrosine) ammonia-lyase (PAL/TAL; EC 4.3.1.5) catalyzes the first committed step in the biosynthesis of phenolics by converting phenylalanine to *trans*-cinnamic acid and tyrosine to *p*-coumaric acid. Phenylalanine usually is the preferred substrate, but the monocot enzyme can use both phenylalanine and tyrosine (Rösler et al. 1997; Andersen et al. 2007).

Since HS stimulate C and N metabolism (Quaggiotti et al. 2004; Nardi et al. 2007), and these in turn cross-talk with phenylpropanoid metabolism, we hypothesized that HS might also directly or indirectly activate PAL and consequently alter phenolic levels. To test this hypothesis, high molecular weight HS derived from earthworm feces were supplied to maize plants growing in hydroponic solution, and the effects on phenylpropanoid metabolism were analyzed in terms of PAL/TAL activity, gene expression, content of amino acids and phenolic compounds.

Methods and Materials

Earthworm Culture Conditions The feces of *Nicodrilus* (=*Allolobophora* (Eisen) = *Aporrectodea* (Oerley)) *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) were collected in September for 3 yr from the surface of uncultivated couchgrass (*Agropyron repens* L.) fields at the College of Agriculture farm (University of Padova). The soil was classified as Calcaric Cambisol (FAO-ISSDS 1999). After collection, the feces were immediately air-dried.

Extraction of HS Feces (20 g) were extracted with 0.1 N KOH (200 ml). The extract was dialyzed against distilled water with a 14 kDa molecular weight cut-off Visking membrane (Medicell, UK) (Muscolo et al. 2007), desalted with ion exchange Amberlite IR-120 (H^+ form) (Stevenson 1994), adjusted to pH 2.1 with glacial acetic acid, and dialyzed against distilled water with a 3,500 Da molecular weight cut-off Spectra/Por® 3 tubing (Spectrum, Gardena, CA, USA). The dialyzed solution was vacuum distilled to remove acetic acid, reduced in volume to about 50 ml, and freeze-dried. From 20 g of feces, 0.76 g of humic carbon was obtained. C, H, N, and S were determined with an elemental analyzer (Thermo Electron model EA 1110 Waltham, MA, USA). Oxygen was calculated by difference, and the ash content was determined by incineration at 700°C (Stevenson 1994). The concentration of COOH groups was determined by potentiometric titration with a VIT 90 AutoTitrator (Radiometer, Copenhagen, DK) using first derivative method to analyze the data. All determinations were performed in triplicate.

^1H NMR Spectra were recorded with a Bruker ACF 250 spectrometer (Bruker, Karslruhe, Germany) with 40 mg/ml samples of HS in D_2O and a 5-mm multinuclear probe. Spectra were accumulated with 16 K data point, one pulse sequence, 40° pulse angle, 3 s relaxation delay, and a sweep width of 2.5 kHz. To obtain a satisfactory signal to noise ratio 1000–2000 scans were needed. Gated irradiation was applied between acquisitions to pre-saturate the residual water peak. Chemical shifts were measured from sodium 3-trimethylsilyl-propionate-2,2,3,3-d_4 (TSP).

DRIFT Spectra were recorded with a Bruker TENSOR series FT-IR Spectrophotometer (Ettlingen, Germany) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT, USA). The spectra were collected from 4000 to 400 cm^{-1} and averaged over 100 scans at a resolution 4 cm^{-1} and converted into Kubelka-Munk units. KBr powder (Aldrich Chemical Co. Milwaukee, WI, USA) was used for the background reference. Analyses of spectral data were performed with Grams/386 spectral software (Galactic Industries, Salem, NH, USA). Spectral sections from 1850 to 600 cm^{-1} were baseline-corrected to an absorbance value of 0.00 at 1850 cm^{-1}. The 2nd derivatives of the IR spectra were used for wave-number determination of overlapped bands.

Indoleacetic Acid (IAA) Determination IAA was determined by using an enzyme linked immuno-sorbent assay (ELISA) standardized with methylated IAA (Phytodetek-IAA, Sigma, St. Louis, MO, USA). The ELISA test utilized a monoclonal antibody to IAA and was sensitive in the range of 0.05–100 picomoles. The tracer and standard solutions were prepared following the manufacturer's instructions, and the absorbances were read at 405 nm with a Biorad microplate reader (Hercules, CA, USA). Samples containing HS at 150–750 µg C/ml were analyzed in duplicate.

Plants Maize seeds (*Zea mays* L. var. DK 585) were soaked for one night in distilled running water and germinated for 60 h in the dark at 25°C on filter paper wet with 1 mM $CaSO_4$. Seedlings were grown for 14 d in hydroponic conditions using a Hoagland no. 2 modified solution (Hoagland and Arnon 1950) with the following composition (µM): KH_2PO_4 (40), $Ca(NO_3)_2$ (200), KNO_3 (200), $MgSO_4$ (200),

FeNaEDTA (10), H_3BO_3 (4.6), $CuCl_2$ (0.036), $MnCl_2$ (0.9), $ZnCl_2$ (0.09), NaMoO (0.01). Plantlets were grown in a climate chamber with a 14:10 h, L:D cycle, air temperature of 27/21°C, relative humidity of 70/85%, and at a photon flux density (PFD) of 280 mol/m^2/s. When plants were 12 d old, they were treated for 48 h with concentrations of HS equivalent to 0, 0.5, 1, or 2 mg C/l in the hydroponic solution. Leaves of 20 control or treated seedlings were harvested, frozen in liquid nitrogen, and stored at −80°C for gene expression analysis. For enzyme activity, amino acid, phenolic, and flavonoid measurements, fresh tissue from 20 plants per treatment was used. For fresh weight measurements, 10 plants per treatment were collected and weighed. The experiment was replicated five times.

Glutamine Synthetase Assay Glutamine synthetase (EC 6.3.1.2) was extracted by crushing 1 g leaf tissue in 3 ml of ice-cold buffer containing 100 mM Hepes-NaOH pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.10 g polyvinylpyrrolidone. After filtering through two layers of muslin, and centrifuging at 20,000x*g* for 15 min, 150 μl of extract were added to 600 μl pH 7 buffer containing 90 mM imidazole-HCl, 60 mM hydroxylamine, 20 mM Na_2HAsO_4, 3 mM $MnCl_2$, 0.4 mM ADP, and 120 mM glutamine. The mixture was incubated 15 min at 37°C and then stopped by adding 250 μl of a mixture of equal parts 10% (w/v) $FeCl_3$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid (TCA), and 50% (w/v) HCl. The γ-glutamyl hydroxamate was determined at 540 nm (Cánovas et al. 1991), and activity was expressed as μmol product/g fresh weight/min.

PEP-carboxylase Assay PEPC (EC 4.1.1.31) was extracted by homogenizing 1 g leaf tissue in 10 ml ice-cold buffer (pH 8.2) containing 0.1 M Tris-HCl, 1 M Na_2EDTA, 10% glycerol, 5 mM β-mercaptoethanol, and 0.10 g polyvinylpyrrolidone. After the extract was filtered through 2 layers of muslin and centrifuged at 4°C for 20 min at 17,000x*g*, the PEPC reaction was started by adding PEP to bring the final concentration to 2 mM in the crude extract. The oxidation of NADH was monitored at 340 nm at 30°C (Schweizer and Erismann 1985). The activity was expressed as μmol NAD^+/g fresh weight/min.

Phenylalanine (Tyrosine) Ammonia-lyase Assay PAL/TAL (EC 4.3.1.5) was extracted by homogenizing 1 g leaf tissue in 5 ml ice cold 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol and 0.10 g polyvinylpyrrolidone. After centrifuging at 4°C for 15 min at 15,000x*g*, the supernatant was chromatographed on Sephadex G-25 equilibrated with the same buffer. Total protein was measured using the Bradford method (Bradford 1976). A mixture of 0.4 ml of 100 mM Tris–HCl buffer (pH 8.8), 0.2 ml of 40 mM phenylalanine, and 0.2 ml of enzyme extract was incubated for 30 min at 37°C and stopped with 0.2 ml 25% TCA (Mori et al. 2001). Phenylalanine was added to the control after incubation and the addition of the acid. After centrifuging 15 min at 4°C at 10,000x*g*, the absorbance of the supernatant was measured at 280 nm relative to the control. TAL activity was assayed at 40°C by monitoring *p*-coumaric acid formation at 310 nm in the same buffers containing 1.9 mM L-tyrosine (Rösler et al. 1997). PAL and TAL activities were expressed as nmol product/mg protein/min.

Semi-quantitative RT-PCR Total RNA was extracted from frozen leaves as described in Quaggiotti et al. (2004) using the Nucleon Phytopure kit (Amersham-Pharmacia, UK). The RNA was analyzed spectrophotometrically (A_{280}/A_{260}), and electrophoresis (1% agarose gel) was used to verify intact nucleic acid. RNA was treated with 10 U of Dnase RQ1 (Promega, Milano, Italy) per 50 μg RNA at 37°C for 30 min. Five μg of RNA from each treatment were used in the reverse transcriptase reaction, using 200 U of ImProm-II™ Reverse Transcriptase (Promega, Milano, Italy) and oligodT as primers in 20 μl reaction mixtures. Mixtures were incubated at 37°C for 60 min, 70°C for 5 min, and 4°C for 5 min to stop the RT reaction.

RT-PCR experiments were performed with specific primers (forward: 5′-CGCATCAACACCCTCCTC-3′; reverse: 5′-GATGTAGGAGAGCGGGACCA-3′) to evaluate the expression level of PAL (*ZmPAL1*: GenBank Accession number L77912). For all PCR reactions, 1 μl of the cDNA was used in a 20 μl reaction mixture with 2 μl of a 0.025 U/μl Taq-polymerase (Amersham-Pharmacia-Biotech, Piscataway, NJ, USA). Between 14 and 30 cycles were tested to determine the optimal number of cycles, corresponding to the exponential phase in the amplification for each gene. Each PCR cycle consisted of: 3 min initial denaturation at 95°C, 30 s denaturation at 95°C, 30 s annealing at 61°C, 30 s extension at 72°C, 7 min final extension at 72°C.

The constitutively expressed actin gene (GenBank Accession number J0128) was used as an internal control to normalize the obtained gene expression results. The primers were: forward: 5′-TGTTTCGCCTGAAGATCAC CCTGTG-3′; reverse: 5′-TGAACCTTTCTGACCCAAT GGTGATGA-3′.

PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and analyzed with the ImageJ program (ImageJ 1.23 J, Wayne Rasband, National Institute of Health). To confirm the expression analysis results, PCR reactions were carried out on cDNAs obtained from two different RNA extractions from two independent experiments, and were repeated at least 4 times for each cDNA.

PCR products obtained from the gene expression analysis were sequenced to verify specific amplification of

each gene. DNA was eluted from the agarose gel with the QIAquick Gel Extraction-Kit Protocol (QIAGEN, Valencia, CA, USA) kit. Gene sequencing was carried out at the BMC Genomics at Padova University, using the ABIPRISM original Rhodamine Terminator kit (PE Biosystems, Branchburg, NJ, USA) and specific and universal primers. Gene sequences were compared with Blastx and Blastn (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi).

Amino Acid Content Amino acids were extracted according to Maggioni and Renosto (1980). Qualitative and quantitative determinations were carried out with a Beckman Amino Acid Analyzer (Model 118 CL La Brea, CA, USA) using amino acid standards Type H (Pierce-Thermo-Fisher Scientific, Rockford, IL, USA).

Extraction and Measurement of Soluble Phenolics and Flavonoids Leaves were crushed with 3 ml methanol per g leaf. After 30 min on ice, the samples were centrifuged for 30 min at 4°C at 5,000x*g*, and the supernatants were stored at −20°C until use. Phenolics were measured by the Folin method (Arnaldos et al. 2001) standardized with gallic acid. Flavonoids were extracted from 1 g leaves with 50 ml of acidified methanol solution and stored at 4°C for 16 h before recording absorbance at 300 nm. Flavonoids were expressed as gallic acid equivalents (Meenakshi et al. 2009).

Statistical Analysis All data were the means of five replicates, and the standard deviations did not exceed 5%. Results were processed statistically with the Student-Newman-Keuls test ($P \leq 0.05$) (Sokal and Rohlf 1969).

Results

HS Characterization The elemental analysis of HS indicated that the sample had a low H/C ratio (Table 1), which is typical of highly condensed aromatic rings (Visser 1983). The HS contained 27±0.72 nmol of IAA per mg C.

The ^{1}H NMR spectrum was divided into three main regions: aromatic hydrogens from 6.0 to 8.0 ppm; hydrogens attached to oxygen groups in carbon a (defined as sugar-like) from 4.2 to 3.0 ppm, and aliphatic hydrogens from 3.0 to 0.5 ppm (Fig. 1). HS had a poorly resolved aromatic proton region and an intense and broad region attributed to sugar-like and polyether components. Other broad and intense resonances at 0.83 ppm and 1.3 ppm were due to the protons of terminal methyl groups of methylene chains, respectively (Fan 1996). Two further intense resonances appeared at 2.9 ppm and 3.40 ppm and were assigned to protons in α CH_3 of acetoacetate and ether aliphatics, respectively (Fan 1996). These groups presumably were bound chemically to the HS macrostructure (Muscolo et al. 2007).

The DRIFT spectrum was characterized by broad and overlapped bands (Fig. 2), which were sharpened and resolved by recording in the second derivative mode (Fig. 2). The amide I (1660 cm^{-1}) and amide II (1540 cm^{-1}) bands were resolved clearly. The intense band at 1726 cm^{-1} was assigned to the C = O stretching mode of COOH groups (Niemeyer et al. 1992). The bands at 1459 and 1418 cm^{-1} were due to CH_2 bending and COO^- symmetric stretching modes, respectively. Strong negative peaks were near 1164, 1078, 1033, 983, and 893 cm^{-1} in the secondary derivative mode. They were attributed mainly to C-O stretching of carbohydrates (Stevenson 1994), as well as to C-C stretching motions of aliphatic groups and in-plane C-H bending of aromatic rings. The band characteristic of aromatic rings was found at 1514 cm^{-1}, while that at 1230 cm^{-1} was due to C-O stretching vibrations in phenols and carboxyl groups.

Plant Growth, Enzyme Activities and Amino Acid Content HS significantly increased plant fresh weight up to the concentration of 1 mg C/l (+115%, $P \leq 0.05$). However, plants treated with HS at 2 mg C/l had a fresh weight (+109%, $P \leq 0.05$) similar to that of plants treated with HS at 0.5 mg C/l (+110%, $P \leq 0.05$).

Application of HS to maize seedlings significantly increased the activity of GS and PEPC (Table 2). Doses up to 1 mg C/l HS increased the content of some amino acids (glutamate, aspartate, serine, proline, glycine, methionine) in the leaves (Table 3). When HS was applied at 2 mg C/l, the content of amino acids decreased, except for aspartic acid, threonine, and alanine (Table 3). Phenylalanine and tyrosine, the amino acid precursors of the phenylpropanoids, decreased in plants treated with HS at 1 mg C/l, while they were unchanged (phenylalanine) or higher (tyrosine) in presence of HS at 0.5 and 2 mg C/l (Table 3). Alanine, valine, and leucine levels changed in a pattern similar to that noted for tyrosine.

Table 1 Composition characteristics of (C, H, N, O, S) the high molecular weight humic fraction (HS). Sulfur was not detected

	C	H	N	O	C/N	Ash	Total acidity meq/g	IAA[a] nmol
HS	52.3±0.1	5.1±0.4	2.2±0.3	40.4±0.2	23.7	0.53±0.05	4.6±0.5	27±0.72

[a] Indoleacetic acid (IAA) in 1 mg C high molecular weight humic fraction (HS)

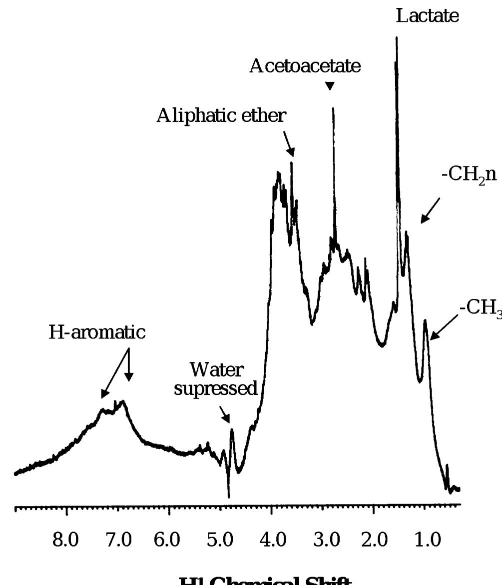

Fig. 1 ^{1}H NMR spectrum of humic fraction extracted from earthworm feces in D_2O. The spectrum shows an aromatic H region (6.0–8.0 ppm) poorly resolved, a broad region (4.2–3.0 ppm) attributed to sugar-like components, polyether materials or methoxy groups, and an intense aliphatic region (3.0–0.5 ppm)

PAL/TAL Gene Expression and Activity, Content of Phenolic Compounds

There was increased accumulation of *ZmPAL1* transcripts in HS-treated plants compared to controls (Fig. 3). In particular, the maximum level of mRNA was observed when plants were supplied with HS at 1 mg C/l (+337%). The activity of PAL/TAL also was significantly higher in maize seedlings treated with HS than the control, and the maximum increase in PAL (+40%) and TAL (+538%) activities was observed when plants were treated with HS at 1 mg C/l (Table 4). Although TAL activity was stimulated by HS more than PAL, the specific activity of PAL was higher under all conditions.

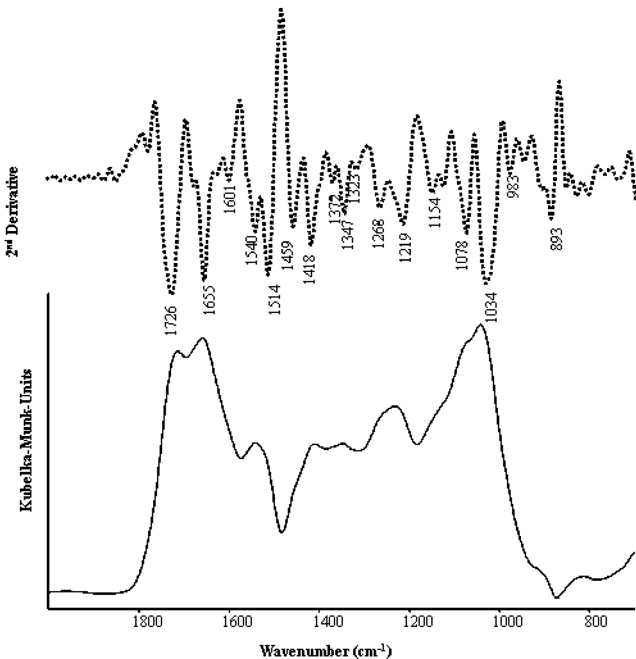

Fig. 2 DRIFT spectrum (*lower line*) of humic substances extracted from earthworm feces. 2nd derivative spectrum (*upper line*) improved the resolution of overlapped and hidden bands in the investigated spectral region

Consistent with the enhancement of PAL expression and activity, the content of total phenolic acids and flavonoids increased following HS treatment, and the maximum values (+45% and +24%, respectively) were measured with HS at 1 mg C/l (Table 4).

Discussion

Despite the considerable amount of physiological and biochemical data on effects of soil HS and earthworm-derived HS on plant growth (Nardi et al. 2009), the effects of HS on secondary metabolism have not been investigated previously.

The earthworm-derived HS used here had a low H/C ratio, which Van Krevelen's diagram suggests is typical of highly condensed aromatic rings (Visser 1983). The presence of hydrophobic domains, such as hydrocarbon chains and aromatic rings, suggested that this fraction was similar to other high molecular weight HS (Muscolo et al. 2007), although it contained less IAA than some preparations of high molecular weight HS (Trevisan et al. 2009). There were physiologically relevant concentrations of IAA in the HS doses applied to maize seedlings in these experiments. How IAA is associated with HS is not well understood. Nevertheless, the high concentration of organic acids released by roots into the rhizosphere may modulate the IAA bioavailability from HS (Nardi et al. 2005). Moreover, HS extracted with acetic acid has a more flexible conformation than bulk humic matter (Nardi et al. 2005;

Table 2 Glutamine synthetase (GS) (μmol/g f.w./ min) and PEP-Carboxylase (PEPC) (μmol NAD^+/g f.w./min) activities in *Zea mays* leaves. Plants were treated for 48 h with high molecular weight humic substances (HS) at the indicated concentrations

HS[a]	GS	PEPC
0.0	4.52±0.12 d[b]	4.00±0.20 d
0.5	5.29±0.07 c	4.72±0.14 c
1.0	5.88±0.11 b	5.60±0.10 b
2.0	6.33±0.08 a	6.00±0.08 a

[a] The unit for HS concentration is mg C/l

[b] Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the Student–Newman–Keuls test

Table 3 Free amino acid contents (mg/g f.wt.) (% of the control, with control=100%) in *Zea mays* leaves. Plants were treated for 48 h with high molecular weight humic substances (HS) at the indicated concentrations[a]

Amino acid	HS							
	0[a]		0.5		1		2	
	mg/g f wt	%	mg/g f wt	%	mg/g f wt	%	mg/g f wt	%
Aspartic acid	9.2±0.2 c[b]	100	17.3±0.3 a	188	18.0±0.3 a	196	15.4±0.4 b	168
Threonine	8.1±0.1 b	100	9.7±0.2 a	120	8.9±0.2 b	110	10.1±0.2 a	125
Serine	12.2±0.3 b	100	12.8±0.4 b	105	14.0±0.4 a	115	10.2±0.3 c	83
Glutamic acid	42.7±0.5 b	100	51.9±1.1 a	122	49.3±2.1 a	115	39.1±3.1 b	92
Proline	6.5±0.2 c	100	9.1±0.2 a	140	7.8±0.4 b	120	5.9±0.3 d	91
Glycine	8.6±0.3 b	100	9.1±0.3ab	106	9.3±0.3 a	108	7.1±0.2 c	82
Alanine	70.5±1.2 b	100	73.5±0.9 b	104	52.9±2.3 c	75	87.5±4.6 a	124
Valine	8.5±0.2 b	100	8.6±0.3ab	101	8.7±0.4ab	103	9.6±0.4 a	113
Methionine	7.0±0.2 b	100	9.1±0.3 a	129	7.8±0.3 b	111	5.8±0.2 c	83
Leucine	11.7±0.4 b	100	13.6±0.4 a	116	11.5±0.5 b	98	12.9±0.4 b	109
Tyrosine	6.6±0.2 b	100	7.9±0.3 a	119	5.2±0.2 c	78	8.0±0.3 a	121
Phenylalanine	15.1±0.3 a	100	15.2±0.5 a	100	12.7±0.3 b	84	15.5±0.2 a	103

[a] The unit for HS concentration is mg C/l

[b] Values in the same row followed by the same letter are not statistically different at $P<0.05$ as per the Student–Newman–Keuls test

Canellas et al. 2008). Thus, the activity of the HS used here likely was related to its chemical composition and molecular conformation in addition to its molecular weight (Canellas et al. 2010).

The high molecular weight HS stimulated phenylpropanoid metabolism, as confirmed by the induction of PAL and TAL enzyme activities and expression, and the increased accumulation of some phenolics.

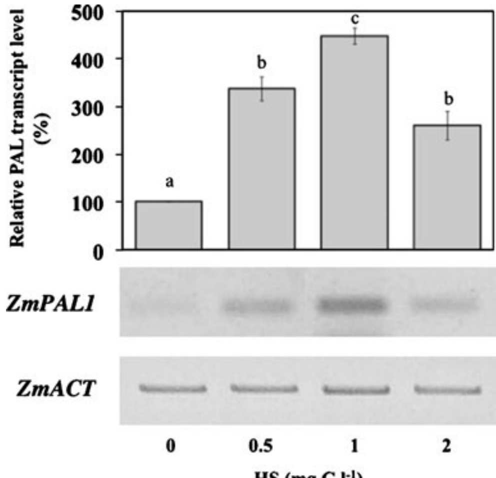

Fig. 3 Gene expression (mRNA level) and relative transcript accumulation of *ZmPAL1* in leaves of *Z. mays* plants grown for 12 d in Hoagland modified complete nutrient solution and treated for 2 d with HS at 0 (control), 0.5, 1, or 2 mg C/l. Transcript amounts were determined using the semiquantitative PCR method in the linear range, and *ZmACT* was used as internal control. The accumulation of the gene transcript was normalized relative to *ZmACT* transcript and expressed relative to the control (100%). Values are shown on bars. Different letters on bars indicate significant differences between treatments ($P<0.05$, ±SD)

HS may have promoted plant growth through the induction of carbon and nitrogen metabolism. PEPC and GS activities were increased, and glutamate and aspartate accumulated in a HS-concentration-dependent manner. The decrease of both phenylalanine and tyrosine in the HS-treated plants (1 mg C/l) may be attributed to consumption of these two amino acids for the synthesis of phenylpropanoids.

Consistent with these findings, PAL/TAL was induced by HS, with maximum activity in plants dosed with HS equivalent to 1 mg C/l. The induction of PAL was noted at the level of gene expression, similar to other studies in which phenylpropanoid synthesis has been enhanced at the transcriptional level by fungal elicitors and hormones (Shinya et al. 2007; Lewis et al. 2008). Flavonoid synthesis,

Table 4 Phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL) activities, total phenolic acid and flavonoid content in *Zea mays* leaves. Plants were treated for 48 h with high molecular weight humic substances (HS) at the indicated concentrations[a]

HS[a]	PAL[c]	TAL[d]	Phenolic acids[e]	Flavonoids[f]
0.0	9.70±0.12 c[b]	0.13±0.06 d	1.03±0.05 d	0.162±0.002 c
0.5	10.67±0.35 b	0.58±0.06 c	1.25±0.13 c	0.186±0.003 b
1.0	13.58±0.41 a	0.83±0.02 a	1.49±0.07 a	0.200±0.005 a
2.0	13.09±0.31a	0.75±0.02 b	1.34±0.05 b	0.188±0.007 b

[a] The unit for HS concentration is mg C/l

[b] Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the Student–Newman–Keuls test

[c] PAL = nmol cinnamic acid /mg protein/min

[d] TAL = nmol *p*-coumaric acid /mg protein/min

[e] Total phenolic acids = mg gallic acid /g f.wt

[f] Flavonoids = mg gallic acid /g f.wt

in particular, is induced by auxins through transcriptional activation (Lewis et al. 2008). In addition, a relationship between flavonoid accumulation and auxin transport has been reported (Buer and Muday 2004; Peer and Murphy 2007). Given that HS contains IAA and exhibits auxin-like activity (Zandonadi et al. 2007; Canellas et al. 2010), the stimulation of phenylpropanoid metabolism likely occured through auxin-mediated signal transduction. High molecular weight HS does not to enter the symplast, but it remains in the apoplast bound to the cell wall where it act as an exogenous signal molecule. As a result, HS is able to induce auxin-like responses including stomata opening (Russell et al. 2006) and lateral root formation (Trevisan et al. 2009). Since HS is poorly characterized, it is possible that its effects on phenylpropanoid metabolism could be ascribed to co-purified fungal elicitors and/or other signalling molecules (hormones, organic acids, peptides, and fatty acids) (Le Floch et al. 2003; Kim et al. 2007). Chemical moieties characteristic of HS, including alcohol, phenol, and carboxyl groups, also could stimulate changes in plant metabolism (Nardi et al. 2009; Zancani et al. 2009).

In summary, the present work shows that application of high molecular weight HS to hydroponically grown maize seedlings changed secondary metabolism associated with the synthesis of phenolic compounds. The stimulatory effects of HS on plant secondary metabolism provide an innovative approach to exploring plant responses to stress.

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Specialist Leaf Beetle Larvae Use Volatiles from Willow Leaves Infested by Conspecifics for Reaggregation in a Tree

Kinuyo Yoneya · Rika Ozawa · Junji Takabayashi

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Abstract Young, gregariously living larvae of the willow leaf beetles *Plagiodera versicolora* are known to exhibit characteristic aggregation-dispersion-reaggregation behavior and local fidelity to a host tree. In this study, we investigated whether plant volatiles induced by feeding *P. versicolora* larvae were involved in the reaggregation behavior. Under laboratory conditions, we conducted dual-choice bioassays and found that the first and second instars discriminated between volatiles from leaves infested by larvae and volatiles from uninfested leaves. The discriminative behavior was dependent on both the time leaves were infested and the age of discriminating larvae. First and second instars preferred odor from 1-d-infested leaves to odor from uninfested leaves, whereas third instars (solitary stage) did not discriminate between these volatile blends. Odor from 2-d-infested leaves was preferred to odor from 1-d-infested leaves by first instars, whereas odor from leaves infested for 3 d was not attractive to these very young larvae. Neither was odor of leaves infested for 1 d and then left uninfested for 1 or 2 d attractive to young larvae. The data suggest that the first and second instars use volatiles from a leaf newly infested by conspecific larvae as one of the reaggregation cues. We detected several herbivore-induced compounds in the headspace of the attractive leaves. Among those, a mixture of synthetic (*E*)-β-ocimene, (*Z*)-β-ocimene, *allo*-ocimene, and linalool was found to attract the larvae.

K. Yoneya · R. Ozawa · J. Takabayashi (✉)
Center for Ecological Research, Kyoto University,
Hirano 2-509-3 Otsu,
Shiga 520-2113, Japan
e-mail: junji@ecology.kyoto-u.ac.jp

Present Address:
K. Yoneya
Freie Universität Berlin,
Angewandte Zoologie / Ökologie der Tiere,
Haderslebener Str. 9,
D-12163 Berlin, Germany

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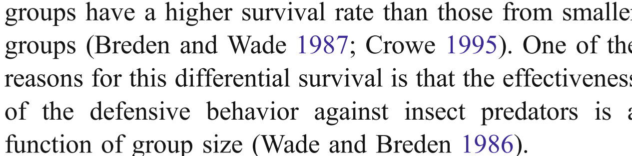
Introduction

Volatile and non-volatile chemicals of food plants play multiple roles in insect—plant interactions (Schoonhoven et al. 2005). Studies on the response of herbivorous arthropods to plant volatiles have focused mainly on adult herbivores finding a host plant for their oviposition. Uninfested leaves emit a blend of volatiles that can be exploited by adult herbivores as information indicating the presence of a host plant (e.g., Bernays and Chapman 1994; Dicke and van Loon 2000; Yoneya et al. 2009a). Foraging adult herbivores also use volatiles from infested plants for host search; some of these odors act as attractants (Bolter et al. 1997; Dicke and van Loon 2000; Kalberer et al. 2001; Horiuchi et al. 2003) and some as repellents (Dicke 1986; Dicke and van Loon 2000; De Moraes et al. 2001; Kessler and Baldwin 2001; Horiuchi et al. 2003; Heil 2004; Yoneya et al. 2009a), thus preventing colonization of food resources that are infested by the same or different herbivore species.

In contrast to arthropods at the adult stage, herbivorous arthropod larvae are rather sedentary, although they may move among host food plants in search of suitable resources. Herbivorous larvae also have been demonstrated to use volatiles from host plants in their foraging behavior (e.g., Huang and Mack 2002; Carroll et al. 2006, 2008; Castrejon et al. 2006; Shiojiri et al. 2006). In dual-choice



tests, saltmarch caterpillar (*Estigmene acrea*) larvae showed a clear preference for volatiles from soybean leaves over those from maize and tomato leaves (Castrejon et al. 2006). By using Y-tube olfactometer bioassays, Carroll et al. (2006) examined the orientation responses of sixth instar fall armyworms (*Spodoptera frugiperda*) to odors from herbivore-damaged and undamaged maize seedlings. The sixth instars preferred odors from damaged maize seedlings to those from undamaged seedlings. Shiojiri et al. (2006) reported that armyworm (*Mythimna separata*) larvae used hostplant volatiles to determine their feeding and hiding behavior. It should be noted that the aforementioned studies focused on generalist herbivores at their larval stages. In contrast, little is known regarding the olfactory responses of the larval stages of specialist herbivores to plant volatiles, particularly those induced by herbivores (but see, Landolt et al. 2000).

Host plant location by leaf beetles (Chrysomelidae) is mediated by cues from both uninfested and infested host plants; furthermore, several leaf beetle species rely on aggregation pheromones (Fernandez and Hilker 2007, and references therein). Most of these studies on chrysomelid host plant location focus on behavior of adults. Knowledge on the chemical cues that are relevant for colonization of host plants by chrysomelid larvae is scarce, even though chrysomelid larvae frequently may move between plants (Bach 1993). For example, the green leaf volatile (*Z*)-3-hexen-1-ol associated with vertical structures has been shown to attract larvae of *Cassida denticollis* (Müller and Hilker 2000). Another example that studied the impact of non-volatile host plant compounds on chrysomelid larval host plant colonization showed that variation in content of phenol glycosides may affect distribution of the willow leaf beetle *Phratora vitellinae,* whereas larval distribution of *Plagiodera versicolora* is not affected (Rowell-Rahier et al. 1987). While larvae of *P. vitellinae* use plant phenolglycosides as precursors for the biosynthesis of their defensive secretion (mainly salicylaldehyde), *P. versicolora* larvae do not use these phenolglycosides for the production of the major compounds of their defensive secretions (mainly cyclopentanoid monoterpenes) (Pasteels et al. 1984).

Plagiodera versicolora is a specialist herbivore that feeds exclusively on Salicaceae species (Hood 1940). The larvae hatch from clutches of 15–25 eggs that are laid on the underside of leaves, and the hatched larvae exhibit gregarious behavior while feeding on leaves (Wade 1994). This gregariousness is typical of the first and second instars (Wade and Breden 1986). Crowe (1995) found that larvae remain aggregated on the natal leaf for 1–5 d. Thereafter, they move singly from the natal leaf to other leaves, and reaggregate on a new leaf within a few hours of dispersal (Wade and Breden 1986; Crowe 1995). Young larvae benefit from reaggregation since individuals from larger groups have a higher survival rate than those from smaller groups (Breden and Wade 1987; Crowe 1995). One of the reasons for this differential survival is that the effectiveness of the defensive behavior against insect predators is a function of group size (Wade and Breden 1986).

Crowe (1995) studied the factors involved in this reaggregation behavior. Although *P. versicolora* larvae respond positively to the presence of conspecific larvae (Crowe 1995), the mechanisms underlying the break-up of the initial aggregation, subsequent dispersion, and reaggregation at a new feeding site on a leaf remain to be elucidated. In the field, we have observed that reaggregated groups consist of both the first and second instars of different egg clutches (Yoneya, unpublished data). Thus, the members of a reaggregated group may not always be from the same egg clutch. We hypothesized that one of the factors promoting settlement, site selection, and subsequent reaggregation on a new willow leaf by dispersing first and second instars is the volatiles emitted from a leaf infested by conspecifics of the dispersing instars. We conducted a combination of dual-choice bioassay and chemical analyses to test whether larvae responded to leaves with different histories of infestation.

Methods and Materials

Insects Adult *P. versicolora* were collected in the field from April to October 2005–2008. Every year, we established a new colony. The colony was maintained in a climate-controlled chamber (25±3°C, 50–70% RH, L18:D6). Larvae from one clutch of eggs (approximately 20) were stored in a plastic cup (diam. 13 cm; height 10 cm), the lid of which was pierced with 50–60 holes (diam. 1–2 mm) to permit air exchange. We offered the beetles fresh field-collected uninfested *Salix eriocarpa* leaves (10 leaves at a time) every 2–3 d until beetles had reached sexual maturity. When beetles were provisioned with fresh leaves, they were placed into a new cup.

Plants We cut a total of 130 shoots (1 or 2 yr-old; length 18 cm) from *S. eriocarpa* trees growing on the floodplain of the Yasu River in Shiga Prefecture, Japan, in early May and mid-August 2004. The shoots were individually potted in humus (using pots that were 15 cm in diam. and 13 cm high) and maintained in a greenhouse (25±3°C, L18:D6) for 2 mo. When the newly emerged plant shoots were approximately 20 cm high, we used them in the bioassay.

For the preparation of infested leaves, we placed two second instar *P. versicolora* on each of five leaves of a potted *S. eriocarpa* plant grown in a greenhouse. We did not place larvae on the first or second leaves from the shoot tip, as eggs in the field generally are found on leaves near

the center of a branch (King et al. 1998). An adjacent uninfested leaf (i.e., a leaf of the same age and the same length, ca. 10 cm) on the same branch was used as the control. Both the infested and uninfested leaves were detached with a razor blade and gently washed with water to remove feces and other associated products. Those leaves were used for experiments within 30 min. after the detachment, and were not used for more than 2 hr.

Dual-choice Bioassay An infested and an uninfested leaf of the same size (length ca. 10 cm) were collected from the same branch by cutting with a razor blade. The leaves were washed gently with water to remove feces and other associated products, and placed immediately on moist paper (8×4 cm) in a Petri dish (diam. 14 cm; height 2.5 cm; Fig. 1). In order to exclude possible visual effects, we covered the infested part with a piece of nylon gauze. We also placed a piece of nylon gauze on an uninfested leaf. A piece of Parafilm was placed so as to bridge the 2 leaves (Fig. 1), with the size of the Parafilm changed according to the larval instar being tested (first instar: 2× 0.4 cm; second instar: 3×0.5 cm; third instar: 5×0.6 cm). A *P. versicolora* larva was placed in the middle of the Parafilm bridge; when it reached one of the two leaves, we recorded this as a choice. When a larva chose neither leaf after 1 hr, we classified it as a no-choice individual. We excluded no-choice individuals from statistical analyses. Each experiment was replicated three to four times on different days. Odor source leaves were used once for each replicate. Individual larvae were used only once. The data were subjected to a binomial test. In order to compare the different data sets, we used Fisher's exact probability test.

The following experiments were conducted:

1. Effects of larvae of different developmental stages. In the field, larvae aggregate during the first and second instars. Thus, we tested the response of first, second, and third instars to a leaf infested by second instars for 1 d and compared this response with the response to an uninfested leaf. In this experiment, a combination of odor sources was offered to larvae of three developmental stages in each replicate.
2. Effects of the duration of infestation. Raupp and Denno (1984) reported that leaves that had been infested by larvae for 3 d no longer provided suitable resources. Thus, we tested whether the first instars responded to volatiles from a leaf that had already been infested by second instars for 2 or 3 d.
3. Effects of the duration of attractive qualities after the termination of the damage. In the field, we did not observe larvae aggregating in a previously infested site that currently lacked larvae (Yoneya, personal observation). We prepared a leaf that had been infested by second instars for 1 d and then left uninfested for 1 or 2 d (previously infested leaf). We tested the response of first instars given a choice between a previously-infested leaf and uninfested leaf.
4. Identification of odor compounds from infested leaves that were attractive to larvae. On the basis of the results of the chemical analyses, we tested the response of first instars to volatile compounds whose amounts were significantly higher in the headspace of attractive infested leaves than in that of uninfested leaves. Hexane was used as the solvent. A hexane solution of test compounds was impregnated into a piece of filter paper (1×3 cm). After evaporation of the solvent, a piece of filter paper with test compounds was placed on one of the two uninfested leaves in a Petri dish. As a control, solvent-impregnated filter paper was placed on the other leaf. The preference of first instars between an uninfested leaf supplemented with the test compounds and an uninfested control leaf was observed. To perform the bioassays, an ocimene and linalool mixture (IFF Chemicals, Florida, U.S.A.) was used. By GC-MS analyses, the mixture was shown to contain (*E*)-*β*-ocimene (74.6% of total ion intensity), (*Z*)-*β*-ocimene (7.2%), *allo*-ocimene (10.8%), and linalool (7.4%). A piece of filter paper (0.5 cm^2) was impregnated with 1 / μl hexane solution of the mixture (100 ng/μl). The amounts of (*E*)-*β*-ocimene emitted from the filter paper (0.1±0.01; peak area of GC/MS normalized with the internal standard: see below) was adjusted to be roughly the same as that emitted from a 1-d-infested leaf during 1 h (0.20±0.02; peak area of GC/MS normalized with the internal standard: see below).

Chemical Analysis of Volatiles from Willow Leaves We compared the headspace volatiles of leaves infested by *P. versicolora* larvae with those of uninfested leaves. We conducted the chemical analyses of volatiles only when the volatiles attracted the larvae. Infested and uninfested leaves were newly prepared for chemical analyses. The preparation of infested and uninfested leaves was the same as those used for the bioassays.

We used 10 uninfested or infested leaves (0.9–1.5 g) detached from 2 infested plants with a razor blade in each sample. The cut-edge of the detached leaf petiole was covered with a piece of moist cotton wool to prevent desiccation. We collected the volatiles in a climate-controlled room (25±2°C). As an internal standard, we used a piece of filter paper impregnated with 1 µl of a hexane solution of tridecane (0.5 µg/µl). We placed the leaves and filter paper in a glass bottle (2 L) fitted with 2 nozzles. One nozzle was connected to an air cylinder and

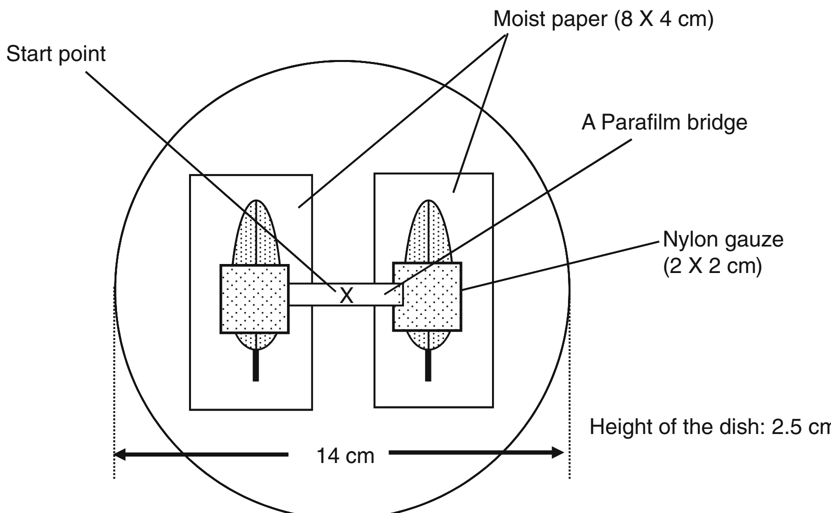


Fig. 1 The bioassay arena used to investigate choices between an infested leaf and an uninfested leaf (each ca. 10 cm long) on moist paper (8 cm×4 cm). We connected the 2 leaves with a Parafilm bridge. The size of the Parafilm bridge varied according to the developmental stage of the tested larvae (first instar: 2×0.4 cm; second instar 3×0.5 cm; third instar: 5×0.6 cm)

the other nozzle to a glass tube packed with Tenax TA adsorbent (GL: Tenax TA 20/35, 100 mg; I.D., 3 mm; length, 160 mm). Air from the cylinder was drawn into the glass bottle, and volatile compounds from the headspace of the bottle were collected on the Tenax TA for 1.5 hr at a flow rate of 100 ml/min.

The collected volatile compounds were analyzed with a gas chromatograph-mass spectrometer (GC-MS) [GC: Hewlett Packard 6890 gas chromatograph (GC) equipped with an HP-5MS capillary column (length, 30 m; I.D., 0.25 mm; film thickness, 0.25 µm); MS: Hewlett Packard 5973 mass selective detector (70 eV) equipped with a thermal desorption cold-trap injector (TCT: CP4010; Chrompack, The Netherlands)]. Headspace volatiles collected on Tenax-TA were released from the adsorbent by heating in the TCT at 220°C for 8 min with He gas flow (1 ml/min). The desorbed compounds were collected in the TCT cold trap unit (SIL5CB-coated fused silica capillary) at−130°C. Flash heating of the cold trap unit induced a sharp injection of the compounds into the capillary column of the GC connected to the unit. The GC oven temperature was programmed to increase from 40°C (5-min hold) to 280°C at 15°C/min. The headspace volatiles were tentatively identified by comparing their mass spectra with those of the Wiley databases (Wiley7N and Wiley275), and the database of National Institute of Advanced Industrial Science and Technology (SDBS compounds and spectral search.) (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi). Further, we compared their retention times with those of authentic compounds (Wako Chemicals, Osaka, Japan, and IFF Chemicals, Florida, U.S.A.). The compounds for which no authentic compounds were available were regarded as tentatively identified with more than 90% of spectra matching with the databases. We normalized the ion intensity of each peak to a "peak area" by dividing the intensity by the ion intensity of the internal standard and the mass (g) of fresh leaves. We compared the peak area of each compound between infested and uninfested leaves on the same plant using the Mann-Whitney *U* test. Chemical analyses were repeated four times for each odor source except for uninfested leaves detached from 2-d-infested plants (N=3).

Results

Both first and second instars preferred a leaf infested by second instars for 1 d to an uninfested leaf (binomial test for larvae of both instars, P<0.01) (Fig. 2). In contrast, third instars showed no preference between the two leaf types (binomial test, P=0.855) (Fig. 2). In the remaining experiments, we used first instars, as the responses of the first and the second instars were not significantly different (Fig. 2; Fisher's exact probability test, P>0.05).

The first instars preferred volatiles from leaves previously infested by second instars for 2 d to uninfested leaf volatiles (binomial test, P=0.015) (Fig. 3a). The first instars showed no preference between an uninfested leaf and a leaf infested by second instars for 3 d (binomial test, P=0.322) (Fig. 3a). The two preference data in Figure 3a were not significantly different (P=0.41, Fisher's exact probability test). First instars showed no preference between an uninfested leaf and a leaf that had been infested by second instar larvae for 1 d and then left uninfested for 1 or 2 d (binomial test, P=0.585, P=0.315, respectively) (Fig. 3b).

We recorded 26 and 29 volatile compounds in the headspace of leaves infested for 1 and 2 d, respectively (Table 1). The tentative identifications of (*E*)-2,6-dimethyl-1,3,5,7-octatetraene, α-copaene, β-gurjinene, (*E*)-β-caryophyllene, β-cubebene, germacrene D, (*syn*)- or (*anti*)- 2-methylbutanal oxime, (*syn*)- or (*anti*)-3-methylbutanal oxime, 2-

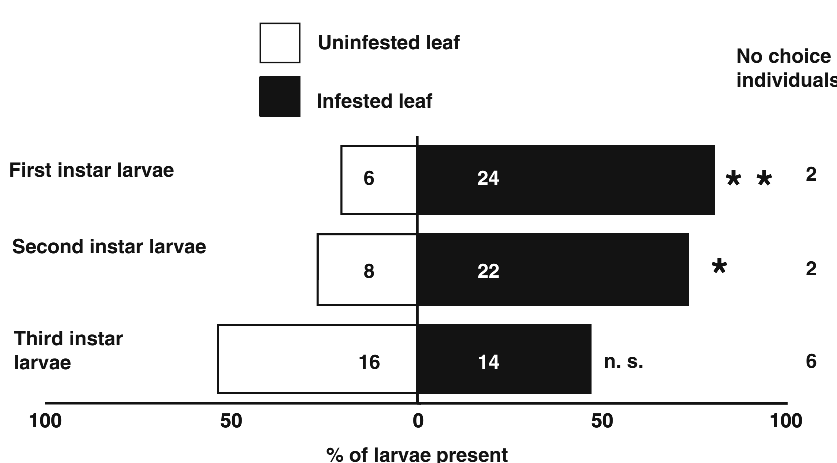


Fig. 2 Olfactory responses of the first, second, and third instar *Plagiodera versicolora* larvae to uninfested vs. 1-d-infested leaves. NS: no significant difference, * :0.01<P<0.05, ** :0.001<P<0.01 (binomial test). Numbers in bars indicate the number of larvae that responded to volatiles

methylbutanenitrile, and 3-methylbutanenitrile were based only on the Wiley databases and on "SDBS compounds and spectral search." The major compounds were (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (*E*)-4-8-dimethyl-1,3,7-nonatriene and (*E,E*)-α-farnesene. The compounds that were present in significantly higher amounts in the headspace of 1- and 2-d-infested leaves than in the respective controls were benzyl alcohol, 2-hydroxybenzaldehyde, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, *allo*-ocimene, (*E*)-2,6-dimethyl-1,3,5,7-octatetraene, α-copaene, germacrene D, (*E,E*)-α-farnesene, and δ-cadinene (Table 1). These were considered as candidate compounds that attracted the larvae.

To test the possible involvement of herbivore-induced volatiles in the attraction of the larvae, we focused on the response of larvae to (*E*)-β-ocimene, as this compound was one of the major induced compounds in the headspace of 1- and 2-d-infested leaves. To perform the bioassays, a mixture of ocimene and linalool, which contained two further candidates (*allo*-ocimene and linalool) in addition to (*E*)-β-ocimene, was used. (*Z*)-β-Ocimene, which was present at a significantly higher level in 1-d-infested leaves than in uninfested leaves, was also in the mixture (see Methods and Materials). Thus, we judged that the ocimene and linalool mixture could be used to test whether the larvae used *P. versicolora*-induced *S. eriocarpa* volatiles for their reaggregation. First instars preferred an uninfested leaf supplemented with the mixture to an uninfested control leaf (binomial test, P=0.022) (Fig. 3c).

Discussion

In our experiments, larvae as well as their associated products (feces, visible secretions, etc.) were removed from leaves. Volatile compounds of larval secretion origin reported in previous studies (e.g., Rowell-Rahier and Pasteels 1986) were not detected in the headspace of larvae-infested willow leaves. Thus, the volatiles that were recorded in the headspace of *S. eriocarpa* plants infested by *P. versicolora* larvae, but not or in trace amounts in that of uninfested plants in this study, were considered to be *P. versicolora* larvae-induced plant volatiles.

The positive responses of first and second instars and the neutral response of third instars (non-aggregation stage) to the 1-d-infested leaf volatiles, compared with their responses to uninfested leaf volatiles, suggest that herbivore-induced leaf volatiles are involved in the reaggregation behavior of the first and the second instars on a tree in the field (Wade and Breden 1986; Crowe 1995). In fact, we did not observe third instars in the field in aggregated *P. versicolora* groups consisting of first and second instars. Crowe (1995) reported that trails made with the secretions of larvae, including feces, and certain leaf-surface chemicals were used by the larvae as cues to follow, suggesting that some contact chemicals are also involved in the reaggregation process.

We found that the first instars preferred volatiles from uninfested leaves supplemented with a synthetic ocimene and linalool mixture that contained three induced volatiles other than (*E*)-β-ocimene. These herbivore-induced volatiles might play a role in evoking the aggregation response of the first instars. Herbivore-induced ocimene and/or linalool are reported to be involved in interactions between plants and carnivorous arthropods (e.g., Dicke et al. 1990; Du et al. 1998; Ishiwari et al. 2007). The adult willow leaf beetle *Phratora vulgatissima* is known to show moderate electrophysiological responses to ocimene released from willow leaves (Fernandez et al. 2007). It remains to be investigated which compound(s) in the mixture attract the larvae. Further, whether the mixture of the same and different concentrations also affects the behavior of the first, and the second instars, but not that of the third instars, remains to be answered. Brilli et al. (2009) reported that constitutive and herbivore-induced monoterpenes emitted from leaves of hybrid popular attracted *Chrysomela populi* beetles.

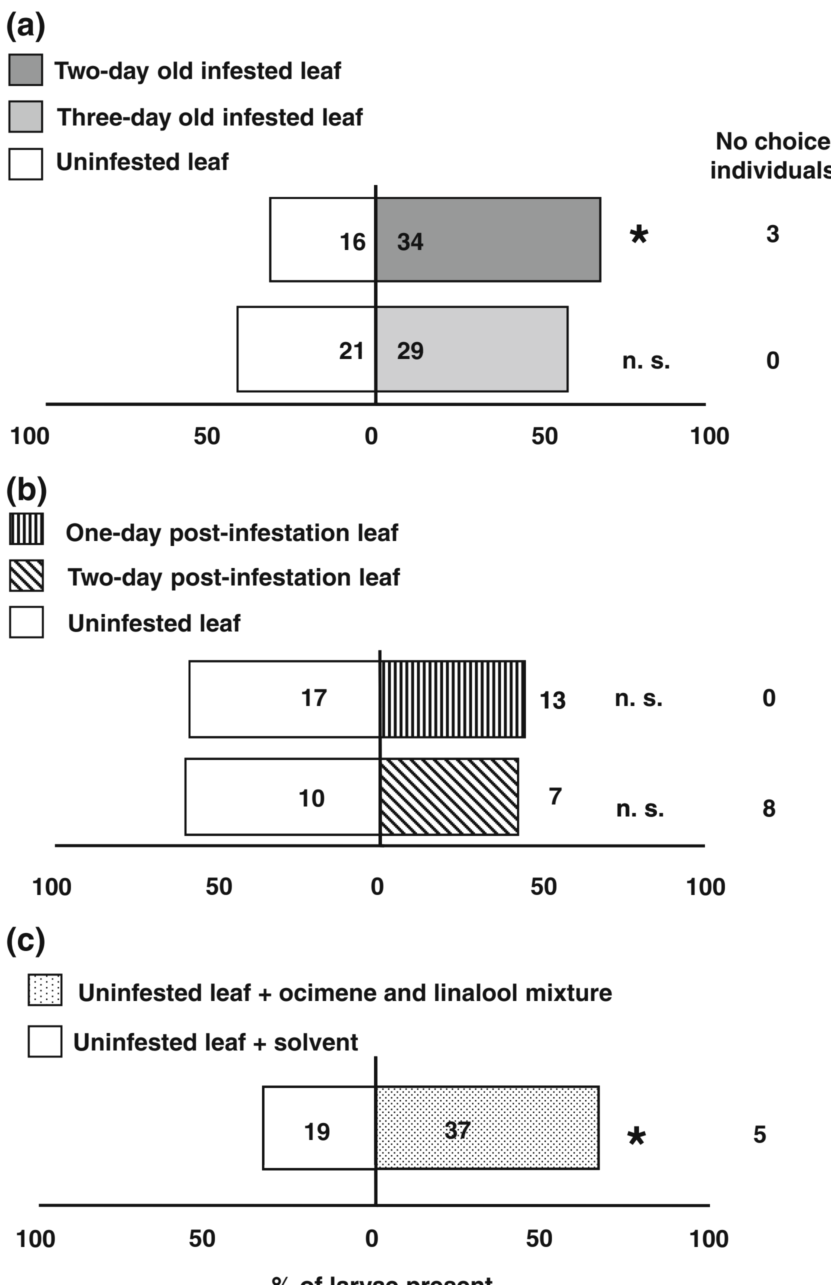


Fig. 3 Olfactory response of first instar *Plagiodera versicolora* larvae to differently treated leaves. NS: no significant difference, * :0.01<P<0.05 (binomial test). Numbers in bars indicate the number of larvae that responded to volatiles. (**a**) Comparisons of uninfested leaves vs. 2-d-infested leaves, and uninfested leaves vs. 3-d-infested leaves. (**b**) Comparison of uninfested leaves vs. 1-d-infested leaves 1 day after the removal of larvae, and uninfested leaves vs. 1-d-infested leaves 2 days after the removal of larvae. (**c**) Comparison of uninfested leaves supplemented with 1 µl hexane vs. leaves supplemented with a hexane solution of 100 ng/µl ocimene and linalool mixture

The first instars showed a preference for 2-d-infested leaves over uninfested leaves, and an equal preference for 3-d-infested leaves and uninfested leaves compared with uninfested leaves. This finding was in accordance with a previous study showing that the quality of the 3-d-infested leaves was not suitable for the larvae within aggregation stages (Raupp and Denno 1984). The volatiles may indicate the suitability to the larvae. Likewise, the first instars showed no preference for previously infested leaves. As the gregarious behavior of early instars is an important life history strategy in terms of defense against predators (Breden and Wade 1987), these differing responses would facilitate their reaggregation at a site where conspecific larvae of the aggregation stage had settled and started feeding. The chemical profiles of herbivore-induced volatiles would be highly dependent on the duration of damage and the time since last damage (Turlings et al. 1990; Maeda and Takabayashi 2001; Arimura et al. 2004, 2008; Hoballah and Turlings 2005). For example, corn seedlings release large amounts of volatile terpenes in response to damage caused by *Spodoptera exigua* larvae (Turlings et al. 1990). They were detected even after the removal of the larvae.

Table 1 Peak area (Mean±SE) of compounds recorded in the headspace of infested and uninfested willow leaves

Compounds	1-d-infested	uninfested	*P*	2-d-infested	uninfested	*P*
2-methylbutane nitrile[a]	0.30±0.10	ND	*	1.00±0.20	ND	*
3-methylbutane nitrile[a]	0.26±0.08	ND	*	0.49±0.10	ND	*
(*E*)-2-hexenal	0.57±0.45	0.2±0.12	n.s.	0.36±0.14	0.07±0.05	n.s.
(*Z*)-3-hexen-1-ol	1.86±0.64	0.6±0.21	n.s.	0.75±0.42	0.39±0.21	n.s.
(*syn*)- or (*anti*)-2-methylbutanal oxime[a]	ND	ND	n.s.	0.06±0.06	ND	n.s.
1-hexenol	0.17±0.08	0.02±0.01	n.s.	0.11±0.07	0.01±0.01	n.s.
(*syn*)- or (*anti*)-2-methylbutanal oxime[a]	ND	ND	n.s.	0.06±0.06	ND	n.s.
(*syn*)- or (*anti*)-3-methylbutanal oxime[a]	ND	ND	n.s.	0.03±0.03	ND	n.s.
n-heptanal	0.07±0.02	0.04±0.02	n.s.	0.02±0.02	0.02±0.01	n.s.
α-pinene	<0.01	<0.01	n.s.	<0.01	<0.01	n.s.
benzaldehyde	0.71±0.34	0.16±0.03	n.s.	1.19±0.23	0.09±0.01	*
(*Z*)-3-hexenyl acetate	4.18±0.12	2.36±0.50	n.s.	2.67±0.88	1.84±0.82	n.s.
(*Z*)-β-ocimene	0.36±0.11	ND	*	0.32±0.11	0.03±0.03	n.s.
benzyl alcohol	0.56±0.27	ND	*	0.96±0.30	ND	*
2-hydroxybenzaldehyde	0.65±0.34	ND	*	0.76±0.17	<0.01	*
(*E*)-β-ocimene	3.72±0.71	0.05±0.05	*	3.65±0.47	0.34±0.34	*
linalool	0.37±0.07	ND	*	0.48±0.15	ND	*
(*E*)-4,8-dimethyl-1,3,7-nonatriene	1.42±0.12	0.09±0.05	*	1.25±0.12	ND	*
allo-ocmene	0.54±0.25	ND	*	0.36±0.03	ND	*
(*E*)-2,6-dimethyl-1,3,5,7-octatetraene[a]	0.58±0.24	ND	*	0.35±0.06	ND	*
(*Z*)-3-hexyl-2-methyl butanoate	0.83±0.03	0.37±0.12	n.s.	0.81±0.10	0.21±0.12	*
α-copaene[a]	0.10±0.01	ND	*	0.17±0.03	ND	*
(*Z*)-jasmone	0.09±0.06	0.02±0.02	n.s.	0.15±0.04	ND	*
β-gurjunene[a]	0.08±0.05	ND	n.s.	0.19±0.04	ND	n.s.
(*E*)-β-caryophyllene[a]	0.09±0.03	ND	*	0.49±0.30	0.06±0.05	n.s.
β-cubebene[a]	0.12±0.09	ND	n.s.	0.2±0.04	ND	*
germacrene D[a]	0.27±0.06	ND	*	0.79±0.14	ND	*
(*E,E*)-a-farnesene	1.31±0.28	0.13±0.07	*	1.35±0.14	002±0.02	*
δ-cadinene	0.24±0.05	0.02±0.02	*	0.02±0.01	ND	*

a: Tentatively identified

The peak areas (ion intensities) were normalized based on the ion intensity of internal standard (tridecane) and gram fresh weight (see Materials and Methods). ND: not detected. * :0.01<P<0.05, n.s.: not significantly different (Mann-Whitney U-test).

Green leaf volatiles [(*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexenol] are detected when larvae are actively damaging the plants. In contrast, some volatile terpenes [α-*trans*-bergamotene, (*E*)-β-farnesene, and (*E*)-nerolidol] are detected several hours after the removal of the larvae. Arimura et al. (2004) studied the temporal patterns of systemic emission of germacrene D in poplar plants infested by forest tent caterpillars. After removal of the caterpillars, emission was reduced during the next 24 hr. The dependence of leaf odor attractiveness on the duration of infestation found in our study with *P. versicolora* larvae might be explained by differential time course emissions of *P. versicolora* larvae-induced volatiles in *S. eriocarpa*.

Yoneya et al. (2009b) reported that 17 compounds were detected in the headspace of infested plants, and four compounds in that of the uninfested plants. By contrast, in the present study, 1-d-infested leaves emitted 26 compounds and uninfested leaves emitted 13 compounds. Yoneya et al. (2009b) used a whole plant as the odor source, while this study used detached leaves as the odor source for chemical analyses, because the dual choice tests were conducted with detached leaves. These data indicate that due to the stress of detachment, the leaves emitted a larger number of volatiles than whole plants. Schmelz et al. (2001) previously reported that detached corn leaves released substantially different amounts of induced volatiles than uninfested ones.

Several studies show that herbivore-induced plant volatiles may have multifunctional effects in ecosystems, such as attracting carnivorous natural enemies of infesting

herbivores, avoiding/attracting conspecific/heterospecific herbivores, and mediating plant-plant communications (Arimura et al. 2009). We recently reported that ladybird (*Aiolocaria hexaspilota*) adults showed no preference for *S. eriocarpa* infested by leaf beetle adults (non-prey) over uninfested plants, but moved more often to the willow plants infested by leaf beetle larvae (prey) than to uninfested plants (Yoneya et al. 2009b). Furthermore, adult *P. versicolora* (either satiated or starved) are attracted to uninfested *S. eriocarpa* leaf volatiles, while only starved female *P. versicolora* are attracted to adult-infested *S. eriocarpa* volatiles in their foraging (Yoneya et al. 2009a). Here, *P. versicolora* larvae-induced volatiles are shown to affect the behavior of conspecific larvae as well. Thus, the volatiles from the larva- and adult-infested willow plants have multiple specific functions in the field. Such volatiles would play important roles in the infochemical networks of a willow community.

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Response of Predatory Mites to a Herbivore-Induced Plant Volatile: Genetic Variation for Context-Dependent Behaviour

Beata Sznajder · Maurice W. Sabelis · Martijn Egas

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Abstract Plants infested with herbivores release specific volatile compounds that are known to recruit natural enemies. The response of natural enemies to these volatiles may be either learned or genetically determined. We asked whether there is genetic variation in the response of the predatory mite *Phytoseiulus persimilis* to methyl salicylate (MeSa). MeSa is a volatile compound consistently produced by plants being attacked by the two-spotted spider mite, the prey of *P. persimilis*. We predicted that predators express genetically determined responses during long-distance migration where previously learned associations may have less value. Additionally, we asked whether these responses depend on odors from uninfested plants as a background to MeSa. To infer a genetic basis, we analyzed the variation in response to MeSa among iso-female lines of *P. persimilis* by using choice-tests that involved either (1) MeSa presented as a single compound or (2) MeSa with background-odor from uninfested lima bean plants. These tests were conducted for starved and satiated predators, i.e., two physiological states, one that approximates migration and another that mimics local patch exploration. We found variation among iso-female lines in the responses to MeSa, thus showing genetic variation for this behavior. The variation was more pronounced in the starved predators, thus indicating that *P. persimilis* relies on innate preferences when migrating. Background volatiles of uninfested plants changed the predators' responses to MeSa in a manner that depended on physiological state and iso-female line. Thus, it is possible to select for context-dependent behavioral responses of natural enemies to plant volatiles.

Key Words *Phytoseiulus persimilis* · Methyl salicylate · I-tube olfactometer · Preference · Genetic variation · Context dependence

B. Sznajder (✉) · M. W. Sabelis · M. Egas
Department of Population Biology, Institute for Biodiversity and Ecosystems Dynamics, University of Amsterdam, Science Park 904, P.O.Box 94248, 1090 GE Amsterdam, the Netherlands
e-mail: b.a.sznajder@gmail.com

Introduction

Predatory arthropods are known to respond to herbivore-induced plant volatile chemicals (Dicke and Van Loon 2000; Sabelis et al. 2007). This phenomenon prompted the hypothesis that first and third trophic levels "conspire" against the second: the infested plants attract the natural enemies that can reduce or eliminate herbivore pressure, whereas the predators acquire information on the location of its prey (Dicke and Sabelis 1988; Dicke and Van Loon 2000; Kessler and Baldwin 2001, but see Allison and Hare 2009). However, for such a system to evolve and function, a number of conditions have to be fulfilled (Van der Meijden and Klinkhamer 2000; Janssen et al. 2002). Among them, it requires that the predators evolve behavioral responses to plant-produced volatiles induced by herbivore feeding. These responses may rely on predators learning to associate herbivore-induced plant volatiles with the presence of prey. Alternatively, predators evolved genetically determined preferences for plant volatiles induced by herbivorous prey, if in the past generations predators innately responding to such volatiles (i.e., prior to any experience) had higher fitness than those that did not show such behavior. Fitness benefit would be obtained because these volatiles were reliably coupled with the presence of prey. Therefore, we expect genetic bases for predator responses to these plant volatiles that are induced

by the feeding of prey, irrespective of the species of infested plant.

We investigated whether there is genetic variation in the response of the predatory mite *Phytoseiulus persimilis* (Athias-Henriot) to a selected herbivore-induced plant volatile, methyl salycilate (MeSa). MeSa is one of the volatile compounds consistently induced by feeding of the two-spotted spider mite (*Tetranychus urticae* Koch)—the prey of *P. persimilis*—on a variety of plant species (van den Boom et al. 2004). Empirical studies showed that it plays a key role in predator attraction to the volatile blends induced by the spider mite (de Boer and Dicke 2004a; de Boer et al. 2004; van Wijk et al. 2008; Ament et al. 2010). A genetic basis for the responses of *P. persimilis* to blends of volatile compounds has been demonstrated in two studies on selection for responses of satiated predators to the complete blend of volatiles released by spider-mite infested lima bean (Margolies et al. 1997; Jia et al. 2002). However, innate preferences for the full volatile blend of infested bean may be the result of the underlying innate response to a single compound shared by the blends of many plant species infested by the spider mite—a condition that is fulfilled by MeSa. In this study, we applied an analysis using iso-female lines (David et al. 2005) to detect genetic variation in response to MeSa among *P. persimilis* lines. A significant difference in the olfactory responses among iso-female lines indicates a genetic basis for this trait, provided that systematic environmental influences are controlled for.

The odor of uninfested plants is a permanent feature of the volatile signal under natural settings. Therefore, we hypothesized that the background odors of uninfested lima bean may affect the predator's perception of MeSa and the strength of response to MeSa. Lima bean leaves heavily infested with spider mites also were used for culturing of the predator population used in this experiment. Therefore, any pre-conditioning of the predator to the odors experienced in the culture would be to the volatile blend of infested lima bean that is quantitatively different from the blend of uninfested bean (e.g., Dicke et al. 1990). Hence we expected that an enhanced predator response to MeSa presented with the background odors of uninfested lima bean would be a result of altered perception to MeSa when presented within a context rather then due to predator pre-conditioning to specific volatiles. Mechanically damaged plants produce increased amounts of a distinct group of volatiles called green leaf volatiles (GLVs), which are also emitted by intact plants in smaller amounts (Hatanaka 1993; Matsui et al. 2000; van den Boom et al. 2004). Thus, we term the background odors provided by the punched leaf discs used in this experiment as GLVs.

Genetically determined responses to herbivore-induced plant volatiles are particularly relevant, and subject to natural selection, in situations where predators cannot yet have learned the association between the presence of specific volatiles and prey. The predatory mite *P. persimilis* may experience such situations during the migratory phase that follows the exploitation of the previous colony of prey. These phases of the predator life history can be approximated by manipulating the predator's hunger level because food conditions provide the proximate cue for the onset of migratory behavior (Sabelis and Afman 1994). Satiation prevails during foraging in dense colonies of spider mites, and starvation induces take-off to aerial dispersal, followed by exploration of the new landing site (Sabelis and van de Baan 1983; Sabelis et al. 1984; Sabelis and van der Meer 1986; Sabelis and van der Weel 1993; Sabelis and Afman 1994; Pels and Sabelis 1999). During the migratory phase, the predators encounter an environment characterized by plant volatiles most likely to be different from those experienced before; the previously learned associations of specific volatiles may be of little value in locating the prey. Therefore, we hypothesized that the innate responses to MeSa depend on the context of the test (satiated vs. starved predators).

We determined the responses of a total of 18 iso-female lines of *P. persimilis* in the olfactory tests where predators were presented with the choice of 1) MeSa in clean air vs. clean air, or 2) MeSa in the background of GLVs vs. GLVs. These tests were performed using either satiated or starved predators. Variable olfactory responses to MeSa among the iso-female lines would indicate genetic variation for this trait in the population under study. In particular, we predicted that this variation would be more pronounced in starved predators, and we asked to what extent this variation depends on the presence of volatiles of uninfested plants in the background.

Methods and Materials

Predatory Mites The base population of *P. persimilis* was maintained in the laboratory (25°C, 70% humidity, and 16:8, L:D conditions) on a diet of two-spotted spider mites (*T. urticae*) on detached leaves of lima bean plants. The culture originated from a sample of predators collected in 2002 at different locations throughout Sicily (Partinico, Scopella, Trappeto, Terrasini, Siculiana, Laghetto, Menfi, Trabia, Alcamo), where they are probably endemic (De Moraes et al. 2004). Iso-female lines were obtained by randomly selecting a number of females from the base population, which from then on were kept separately in Petri dishes. Typically in haplodiploid arthropods such lines are obtained through mating between the virgin female and her sons (oedipal mating). However, *P. persimilis*, just as probably the whole family Phytoseiidae, is pseudo-arrhenotokous, i.e., sons and daughters arise from fertilized

eggs, and male zygotes become haploid due to paternal genome inactivation and elimination during embryogenesis (Helle et al. 1978; Nelson-Rees et al. 1980). Hence, mating is a prerequisite for producing eggs destined to become females, as well as males. For this reason, we selected already mated females, and propagation of the lines occurred through brother-sister mating. There were 18 lines established, which were allowed to propagate for 7 wk before the onset of the olfactory tests (for at least 5 generations). All lines were reared in the same climate room (under conditions as described for the base population) to minimize any effect of environment on predatory behavior. Incidental mixing among the lines was prevented by maintaining each line in a separate rearing enclosed in a plastic container with a small opening covered with gauze (mesh width=0.07 mm, average adult predator size= 0.5 mm, average predator egg size=0.2 mm, small enough to prevent immi- and emigration) to allow for airflow. Additionally, each of these containers was surrounded by its own water barrier. Individual iso-female lines were labelled with numbers (1–18) for identification.

Tests of Olfactory Preference Behavioral responses of predators to MeSa were tested in a so-called I-tube: a single straight glass tube (length 20 cm, 0.5 cm diam) with a small opening (2 mm) in the middle to introduce the predatory mites (Fig. 1). This olfactometer was provided with a trap at the end of either arm of the I-tube to collect predators. Opposite the entrance hole, another opening (0.5 cm diam) was present that was gauze-covered and served as an air-outlet. At the ends of the I-tube, there were plastic trap vials (30 mm diam, height 55 mm), which in turn were connected to jars that either contained a capillary with MeSa or not. The traps were designed as an easy-to-enter-yet-difficult-to-exit vial, and were provided with a water source (wet cotton wool). After purification by activated carbon filters, the air was flowing at 20 l/h through the jars, the vials, and then the arms of the glass tube, leaving the system through the opening in the middle of the I-tube. At release in the middle of the I-tube, a mite found itself in air streams coming from right and left, i.e., one with MeSa and the other without. Subsequently, it could move left or right in the I-tube and ultimately entered one of the trap vials or remained in the I-tube. After release of the test animals, the entrance hole was sealed with Parafilm®. Pilot experiments showed that the I-tube olfactometer produced results consistent with our knowledge of the responses of *P. persimilis* to the full blend of herbivore-induced plant volatiles (Van Wijk, unpublished data).

Per replicate experiment, a total of 25 adult females were released (except for a few cases where numbers were less than 25, yet larger than 20) sequentially. Visual cues play no role in predator orientation, as the predator is blind and orients itself by a means of chemical cues. A previous study that used a Y-tube set-up showed that there is no effect of possible residues, deposited along the path taken by an individual, on the choices of the subsequent individuals (Sabelis and van de Baan 1983). After 25 min, the number of mites in each of the two trap vials was counted. The number that remained in the I-tube, was scored as "no choice". For each consecutive replicate of a line, a new clean I-tube was used, and the side of the arm containing air with MeSa was inter-changed to exclude any unforeseen asymmetries in the experimental set-up. Per line per treatment, roughly 120 mites were tested in 4–8 replicate experiments (in one case, three replicates were performed).

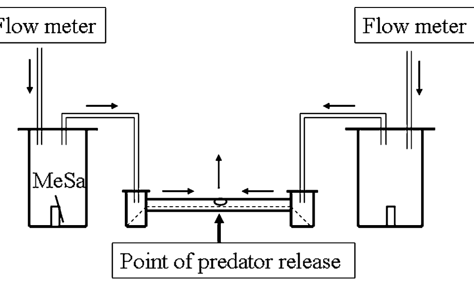

Fig. 1 Experimental set-up of the I-tube olfactometer. The diagram depicts a horizontal tube (the glass I-tube) with two openings, where the bigger one serves as an air-outlet. The *thick vertical arrow* below the I-tube indicates the location of the small opening—the point of release of the predators. The *broken line* indicates a thin capillary inside the glass tube to provide structure, on each side ending in a metal pin leading down into the trap vial. The trap vials were connected with plastic tubes to jars that either contain a capillary with MeSa or not. The remaining *arrows* indicate the direction of air-flow. Above the set-up there was a source of dispersed light (not shown)

Synthetic MeSa (Sigma-Aldrich Fluka, pure; assay ≥99%) was offered undiluted in a small capillary (9 µl, 0.60 mm diam, Omnilabo) placed in one of the jars connected to the I-tube (Fig. 1). The air flow was led through the set-up at least 2 h before the start of the test. The MeSa evaporation rate was ±30 µg/h. This evaporation rate was chosen based on preliminary experiments to find a concentration to which the base population exhibited a neutral (50:50) response. Such a set-up allows detection of either increased or decreased responses of the iso-female lines to MeSa with reference to the response of the base population. It is difficult to compare this evaporation rate with previous studies that attempted to measure the dose-response relations, because these assessments were based on filter paper as a substrate (de Boer and Dicke 2004b; van Wijk et al. 2008). Unlike filter paper, capillaries generate a constant evaporation rate. It is also difficult to compare this evaporation rate with evaporation from infested Lima bean plants, because there are hardly any data available. The only data we are aware of is 0.4–0.8 µg/h estimated by de Boer and Dicke (2004b) from data in Dicke et al. 1999, evaporated from 10 leaves after 3 d of infestation

by 50 adult female spider mites per leaf with a flow rate of 30 l/h. It is unclear at what distance from the plant this concentration was measured, and also how such concentrations of MeSa changed over the time of infestation.

Experimental Design We assessed olfactory preferences of 18 iso-female lines by subjecting satiated and starved adult female predators to two types of olfactory tests that involved choices between either (1) MeSa in clean vs. clean air, or (2) MeSa with a background of green leaf volatiles (GLVs) vs. green leaf volatiles (GLVs). The background odors of GLVs were provided by discs (1.5 cm diam) punched from the leaves of 2-wk-old, uninfested Lima bean plants (var. *Phaseolus lunatus*, Big Lima). New leaf discs were punched for each test from fresh primary leaves, and placed inside the plastic trap vials of the I-tube—one disc in each of the vials—on a ball of wet cotton wool. Airflow was allowed to pass through the I-tube for an additional 15 min before the start of the olfactory test to allow the GLVs to reach the middle of the I-tube. The responses of the satiated predators were determined by testing females taken straight from the culture where they were kept in a well fed state. Starved predators were obtained by food-deprivation for 24 h prior to the olfactory tests. During the period of food deprivation, mites were kept in a closed Eppendorf vial placed in a climate box at 18°C, with water provided via a strip of wet filter paper.

The total of 18 olfactory tests performed daily on each of the 18 lines was about the maximal number feasible under the experimental protocol. The main aim of this study was to sample a large number of iso-female lines. Therefore, we preferred to test as many lines as possible per day, at the expense of obtaining simultaneous replicates for each line. To minimize effects of the time of day on predatory responses, we randomized the order of testing of the 18 lines each day. The number of replicates varied between four and six per hunger level for each olfactory test (i.e., tests with or without GLVs context). Tests were performed within a period of 5 wk.

Statistical Analysis We aimed to determine (1) whether there is a genetic component in predator response to MeSa, and (2) whether this behavior is dependent on the nutritional status or the environmental context provided by the volatiles of uninfested plants in the background. To address the first question we constructed a mixed-effects model that included the iso-female line effect as the random effect; hence, the response variable (i.e., a single data point) was the mean response per replicate per line based on the numbers of individuals that made a choice (hence, excluding the no-choice individuals). In this analysis, we tested whether the amount of variation explained by the random effect of iso-female line is different from zero. The variation due to iso-female line is a measure of the total amount of variation among tested lines that is due to variation in their genes, provided that the differences among the lines arising from heterogeneity in rearing conditions can be excluded (David et al. 2005). It reflects broad-sense heritability that comprises the additive genetic variation as well as the non-additive effects of dominance and epistasis. The amount of this variation was estimated by using restricted-maximum likelihood method of the SPlus 6.2 software (Pinheiro and Bates 2000; Venables and Ripley 2002), which adjusted for the fixed effects of hunger level (satiated or starved) and GLVs context (MeSa presented alone or MeSa in the background of GLVs). Additionally, we tested whether the responses of the lines changed within the period of testing such that there was a decreasing or increasing trend. To this end we also tested the covariate representing the number of days elapsed from the onset of testing the first replicate of a line within a treatment. A significant covariate would indicate that environmental effects, consistent in time, contribute to the differences among the lines. A non-significant covariate, on the other hand, would indicate that no effect of consistent differences among the rearing environment of the lines was detected, thus strengthening the argument that the variation among the lines reflects genetic differences. The number of days elapsed since the onset of testing encompassed a period of 1–2 wk. Thus, it may have encompassed the responses of more than one generation of predators (Sabelis 1981). A non-significant covariate would be consistent with a positive correlation between mean values in different generations (a result that supports a genetic basis for preference within iso-female lines, David et al. 2005), although the design of this study did not address this correlation explicitly. The diagnostics plots showed that the assumptions of the mixed effect model were satisfied (the random variables were normally distribution with mean zero and independent for different groups, and the within-group errors were independent and normally distributed with a mean of zero), hence, the response variable was not transformed.

To address the second question we tested for the situation where different genotypes respond to MeSa differently, dependent on predator satiation level or volatile presentation (as reflected in two- and three-way interactions between the line effect and the experimental factors). Therefore, in the second part of the analysis, we also analyzed the probability of an individual choosing MeSa in relation to three explanatory variables: iso-female line (18 lines), hunger level (satiated or starved), and GLVs context (MeSa presented alone or MeSa in the background of GLVs) by fitting a logistic regression model with logit link function and binomial error variance in SPlus 6.2 for Windows. The response variable in this analysis was the

response of an individual predator. The relevance of the explanatory variables was assessed based on the comparison of values of deviance and Akaike's Information Criterion for the models that included combinations of one or more of the three explanatory variables (Agresti 1990; Quinn and Keough 2002). We further tested the fit of the regression model with respect to given explanatory variable by comparing this model with an appropriate reduced model (i.e., a model that contains all terms of the complete model but the explanatory variable tested) by using the log-likelihood ratio test (Agresti 1990). Additionally, we analyzed the effect of the three explanatory variables on the probability of an individual making a choice (i.e., the probability of individual entering any trap vial in the I-tube).

Results

The first part of this section presents the analysis of the variation detected among the iso-female lines. The second part deals with the analysis of the probability of predators choosing MeSa using the factors iso-female line, hunger level, and GLVs context. Finally, we discuss the probability of predators making a choice (i.e., the probability of choosing either arm).

Variation among Lines The variation explained by the iso-female line was different from zero (P=0.02, Table 1), thus supporting the hypothesis that there is a genetic component in predator responses to MeSa. It is a measure of the total amount of variation among the tested iso-female lines that is due to genetic variation, provided that the differences among the lines due to heterogeneity in rearing conditions are negligible. We detected no systematic change in the average responses of the lines within the period of testing (P=0.56, Table 1). This result supports the conclusion that the observed differences among the lines were not due to systematic differences among the rearing environment of the lines, but rather reflect genetic variation.

In this analysis, the effect of hunger level was significant, but not the GLVs provided as context. However, inspection of the data (Fig. 2; in each panel the ordering of lines from low to high response results in a different sequence of lines) indicates that there is variation in the responses of different lines dependent on the level of these two factors. This interaction was explored further in the second part of the analysis.

Probability of Choosing MeSa We used the logistic regression method to analyze the probability of predators choosing MeSa in relation to the factors: iso-female line, hunger level, and GLVs as context. Comparison of deviance and AIC values throughout all models (that included combinations of one or more of the three effects) revealed that iso-female line, hunger level, and GLVs as context, all affected the probability that a predator would choose MeSa (log-likelihood ratio test, P_{line}<0.001, P_{hunger}<0.001, P_{GLVs}=0.02). Figure 2 shows the variation among iso-female lines in the proportion of individuals choosing MeSa, categorized by hunger level, when MeSa is presented alone and when it is presented with the background of GLVs. Visual inspection of these figures indicates an interaction between iso-female line, hunger level, and GLVs as context. Therefore, we also compared the deviances and AIC values of the models that included the effects as well as their interaction terms. The final model that best described predator responses included the interaction between the iso-female line and the two fixed effects (log-likelihood ratio tests; $P_{line\ x\ hunger}$<0.001, $P_{line\ x\ GLVs}$<0.001, $P_{hunger\ x\ GLVs}$=0.41, $P_{line\ x\ hunger\ x\ GLVs}$<0.001). Figure 3 exemplifies the three-way interaction by presenting the responses of some of the iso-female lines.

Probability of Making a Choice We constructed a logistic regression model describing the probability that a predator

Table 1 Mixed-effects model of predator responses to MeSa; iso-female line was the random effect and the fixed effects included the hunger level (2 levels: satiated, starved) and GLVs context (2 levels: MeSa presented alone, MeSa in the background of GLVs). The number of days from the onset of testing the line within a given treatment was added as covariate. The significance of fixed effects, their interaction and the significance of the random effect (i.e., the hypothesis that the variance of random effect ≠ 0) was tested using the likelihood ratio tests (Pinheiro and Bates 2000; Venables and Ripley 2002)

Effect		Likelihood ratio (df_1, df_2)	P value	Variance
Random	Iso-female line	5.25 (7, 6)	0.02	0.003
	Residual			0.05
Fixed	Hunger level	6.75 (6, 5)	0.009	
	GLVs context	0.05 (6, 5)	0.81	
	Days from onset of testing	0.34 (6, 5)	0.56	
Interaction	Hunger level: GLVs context	0.03 (7, 6)	0.86	

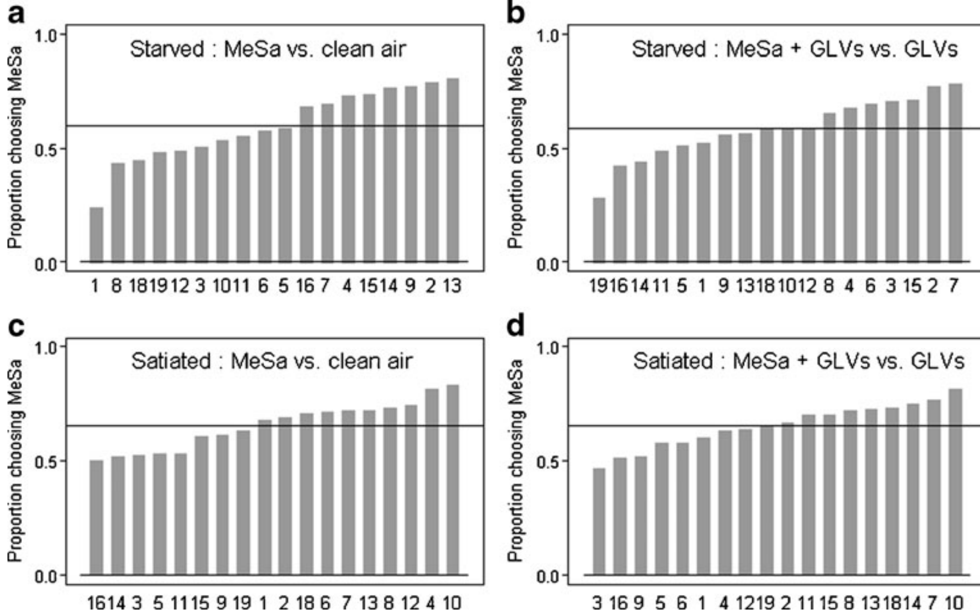

Fig. 2 The responses of eighteen iso-female lines of *Phytoseiulus persimilis* by starved predators in the tests (**a**) MeSa plus clean air vs. clean air, (**b**) MeSa plus the background of GLVs vs. GLVs, and by satiated predators in the tests (**c**) MeSa plus clean air vs. clean air, (**d**) MeSa plus the background of GLVs vs. GLVs. The bars show the proportion of individuals choosing the arm containing MeSa (y-axis) calculated for each line (x-axis). The *horizontal lines* indicate the average responses obtained by pooling replicates over all iso-female lines within each of the experimental treatments. Note that in each panel the ordering of lines from low to high response results in a different sequence of lines, e.g., line 10 is on the right in (**c**) and (**d**) but on the left in (**a**) and in the middle in (**b**)

will make a choice in the olfaction test (i.e., it will choose either arm). The full model (i.e., line + test + hunger) explained the responses better than the null model (log-likelihood ratio, $P<0.001$). All main effects were individually significant ($P_{\text{line}}<0.001$, $P_{\text{test}}<0.001$, $P_{\text{hunger}}=0.03$, see Fig. 4).

Discussion

We asked whether there is genetic variation in responses of *P. persimilis* to the plant volatile MeSa in a field-collected population. To this end we employed the iso-female line technique where the variation among iso-female lines is

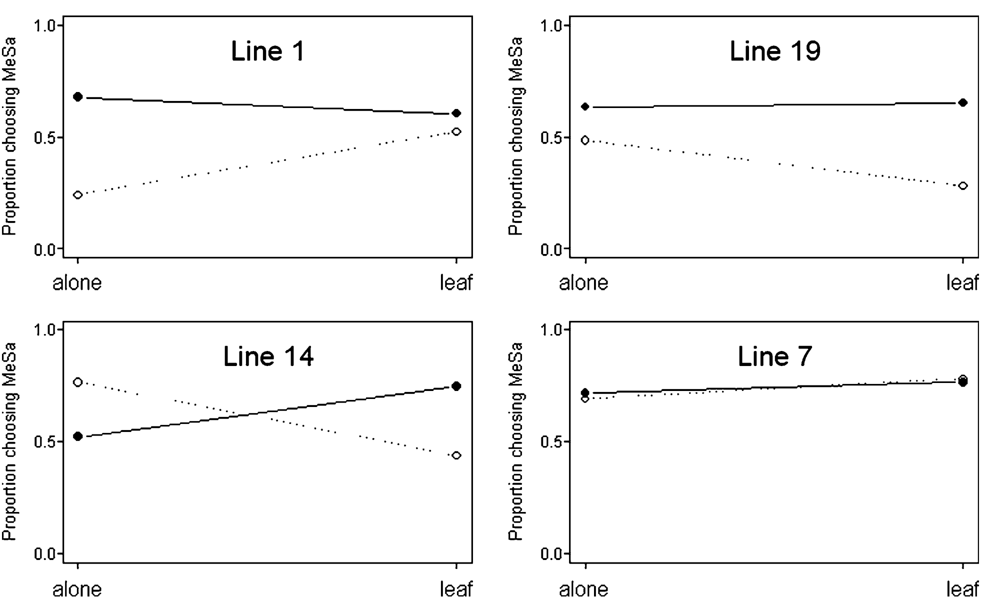

Fig. 3 Illustration of the interaction between iso-female line, hunger level and GLVs context in selected iso-female lines; the graphs present the mean proportions of individuals choosing MeSa (values on the y-axis) categorized by the factors. *Open circles* connected by *dashed line* refer to the responses of the starved predators, *closed squares* connected by *solid line* to the responses of the satiated predators, and the x-axis presents the categorization with respect to GLVs context

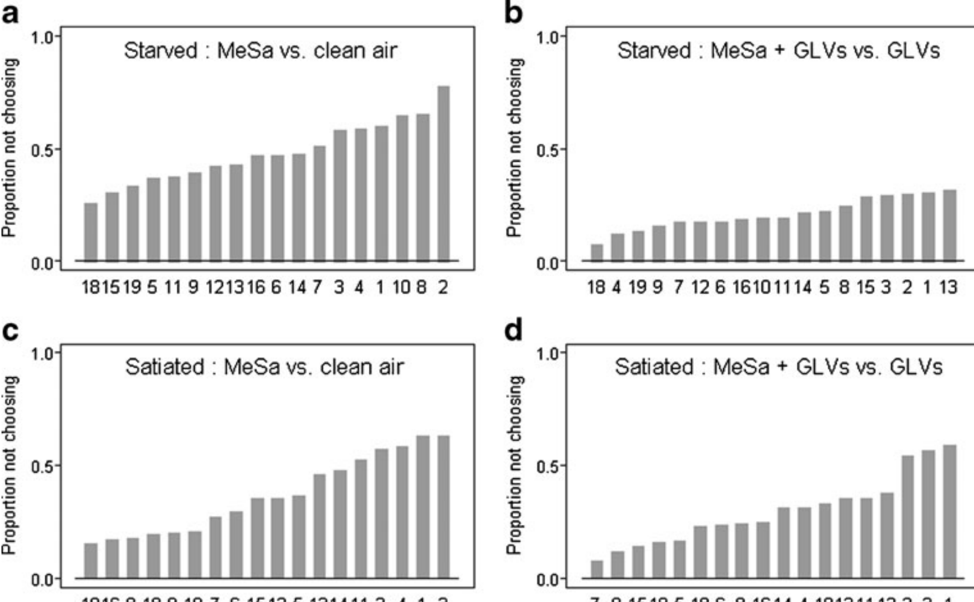

Fig. 4 Variation in the mean proportion of no-choice individuals (y-axis) in the 18 iso-female lines (x-axis) of *Phytoseiulus persimilis* categorized by hunger level and GLVs context. No-choice individuals are those that remained in the glass tube for the duration of the test, i.e. did not make a choice. The panels present the proportions observed for starved predators in the tests (**a**) MeSa plus clean air vs. clean air, (**b**) MeSa plus the background of GLVs vs. GLVs, and for satiated predators in tests (**c**) MeSa plus clean air vs. clean air, (**d**) MeSa plus the background of GLVs vs. GLVs

interpreted as the variance due to differences in their genes provided the heterogeneity among the rearing environments does not systematically influence the behavior of individual lines. We showed that the variation in predator responses to MeSa explained by the iso-female line was significantly different from zero. From this result, we infer that the studied population harbors genetic variation in olfactory responses to MeSa. There is more variation among the lines when the predators are starved, yet variation is also present in satiated mites. However, it is only when predators are starved that we observe the full range of responses ranging from avoidance to preference. This pattern occurs when predators have a choice of both MeSa presented alone and MeSa against a background of GLVs.

Using the variances quantified from the mixed-effects model for response to MeSa (see Statistical Analysis) we were able to obtain an estimate of broad-sense heritability defined as $H^2 = \frac{\sigma^2_{line}}{\sigma^2_{line}+\sigma^2_{residual}} = 0.05$, where $\sigma^2_{residual}$ is the remaining amount of unexplained variation that represents the environmental variance (Table 1). The value of estimated broad-sense heritability is relatively small and comparable with most values of narrow-sense heritability of fitness traits (Houle 1992; Visscher et al. 2008; Tien et al. 2009). Moreover, our results indicate that any estimate of heritability may be deflated due to large values of residual, i.e., unexplained, variance detected in this study (Table 1). The residual variance represents the environmental variance, and its magnitude indicates heterogeneity in other uncontrolled factors, such as predator age, and correlated with it the amount of experience.

To date, studies that have measured the attractiveness of MeSa (or other herbivore-induced plant volatiles) to natural enemies have not controlled for genetic background (e.g., de Boer and Dicke 2004b; van Wijk et al. 2008). However our results show that predator populations may be genetically variable for the tested responses, and that the behavior of various genotypes may influence the average response measured at the population level. Thus, the average population response may be characterized by a weak attraction or no clear preference if genotypes of extreme innate preferences are tested together and their responses pooled. To illustrate this, we calculated the proportions of individuals choosing MeSa by pooling the replicates over all iso-female lines for each of the experimental treatments. These values (shown in Fig. 2 as horizontal lines) may be treated as estimates of the responses to MeSa measured at the level of the base population from which the lines originated, under the assumption that different iso-female lines (i.e., genotypes) are represented. Indeed, there is little difference among the experimental treatments.

The inclusion of GLVs in the background of MeSa presentation does not, at first glance, appear to change the overall result that a wider range of responses is expressed by starved predators (compare Fig. 2a, c and Fig. 2b, d). However, closer inspection reveals that the addition of the

background of GLVs does change the behavior of individual lines. We observed a whole range of patterns in the responses of the lines that exemplify a significant three-way interaction between iso-female line, hunger level, and GLVs as background (except for line 5 that shows indifferent responses in all tests without exception). In particular, there are iso-female lines characterized by an extreme response to MeSa if the predators are starved and the choice-test involves MeSa in the background of GLVs, whereas their responses in all the other treatments are indifferent (lines 19 and 3). The example of these lines may indicate that testing the responses of starved predators to MeSa presented in the background of GLVs are essential conditions for eliciting an innate response of the predators. However, there are also lines characterized by extreme responses to MeSa if the predators are starved and the choice-test involves MeSa presented alone (e.g., lines 1 and 9), and predator responses in all the other treatments are indifferent. The example of these lines may thus indicate that the adding of GLVs as a background to MeSa confounds the predator's perception of this volatile. The remaining iso-female lines show yet other patterns of interaction among the factors iso-female line, hunger level, and GLVs as background (see Fig. 3 for examples).

We conclude therefore that the responses of *P. persimilis* to MeSa are context-dependent in that they depend on the genetically determined preference of the tested individuals (i.e., their genotypes) as well as on the environmental context in which the volatile is presented. Further research is needed to understand how a biologically relevant single compound cue is perceived and interpreted when presented in a mixture (van Wijk 2007; Shröder and Hilker 2008), a more realistic situation in nature. In our study, the addition of a background odor of GLVs increased the proportion of individuals making a choice in olfaction tests (Fig. 4). Thus, we conclude that GLVs affected the predator's perception of MeSa. This effect of GLVs on the willingness to choose varied among individual lines (which indicates a genetic basis also in this aspect of behavior).

Given the genetic basis in the responses to MeSa in this population collected from the field, we conclude that there is opportunity for an evolutionary response on the part of the predators to using MeSa as a plant signal indicating prey presence. The distribution of prey is patchy and unpredictable under natural conditions. Hence, it is expected that *P. persimilis* undergoes long-lasting food deprivation during dispersal (Sabelis and Afman 1994). Our findings may indicate stronger reliance of the predator on genetic predispositions during this migratory phase in which the predator alternates passive aerial dispersal and local exploration for prey patches. Thus, natural selection may be more efficient in shaping the olfactory responses when the predators are starved and therefore probably migrating.

Our results show that the responses of *P. persimilis* to plant volatiles are far from simple. Even in the case of a single plant volatile, we observed genetic variation expressed in a context-dependent manner. Iso-female lines created from a field-collected base population showed responses to MeSa ranging from preference to avoidance (although the latter response was much less common). The variation is still present in the population and conflicts with the prediction that preference for environments associated with the presence of MeSa has fitness benefits (i.e. helps locate the prey) and should therefore prevail. This raises the question of the mechanism that maintains the observed genetic variation in the field; the question could be addressed with selection experiments for olfactory responses to single volatile compounds and mixtures. Our results suggest that such experiments will be more successful if conducted on starved predators.

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Changes in Monoterpene Emission Rates of *Quercus ilex* Infested by Aphids Tended by Native or Invasive *Lasius* Ant Species

Carolina I. Paris · Joan Llusia · Josep Peñuelas

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Abstract The emission of volatile organic compounds (VOCs) depends on temperature and light. Other factors such as insect herbivory also may modify VOC emission. In particular, aphid feeding promotes the release of new compounds and changes the composition of plant volatile blends. Given that some aphids are tended by ants, we investigated whether ants change the emission of VOCs indirectly through attendance on aphids. The effect of *Lachnus roboris* aphids and two different tending ant species on terpene emission rates of 4-year-old holm oak (*Quercus ilex*) saplings was investigated during a field experiment. There were five treatments: saplings alone (T1), saplings infested with *L. roboris* aphids (T2), saplings infested with aphids tended by the local ant *Lasius grandis* (T3), those tended by small colonies of the invasive ant *Lasius neglectus* (T4), and those tended by large colonies of the same invasive ant species (T5). The infestation by *L. roboris* elicited the emission of Δ^3-carene and increased the emission of myrcene and γ-terpinene. Terpene emissions were modified depending on the tending ant species. Attendance by the local ant *L. grandis* increased α and β-pinene and sabinene. Attendance by the invasive ant *L. neglectus* only decreased significantly the emission of myrcene, one of the major compounds of the *Q. ilex* blend. Aphid abundance decreased with time for all treatments, but there was no difference in aphid abundance among treatments. Total terpene emission rates were not correlated with aphid abundance. These results highlight that aphids and tending ants may change terpene emission rates, depending on the ant species.

Key Words Aphid-ant interaction · Induced volatiles · Indirect effect · Invasive ant species · Terpene emissions · Biotic factors

C. I. Paris (✉)
Department of Animal Biology, Plant Biology and Ecology,
Autonomous University of Barcelona,
Edifici C, Campus de Bellaterra (UAB),
Cerdanyola del Vallès (Bellaterra),
08193 Barcelona, Spain
e-mail: baikibadai@yahoo.com

J. Llusia · J. Peñuelas
Global Ecology Unit CREAF-CEAB-CSIC, Center of Ecological Research and Forestry Applications (CREAF),
Edifici C, Campus de Bellaterra (UAB),
08193 Bellaterra (Barcelona), Spain

J. Llusia
e-mail: j.llusia@creaf.uab.cat

J. Peñuelas
e-mail: josep.penuelas@uab.cat

Present Address:
C. I. Paris
Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires,
Intendente Güiraldes 2160 Lab 28 4to piso - Ciudad Universitaria,
C1428EGA Buenos Aires, Argentina

Introduction

Plants emit a broad spectrum of volatile organic compounds (VOCs), mainly isoprene and monoterpenes, which have multiple protective and signaling functions (Peñuelas and Llusia 2004), including defensive and alellopathic roles. For example, they act as herbicides and may inhibit seed germination, cytochromic respiration, and growth of annual plants (Peñuelas et al. 1996). Under certain conditions, i.e., abiotic stress or herbivore attack, new volatiles may be produced *de novo* or released

from storage organs (Kesselmeier and Staudt 1999). These induced emissions have received increasing attention since the first studies that showed emission in response to herbivore attack (Dicke and Sabelis 1988). Some compounds of the emitted blend act as deterrents of the herbivore or as signals to attract predators or parasitoids that protect the plant against insects.

Holm oak (*Quercus ilex* L.) is a widespread tree of the Mediterranean basin, and it is a major source of VOCs (Staudt et al. 2001). In contrast to many deciduous oak species that emit large amounts of isoprene, *Q. ilex* emits huge amounts of monoterpenes, although it has no specific VOC storing organs (Staudt and Seufert 1995), and there is no storage pool of VOCs in the leaves or bark (Pasqua et al. 2001). The compositional fingerprint of the emissions is controlled mainly by genetics (Staudt et al. 2003). However, the synthesis and emission of these VOCs are modulated continuously by environmental factors such as light, temperature (Staudt and Bertin 1998; Llusia and Peñuelas 1999) and severe drought (Llusia and Peñuelas 1998), or by biological factors such as herbivore attacks (Staudt and Lhoutellier 2007). Identifying those biotic factors that promote changes of VOC emissions from holm oak is crucial for Mediterranean ecosystems because the monoterpenes released play a key role both in the biology of the community and in the formation of oxidants and secondary aerosols in the troposphere (Kesselmeier and Staudt 1999; Peñuelas and Staudt 2010). This has consequences for air quality and visibility, and even for the climate on a regional scale (Peñuelas and Llusia 2003).

In Spain there are many aphids that feed on *Q. ilex* (Nieto-Nafría and Mier-Durante 1998), in particular *Lachnus roboris* L., which is an obligate myrmecophilous aphid (Michel 1942; Sudd and Sudd 1985), and is tended by several ant species that collect honeydew as food (Paris and Espadaler 2009). Honeydew, a sugar solution excreted by aphids while feeding on sap plants, is the main food of several ant species (Carroll and Janzen 1973). Through aphid attendance, ants may exert indirect effects on plants, such as a decrease in seed production (Rico-Gray and Castro 1996), plant herbivory (Suzuki et al. 2004), and pollination (Lach 2007). The degree of these indirect effects varies among native ants, primarily as a result of differences among species in aggressiveness, territoriality, colony density, worker abundance, and the strength and persistence of the interactions between particular ant species (Styrsky and Eubanks 2007). In the case of invasive ants, it is assumed that the indirect effect on plants through aphid attendance may increase in magnitude because invasive ants frequently cause local hemipteran outbreaks (Styrsky and Eubanks 2007). The aggressive behavior and unicolonial structure of invasive ants may achieve higher colony densities and worker abundance compared with those of native ants (Passera 1994). As a consequence, native ants are displaced, and honeydew sources are dominated by tending invasive ants (Holway et al. 2002). In response to ant attendance, aphids may increase sap feeding (Buckley 1987; but see Yao and Akimoto 2001) as well as the frequency of honeydew excretion, and they may change the sugar composition of honeydew (Fischer et al. 2002). Previous studies have shown that *L. roboris* excretes more drops of honeydew per minute when tended by the invasive ant *Lasius neglectus* than when tended by the native ant *Lasius grandis* (Paris and Espadaler 2009). This apparently is related to the higher intensity of attention shown by this invasive ant (Paris and Espadaler 2009).

As a consequence of aphids feeding on plants, VOC emissions may change and new compounds may appear (Du et al. 1998; Powell et al. 1998). Considering the direct effect of ants on aphid feeding and the indirect effect on plants, we hypothesized that the composition of the VOC blend of *Q. ilex* changes when the aphid *L. roboris* is tended by the invasive rather than the native *Lasius* ant species. Given that the effect of invasive ants on other components of the ecosystem is in part related to their abundance (Holway et al. 2002), we investigated whether the VOCs emitted by holm oak change according to the size of the *L. neglectus* colony.

We hypothesized that VOCs emitted by holm oaks would change qualitatively and quantitatively (i) according to the presence of the aphid *L. roboris*, (ii) depending on which ant species tended the aphids (invasive *L. neglectus* or native *L. grandis*), and (iii) depending on the size of the invasive ant colony. In this study, we aimed: a) to test these three hypotheses and to discover whether such changes occurred; b) to identify the compounds whose emission rates changed when holm oaks became infested with aphids; and c) to determine the compounds linked to ant attendance. We sampled volatiles emitted by holm oak saplings alone or infested with *Lachnus roboris* that were tended or not tended by *L. grandis* or *L. neglectus*.

Methods and Materials

Study Site

This experiment was performed at a field close to the Center of Ecological Research and Forestry Applications (CREAF) at the Autonomous University of Barcelona (41° 30′ N, 2° 6′ E). We used 4-yr-old saplings of *Quercus ilex* (mean±SE diameter, 13.08±0.62 mm) placed in 10-L pots the previous year that were filled with a mixture of commercial humus and a sandy soil (1:1). We used this mixture because previous work showed that in commercial humus alone, ant digging promotes rapid loss of water and consequent drying of the plant.

Ant and Aphid Colonies

At the beginning of June, when new inseminated queens of *L. neglectus* were particularly easy to find under flat rocks and leaf litter, we sampled several queens and workers from an invaded area of the University campus. Fertilized queens are easy to identify because they are huge compared with workers and because they lack wings. Young queens are adopted by workers (Espadaler and Rey 2001). The queens began to lay eggs in the first few days after their capture (Paris, pers. observ.). Given that *L. neglectus* is a polygynic ant species (Boomsma et al. 1990), we placed three queens in each nest to ensure that the colony remained polygynic. In a preliminary experiment, we placed 30, 65, and 150 workers plus 3 queens in artificial nests. We realized that in small colonies of *L. neglectus* the queens were decapitated, but this did not occur in colonies with 150 workers. Therefore, we tried 30 workers per queen and found that with this proportion the colony grew. Finally, we decided to place three queens with 90 or 210 workers, to simulate small and large colonies, respectively. Newly inseminated queens of the native ant *L. grandis* were collected in June of the previous year during a nuptial flight in the campus. Recently landed queens were sampled immediately and brought to the laboratory to rear colonies for the planned experiment. One queen of the native ant *L. grandis* was placed in each nest because this is a monogynic ant species.

Until the experiment started, ants were kept in artificial nests consisting of 3.77 cm^3 (7.5×0.8 cm) nesting tubes, one-third filled with water, plugged with cotton wool, and covered with aluminium foil. The tubes were placed inside plastic boxes (17.7×11.2×3.5 cm) which were set in a growth chamber adjusted to a photoperiod and temperature regime that simulated the ongoing season. We fed the ants *ad libitum* with an artificial diet, according to Bhatkar and Whitcomb (1970), and with Tenebrionidae larvae that had been killed previously by freezing.

In the middle of June, we sampled *L. roboris* aphids, larvae and adults from holm oaks in invaded and control areas. The aphids were pooled and distributed evenly among saplings. We placed 10.5±1.1 (mean±SE aphids per sapling) aphids on saplings 2 d before transferring the artificial ant nests to the pots. Previous work to develop the general design of this experiment showed that after 4–9 days only between 12.5 and 20% of the aphid colonies (a colony = more than five individuals) survived without attention from ants. The ants were starved for 48 hr before transferring the artificial ant nests to the pots. The artificial nest was placed on the soil of the pot without the aluminium paper, to motivate the ants to move into the soil. At that moment, we checked how many queens were still inside the artificial nest. The colonies of the invasive ant began to move immediately, while the colonies of the native ant began to move 3 to 5 hr after placing the tube on the soil. After one day, all the colonies had moved into the soil. During the experiment, Tenebrionidae larvae were provided to avoid aphid predation by the ants. We placed on the soil, close to the sapling trunk, a glass with a freshly killed larva on it. The ants tended the aphids for 1 wk prior to the first measurements of VOCs.

The number of aphids added to each sapling was chosen as a trade-off among several variables: mean aphid abundance per tree, the percentage of infested twigs and mean ant activity per tree that had been estimated under natural conditions on mature holm oaks (oaks that produce acorns), and the size and age of the saplings. Previous data showed that at the University campus, *L. roboris* were scarce on holm oaks colonized by the native or by the invasive ant species (mean aphid abundance per tree ±SE, *L. grandis*: 28.54±11.92, *L. neglectus*: 59.43±16.90; % of infested twigs ±SE, *L. grandis*: 1.37±0.63%, *L. neglectus*: 2.75±0.91%) (Paris and Espadaler 2009). If we had extrapolated these estimations to the saplings used for this experiment, which had a mean of 16 twigs per sapling, only three aphids should have been added to each sapling. This aphid abundance would not have provided enough honeydew for ant colonies and would probably not have exerted any effect on VOC emissions from the holm oak.

On other hand, the aphid abundance should be related to the size of the ant colonies. Larger colonies than those used for this experiment may need a greater supply of honeydew, which would require more aphids. We considered that a 4-yr-old *Q. ilex* sapling would not be able to carry more than 20 aphids without becoming stressed by aphid feeding.

Experimental Design

Five treatments with 4 saplings per treatment were used: sapling alone (T1); sapling + aphids (T2); sapling + aphids + *L. grandis* (native ant) colony (T3); sapling + aphids + *L. neglectus* (invasive ant) colony of 90 workers (small colony) (T4); and sapling + aphids + *L. neglectus* colony of 210 workers (large colony) (T5). T1 represented the situation of basal emissions of VOCs (without any biotic stress). T2 simulated an aphid infestation without ant attendance, to determine which VOCs are elicited by aphid feeding alone (first hypothesis). To investigate the effect of the attendance of different ant species (second hypothesis), we established T3, T4, and T5. The number of *L. neglectus* workers in T4 and T5 was altered to test the effect of colony size (third hypothesis). The morphological traits (height, trunk diameter, dry weight, number and area of leaves) of the saplings did not differ among treatments (Kruskall Wallis, $P>0.324$).

Each pot was placed in a plastic box (49×29×25 cm) filled with the same soil mixture as that used for the pots. The plastic boxes were surrounded by two barriers of non-

toxic sticky resin (Tanglefoot®, Tanglefoot Company, MI, USA) to prevent ants escaping and other ant species entering. This device worked as a buffer area, which developed a gradient of humidity, from the pot, where we watered the plant, to the plastic box. This gradient of humidity was necessary to allow the ants to establish in an environment with sufficient humidity according to their preferences.

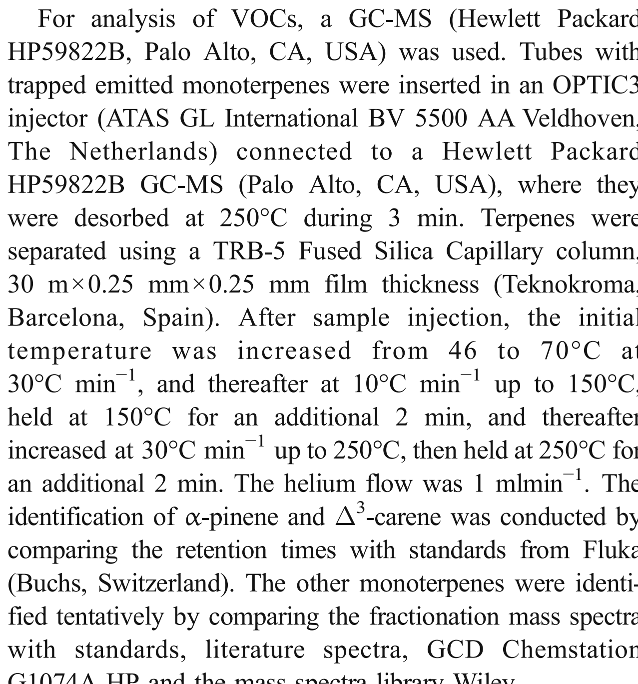
VOC Sampling and Analysis

Before sampling VOCs, we counted the number of aphids. We sampled VOCs 1 wk after the ants had begun to tend the aphids. During the daily sampling protocol, we sampled VOCs from the empty chamber to test whether adsorption occurred on the enclosure walls of the chamber. Given that soil may act as a source or sink for VOCs (Asensio et al. 2007), we sampled VOCs from extra boxes filled with soil but without plants or roots (termed soil boxes). The values obtained from soil boxes represent the blank for the system, so they were subtracted from the measured plant VOC emissions. The plants and soil boxes were watered the evening before sampling of VOCs.

Five saplings, belonging to different treatments, were sampled for VOC emissions each day on 4 different days between 9:30 and 14.00 hr. VOCs were sampled from the soil boxes at 3 different times during the morning: at the beginning of the daily sampling, after 2 samplings of holm oak VOCs had been made, and at the end of the daily sampling.

Sampling of VOC emissions was conducted by enclosing the sapling in a cylindrical dynamic chamber made of Tedlar, which was mounted in a cylindrical aluminium frame (65 cm high, 35 cm diam) and had a volume of 62.5 L. After the cylindrical chamber had been installed on each tree, we waited 10 min before sampling the VOCs. Inside the chamber was a fan that homogenized the air. The air entering and leaving the chamber passed through respective glass tubes (11.5 cm long and 0.4 cm internal diam) filled with Carbotrap C (300 mg), Carbotrap B (200 mg) and Carbosieve S-III (125 mg) adsorbents from Supelco (Bellefonte, PA, USA), which were separated by plugs of quartz wool. These tubes were conditioned previously for 20 min at 350°C with a stream of purified helium.

In order to obtain comparable results, the emission rates were expressed on a leaf dry weight basis. The sampling time was 10 min, and the flow was adjusted to 500 ml min^{-1}. The glass tubes (with trapped VOCs) were stored in a portable refrigerator at 4°C, and taken to the adjacent laboratory. At the laboratory, the glass tubes were stored at−30°C before analysis, for no longer than 48 hr.



For analysis of VOCs, a GC-MS (Hewlett Packard HP59822B, Palo Alto, CA, USA) was used. Tubes with trapped emitted monoterpenes were inserted in an OPTIC3 injector (ATAS GL International BV 5500 AA Veldhoven, The Netherlands) connected to a Hewlett Packard HP59822B GC-MS (Palo Alto, CA, USA), where they were desorbed at 250°C during 3 min. Terpenes were separated using a TRB-5 Fused Silica Capillary column, 30 m×0.25 mm×0.25 mm film thickness (Teknokroma, Barcelona, Spain). After sample injection, the initial temperature was increased from 46 to 70°C at 30°C min^{-1}, and thereafter at 10°C min^{-1} up to 150°C, held at 150°C for an additional 2 min, and thereafter increased at 30°C min^{-1} up to 250°C, then held at 250°C for an additional 2 min. The helium flow was 1 mlmin^{-1}. The identification of α-pinene and Δ^3-carene was conducted by comparing the retention times with standards from Fluka (Buchs, Switzerland). The other monoterpenes were identified tentatively by comparing the fractionation mass spectra with standards, literature spectra, GCD Chemstation G1074A HP and the mass spectra library Wiley.

Frequent calibration (once every 3 analyses) with external standards (α-pinene, Δ^3-carene, *p*-cymene, limonene) was used during the quantification. The detection limit was about 0.6 ng. The calibration curves were always highly significant ($r^2 > 0.99$). At the end of the experiment, the leaves were harvested from all the saplings to determine the dry weight after drying at 70°C in a ventilated oven until the weight was constant. Monoterpene emission rates were calculated as the difference between the amount of each compound emitted in samples from plants and the amount emitted from soil boxes without the plant (blank). The VOC emissions were expressed as μg per g of dry weight of leaves per hour.

Statistical Analysis

We applied Mann-Whitney and Kruskall-Wallis procedures because the data did not fit a normal distribution. To test the first hypothesis, we compared the VOC emissions of holm oaks alone (T1) against the emissions of saplings whose aphids were not tended (T2) by using the Mann-Whitney procedure. Given that the colony size of the invasive ant *L. neglectus* (T4 vs. T5, third hypothesis) did not produce differences in any emitted VOCs (Mann-Whitney, $P>0.2$), these treatments were combined. To test the effect of different tending ant species on VOC emissions (T2 vs. T3 vs. T4+T5, second hypothesis) we used the Kruskall-Wallis test. Two tailed exact *P* values are reported. When significant values arose, we performed the nonparametric Dunn's test for multiple comparisons, according to the process proposed by Zar (1996). Finally, aphid abundance was compared among treatments and by day using the

Kruskall-Wallis procedure and correlated with the total emission of VOCs using the Spearman procedure. All analyses were conducted using STATISTICA 6.0 for Windows (StatSoft, Inc. Tulsa, OK, USA, 1996).

Results

One day after placing the artificial nests on soil pots, all ant colonies had moved into the soil and begun to tend aphids. Total emissions from the holm oak increased 1.31-fold when aphids were present (range 0.62–2.13 $\mu g.g^{-1}.h^{-1}$) while the attendance by *L. grandis* and *L. neglectus* increased the total emissions 5- and 1.19-fold, respectively (*L. grandis* range: 3.60–5.06 $\mu g. g^{-1}.h^{-1}$; *L. neglectus* range: 0.27–2.19 $\mu g.g^{-1}.h^{-1}$). In all treatments, α- and β-pinene, myrcene, and sabinene comprised the main compounds of the *Q. ilex* blend (Fig. 1). Δ^3-Carene, a minor compound, was not emitted by uninfested saplings and was produced only by infested plants, independent of whether the aphids were tended or not (Fig. 2). The emission of myrcene and γ-terpinene increased significantly when holm oak saplings were infested with *L. roboris* (Mann-Whitney, T1 vs. T2, $U_{4,\ 4}$=0, P<0.03 for both components) (Fig. 2).

The terpene emissions of holm oak saplings changed according to the tending ant species (Table 1). In particular, α- and β-pinene, camphene, sabinene and Δ^3-carene increased when aphids were tended by the native ant *L. grandis* compared with unattended aphids or those tended by the invasive ant *L. neglectus* (except camphene and Δ^3-carene) (Kruskall-Wallis, α- and β-pinene: $H_{2,\ 16}$=8.74, P<0.01; sabinene: $H_{2,\ 16}$=8.49, P<0.01, camphene: $H_{2,\ 16}$= 7.65, P<0.02; Δ^3-carene: $H_{2,\ 16}$=7.50, P<0.02; Dunn's test $Q_{0.05,\ 3}$=2.39) (Table 1). The attention of the invasive ant decreased the emission of myrcene (Kruskall-Wallis, $H_{2,\ 16}$= 11.31, P<0.003) but no changes were detected for the other compounds (Table 1).

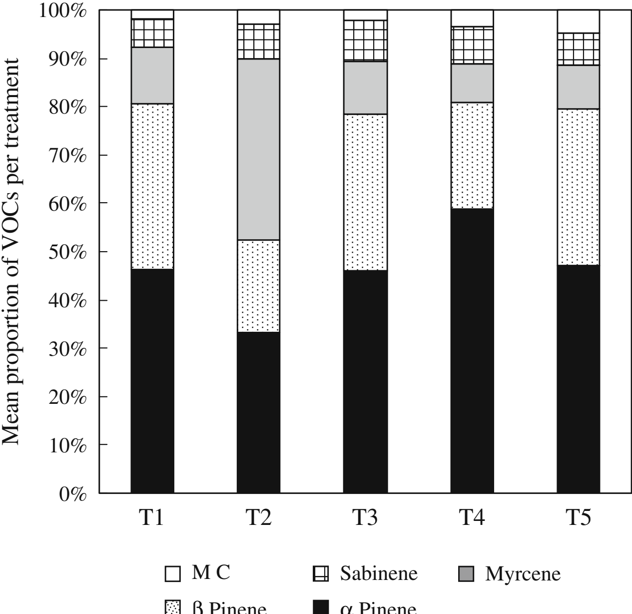


Fig. 1 Proportion of terpenes emitted by *Quercus ilex* saplings alone (T1), from saplings infested with *Lachnus roboris* aphids (T2), saplings with aphids tended by the native ant *Lasius grandis* (T3) or tended by the invasive ant *Lasius neglectus* from small colonies (T4) or from larger colonies (T5). MC: minor compounds (less than 1.5 % of total emission). They were γ-terpinene, terpilone, α-thujene, Δ^3-carene, Camphene and α-terpine

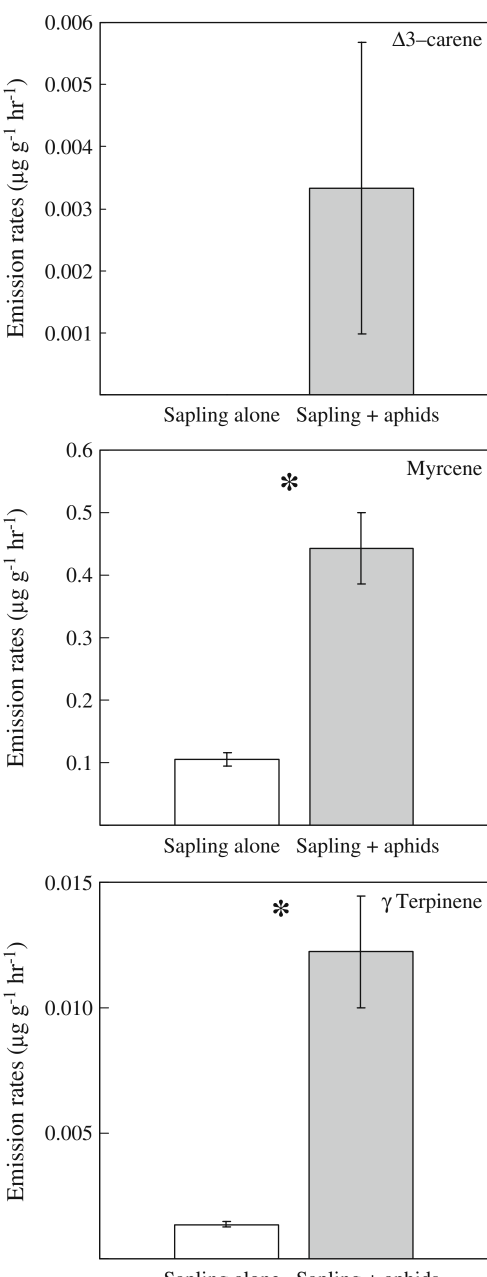


Fig. 2 Mean and standard error of volatile organic compounds emitted at different rates by *Quercus ilex* saplings alone (T1) or infested with *Lachnus roboris* aphids (T2). Asterisk shows statistical significance between treatments according to Mann Whitney's test (P<0.05)

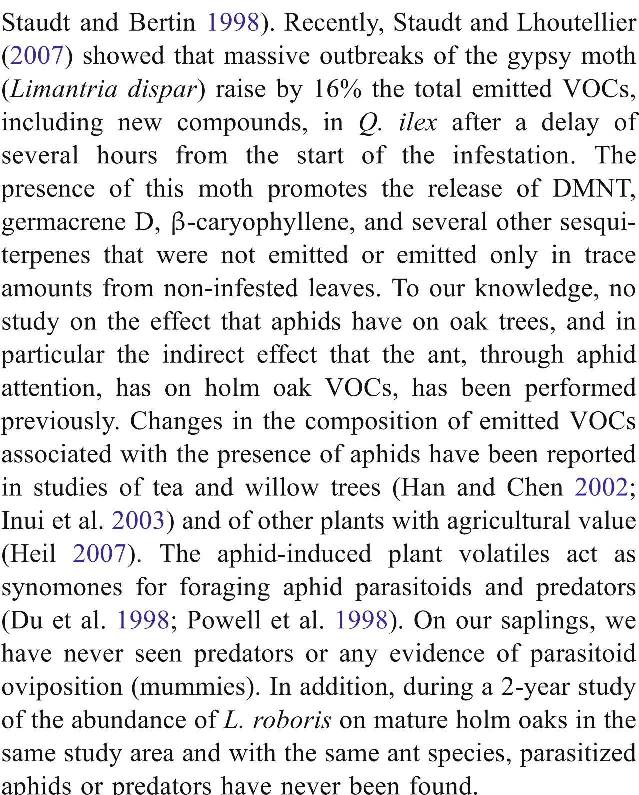
Table 1 Means and standard error (μg. $g^{-1}.h^{-1}$) of main compounds emitted by *Quercus ilex* saplings (N=4 for T2 and T3 and N=8 for T4+T5) infested with not tended aphids (T2) or infested with aphids tended by the native ant *Lasius grandis* (T3) or by the invasive ant *Lasius neglectus* (T4 and T5 coupled). Different letters indicate statistical differences of non parametrical comparisons treatments (Dunn's test, P<0.05)

Compound	T2	T3	T4+T5
α-Thujene	0.007±0.004 a	0.015±0.005 a	0.005±0.001 a
α-Pinene	0.391±0.182 a	2.072±0.033 b	0.551±0.105 a
Camphene	0.007±0.003 a	0.047±0.014 b	0.012±0.004 ab
Sabinene	0.084±0.042 a	0.387±0.045 b	0.075±0.017 a
β-Pinene	0.227±0.105 a	1.454±0.269 b	0.306±0.075 a
Myrcene	0.443±0.057 a	0.482±0.044 a	0.093±0.020 b
Δ^3-Carene	0.003±0.002 a	0.018±0.001 b	0.010±0.003 ab
α-Terpine	0.004±0.001 a	0.003±0.001 a	0.001±0.000 a
γ-Terpinene	0.012±0.002 a	0.011±0.004 a	0.013±0.005 a
Terpinolene	0.001±0.000 a	0.002±0.001 a	0.005±0.002 a

The abundance of aphids decreased with time for all infested saplings (mean aphids per day ±SE, day 1: 10.5±1.1, day 2: 17.8±0.9, day 3: 6.5±1.0, day 4: 3.3±0.7) ($H_{2,\ 16}$= 11.59, P<0.01), but aphid abundance did not differ among treatments (mean aphids per treatment ±SE, T2: 13±4.2; T3: 9.4±1.6; T4+T5: 11.9±1.4; $H_{2,\ 16}$=0.02, P<0.99). No correlation was found between aphid abundance and total emitted VOCs (r_s=−0.01, P<0.9, N=12).

Discussion

The infestation by *L. roboris* elicited the emission of Δ^3-carene and increased significantly the emissions of myrcene and γ-terpinene. The most important biogenic volatile organic compounds (BVOCs) produced by terrestrial plants are isoprene and monoterpenes, such as those mentioned above (Karl et al. 2009). In the Mediterranean basin there are several plants that emit isoprenoids, particularly those of the *Quercus* genus (Owen et al. 2002). Among them is the holm oak (*Quercus ilex*), which despite lacking storage organs is a strong emitter of monoterpenes in comparison with other *Quercus* spp. that emit mainly isoprene (Kesselmeier and Staudt 1999). The blend produced by holm oak has been studied mainly in relation to its modulation by abiotic factors (temperature, light, drought, different CO_2 concentrations) (Staudt and Bertin 1998; Llusia and Peñuelas 1999; Rapparini et al. 2004; Peñuelas and Staudt 2010). In accordance with previous reports of holm oak terpene emissions, we found that the blend produced by holm oak saplings was composed mainly of α- and β-pinene, sabinene and myrcene (Street et al. 1997;



Staudt and Bertin 1998). Recently, Staudt and Lhoutellier (2007) showed that massive outbreaks of the gypsy moth (*Limantria dispar*) raise by 16% the total emitted VOCs, including new compounds, in *Q. ilex* after a delay of several hours from the start of the infestation. The presence of this moth promotes the release of DMNT, germacrene D, β-caryophyllene, and several other sesquiterpenes that were not emitted or emitted only in trace amounts from non-infested leaves. To our knowledge, no study on the effect that aphids have on oak trees, and in particular the indirect effect that the ant, through aphid attention, has on holm oak VOCs, has been performed previously. Changes in the composition of emitted VOCs associated with the presence of aphids have been reported in studies of tea and willow trees (Han and Chen 2002; Inui et al. 2003) and of other plants with agricultural value (Heil 2007). The aphid-induced plant volatiles act as synomones for foraging aphid parasitoids and predators (Du et al. 1998; Powell et al. 1998). On our saplings, we have never seen predators or any evidence of parasitoid oviposition (mummies). In addition, during a 2-year study of the abundance of *L. roboris* on mature holm oaks in the same study area and with the same ant species, parasitized aphids or predators have never been found.

The volatiles induced by insect damage depend on the feeding habits (Delphia et al. 2007) and on the density of herbivores (Dicke et al. 1993; Tumlinson et al. 1993). In general, phloem feeders induce lower emissions of plant volatiles compared with chewing insects, even under high levels of plant infestation (Staudt and Lhoutellier 2007). Aphids are all phloem feeders, and their feeding behavior comprises two phases. The first is the probing phase, during which the aphid inserts its stylet and tests sap quality, and the second is the ingestion phase, during which the aphid regulates sap intake using its cybarial pump (Miles 1987). During probing, the stylet of the aphid transiently punctures epidermal, mesophyll, and parenchyma cells. For the most part, however, aphids penetrate plant tissues via a primarily intercellular route, and their impact on plants is thought to be largely due to removal of photosynthesis products and injection of saliva (Goggin 2007). After identifying a suitable plant and locating on the plant, aphids begin to ingest sap from the sieve tube (Miles 1987). The ability to prevent sieve tube plugging is an important adaptation that allows aphids to remain at a single feeding site for hours at a time (Goggin 2007). Despite the low level of cell damage that aphids produce when inserting their stylets between cells (Goggin 2007), some aphid species may trigger large responses in plant volatiles shortly before starting to feed. The plant response induced by aphids usually appears with a delay of several hours after infestation (El-Aouni et al. 2007) and may cause systemic production of volatiles for up 24 hrs after the aphids have been removed from the

plant (Guerrieri et al. 1996). Du et al. (1998) found that 6-methyl-5-hepten-2-one, linalool, geranic acid, and (E)-β-farnesene appeared during the first day after *Acyrthosiphon pisum* infestation of broad bean plants and increased in concentration with increasing duration of aphid feeding. These facts highlight an immediate plant response that is amplified with time and does not stop immediately.

In our study, we began to sample VOCs 1 week after aphids began to feed on *Q. ilex* saplings. This suggests that we probably sampled VOCs when the response of the saplings was amplified. Moreover, the response of the saplings may have continued, because aphid abundance decreased with time in all treatments, but no correlation between aphid abundance and total emitted VOCs was found. The decrease in the number of aphids could be due to ant predation, despite the fact that ants were provided with freshly killed Tenebrionidae to prevent aphid predation. On holm oak, the main prey of *L. grandis* and *L. neglectus* are Psocoptera (33.8% and 31.1% of total predated insects, respectively) and the untended aphid *Hoplocallis picta* (35.4% and 37.8% of total predated insects, respectively) (Paris and Espadaler 2009). *Lachnus roboris* represents only 1.5% and 2.5% of total predated insects for *L. grandis* and *L. neglectus*, respectively (Paris, unpublished data). We have never seen ants carrying aphids to the nest but, as mentioned, both ant species prey on *L. roboris* so we cannot completely discount aphid predation.

As stated above, our results showed that feeding by *L. roboris* induced the emission of Δ^3-carene and increased the emission of myrcene and γ-terpinene. It has already been reported that mature holm oaks release Δ^3-carene in very low quantities (1.9–8.3% of total emissions depending on the month) (Street et al. 1997), but previous studies did not report whether or not the sampled holm oaks were infested by aphids because this trait was not related to the aims of those studies. The reported temporal variations of Δ^3-carene emissions in these studies coincided with the population dynamics of *Lachnus roboris* on holm oaks (Street et al. 1997), which shows a main peak abundance at the end of spring or beginning of summer and a secondary peak abundance at the beginning of autumn (Paris and Espadaler 2009). This leads us to think that the sampled mature holm oaks could have been infested by aphids.

Why Different Species of Tending Ants Resulted in Different Emissions

Our results also showed that the attendance of ants may change the VOC emissions of plants, and that their effect is dependent on the ant species. When the local ant *L. grandis* was present, α- and β-pinene, sabinene, camphene, and Δ^3-carene increased significantly compared with the emissions of saplings with untended aphids. On the other hand, attention from the invasive ant *L. neglectus* decreased only the emission of myrcene, one of the main VOCs emitted by holm oak. However, when the effect of *L. neglectus* colony size was tested, no difference was found in BVOC emissions. We suspect that this finding could be explained by the adjustment that *L. roboris* can make to its rates of feeding and excretion when tended by different ant species. According to electrical penetration graphs obtained during feeding, aphids are able to regulate the ingestion of sap (Tjallingii 1995). Once a plant is accepted as a suitable source, the total time of penetration of sieve elements is the same with or without the presence of tending ants (Rauch et al. 2002). The total duration of penetration is composed of two waveforms: E1 (salivation) and E2 (ingestion of sap). Rauch et al. (2002) suggested that it is the duration of ingestion of sap that aphids change when they are tended. However, these authors were unable to distinguish between E1 and E2 waveforms but suggested that an increase in sap ingestion may occur as honeydew excretion increases when ants tend aphids. Therefore, *L. roboris* probably adjusted its feeding rate, not necessarily by increasing it but by modifying its frequency and the opening time for the cybarial pumping of sap. In addition, aphids are able to regulate the number of drops excreted per minute and the mean volume of honeydew drops, depending on whether or not they are tended. It has been demonstrated that when aphids are tended by ants, they increase the number of drops per minute but decrease the volume of drops (Yao and Akimoto 2001). This evidence shows that aphids apparently maintain a constant excretion rate (drops* $hour^{-1}$ * $aphid^{-1}$ per drop volume) regardless of whether tended or not. The excretion frequency (drops* $hour^{-1}$ * $aphid^{-1}$) was significantly higher when *L. roboris* was tended by the invasive ant (Paris and Espadaler 2009). However, we cannot determine whether or not the volume of the drops changes when aphids become tended by the invasive or by the native ant. Therefore, we cannot be totally sure whether the rate of honeydew excretion changes. However, some kind of regulation of excretion rate should have occurred because the frequency (drops per minute) changed. This change in the frequency of excretion of *L. roboris* could reflect an adjustment of aphid feeding when tended by *L. neglectus* instead of *L. grandis*, which may affect the VOC emissions of the plant.

Ants and Aphids as Sources of VOCs

Volatiles are involved in ant social behaviors such as recruitment, foraging, alarm, caste and nestmate recognition (Hölldobler and Wilson 1990), as well as in their interactions with other species. On the other hand, aphids emit volatiles in association with crowding, dispersion, alarm, and sexual attraction (Pickett et al. 1992). As a

result, in our experiment both insects may have been sources of BVOCs. We searched each compound detected in this study in the literature and in Pherobase, a web-based library of BVOCs elicited from plants and insects (http://www.pherobase.com/). Terpenes can be emitted in large amounts by aphids as a component of the alarm blend (Francis et al. 2005). In a study of the volatile compounds emitted by 23 different aphid species, myrcene, α- and β-pinene, camphene, and γ-terpinene were emitted by several aphid species after crushing but not during their feeding on plants (Francis et al. 2005). Total emission of the main component of the alarm pheromone in the pea aphid (*Acyrthosiphon pisum*), when attacked by predators, averages 16.33±1.54 ng per aphid and ranges from 1.18 to 48.85 ng per aphid (Schwartzberg et al. 2008).

Concerning ants, α- and β-pinene, sabinene and camphene have been detected as components of the alarm blend in the ants *Crematogaser laboriosa*, *Pristomyrmex pungens* (except myrcene), *Lasius niger* (except camphene), *Technomyrmex albipes* (except camphene and β-pinene), and *Tetramorium caespitum* (except camphene and β-pinene) (Hayashi and Komae 1980). However, these authors gave information about the proportion of each compound represented in the blend and not the absolute values. Janssen et al. (1997) found that β-pinene occurs only in picogram quantities at foraging trails of *P. pungens* and is a major component of monoterpene secretions from the poison gland of this species. In leaf cutting ants, when a foraging trail is made by dribbling volatile trail pheromone, only 40 pg/μl–0.4 ng/μl is necessary to trigger trail-following activity (Riley et al. 1974). At high concentrations (4 μg/μl), the ants are repelled from the trail (Riley et al. 1974). In ants of *Tetramorium* sp., the Dufour's gland (a gland that produces several volatiles) may contain between 30 and 70 ng of a chemical blend per worker (Hölldobler and Wilson 1990). In workers of *Pogonomyrmex badius*, the main alarm pheromone is stored in quantities of 0.2–34 μg in the mandibular gland reservoir (Hölldobler and Wilson 1990). These examples highlight that the emission of VOCs by aphids and ants occurs at a level three or four orders of magnitude below those detected in this study, although ants can store high quantity of VOCs. Concerning Δ^3-carene, there is no evidence that this component has been secreted by ants or by aphids.

Should Aphids and Tending Ants be Considered as a New Driving Factor for Monoterpene Emissions in BVOC Inventories of the Mediterranean Region?

To answer this question we should consider the seasonality of monoterpene emissions by *Q. ilex*, the abundance of trees infested with aphids and the amount of honeydew collection by ants, whether these factors overlap in time, and the intensity of ant-aphid mutualism. In Europe, forests cover 40.4% of the vegetated surface and are the major source of monoterpene emissions (Karl et al. 2009). Monoterpene emissions arise in summer, and the main contribution comes from forests of the Boreal and Mediterranean regions where *Pinus* spp. and *Q. ilex* are the main emitters as a result of the area they cover (Karl et al. 2009). According to Köble and Seufert (2002), *Q. ilex* contributes 2.1% to the forested area and is the first tree species of newly synthesized monoterpene emissions (standard emissions factor of 43 μg $g^{-1}h^{-1}$). Monoterpenes are emitted mainly by *Q. ilex* during the summer, especially in July and August, owing to the light and temperature dependence of their production (Staudt and Bertin 1998) and the seasonality shown in the monoterpene synthase activity of *Q. ilex* (Fischbach et al. 2002). At fragments of mixed forest colonized by *L. neglectus* or by local ants, the number of trees colonized by ants peaks in June or July depending on the year (Paris 2007). Honeydew collection per holm oak, by *L. neglectus* and *L. grandis*, follows the same temporal variation (Paris and Espadaler 2009). This evidence suggests that the period of maximum monoterpene emission from forests, the number of trees infested with aphids, and honeydew collection by ants overlap in time. At forest stand level, it is the intensity of aphid infestation, i.e., the area covered by infested trees and the abundance of different tending ant species that could change the rate of monoterperne emission for the purposes of the BVOC inventories. In Spain, *L. roboris* seems to be a rare aphid (Paris and Espadaler 2009) that does not exceed a level of 10% of infested trees at stand level (Melia et al. 1993), in contrast to the situation in temperate forests, where this aphid species is more frequent (Sudd and Sudd 1985). However, *Lasius* spp. and other tending ant species tend many aphid species on different tree species, especially *Pinus pinaster* and *P. halepensis*, two monoterpene emitters that are broadly distributed in the Mediterranean region (Karl et al. 2009). Thus, monoterpene emission by other tree species apart from *Q. ilex* could be modified also by tending ants.

Monoterpenes play different roles in the troposphere and for plants. In the troposphere, monoterpenes can contribute to the formation of O_3 from a series of reactions with NOx emitted from anthropogenic and natural sources, and to the formation of secondary organic aerosols as a result of the gas/particle partitioning of their reaction products (Karl et al. 2009). At the plant surfaces, as well as in the mesophyll monoterpenes quench O_3 that protects the plant against oxidative stress (Holopainen 2004), and protects the plant against higher temperatures (Copolovici et al. 2005). Taking into account the facts that the invasive ant *L. neglectus* decreased total monoterpene emissions by 76% in comparison with *L. grandis*, that this invasive ant is

expanding its range in Europe (Espadaler et al. 2007), and that it is displacing native ants we suggest that massive aphid outbreaks in areas where this invasive ant occurs should be considered when modelling the emission of VOCs. A rapid way to include this information in inventories will be to estimate the proportion of trees colonized only by *L. neglectus*. Moreover, it will be necessary to know how monoterpene emission changes in other tree species colonized by *L. neglectus*, in particular other *Quercus* and *Pinus* spp., which are the main monoterpene emitters in the Mediterranean region (Karl et al. 2009).

In summary, the results of this study highlight that the amount of various terpenes emitted by holm oaks can increase or decrease (depending on the compound), or new compounds can be released, according to the presence of aphids and the identity of the tending ant species. However, the range of ant colony sizes tested here had no effect on terpene emission rates. We discard the possibility that changes in α- and β-pinene, sabinene, and camphene emissions were caused by emissions from ants or aphids because of the scale at which these insects emit VOCs. Therefore, from an ecological perspective, the altered monoterpene composition of the *Q. ilex* blend associated with the feeding of aphids and tending ants may affect the protection that monoterpenes exert against episodes of high O_3 and temperatures during the Mediterranean summer (Pinto et al. 2007).

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Cucumber Volatile Blend Attractive to Female Melon Fly, *Bactrocera cucurbitae* (Coquillett)

Matthew Samuel Siderhurst · Eric B. Jang

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Abstract The melon fly, *Bactrocera cucurbitae* (Coquillett), is a serious crop pest throughout the Asia Pacific subcontinent and Southeast Asia, causing damage to tree fruits, cucurbits, and related crops. Attractants for female melon flies are of particular interest as they could be used in control tactics to reduce pest levels. Previous work has shown that freshly sliced cucumbers are attractive to female melon flies, but the compounds responsible for this attraction were not identified. The objective of the present study was to create a synthetic lure for female *B. cucurbitae* based on its close association with Cucurbitaceae. Coupled gas chromatography-electroantennogram detection (GC-EAD) analysis of fresh and aged puréed cucumbers identified 31 compounds that were detected by females. Compounds that elicited EAD responses initially were screened as single components in glass McPhail traps in outdoor rotating olfactometer experiments. Four criteria were used to select compounds for testing in blends: a) strength of EAD response elicited; b) amount of compound present; c) relative attractiveness of a single compound; and d) compound novelty to Cucurbitaceae. Several synthetic blends attracted significant numbers of females in outdoor rotating olfactometer experiments; a nine-component blend (lure #7) was the most attractive. Field captures of female *B. cucurbitae* in traps baited with lure #7 were twice those in traps baited with Solulys protein bait. Besides having a female-biased attraction, this lure may have several advantages over protein baits: it can be used with a dry trap, is long lasting, and it captured low numbers of non-target species. Possible applications of this lure include trapping (for detection and/or monitoring/delimitation) and control/eradication (e.g., mass trapping, attract-and-kill, or as an attractant for existing protein insecticide bait sprays such as GF-120).

Key Words Melon fly · Gas chromatography-electroantennogram detection · Kairomone · Female attractant · Tephritidae · Cucumber · Volatiles · Bioassay · *Bactrocera cucurbitae*

M. S. Siderhurst (✉)
Department of Chemistry, Eastern Mennonite University,
1200 Park Rd.,
Harrisonburg, VA 22802, USA
e-mail: ms826@emu.edu

E. B. Jang
United States Department of Agricultural, U.S. Pacific Basin
Agricultural Research Center, Agricultural Research Service,
P.O. Box 4459, Hilo, HI 96729, USA

Introduction

The melon fly, *Bactrocera cucurbitae* (Coquillett), is a serious pest of tropical agricultural crops, causing damage to at least 81 host species (Dhillon et al. 2005). Infestations are particularly high in cucurbit crops, in which fruit losses can range from 30 to 100% (Dhillon et al. 2005). The melon fly was the first (in 1895) of four tephritid fruit flies to invade Hawai'i. Melon fly, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the oriental fruit fly, *Bactrocera dorsalis* (Hendel), cause up to an estimated 15 million USD/year in direct costs to Hawaiian agriculture (Nakahara 1977), and 300 million USD/year in lost markets (Miller et al. 1992). Melon fly also is considered a major quarantine pest for subtropical agricultural states, such as California, Florida, and Texas, which deploy grid trapping for early detection of this pest.

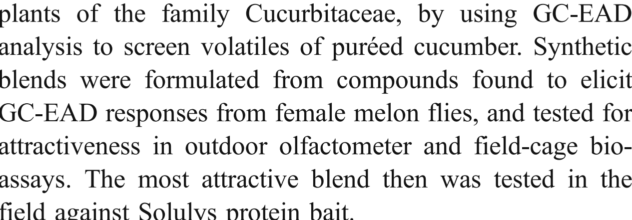
Plant semiochemicals are critical to fruit fly behaviors, such as host finding and oviposition, and have been studied

as sources of attractants for detection and control (Jang and Light 1996; Light and Jang 1996). While several attractants for males currently are known (raspberry ketone and its derivatives cuelure and melolure), chemicals that attract females are limited to food-type attractants, such as fermenting sugars, hydrolyzed protein, and yeast (Jang and Light 1996). Unfortunately, these liquid lures lack potency, have limited field life, are difficult to handle, and attract non-target species. Non-food-based female attractants are a promising, if elusive, goal for control programs, given that such attractants have the potential to kill individual females and prevent future offspring (Jacobson et al. 1971; Jang and Light 1996; Light and Jang 1996). The most promising sources for female attractants are plant materials, such as host fruits and leaves. Host fruits are of particular interest since some tephritid females prefer host fruit volatiles when searching for oviposition sites (Jang and Light 1996; Light and Jang 1996). However, isolation and identification of attractant compound(s) from fruits often is difficult, as the fruit odors can be complex blends of chemicals, which may change composition during fruit ripening. Additionally, it is possible that polyphagous fruit flies, such as the melon fly, detect and/or respond to a wider array of chemical cues than do oligophagous fruit flies, such as the apple maggot, *Rhagoletis pomonella* (Walsh) (Siderhurst and Jang 2006).

Rapid screening of multiple compounds of interest and/or analysis of complex blends has been facilitated by coupled gas chromatography-electroantennogram detection (GC-EAD) systems, which have been successfully used to identify fruit fly attractants from several plant sources (Cosse et al. 1995; Zhang et al. 1999; Nojima et al. 2003a, b; Malo et al. 2005; Siderhurst and Jang 2006; Alagarmalai et al. 2009). Identification of electroantennogram-active fruit volatiles for *R. pomonella* has led to the development of agriculturally useful control lures (Zhang et al. 1999; Stenliski and Liburd 2002). Attractive plant-host volatiles also have been identified without use of electrophysiology by gas chromatography-mass spectrometry (GC-MS) and behavioral bioassays (Nigg et al. 1994; Hwang et al. 2002; Massa et al. 2008).

Melon fly has a strong host association with a variety of cucurbits, and its attraction to cucumber, zucchini, bitter melon, kabocha, cantaloupe, and ivy gourd has been investigated (Miller et al. 2004; Piñero et al. 2006). Freshly sliced cucumber is especially attractive (Miller et al. 2004; Piñero et al. 2006), and has been utilized to investigate melon fly behavior in field situations (Prokopy et al. 2003, 2004). Additionally, farmers in Hawai'i have used fresh-sliced or mashed cucumber for many years to attract and control female melon fly.

The objective of the present study was to find a female attractant for melon fly, based on its close association with



plants of the family Cucurbitaceae, by using GC-EAD analysis to screen volatiles of puréed cucumber. Synthetic blends were formulated from compounds found to elicit GC-EAD responses from female melon flies, and tested for attractiveness in outdoor olfactometer and field-cage bioassays. The most attractive blend then was tested in the field against Solulys protein bait.

Methods and Materials

Insects Adult flies used in bioassays were obtained as pupae from the mass-rearing unit at the USDA ARS Pacific Basin Agricultural Research Center, Mass-Rearing Facility in Honolulu, Hawai'i. Larvae were reared on a standard wheat, sugar, yeast diet (Tanaka et al. 1969). Pupae were shipped by air to Hilo, HI, where they were placed into 30× 30×30 cm aluminum screen cages, containing sugar, water, and hydrolyzed protein. Flies were held at 24°C, 60–80% relative humidity, and 12 L:12D photoperiod until use. Prior to testing, flies were immobilized at 5°C, separated into groups of either 50 males or females, and then held at room temperature (24°C) for at least 1 h before use. Flies were approximately 9–11 d old when tested and presumed to be mated (>95% of females from mixed cages are mated by d 7; Jang et al. 1997).

Fruits Cucumber fruit (variety unknown) was purchased from local supermarkets and stored no longer than 1 wk at 4°C before use.

Volatile Sampling Headspace sampling from fresh, 1-, 3-, and 5-d-old puréed cucumber slurries was conducted using Porapak Q absorbent (50/80 mesh; Alltech, Deerfield, IL, USA). The absorbent (1 g) was packed between glass wool in a Pasteur pipet, preconditioned at 70°C for 16 h, washed with dichloromethane, and then dried under carbon-filtered nitrogen. Individual, intact cucumbers (~200 g) were puréed and immediately transferred to a 4-l, round-bottom aeration chamber for aging (at 28°C) and volatiles collection. Carbon-filtered nitrogen (1 l. min^{-1}) was used to sweep volatiles from the chamber through the collection pipet for 1 h. Collections from cucumber slurries were made immediately following maceration (fresh), and at 1-, 3-, and 5-day intervals. No air flow was passed through the aeration chamber during the aging periods. Volatiles were eluted from the absorbents with 1 ml of dichloromethane, concentrated under a purified nitrogen stream and stored at −8°C until analysis.

Headspace collections also were made with three different solid phase microextraction (SPME) fibers: polydimethylsiloxane (PDMS; film thickness 100 µm; Supelco Inc., Bellefonte, PA, USA), polyacrylate (PA; film thick-

ness 85 μm), and carbowax/divinylbenzene (CW/DVB; film thickness 70 μm). SPME collections were conducted to assess whether additional cucumber volatiles, not collected by Porapak Q, elicited EAD responses from melon fly antennae. SPME fibers were conditioned before use in a GC injector (250°C) for 30 min. Puréed cucumber (~100 g) immediately was transferred after blending to a 500-ml clear, straight-sided jar, with a screw-top lid fitted with a Teflon liner. Collections from cucumber slurries were made immediately following maceration (fresh), and at 1-, 3-, and 5-day intervals. No air flow was passed through the aeration chamber during the aging periods. The SPME needle was inserted through a small hole in the lid, and the fiber was exposed for 1–24 h to absorb volatiles. SPME fiber exposure time was varied to ensure detection of minor volatile components. The fiber was inserted into the GC injection port to desorb volatiles.

Instrumentation Electroantennogram responses to cucumber volatiles were recorded using an Agilent Technologies (Palo Alto, CA, USA) 6890 GC coupled to a Syntech (Hilversum, The Netherlands) EAD system. The GC was equipped with an HP-5 column (30 m×0.32 mm ID 0.25 μm film thickness), with hydrogen as carrier (2.3 ml. min^{-1}) and makeup (added before the splitter, at 10 ml. min^{-1}) gas. A Graphpack-3D/2 flow splitter was attached to the end of the column, and the effluent was split 1:1 between the flame ionization detector (FID) and the EAD (via a 250°C transfer line). The injector, in splitless mode, and FID were held at 250°C and 275°C, respectively. The oven temperature program began at 80°C (held for 1 min), then was increased at 10°C.min^{-1} to 240°C, and held for 13 min. Whole female fly heads were removed with forceps and secured with electrode gel (Spectra 360, Parker Laboratories, Inc., Fairfield, NJ, USA) between the electrodes of a Syntech EAG probe antenna holder. Antennal preparations were discarded after one or two runs. Humidified air (400 ml.min^{-1}) was used as a carrier for effluent from the EAD transfer line to the antennal preparation. The signals generated by the EAD and FID were passed through a Syntech NL 1200 high-impedance amplifier and analyzed by using Syntech GC-EAD2000 software version 2.5. For Porapak Q collections, four runs with high signal:noise ratios were conducted to determine consistent EAD responses for each treatment. Peaks eliciting EAD responses in at least 3 of the runs were subjected to identification by GC-MS. SPME collections were not replicated more than twice per fiber type or collection time, with peaks with reproducible EAD responses subjected to identification by GC-MS.

GC-MS analysis was performed on an Agilent 6890N GC interfaced with a Hewlett-Packard 5973 Mass Selective Detector. The GC was equipped with an HP-5MS column (30 m×0.25 mm ID 0.25 μm film thickness), which was temperature programmed from 60 to 300°C at 10°C.min^{-1} following a 1 min. delay. The injector temperature was 250°C, and helium was the carrier gas (1.1 ml.min^{-1}.). Compounds from cucumber were identified on the basis of their retention times and mass spectra (NIST98 mass spectral database) and in comparison with authentic standards.

Chemicals Chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Compound purities were >98% based on GC-FID analysis. In all bioassay and field experiments, compounds were presented neat in 1- or 5-μl glass capillary tubes, either as single components in capillaries or as groupings of capillaries with each component in a separate capillary. Compounds used in lure compositions were selected according to three parameters: they must have evoked reproducible and relatively high amplitude EAD responses, their relative amounts in cucumber volatiles (determined by GC-FID), and their relative attractiveness (trap captures) in olfactometer bioassays using single compounds.

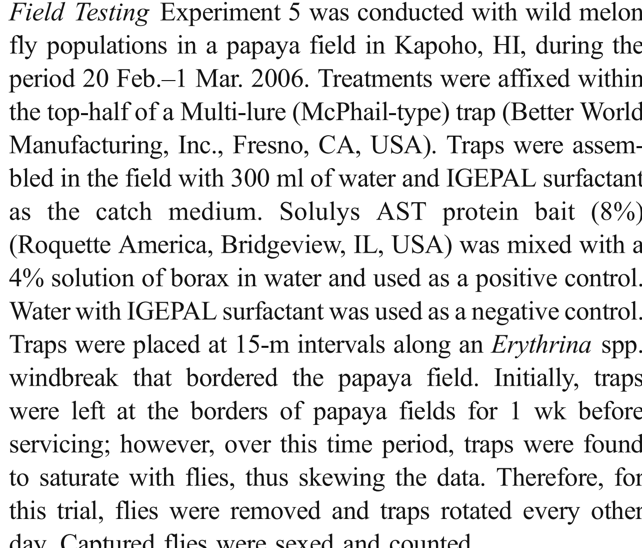
Bioassays An outdoor multiple-trap rotating olfactometer was used to evaluate the attractiveness of single compounds and blends of cucumber volatiles to melon flies. The outdoor olfactometer consisted of a 3×3×2.5 m rectangular wooden-framed screen cage (Jang et al. 1997), used to compare different treatments in competitive tests. A rotating hub with 10 arms, each ca 62 cm long, was hung from the center of the cage, ca 1.8 m from the floor. The arms of the motorized hub unit revolved at 1.5 rpm. Treatments were prepared by affixing, with tape, glass capillaries containing synthetic compounds within small invaginated glass McPhail traps. The bottoms of these traps were filled with 30 ml of water containing 2 drops of the nonionic surfactant IGEPAL (Sigma-Aldrich). Traps were assigned randomly to hub arms. Approximately 500 flies of each sex were placed in the outdoor cage and left for approximately 4 h (initiated 09:00–10:00; terminated 13:00–14:00). Tests were conducted under ambient outside conditions (usually 23–28°C) under natural (indirect) light. Tests never were conducted on days when rain was persistent and heavy. At the end of each test, flies were removed from the traps, and counted by sex. Between replicates, capillaries were removed from the traps, and the traps washed in hot soapy water. Each experiment was replicated four times.

Experiment 1 evaluated the attractiveness of individual chemicals from cucumber volatiles to melon flies. Five or six treatment traps, containing 1-μl capillaries, and a control were assigned randomly to hub arms and run together as a replicate group. To standardize for different

ambient conditions (e.g., temperature, humidity, light levels, etc.), the number of flies caught by the negative control (water plus surfactant only) was subtracted from all other trap catches.

Experiments 2 and 3 tested melon fly attraction to blends of six nine-carbon aldehydes or alcohols. The structurally similar (*Z*)-6-nonenal (*Z*6-9:al), (*E*,*Z*)-2,6-nonadienal (*E*2*Z*6-9:al), (*E*)-2-nonenal (*E*2-9:al), and (*Z*)-6-nonen-1-ol (*Z*6-9:1-ol) were among the most abundant chemicals collected on Porapak Q, while nonanal (9:al) and 1-nonanol (9:1-ol) were apparent in SPME collections, and both elicited EAD responses from female melon flies. Experiment 2 tested two blends of all six compounds: four compounds (*Z*6-9:al, *Z*6-9:1-ol, 9:al, 9:1-ol) emitted from 1-μl capillaries and two compounds (*E*2*Z*6-9:al, *E*2-9:al) from 5-μl capillaries (lure #1), and all six from 5-μl capillaries (lure #2), along with a control. Experiment 3 was a subtraction bioassay based on lure #1. In addition to lure #1, four blends (lures #3–6) with various compounds missing were tested.

Experiment 4 tested melon fly attraction to lures containing an expanded set of compounds, all shown to elicit both high EAD and behavioral responses from *B. cucurbitae*. Given the preference of *B. cucurbitae* for freshly sliced cucumber (Miller et al. 2004; Piñero et al. 2006), chemicals released from fresh cucumber were selected over those from aged cucumber. Lure #7 was based on lure #3 plus five additional compounds: 1-octen-3-ol (1-8:3-ol), acetic acid (AA), hexanal (6:al), (*E*)-2-octenal (*E*2-8:al), and 1-hexanol (6:1-ol). Lure #8 was based on lure #3 plus 2 additional compounds, 1-8:3-ol and AA. Lure #9 was composed of *Z*6-9:al, *Z*6-9:1-ol, 1-8:3-ol, and AA.



Field Testing Experiment 5 was conducted with wild melon fly populations in a papaya field in Kapoho, HI, during the period 20 Feb.–1 Mar. 2006. Treatments were affixed within the top-half of a Multi-lure (McPhail-type) trap (Better World Manufacturing, Inc., Fresno, CA, USA). Traps were assembled in the field with 300 ml of water and IGEPAL surfactant as the catch medium. Solulys AST protein bait (8%) (Roquette America, Bridgeview, IL, USA) was mixed with a 4% solution of borax in water and used as a positive control. Water with IGEPAL surfactant was used as a negative control. Traps were placed at 15-m intervals along an *Erythrina* spp. windbreak that bordered the papaya field. Initially, traps were left at the borders of papaya fields for 1 wk before servicing; however, over this time period, traps were found to saturate with flies, thus skewing the data. Therefore, for this trial, flies were removed and traps rotated every other day. Captured flies were sexed and counted.

Data Analysis Results were analyzed by ANOVA, and means compared using Tukey's HSD studentized range test (Proc GLM, SAS 2000). Trap catches from Experiments 4 and 5 were transformed by $\sqrt{X+0.5}$ to normalize the distribution and stabilize variance, before statistical analysis.

Results

GC-EAD analysis of headspace volatiles from puréed cucumber revealed 31 compounds that elicited consistent electroantennogram responses from *B. cucurbitae* females (Fig. 1, Tables 1, 2). GC-MS analysis, along with

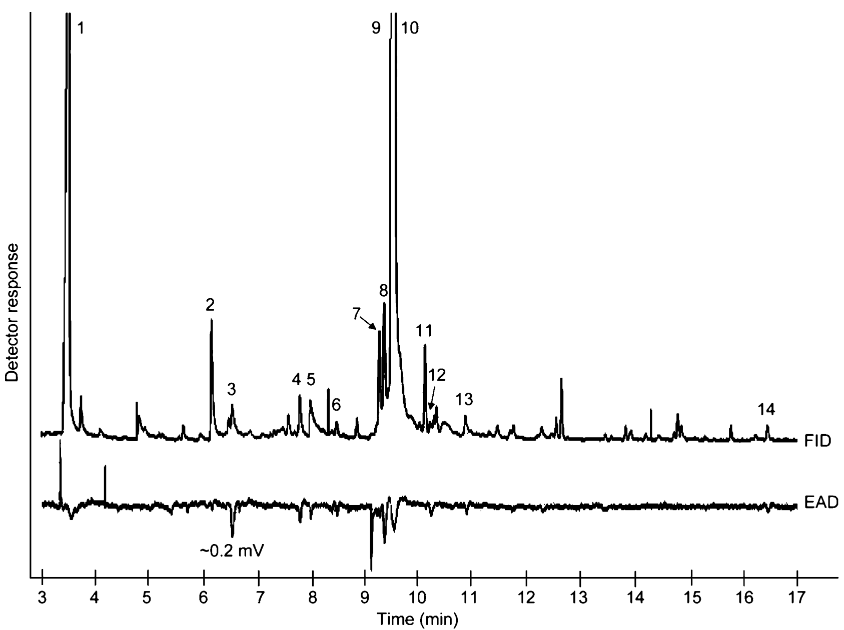


Fig. 1 Coupled gas chromatograph (flame ionization detection; *FID*) and electroantennogram detection (*EAD*) run of volatiles from 3-d-old puréed cucumber, using female *Batrocera cucurbitae*. Volatile collections were made using a SPME CW/DVB fiber. *1* = acetic acid; *2* = (*E*)-2-heptenal; *3* = 1-octen-3-ol; *4* = (*E*)-2-octenal; *5* = (*E*)-2-octen-1-ol; *6* = nonanal; *7* = (*E*,*Z*)-2,6-nonadienal; *8* = (*E*)-2-nonenal; *9* = (*E*,*Z*)-2,6-nonadien-1-ol; *10* = (*Z*)-6-nonen-1-ol; *11* = decanal; *12* = (*E*,*E*)-2,4-nonadienal; *13* = (*E*)-2-decenal; *14* = unknown 4

Table 1 Female melon fly electroantennogram detection (EAD) responses to Porapak Q collections of cucumber volatiles

Compound	Rt (min)	Porapak Q headspace collections from cucumber								Prev. ref.[d]
		Fresh		1 day old		3 day old		5 day old		
		EAD (mV)[a]	Amount[b]	EAD (mV)[a]	Amount[b]	EAD (mV)[a]	Amount[b]	EAD (mV)[a]	Amount[b]	
Acetic acid	3.2	–	–	–	–	0.19±0.05	1	0.35±0.11	51	Bd
Hexanal[c]	3.8	0.22±0.04	14	0.17±0.01	1	–	–	–	–	Bd, Cc
(*E*)-2-Hexenal[c]	4.6	0.17±0.02	14	–	–	–	–	–	–	Bd, Cc
1-Hexanol[c]	4.9	–	–	0.28±0.04	51	0.42±0.05	51	0.39±0.12	1	Al, Bd, Cc
Unknown 1	6.4	–	–	0.22±0.04	4	0.25±0.07	1	0.25±0.17	–	
1-Octen-3-ol[e]	6.6	0.24±0.02	trace	0.37±0.04	4	0.44±0.08	3	0.55±0.12	8	Cc, Rp
(*E*,*E*)-2,4-Heptadienal	6.9	0.14±0.06	2	–	–	–	–	–	–	
Benzyl alcohol	7.6	–	–	0.13±0.04	2	0.55±0.14	3	0.29±0.17	12	
(*E*)-2-Octenal	7.7	0.11±0.01	1	–	–	–	–	–	–	
(*E*)-2-Octen-1-ol	8	0.18±0.04	1	–	–	–	–	–	–	Bd
3,5-Octadien-2-one	8	–	–	0.20±0.04	4	0.26±0.08	4	0.19±0.08	5	
(*E*)-4-Nonenal[c]	8.3	0.18±0.02	6	–	–	–	–	–	–	
Linalool[e]	8.5	–	–	–	–	0.23±0.04	5	0.27±0.11	6	Cc
(*Z*)-6-Nonenal[c]	8.5	0.15±0.02	14	–	–	–	–	–	–	
2-Phenylethyl alcohol	8.8	–	–	–	–	0.15±0.01	1	0.23±0.08	33	
(*E*,*Z*)-2,6-Nonadienal[c]	9.2	0.18±0.02	100	0.28±0.06	4	0.29±0.05	5	0.26±0.10	6	
(*E*)-2-Nonenal[c]	9.3	0.36±0.04	94	0.38±0.04	6	0.49±0.05	9	0.35±0.09	4	
(*E*,*Z*)-2,6-Nonadien-1-ol[c]	9.5	0.28±0.14	9	0.27±0.04	19	0.42±0.05	28	0.27±0.11	4	
(*Z*)-6-Nonen-1-ol[c]	9.6	0.28±0.14	10	0.27±0.04	38	0.42±0.05	64	0.27±0.11	27	
Unknown 2	9.8	–	–	–	–	–	–	0.19±0.06	8	
Methyl geranate	11.7	0.15±0.03	3	0.24±0.05	3	0.14±0.03	trace	0.17±0.08	4	Cc
Geranyl acetone	13.4	0.11±0.03	trace	0.19±0.04	trace	0.26±0.06	trace	0.15±0.05	trace	
Unknown 3	14.1	–	–	0.13±0.01	trace	0.15±0.04	trace	–	–	
Tetradecanal	15.3	0.12±0.01	trace	0.15±0.01	2	0.16±0.04	2	–	trace	
Unknown 4	16.4	0.12±0.01	3	0.17±0.05	13	0.13±0.02	15	–	4	

[a] Means (± SE) of the four largest depolarizations recorded for each compound that elicited EAD responses from female melon flies

[b] Relative abundance for a single compound by flame ionization detector peak area (solvent peak excluded)

[c] Compounds likely produced by the lipoxygenase (LOX) pathway

[d] Compounds eliciting EAD responses from other tephritids are indicated by species (*Anastrepha ludens* = Al, *Batrocera dorsalis* = Bd, *Ceratitis capitata* = Cc, *Rhagoletis pomonella* = Rp) (Light and Jang 1987; Light et al. 1988; Jang et al. 1989; Lee et al. 1998; Nojima et al. 2003a; Malo et al. 2005)

[e] Absolute configurations not determined

comparison with authentic standards, was used to identify these compounds, with the exception of 3,5-octadien-2-one, which was only tentatively identified by mass spectral pattern, and four compounds that could not be identified. Of the 31 compounds, twenty-five were present in the Porapak Q collections, while SPME sampling revealed an additional six compounds. There was a high level of overlap between the collection methods, with 13 of the EAD-active compounds detected in both. Given the small number of SPME replications, and variations between collections, we did not quantify differences between the fibers. Volatiles collected by Porapak Q were not quantified but relative abundances were used as a rough guide for lure formulation. For the purpose of this comparison FID detector response to all compounds was assumed to be equal.

Headspace collections from fresh cucumber consisted predominantly of *E*2*Z*6-9:al and *E*2-9:al, while the most abundant components of aged samples were *Z*6-9:1-ol, 6:1-ol, and AA. The number of EAD responses observed was roughly constant among the different-age cucumber preparations, although, as noted, the amounts of collected material varied greatly (Tables 1, 2). The chemicals, 1-8:3-ol, *E*2-9:al, and 6:ol elicited the largest EAD responses

Table 2 Female melon fly electroantennogram detection (EAD) responses to solid phase microextraction (SPME) collections of cucumber volatiles

Compound	EAD responses to SPME collection			Prev. ref.[b]
	Fresh	1 day old	3 day old	
Acetic acid	–	X	X	Bd
(*E*)-2-Heptenal	–	–	X	
1-Octen-3-ol[a]	–	X	X	Cc, Rp
(*E*)-2-Octenal	–	–	X	
(*E*)-2-Octen-1-ol	–	–	X	
Nonanal	X	X	X	Bd, Cc
1-Nonanol	X	X	X	Bd, Cc
(*Z*)-6-Nonenal	X	X	X	
(*E,Z*)-2,6-Nonadienal	X	X	X	
(*E*)-2-Nonenal	X	X	X	
(*E,Z*)-2,6-Nonadien-1-ol	–	X	X	
(*Z*)-6-Nonen-1-ol	X	X	X	
Decanal	–	–	X	Bd, Cc
(*E,E*)-2,4-Nonadienal	–	–	X	
(*E*)-2-Decenal	–	–	X	
Geranyl acetone	X	X	–	
Unknown 3	X	X	–	
Tetradecanal	X	X	–	
Unknown 4	X	X	X	

[a] Absolute configurations not determined

[b] Compounds eliciting EAD responses from other tephritids are indicated by species (*Anastrepha ludens* = *Al, Batrocera dorsalis = Bd, Ceratitis capitata = Cc, Rhagoletis pomonella = Rp) (Light and Jang 1987; Light et al. 1988; Jang et al. 1989; Lee et al. 1998; Nojima et al. 2003a; Malo et al. 2005)*

when the data from all different-age cucumber preparations were averaged (Table 1). However, strict comparison of EAD responses among several partially co-eluting compounds is complicated by the fact that the EAD voltage did not return to baseline before the antenna was stimulated by the next peak (e.g., Fig. 1, *Z*6-9:al, *E*2*Z*6-9:al, *E*2-9:al, and *Z*6-9:1-ol).

Experiment 1 showed a wide variation in single compound attractancy in the outdoor olfactometer bioassay (Table 3),with 1-8:3-ol and *Z*6-9:al being the most attractive. In general, more females than males were caught, although the bias to an individual compound did not exceed a ratio of 2:1 (females: males).

In Experiment 2, only the mean number of female flies caught to lure #1 was greater than the mean number of flies caught in the control (ANOVA: $F=7.017$, $df=5$, $P=0.001$). Both lures #1 and #2 caught female and male flies in a roughly 2:1 ratio (Fig. 2).

In the third experiment (Experiment 3), female melon fly catches to lure #3 were greater than to all other catches (ANOVA: $F=15.441$, $df=11$, $P<0.001$; Fig. 3). The number of males caught by this lure was the only other catch significantly greater than that of the control. The compounds 9:al and 9:1-ol appeared to have an antagonistic effect on attractiveness, as their removal (i.e., lure #1 compared to lure #3) led to increased trap captures. In contrast, the removal of *Z*6-9:1-ol (i.e., lure 6) from lure 3 resulted in decreased fly attraction. Neither of the binary blends tested (lure #4 = *E*2*Z*6-9:al plus *E*2-9:al; lure #5 = *Z*6-9:al plus *Z*6-9:1-ol) was attractive.

In the experiment (Experiment 4) testing additional EAD-active compounds, female melon fly catches to lure #7 were greater than all other catches (ANOVA: $F=8.444$, $df=7$, $P<0.001$; Fig. 4). This increase indicates that one, or more, of 6:al, *E*2-8:al and 6:1-ol are critical lure components in the attraction of females to lure #7.

Wild female melon fly catches in a papaya field (Experiment 5) were greater (ANOVA: $F=27.367$, $df=5$, $P<0.001$) to lure #7 than to the other lures tested (Fig. 5). Male melon fly catches to lure #7 were not significantly greater than male catches to Solulys, but both were greater than catches in the negative control. Lure #7 also appeared to be highly species specific with traps baited with this lure containing only ca 1% *B. dorsalis*, the other major *Bactrocera* sp. abundant in the papaya fields. In comparison, Solulys-baited traps captured ca 10% *B. dorsalis*. The only non-target insects regularly found in traps baited with lure #7 were species of Ceratopogonidae, Phoridae, Gryllidae and Tettigoniidae.

Concurrent with experiment 5, there were also Cuelure traps in an adjoining field, and catches from those traps are shown for rough comparison (Fig. 5). Strict comparison with the Cuelure catches is not possible as the Cuelure treatments used a different trap type (a bucket trap instead of a Multilure trap) and kill agent (volatile pesticide instead of water); however, it is interesting to note that female

Table 3 Melon fly attraction to individual cucumber volatiles in cage olfactometer

Compound	Flies caught per trap[a]	
	Females	Males
(+/-)-1-Octen-3-ol	17.0±5.6	10.4±2.3
(*Z*)-6-Nonenal	11.8±2.5	15.4±1.4
(+/-)-Linalool	7.9±2.1	3.3±0.7
Acetic acid	5.7±1.4	8.2±3.0
(*E*)-2-Hexenal	5.1±1.9	4.6±1.3
Hexanal	3.4±1.5	1.2±0.9
(*E*,*E*)-2,4-Nonadienal	3.3±1.5	1.8±1.3
(*Z*)-6-Nonen-1-ol	2.8±0.3	5.1±0.5
(*E*,*Z*)-2,6-Nonadien-1-ol	2.6±1.5	2.0±1.7
(*E*)-2-Octenal	1.9±0.1	0.3±0.3
Decanal	1.9±0.4	1.0±0.5
(*E*)-2-Heptenal	1.6±1.0	1.0±0.8
(*E*)-2-Octen-1-ol	1.5±1.1	0.8±0.8
1-Hexanol	1.1±0.9	−0.2±0.3
(*E*)-2-Nonenal	0.9±0.2	1.1±0.5
Nonanal	0.6±0.4	2.0±0.5
2-Phenylethyl alcohol	0.5±0.4	0.3±0.3
1-Nonanol	0.3±0.6	4.2±3.1
Geranyl acetone	0.0±0.6	−0.5±0.1
(*E*,*Z*)-2,6-Nonadienal	−0.2±0.2	0.8±0.5
(*E*)-2-Decenal	−0.2±0.3	−0.4±0.4
Methyl geranate	−0.3±0.2	−0.8±0.1
Benzyl alcohol	−0.3±0.3	−0.3±0.2

[a] Relative to control. Trap catches reflect the number of flies caught minus the number of flies caught in the negative control

catches to lure #7 are roughly comparable to male catches using Cuelure in its standard trap.

Discussion

Thirty-one compounds, among the volatiles collected from puréed cucumber, elicited consistent electroantennogram responses from melon fly females in GC-EAD runs. The identified compounds were mostly alcohols and aldehydes, many of which are commonly produced by fruits and flowers of various plants. A number of the compounds [e.g., AA, 6:al, (*E*)-2-hexenal (*E*2-6:al), 6:1-ol, 1-8:3-ol, (*E*)-2-octen-1-ol, linalool, and methyl geranate] that elicited EAD responses from female melon flies also are known to stimulate responses in other fruit fly species, such as *Anastrepha ludens* (Malo et al. 2005), *B. dorsalis* (Light and Jang 1987; Lee et al. 1998), *C. capitata* (Light et al. 1988; Jang et al. 1989) and *R.. pomonella* (Nojima et al. 2003a). Another group among these 31 compounds, are the six- and nine-carbon alcohols and aldehydes not previously shown to elicit electrophysiological responses from tephritids. These oxygenated compounds are produced by the enzymatic breakdown of linolenic and linoleic acids, initiated by plant-issue damage, via the lipoxygenase (LOX) pathway (Hornostaj and Robinson 1999; Feussner

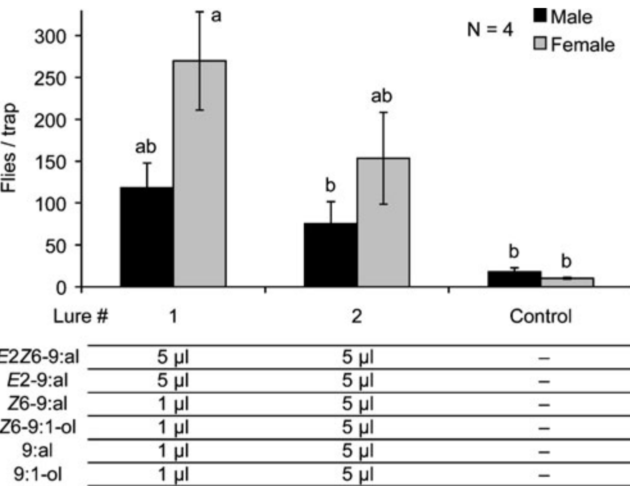


Fig. 2 Numbers (mean/trap ± SE) of *Batrocera cucurbitae* caught in traps, baited with blends of six nine-carbon aldehydes and alcohols [(*E*,*Z*)-2,6-nonadienal (*E*2*Z*6-9:al), (*E*)-2-nonenal (*E*2-9:al), (*Z*)-6-nonenal (*Z*6-9:al), (*Z*)-6-nonen-1-ol (*Z*6-9:1-ol), nonanal (9:al), and 1-nonanol (9:1-ol)], in a rotating outdoor olfactometer (Experiment 2). *Different letters* represent significant differences ($P<0.05$) between the mean numbers of flies caught (ANOVA, followed by Tukey's HSD)

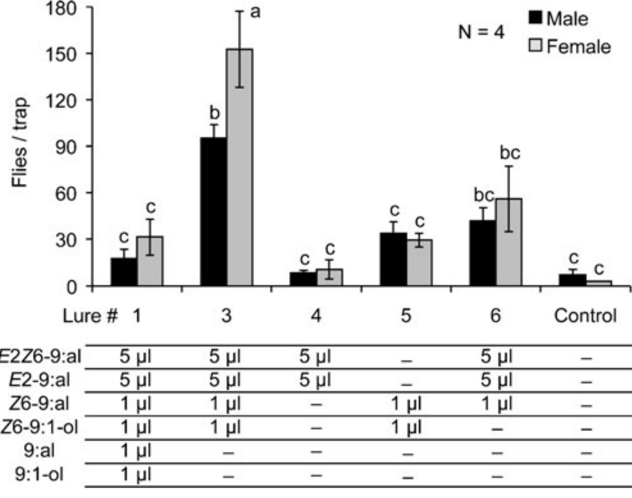
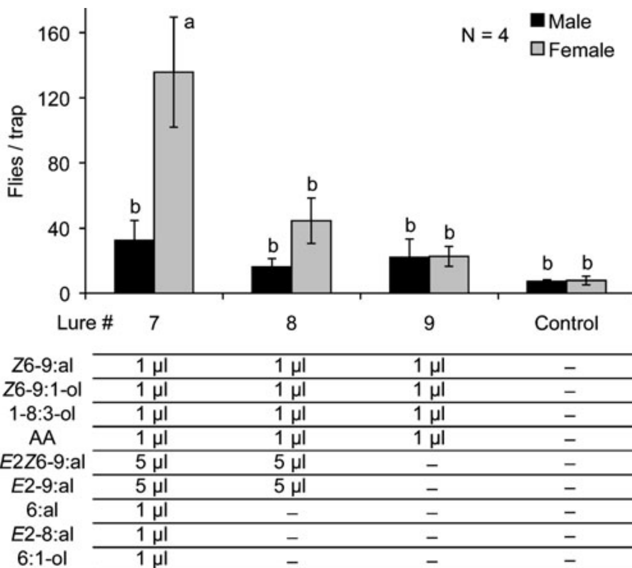


Fig. 3 Numbers (mean/trap ± SE) of *Batrocera cucurbitae* caught in traps, baited with blends of six nine-carbon aldehydes and alcohols alcohols [(*E*,*Z*)-2,6-nonadienal (*E*2*Z*6-9:al), (*E*)-2-nonenal (*E*2-9:al), (*Z*)-6-nonenal (*Z*6-9:al), (*Z*)-6-nonen-1-ol (*Z*6-9:1-ol), nonanal (9:al), and 1-nonanol (9:1-ol)], in a rotating outdoor olfactometer (Experiment 3). *Different letters* represent significant differences ($P<0.05$) between the mean numbers of flies caught (ANOVA, followed by Tukey's HSD)



	7	8	9	Control
Z6-9:al	1 µl	1 µl	1 µl	–
Z6-9:1-ol	1 µl	1 µl	1 µl	–
1-8:3-ol	1 µl	1 µl	1 µl	–
AA	1 µl	1 µl	1 µl	–
E2Z6-9:al	5 µl	5 µl	–	–
E2-9:al	5 µl	5 µl	–	–
6:al	1 µl	–	–	–
E2-8:al	1 µl	–	–	–
6:1-ol	1 µl	–	–	–

Fig. 4 Numbers (mean/trap ± SE) of *Batrocera cucurbitae* caught in traps in a rotating outdoor olfactometer (Experiment 4) baited with various blends [(*Z*)-6-nonenal (*Z*6-9:al), (*Z*)-6-nonen-1-ol (*Z*6-9:1-ol), 1-octen-3-ol (1-8:3-ol), acetic acid (AA), (*E*,*Z*)-2,6-nonadienal (*E*2*Z*6-9:al), (*E*)-2-nonenal (*E*2-9:al), hexanal (6:al), (*E*)-2-octenal (*E*2-8:al), and 1-hexanol (6:1-ol)]. *Different letters represent* significant differences ($P<0.05$) between the mean numbers of flies caught

and Wasternack 2002), and have been found previously in both fresh and fermented cucumbers (Kemp et al. 1974; Zhou and McFeeters 1998). The predominant volatile isolated from fresh blended cucumber is *E*2*Z*6-9:al, which is also the most important compound in producing the characteristic cucumber smell perceived by humans (Forss et al. 1962; Schieberle et al. 1990). Interestingly, several LOX pathway derivatives, or close analogs, have been

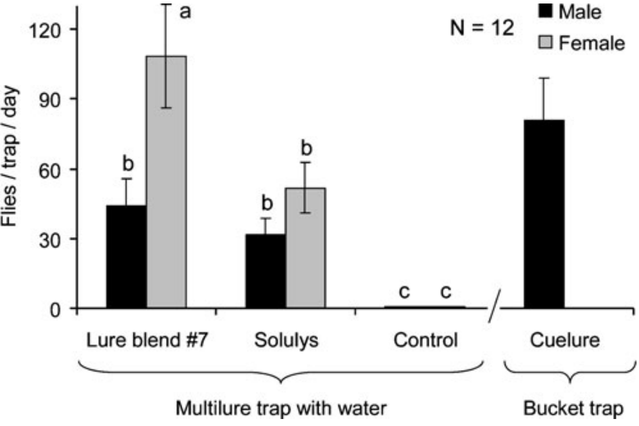


Fig. 5 Numbers (mean/trap ± SE) of wild *Batrocera cucurbitae* caught in traps in a papaya field in Kapoho, HI (Experiment 5) baited with the best synthetic cucumber blend (Lure #7) or Solulys protein bait. Different letters represent significant differences ($P<0.05$) between the mean numbers of flies caught (ANOVA, followed by Tukey's HSD). Cuelure trap captures from an adjoining field, cannot be compared with the other catches, but are shown for rough comparison

implicated previously in melon fly attraction. One of these, (*E*)-6-nonenyl acetate, attracts female melon flies (Jacobson et al. 1971) as well as stimulates oviposition (Keiser et al. 1973). Other compounds, including (*Z*)-3-hexen-1-ol and *E*2-6:al, are prominent in extracts of bittermelon, *Momordica charantia* L., and attract *B. cucurbitae* (Binder et al. 1989).

Our bioassay approach allowed us to identify which of these 31 compounds were required for eliciting male and female melon fly attraction. After first screening single compounds in a rotating olfactometer trap (Experiment 1), six nine-carbon LOX pathway derivatives were tested further in blend combinations. Catches in these trials (Experiments 2 and 3), showed that a blend of *E*2*Z*6-9:al, *E*2-9:al, *Z*6-9:al, and *Z*6-9:1-ol was attractive to melon fly, while addition of 9:al and 9:1-ol to this blend had behavioral antagonistic effects. In a further trial (Experiment 4), several additional compounds were tested, with the blend (lure #7) containing five additional (to the previous four) compounds proving to be the most attractive to female flies. This nine-component blend was attractive to melon flies when tested in papaya fields, catching more female flies than Solulys protein bait traps. Although our approach has proven successful in identifying an attractive lure, further refinement of components and blend ratios, perhaps employing the mixture principles and response surface methodology recently described by Lapointe et al. (2008), could produce a more effective attractant for female melon flies.

Our success in producing a synthetic lure for female melon fly holds promise for future applications in trapping for detection (new infestations/biosecurity) or monitoring (for treatment timing), and in control or eradication (e.g., mass trapping, attract-and-kill, an attractant added to existing protein insecticide bait sprays such as GF-120) programs. The ability of the lure to attract females is advantageous over other attractants (such as Cuelure) that only attract males, because removal of females should have a more significant impact on a pest population. That catches of females to the lure in the field trial were roughly similar to catches of males in Cuelure-baited traps, not withstanding differences between the respective fields and trap types, was encouraging. However, more rigorous comparison between the two lures will be needed to establish their relative attractancy, and their impact on pest populations.

Apart from female-biased attraction, synthetic cucumber lures, such as our nine-component lure #7, have the potential to improve the ease of use of traps for catching female melon flies. Current lures that attract females are limited to food-type attractants, which often lack potency, have limited field life, are difficult to handle, and attract non-target species. Synthetic cucumber lures have the potential to be longer lasting (after formulation into a

matrix), may be adaptable to use with a dry trap, and from our limited study, appear to capture fewer non-target species than food-type attractants.

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Oviposition Responses of the Mosquitoes *Aedes aegypti* and *Aedes albopictus* to Experimental Plant Infusions in Laboratory Bioassays

Loganathan Ponnusamy · Ning Xu · Katalin Böröczky · Dawn M. Wesson · Luma Abu Ayyash · Coby Schal · Charles S. Apperson

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Abstract Attraction of the mosquitoes *Aedes aegypti* and *Ae*. *albopictus* to plant infusions was evaluated by using a modified sticky-screen bioassay that improved the resolution of mosquito responses to odorants. Under bioassay conditions, solid-phase microextraction-gas chromatographic analyses of the volatile marker chemical indole showed that odorants diffused from bioassay cups, forming a concentration gradient. Infusions were prepared by separately fermenting senescent leaves of eight plant species in well water. Plant infusions were evaluated over an 8-fold range of leaf biomass and/or a 28 d fermentation period. The responses of gravid females of both mosquito species varied with the plant species and biomass of plant materials used to make infusions, and with the length of the fermentation period. Infusions made from senescent bamboo (*Arundinaria gigantea*) and white oak (*Quercus alba*) leaves were significantly attractive to both mosquitoes. In general, infusions prepared by using low biomass of plant material over a 7–14 d fermentation period were most attractive to *Ae*. *aegypti*. In contrast, *Ae*. *albopictus* was attracted to infusions made using a wider range of plant biomass and over a longer fermentation period. Both mosquito species were more attracted to a non-sterile white oak leaf infusion than to white oak leaf infusion that was prepared using sterilized plant material and water, thus suggesting a role for microbial activity in the production of odorants that mediate the oviposition response of gravid mosquitoes.

Key Words *Aedes aegypti* · *Aedes albopictus* · Oviposition · Attractants · Plant infusion · Fermentation · Microbe-insect interactions

L. Ponnusamy · N. Xu · K. Böröczky · L. Abu Ayyash · C. Schal · C. S. Apperson (✉)
Department of Entomology, North Carolina State University, Box 7647, Raleigh, NC 27695, USA
e-mail: charles_apperson@ncsu.edu

D. M. Wesson
Department of Tropical Medicine, Tulane University, New Orleans, LA 70112, USA

Introduction

Aedes aegypti L. and *Ae*. *albopictus* (Skuse) are the principal vectors of dengue and dengue hemorrhagic fever viruses on a global basis (Gubler 2002). Immature stages of both mosquito species inhabit human-made containers placed in residential landscapes. Gravid females lay eggs in water-filled containers. The oviposition behavior of these mosquitoes is mediated by visual, olfactory, tactile, or chemo-tactile cues associated with their container habitats (Bentley and Day 1989; Millar et al. 1992; Navarro et al. 2003; Ponnusamy et al. 2008). Chemical cues that mediate oviposition behavior originate from organic infusions in containers as metabolic products of microbial origin (Benzon and Apperson 1988; Isoe and Millar 1995; Sumba et al. 2004; Sant'Ana et al. 2006; Ponnusamy et al. 2008, 2010).

A variety of plant species and plant-associated materials have been used to produce organic infusions for investigating the oviposition behavior of mosquitoes or for monitoring oviposition activity in the field. Infusions made from a variety of grasses (Reiter et al. 1991; Chadee et al. 1993; Rawlins et al. 1998; Polson et al. 2002; Sant'Ana et al. 2006) and from oak leaves (*Quercus* spp.) (Szumlas et al. 1996; Trexler et al. 1998) have been used in oviposition

traps (ovitraps) for monitoring the egg-laying activity of container-inhabiting *Aedes* mosquitoes in the field. Other organic materials, such as sod and pelletized plant-based animal feeds, have been fermented to create infusions that were attractive to gravid *Aedes* (Lampman and Novak 1996a; Ritchie 2001) and *Culex* mosquitoes (Lampman and Novak 1996b). Gravid females of other mosquitoes, such as *Culex nigripalpus* (Theobald) (Ritchie 1984), *Cx. quinquefasciatus* (Say) (Isoe et al. 1995b; Mboera et al. 2000), and *Cx. tarsalis* Coq. (Isoe and Millar 1995) are attracted to hay and grass infusions. Ovitraps baited with organic infusions have been used to monitor arbovirus vectors during disease outbreaks or to collect mosquitoes for arbovirus testing (Tsai et al. 1989; Savage et al. 1993; Nasci et al. 2002; Polson et al. 2002).

Notably, there are no standard methods for producing infusions. Tightly closed plastic garbage bags (Reiter et al. 1991; Chadee et al. 1993; Trexler et al. 1998), fiberglass tubs (Isoe et al. 1995a), open containers (Rawlins et al. 1998), and closed plastic containers (Sant'Ana et al. 2006) have been used to make infusions from plant materials. Moreover, the attractiveness of infusions to gravid mosquitoes changes over the fermentation time (Isoe et al. 1995b; Sant'Ana et al. 2006; Ponnusamy et al. 2010) and with the species and amounts of plant materials fermented (Ponnusamy et al. 2010). Maw (1970) concluded that these changes were because of variations in bacterial activity, and Reiter (1986) recommended that infusions should be prepared by a standard protocol because rapid changes in the microbial population and chemical composition could change the attractiveness of infusions with time.

There also appears to be a lack of standard methods of evaluating and interpreting the biological activity of organic infusions. Most commonly, infusions are evaluated based on the numbers of eggs laid in containers holding test and control media. However, this measure represents an endpoint of the biological activity of various cues, and it fails to separate the effects of attractants and contact chemo-stimulants on the oviposition response (Benzon and Apperson 1988; Isoe et al. 1995a).

The objectives of our research were to: (1) refine a sticky-screen bioassay used previously (Trexler et al. 1998) for measuring the attraction of *Aedes* mosquitoes to plant infusions; (2) compare several methods of preparing infusions that were attractive to gravid *Ae. aegypti* and *Ae. albopictus*; (3) validate that females can be guided within our 2-choice bioassay cages by an odor gradient emanating from the cup holding plant infusion; (4) evaluate effects of plant species, plant biomass, and fermentation time of infusions on the responses of gravid mosquitoes; and (5) determine the significance of microbial activity to the production of attractive infusions.

Methods and Materials

Origin and Maintenance of Mosquito Colonies Aedes aegypti and *Ae. albopictus* colonies were established from eggs collected in New Orleans, LA, USA in 2003. At 6–8 month intervals, adults were added to each mosquito colony to sustain genetic diversity. Larvae of both species were reared as described by Trexler et al. (2003). Mosquito colonies were maintained in separate insectaries at ~28°C, ~75% RH, and a photoperiod of 14:10, L:D, including two twilight periods (60 min each). Eggs for maintenance of mosquito colonies were obtained from females that were blood-fed on a human forearm. Gravid females for oviposition bioassays were produced by feeding 7–14-d-old females on a human forearm 4–5 d prior to the setup of an experiment. The protocol for feeding was in compliance with IRB Guidelines of North Carolina State University.

Preparation of Plant Infusions We hypothesized that production of odorants that attract gravid mosquitoes would be influenced by many factors, including the type and amount of fermented organic material, the amount of headspace available, and duration of the fermentation. Initially, the ratio of plant biomass to well water (33.6 g per 4 L = 1X infusion) described by Trexler et al. (1998) was used to make white oak leaf (WOL) infusion in black plastic trash can liners (14 µm thickness, No. 386014, Central Polybag, Corp., Linden, NJ, USA). We compared three fermentation methods in which: (1) the plastic bag was left open; (2) the plastic bag was tightly sealed with no headspace above the infusion; or (3) the bag was tightly sealed, but ballooned to create a headspace above the infusion equal to approximately 50% of the capacity of the bag. Methods 1 and 3 were intended to achieve aerobic decomposition, while method 2 was intended to accomplish facultative anaerobic fermentation of plant materials. After 1, 2, and 4 week fermentation periods, each type of infusion was tested for attractive odorants against gravid *Ae. aegypti* in a sticky-screen bioassay (Trexler et al. 1998) modified as decribed below.

The potential chemical reactivity of the plastic trash bags was of concern. Therefore, we used Teflon bags (TFM Modified PTFE homopolymer inert bag, No. P-00021-2, 63.5 µm thickness, Big Science, Inc., Huntersville, NC, USA) as an alternative container for producing plant infusions. Infusions were made in tightly clamped Teflon bags, leaving a headspace equivalent to ~50% of the bag volume for the entire fermentation period. Plant infusions were prepared by using senescent leaves of bamboo (*Arundinaria gigantea*), white oak (*Quercus alba*), live oak (*Quercus virginiana*), pecan (*Carya illinoensis*), hackberry (*Celtis occidentalis*), red maple (*Acer rubrum*),

redtop panicgrass (*Panicum rigidulum*), and harvested Bermuda grass (*Cynodon dactylon*) that were obtained from multiple sites in Raleigh, NC and New Orleans, LA. For each plant infusion, there were 6–8 replicate bioassays per trial with three trials completed on different dates.

Optimization of Sticky-screen Attractant Bioassay In preliminary experiments, the original and a modification of the sticky-screen bioassay method of Trexler et al. (1998) were evaluated using white oak leaf (WOL) (*Quercus alba*) infusion (Trexler et al. 1998) and gravid *Ae. aegypti* and *Ae. albopictus*. The original method involved placing a screen coated with insect glue (Tanglefoot®, Tangle Foot Co., Grand Rapids, MI, USA) directly on top of the bioassay cup (120 ml, polypropylene, No.13-711-57, Fisher Scientific) that was spray-painted flat black on the exterior. Gravid females attracted to volatiles and attempting to enter the test cup were trapped on the sticky-screen. However, some females were routinely found free in the cage, indicating that the capture efficiency of this design needed improvement. Therefore, we modified the sticky-screen method by placing the screen approximately 4.2 cm below the cup rim on top of a clear polycarbonate ring (4.2 cm diam.×3.2 cm high) that was cut from a fluorescent tube lamp guard (No. 1743564, Copper Lighting, Peachtree City, GA, USA); this ring held the sticky-screen just above the surface of the water. In each bioassay, two black cups (test and control) were placed randomly in diagonal corners of a reach-in cage (30×30×30 cm, Lucite® CP acrylic, Lucite International, Inc.) fitted with a stockinette sleeve (No. 081620, Albahealth LLC, Rockwood, TN, USA). Cages were placed on shelves in a room where environmental conditions were the same as in the insectary. Ten gravid females were transferred into each cage, and after a 24 or 48 hr bioassay, females trapped on each sticky screen were counted. For the original or modified bioassay, there were 6 replicate cages per trial with three to four trials completed on different dates.

Reach-in Cage Bioassay of Plant Infusions The modified sticky screen bioassay described above proved to be superior to the conventional method for evaluating plant infusions in reach-in cages. Consequently, the modified method was used to bioassay plant infusions. In setting up a bioassay, the contents of the Teflon bag were mixed vigorously prior to removing infusion. The plant infusions were diluted to 50% with well water before the test cup was filled (30 ml) and well water (30 ml) was placed in the control cup. The 1X Bermuda hay infusion was evaluated undiluted (100% infusion) and diluted to 50% and 10% with well water.

Evaluation of Odorant Diffusion Patterns To examine diffusion patterns of odorants in bioassay cages, indole was used as a marker chemical to quantify volatiles in and around a bioassay cage. Specifically, we were interested in measuring the concentrations of indole inside the bioassay cage at various distances from the test cup and above the cage at two time points: immediately after setting up the bioassay (0 hr) and 24 hr later (24 hr). Two cups, a test cup, and a control cup were placed diagonally at the corners inside a standard bioassay cage. The test cup contained 30 ml of distilled water to which 90 μl of a 10 μg/μl indole (CAS No. 120-72-9, Sigma Aldrich Co) solution in dichloromethane were added. The control cup contained 30 ml of distilled water. The headspace was sampled simultaneously at various positions inside and outside the bioassay cage with solid phase microextraction (SPME) (DVB/CAR/PDMS, Supelco) for 10 min. Prior to placement, SPME fibers were conditioned at 250°C for 20 min in a gas chromatograph (GC) inlet (Agilent) purged with helium. Volatiles were captured at five positions inside the bioassay cage (at the surface of the sticky screen inside the test cup, in the middle at the lip of the test cup, 10 cm above the test cup, in the middle of the cage, and at the surface of the sticky screen inside the control cup) and at one position outside the cage, 1 cm above the center of the cage. These experiments were replicated three times.

Indole captured on SPME fibers at both time points (0 hr and 24 hr) was analyzed by using an Agilent 7890A GC with a flame ionization detector (FID), equipped with a DB-5 (30 m×0.25 mm×0.25 μm) column (J&W Scientific) and a 2-m deactivated guard column (Alltech) using helium as a carrier gas. The injector and the detector were kept at 260°C and 280°C, respectively. The oven was held at 45°C for 1 min, then heated to 180°C at 10°C/min, and finally heated at 20°C/min to 280°C and held at the final temperature for 5 min. Samples were injected splitless with an inlet purge time of 0.75 min. Quantification of samples was based on external calibration using a dilution series of indole in the range of 0.1–100 ng of compound injected in the GC.

Comparison of Sterile and Non-sterile Infusions We evaluated the attraction of gravid females to non-sterile and sterile WOL infusions based on the hypothesis that non-sterile infusions would be significantly more attractive to gravid mosquitoes. In behavioral assays, we compared 2-week old WOL (0.5X) infusion (4.2 g leaves/L water) that was produced using sterilized oak leaves and well water (autoclaved for 45 min at 120°C) against unsterilized infusion (unsterilized leaves and well water). After autoclaving, well water (1 L) and WOL (4.2 g) were combined in sterile glass jars (2 L) fitted with threaded plastic lids. Similarly, non-sterile infusion was produced in sterile jars but with leaves and water that were not sterilized. Jars were held at 28°C for 2 weeks before infusions were tested in

modified sticky-screen bioassays. Test cups filled with sterile WOL infusion (30 ml, 50% dilution) were evaluated against control cups containing non-sterile WOL infusion (30 ml, 50% dilution). After a 48-hr bioassay, females trapped on screens in test and control cups in each cage were counted separately.

Walk-in Cage Bioassay of Plant Infusions Based on results from small cage experiments, 1-wk-old bamboo leaf (BL) (1X) and 2-wk-old WOL (0.5X) infusions were evaluated against gravid *Ae. aegypti* and *Ae. albopictus* in a two-choice sticky screen bioassay in a walk-in cage (4×4×2 m height) (Lumite, Inc., Gainesville, GA, USA) that was set up indoors using a wooden frame. Environmental conditions in the walk-in cage were variable, with mean daily temperature and humidity ranging from 24–27°C and 66–83%, respectively. Humidity was provided by a household humidifier (Cool-Moisture Humidifier, No. 564B, Kaz Inc., Southborough, MA, USA). A light regimen of 14:10, L:D was used with the photophase provided by 4 twin bulb (35 W) fluorescent fixtures placed at each corner of the cage. At the beginning and end of the dark period, partial light (1 hr) was provided by an incandescent bulb (25 W) placed in the center of the cage. To reduce penetration of external light into the cage, it was covered with white cotton bed sheets, which were then covered with black plastic sheeting.

Each infusion was evaluated four times on separate dates. With each replicate trial, the positions of treatment and control containers were exchanged. Number 10 tin cans (17 cm high×15 cm diam., 4 L nominal) painted black inside and out with Rustoleum® were used as bioassay containers. Infusion was added to one can, and the same volume of well water was added to the control can. The volume of infusion was 0.5 L or 1.23 L, either undiluted 1x infusion (100% infusion) or 1X infusion diluted with an equal volume of well water (50% infusion). Sticky screens (15 cm diam.) then were placed on top of clear polypropylene cups (7.5 cm high×6 cm diam.) inside each can approximately 15 cm from the lip. At approximately noon, the test and a control container were placed 1 m apart in the middle of the cage, and 30 gravid mosquitoes were released in the cage. After 24 hr, the bioassay was terminated and females trapped on each screen were counted.

Statistical Analyses The Oviposition Activity Index (OAI), described by Kramer and Mulla (1979), was used to evaluate the response of gravid mosquitoes to plant infusions. The OAI standardizes the data by converting the number of females trapped on the sticky screen in the test cup to a proportion after correcting for the number of females trapped on the screen in the control cup. The OAI ranges from −1 to +1, with 0 indicating neutral response. Within each experiment, an OAI was calculated for each replicate as follows:

$$\mathrm{OAI} = \frac{N_t - N_c}{N_t + N_c},$$

where N_t is the number of females trapped on the screen in the test container and N_c is the number of females trapped on the screen in the control container. The SAS procedure PROC UNIVARIATE (SAS for Windows ver. 9.1, SAS Institute, Cary, NC, USA) was used to generate an approximate t statistic which was used to test the hypothesis that the mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0 at $\alpha \leq 0.05$.

Results

Optimized Sticky-screen Bioassay Modifying the sticky-screen bioassay by placing the glue-coated screen inside the cup increased the number of gravid females trapped in the test cup containing WOL infusion (Fig. 1). Additionally, fewer free females remained in cages in which the sticky-screen was placed inside of the bioassay cup than in cages with the screen on top of the bioassay cup (Fig. 1).

Fermentation Methods for Plant Infusions Generally, 7-d-old WOL infusions were less attractive to gravid *Aedes* than 14- and 28-d-old infusions (Fig. 2). For both mosquito species, infusions prepared in closed bags with a headspace above the infusion elicited consistently higher responses from gravid females than infusions prepared in open bags or closed bags without a headspace (Fig. 2).

Evaluation of Odorant Diffusion Patterns Under "still air" bioassay conditions, the amount of indole detected using 10 min of headspace sampling with SPME was highest at the position nearest to its source and declined toward the center of the cage as well as upward (Table 1). Only trace amounts of indole were detected in the center of the cage, in the control cup, and above the cage. The pattern was the same at the two time points indicating that the bioassay arrangement maintained an odor gradient for at least 24 hr.

Response to Plant Infusions: White Oak In general, the number of gravid *Ae. aegypti* and *Ae. albopictus* caught on the sticky screen decreased as leaf biomass increased, but this effect was more pronounced for *Ae. aegypti* (Fig. 3). Gravid *Ae. aegypti* were attracted to low biomass infusions (0.5X=4.2 g leaves/L well water) across the entire 28-d fermentation period. The highest OAI (0.42) was achieved with 0.5X biomass infusions after 14 days of fermentation. In contrast, with the exception of the first week, *Ae. aegypti*

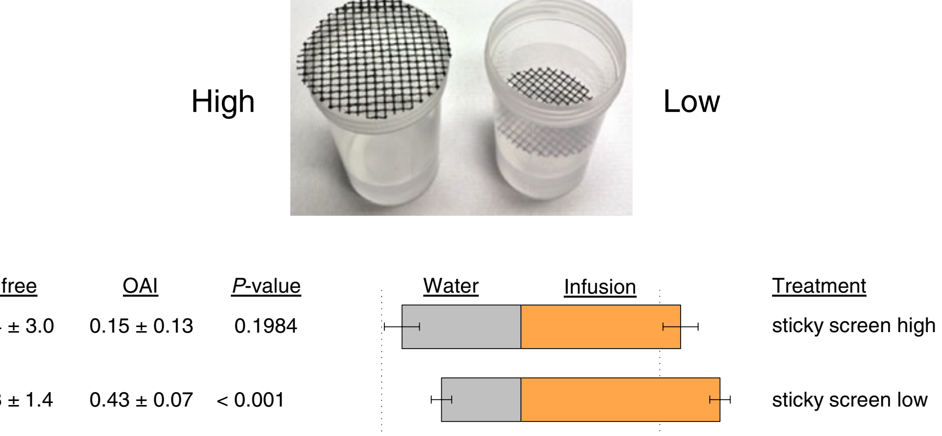

Fig. 1 High and low placement of glue-coated screens in bioassay cups used in modified and original sticky-screen bioassay, respectively. Clear cups are shown here to demonstrate the placement of the screens, but for bioassays the cups were spray-painted flat black on the exterior. Sticky-screen bioassay results for 0.5X (4.2 g leaves/L water) white oak leaf infusion showing higher capture of *Aedes aegypti* in the modified assay. There were six replicate cages per trial and three trials completed on different dates for both the modified and original bioassays

females were repelled by high biomass (4X) infusions over the 28 day fermentation period (Fig. 3).

In comparison, *Ae. albopictus* was attracted to WOL infusions over a broader range of leaf biomass and fermentation times (Fig. 3). The highest mean OAI for *Ae. albopictus* was achieved using 0.5X biomass infusions after 14 and 28 days of fermentation, but the 1X biomass infusions also elicited responses from *Ae. albopictus* across

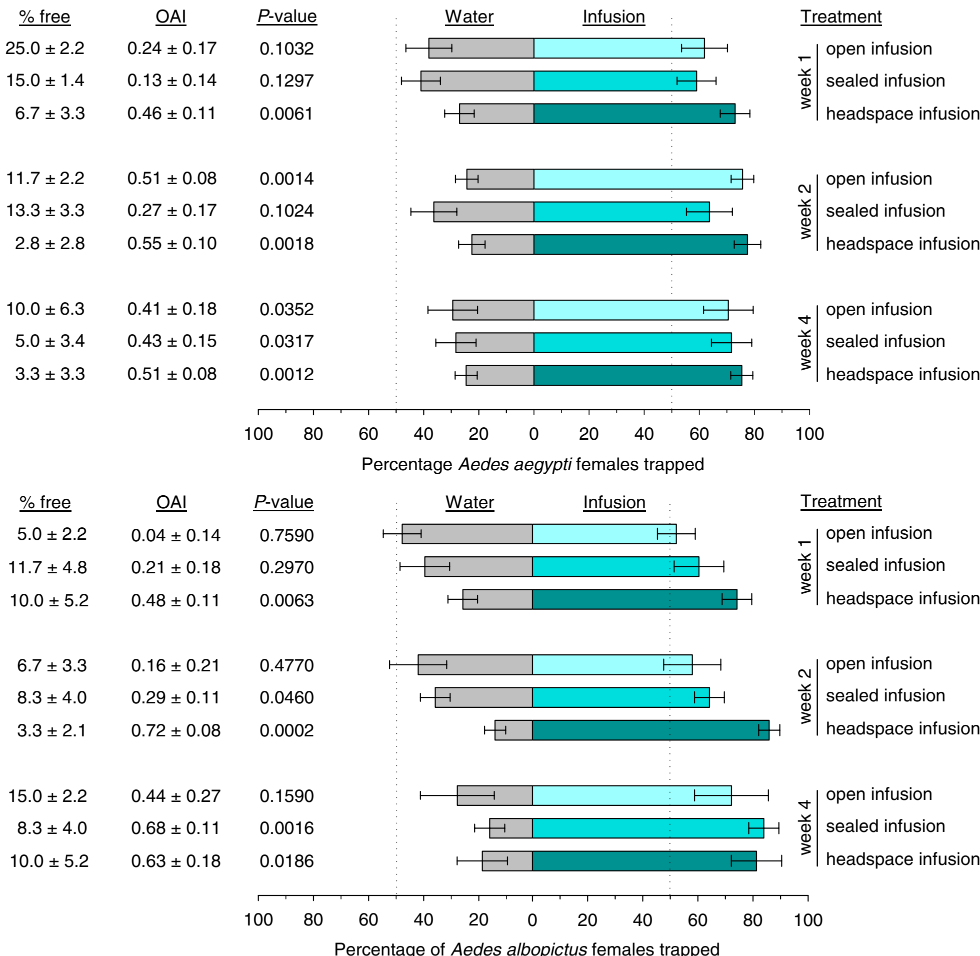

Fig. 2 Evaluation of fermentation procedures and infusion age on attraction of gravid *Aedes aegypti* and *Ae. albopictus* to 0.5X (4.2 g leaves/L water) white oak leaf infusion. There were six replicate bioassay cages per infusion age within each fermentation procedure

Table 1 Amount of indole detected in 10-min SPME collections inside and above the bioassay cage

Sampling position	Amount of indole (ng)			
	0hr		24hr	
	Mean[a]	SE	Mean[a]	SE
Test cup, at the screen	1.27	0.67	0.91	0.26
Test cup, at cup lip	0.43	0.16	0.49	0.14
Test cup, 10 cm above	0.12	0.01	0.20	0.07
Cage center	LOD[b]		LOD	
Control cup, at the screen	LOD		LOD	
Outside the cage, 1 cm above in the center	LOD		LOD	

The test cup contained 30 μg/ml of indole. Headspace samples were collected immediately after setting up the experiment (0 hr) and 24 hr later. Samples were analyzed using GC-FID. Quantification was based on external calibration.

[a] $n=3$

[b] Amounts below the limit of detection (0.10 ng) are indicated by 'LOD'

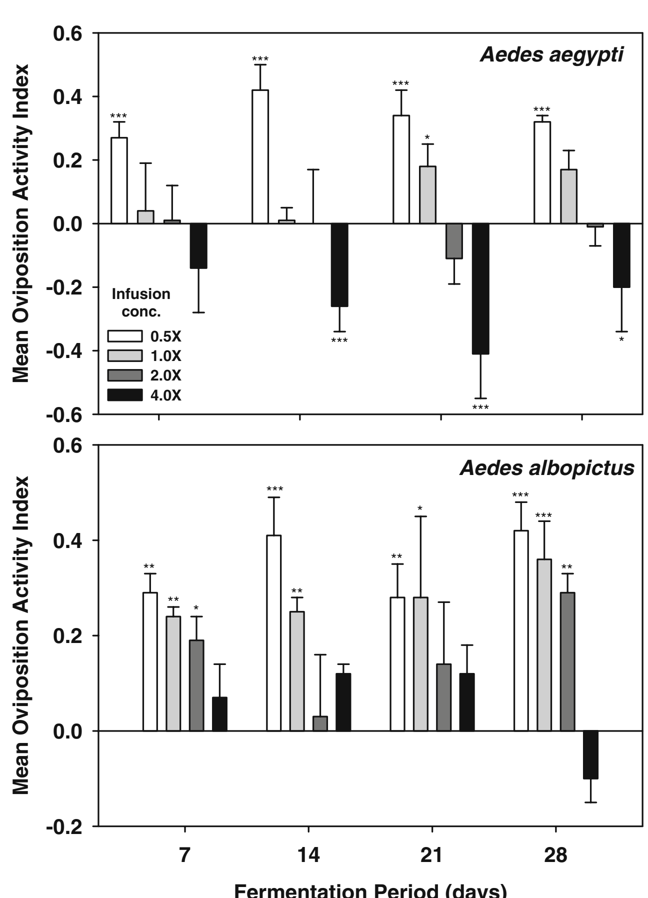

Fig. 3 Effects of leaf biomass and fermentation duration on attraction of gravid mosquitoes to white oak leaf infusions. For each infusion age, there were six replicate cages per trial and three trials completed on different dates. Student's *t*-test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0. ***$P \leq 0.001$, **$0.01 \leq P > 0.001$, *$0.05 \leq P > 0.01$. 1X infusion = 8.4 g leaves per L water

28 days of fermentation. *Aedes albopictus* was not significantly attracted to 4X biomass infusion (Fig. 3).

Bamboo The largest response from both mosquito species to 1X biomass infusions was to bamboo infusion that had been fermented for 7 days (Fig. 4). Attraction of *Ae. aegypti* decreased with longer fermentations. The responses of gravid *Ae. aegypti* were significantly greater than 0 after 7, 14, or 28 days of fermentation; however, the OAI was not significantly different from 0 after 21 days of fermentation ($P=0.288$). *Aedes albopictus* females were attracted to 1X biomass infusion at each bioassay interval over the 28-d time course of fermentation (Fig. 4).

Hackberry Aedes aegypti was attracted to a 1X biomass infusion made from hackberry tree leaves only at the 28 day time point (Fig. 5). For other fermentation time points, mean OAI values were <0.15 and not significantly different from zero ($P>0.05$). In contrast, *Ae. albopictus* responded to 1X biomass infusion at each bioassay interval in the 28 day fermentation period (Fig. 5) and responded most strongly to infusion that had been fermented for 14 days (Fig. 5).

Bermuda Hay In general, *Ae. aegypti* exhibited repellent or neutral responses to 1X Bermuda hay infusions and two dilutions of this infusion, regardless of the age of the infusion (Fig. 6). In contrast, *Ae. albopictus* was attracted to all dilutions and ages of Bermuda hay infusion, except for the undiluted infusion after 14 days of fermentation (Fig. 6).

Live Oak, Red Maple, Pecan, and Panicgrass A live oak infusion at 1X biomass was not attractive to *Ae. aegypti* or *Ae. albopictus* in any of the bioassays completed during the

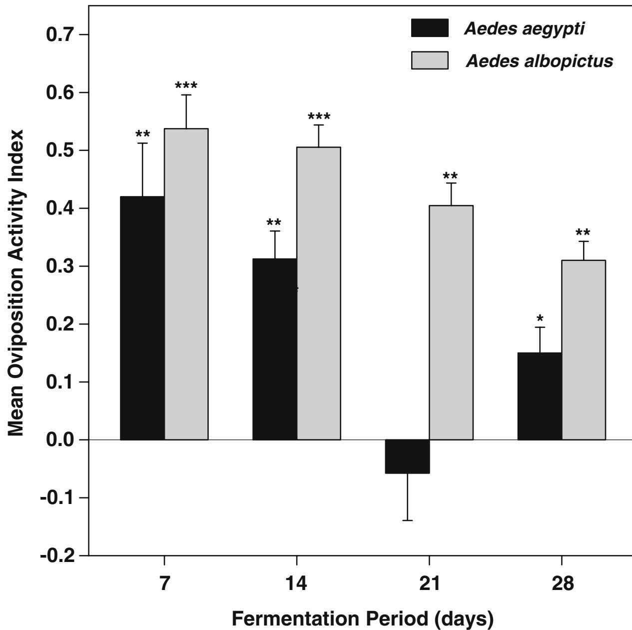

Fig. 4 Effects of infusion age on attraction of gravid *Aedes* mosquitoes to 1X bamboo leaf infusion. For each infusion age, there were six replicate cages per trial and four trials completed on different dates. Student's *t*-test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0. $***P \leq 0.001$, $**0.01 \leq P > 0.001$, $*0.05 \leq P > 0.01$. 1X infusion = 8.4 g leaves per L water

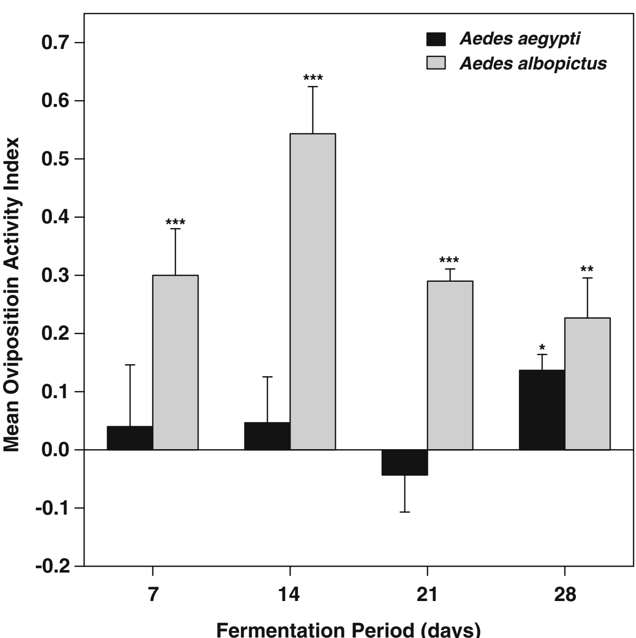

Fig. 5 Effects of infusion age on attraction of gravid *Aedes* mosquitoes to 1X (8.4 g leaves/L water) hackberry leaf infusion. For each infusion age, there were six replicate cages per trial and three trials completed on different dates. Student's *t*-test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0. $***P \leq 0.001$, $**0.01 \leq P > 0.001$, $*0.05 \leq P > 0.01$

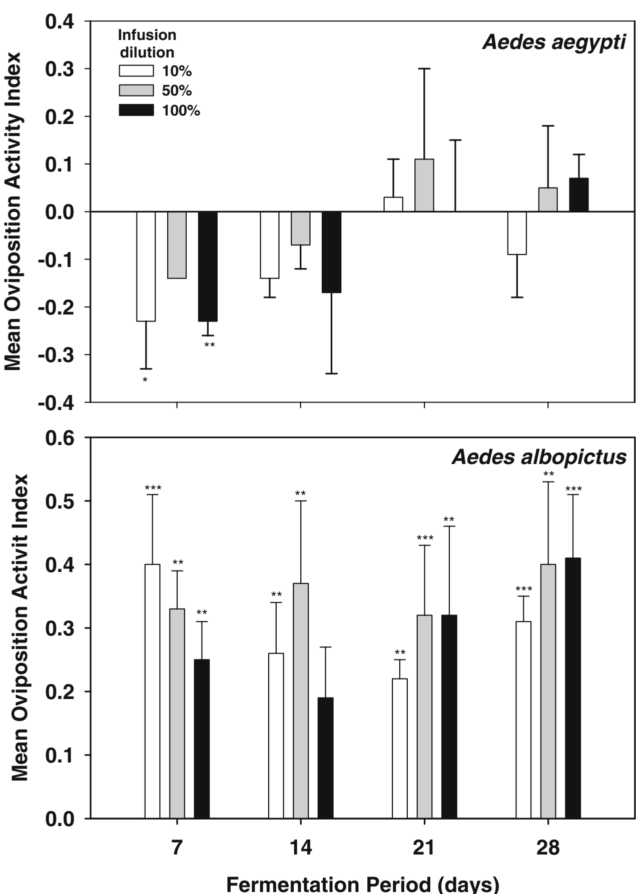

Fig. 6 Effects of infusion concentration and fermentation duration on attraction of gravid mosquitoes to 1X Bermuda hay infusion. Student's *t*-test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0. $***P \leq 0.001$, $**0.01 \leq P > 0.001$, $*0.05 \leq P > 0.01$. 1X infusion = 8.4 g leaves per L water

28 day period of fermentation. For both species, mean OAI values were ≤ 0.15 and not significantly different from zero ($P > 0.05$). Similarly, OAI values derived from responses of both mosquito species to 1X biomass infusions prepared from red maple leaves, pecan leaves, and panicgrass were not significantly different from zero.

Responses to Non-sterile and Sterile WOL Infusions Non-sterile WOL infusion was significantly more attractive to *Ae. aegypti* and *Ae. albopictus* than was sterile infusion (Table 2). The mean OAI for both mosquito species was highly significant, reflecting the high level of attraction of these mosquitoes to the non-sterile infusions and the possible role of microbial fermentation (Table 2).

Walk-in Cage Boassays Of the plant infusions that were tested, BL and WOL infusions elicited the strongest oviposition responses and were further evaluated in walk-in cage bioassays. Both mosquito species exhibited signif-

Table 2 Response of gravid mosquitoes to non-sterile and sterile white oak leaf infusions in sticky-screen bioassays

Species	Mean total no. females (SE) captured per trial[a]		Mean OAI[b] (SE)	$P \geq \|t\|$[c]
	Non-sterile infusion	Sterile infusion		
Aedes aegypti	41.6 (1.6)	16.3 (0.4)	0.43 (0.05)	< 0.001
Aedes albopictus	45.6 (0.4)	13.0 (0.4)	0.55 (0.03)	< 0.001

[a] In each of three experimental trials, there were six replicate cages, each containing 10 gravid females

[b] *OAI* Oviposition Activity Index

[c] Student's *t* test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0

icant, albeit variable, responses to the dilutions and volumes of both plant infusions (Table 3). The responses of *Ae. aegypti* generally were more consistent to undiluted infusions of both plant species. *Aedes albopictus* was highly attracted to undiluted infusions of both plant species, but exhibited the strongest response to BL infusion (Table 3).

Discussion

Our investigation is the first comprehensive analysis of methods used to produce and optimize organic infusions for production of oviposition attractants for *Ae. aegypti* and *Ae. albopictus*. With indole as a marker odorant, we determined that volatiles from test containers placed inside bioassay cages would provide females with differential cues to guide them to attractive infusions. In contrast to our present research with WOL infusion, Trexler et al. (1998) reported that WOL infusions mediated oviposition of *Ae. triseriatus* (Say) and *Ae. albopictus* through contact with nonvolatile arrestants or oviposition stimulants rather than through odorants. However, we found that lowering the sticky screen deeper into the bioassay cup significantly increased the ability of females to differentiate between treatments, presumably by creating more headspace or through a yet unknown interaction of olfactory and visual cues at close-range as the female enters the infusion container.

The importance of microbial metabolic activity in breaking down organic matter and producing semiochemicals that mediate the oviposition responses was suggested by the lack of significant attraction of *Ae. aegypti* and *Ae. albopictus* to a sterile WOL infusion. However, it should be noted that the leaves used to construct the sterile WOL infusion were autoclaved. It is possible that high temperature would have affected the chemistry of the resulting infusion, altering the response of gravid mosquitoes by chemical changes rather than biological changes. Recently, however, we showed that microbe-associated contact chemical cues in the infusion elicited *Ae. aegypti* females to oviposit more when microbes were present than when they were filtered out (Ponnusamy et al. 2008). Similarly, *Culex* laid fewer eggs in response to Bermuda grass infusion that had been filtered to remove microorganisms (Isoe and Millar 1995).

Table 3 Effects of volume and dilution on attraction of gravid *Aedes* mosquitoes to bamboo leaf (1X) and white oak leaf (0.5X) infusions in walk-in cages using the sticky-screen bioassay procedure. Infusions were prepared as 1X (8.4 g/L well water) or 0.5X (4.2 g/L well water) infusions, and were used either at 100% or after dilution with an equal volume of well water (50%)

Infusion	Volume (ml)	Dilution (%)	Mean Oviposition Activity Index (± SE)[a]			
			Ae. aegypti	$P > \|t\|$[b]	*Ae. albopictus*	$P > \|t\|$[b]
Bamboo leaf	500	50	−0.12 (0.13)	0.4287	0.44 (0.09)	0.0159
		100	0.34 (0.01)	< 0.001	0.72 (0.13)	0.0125
	1230	50	0.36 (0.09)	0.0310	0.63 (0.14)	0.0193
		100	0.35 (0.11)	0.0557	0.50 (0.13)	0.0305
White oak leaf	500	50	0.47 (0.08)	0.0116	0.08 (0.11)	0.5486
		100	0.32 (0.13)	0.0869	0.56 (0.04)	< 0.001
	1230	50	0.22 (0.04)	0.0125	0.16 (0.06)	0.0718
		100	0.41 (0.10)	0.0276	0.32 (0.15)	0.1151

[a] OAI (Oviposition Activity Index) is the mean of four trials

[b] Student's *t* test of the hypothesis that mean OAI for each treatment is significantly greater (attraction) or less (repulsion) than 0

The importance of microbial metabolism is also suggested by our finding that infusions prepared in closed Teflon bags with some headspace were more attractive to gravid mosquitoes than infusions produced in open bags or closed bags without headspace. In closed bags with headspace, microbial breakdown of organic materials likely occurred through facultative anaerobic fermentation. Similarly, Sant'Ana et al. (2006) found that fermentation of *Panicum maximum* grass in a sealed plastic container produced infusions that were more active in eliciting an oviposition response from *Aedes* (*Stegomyia*) mosquitoes than infusions prepared in an open container.

Even under uniform fermentation conditions, there were large batch-to-batch variations in the response of gravid mosquitoes to some infusions. We attribute this variation to differences in the quality and amounts of attractive odorants released from infusions. Since we used senescent plant materials that were collected from the ground to make infusions, it is likely there were differences in the leaf condition as well as differences in the species composition of the microbial community associated with plant materials. Significant variation in the abundance and species composition of microbial populations is known to occur on the surfaces of leaves from the same trees (Brunel et al. 1994; Yang et al. 2001; Lambais et al. 2006). Such differences would be expected to result in variations in microbial populations in our experimental plant infusions and in corresponding differences in the amounts and kinds of odorants that were produced. Notably, infusions made from live oak, red maple, pecan, and panicgrass leaves were not attractive to *Ae*. *aegypti* or *Ae*. *albopictus*. These results reinforce the critical importance of the plant species used to produce infusions. The implication of our findings is that plant infusions can be an inconsistent source of attractive odorants, and that each batch of infusion should be evaluated to verify that it is behaviorally active before it is used in the field. Ideally development of a mosquito monitoring or surveillance system would involve controlled release of specific chemically defined oviposition cues.

In sticky-screen bioassays in reach-in cages, infusions were differentially attractive to each mosquito species depending on the plant species, biomass used and length of fermentation period. In general, *Ae*. *aegypti* exhibited higher attraction to low biomass infusions produced over short fermentation times from just a few plant species. In contrast, *Ae*. *albopictus* was attracted to infusions that were produced using a broader range of plants species, biomass, and fermentation periods. For example, *Ae*. *aegypti* was either repelled or exhibited a neutral response to infusions made from Bermuda hay, while *Ae*. *albopictus* was significantly attracted to Bermuda hay infusions regardless of the age of the infusion. Walk-in bioassay cage results were concordant with results of reach-in cages. In walk-in cage bioassays, both mosquito species were significantly attracted to WOL and BL infusions. Generally, *Ae*. *aegypti* was attracted to diluted and low volume infusions. Surprisingly, *Ae*. *albopictus* was attracted to 1X WOL infusion over a narrower range of infusion volumes compared to *Ae*. *aegypti*.

In previous investigations, infusions made by fermenting a variety of grass and hay species have been reported to be active towards gravid *Ae*. *aegypti* in laboratory and field bioassays (Reiter et al. 1991; Chadee et al. 1993; Rawlins et al. 1998; Polson et al. 2002; Sant'Ana et al. 2006). In these investigations, the response of mosquitoes was evaluated by using an end point; the numbers of eggs deposited in test containers holding infusions compared to control containers holding water. Higher numbers of eggs deposited in test containers was interpreted to indicate that more gravid mosquitoes were attracted to the test infusion. With an olfactometer, Hazard et al. (1967) showed that gravid *Ae*. *aegypti* were not attracted to any of the volatile chemicals released from alfalfa hay infusion. However, the hay infusion contained nonvolatile chemicals that arrested females at the surface of the water and stimulated them to oviposit. Similar negative results of olfactometer experiments involving hay infusion were reported by Allan and Kline (1995) for gravid *Ae*. *aegypti*. Isoe et al. (1995a) and Isoe and Millar (1995), in bioassays of gravid *Culex* mosquitoes, cautioned against using the numbers of eggs deposited as an endpoint for assessing attraction to plant infusions. They emphasized that differentiating oviposition responses mediated by olfactory attractants from nonvolatile oviposition stimulants cannot be done simply by counting eggs. Thus, the increased oviposition previously reported for *Ae*. *aegypti* in response to hay and grass infusions likely resulted from nonvolatile chemicals that arrested gravid females at the surface of the water and stimulated them to lay eggs, rather than from odorants that attracted gravid females from a distance. The intent of our study was to evaluate olfactory-mediated responses of gravid females to volatiles from plant infusions. It should be noted that attraction of gravid females to odorants that emanate from a plant infusion might not result in increased oviposition because volatile chemicals that attract females may not necessarily function also as oviposition stimulants. However, some species of cultivable bacteria in bamboo leaf infusion produce metabolites that attract gravid females and also stimulate them to lay eggs. With bioassay-guided fractionation of bacterial extracts, carboxylic acids and methyl esters associated with 14 species of bacteria cultured from bamboo leaf infusion were shown to be potent oviposition stimulants (Ponnusamy et al. 2008). A mix of these same 14 species of bacteria produces odorants that are highly attractive to gravid *Ae*. *aegypti* (Ponnusamy et al. 2010).

Optimally attractive infusions for *Ae*. *aegypti* and *Ae*. *albopictus* required fermentation periods of different

lengths depending on the plant species. Similar results were reported by Isoe et al. (1995b) for response of *Culex quinquefasciatus* and *Cx*. *tarsalis* to Bermuda grass infusion. *Culex quinquefasciatus* continued to respond significantly to Bermuda grass infusions that were fermented over a longer period of time compared to *Cx*. *tarsalis*, which preferred infusions that were fermented over a shorter period. Sant'Ana et al. (2006) found that *Aedes* (*Stegomyia*) mosquitoes exhibited the highest oviposition responses to 15–20-d-old infusions produced from the grass *Panicum maximum*. On average, ovitraps containing younger or older infusions received lower numbers of eggs. Ponnusamy et al. (2010) showed that the attraction of gravid *Ae*. *aegypti* and *Ae*. *albopictus* to WOL and BL infusions was significantly associated with changes in the abundance and diversity of bacterial species, which in turn was affected by plant species, leaf biomass and fermentation time.

Currently, we are characterizing the attraction and egg-laying response of gravid *Ae*. *aegypti* and *Ae*. *albopictus* to individual bacterial species cultured from BL and WOL infusions and characterizing semiochemicals that mediate attraction and oviposition. An understanding of the bioactivity of bacterial species may explain why *Ae*. *aegypti* and *Ae*. *albopictus* are differentially attracted to the two types of infusions. Pickett et al. (2010) emphasized the importance of investigating the chemical ecology of disease vectors with the aim of developing efficient tools for surveillance and control. In this regard, the optimized infusions, specific bioactive microbes isolated from them, and specific compounds that attract and stimulate females to oviposit can be used in mosquito and virus surveillance, mass trapping, and other intervention approaches to reduce the human burden of *Aedes*-transmitted arboviral diseases.

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Antitermite Activities of Coumarin Derivatives and Scopoletin from *Protium javanicum* Burm. f.

Morina Adfa · Tsuyoshi Yoshimura · Kenichi Komura · Mamoru Koketsu

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Abstract The antitermite (termiticidal and antifeedant) activity of *Protium javanicum* Burm. f. extract was investigated. The ethyl acetate fraction was active. Scopoletin (**1**), quercetin, and stigmasterol were isolated by bioassay-guided fractionation. Scopoletin had the highest activity among the three compounds. In order to investigate the structure-activity relationship (SAR) of the methoxy and hydroxy groups at the C-6 and C-7 positions of the coumarin skeleton, we synthesized several coumarin derivatives whose chemical structures are similar to scopoletin. Scopoletin exhibited the strongest termiticidal activity among the 10 compounds tested, followed by 6-methoxycoumarin (**3**), 6-hydroxycoumarin (**7**), and umbelliferone (**8**). All compounds except coumarin (**9**) showed antifeedant activity.

Key Words *Protium javanicum* Burm. f. · *Coptotermes formosanus* Shiraki · Scopoletin · 6-Methoxycoumarin 6-Hydroxycoumarin · Umbelliferone · SAR

M. Adfa · K. Komura · M. Koketsu (✉)
Department of Materials Science and Technology,
Faculty of Engineering, Gifu University,
1–1 Yanagido,
Gifu 501–1193, Japan
e-mail: koketsu@gifu-u.ac.jp

T. Yoshimura
Research Institute for Sustainable Humanosphere (RISH),
Kyoto University,
Gokasho,
Uji, Kyoto 611–0011, Japan

M. Adfa
Department of Chemistry, Bengkulu University,
Jl. Raya Kandang Limun, Bengkulu 38371, Indonesia

Introduction

Termites pose a serious threat to both the plant kingdom and buildings. There are over 2,800 described species with about 185 considered to be pests. They cause considerable damage to annual and perennial crops and the wooden parts in buildings, especially in the semi-arid and sub-humid tropics (Verma et al. 2009). Among the many species, *Coptotermes formosanus* Shiraki is the subterranean termite responsible for major destruction of wooden materials in countries such as Japan, and Taiwan, and the southern parts of the United States. For this reason, *Coptotermes formosanus* Shiraki has been bioassayed in many research studies (Chang and Cheng 2002; Zhu et al. 2003; Cheng et al. 2007; Katsumata et al. 2007; Mozaina et al. 2008).

Synthetic chemicals currently are used to control termites. However, synthetic termiticides have a negative impact on the global environment, including causing health hazards to humans. To avoid these impacts, naturally occurring compounds that are bio-active against termites are being identified. A number of diverse compounds derived from termite-resistant wood species have been reported to be toxic to termites: these include the abietane-type diterpene, 6,7-dehydroroyleanone, from *Taxodium distichum* (Kusumoto et al. 2009); abietane diterpenes, plumbagin, isodiospyrin, and microphyllone, from *Diospyros sylvatica* (Ganapaty et al. 2004); naphthoquinone, 7-methyljuglone, from *Diospyros virginiana* L. (Carter et al. 1978); and iridoid glycoside, loganin, from *Guettarda speciosa* L. (Yaga and Kinjo 1985).

Protium javanicum Burm. f. (Burceracea) is known locally as "*kayu bawang*" and "*kayu pahit*" in Bengkulu-Indonesia. Kayu bawang has been utilized as a building material in desks, ground sills, and exterior walls because of its biological durability. Species of the genus *Protium*

comprise an attractive commercial botanical source of aromatic compounds. In folk medicine, gum and oleoresins from *Protium* species, among other medicinal applications, are used for preparing tonics, stimulants, and anti-inflammatory agents; treating ulcers (Rüdiger et al. 2007); and treating headaches, eyelid inflammation, and rheumatic pains (Deharo et al. 2001). The resin also is often burned to illuminate houses in the forest and repel undesirable insects (Rüdiger et al. 2007).

Chemical investigation of *Protium* species has led to the identification of steroids, triterpenes, coumarins, lignans, and other derivatives. Propacin and sitosterol have been found in wood of *P. opacum* (Zoghbi et al. 1981), and cleomiscosin, along with *p*-coumaric ethyl ester, fraxetin, scopoletin, and lupeol, has been isolated from the stems and bark of *P. heptapyllum* (Almeida et al. 2002).

Thus far, to the best of our knowledge, no phytochemical investigation has been reported on *P. javanicum*. In this study, we isolated compounds from *P. javanicum* by bioassay-guided fractionation, and investigated their anti-termite activities against *C. formosanus*. In addition, we examined the antitermite activities of scopoletin and coumarin derivatives whose chemical structures are similar to scopoletin to evaluate the effect of chemical structure on the antitermite properties.

Methods and Materials

General Experimental Procedures ^{1}H and ^{13}C-NMR spectra were recorded on a JOEL ECA 600 spectrometer with tetramethylsilane (TMS) as an internal standard. MS spectra were recorded using a JOEL JMS-700/GI mass spectrometer. Column chromatography (CC) was performed on a neutral silica gel (Silica Gel 60 N, spherical, neutral, 40–50 μm) (KANTO Chemical Co., Inc.). Melting points were determined on a Yanagimoto micro melting-point apparatus. Compounds **2**, **3**, **4**, **5**, and **6** were synthesized in our laboratory according to previously described methods (Crosby and Berthold 1962; Castillo et al. 1986). Compound **7** was purchased from Sigma-Aldrich. Compounds **8**, **9**, and **10** were purchased from Tokyo Chemical Industry Co., Ltd.

Plant Material The leaves of *P. javanicum* were collected from Bengkulu Utara, Indonesia. Identification of the plant material was performed by the Herbarium of Andalas University, where voucher specimens are deposited.

Extraction and Isolation of Antitermite Compounds Fresh leaves of *P. javanicum* (10 kg) were macerated at room temperature in methanol (3×30 L). The mixture was subsequently filtered and concentrated *in vacuo* to yield a methanol extract (614 g). This extract was suspended in water and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH to yield *n*-hexane (69 g), EtOAc (118 g), *n*-BuOH (56 g), and water (290 g) fractions, respectively. Each stage of the isolation process and all column fractions were monitored for antitermite activity. The ethyl acetate fraction contained the highest activity among all fractions as assessed by the no-choice feeding test.

A portion of the ethyl acetate fraction (69 g) was subjected to silica gel column chromatography with *n*-hexane-EtOAc, EtOAc-acetone, and acetone-MeOH step-wise (100:0 to 0:100) to obtain 12 fractions (E1–E12), which were combined for TLC analysis. Fractions E3, E9, and E10 were the most active. Stigmasterol colorless crystals (19.9 mg) were purified from fraction E3 by re-crystallization with *n*-hexane, mp. 147–148°C, EI-MS m/z (rel. int. %): 412 (M^+, 80), the ^{1}H-NMR and ^{13}C-NMR spectra were in good agreement with reported data (Farines et al. 1981; Akihisa et al. 1987). Fraction E9 (1.46 g) was subjected to CC on silica gel, eluted with *n*-hexane-EtOAc, and EtOAc-acetone (100:0 to 0:100) to obtain 6 sub fractions (E9a–E9f). Sub fraction E9d was subjected further to flash CC on silica gel, eluting with $CHCl_3$-MeOH mixtures of increasing polarity (100:0 to 0:100), affording quercetin as a yellow amorphous powder (10.2 mg). Compound **1**, a pale yellow crystal (23.1 mg), was isolated from fraction E10 (2.58 g). CC on silica gel was performed on fraction E10, which was eluted with *n*-hexane-EtOAc (100:0 to 0:100), and then EtOAc-acetone (100:0 to 0:100) to give 5 sub-fractions (E10a–E10e). Flash CC on silica gel was performed subsequently on sub-fraction E10b, which was eluted with *n*-hexane-$CHCl_3$ (100:0 to 0:100). This, in turn, was followed by re-crystallization with chloroform. The structure of **1** was confirmed by ^{1}H and ^{13}C-NMR; DEPT, COSY, HMBC, HMQC, and MS; and by comparing the data with the literature (Steck and Mazurek 1972; Barberá et al. 1986b; Razdan et al. 1987). Scopoletin (**1**), quercetin, and stigmasterol were reported previously in *P. heptapyllum* (Almeida et al. 2002; Rüdiger et al. 2007), but this is the first time they have been isolated from *P. javanicum*.

Quercetin Yellow amorphous powder, mp. >300°C; ^{1}H-NMR δ (Acetone-d_6): 6.27 (1H, d, *J*=2.0 Hz, H-6), 6.53 (1H, d, *J*=2.0 Hz, H-8), 6.99 (1H, d, *J*=8.2 Hz, H-5′), 7.70 (1H, dd, *J*=8.58 and 2.8 Hz, H-6′), 7.83 (1H, d, *J*=2.8 Hz, H-2′), 12.16 (1H, s, OH-5); ^{13}C-NMR δ (CD_3OD): 94.5 (C-8), 99.3 (C-6), 104.6 (C-10), 116.1 (C-2′), 116.3 (C-5′), 121.7 (C-6′), 124.2 (C-1′), 137.3 (C-3), 146.3 (C-3′), 148.1 (C-2), 148.8 (C-4′), 158.3 (C-9), 162.6 (C-5), 165.6 (C-7), 177.4 (C-4). The melting point, as well as the ^{1}H and ^{13}C-NMR data were in agreement with reported data (Barberá et

al. 1986a; Fossen et al. 1998; Tatsis et al. 2007; Luo et al. 2009).

Compound **1** *(Scopoletin)* Pale yellow crystals, mp. 181–182°C; ^{1}H-NMR δ (CD_3OD): 3.91 (3H, s, CH_3), 6.20 (1H, d, J=9.6 Hz, H-3), 6.77 (1H, s, H-8), 7.11 (1H, s, H-5), 7.85 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR δ (CD_3OD): 56.8 (OCH_3), 104.0 (C-8), 110.0 (C-5),112.56 (C-10), 112.62 (C-3), 146.1 (C-4), 147.1 (C-6), 151.4 (C-9), 152.9 (C-7), 164.0 (C-2). FAB-MS m/z: 193 $[M+H]^+$ (matrix: mixture of dithiothreitol and dithioerytritol 3:1).

Synthesis Procedure and Spectral Data of Coumarin Derivatives 2,5-Dihydroxyanisole (**a1**, 1.5 g, 11 mmol) and ethyl 3,3-diethoxypropionate (2.44 g, 13 mmol) were dissolved in liquid phosphoric acid (5 ml) in a small flask and heated at 100°C for 2 h. The mixture was poured over 25 g of crushed ice; the precipitate was recovered by filtration and washed with cold water. The solid product was subjected to CC on silica gel with the eluent $CHCl_3$-EtOAc (8:2) to give isoscopoletin (**2**). Compounds **3**, **4**, and **5** were synthesized in similar manner with the exception that 4-methoxyphenol (**a2**), 3-methoxyphenol (**a3**), and 3,4-dimethoxyphenol (**a4**), respectively, were used instead of **a1** (Scheme 1).

Compound **2** *(Isoscopoletin)* Pale yellow needles, mp. 183–184°C (96% yield); ^{1}H-NMR δ ($CDCl_3$:CD_3OD=4:1): 3.96 (3H, s, CH_3), 6.27 (1H, d, J=9.6 Hz, H-3), 6.85 (1H, s, H-8), 6.94 (1H, s, H-5), 7.65 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR δ ($CDCl_3$:CD_3OD=4:1): 56.2 (OCH_3), 99.5 (C-8), 111.4 (C-5), 112.1 (C-10), 113.1 (C-3), 143.4 (C-6), 144.0 (C-4), 149.1 (C-9), 151.3 (C-7), 162.4 (C-2); EI-MS m/z (rel. int. %): 192 (M^+, 60), 177 (13), 164 (50), 149 (80), 122 (25), 118 (100).

Compound **3** *(6-Methoxycoumarin)* Colorless crystals, mp. 95–96°C (58% yield); ^{1}H-NMR δ ($CDCl_3$): 3.85 (3H, s, CH_3), 6.43 (1H, d, J=9.6 Hz, H-3), 6.92 (1H, d, J=2.8 Hz, H-5), 7.11 (1H, dd, J=9.3 and 2.8 Hz, H-7), 7.27 (1H, d, J=8.9 Hz, H-8), 7.66 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR δ ($CDCl_3$): 56.0 (OCH_3), 110.1 (C-5), 117.2 (C-3), 118.0 (C-8), 119.3 (C-10), 119.6 (C-7), 143.3 (C-4), 148.6 (C-9), 156.2 (C-6), 161.1 (C-2); FAB-MS m/z: 177 $[M+H^+]$ (matrix: mixture of dithiothreitol and dithioerytritol 3:1).

	R^1	R^2
a1	OCH_3	OH
a2	H	OCH_3
a3	OCH_3	H
a4	OCH_3	OCH_3

H_3PO_4, 100 °C, 2 hr

	Yield (%)
2	96
3	58
4	88
5	59

Scheme 1 Synthesis of coumarin derivatives (**2**–**5**)

Compound **4** *(Herniarin)* Colorless crystals, mp. 115°C (88% yield); ^{1}H-NMR δ ($CDCl_3$): 3.88 (3H, s, CH_3), 6.25 (1H, d, J=9.6 Hz, H-3), 6.81 (1H, d, J=2.8 Hz, H-8), 6.85 (1H, dd, J=8.6 and 2.8 Hz, H-6), 7.38 (1H, d, J=8.2 Hz, H-5), 7.64 (1H, d, J=9.7 Hz, H-4); ^{13}C-NMR δ ($CDCl_3$): 55.9 (OCH_3), 101.0 (C-8), 112.65 (C-10), 112.68 (C-6), 113.2 (C-3), 128.9 (C-5), 143.5 (C-4), 156.0 (C-9), 161.3 (C-2), 163.0 (C-7); EI-MS m/z (rel. int. %): 176 (M^+, 95), 148 (100), 133 (60), 119 (35), 105 (16).

Compound **5** *(Scoparone)* Yellow needles, mp. 122–123°C (59% yield); ^{1}H-NMR δ ($CDCl_3$): 3.92 (3H, s, CH_3), 3.95 (3H, s, CH_3), 6.28 (1H, d, J=9.6 Hz, H-3), 6.83 (1H, s, H-8), 6.87 (1H, s, H-5), 7.64 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR δ ($CDCl_3$): 56.4 (OCH_3), 100.0 (C-8), 108.1 (C-5), 111.5 (C-10), 113.5 (C-3), 143.4 (C-4), 146.4 (C-6), 150.0 (C-9), 152.9 (C-7), 161.4 (C-2); EI-MS m/z (rel. int. %): 206 (M^+, 100).

Compound **6** *(Ayapin)* Compound **6** was obtained from esculetin (**10**) (Castillo et al. 1986). Briefly, the reaction of **10** (232 mg, 1.3 mmol) with diiodomethane (0.21 ml, 2.6 mmol) in the presence of sodium hydride (62.5 mg, 2.6 mmol) in hexamethylphosphoric triamide (4 ml) at room temperature to afford **6** (59% yield) as colorless crystals, mp. 199.5°C; ^{1}H-NMR δ ($CDCl_3$): 6.07 (2H, s), 6.28 (1H, d, J=9.6 Hz, H-3), 6.82 (1H, s, H-8), 6.83 (1H, s, H-5), 7.58 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR δ ($CDCl_3$): 98.4 (C-8), 102.4 (CH_2), 105.0 (C-5), 112.7 (C-10), 113.4 (C-3), 143.5 (C-4), 144.9 (C-6), 151.27 (C-9), 151.29 (C-7), 161.2 (C-2); EI-MS m/z (rel. int. %): 190 (M^+, 100), 162 (73), 161 (43), 113 (37).

The structure of compounds (**2**–**6**) were elucidated and confirmed by ^{1}H and ^{13}C-NMR, MS, and by subsequent comparisons with reference data from the available literature (Duddeck and Kaiser 1982; Tsukamoto et al. 1984; Afek et al. 1986; Debenedetti et al. 1998; Maes et al. 2005).

Termites Workers and soldiers of *C. formosanus* Shiraki were obtained from a laboratory colony maintained at 28°C ± 2 and >85% Relative Humidity (RH) at the Deterioration Organisms Laboratory of the Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Japan.

Termiticidal and Antifeedant Tests A no-choice test was employed for evaluating termiticidal and antifeedant activities (Watanabe et al. 2005). Test samples [(10 mg for every fraction from the leaves of *P. javanicum* extract, 0.5 mg for isolated compounds, and 5 μmol for compound **1**, and the coumarin derivatives (**2**–**10**)] were dissolved in 0.05 ml acetone. The resulting solution was applied to paper discs (1.5 mm thick 8 mm diam, Advantec, Tokyo, Japan), and the paper discs were dried overnight in a vacuum

desiccator. Subsequently, each disc was placed onto a plastic spacer in an acrylic resin cylinder with a plaster bottom (80 mm diam × 60 mm height), and thirty-three active termites (30 workers and 3 soldiers) were added to each cylinder. The cylinders were placed on a cotton sheet in a plastic tray and kept in a dark incubator at 28°C ± 1 and 80% ± 5 RH for 2 wk. The number of dead termites was counted daily. The mass loss of the paper disc was measured at the end of the test. Paper discs treated with acetone only were used as a negative control. Three replications were performed for each sample. Termite mortality (%) and mass loss of the paper disc (%) were calculated using the following equations:

$$Termite\,Mortality(\%) = \frac{Number\ of\ Dead\ Termites}{Total\ Number\ of\ Test\ Termites} \times 100\%$$

$$Mass\,Loss(\%) = \frac{Vacuum\ Dried\ Weight\ Before\ Test - Vacuum\ Dried\ Weight\ After\ Test}{Vacuum\ Dried\ Weight\ Before\ Test} \times 100\%$$

Statistical Analysis Tukey's multiple comparison test was used to evaluate differences in percent mortality and mass loss in antitermite activities. Results with $P<0.05$ were considered significant. Calculations were performed using statistical software (Stats Direct, UK).

Results and Discussion

Antitermite Activities of Protium javanicum Fractions Against Coptotermes formosanus Shiraki We evaluated the termiticidal and antifeedant activities of *n*-hexane, EtOAc, *n*-BuOH, and water fractions of *P. javanicum* leaves methanol extract. Among all fractions, the ethyl acetate fraction showed the highest activity against *C. formosanus*, when tested initially at 10 mg/0.05 ml acetone in the no-choice feeding test. After 14 days, 42.4% of termites given the ethyl acetate fraction died on average. This percentage was significantly different from that of the control at the $P<0.05$ level (Tukey's multiple comparison test). Moreover, when testing antifeedant activity, the average mass loss of the paper disc treated with the ethyl acetate fraction reduced five-fold as compared to the control (Table 1).

Table 1 Antitermite activities of *Protium javanicum* fractions against *Coptotermes formosanus* Shiraki[a]

Fraction	Mortality of termite (%)	Mass loss of paper disc (%)
n-Hexane	26.3±9.7a,b	0.6±0.3b
EtOAc	42.4±8.0a	8.5±9.8b
BuOH	15.1±0b,c	10.9±8.9b
H_2O	12.1±5.2b,c	12.7±4.3b
Control	1.0±1.8c	41.6±3.1a

[a] Data are given as mean ± standard deviation of triplicate tests (N=3). Numbers followed by different letters (a–c) are significantly different at the level of $P<0.05$ according to Tukey's multiple comparison test

Antitermite Activities of Isolated Compounds The isolation of scopoletin (**1**) together with quercetin and stigmasterol from the ethyl acetate fraction was achieved by CC followed by re-crystallization with chloroform, and we then evaluated the antitermite activities, the results of which are summarized in Figs. 1 and 2. Average termite mortality of scopoletin (**1**) (55.6%) was higher than those of control, quercetin (15.2%), and stigmasterol (2.0%) after 14 days' exposure at the $P<0.05$ level (Tukey's multiple comparison test) (Table 2). The termites least consumed the paper disc treated with scopoletin (**1**), with the mass loss of the disc (avg. 9.7%) being significantly different from the control (avg. 47.6%), quercetin (avg. 32.4%), and stigmasterol (avg. 41.2%). These results show that scopoletin (**1**) has the highest activity among the 3 compounds.

Structure-Activity Relationship (SAR) of Scopoletin and Coumarin Derivatives to Antitermite (Termiticidal and

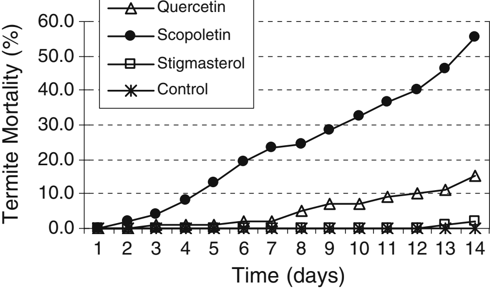

Fig. 1 Termite mortalities (%) of isolated compounds against *Coptotermes formosanus* Shiraki in no-choice test. Each experiment was performed in triplicate. 33 termites were used per replication, and the data were averaged (N=3)

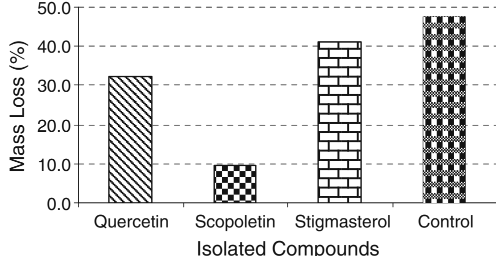

Fig. 2 Paper disc consumption of *Coptotermes formosanus* Shiraki after 14 d exposure to isolated compounds in no-choice test. Each experiment was performed in triplicate. 33 termites were used per replication, and the data were averaged (N=3)

Antifeedant) Activities By comparing the activity of latifolin and its derivatives, it has been suggested previously that the phenolic hydroxyl group at the C-5 position of the A ring is responsible for a compound's antitermite activities (mortality and mass loss) (Sekine et al. 2009). The effect of stilbene glucosides and their related compounds on termite feeding behavior also has been investigated, with piceid exhibiting the highest feeding-deterrent effect followed by isorhapontin and astringin. An increase in the termite mortality rate also has been reported for isorhapontigenin when the compound is methylated (Shibutani et al. 2004). The antifeedant activity of some flavonoids was influenced by the position of hydroxyl groups (Ohmura et al. 2000). Several of the monomethoxycoumarins (5-alkoxycoumarins and their derivatives) have been reported as having nematicidal activity. Among these, 5-ethoxycoumarin showed the highest activity against the phytopathogenic nematode, *Bursaphelenchus xylophilus* (Takaishi et al. 2008). On the other hand, coumarin and its derivatives are isolated chiefly from higher plants, especially the Rutaceae family, and exhibit various biological and pharmacological activities, such as anti-bacterial, anti-oxidant, anticoagulant, anti-inflammatory, rodenticidal, and analgesic properties (Murray et al. 1982). Until now, however, studies on the SAR of coumarin derivatives to termiticidal activity have not been performed.

In this study, for scopoletin (**1**), our interest focused on the modification of the position of the methoxy and hydroxy groups at C-6 and C-7, respectively, of the coumarin skeleton. In order to investigate the structure-activity relationship (SAR), we synthesized several coumarin derivatives whose chemical structures are similar to scopoletin (compounds **2**, **3**, **4**, **5**, and **6**) in good yields and four compounds were purchased (compounds **7**, **8**, **9**, and **10**). Finally we collected 10 compounds (Fig. 3) and tested them for antitermite (termiticidal and antifeedant) activities.

The daily termite mortality against *C. formosanus* with scopoletin (**1**) and coumarin derivatives (**2**–**10**) was determined for 2 weeks. The results are summarized in Fig. 4. Compound **1** showed the highest mortality (avg. 59.6%), while the mortality of compound **2** (avg. 11.1%) decreased five-fold compared to compound **1**. Compound **3**, bearing a substituent OCH_3 at the C-6 position, showed 46.5% mortality on average. Compound **7**, bearing an OH group at the C-6 position, showed an average mortality of 45.5%. Compound **8**, bearing a substituent OH group at the C-7 position, killed 36.4% of termites on average. Other compounds had lower activities and exhibited different patterns of mortality change. The mortality of compound **1** showed a steady increase for 2 weeks, while the mortality

Table 2 Statistical analysis of antitermite activities of isolated compounds and coumarin derivatives[a]

Compound	Mortality of termite (%)	Mass loss of paper disc (%)
Isolated compounds		
Scopoletin	55.6±6.3a	9.7±1.5c
Quercetin	15.2±5.2b	32.4±4.8b
Stigmasterol	2.0±1.7c	41.2±5.9a,b
Control	0.0±0c	47.6±2.9a
Scopoletin and coumarin derivatives		
Scopoletin (**1**)	59.6±11.5a	2.0±1.6d
Isoscopoletin (**2**)	11.1±4.6d,e	21.7±5.7b
6-Methoxycoumarin (**3**)	46.5±7.6a,b	4.7±0.9c,d
Herniarin (**4**)	13.1±1.7d,e	3.5±3.3d
Scoparone (**5**)	21.2±3.0c,d	8.3±3.8b,d
Ayapin (**6**)	11.1±4.6d,e	19.4±3.6b,c
6-Hydroxycoumarin (**7**)	45.5±9.1a,b	4.8±1.4c,d
Umbelliferone (**8**)	36.4±5.2b,c	21.0±9.3b
Coumarin (**9**)	12.1±0d,e	47.7±10.6a
Esculetin (**10**)	11.1±1.7d,e	18.4±2.9b,c
Control	0.0±0e	57.5±1.7a

[a] Data are given as mean ± standard deviation of triplicate tests (N=3). Numbers followed by different letters (a–c) are significantly different at the level of $P<0.05$ according to Tukey's multiple comparison test

1) Scopoletin **2**) Isoscopoletin **3**) 6-Methoxycoumarin **4**) Herniarin **5**) Scoparone

6) Ayapin **7**) 6-Hydroxycoumarin **8**) Umbelliferone **9**) Coumarin **10**) Esculetin

Fig. 3 Chemical structure of scopoletin (**1**) and coumarin derivatives (**2**–**10**)

started to increase sharply after 5 days for compounds **3** and **7**, and after 7 days for compound **8**. In addition, termiticidal activity was retarded in compounds **2**, **4**, **6**, **9**, and **10**, and the mortality of compound **5** increased slightly after 12 days. The results of statistical analyses of the mortalities of compound **1** and of the coumarin derivatives are shown in Table 2.

Exposure of termites to the treated paper discs for 14 days resulted in the following average mass losses for compound **1** and the coumarin derivatives (**2**–**10**): 2.0%, 21.7%, 4.7%, 3.5%, 8.3%, 19.4%, 4.8%, 21.0%, 47.7%, and 18.4%, respectively (Fig. 5). Compound **1** elicited the lowest loss, showing a significantly greater reduction in feeding activity compared to the control and nine other compounds (Tukey's multiple comparison test) (**2**–**10**). The average mass losses for paper discs treated with compounds **2**–**8**, or **10** were lower than that of the control (avg. 57.5%). Compound **9**, unsubstituted at the C-6 and C-7 positions of the coumarin skeleton, showed no significant difference from control, exhibiting the lowest antifeedant activity with a higher average mass loss than those of other compounds (Table 2). Among the compounds active for termite mortality (**1**, **3**, **7**, and **8**), the average mass loss for compound **1** (2.0%) was lowest, followed by losses for compounds **3** (4.7%) and **7** (4.8%). Interestingly, compound **4** did not show any mortality in particular, although the average mass loss (3.5%) was lower than those for **3** and **7**.

In summary, we isolated scopoletin (**1**), quercetin, and stigmasterol from the ethyl acetate fraction of *P. javanicum* Burm. f. leaf extract by bioassay-guided fractionation. We evaluated the antitermite activities of scopoletin (**1**), quercetin, and stigmasterol against *C. formosanus* Shiraki and found that scopoletin (**1**) showed the highest activity among the three compounds. In order to investigate the SAR of the methoxy and hydroxy groups at the C-6 and C-7 positions of the coumarin skeleton, respectively, we synthesized several coumarin derivatives whose chemical structures are similar to scopoletin. The comparison of termite mortalities for compound **1** and coumarin derivatives (**2**–**10**) suggested that compound **1** showed the strongest termiticidal activity among the 10 compounds tested, followed by **3**, **7**, and **8**, in that order (Fig. 4). The other compounds showed weak activity. Further, all compounds except compound **9** showed antifeedant activity (Fig. 5). These results suggest that scopoletin and other coumarin derivatives whose chemical

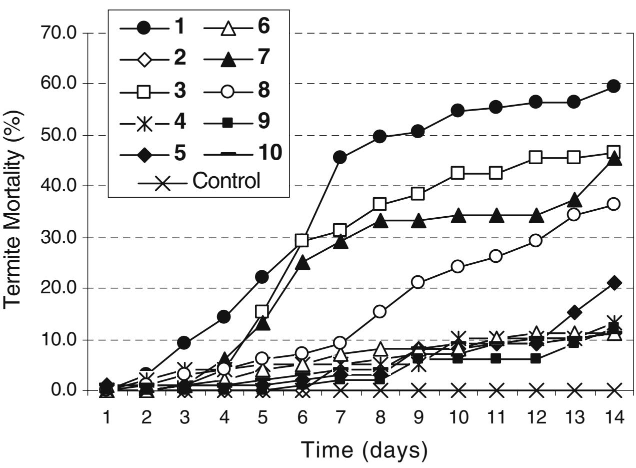

Fig. 4 Termite mortalities (%) of compound **1** and coumarin derivatives (**2**–**10**) against *Coptotermes formosanus* Shiraki in no-choice test. Numbers 1–10 refer to the compounds shown in Fig. 3. Each experiment was performed in triplicate. 33 termites were used per replication, and the data were averaged (N=3)

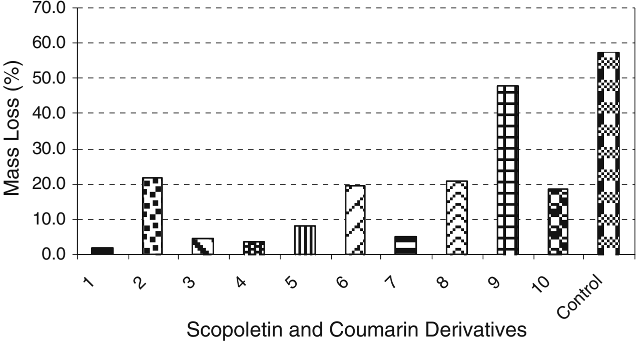

Fig. 5 Paper disc consumption of *Coptotermes formosanus* Shiraki after 14 d exposure to compound **1** or coumarin derivatives (**2**–**10**) in no-choice test. Numbers 1–10 refer to the compounds shown in Fig. 3. Each experiment was performed in triplicate. 33 termites were used per replication, and the data were averaged (N=3)

structures are similar to scopoletin might be useful for termite control agent, because they are abundant in plants or synthesized using well established procedures.

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A Metabolomic Approach to Identifying Chemical Mediators of Mammal–Plant Interactions

David J. Tucker · Ian Robert Wallis · Jessica M. Bolton · Karen J. Marsh · Adam A. Rosser · Ian M. Brereton · Dean Nicolle · William J. Foley

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Abstract Different folivorous marsupials select their food from different subgenera of *Eucalyptus*, but the choices cannot be explained by known antifeedants, such as formylated phloroglucinol compounds or tannins, or by nutritional quality. Eucalypts contain a wide variety of plant secondary metabolites so it is difficult to use traditional methods to identify the chemicals that determine food selection. Therefore, we used a metabolomic approach in which we employed ^{1}H nuclear magnetic resonance spectroscopy to compare chemical structures of representatives from the two subgenera and to identify chemicals that consistently differ between them. We found that dichloromethane extracts of leaves from most species in the subgenus *Eucalyptus* differ from those in *Symphyomyrtus* by the presence of free flavanones, having no substitution in Ring B. Although flavanoids are known to deter feeding by certain insects, their effects on marsupials have not been established and must be tested with controlled feeding studies.

Key Words Metabolomics · *Eucalyptus* · *Symphyomyrtus* · Folivorous marsupials · Common brushtail possum · ^{1}H NMR spectroscopy · Flavanones · Herbivory

D. J. Tucker · A. A. Rosser
School of Science & Technology, University of New England,
Armidale 2351, Australia

I. R. Wallis (✉) · J. M. Bolton · K. J. Marsh · W. J. Foley
Evolution, Ecology and Genetics, Research School of Biology,
Australian National University,
Canberra 0200, Australia
e-mail: ian.wallis@anu.edu.au

I. M. Brereton
Centre for Magnetic Resonance, University of Queensland,
Brisbane, QLD 4072, Australia

D. Nicolle
Currency Creek Arboretum,
15 Rousillion Promenade,
Old Reynella, SA 5161, Australia

Introduction

Plants contain a wide array of chemicals and nutrients that influence how animals respond to them. Although ecologists have long understood the value or liability of general classes of compounds, such as "tannins" or "terpenes" (Swihart et al. 2009), they are just beginning to recognize how specific compounds or particular molecular structures influence how animals choose their diets. Without this knowledge of structure and function, it is impossible to evaluate ideas about dietary constraints on an herbivore's life history, habitat selection, fitness, competition, and coevolution. Although this applies to all herbivores, it is particularly acute in studies of larger mammalian herbivores. It is widely believed that differential responses to secondary compounds allow mammalian herbivores to partition the available resource to reduce interspecific competition (Marsh et al. 2003a). Without detailed chemical knowledge, these ideas cannot be assessed and incorporated into conservation and monitoring programs.

Several factors make it difficult to understand interactions between animals and plants. The first is the complexity of the chemical profile in plants. Related to this is the need to consider intraspecific as well as temporal and spatial variation in chemical makeup. This variation makes it hard to design specific assays for compounds that might be active. Bioassay guided fractionation has been used successfully to identify specific antifeedant compounds in arctic plants (Reichardt et al. 1985; Bryant et al. 1989) and in Australian eucalypts (Pass et al. 1998). In

this procedure, compounds are isolated progressively from a plant and fed to animals to identify the compound of interest. Among other problems, this approach requires great effort to fractionate large volumes of extracts to use in long-term feeding tests with mammals.

McIlwee et al. (2001) suggested that spectra of plants obtained with near infrared reflectance spectroscopy (NIRS) could be related to how much animals eat of those plants. In this way, NIRS could be used to predict feeding in vertebrate browsers independent of a detailed understanding of the underlying chemistry. While that approach has been useful in eucalypts (Foley et al. 1998; Wallis and Foley 2003), NIRS is not suitable for deciphering the underlying chemistry that contributes to differences in the NIR spectra of plants. Nonetheless, spectroscopic approaches are attractive because they give a more comprehensive view of plant chemistry than do assays of broad classes of compounds. In contrast to NIRS, ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) is a spectroscopic approach that can provide structural information about the underlying chemical differences between groups of plants. In this paper, we evaluate the use of ^{1}H NMR spectroscopy combined with principal components analysis (PCA) of the spectra to identify significant chemical differences between subgenera of *Eucalyptus* that support known differences in feeding behavior.

Within *Eucalyptus* there are distinct differences among the subgenera in the extent to which marsupial (and insect) herbivores use the foliage as food. In particular, the two largest subgenera, *Symphyomyrtus* (common name symphyomyrtles) and *Eucalyptus* (common name monocalypts) are used differentially by marsupial browsers. Koalas (*Phascolarctos cinereus*) and common brushtail possums (*Trichosurus vulpecula*) feed predominately on foliage from species in the *Symphyomyrtus*, whereas common ringtail possums (*Pseudocheirus peregrinus*) and greater gliders (*Petauroides volans*) eat leaves mostly from the subgenus *Eucalyptus* (Moore et al. 2004a).

The aversion of ringtail possums to symphyomyrtles is due to their sensitivity to the nauseating effects of formylated phloroglucinol compounds (FPCs) such as sideroxylonal-A (Fig. 1, structure **1**) (Moore et al. 2004b). These compounds are absent from the monocalypts but variably present in the symphyomyrtles, which explains diet selection by koalas and brushtail possums in both the laboratory and the field (Wallis et al. 2002; Scrivener et al. 2004; Moore and Foley 2005) as follows. Both species feed mainly from symphyomyrtles but select leaves to limit their ingestion of FPCs. In contrast, captive brushtail possums eat very little monocalypt foliage but they eat much more when the foliage is coated with the tannin-blocking agent, polyethylene glycol (PEG), while PEG

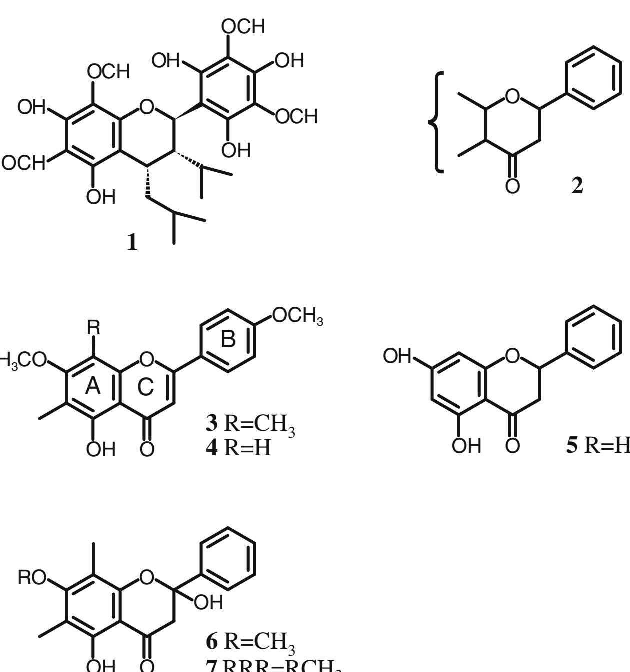


Fig. 1 The structures and partial structures of compounds referred to in the text. **1** sideroxylonal-A; **2** proposed partial structure of a compound in the extract of *E. agglomerata*; **3** eucalyptin; **4** demethyleucalyptin; **5** pinocembrin; **6** and **7** novel 2-hydroxyflavanone structures from *E. rossii*

has no effect on feeding by the common ringtail possum (Marsh et al. 2003b). In mixed stands of eucalypt trees, representatives from both major subgenera tend to co-dominate (Pryor 1959). Thus, by specializing on the PSMs produced by a particular eucalypt subgenus, different folivores could inhabit the same tracts of forest without competing for food (Marsh et al. 2003a). Along with hyrax (Hoeck 1975) and bamboo lemurs (Tan 1999; Yamashita et al. 2009), this is one of the few clear examples of dietary partitioning by vertebrates. If we could identify the factors in monocalypts that deter feeding by the koala and common brushtail possum or the mechanism by which the common ringtail possum processes the monocalypt compounds, then we would have a better understanding of niche partitioning by sympatric vertebrate herbivores.

In this research, we compared extracts from the leaves of a wide variety of monocalypt and symphyomyrtle species. We hypothesized that, unlike the symphyomyrtles, the monocalypts contain specific compounds that are unpalatable to koalas and brushtail possums, and that ^{1}H NMR spectra could reveal these compounds. We added additional control to the study by including several *E. melliodora* (*Symphyomyrtus*) with varying concentrations of sideroxylonals, expecting those with low concentrations of sideroxylonals to group closer to the monocalypts than do those with higher concentrations.

Methods and Materials

Preliminary Collection We did an initial study of a small number of species growing around Canberra, ACT, Australia, to see if ^{1}H NMR spectroscopy would detect consistent differences between the eucalypt subgenera and, if so, would identify the types of compounds that contribute to the differences. By beginning with a small number of species, we could also examine intraspecific variance. Leaf samples were collected from mid-canopy from five individuals of each of the following species: *E. agglomerata, E. dives, E. elata, E. macrorhynca,* and *E. rossii* (monocalypts); *E. globulus ssp bicostata, E. mannifera*, and *E. polyanthemos* (symphyomyrtles). We also collected leaves from 10 individuals, known to vary in FPC concentration, of another symphyomyrtle, *E. melliodora*. Leaves were frozen upon collection and later freeze dried but left unground.

Large-scale Collection The preliminary study gave promising results so we undertook a larger study of a diversity of taxa from *Eucalyptus* and *Symphyomyrtus*. In this way, we could determine whether there are consistent chemical differences between the subgenera. All leaf samples were collected during a single field trip to the Currency Creek Arboretum 80 km south of Adelaide in South Australia in April 2006. The collection included 83 symphyomyrtles (11 of 15 sections that did not include species in the monotypic *Racemus*, *Similares,* or *Pumilio,* or any of the six species in *Platysperma*) and 31 monocalypts (10 of 11 sections that excluded the monotypic *Nebulosa*). A small branch from mid-canopy height of each shrub or tree was cut, and from it about 60 g of mature leaf were stripped, sealed in a plastic bag, and placed in a portable freezer. Upon returning to Canberra, the leaves immediately were freeze dried and ground to pass a 1 mm sieve by using a Cyclotec 1093 Mill (Tecator, Sweden). The resulting powder was stored in the dark in clear plastic 50 ml specimen containers.

Extraction For the preliminary study, we crushed 2.00± 0.05 of leaves into a conical flask, added 40±1 ml of AR grade dichloromethane (DCM), stoppered the flask, and extracted the sample for 48 hr. The solvent was decanted into a round-bottomed flask through a sintered glass funnel fitted with a Whatman No. 41 filter paper and rotary-evaporated at 40°C. The resulting material was redissolved in a small amount of DCM, transferred to a glass vial, dried under a stream of air for 48 hr, and freeze–dried.

For the large-scale collection, we weighed 2.00±0.01 g of dry ground leaves into a conical flask, containing 40± 1 ml DCM. After 5 min of sonication, the extraction was continued for 23 hr with occasional stirring. The solvent was decanted into a round-bottomed flask through a sintered glass funnel fitted with a Whatman No. 41 filter paper, and the leaf residue was re-extracted for about 1 hr in 10 ml DCM. The extract was filtered, added to the first extract, and the solvent was removed by rotary evaporation at 40°C. The resulting crude material was transferred quantitatively with five aliquots of DCM (2 ml each) to a pre-weighed glass vial. We then dried the extracts under a gentle stream of N_2 for about 24 hr, freeze–dried them to remove any remaining solvent, and reweighed them to give the amount of extract per g of dry leaf.

NMR Spectroscopy ^{1}H NMR spectra were measured using a Bruker Avance 750 spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) with a 5 mm TXI XYZ ^{1}H -^{13}C -^{15}N probe, operating at 749.66 MHz (^{1}H). Crude leaf extracts were dissolved in $CDCl_3$ at a concentration of 10 mg/ 750 µl. Spectra were recorded at 298 K and referenced to the residual $CHCl_3$ signal (δ7.26), and chemical shifts are expressed in ppm downfield from TMS. The following parameters were used for ^{1}H spectra: (pulse program:zg) 9.014 KHz spectral width, 64 K data points, 32 scans, 90° pulse (8.0 µs), 1 sec recycle delay. No exponential line broadening was applied prior to Fourier transformation. The 2D-NMR spectra were measured using either a Bruker Avance 750 spectrometer, operating at 749.66 MHz (^{1}H) and 188.52 MHz (^{13}C) with conditions as described above and using the following parameters: COSY: (pulse program: cosydfph) 4 K × 760 data points, 8 scans; HSQC: (pulse program: hsqcetgpsi2) 2 K × 512 data points, 24 scans; HMBC: (pulse program: hmbcgplpndqf) 4 K × 512 data points, 24 scans, or a Bruker Avance 300 spectrometer with a 5 mm BBIz probe, operating at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C) using the following parameters: COSY: (pulse program: cosygpmftp) 1 K × 512 data points, 4 scans; HSQC: (pulse program: invieagssi) 1500 × 512 data points, 16 scans; HMBC: (pulse program: inv4gplplrnd) 1500 × 512 data points, 32 scans.

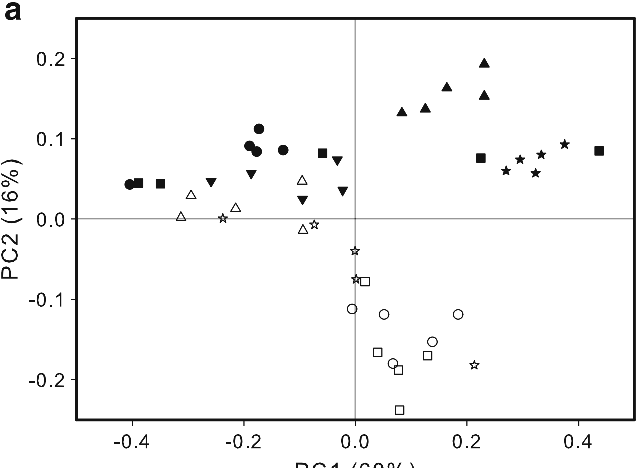
Statistical Analysis Spectra were binned and Principal Component Analysis (PCA) was done using the Bruker Amix software package (version 3.6.8). Spectra were binned from 10.6 to 0 ppm, excluding the regions from 7.3 to 7.2 and 1.3 to 1.2 ppm, with a bin width of 0.1 ppm or from 10.6 to 7.3 ppm with a bin width of 0.05 ppm. Bins were scaled relative to the largest bin. A number of principal components sufficient to explain at least 95% of the variance were calculated (typically 6–12 principal components).

Large-scale Extraction of E. rossii (monocalypt) and Fractionation of the Dichloromethane Extract Freeze–dried intact leaves (260 g) were extracted by soaking (twice) for 24 hr with petroleum spirit (bp 60–70°C, about

1.5 l per extraction) at room temperature, followed by two extractions with redistilled DCM under the same conditions. The two DCM extracts were combined to yield, after rotary evaporation, a green gum (4.6 g, *ca* 2% of dry leaf mass). On redissolving the extract in DCM, a small quantity of a white, DCM-insoluble residue remained in the flask. This material was identified as ursolic acid by 2D-NMR spectroscopy and confirmed by comparison of NMR and melting point data with literature values. The soluble portion of the extract was subjected to flash column chromatography on silica gel (Merck Keiselgel 60, 40–63 μm, 100 g). Elution with DCM yielded a fraction containing crude 2,5-dihydroxy-7-methoxy-6,8-dimethylflavanone (35 mg), while further elution with 10% ethyl acetate/DCM yielded a fraction from which yellow crystals of 2,5,7-trihydroxy-6,8-dimethylflavanone (10 mg) were obtained.

Results

Preliminary Collection A large broad singlet peak at δ1.29, from lipid and/or wax components dominated the ^{1}H NMR spectra of the DCM extracts of the leaves of all species. As this peak was common to both subgenera, it was considered unlikely to be associated with antifeedant properties and it was omitted from the spectra prior to PCA to avoid its effect masking the contribution from minor constituents. The scores plot from the PCA reasonably separates the subgenera along the Principal Component 2 (PC2) axis (Fig. 2a), the major loadings being 1.05 (i.e., 1.10–1.00 ppm in the negative direction, correlating to the symphyomyrtle species) and 1.35 (positive, monocalypts) (Fig. 2b). Both of these bins may contain lipid methylene (δ1.35) and methyl (δ1.05) resonances, particularly those of unsaturated lipids, and so they were considered to be poor potential indicators of chemical differences between the subgenera. Further analyses showed that optimum separation occurred only when the regions of the ^{1}H NMR spectra between δ 10.6 and 7.3 ppm were included in the analysis (Fig. 3a). Here, PC1 produces a tight grouping of monocalypt species, with the exception of *E. elata* and two individuals of *E. macrorhynca*. Although these individuals have a similar score in PC1, they are separated from the remaining monocalypts in PC2. The symphyomyrtles also show a tight grouping with the exception of four out of five of the individuals of *E. melliodora* known to have a low FPC content (Fig. 3a). The major loadings in the positive direction (*Symphyomyrtus*) of PC1 were 10.13 (10.15–10.10 ppm) and 10.23, due to the aldehyde proton resonances of FPCs (Fig. 3b), while the monocalypts were typified by loadings at 7.48 and 7.43 (Fig. 3b).



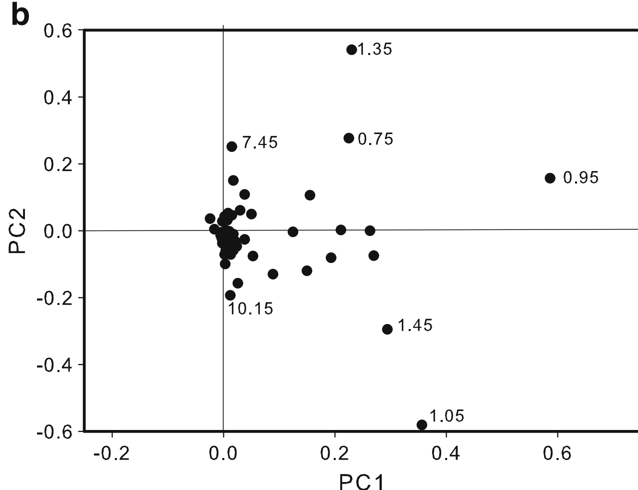
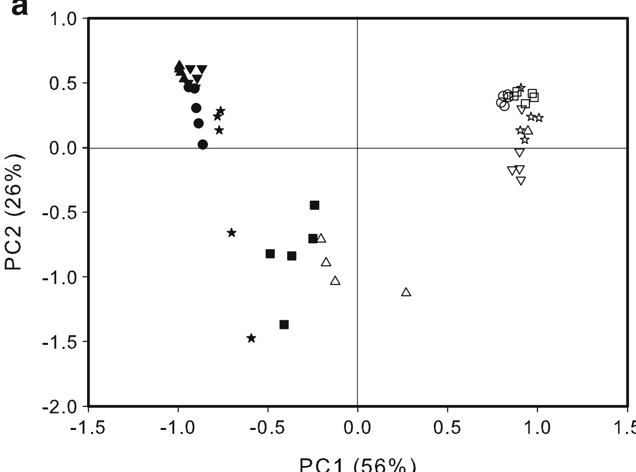


Fig. 2 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves. Bins: 0.1 ppm wide, δ10.6–δ0.0 (δ7.3–7.2, δ1.3–1.2 excluded). **a** Scores plot. Filled symbols: *Eucalyptus* species. ▲ *E.agglomerata;* ▼ *E. rossii*; ● *E. dives*; ★ *E. macrorhynca*; ■ *E. elata*. Open symbols: *Symphyomyrtus* species. Δ *E. melliodora* (low FPC variety); ∇ *E. melliodora*; ○ *E. globulus*; ☆ *E. polyanthemos;* □ *E. mannifera*. **b** Loadings plot

The region between δ7.5 and δ7.3 of most of the monocalypt ^{1}H NMR spectra revealed a pattern of one doublet and two triplets, with integrals of 2, 2, and 1, respectively (Fig. 4), typical of a monosubstituted aromatic ring. There were two exceptions: the spectra from *E. rossii*, in which the doublet signal was further downfield (δ7.71), and those of *E. elata*, which had no such resonances (Fig. 4). These signals were absent from the spectra of the symphyomyrtle species, as typified by *E. globulus* (Fig. 4), which suggests that monocalypt species differ from symphyomyrtle species by the presence of a phenolic compound bearing a monosubstituted ring. However, the small differences between species in the chemical shifts of these resonances suggest that the monocalypts share a class



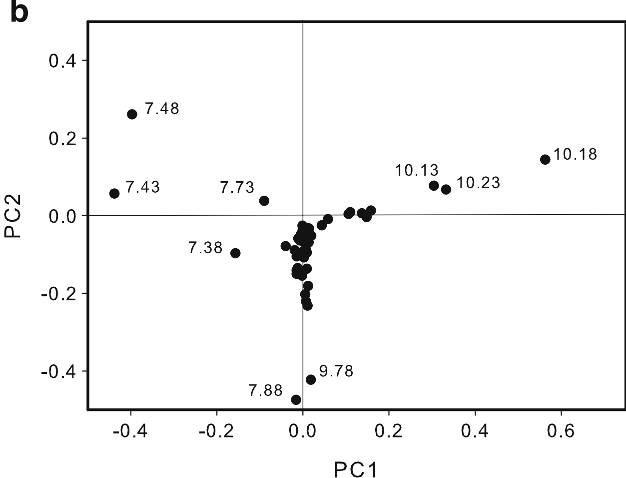


Fig. 3 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves. Bins: 0.05 ppm wide, δ10.6–δ7.3. **a** Scores plot. Filled symbols: *Eucalyptus* species. ▲ *E. agglomerata;* ▼ *E. rossii*; ● *E. dives*; ★ *E. macrorhynca*; ■ *E. elata*. Open symbols: *Symphyomyrtus* species. Δ *E. melliodora* (low FPC variety); ∇ *E. melliodora*; ○ *E. globulus*; ☆ *E. polyanthemos;* □ *E. mannifera*. **b** Loadings plot

of compounds having similar structures, rather than a specific compound.

To gain more information about the structure of these phenolic compounds, 2D-NMR spectra were run on one sample of the DCM extract of *E. agglomerata*. The COSY, HSQC, and HMBC spectra confirmed that the three resonances, at δ7.47, 7.44, and 7.38, were due to a monosubstituted aromatic ring. However, apparent splitting of the peaks at δ7.44 and 7.38 suggested that these signals were due to at least two sets of overlapping resonances. The HMBC spectrum allowed the assignment of the quaternary carbon of the aromatic ring (at δ138.8) and revealed a connection from that resonance to another three proton coupling system (again overlapping pairs of almost equal intensity) consisting of resonances at δ5.42, a doublet of doublets (dd) (J 13.2 and 3.2 Hz, overlapped with a similar resonance at δ5.40), δ3.05 (dd, J 13.2 and 17.0 Hz, overlapped with δ3.02) and δ2.85 (dd, J 3.2 and 17.0 Hz, overlapped with δ2.845). Those resonances were coupled further to a carbon resonance at δ196.1. This evidence leads to the partial structure (Fig. 1, structure **2**) and suggests that the major phenolic constituents of the extract were a mixture of two flavanone compounds in approximately equal proportions, both having no substitution on Ring B and differing only in their substitution in Ring A. The complexity of the HMBC spectrum, due to overlap of some aromatic resonances and the presence of many similar minor constituents, prevented the full elucidation of the Ring A substitution patterns of these compounds. However, the ^{1}H NMR data were largely compatible with a mixture of two compounds having Ring A substitution patterns analogous to those of the commonly occurring flavones (ring-C unsaturated analogues of flavanones), eucalyptin (Fig. 1, structure **3**) and demethyleucalyptin (Fig. 1, structure **4**), although there were some discrepancies between the ^{1}H chemical shifts (and those of connected carbons found from the HMBC) for these compounds and those reported for the corresponding flavanone analogues (Diaz et al. 1987; Hsieh et al. 1998; Mayer 1990).

In summary, PCA analyses performed only on the ^{1}H NMR spectra identified the region between 7.5 and 7.3 ppm as being the major point of difference between species of the two subgenera. Inspection of 1H NMR spectra reveals

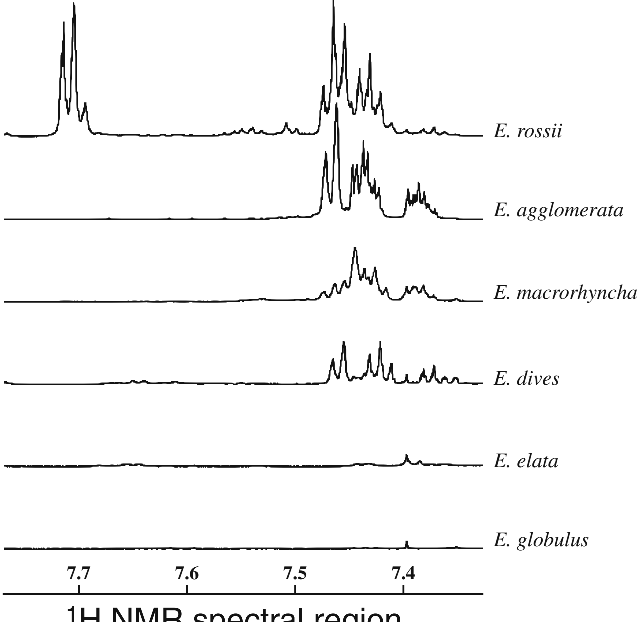
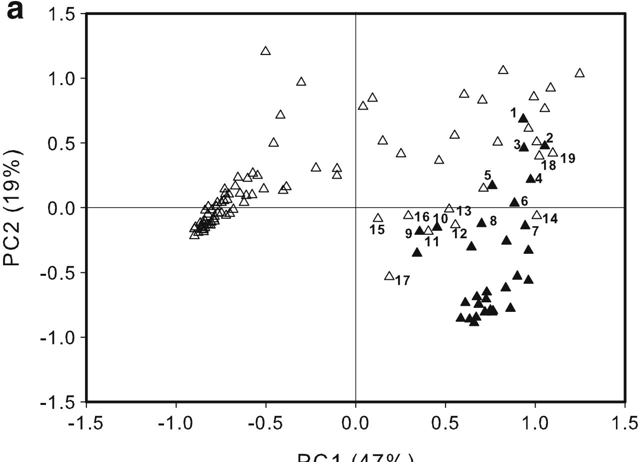


Fig. 4 The region of the ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves between δ 7.75 and δ 7.35. All are monocalypts except *E. globulus*, which gives a trace typical of *Symphyomyrtus.* One monocalypt, *E. elata*, tends to group away from other monocalypts

that the resonances present in this region can be attributed to a monosubstituted aromatic ring, while the 2D NMR data prove that the monosubstituted aromatic ring is the B ring of a flavanone. However, even within single species, a complex mixture of compounds with varying A-ring substitution patterns occurs. The exact nature of these substituents remains uncertain until the compounds are isolated from a larger collection of the plant material. That said, structures similar to that of the widely occurring unsubstituted B-ring flavanone, pinocembrin (Fig. 1, structure 5) seem likely.

Large-scale Collection A PCA of the ^{1}H NMR data of extracts from the large scale collection of 83 symphyomyrtle and 31 monocalypt species produced results (Fig. 5a) similar to those obtained for the preliminary collection. Again, the monocalypts largely group together with major loadings at 7.48 and 7.43 ppm (Fig. 5b). This suggests that there are consistent chemical differences between the leaves of species from these two major eucalypt subgenera.

A few symphyomyrtle species group close to the monocalypts in the PCA (Fig. 5). Inspection of their ^{1}H NMR spectra reveals that this is due to a combination of two features. First, they show very small or no resonances corresponding to FPCs (e.g., *E. nitens*), similar to our observations in the preliminary experiment for *E. melliodora* with low concentrations of the FPC, sideroxylonal (Fig. 3a). Second, the symphyomyrtles that grouped close to the monocalypts show one or more peaks in the region from δ 7.5–7.4. However, the chemical shift and multiplicity of these resonances suggest that they are not due to unsubstituted B-ring flavanones. In some cases, the resonance was a doublet, with a coupling partner at lower field (ca. δ 7.8–7.7) suggesting a 4′-substituted flavone, such as eucalyptin (Fig. 1, structure 3) or related compounds. In contrast, the spectra of three species, *E. dundasii*, *E. salubris,* and *E. pleurocorys*, had resonances with multiplicity typical of a monosubstituted aromatic system (i.e., a one proton triplet, a two proton triplet, and a two proton doublet) with chemical shifts of δ 7.55, 7.44, and 8.04, respectively, but the latter shift suggests a flavone with an unsubstituted B-ring rather than a flavanone derivative. The presence of a resonance at δ 6.6 in the spectra of these species, perhaps due to H-3, supports this suggestion. Another species of interest is *E. grandis*, which also has resonances in its spectrum due to a monosubstituted aromatic ring. However, in this case the shifts (δ7.30 (2H, t), 7.26 (2H, d) and 7.21 (1H, t) are not compatible with those of the B-ring of a flavone or a flavanone. Leaves of *E. grandis* contain the β-triketones, flavesone, leptospermone, and isoleptospermone (Boland et al. 1991), and it seems likely that these aromatic resonances may be due to the aromatic analogue, grandiflorone, which has not been



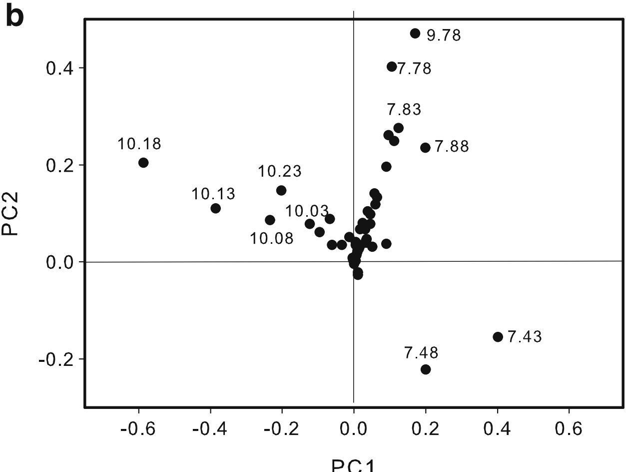


Fig. 5 Results of principal components analysis for ^{1}H NMR spectra of dichloromethane extracts of eucalypt leaves from the large scale collection. Bins: 0.05 ppm wide, δ 10.6–δ 7.3. **a** Scores plot. ▲ *Eucalyptus* species, *N*=31; Δ *Symphyomyrtus* species, *N*=83. Some species are labelled as follows: (*Eucalyptus*) 1—*E. dives*; 2—*E. brevistylis*; 3—*E. staeri*; 4—*E. buprestium*; 5—*E. marginata*; 6—*E. lacrimans;* 7—*E. moorei*; 8—*E. amydalina;* 9—*E. elata;* 10—*E. laevopinea;* (*Symphyomyrtus*) 11—*E. nitens*; 12—*E. dundasii*; 13—*E. salubris*; 14—*E. caleyi*; 15—*E. brunnea*; 16—*E. angustissima*; 17—*E. dawsonii;* 18—*E. pleurocorys;* 19—*E. grandis*. **b** Loadings plot

reported previously in eucalypts. The only ^{1}H NMR data reported for grandiflorone was at 60 MHz (Hellyer and Pinhey 1966), at which frequency the aromatic protons are unresolved, but the shift of δ 7.22 agrees reasonably well with those found at 750 MHz in this work.

Of the outlying monocalypts, two species, *E. amygdalina* and *E. moorei*, contained substantial quantities of unsubstituted B-ring flavanones, but a large score in PC2 separated them from most monocalypts. The loadings responsible for this score were at 7.83 and 7.87 ppm due to eucalyptin and 8-demethyleucalyptin (confirmed by comparison of the ^{1}H NMR spectra of these species with literature data) (Sarker et al. 2001). Horn and Lamberton

(1963) found these flavones in both monocalypt and symphyomyrtle species, while we found NMR evidence of many further occurrences in both sub-genera, thus suggesting that they have no chemotaxonomic significance. The other outlying monocalypt species (*E. brevistylis*, *E. buprestium*, *E. dives*, *E. elata*, *E. lacrimans, E. laevopinea, E. marginata,* and *E. staeri*) contained no unsubstituted B-ring flavanones.

The literature provides some support for our observation that flavanones with unsubstituted B-rings occur in monocalypts but not in symphyomyrtles. A wide variety of flavonoids have been isolated from symphyomyrtle species, including C-methylated flavones, C-methylated flavonols and flavonols (Wollenweber and Kohorst 1981; Conde et al. 1997). In contrast, the unsubstituted B-ring flavanones pinocembrin, alpinetin, and O,O-dimethylpinocembrin, have been reported only in the monocalypt *E. sieberi* (Bick et al. 1972). A reanalysis of negative ion ESIMS data published by Eschler et al. (2000) revealed that the spectra of all the monocalypt species contained an ion at m/z 255, which plausibly could be the $M\text{-}H^+$ ion of pinocembrin. The m/z 255 ion was present in substantially lower relative abundance in the two species *E. andrewsii* and *E. haemastoma*.

Large-scale Extraction of E. rossii and Fractionation of the DCM Extract We attempted to isolate and characterize the compounds responsible for the major aromatic resonances observed in the ^{1}H NMR spectrum of the leaf extract of *E. rossii* leaves. The appeal of the species was that its ^{1}H NMR spectrum suggested the presence of a flavonoid with an unsubstituted B-ring that differed in the aromatic region from that of most other monocalypt species. The DCM extract yielded two compounds tentatively assigned as novel 2-hydroxyflavanones (Fig. 1, structures **6** and **7**). The structural elucidation of these compounds will be reported elsewhere.

Discussion

We demonstrated that a broad metabolomic approach using ^{1}H NMR spectra of the DCM extracts of leaves can detect consistent chemical differences between the two major eucalypt subgenera. In particular, monocalypt leaf extracts contain flavanones having an unsubstituted B-ring, typified by resonances in the region from δ 7.4–7.3, while species of symphyomyrtle do not contain this class of compounds. In contrast, the symphyomytles have unique signals in the vicinity of 10 ppm that correspond to FPCs. These differences provide a correlative explanation for why brushtail possums refrain from eating monocalypt foliage (Marsh et al. 2003a). Furthermore, these differences help explain dietary niche partitioning in marsupial folivores and provide clues about the evolutionary relationships between eucalypts and the animals that eat them. As pointed out previously, most eucalypt forests contain mixed stands with representatives from both major subgenera tending to co-dominate (Pryor 1959). This, together with the high intraspecific chemical variation in eucalypts (Moore et al. 2004b) that is largely under genetic control (Andrew et al. 2007), creates a patchy landscape for folivores with likely effects on the ecosystem (Whitham et al. 2008).

While we have no direct evidence that unsubstituted B-ring flavanones are responsible for the feeding behavior of brushtail possums, many flavonoids have been reported to deter feeding by insects (Treutter 2006). The reported isolation of free flavonoids from eucalypt epicuticular wax may support an antifeedant role for these compounds (Wollenweber and Kohorst 1981). Furthermore, flavonoids with an unsubstituted B-ring differ, both in biological activity and chemical reactivity, from flavonoids with mono or di-substitution in Ring B (Pannala et al. 2001; Jia et al. 2003). Thus, such compounds may have antifeedant activity that differs from that of other flavonoids, which are more widely distributed across the eucalypt genera. These results suggest two worthwhile directions for feeding studies. It would be reasonable to compare feeding by brushtail possums offered monocalypt species that have unsubstituted B-ring flavonoids to those few species that do not have these compounds (*E. brevistylis*, *E. buprestium*, *E. dives*, *E. elata*, *E. lacrimans, E. laevopinea, E. marginata,* and *E. staeri*). In addition, we suggest a comparison of feeding by possums offered artificial diets containing isolated pinocembrin (an unsubstituted B-ring flavanone, Fig. 1, structure 5) to those offered diets containing flavonoids with B-ring substitution.

We acknowledge that monocalypts may contain compounds with feeding deterrent activity that were not extracted with DCM and were thus not detected in our study. Indeed, more polar compounds including flavonoid glycosides and proanthocyanidins (condensed tannins) are well known to have antifeedant activity (Marsh et al. 2003b) but would not be present in a DCM extract. On the other hand, the exclusive production of unsubstituted B-ring flavanones by monocalypts, revealed in this research, indicates that these species are capable of biosynthetic pathways unavailable to symphyomyrtles. Such processes may produce unique flavonoid glycosides and proanthocyanidins, as these compounds share common biosynthetic precursors with flavanones (Veitch and Grayer 2008). This prompts a similar study using methanol extracts to determine whether the more polar metabolites of these subgenera show similar differentiation to that seen in DCM extracts.

A key message of this paper is the value of spectral approaches and metabolomics for elucidating ecological problems. The literature contains numerous examples in which the study design was based on the assumption that a particular nutrient, especially nitrogen, was limiting. One advantage of spectral approaches is that they require no preconceived ideas about chemical or nutrient composition, but provide a holistic view of the material. Approaches such as NIRS have been used to discriminate groups, such as leaf samples from different species or with differing susceptibility to herbivory, but this method is of limited value for deciphering the underlying chemistry. The better method is broad metabolite profiling, in which one aims to identify as many metabolites as possible in a single extract to provide the biochemical phenotype of the organism (Allwood et al. 2008). One of the preferred methods, gas chromatography-mass spectrometry, can be used routinely to determine the concentrations of 300–500 polar and non-polar metabolites per sample (Lisec et al. 2006). Its disadvantages include the need for compounds to be in mass spectral libraries (i.e., known compounds), the need to derivatize non-volatile compounds, and the need for a calibration system to account for differences in detection sensitivity among compounds. Therefore, GC-MS is not suitable for poorly understood systems, such as *Eucalyptus*. In contrast, ^{1}H NMR spectroscopy offers several benefits (Nicholson et al. 1998): 1) samples require minimal treatment; 2) detection is universal, since virtually all organic compounds give ^{1}H NMR signals; 3) resonance intensity is directly proportional to the concentration of compound; 4) the bins responsible for the major loadings in the PCA provide structural information about compounds.

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No Major Role for Binding by Salivary Proteins as a Defense Against Dietary Tannins in Mediterranean Goats

Michal Hanovice-Ziony · Nathan Gollop · Serge Yan Landau · Eugene David Ungar · Hussein Muklada · Tzach Aharon Glasser · Avi Perevolotsky · John Withers Walker

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Abstract We investigated whether Mediterranean goats use salivary tannin-binding proteins to cope with tannin-rich forages by determining the affinity of salivary or parotid gland proteins for tannic acid or quebracho tannin. Mixed saliva, sampled from the oral cavity, or parotid gland contents were compared to the intermediate affinity protein bovine serum albumin with a competitive binding assay. Goats that consume tannin-rich browse (Damascus) and goats that tend to avoid tannins (Mamber) were sequentially fed high (*Pistacia lentiscus* L.), low (vetch hay), or zero (wheat hay) tannin forages. Affinity of salivary proteins for tannins did not differ between goat breeds and did not respond to presence or absence of tannins in the diet. Proteins in mixed saliva had slightly higher affinity for tannins than those in parotid saliva, but neither source contained proteins with higher affinity for tannins than bovine serum albumin. Similarly, 3 months of browsing in a tannin-rich environment had little effect on the affinity of salivary proteins for tannin in adult goats of either breed. We sampled mixed saliva from young kids before they consumed forage and after 3 months of foraging in a tannin-rich environment. Before foraging, the saliva of Mamber kids had higher affinity for tannic acid (but not quebracho tannin) than the saliva of Damascus kids, but there was no difference after 3 months of exposure to tannin-rich browse, and the affinity of the proteins was always similar to the affinity of bovine serum albumin. Our results suggest there is not a major role for salivary tannin-binding proteins in goats. Different tendencies of goat breeds to consume tannin-rich browse does not appear be related to differences in salivary tannin-binding proteins.

Key Words Defense mechanism against tannins · Tannin-binding capacity · Parotid · Lentisk · *Pistacia lentiscus*

M. Hanovice-Ziony · S. Y. Landau (✉) · E. D. Ungar · H. Muklada · T. A. Glasser · A. Perevolotsky
Department of Agronomy and Natural Resources, Institute of Plant Sciences, Agricultural Research Organization, P.O. Box 6, Bet Dagan 50250, Israel
e-mail: vclandau@agri.gov.il

N. Gollop
Department of Food Sciences, Institute of Technology and Storage of Agricultural Products, Agricultural Research Organization, P.O. Box 6, Bet Dagan 50250, Israel

J. W. Walker
Texas A&M University Agricultural Research and Extension Center, 7887 U.S. Hwy. 87 N., San Angelo, TX 76901, USA

Introduction

Although dietary tannins may have some positive effects (Mueller-Harvey 2006), the ingestion of large amounts of tannins by most herbivores is associated with reduced feed intake and digestibility, and decreased availability of a wide array of nutrients, the most important of which is protein. Condensed tannins can be excreted via the feces, but some are metabolized and excreted as urinary metabolites; hydrolyzable tannins can be partially absorbed, and some of them can be toxic (Mueller-Harvey 2006). Domestic ruminants employ a variety of behavioral mechanisms to cope with dietary tannins, such as avoidance (sheep; Titus et al. 2000), modified meal patterns (cattle; Landau et al. 2000), and foraging selectivity that maintains dietary tannins all year-long in a narrow range of concentration (goats; Kababya et al. 1998) that may be beneficial (Mueller-Harvey

2006). In addition, herbivores may use associations between classes of PSMs to reciprocally neutralize their deleterious effects (Lyman et al. 2008; Mote et al. 2008). Physiological adaptations also exist, encompassing tannin-resistant rumen flora (Odenyo et al. 1999), improved liver capacity to detoxify tannins (Dearing et al. 2005), and the secretion of tannin-binding salivary proteins (TBSPs; Shimada 2006). TBSPs have a binding affinity for tannins that is 5–80 times that of bovine serum albumin (BSA).

Mehansho et al. (1983) discovered that in rats, parotid TBSPs are induced by tannin ingestion. Subsequently TBSPs in saliva have been reported for many species of rodents (mouse, root vole), lagomorphs (rabbit, mountain hare, North American pika), ungulates (moose, musk ox, mule deer, roe deer, fallow deer), perissodactyls (white, Indian and black rhinoceroses), and primates (man, macaque) (Shimada 2006). The feeding niche theory states that herbivores that consume more browse are more likely to secrete TBSPs (Shimada 2006). Notable exceptions include a variety of marsupials, which are browsers but do not secrete TBSPs (Shimada 2006), and the Indian rhinoceros, a grazer with saliva that has similar or higher affinity for tannins than that of the black rhinoceros, a browser (Clauss et al. 2005). In arid areas, where nitrogen is the first limiting nutrient, animals face a trade-off between the cost of secreting proteins in their saliva and the benefit of preventing protein depletion by tannins.

Since tannin-rich browse is a major component of goat diets in Mediterranean shrublands (Landau et al. 2002), one would expect them to produce TBSPs. Distel and Provenza (1991) did not find TBSPs in the saliva sampled from the oral cavity of goats and Lamy et al. (2008) did not find TBSPs in sheep and goat parotid saliva. Austin et al. (1989) did not identify TBSPs in cattle and the tannin-binding capacity of salivary proteins for tannins did not differ between cows fed high- or low-tannin diets (Makkar and Becker 1998).

The overall objective of our study was to evaluate the affinity of saliva from domestic goats (*Capra hircus*) kept in a tannin-rich environment for tannin. Our specific objectives were: a) to test if salivary proteins have a higher affinity for tannin than that of BSA; b) to compare the affinity for tannins of parotid versus mixed saliva; c) to test if short- and long-term exposure to dietary tannins stimulates salivary affinity for tannins; and d) to test the "feeding niche" theory by comparing the affinity for tannins of saliva from two goat breeds that differ in their propensity to ingest tannin-rich browse.

Methods and Materials

Animals Adult and kid goats were selected from the experimental flock at Ramat HaNadiv, located at the southern tip of the Carmel Ridge, Israel (32°33′ N, 34°57′ E). All procedures were monitored by the Israel Council on Animal Care Guidelines (ICACG 1994).

The region is characterized by an average yearly rainfall of 600 mm, and a rainy season of 180 d, from October to April. The ecosystem is a disturbed Mediterranean shrubland (garrigue) featuring steep, rocky slopes with scarce patches of shallow soil. The vegetation is dominated by low trees (mainly *Phillyrea latifolia* L.) and tall shrubs, mainly lentisk *(Pistacia lentiscus* L.) and *Calicotome villosa* Poir. Link, that can contribute over 60% of the plant matter ingested by goats (Landau et al. 2005; Glasser et al. 2008).

Experiment 1 The response of goats to short-term tannin exposure was compared in two breeds with contrasting tendencies to consume tannin-rich lentisk: Damascus (high) and Mamber (low) (Glasser 2009). Four yearling female goats of each breed were selected at random, weighed and kept in separate 3×3 m dirt floor pens. Average (± SE) animal weight was 29.5±0.28 kg for the Mamber goats and 41.0±0.81 kg for the Damascus goats. After a 14 d period of adaptation to the pens, the feeding trial comprised four 10 d periods during which goats were fed a base ration of 350 g d^{-1} of a commercial concentrate feed (Ambar, Hadera, Israel) and an *ad libitum* allowance of a forage that changed between periods in crude protein (CP) and tannin content (expressed as Polyethylene glycol-binding-tannins, Landau et al. 2004). The forages were as follows: Period 1—vetch hay with high protein and low tannin content (15.8% and 1.5% of DM (dry matter), respectively); Period 2—freshly clipped foliage of lentisk with low protein and high tannin content (7.8% and 19.3% of DM, respectively); Period 3—vetch hay as in Period 1; Period 4—wheat hay with medium protein and no tannin (11.7% and 0% of DM, respectively) (Table 1).

At the end of each feeding period, saliva samples were extracted from the oral cavity ("mixed") and from the parotid gland, and blood was sampled from the jugular vein. Serum from the blood samples was stored at −20°C until it was analyzed for urea, which was used to estimate the effect of lentisk tannins on protein metabolism (Silanikove et al. 1996a).

Experiment 2 The response of goats to long-term tannin exposure was compared in Damascus and Mamber goats. Mixed saliva samples were collected from nine female kids of each breed at 3 months of age, before the animals had any foraging experience, and again after the animals had 3 months of foraging experience in tannin-rich brushland. To further assess the effect of long-term foraging in tannin-rich brushland, mixed saliva was collected from four adult goats of each breed.

Table 1 Chemical composition and rates of intake of forages by goats in experiment 1. Dry matter, DM; Neutral Detergent Fiber, NDF; Acid Detergent Fiber, ADF; Acid Detergent Lignin, ADL; PEG-binding-tannins, PEG-b-T; all as %. Intake rate is g DM d^{-1}

	Vetch hay		Lentisk foliage		Wheat hay	
DM	94.4		52.3		89.5	
On DM basis						
Ash	12.3		4.8		8.2	
Fiber						
NDF	49.5		44.8		55.7	
ADF	32.8		27.7		28.9	
ADL	3.8		2.5		1.2	
Crude Protein	15.8		7.8		11.7	
PEG-b-T	1.5		19.3		0	
Intake rate	Damascus	Mamber	Damascus	Mamber	Damascus	Mamber
Period 1	600	620	–	–	–	–
Period 2	–	–	630	530	–	–
Period 3	710	700	–	–	–	–
Period 4	–	–	–	–	580	380

Saliva Collection Saliva was sampled from the oral cavity in the morning, before the goats or kids were fed, in order to minimize food contamination. A sponge was tied to the animal's mouth for 15 min and then squeezed above a 50 ml tube. The saliva samples were then frozen (−20°C) until analysis.

Extraction of parotid saliva requires anesthesia. The procedure was conducted in the morning, before the goats were fed, to avoid regurgitation and aspiration of rumen contents during unconsciousness. The goats were sedated intravenously by xylazine (Phoenix Pharm Distributors, Oakland, New Zealand; 0.03 mg kg^{-1}) and anaesthetized intravenously by a 1:2 v/v mixture of Ketamine (Clorake-tam1000; Vetoquinal, Lore, France) and Diazepam (Assival, Teva, Petah Tiqwa, Israel). A fine catheter (Angiocath 16-gauge, Becton Dickinson Infusion Therapy Systems Sandy, UT) was inserted, with the help of a guide wire, into the parotid duct, which opens in the inner cheek in front of the 3rd maxillary premolar tooth. Saliva was then aspirated with a syringe while gently massaging the gland, and collected into capped tubes. After removal of the catheter, the goats were given yohimbine (Procomil; Phoenix Pharm Distributors, Oakland, New Zealand) intravenously to counteract the sedative effect of xylazine. The saliva samples were frozen (−20°C) until analysis.

Saliva was prepared for analysis according to Fickel et al. (1998) with minor modification. Saliva was centrifuged at 3,000×g for 5 min at 4°C to remove particulate matter and desalted by dialysis against deionized water for 24 h at 4°C using membrane with a 3 kDa molecular weight cut-off (GeBA, Kfar Hanagid, Israel). Protein concentration was determined in triplicate using the bicinchoninic acid (BCA) method of Smith et al. (1985) with BSA (Thermo Fisher Scientific Inc., Rockford, IL, USA) as a standard. Samples containing less than 100 µg protein ml^{-1} were concentrated by ultrafiltration using 3 kDa molecular weight cut-off membranes (Centricon tubes, Millipore, Bilerica, MA, USA), and more concentrated samples were diluted, so that salivary protein was in a range between 100 and 120 µg protein ml^{-1}.

Affinity for Tannins Affinity for tannins was estimated as described by Fickel et al. (1999) with minor modifications (Fig. 1). The assay is based on the inhibition of trypsin by tannins and reversal of inhibition when tannin-binding proteins such as TPSP are present. N-α-benzoyl-DL-arginine-p-nitroanilide, which is cleaved by trypsin to the chormophore p-nitroaniline, was used as the substrate, since it is not a protein and does not interact with tannins. Our main modification was to dissolve the substrate in dimethyl sulfoxide (DMSO) to increase enzyme efficiency (OD_{405} of the positive control was 1.8 times greater with DMSO than with water as solvent); to increase sensitivity (OD_{405} of the negative control with DMSO as solvent was 0.80 of that with water); to enhance the affinity of BSA; and to improve substrate solubility.

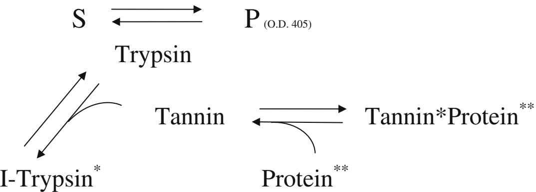

Fig. 1 The tannin-binding assay based on the inhibition of trypsin by tannins and reversal of inhibition by tannin-binding proteins. S = Substrate (Nα-benzoyl-DL-arginine-p-nitroanilide), P = Product, I-Trypsin = Inactivated trypsin, Protein = BSA or Salivary proteins

The assay was done in 96-well plates, with three wells for each control or test sample. Each well was filled with 100 µl pre-incubation mix consisting of: 90 mM Tris-HCl (pH 8.2), 18 µM $CaCl_2$, 100 µg ml^{-1} trypsin, 25 µg ml^{-1} tannic acid (Sigma, Rehovot, Israel) or 50 µg ml^{-1} quebracho extract (Trask Chemical, Marietta, GA, USA) containing 42% PEG-binding tannins (Landau et al. 2004). BSA or protein-standardized saliva (20 µg ml^{-1}) was added, the reactions were pre-incubated for 15 min at room temperature, and substrate was added. After 20 min at 37°C, the reactions were stopped by adding 50 µl of acetic acid, and optical density was read at 405 nm (OD_{405}) with a plate-reader (Lucy 2, Anthos, Imola, Italy). The four controls for each reaction were: Background, all components without trypsin; Positive control, substrate, trypsin, and buffer, without tannin or test protein; Negative control, with trypsin, tannin, and buffer, without test protein; BSA control, with trypsin, tannin, buffer, and BSA, a protein that has moderate affinity for tannin.

Three ratios were used to evaluate the affinity of saliva samples for tannins. Comp max, OD_{405} sample / OD_{405} positive control, is equal to 1.0 when the salivary protein completely scavenges the tannin, and is less than 1.0 when the salivary protein partially protects trypsin. Comp min, OD_{405} sample / OD_{405} negative control, is equal to 1.0 when the salivary protein does not interact with tannin, and is greater than 1.0 when the salivary protein binds tannin. Comp BSA, OD_{405} sample / OD_{405} BSA, is equal to 1.0 when the salivary protein and BSA have the same affinity for tannin, and is greater than 1.0 when the salivary protein has higher affinity for tannin than BSA. Therefore, the value of Comp BSA is a meaningful estimate, which allows categorization of salivary proteins as tannin-binding proteins or proteins with no special affinity for tannin as defined by Shimada (2006).

Statistics In experiment 1, urea serum concentration was analyzed by ANOVA using the GLM procedure of SAS (SAS 1989). Terms in the repeated measures analysis were goat breed and diet as main effects, their interaction, goat within breed as error term, and period as the repeated measure. In the statistical analysis of the tannin binding assay, the ELISA plate effect was significant in both experiments 1 and 2, as noted before by Fickel et al. (1999). Therefore, before data from different plates were combined (Exp.1), we ascertained that OD_{405} values for positive and negative controls, and for BSA did not differ between plates. In experiment 1, analyses of tannin-binding were made separately for tannic acid and quebracho extract using a repeated measures analysis with goat breed, diet, and saliva type as main effects, their interactions, goat within breed as error term, and period as the repeated measure. Statistical separation of means used the studentized Tukey-Kramer range test (SAS 1989). Also, all measurements of tannin-binding from experiment 1 were combined (eight goats, four periods, N=32) and Student's paired t-tests were used to compare the affinity for tannins of parotid and mixed saliva.

In experiment 2, backgrounds for titer plates differed, therefore only effects measured on one micro-titer plate could be analyzed, and the number of wells limited the complexity of analyses for kids (N=9 for each breed). Thus, the effects of grazing experience and breed were analyzed separately, without interaction. Paired t-tests were used to evaluate the effects of kid grazing experience and breed on the affinity for tannins of saliva proteins, and a one-way ANOVA was used to compare kids to mature goats.

Results

Experiment 1 Goats were not deterred by tannins in lentisk foliage (Table 1). Intake was similar for periods 1 (vetch) and 2 (lentisk) in Damascus goats, and a small decrease was noted in Mamber goats. However, protein status, as assessed from blood urea concentration, was markedly affected by diets during these periods: blood urea concentrations were lowest (P<0.001; Fig. 2) with lentisk foliage and highest with vetch hay. In period 4 (wheat hay), intake decreased more in Mamber goats than in Damascus goats (Table 1), and blood urea concentrations in both were intermediate (Fig. 2). Damascus goats had higher (P<0.05) blood urea concentration than Mamber goats in period 3 (vetch), but not in the other periods, resulting in a significant breed × diet interaction (P<0.05).

Protein concentration was higher (P<0.001) in mixed saliva than in parotid saliva (332±32 and 145±19 µg ml^{-1}, respectively). For mixed saliva, but not parotid saliva, the concentration of protein after dialysis was affected by diet

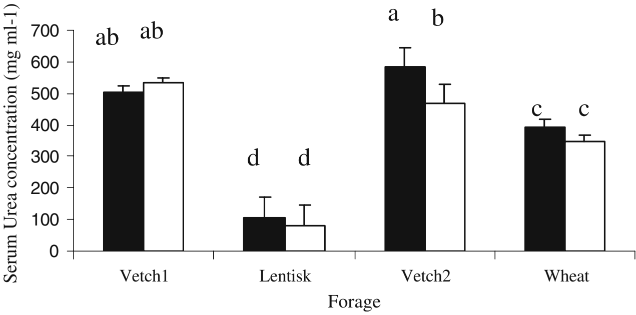

Fig. 2 Blood serum concentration of urea (mg l^{-1}) in Damascus (*black bars*) and Mamber (*white bars*) goats fed sequentially diets with vetch hay, lentisk foliage, vetch hay, and wheat hay (*bars*, means; *error bars*, standard error; N=4). Means with the same letters do not differ at P<0.05 (Adjusted Tukey-Kramer multiple range test)

($P<0.05$) or breed ($P<0.05$), but not their interaction (Table 2). Mixed saliva protein concentration was higher for Mamber goats than for Damascus goats (485±71 and 268±50 μg ml^{-1}, respectively, $P<0.05$) and feeding wheat hay to Mamber goats resulted in the highest salivary protein concentration in experiment 1.

The paired comparison of Comp min, Comp max, and Comp BSA data of experiment 1 by paired *t*-tests showed that the affinity of mixed saliva was higher for both quebracho and tannic acid than the affinity of parotid saliva (Table 3).

The ANOVA models for Comp min and Comp max were not significant. The only significant ($P<0.05$) ANOVA model was for Comp BSA, in which saliva source significantly affected tannin-binding ($P<0.01$), but diets and breeds did not. In other words, neither goat breed nor diet affected Comp min, Comp max, or Comp BSA. Therefore, the values shown in Table 3 are averages of all goats and dietary treatments by saliva and tannin type. The average Comp max values for quebracho and for tannic acid indicated a low level of protection of trypsin from tannin by saliva protein (Table 3).

The average Comp BSA values for quebracho tannin indicated that salivary protein had a lower affinity for quebracho than BSA (Table 3). The corresponding values for tannic acid indicated that salivary protein had affinity for tannins similar to BSA. Therefore, salivary proteins did not belong to the family of TBSPs, which have an affinity for tannins 5−80 times higher than that of BSA.

Experiment 2 Three months of exposure to a tannin-rich foraging environment by goat kids with no prior foraging experience only affected Comp min measured with quebracho. A small increase was noted for Mamber kids after exposure to tannin-rich pasture (Table 4).

Before grazing, the saliva of Mamber kids had higher Comp min values, when measured with tannic acid, than the saliva of Damacus kids ($P<0.01$; Table 5) but that difference faded ($P>0.08$) after adaptation to grazing.

Table 2 Protein concentration (μg ml^{-1}) of mixed saliva in Damascus and Mamber goats in the four consecutive periods of experiment 1 (means±SE)

	Damascus	Mamber
Period 1 (vetch hay)	316±143 ^{b}	493±29 ^{ab}
Period 2 (lentisk foliage)	173±37 ^{b}	279±32 ^{b}
Period 3 (vetch hay)	289±27 ^{b}	393±56 ^{ab}
Period 4 (wheat hay)	296±137 ^{b}	775±127 ^{a}

Means with the same superscripts do not differ at $P<0.05$ (Tukey-Kramer adjusted multiple range test); Main effects: breeds, Mamber> Damascus, $P<0.05$; Diets, Vetch and wheat hay>lentisk, $P<0.05$; Breed × Diet, $P=0.14$

Table 3 Comparison of Comp min, Comp max, and Comp BSA, between mixed and parotid saliva in experiment 1 (means±SE)

	Parotid saliva	Mixed saliva
Quebracho extract		
1.24±0.03 ^{b}	1.32±0.03 ^{a}	Comp min
0.40±0.01 ^{b}	0.43±0.01 ^{a}	Comp max
0.80±0.02 ^{b}	0.85±0.02 ^{a}	Comp BSA
Tannic acid		
1.36±0.03 ^{b}	1.47±0.03 ^{a}	Comp min
0.27±0.007 ^{b}	0.29±0.005 ^{a}	Comp max
1.00±0.03 ^{b}	1.08±0.03 ^{a}	Comp BSA

Row-wise, means with different superscripts differ at $P<0.01$ (paired *t*-tests)

There were no differences between breeds in measurements of salivary affinity for tannins when quebracho was used in the assay.

When we compared the six-month-old kids to the adults of both breeds (Fig. 3), the breed × age interaction was significant ($P<0.05$), the effect of age was highly significant ($P<0.001$), but breed was not significant. Comp min with tannic acid tended to be greater for adults than for kids (($P=0.06$) in Damascus goats, but not Mamber goats (Fig. 3).

Discussion

The sharp decrease in serum urea following ingestion of lentisk foliage (Fig. 2) agrees with the increased fecal excretion of proteins and decreased N retention reported before for animals ingesting lentisk (Silanikove et al. 1996b; Decandia et al. 2000). This suggests that lentisk foliage was a suitable choice of forage for our investigation of salivary tannin-binding proteins.

Here, we present three arguments against a prominent role of salivary tannin-binding as a defense against dietary tannins in goats: a. the lack of a tannin-induced increase in

Table 4 The effect of 3 months grazing experience on the affinity of mixed saliva for quebracho tannin and tannic acid, measured as Comp min, in Damascus and Mamber kids: means±standard error; $N=9$

	Before Grazing	After grazing	P (paired *t*-test)<
Tannic acid			
Mamber	2.71±0.18	2.83±0.08	0.49
Damascus	2.19±0.44	1.91±0.23	0.07
Quebracho			
Mamber	1.30±0.04	1.43±0.03	0.02
Damascus	1.15±0.04	1.13±0.03	0.68

Table 5 Effects of kid breed on the affinity of mixed saliva for tannins, measured as Comp min, before and after 3 months grazing experience: means±standard error; N=9

	Mamber	Damascus	P (paired t-test) <
Tannic acid			
Before	2.35±0.18	1.65±0.12	0.01
After	2.27±0.10	2.04±0.04	0.08
Quebracho			
Before	1.17±0.04	1.14±0.02	0.55
After	1.25±0.04	1.18±0.05	0.28

salivary tannin-binding (experiment 1); b. the similar affinity for tannins of parotid and mixed saliva (experiment 1); and c. the similar affinity for tannins of saliva protein and BSA (experiments 1 and 2).

The goats used in experiment 1 were habituated by many months of earlier exposure to tannin foliage while in pasture. In addition, they consumed lentisk for 10 days before saliva collection. Therefore, if short-term exposure to tannins induces the secretion of TBSPs, the transition from vetch hay (period 1) to lentisk foliage (period 2) should have resulted in an increased affinity of the goat saliva for tannins. Likewise, the reverse transition (lentisk in period 2 to vetch in period 3) should have decreased this affinity. Neither the increase nor the decrease occurred. Also, in experiment 2, there was no effect of 3 months exposure of naïve Damascus kids to a tannin-rich environment. In Mamber kids, there was a modest effect that was significant for Comp min only (Table 4). This casts a serious doubt on the ability of dietary tannins to induce salivary tannin-binding in goats, similar to the results for cattle (Makkar and Becker 1998) and sheep (Austin et al. 1989).

In rodents, tannin ingestion can induce parotid hypertrophy and production of TBSPs in the parotid (Bennick 2002; Gho et al. 2006). In roe deer, the affinity of mixed saliva for tannin is only 50% (tannic acid) to 65% (quebracho) of that of parotid saliva (Fickel et al. 1998). In contrast, our study showed that mixed saliva had higher affinity for tannins than parotid saliva (Table 3). We regard limited tannin-binding by parotid saliva as evidence that salivary protein is not a line of defense against dietary tannins in goats.

The affinity of mixed saliva and parotid saliva for tannin was approximately equal to that of BSA in experiment 1 of the present study (Table 3); and was less than that of BSA in experiment 2. In comparison, TBSPs have affinity for tannins 5–80 times higher than that of BSA (Shimada 2006). This constitutes a third argument against the role of salivary tannin-binding as a defense against tannins in goats.

It is widely believed that because grazers such as cattle and sheep consume diets virtually free of tannins, they do not produce TBSPs (Austin et al. 1989). According to the "niche theory", goats, which include a significant amount of tannin-rich browse in their diets (Kababya et al. 1998; Landau et al. 2002), should produce salivary tannin-binding proteins. In particular, Damascus goats, which behave more as "browsers" than Mamber goats (Glasser 2009), should produce more TBSPs. Our data showed that the tendency to consume tannin-rich browse was not reflected in higher salivary tannin-binding capacity in either young animals (Table 5) or in adults (Fig. 3). This is in agreement with Lamy et al. (2008), who reported considerable similarity in SDS-PAGE profiles between sheep and goat parotid salivary proteins, and who did not identify proline-rich proteins (a category of TBSPs) in either type of saliva.

Tannic acid and quebracho tannins are models of hydrolyzable and condensed tannins and thus have limitations (Rautio et al. 2007). Commercial preparations of tannic acid may contain simple galloylglucoses or even gallic acid (the hydrolysis product of gallotannins) in addition to true "tannins". Lentisk foliage contains monogalloyl glucose and gallic acid (Romani et al. 2002), suggesting tannic acid is not a bad model for phenolics in this plant. Quebracho is a profisetinidin condensed tannin that is structurally and functionally different from the procyanidin typically contained in Northern hemisphere plants, including those found in Mediterranean environments (Rautio et al. 2007). In other words, both tannin preparations tested here, but in particular quebracho, may have contained tannin chemical structures that were not encountered in the diets consumed by goats in experiments 1 (lentisk foliage) and 2 (Mediterranean brushland).

It is possible that the salivary proteins of goats bind only to specific tannins, and not to tannic acid or quebracho, which were tested here. TBSPs in moose (*Alces alces* L.) and beaver (*Castor canadensis* Kuhl) bind specifically to linear condensed tannins that are common in their diets, and not to others, while in black bear (*Ursus americanus* Pal.), an omnivore, TBSPs have affinity for a great variety of

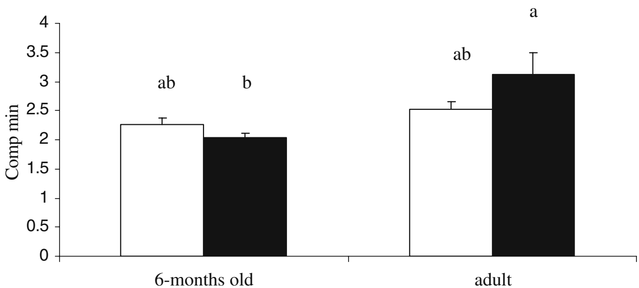

Fig. 3 Affinity of saliva for tannic acid measured as Comp min. Saliva from Damascus (*black bars*) and Mamber (*white bars*) kids (N=9) or adult goats (N=4). *Bars*, means; *error bars*, standard errors; means with the same letters do not differ at P<0.05 (Adjusted Tukey-Kramer multiple range test)

tannins (Hagerman and Robbins 1993). Goats are opportunistic feeders that are exposed to a wide array of tannins in a variety of habitats. Under free ranging conditions, Damascus and Mamber goats may forage on more than twenty tanniferous plants throughout the year (Glasser et al. 2008), each of them containing a great variety of tannins and other phenolics. For instance, thirteen different galloyl derivatives, flavonoid glycosides, and anthocyanins, have been identified in lentisk foliage (Romani et al. 2002). Because goats consume such a wide variety of tannins, we would not expect their TBSPs, if any, to be specific to a narrow range of tannin structures, but this hypothesis needs further research. Indeed, where TBSPs exist, for example in roe deer (*Capreolus capreolus* L.), tannin-binding by salivary proteins can be demonstrated by using both quebracho and tannic acid as tannin sources (Fickel et al. 1998), even though roe deer select diets with low amounts of tannic acid but reject quebracho tannin, when given a choice (Clauss et al. 2003).

Our study confirms the claim of Shimada (2006) that the ingestion of tannins in browse does not necessarily imply that salivary tannin-binding is a mechanism used to counteract their deleterious effects. In summary, explanations for the resilience of Mediterranean goats to lentisk tannins must involve other mechanisms such as microbial adaptations in the distal parts of the gastro-intestinal tract or detoxification in the liver

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Trail Pheromone Disruption of Red Imported Fire Ant

David M. Suckling · Lloyd D. Stringer · Barry Bunn · Ashraf M. El-Sayed · Robert K. Vander Meer

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Abstract The fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), is considered one of the most aggressive and invasive species in the world. Toxic bait systems are used widely for control, but they also affect non-target ant species and cannot be used in sensitive ecosystems such as organic farms and national parks. The fire ant uses recruitment pheromones to organize the retrieval of large food resources back to the colony, with *Z,E*-α-farnesene responsible for the orientation of workers along trails. We prepared *Z,E*-α-farnesene, (91% purity) from extracted *E,E*-α-farnesene and demonstrated disruption of worker trail orientation after presentation of an oversupply of this compound from filter paper point sources (30 µg). Trails were established between queen-right colony cells and food sources in plastic tubs. Trail-following behavior was recorded by overhead webcam, and ants were digitized before and after presentation of the treatment, using two software approaches. The linear regression statistic, r^2 was calculated. Ants initially showed high linear trail integrity (r^2=0.75). Within seconds of presentation of the *Z,E*-α-farnesene treatment, the trailing ants showed little or no further evidence of trail following behavior in the vicinity of the pheromone source. These results show that trailing fire ants become disorientated in the presence of large amounts of *Z,E*-α-farnesene. Disrupting fire ant recruitment to resources may have a negative effect on colony size or other effects yet to be determined. This phenomenon was demonstrated recently for the Argentine ant, where trails were disrupted for two weeks by using their formulated trail pheromone, *Z*-9-hexadecenal. Further research is needed to establish the long term effects and control potential for trail disruption in *S. invicta*.

Key Words Red imported fire ant · Ant · Trail pheromone · Trail disruption · Invasive species · *Z,E*-α-farnesene · *Solenopsis invicta*

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D. M. Suckling (✉) · L. D. Stringer · B. Bunn · A. M. El-Sayed
The New Zealand Institute for Plant and Food Research Ltd.,
PB 4704, Christchurch, New Zealand
e-mail: Max.Suckling@plantandfood.co.nz

R. K. Vander Meer
USDA ARS,
1600 SW 23rd Drive,
Gainesville, FL, USA

Introduction

In the early 1960s, Wright proposed that synthetic sex pheromone could disrupt mating of moths (Wright 1964). The feasibility of this was soon demonstrated (Gaston et al. 1967). Since then, the identification of sex pheromones of many moth species has been achieved along with the development of successful direct pest control options based on pheromones and other attractants (El-Sayed 2010; Witzgall et al. 2010). Despite the discovery and characterization of ant trail pheromones over the past several decades (Tumlinson et al. 1971; El-Sayed 2010), surprisingly little examination of these compounds has been undertaken for pest management. Research on the potential for using odorants in this way has targeted the control of leaf cutting ants (Robinson and Cherrett 1978) and the red imported fire ant (Vander Meer 1983, 1996), but the current paradigm remains largely confined to improving the performance of toxic baits (e.g., Rust et al. 2004).

New application technologies that deliver pheromones against invasive pest ants could help reduce our reliance on

the use of insecticides for ant pest control in sensitive ecosystems or where insecticides are undesirable. Trail pheromone disruption that affects recruitment is an example of a novel tactic for ant pest management. The Argentine ant (*Linepithema humile*) trail pheromone, *Z*-9-hexadecenal (Cavill et al. 1979), is being examined for disruption of trails by using either polyethylene tubing dispensers (Tatsuki et al. 2005; Tanaka et al. 2009) or widely dispersed point sources of pheromone (Suckling et al. 2008, 2010). This compound also is a moth pheromone (El-Sayed 2010). Tubing dispensers showed some disruption of traffic rates on the ground or tree trunks, when placed on bamboo at a 50 cm height above the ground (Tatsuki et al. 2005). The mechanism of disruption of trailing behavior observed for individuals at close range before and after exposure involved changes in walking track angles (Suckling et al. 2008). This single component was shown at a laboratory scale to be capable of disorienting trail following workers when presented in greater than physiologically normal quantities (Suckling et al. 2008). Measurement of trail disruption was achieved by assessing trail linearity as measured by the square of the linear correlation coefficient (r^2) (Suckling et al. 2008). The disruption concept was extended successfully to the field by using widely-dispersed wax-covered sand laden with the pheromone (Suckling et al. 2008) and formulated as sprayable microencapsulated particles (Suckling et al. 2010). Treatment achieved up to 90% disruption of visible Argentine ant trails for two weeks in 400 m^2 plots under hot and windy conditions in Hawaii's Volcanoes National Park (Suckling et al. 2010). Foraging to baits also was significantly reduced. These interesting developments suggest that this approach could have applicability to other invasive pest ant species, if impacts on nests can be demonstrated.

The Red Imported Fire Ant, *Solenopsis invicta*, is one of the 100 worst invasive alien species in the world (Lowe et al. 2000). Imported fire ants infest more than 330 million acres in 13 southern tier US states and Puerto Rico and are spreading northward (USDA APHIS 2009). This invasive ant is estimated to be responsible for almost $7 billion annually in damage repair, medical care, and control costs. The affected economic sectors are broad ranging and include households, electric service and communications, agriculture, schools, and recreation areas (Lard et al. 2006). In the last decade, *S. invicta* has changed from an invasive pest ant in the United States to a global problem, with infestations occurring in New Zealand (now eradicated, Christian 2009), Australia (Henshaw et al. 2005), Mexico (Sánchez-Peña et al. 2005), Taiwan (Chen et al. 2006), China (Zhang et al. 2007), and many Caribbean Island countries (Davis et al. 2001). The monogyne social form of *S. invicta* in the USA has population densities up to 100 colonies/ha and, at 250 thousand workers per colony, the resource demands are great.

Toxic ant baits have been developed and refined over the past decades in response to a need for fire ant control over large areas with minimal amounts of toxicant (Williams et al. 2001). Toxic baits are more environmentally friendly in that they introduce significantly less insecticide into the environment than mound drenches. However, fire ant baits also have a negative effect on non-target ant species that could otherwise slow fire ant reinfestation rate by feeding upon newly mated fire ant queens (Nichols and Sites 1991). More environmentally-friendly control methods are needed for management and, under certain circumstances, eradication of this invasive ant. Disruption of the critical recruitment process potentially could decrease colony resources and concomitantly population densities, and/or induce colonies to move.

Ants exhibit a wide variety of recruitment and orientation methods by using pheromones that direct worker movement between the colony and food sources or during colony migration. Similarly, the glandular sources and the chemistry of recruitment pheromones is diverse (Vander Meer and Alonso 1998). The Dufour's gland of fire ant workers is the source of its recruitment pheromone, which has been divided into three behavioral categories: orientation induction (Vander Meer et al. 1990), attraction (Vander Meer et al. 1988), and orientation (Vander Meer et al. 1981). Orientation induction requires essentially all Dufour's gland components, and acts to increase the sensitivity of workers to the orientation part of the pheromone. Two components, *Z,E*-α-farnesene and a bicyclic homosesquiterpene-diene, homoeudesmandiene, but not a tricyclic homosesquiterpene monoene as previously suggested (Vander Meer et al. 1988), account for 100% of the attractive element of the recruitment pheromone. *Z,E*-α-Farnesene accounts for 100% of the orientation (movement of worker ants back and forth along a trail) part of the recruitment process. The ants are sensitive to *Z,E*-α-farnesene trails as low as fg/cm levels. Laboratory trials were the first step in assessment of the potential for developing trail pheromone disruption against the Argentine ant (Suckling et al. 2008), before more expensive field trials (Suckling et al. 2010). Since behavioral responses to synthetic chemically-based disruption are concentration-dependent, we considered that it was sufficient to record statistical significance with the phenomenon in the laboratory as a first step before formulation development. This paper focuses on demonstration of the potential for trail pheromone disruption of red imported fire ants by using *Z,E*-α-farnesene, the recruitment orientation pheromone.

Methods and Materials

Chemicals and Synthesis A mixture of Z,E-α-farnesene (91% purity) and *E,E*-α-farnesene (9%) was synthesized

and maintained in a petroleum ether (115 mg/ml) stock solution until use. The preparation of *E,E*-α-farnesene from 60 kg of cv. Granny Smith apples (*Malus domestica* × *M. sylvestris*) was done following Murray (1969). The 60 kg of apples were divided into 5×12 kg batches that were washed, adding the apples in portions in 2 L of petroleum ether (boiling range 35–60°C). The apples were stirred for approximately 1 min then transferred into another 1.5 L of solvent where the stirring process was repeated. The solvent from the combined extracts of all the apples was removed *in vacuo* to give 36.2 g of a waxy solid. The solid was analyzed by TLC (10% EtOAC, 90% petroleum ether), which indicated the presence of *E,E*-α-farnesene by comparison with an authentic standard. The waxy solid was then dissolved in 400 ml petroleum ether (boiling range 35-60°C) and refrigerated overnight at 0°C. Solids were removed by filtration and discarded. The solvent extracts then were divided into two equal portions, and to each portion urea (130 g) was added. The material was refrigerated again overnight at 0°C. Solids were removed by filtration, and the solvent removed *in vacuo*. The crude product thus formed was purified by column chromatography using 5% EtOAc / 95% petroleum ether as eluant. The appropriate fractions were collected and repurified under the same conditions. The product (1.05 g), verified by ^{1}H-NMR (Spicer 1994), was collected as a colorless oil.

Following the procedure of Ramaiah et al. (1995), photo-isomerization of *E,E*-α-farnesene to *Z,E*-α-farnesene entailed dissolving *E,E* -α-farnesene (385 mg, 1.88 mmol) in benzene (12 ml), and dividing the solution into 4×3 ml portions that were put into 4 quartz spectrophotomer cells (1×1×4 cm). A drop of acetophenone was added to each of the samples. The solutions were sealed with a plastic lid and then photo-irradiated at 254 nm using a Hoefer UVC 500 UV Crosslinker. After 4 h, the photo-isomerization had reached approximately 40% conversion, and further irradiation had no effect. The solvent then was removed *in vacuo*, the crude material was redissolved in diethyl ether (10 ml), and tetracyanoethylene was added in small portions (50 mg at a time) until monitoring by GC-MS indicated that most of the unwanted *E,E* isomer had been consumed. It was not possible to remove all of the *E,E* isomer by addition of further tetracyanoethylene. The solvent was removed *in vacuo*, and the product was purified by column chromatography using petroleum ether as eluant. The product was obtained as a colorless solution, which was a mixture of *Z,E:E,E* isomers in the proportion 91:9 as determined by GC-MS, with a final yield of 115 mg.

Insects Queen-right colonies of *S. invicta* were collected in the field or started from field-collected newly-mated queens and maintained by standard methods (Banks et al. 1981). Prior to experiments, a colony cell (15 cm Petri dishes with a central hole in the lid and a layer of moistened Castone® in the bottom) was placed at one end of a white plastic tub (50×37 cm and 12 cm deep) that had the inner sides coated with Fluon® (Asahi Glass Co. Tokyo) to prevent escapes. Each colony cell was allowed to rest for at least 16 h before experiments. The ants were starved, but had continual access to water. Trail formation was achieved by placing one thawed house cricket (*Acheta domesticus*) and an absorbent cotton ball (Johnson and Johnson) dampened with 10% sugar water at the opposite end of the tub from the colony cell. Trials began once the trail had formed, with workers visibly trailing back and forth.

Video Recording A Logitech webcam (640×480 pixels, Notebook Pro, Logitech, Freemont, CA, USA) supported by retort stand was used to record a vertical view of fire ant workers trailing to and from a food source. Filming generated Audio Video Interleave (avi) files at a rate of 15 frames/s onto a laptop computer (actual screen size 10×12 cm at 480×640 pixels) to record fire ant walking tracks, with the method developed on Argentine ants (Suckling et al. 2008).

Experiment 1 Point source trail disruption was tested by placing treated (30 µg *Z,E*-α farnesene in 10 µl petroleum ether) or control filter paper (10 µl petroleum ether) within ca. 1 cm of an established trail. The treatment and control solutions were applied to filter paper squares (Whatman No. 1, 1 cm^{2}) by gas tight syringe and allowed to evaporate for ca. 1 min in a fume hood before use. The fume hood was left switched on for the entire experiment to remove any excess pheromone from the laboratory. Furthermore, control papers were tested before treated papers to reduce the likelihood of pheromone contamination. The experiment was repeated four times (*N*=4 colonies). Movies were recorded for 1 min before, and 1 min after, paper squares were placed within 1 cm of an existing trail.

Experiment 2 This experiment aimed to test the effect of trail disruption at two concentrations (solvent control plus 30 or 300 µg *Z,E*-α farnesene), and was repeated four times (*N*=4 colonies), using the same methods as above. Movies were recorded for 1 min before, and up to 10 min after paper squares were placed within 1 cm of an existing trail.

Statistical Analysis Two software approaches were used for track analysis, by instantaneous and continuous tracking. The instantaneous position of individual ants was recorded using MaxTraq v1.92 trial edition (Innovision Systems, Lapeer, MI, USA) (Suckling et al. 2008). The x-y positions of all the ants present were recorded from individual frames at 5 s intervals, as this was sufficient time for the ants to leave the video frame under control conditions, rendering

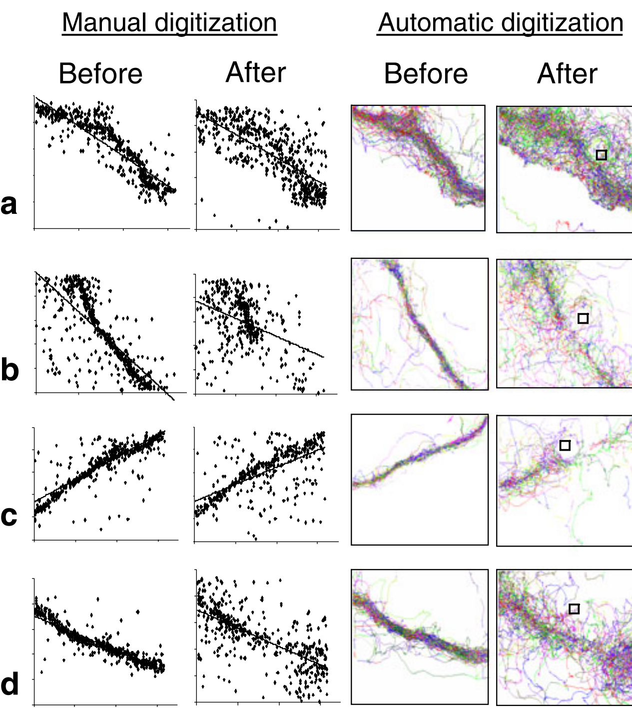

Fig. 1 Superimposed positions of red imported fire ants, *Solenopsis invicta*, in four replicate trails (**a**–**d**), with either manual digitization (left two columns) sampled instantaneously at 5 s intervals from 1 min before and 1 min after treatment, or continuously using automatic digitization (right two columns). The small square shows approximate placing near the ant trail of 1 cm filter paper loaded with 30 µg *Z,E*-α-farnesene. Trail integrity (r^2, calculated from manual digitizations) dropped from the minute before to the minute after in each replicate (**a**: r^2=0.77–0.49, **b**: r^2=0.58–0.16; **c**: r^2=0.72–0.40; **d**: r^2=0.81–0.45)

the individual samples independent. Digitizing required one mouse click per ant, but it was possible to capture the position of every ant in the frame (a labor intensive process), which enabled the determination of the linearity of trails in each frame, by calculation of r^2.

However, it was realized that this could be replaced with an approach that sub-sampled the movements of multiple individual ants, frame by frame, for differences with the preceding reference frame.

Thus, continuous analyses of the majority of ants walking (up to 25 individuals per frame) were conducted after processing, by using novel software that was developed in house with HALCON/C languages interface (MvTech Software GmbH, Munich, Germany, 2009) for the Microsoft Windows operating system. The Multiple Individual Tracking Software enables the implementation of high-speed video tracking systems capable of accurately tracking multiple objects (2–25 insects), at high speeds (>60 frames/sec). It allows for offline processing of digitally recorded videos as well as real-time processing of images from fast video streams (where the video stream does not require storage except as a text file). Identification of the target is based on both the morphological features, and its temporal speed in successive images. It utilizes a subtraction algorithm to construct tracks for each target insect. The target of the previous image is matched with the target of the current image, creating unique motion estimation for each moving object. During this process, any target estimation errors, such as when insects occlude and split, are detected and corrected. Tracking is activated once an insect enters the camera field of view. The insect is detected immediately by the change in the detection modules. While tracking insects, the tracking module keeps looking for new insects entering the field of view. The digitized images recorded per frame as X,Y coordinates for each insect were stored as a text file on the hard disk. Another program, written in Borland Delphi (version 7; Borland Software Corporation, Austin, TX, USA), was used to visualize the tracks and to export the images of resulting tracks as bitmap files. In addition, this program was used to analyze and evaluate the tracks and to calculate movement parameters.

Results

Ant trails showed clear visual evidence of a trail disruption effect well within a minute of exposure to 30 µg *Z,E*-α-

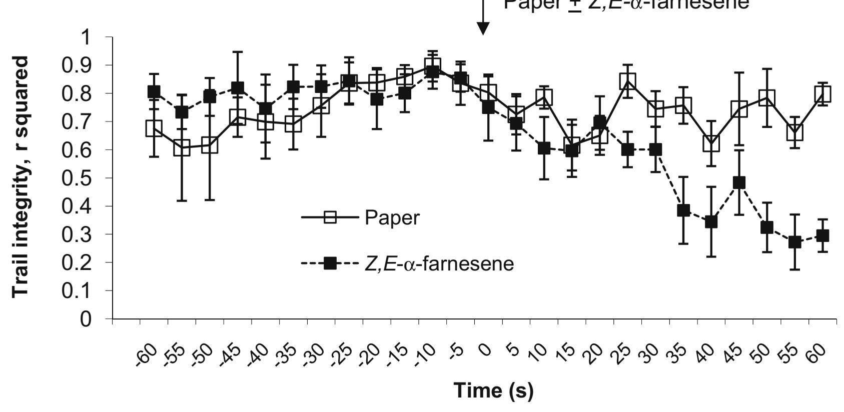

Fig. 2 Red imported fire ant, *Solenopsis invicta,* trail integrity (mean±SEM), r^2, sampled at 5 sec intervals for 1 min before and after introduction of 30 µg *Z,E*-α-farnesene (statistical significance is presented in Table 1)

Table 1 Two way analysis of variance of red imported fire ant (*Solenopsis invicta*) trail integrity (measured as r^2), 1 min before and 1 min after treatment with filter paper loaded with 30 µg *Z,E*-α-farnesene

Source	DF	Sequential Sums of Squares	Adjusted Sums of Squares	Adjusted mean square	*F*	*P*
Treatment	1	0.5440	0.5951	0.5951	15.48	<0.001
Time	1	1.7389	1.4446	1.4446	37.59	<0.001
Treatment * Time	1	1.0880	1.0880	1.0880	28.31	<0.001
Error	224	8.6089	8.6089	0.0384		
Total	227					

farnesene presented as a point source on filter paper (supplementary material, downloadable movie of trail disruption and software). Two methods extracted similar behavioral effects. The width of the trail was broadened visibly in the minute after treatment, and the effect was quantified using the trail integrity statistic r^2 (Suckling et al. 2008) derived from locations of ants sampled every 5 sec for a minute before or after presentation of treated paper (Fig. 1). The software using continuous recording of the actual trails of ants showed a similar effect, but in more detail. Random movement away from the previous trail was more evident.

Obvious trails, with significant r^2 values, were evident only before treatment, and after treatment only with solvent controls (Fig. 2). With the trail pheromone treatment, the ants were less able to follow the trail they had sensed moments before, and this was demonstrated by the reduction of the r^2 value after treatment (Fig. 2). The mean (±SEM) values before treatment (r^2=0.75±0.03) were reduced after treatment (r^2=0.48±0.03), compared to the controls that did not change (before r^2=0.75±0.03; after r^2=0.73±0.02). The two-way analysis of variance showed a significant effect from pheromone treatment and time (before and after treatment), as well as an interaction between these variables (Table 1) because of declining trail integrity over time (Fig. 2). A short delay occurred after treatment before the trail showed evidence of disruption as diffusion occurred, so assessment in the first minute is a conservative measure of an effect.

The effect of increasing disruption over time and with an increased concentration of the pheromone is evident in the next experiment (Fig. 3), displayed using the Multiple Individual Track System. When post-treatment recordings of longer duration are examined, the trails show increasing evidence of disruption over time. In the case of experiments with 300 µg loadings of *Z,E*-α-farnesene on filter paper (Fig. 3), the trails appear to literally melt away over time as diffusion spreads the oversupply of trail pheromone over existing trails.

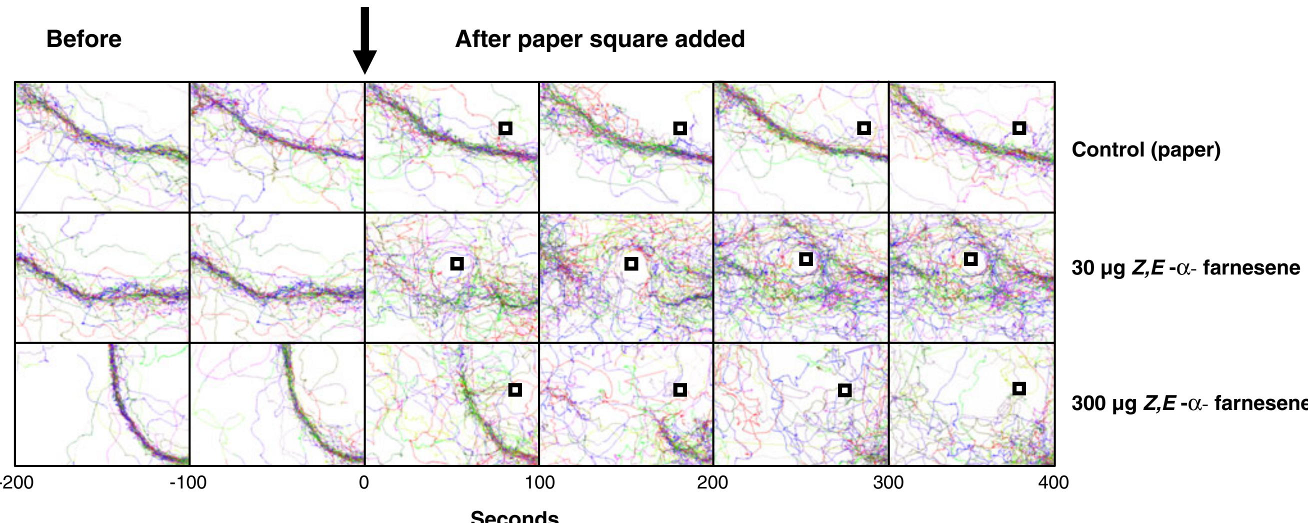

Fig. 3 Trail formation in red imported fire ant, *Solenopsis invicta,* before treatment (-200-0 s) and at 0–400 s after trail disruption, from the addition of an oversupply of trail pheromone *Z,E*-α-farnesene (solvent control, 30 or 300 µg on 1 cm² of filter paper). Walking tracks of ants were recorded by webcam and analyzed by Multiple Individual Tracking Software, written in-house

Discussion

We have demonstrated for the first time that the trail-following behavior of *S. invicta* can be disrupted by an oversupply of the trail pheromone, *Z,E*-α-farnesene. This is the first step in a new line of enquiry against this pest species. The precedent case of trail pheromone disruption in one other species (Argentine ant) has advanced to a formulation lasting up to two weeks in the field under windy conditions, and field tests over several months in a combination treatment with toxic baits as part of the Japanese eradication program at the port of Yokohama (S. Tatsuki pers. comm.). A single point source can be used for demonstration of disruption of the nearest trails (Tatsuki et al. 2005; Suckling et al. 2008), although demonstration of sustained nest level effects is needed (Suckling et al. 2010). The potential to use trail disruption as a management tool or in some circumstances, eradication, against fire ants is a worthy goal, but will require suitable technology to release *Z,E*-α-farnesene or other compounds at an effective rate and cost.

The analysis of ant trail movies into statistical parameters such as r^2 for trail integrity sampled at regular intervals (Suckling et al. 2008) enabled a new approach to measuring the impact of disrupting trailing behavior. The bioassay uses a statistical definition of trail following (r^2), and trail disruption is defined when this statistic becomes insignificant. This offers a number of advantages, including an objective statistical basis and lack of bias as to what constitutes a trail, disrupted or otherwise. However, it is labor intensive. In contrast, the development of software that automatically performs the digitization of multiple individuals and tracks their movements without requiring mouse clicks offers greater digitization speed. These advanced functions expedite recording and analysis of insect behavior, and have enabled our experiments to investigate trail behavior from many individuals quickly.

Trail pheromone disruption of *Solenopsis invicta* warrants further examination, but any eventual use would rely on appropriate technology for delivery of the necessary concentrations, as well as an understanding of the biological implications, if any, of reducing the efficiency and potentially information and energy (food) content of workers returning to the nest. The chemical instability and likely cost of the trail pheromone compounds could prove to be an insurmountable problem to achieving this, although further research may overcome these barriers, particularly since plant-based production via synthase has been established (Green et al. 2007). If this is the case, there could be various ways of using the phenomenon of trail disruption, depending on how well it works and what effect it has on the insects over time. There are also other related ant species which use this compound (Barlin et al. 1976; El-Sayed 2010). It is potentially useful in integrated pest management, for example in combination with poison baits, possibly before baiting treatment to improve bait removal to the nest after starvation, or after insect densities have been lowered by baiting. Trail disruption could prove to be useful in the event of detection of new incursions of this species, for example to island nations (Davis et al. 2001; Henshaw et al. 2005; Chen et al. 2006; Christian 2009), where the prospects of eradication may be greater.

Certainly, there is a high risk of failure in establishing an entirely new pest management tactic against ants based on trail pheromones, but there is also a great reward for success if they can be successfully developed and deployed for these pests. The very low eco-toxicological hazard of these compounds presented in such a low quantity means that they could become acceptable tools for use in sensitive environments, and wherever conventional pesticides are undesirable. We have shown that a trail pheromone compound can locally disrupt *S. invicta* trails, and other active compounds should also be investigated for their potential effects. Trail pheromone disruption against this species will require the development of sustained release formulations to determine if the approach can be made practical in the field.

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Deciphering the Chemical Basis of Nestmate Recognition

Ellen van Wilgenburg · Robert Sulc · Kenneth J. Shea · Neil D. Tsutsui

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Abstract Social insects maintain colony cohesion by recognizing and, if necessary, discriminating against conspecifics that are not part of the colony. This recognition ability is encoded by a complex mixture of cuticular hydrocarbons (CHCs), although it is largely unclear how social insects interpret such a multifaceted signal. CHC profiles often contain several series of homologous hydrocarbons, possessing the same methyl branch position but differing in chain length (e.g., 15-methyl-pentatriacontane, 15-methyl-heptatriacontane, 15-methyl-nonatriacontane). Recent studies have revealed that within species these homologs can occur in correlated concentrations. In such cases, single compounds may convey the same information as the homologs. In this study, we used behavioral bioassays to explore how social insects perceive and interpret different hydrocarbons. We tested the aggressive response of Argentine ants, *Linepithema humile*, toward nest-mate CHC profiles that were augmented with one of eight synthetic hydrocarbons that differed in branch position, chain length, or both. We found that Argentine ants showed similar levels of aggression toward nest-mate CHC profiles augmented with compounds that had the same branch position but differed in chain length. Conversely, Argentine ants displayed different levels of aggression toward nest-mate CHC profiles augmented with compounds that had different branch positions but the same chain length. While this was true in almost all cases, one CHC we tested elicited a greater aggressive response than its homologs. Interestingly, this was the only compound that did not occur naturally in correlated concentrations with its homologs in CHC profiles. Combined, these data suggest that CHCs of a homologous series elicit the same aggressive response because they convey the same information, rather than Argentine ants being unable to discriminate between different homologs. This study contributes to our understanding of the chemical basis of nestmate recognition by showing that, similar to spoken language, the chemical language of social insects contains "synonyms," chemicals that differ in structure, but not meaning.

Key Words Nest-mate recognition · Cuticular hydrocarbons · Argentine ants · *Linepithema humile*

E. van Wilgenburg (✉) · N. D. Tsutsui
Department of Environmental Science, Policy and Management, University of California,
137 Mulford Hall, #3114,
Berkeley, CA 94720-3114, USA
e-mail: ellenvw@berkeley.edu

E. van Wilgenburg
Department of Zoology, University of Melbourne,
Melbourne, Victoria 3010, Australia

R. Sulc · K. J. Shea
Department of Chemistry, University of California,
5042D Fredrick Reines Hall,
Irvine, CA 92697, USA

Introduction

Recognition of group members is considered a key innovation in the evolution of sociality, as the direct and indirect fitness benefits of social living can be gained only if altruistic behaviors are directed toward the appropriate recipients. Some of the fundamental contributions to our understanding of social evolution, and the underlying social recognition systems, have come from research on social insects. Social insects have evolved a highly sophisticated recognition system that allows individuals to identify a non-colony mate among large numbers of colony mates. These insects are able to achieve such precise discrimination with a single sweep of the antenna across the cuticle of another

individual. The cues used for nest-mate recognition are typically hydrocarbons on the exoskeleton of workers (Thomas et al. 1999; Wagner et al. 2000; Torres et al. 2007). These cuticular hydrocarbons (CHCs) usually are a complex mixture of alkanes, alkenes, and methyl-branched alkanes (Martin and Drijfhout 2009b). The number of CHCs that have been recorded for an ant species ranges from as few as 8, in *Formica exsecta* (Martin and Drijfhout 2009b), to as many as 110, in *Myrmica incompleta* (Elmes et al. 2002). In total, almost 1,000 different CHCs have been described for ants (Martin and Drijfhout 2009b). CHC profiles usually are colony specific, with colonies having different relative concentrations of compounds (Nielsen et al. 1999; Tentschert et al. 2002; van Wilgenburg et al. 2006; Foitzik et al. 2007; Martin et al. 2008a; Brandt et al. 2009). Studies have shown that there is a positive relationship between the level of aggression among colonies and their differences in CHC profiles (Suarez et al. 2002; Zweden et al. 2009). Moreover, behavioral studies have shown that workers often act aggressively toward dummies or workers that have been treated with CHCs of non-colony mates (Thomas et al. 1999; Wagner et al. 2000; Akino et al. 2004; Ozaki et al. 2005; Torres et al. 2007; Martin et al. 2008a). However, little is known about the mechanisms that allow individual social insects to discriminate between the complex cocktails of chemicals emitted by members of their own colony versus those of other colonies.

The primary function of CHCs is to prevent water loss, with not all CHCs functioning for recognition (Hadley 1984; Singer 1998). For example, it appears that methyl-branched alkanes and alkenes, but not *n*-alkanes, are used as nest-mate recognition cues (Bonavita-Cougourdan et al. 1987; Dani et al. 2005; Lucas et al. 2005; Martin et al. 2008b; but see Greene and Gordon 2007). Additionally, some cues may be used for intra-colony recognition. Workers performing different tasks may have different CHC profiles (Kaib et al. 2000; Wagner et al. 2001; Martin and Drijfhout 2009c), and can detect and respond to these intra-colony differences (Greene and Gordon 2003). Finally, not all compounds within a profile may function independently as recognition cues. A recent study of *Formica* ants and *Vespa* hornets has shown that CHC profiles are a mixture of correlated and non-correlated compounds (Martin et al. 2008a; Martin and Drijfhout 2009b). Within a CHC profile, many of the hydrocarbons are structurally related, being part of a homologous series, in which all members possess the same methyl group or double bond, with a backbone that varies in length by two carbons [e.g., 15-methyl-pentatriacontane (15-MeC35), 15-methyl-heptatriacontane (15-MeC37), 15-methyl-nonatriacontane (15-MeC39)]. The relative amounts of compounds within a homologous series often are constant, both within colonies (Martin et al. 2008a; Martin and Drijfhout 2009b) and species (Martin and Drijfhout 2009b). Consequently, a single compound may provide the same information as its homologs.

It also is possible that social insects may not be able to distinguish between structurally related chemicals. Several studies of honey bees have shown that workers are able to discriminate compounds that differ in functional group better than those that differ in carbon chain length, especially when the latter differ by only a few carbons (Vareschi 1971; Getz and Smith 1990; Chaline et al. 2005; Guerrieri et al. 2005). Taken together, these finding suggest that the chemical basis of nest-mate recognition may be simpler than it appears, and that specific features of a few key hydrocarbons may allow social insects to identify colony mates. If so, we would predict that homologous CHCs that are correlated in abundance, and thus contain redundant information, should elicit similar behavioral responses.

The aim of this study was to investigate the aggressive response to homologous CHCs by using the Argentine ant, *Linepithema humile*, as a model species. The Argentine ant, native to South America, is an introduced and invasive species in many temperate regions of the world (Suarez et al. 2001; Tsutsui and Case 2001). Although native populations display intraspecific (inter-colonial) aggression across spatial scales, ranging from tens to hundreds of meters (Suarez et al. 1999; Tsutsui et al. 2000; Pedersen et al. 2006), introduced populations are characterized by the formation of much larger supercolonies, up to several thousand kilometers long (Suarez et al. 1999; Tsutsui et al. 2000; Giraud et al. 2002; Corin et al. 2007; Bjorkman-Chiswell et al. 2008; Sunamura et al. 2009). This unicolonial structure is one of the keys to the success of introduced populations (Holway et al. 1998).

We showed previously that Argentine ants can perceive and respond aggressively to colony mates treated with a single hydrocarbon, and the intensity of the response varies from colony to colony (Brandt et al. 2009). We also showed that Argentine ants show a higher aggressive response when several different hydrocarbons are applied simultaneously. In this study, we tested whether compounds within a homologous series elicit correlated nest-mate recognition responses. For this, we conducted a series of field-based bioassays, in which we compared the behavioral response of workers to hydrocarbon extracts augmented with one each of eight synthetic hydrocarbons that were either homologs or non-homologs of each other. In addition, we investigated the relationships among these compounds in CHC profiles within and among 14 Argentine ant colonies.

Methods and Materials

For the behavioral assays, we used Argentine ants from three supercolonies in California: Berkeley (B), Lake

Fig. 1 Synthesis of unsymmetrical mono-methyl hydrocarbons 15-methyl-pentatriacontane (**1**), 17-methyl-pentatriacontane (**2**), 15-methyl-heptatriacontane (**3**) and 17-methyl-heptatriacontane (**4**)

Me–C(=O)–R_1 —(BrMg—R_2, THF)→ R_3–C(Me)(OH)–R_4 (**8a-d**) —(TsOH, benzene)→ **9a-d** —(Pd/C, Et_2O)→ **1-4**

6a $R_1 = (CH_2)_{13}Me$
6b $R_1 = (CH_2)_{15}Me$

7a $R_2 = (CH_2)_{17}Me$
7b $R_2 = (CH_2)_{19}Me$
7c $R_2 = (CH_2)_{21}Me$

a $R_3 = (CH_2)_{13}Me$ $R_4 = (CH_2)_{19}Me$
b $R_3 = (CH_2)_{15}Me$ $R_4 = (CH_2)_{17}Me$
c $R_3 = (CH_2)_{13}Me$ $R_4 = (CH_2)_{21}Me$
d $R_3 = (CH_2)_{17}Me$ $R_4 = (CH_2)_{19}Me$

Hodges (LH), and Cottonwood (CW). These supercolonies are behaviorally and genetically distinct from each other (Tsutsui et al. 2000, 2003). To test whether the hydrocarbons of a homologous series triggered similar responses, we tested the behavior of workers to filter papers treated with hydrocarbon extracts of colony mates, augmented with one of eight synthetic HCs (see hydrocarbon synthesis and behavioral assays below). We tested two homologous pairs of methyl-branched alkanes [15-MeC35 and 15-MeC37; 17-methyl-pentatriacontane (17-MeC35) and 17-methyl-heptatriacontane (17-MeC37)], and one methyl-branched alkane with a different methyl branch position [19-methyl-heptatriacontane (19-MeC37]. We also tested three homologous tri-methyl alkanes [5,3,17-trimethyl-tritriacontane (5,13,17-triMeC33), 5,13,17-trimethyl-pentatriacontane (5,13,17-triMeC35), and 5,13,17-trimethyl-heptatriacontane (5,13,17-triMeC37)]. All behavioral assays were conducted in the field. For controls, we tested the behavioral response of Argentine ant workers to filter papers treated with CHCs of their own colony mates (negative control) or of workers from a foreign colony (positive control).

Synthesis of Monomethyl Hydrocarbon Nestmate Recognition Cues We synthesized the mono-methyl hydrocarbons **1–5** [15-MeC35 (**1**), 17-MeC35 (**2**), 15-MeC37 (**3**), 17-MeC37 (**4**), and 19-MeC37 (**5**)] according to the schemes in Figs. 1 and 2 (see Table 1 for yields). 2-Ketone precursors **6a–b** were reacted with α-Grignard reagents **7a–c** of desired length to produce 3° alcohols **8a–d**, which were separated from hydrocarbon byproducts by silica column chromatography. Dehydration of **8a–d** with catalytic TsOH created mixtures of isomers of alkenes **9a–d**, which were purified by a second silica column. The alkenes **9a–d** were subsequently hydrogenated with catalytic Pd/C under hydrogen (1 atm) to yield the desired mono-methyl hydrocarbons **1–4**.

We began synthesis of the symmetrical hydrocarbon **5** by the double addition of an α-C18-Grignard reagent **7a** to ethyl acetate (**10**) to form 3° alcohol **8e**. Dehydration of **8e** with catalytic TsOH produced a mixture of isomers **9e.** Both products **8e** and **9e** were purified by silica gel chromatography from hydrocarbon byproducts. The alkene **9e** was hydrogenated with catalytic Pd/C under hydrogen to yield hydrocarbon **5**.

Products (and purity) were characterized by NMR and gas chromatography-mass spectrometry (GC-MS). NMR was not particularly useful to confirm the exact structures, since there were so many similar carbons and protons in the compounds. The alcohol products **8a–e** appeared as a single peak on GC, and the diagnostic ion as M^+-H_2O in the mass spectrum. The alkene products **9a–e** appeared as two peaks on GC, with the M^+ ion diagnostic in the mass spectrum and a ^{1}H NMR multiplet at ~5 ppm corresponding to the expected alkene protons. We observed the final products **1**–**5** as single peaks in the GC with $(M\text{-}Me)^+$ as one of the main fragments in the mass spectrum. A $(M\text{-}Me)^+$ peak is a common fragment of saturated linear hydrocarbons. Other main diagnostic mass spectral fragments arose from fragmentation at the tertiary carbon. The ^{1}H NMR spectrum, although not diagnostic, supported the structure of the final products.

Synthesis of Tri-methyl Hydrocarbon Nestmate Recognition Cues We synthesized the tri-methyl compounds **11**, **12,** and **13** [5,13,17-triMeC33 (**11**), 5,13,17-triMeC35 (**12**), and 5,13,17-triMeC37 (**13**)] by sequential Grignard reactions (Figs. 3, 4). Treatment of 8-bromooctanoic acid (**14**) with two equivalents of methyl lithium provided methyl ketone **15**, which was converted to ketal **16** with TsOH and ethylene glycol. Grignard reagent, derived from bromo ketal **16**, was added to 2-hexanone (**17**) to produce tertiary alcohol **18**. Alcohol **18** was dehydrated with TsOH and, following a H_2O work up, gave the deprotected methyl ketone **19**. Addition of the Grignard reagent, derived from protected ketal **20** to ketone **19**, produced alcohol **21**. The 5,13,17-trimethyl core **22** was produced following dehydration, and subsequent deprotection of alcohol **21**. To eliminate the possibility of cyclization, the intermediate **22**

Fig. 2 Synthesis of symmetrical mono-methyl hydrocarbon 19-methyl-heptatriacontane (**5**)

Me–C(=O)–OEt (**10**) —(BrMg—R, THF; **7a** $R = (CH_2)_{17}Me$)→ R–C(Me)(OH)–R (**8e**) —(TsOH, benzene)→ **9e** —(Pd/C, Et_2O)→ **5**

Table 1 Summary of reaction yields for mono-methyl hydrocarbons. For identity reactants see Fig. 1

Product	Reactants	8 a–d (%)	9 a–d (%)	Product (%)
15-methyl-pentatriacontane	6a+7b	61	89	89
17-methyl-pentatriacontane	6b+7a	66	65	87
15-methyl-heptatriacontane	6a+7c	84	91	97
17-methyl-heptatriacontane	6b+7b	87	91	93
19-methyl-heptatriacontane	10+7a+7a	38	47	89

was reacted with Wittig reagent **23a–c** to form triene **24a–c**. The hydrogenation of **24a–c**, with Pd/C H_2 at elevated pressure, produced the saturated alkanes **11**–**13**. The structures of hydrocarbons **11**–**13** were confirmed by the fragmentation patterns of the mass spectra. The tri-methyl compounds produced were a mixture of the chiral forms.

Preparation of Filter Paper To prepare the treatment solution for the filter papers used in the behavioral assays, we extracted the CHCs of ca 8000 frozen, field-collected workers by immersing the ants in n-hexane for 10 min. Hydrocarbons were separated from polar surface lipids by running samples through a Pasteur pipette filled with silica gel. The eluate was divided into 10 aliquots, the solvent evaporated, and the extract reconstituted in 267 µl hexane. To each of the vials we added either 218 µl of a synthetic hydrocarbon (1 mg/ml hexane) or 218 µl of hexane (control). The amount of synthetic hydrocarbon solution used was a 2–7-fold increase of that in the CHC profile (Brandt et al, unpublished data). Next, we added 9 µl of treatment (about 15 ant equivalents) or control solution to each filter paper (6 x 4 mm, with three slits). We allowed the hexane to evaporate, and stored the filter papers in the freezer (−20°C) until used in the behavioral assays.

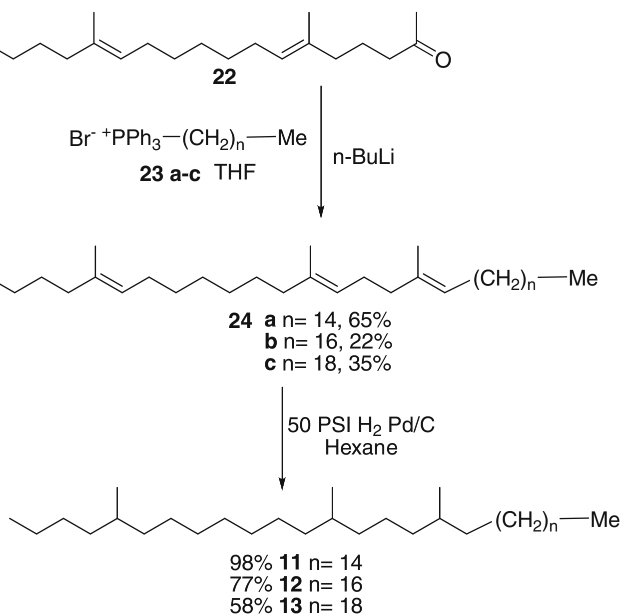

Fig. 4 Synthesis of hydrocarbons 5,13,17-trimethyl tritriacontane (**11**), 5,13,17-trimethyl pentatriacontane (**12**) and 5,13,17-trimethyl heptatriacontane (**13**)

Behavioral Assays Seven Argentine ant workers were collected from a foraging trail, and their responses to a filter paper treatment recorded for 3 min. in a 35 mm, Fluon-coated Petri dish. We scored behavioral assays as either aggressive (flaring of mandibles, biting or grabbing of the filter paper) or non-aggressive. We repeated this 45 times for each treatment, and tested 10 treatments, for colonies LH and LP, and nine treatments for colony CW (a total of 1,305 trials). All behavioral assays were conducted blind with regard to the treatment.

Statistical analyses were carried out using Genstat (version 6). We used generalized linear mixed models (GLMM), with the presence or absence of aggression as the

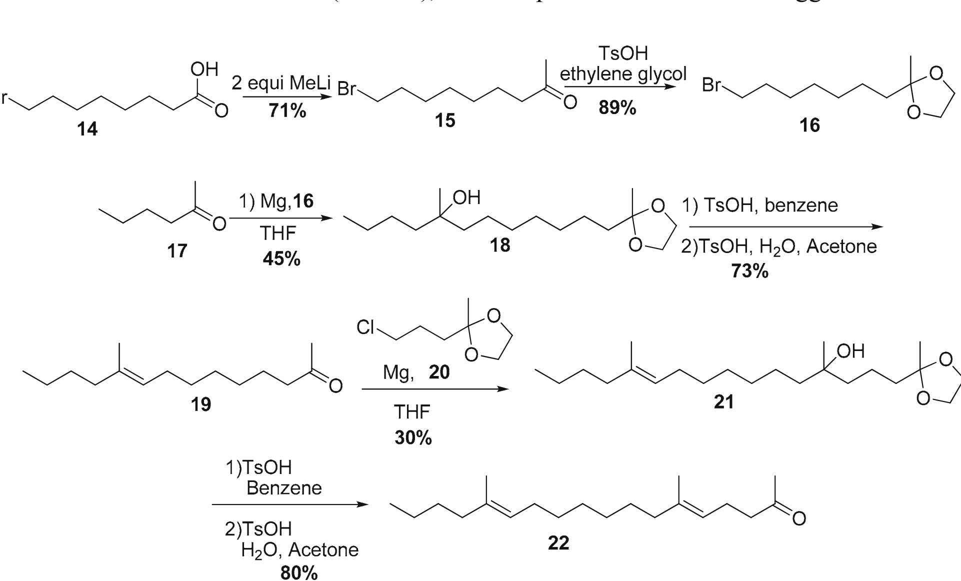

Fig. 3 Synthesis of the 5,13,17-trimethyl core compounds (**22**)

response variable and colony identity as a random factor, to accommodate repeated measures of each colony.

Chemical Analysis Cuticular lipids, from at least 12 workers from each colony, were extracted by immersing an individual ant in 45 μl of hexane for 10 min, and the individual's CHC profile analyzed by GC–MS. For quantification, 1 μl of each sample was injected splitless (temperature 250°C) into an Agilent 7890 GC equipped with an HP5-MS capillary column (30 m×250 mm× 0.25 μm, Agilent Technologies). Helium, at 1 $ml.min^{-1}$, was the carrier gas, and the column oven was programmed at 80°C, for 2 min., then to 270°C at $20°C.min^{-1}$, then to 310°C at $3°C.min^{-1}$. Electron impact mass spectra were obtained with an ionization voltage of 70 eV and a source temperature set to 250°C. Chromatograms were integrated using the program ACD SpecManager (version 10.0, Advanced Chemistry Development), and relative proportions of each peak area, to that of the total sample, were calculated. Selected ion monitoring was used to record m/z 99, 113, and 127. Full mass spectra of all the peaks in the cuticular extracts from a pool of 50 ants of each colony also were recorded, enabling characterization of the compounds based on diagnostic ions and Kovat's indices.

Inter- and intra-colony ratios (relative amounts) of the compounds used in the behavioral assays were based on quantitative analyses of individuals of 14 colonies, three of which were used in our study and the others published in Brandt et al (2009). To avoid pseudo-replication we included colonies only that are chemically and genetically distinct from each other. Data were analysed using SPSS 12.0.

Methyl-branched hydrocarbons with the same carbon-chain backbone often co-elute on a non-polar column as a single peak. While others have determined the ratios of the different hydrocarbons within a given peak by the ratios of the respective diagnostic ions (Martin et al. 2008a), this method failed to provide consistent results in our study. Therefore, we correlated relative amounts of whole peaks (i.e., all the co-eluting compounds) with other whole peaks.

Results

Synthetic Hydrocarbon Experiment In general, workers frequently were more aggressive ($F_{1,1164}$=14.68, P<0.001) to filter papers augmented with a single synthetic hydrocarbon than to filter papers treated only with nest mate hydrocarbons (negative control), but were less frequently aggressive ($F_{1,1164}$=5.76, P=0.017) to filter papers augmented with synthetic hydrocarbons than to filter papers treated with non-colony mate hydrocarbons (i.e., positive control; Fig. 5). Workers showed similar frequencies of aggression to each of the compounds in the respective pairs of homologous monomethyl hydrocarbons (i.e., to 15-MeC35 and 15-MeC37, and to 17-MeC35 and 17-MeC37). The location of the methyl branch of a monomethyl alkane had an effect on the frequency of aggression of workers, but carbon chain did not (Fig. 5; branch position, $F_{2,621}$=11.60, P<0.001, chain length $F_{1,621}$=1.49, P=0.222). In contrast, workers showed different frequencies of aggression to filter papers treated with the three homologous trimethyl hydrocarbons; workers were more often aggressive to filter papers treated with 5,13,17-triMeC33 than to those treated with 5,13,17-triMeC35 or 5,13,17-triMeC37. For these compounds, there was an effect of chain length ($F_{2,402}$=4.18, P=0.016).

Relationship Among Compounds Monomethyl C35 and C37 peaks were correlated both across and within colonies (Fig. 6, Table 2). Similarly, 5,13,17-triMeC35 was highly correlated with 5,13,17-triMeC37 across, but to a lesser extent within, colonies (Fig. 6, Table 2). There was a weaker correlation between the 5,13,17-triMeC33 and the 5,13,17-triMeC35 peaks both across and within colonies. The 5,13,17-triMeC33 and the 5,13,17-triMeC37 peaks were not correlated across or within colonies (Fig. 6, Table 2).

Discussion

The recognition code of social insects generally is embedded in a complex mixture of chemicals. This study provides a significant step forward in the understanding of the

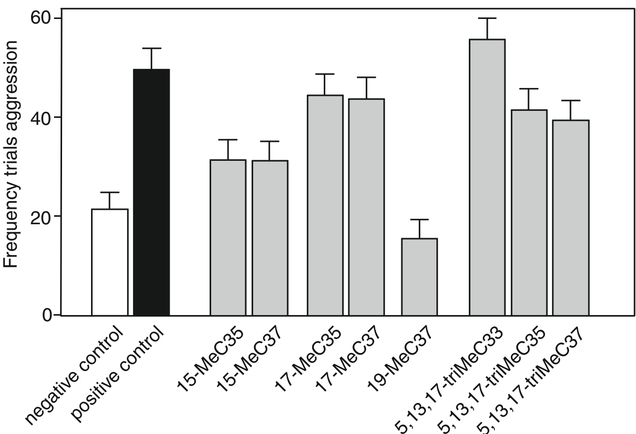

Fig. 5 Mean frequencies of aggression (±SE) exhibited by *Linepithema humile* workers across colonies to filter papers treated with cuticular hydrocarbons of the same colony augmented with a synthetic hydrocarbon (*grey bars*) and filter paper controls (*black* = cuticular hydrocarbons of a foreign colony only, and *white* = cuticular hydrocarbons of the same colony only). 13-methyl-pentatriacontane (13-MeC35), 15-methyl-pentatriacontane (15-MeC35), 17-methyl-pentatriacontane (17-MeC35), 13-methyl-heptatriacontane (13-MeC37), 15-methyl-heptatriacontane (15-MeC37), 17-methyl-heptatriacontane (17-MeC37), 5,3,17-trimethyl-tritriacontane (5,13,17-triMeC33), 5,13,17-trimethyl-pentatriacontane (5,13,17-triMeC35), and 5,13,17-trimethyl-heptatriacontane (5,13,17-triMeC37)

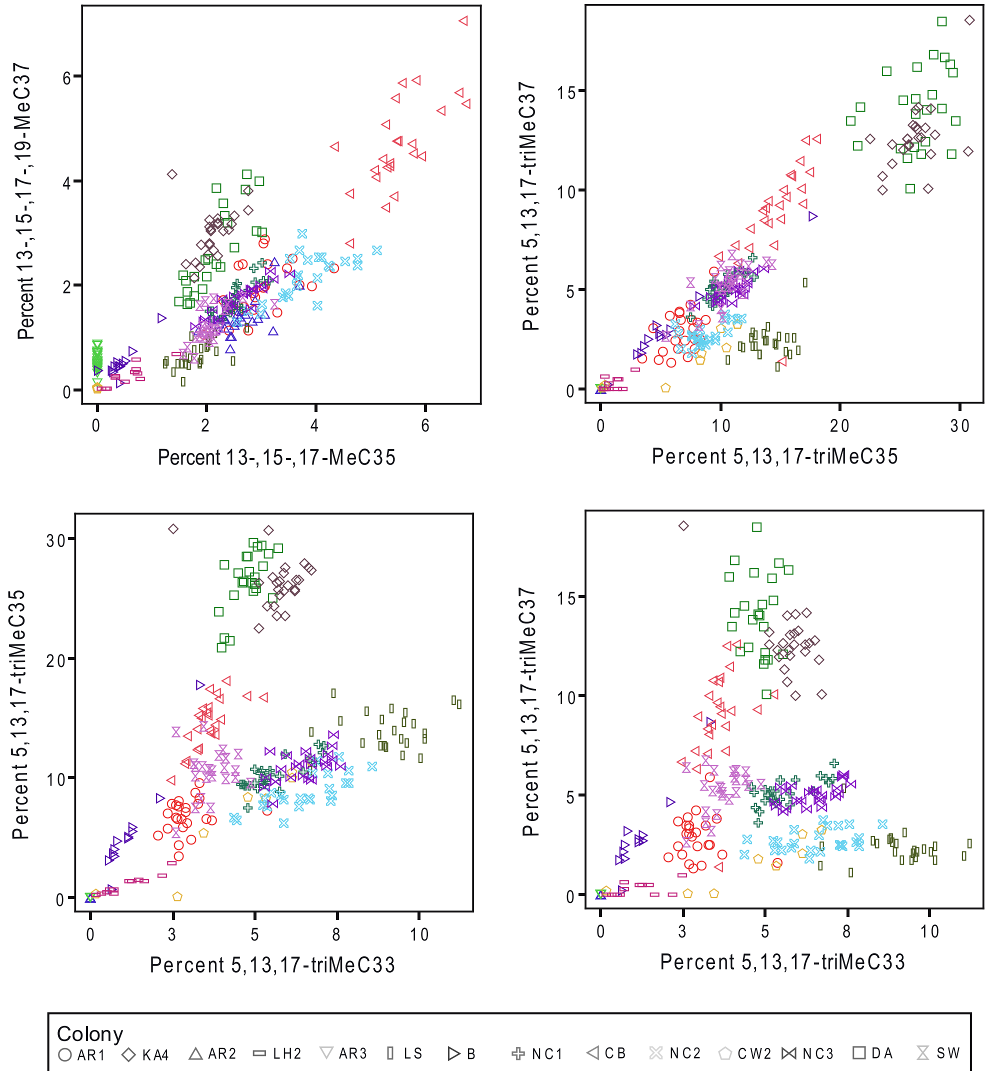

Fig. 6 Correlations between relative proportions of hydrocarbon peaks (consisting of co-eluting compounds) within cuticular hydrocarbon profiles of *Linepithema humile*. Distinct supercolonies are encoded for individuals within the same supercolony. Sampling locations and abbreviations for supercolonies are described in the methods and in Brandt et al. (2009). 13-,15-,17-MeC35 = total of 13-methyl-pentatriacontane, 15-methyl-pentatriacontane, and 17-methyl-pentatriacontane; 13-,15-,17-MeC37 = total of 13-methyl-heptatriacontane, 15-methyl-heptatriacontane, and 17-methyl-heptatriacontane; 5,13,17-triMeC33 = 5,3,17-trimethyl-tritriacontane; 5,13,17-triMeC35 = 5,13,17-trimethyl-pentatriacontane; 5,13,17-triMeC37 = 5,13,17-trimethyl-heptatriacontane

chemical basis of nest mate recognition by suggesting that, similar to spoken language, the chemical language of social insects contains "synonyms": chemicals that differ in structure, but are interpreted as being equivalent.

In general, Argentine ants showed similar levels of aggression to nest-mate CHC profiles augmented with compounds that differed in chain length but with the same methyl-branch position. There are at least two possible explanations for this. First, ants may not be able to discriminate among roughly similar homologs. It has been shown that honey bees often generalize odors with the same functional group, and that the ability to discriminate among compounds decreases with decreasing differences in chain length and increasing chain length (Vareschi 1971; Guerrieri et al. 2005). We tested compounds of very long carbon-chain length (33, 35, and 37), differing only by two or four carbons. Thus, it is possible that Argentine ants respond similarly to these homologs because they perceive them as

Table 2 Correlations (r^2) between relative amounts of hydrocarbons present in the cuticular hydrocarbon profile of *Linepithema humile*

Compounds[a]	Between colonies	Within colonies +/− s.d.
13-,15-,17-MeC35 vs. 13-,15-,17-,19-MeC37	r^2=0.863, P<0.001	r^2=0.71 +/− 0.16
5,13,17-triMeC35 vs. 5,13,17-triMeC37	r^2=0.943, P<0.001	r^2=0.63 +/− 0.20
5,13,17-triMeC33 vs. 5,13,17-triMeC37	r^2=0.375, P=0.187	r^2=0.30 +/− 0.48

[a] 13-methyl-pentatriacontane (13-MeC35), 15-methyl-pentatriacontane (15-MeC35), 17-methyl-pentatriacontane (17-MeC35), 13-methyl-heptatriacontane (13-MeC37), 15-methyl-heptatriacontane (15-MeC37), 17-methyl-heptatriacontane (17-MeC37), 5,3,17-trimethyl-tritriacontane (5,13,17-triMeC33), 5,13,17-trimethyl-pentatriacontane (5,13,17-triMeC35), and 5,13,17-trimethyl-heptatriacontane (5,13,17-triMeC37)

the same compound. Alternatively, homologous CHCs may elicit the same aggressive response because they convey the same information. The data from our and other studies (Martin et al. 2008a; Martin and Drijfhout 2009b) show that homologs often occur in concentrations correlated within the CHC profile; colonies or individuals that produce relatively high or low concentrations of one compound also produce relatively high or low concentrations of a homolog. Increasing the concentration of one compound within the CHC profile may thus change the profile to a degree similar to increasing the concentration of the homolog. Although the exact biosynthetic mechanisms that regulate the number and position of methyl branches are unknown (Howard and Blomquist 2005), it has been suggested that amounts of homologous CHCs are correlated because they share a biosynthetic pathway (Martin and Drijfhout 2009b). However, we found that the relative amount of one of the compounds did not correlate with the amounts of its homologs; the amount of 5,13,17-triMeC33 was only weakly correlated with homologous 5,13,17-triMeC35, and not at all with 5,13,17-triMeC37. Interestingly, this lack of correlation was matched by responses to these compounds; workers were more frequently aggressive to nest-mate CHC profiles augmented with 5,13,17-triMeC33 than to those augmented with 5,13,17-triMeC35 or 5,13,17-triMeC37. Thus, it appears that the amounts of some homologous CHCs are not strictly related biosynthetically, and that Argentine ants are able to distinguish between certain homologs of similar carbon length.

In spite of this, our data show that individual compounds within a profile do not necessarily function as independent recognition cues, but rather that groups of homologs may do so. Consequently, the diversity of recognition cues likely may be far smaller than the number of CHCs in a profile. Studies on colony-cue recognition by social insects typically perform statistical tests, such as discriminate and principal component analysis, using all the CHCs in a profile as variables (Nielsen et al. 1999; Tentschert et al. 2002; van Wilgenburg et al. 2006; Foitzik et al. 2007). Our findings, as well as those of other studies (Martin and Drijfhout 2009a), indicate that it may be more appropriate to treat highly correlated compounds as a single variable. Moreover, when testing the function of individual CHCs in colony recognition, specificity of response may be elucidated more readily by testing non-homologous CHCs.

The evolution of complex signals, such as CHC profiles, can be driven by the value of providing additional and more reliable information. However, signal evolution is expected to be constrained by the interaction of physiological factors and the chemoreception system. Ants may not have evolved additional pathways that allow for variation in concentration among homologous CHCs because they either cannot detect or generate this type of variation.

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Chemical Communication in *Schizocosa malitiosa*: Evidence of a Female Contact Sex Pheromone and Persistence in the Field

Luciana Baruffaldi · Fernando G. Costa · Alejandra Rodríguez · Andrés González

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Abstract Mate finding in many spider species is mediated, at least in part, by chemical cues. Although few have been characterized, most spider sex pheromones seem to be associated with the silk threads of adult females, attracting and/or triggering sexual behaviors in males. *Schizocosa malitiosa* (Araneae: Lycosidae) is a wolf spider common in dry environments in Southern Uruguay. Here, we report evidence for the occurrence of a female sex pheromone in the silk of virgin *S. malitiosa* females. The pheromone elicits typical courtship displays by conspecific males (palpal drumming and leg shaking), when it is applied to an artificial substrate. We also showed that this pheromone is quickly inactivated under field conditions, possibly due to the effect of dew, and that it is readily extracted from the silk by water, which renders the silk threads inactive. Preliminary chemical analyses by mass spectrometry suggest that the pheromone is a low molecular weight, highly oxygenated polar compound, present in a high concentration only in older virgin females. Quick inactivation in the field of the pheromone would allow males to discriminate between recent and old cues, thus facilitating mate searching and sexual meeting, by preventing males from following old cues for wandering females that could be distant in space and/or time.

Key Words *Schizocosa malitiosa* · Wolf spider · Contact sex pheromone · Persistence · Polarity · Mass spectrometry analysis · Araneae · Lycosidae

L. Baruffaldi · F. G. Costa
Laboratorio de Etología, Ecología y Evolución.
Instituto de Investigaciones Biológicas Clemente Estable,
Av. Italia 3318,
Montevideo CP 11600, Uruguay

A. Rodríguez
Laboratorio de Espectrometría de Masa, Polo Tecnológico,
Facultad de Química, Universidad de la República,
Av.Aparicio Saravia s/n,
Pando CP 91000, Uruguay

A. González
Laboratorio de Ecología Química, Facultad de Química,
Universidad de la República,
Gral. Flores 2124,
Montevideo CP 11800, Uruguay

L. Baruffaldi (✉)
Sección Entomología, Facultad de Ciencias,
Universidad de la República,
Iguá 4225,
Montevideo CP 11400, Uruguay
e-mail: lubaru@iibce.edu.uy

Introduction

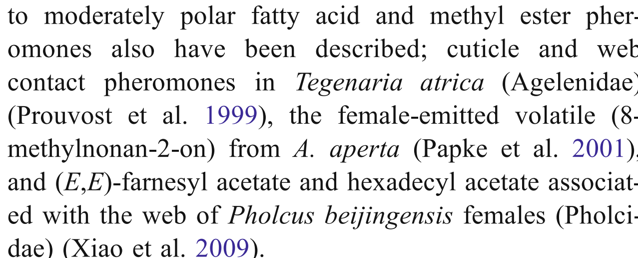
Sex pheromones are widespread chemical substances involved in sexual communication, sex recognition, and species-specific recognition (Wyatt 2003). In spiders, knowledge of their action is limited mainly to inferences from behavioral observation (Gaskett 2007). There is evidence for volatile pheromones in spiders (Olive 1982; Riechert and Singer 1995; Searcy et al. 1999; Gaskett et al. 2004; Aisenberg et al. 2010), and one such pheromone has been characterized chemically in *Agenelopsis aperta* (Agelenidae) (Papke et al. 2001). However, most studies involve sex pheromones associated with the web or draglines released by the females during locomotion (Tietjen and Rovner 1982; Schulz 2004; Huber 2005; Gaskett 2007). There also is indirect evidence for the occurrence of sex pheromones, mainly descriptions of courtship behaviors observed when males are placed on a substrate previously exposed to a conspecific female (Hegdekar and Dondale 1969; Tietjen

1977, 1979; Rypstra et al. 2003; Roberts and Uetz 2005; Baruffaldi and Costa 2010). Other properties of the female silk threads, however, such as the mechanical or physical characteristics of its surface, may provide males with information about the species identity and/or female mating status, thus eliciting sexual behavior (Anderson and Morse 2001; Schulz 2004; Annes and Morse 2006).

To establish the presence of a sex pheromone associated with silk threads, and exclude the possibility of tactile cues, one must: 1) show that males sexually respond to untreated silk threads in the absence of a female; 2) extract the chemical signal from the silk thread; 3) show that activity is lost from washed silk threads; and 4) recover male sexual response once the solvent from an extract is evaporated from an inactive substrate (Schulz 2004). Some researchers have obtained clear responses when they expose male spiders to female silk extracts (Roberts and Uetz 2004; Stoltz et al. 2007; Perampaladas et al. 2008), and often the loss of a male response when female silk threads are washed with solvent has been reported (Hegdekar and Dondale 1969; Dondale and Hegdekar 1973; Lizotte and Rovner 1989). Solvent treatment of the silk, however, may alter a variety of its physicochemical surface properties, which may in turn affect the response of males (Schulz 1999; Shao and Vollrath 1999).

Extraction of a chemical signal from its natural support, the silk thread, and the recovery of its attractive properties from the extract is not only a good indicator of the presence of a pheromone, but also may provide hints about the chemical characteristics of the semiochemical. The solvent, for example, provides information about the polarity of the pheromone (Schulz 2004). Similarly, the persistence of the biological activity of a pheromone once it has been extracted may be studied as a function of time, and this may correlate with structural features that affect the stability of the compound in a specific solvent. When the solvent is water, experimental data on the persistence of the pheromone may bear ecological and adaptive significance. Information about the persistence of chemical signals under natural conditions provides fundamental data for understanding how males respond to such cues over time in the field, and for deducing the sexual strategies involved in mate location for males and females.

Solvents used for extraction of spider pheromones from silk have varied from polar (water, methanol, ethanol) to medium or non-polar (ether, hexane). Most studies have reported polar solvents as more suitable (Gaskett 2007), and in fact the two characterized contact pheromones associated with female web threads from the linyphiid, *Linyphia tringularis* (Schulz and Toft 1993), and the ctenid, *Cupiennius salei* (Papke et al. 2000), are both polar compounds bearing free hydroxyl and carboxyl groups, and therefore readily soluble in methanol or water. Nonetheless, non-polar to moderately polar fatty acid and methyl ester pheromones also have been described; cuticle and web contact pheromones in *Tegenaria atrica* (Agelenidae) (Prouvost et al. 1999), the female-emitted volatile (8-methylnonan-2-on) from *A. aperta* (Papke et al. 2001), and (*E*,*E*)-farnesyl acetate and hexadecyl acetate associated with the web of *Pholcus beijingensis* females (Pholcidae) (Xiao et al. 2009).



Schizocosa malitiosa (Araneae: Lycosidae) is a wandering wolf spider that occurs at high densities on the Southern coasts of Uruguay. Females are frequent throughout the year, showing a biannual life cycle, whereas males mainly reach adulthood in autumn, then search for females (Costa 1991). Male density decreases during winter and remains low thereafter, suggesting mortality is high at the end of the winter. Mate attraction is accomplished by the presence of silk from a female, which elicits male searching behavior (slow locomotory movements contacting the substrate with the palpal cymbium), as well as courtship displays (Costa 1975; Costa and González 1986). Courtship in *S. malitiosa* includes visual (foreleg shaking), vibratory (palpal drumming), and tactile (touching and mounting attempts) signals (Costa 1975). Males can assess the reproductive status of females by putative contact semiochemicals on their silk (Baruffaldi and Costa 2010). Aisenberg and Costa (2005) showed that mated females of *S. malitiosa* are partially reluctant to remate. González and Costa (2008) found that this reluctance is acquired immediately after mating, and persists for at least 1 month under laboratory conditions. In experiments with silk from females of different ages, mating status, and egg-sac occurrence, males displayed the most intense courtship towards silk threads of older virgin females (20–40 days after last molt) (Baruffaldi and Costa 2010).

Here, we report evidence of the presence of a female sex pheromone in the silk threads of *Schizocosa malitiosa*, including a preliminary structural study based on mass spectrometry of female silk extracts. In addition, we report a study of pheromone persistence under laboratory and field conditions.

Methods and Materials

Spider Collection and Maintenance Seventy sub-adult females and 130 sub-adult males of *S. malitiosa* were collected in Marindia (34° 46′ 49.9″ S, 55° 49′ 34.1″ W), Canelones, Uruguay, from March to August of 2007 and 2008. They were individually housed in plastic Petri dishes (9.0×5 cm), each with a moistened cotton ball. Spiders were fed *ad libitum* with *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae). To accelerate their development, sub-adults were kept in a warmed room (24.2±1.0°C; mean±SD; range: 21–27°C) until they molted into adults. Molting was

checked daily, and maturity was determined by observing male and female genitalia under a dissecting microscope. Adults were maintained at room temperature (20.1±4.2°C, range: 11–26°C). Males were used for experiments 7 d after their molting to adulthood, and were randomly assigned to a treatment. Females were used 20 d after molting, as previous results indicated that the silk threads of older females elicited higher male sexual response (Baruffaldi and Costa 2010). Voucher specimens were deposited in the arachnological collection of Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.

Silk Collection Silk for the pheromone persistence and solvent-extraction experiments was obtained from 20 individual virgin females placed in cylindrical plastic containers (9 cm diam × 5 cm height). The substrate was a cardboard disc (9 cm diam) covered with gauze; four wooden toothpicks were affixed vertically onto the cardboard, forming a square. Two centimeters were left between the toothpicks and the container wall, so as to allow females to entangle their silk around the toothpick bases. After 48 h, the spiders were removed, and the gauze (with the silk) was lifted off the toothpicks.

Silk for chemical analysis and for testing the activity of pheromone extracts was obtained similarly, but cardboard and gauze were not used as substrate; instead, the toothpicks were replaced by glass capillary tubes (2 mm diam) affixed vertically to the base of the dish. The silk was collected by spinning a capillary tube so as to wind the silk threads at the tip. The capillary was broken immediately into a V-shaped vial, and the silk was immersed in 0.1 ml of distilled water for 3 h to extract pheromone.

Pheromone Persistence To investigate persistence of the chemical signal, gauze with silk from females was kept under laboratory and outdoor conditions, and the response of males (see below) to silk from both treatments was evaluated through time. Gauze left outdoors was pinned onto discs of expanded polystyrene, and covered with a screened cage (2 cm mesh). Silk left under laboratory conditions was placed on an open plastic Petri dish.

Solvent Extraction of Pheromone Silk from virgin females first was tested for activity with a male (palpal drumming and leg shaking, see below), then extracted with solvent, and finally re-tested with a different male after air drying the gauze. Gauze was extracted in a glass Petri dish with 3×10 ml of solvent, each for 10 min. Hexane, methanol, and distilled water were used as solvents to investigate pheromone solubility.

Activity of Extracts To show unambiguously that the response of males was triggered by a chemical signal, silk extracts were evaporated onto clean gauze, and the sexual response of males was evaluated (see below). Only water extracts were used, given that water-extracted gauze from the previous experiment was clearly inactive, indicating that water was the most efficient solvent for extraction. A piece of clean gauze was placed on a glass plate, which was marked at the bottom for pinpointing the location of the pheromone. The silk extract (0.1 ml) from an individual female was added to the gauze on top of the mark, and the water was left to evaporate.

Male Behavioral Responses The measured sexual responses of males to the silk and the extract from virgin females were palpal drumming and leg shaking. Palpal drumming is characterized by bouts of rapid oscillations of the palps while contacting the substrate with the tips. Leg shaking also entails bouts of rapid up-and-down oscillations of the two extended forelegs. Data recorded were: a) the occurrence (presence/absence) of palpal drumming and leg shaking during each trial, and b) the frequency of the male behavior when it occurred, i.e., the number bouts of drumming and the number of leg shakes performed during a 5-min period.

In the pheromone persistence and solvent extraction experiments, the gauze with silk was placed in the bottom of a plastic Petri dish (9 cm diam), and a cylindrical cage (10 cm diam, 8 cm height) was placed upside down around the dish to enclose one male spider. This cage had a 3-cm round hole near the base through which the male was introduced by using a cylindrical tube (2.8 cm diam, 10 cm length). The tests were performed under laboratory conditions during the daytime. The behavior of the male was recorded continuously for 5 min. For the persistence trials, if two consecutives males did not show any sexual response, the silk was considered unattractive.

For the solvent-extraction trials, in order to restrict movement of the male and to ensure that he would walk over the pheromone extract, two glass cylinders were placed concentrically on the gauze (10 and 8 cm diam, 16 and 8 cm height, respectively), so as to leave the pheromone extract between both cylinders. The male then was released within this circular corridor, and his behavior was recorded. The same procedure was carried out with 20 independent samples of extract, using 0.1 ml of distilled water as control. Half of the males were tested with the pheromone extract first and the control second (24 h in between), and the other half was tested in the reverse order.

Mass Spectrometry A MicroTof-Q mass spectrometer (Burker Daltonics, Billerica, MA, USA) was employed for accurate mass measurements of metabolites. The MS was operated in negative-ion electrospray mode, and was calibrated on the experimental day using Tunemix (Burker Daltonics, Billerica, MA, USA) for a low mass range. The

electrospray source conditions were as follow: endplate off-set voltage −500 V, capillary voltage −4500 V, nebulizer 0.4 bar, dry gas flow 4.0 L/min, dry gas temperature 180°C.

Full-scan TOF MS spectra were acquired for the measurement of accurate mass of ions.

Tandem mass spectrometry analysis was performed in an Esquire 6000 Ion Trap (Bruker Daltonics, Billerica, MA, USA) operated in a negative ion electrospray mode, with endplate off set voltage −500 V, capillary voltage −4000 V, nebulizer 10 psi, dry gas flow 4.0 L/min, and dry gas temperature 300°C. Aqueous silk extracts from adult males, mated females, and virgin females were diluted with methanol (1:1), and analyzed by direct injection.

Statistical Analyses The PAST package (Hammer et al. 2003) for statistical analyses was used. Non-parametric statistics were used throughout; specific tests employed are given in the results section.

Results

Pheromone Persistence The silk under outdoor conditions remained active for 3.2±1.3 d (mean±SD, range 1–5, N=20), while indoors the activity lasted for 18.7±5.4 d (range 8–28, N=20). The percent of males responding with either drumming or leg shaking decreased sharply for silk left outdoors, while more than 50 % of the males courted silk even after 15 d of indoor exposure (Fig. 1). Moreover, when comparing the occurrence of each male's sexual behavior on a daily basis, we found that both leg shaking and palpal drumming occurred in higher ratios toward indoor-treated silk after 3 and 4 days, respectively (Table 1). Similarly, the frequencies of bouts of palpal drumming and leg shaking were significantly lower as soon as 2 days after the silk was exposed outdoors (Table 1).

Solvent Extraction Before the solvent treatments, most males displayed both sexual behaviors, with no statistical differences among treatment groups (Cochan-Q test, P>0.9; Fig. 2). After silk extraction with methanol and water, we found a reduction in male sexual response (McNemar test, P<0.001 for both behavior/solvent combinations), whereas no difference was observed for either behavior after hexane extraction (McNemar test, P>0.20) (Fig. 2). When comparing male sexual responses after methanol and water extraction, the water-extracted silk elicited less leg shaking (χ^2=10.13, P<0.003), while palpal drumming was not statistically different between either solvent treatment (χ^2=3.13, P>0.05) (Fig. 2).

Hexane treatment of the silk did not affect the frequencies of palpal drumming nor leg shaking, while both polar solvents decreased the frequencies of the courtship displays (Fig. 3).

Activity of Extracts Fifteen males responded to the pheromone extracts while only one showed courtship behavior in the control trails (N=20 for each treatment). The majority of males that responded to the extract (8 out 15) displayed both courtship behaviors (Fig. 2). Both leg shaking and palpal drumming were more frequent in tests with aqueous extracts of silk from females than from the control (McNemar test, P<0.001)

Single males that showed leg shaking with the water control performed a single up-and-down movement. The response to the silk extract was more typical, although the number of bouts of palpal drumming and the frequency of leg shaking was significantly lower than that for the untreated silk of females (Mann-Whitney test, U=26.4, P<0.01 for palpal drumming; U=6, P<0.001 for leg shaking) (Fig. 3).

Mass Spectrometry While all samples were extremely dilute, and showed a variety of background ions in their mass spectra, we found differences in the mass spectra of

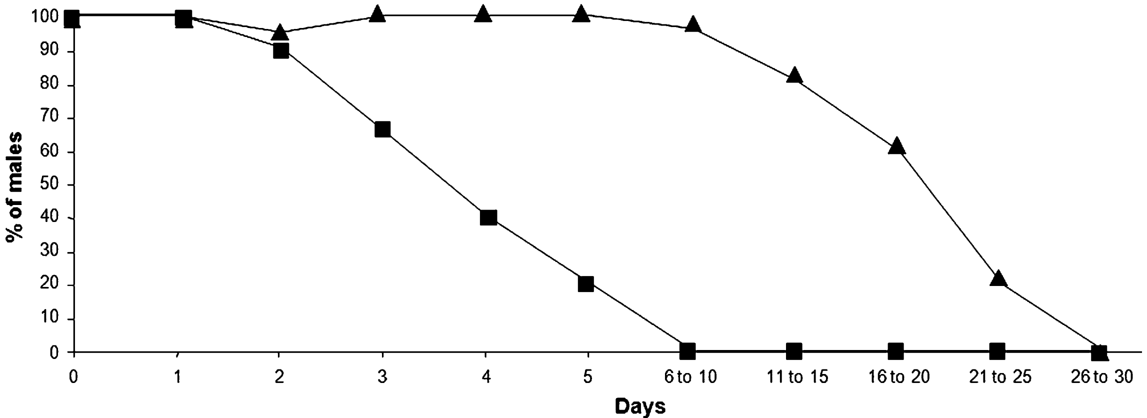


Fig. 1 Percent of *Schizocosa malitiosa* males displaying courtship behavior toward female silk kept under indoor (▲) or outdoor (■) conditions. The persistence of the chemical cue was significantly longer indoors (Mann-Whitney test; U=0, P<0.001)

Table 1 Male *Schizocosa malitiosa* sexual responses to silk exposed to outdoor/indoor conditions. Data are shown for the period during which both silk types were active (*N*=20 for each day/treatment combination)

Days	Palpal drumming				Leg shaking			
	Outdoor		Indoor		Outdoor		Indoor	
	% occurrence	bouts	% occurrence	bouts	% occurrence	shakes	% occurrence	shakes
0	95	6.7±2.7	100	7.9±4.0	100	20.8±7.9	100	18.8±4.3
1	80	4.8±3.4	80	4.9±3.3	100	13.7±6.1	100	14.4±6.4
2	50	2.0±1.7†	75	5.1±4.0†	90	6.9±4.3†	95	10.3±5.3†
3	45	1.7±1.0††	70	5.1±3.3††	65*	6.2±4.1††	100*	12.1±4.4††
4	10**	2.5±2.2‡	75**	4.1±2.8‡	40**	4.0±4.4††	100**	11.2±6.5††
5	20**	2.7±2.1‡	80**	4.1±2.2‡	20**	5.2±4.1‡	100**	9.8±6.2‡

* $P<0.01$ or ** $P<0.001$ for the occurrence of the male response (χ^2 test between indoor/outdoor treated silk).

† $P<0.05$ or †† $P<0.01$ for the number of bouts or shakes in the male response (Mann-Withney test between indoor/outdoor treated silk)

‡ Not statistically tested because less than 5 males responded to the outdoor-treated silk.

silk extracts of males and mated females (Fig. 4 a, b) from those of virgin females (Fig. 4 c, d). In the latter, two ions at *m/z* 205 and 111 were present in all (12) samples analyzed from different individuals in older virgin females, and in newly-molted females (Fig. 4 c, d, respectively). These ions were absent from mass spectra of silk extract from males, and present only in low amounts in mass spectra of silk extracts from mated females. HR-MS of these ions gave the exact masses of 205.0354 and 111.0084, which suggest the formulae $C_7H_9O_7$ and $C_5H_3O_3$, respectively (calculated: 205.0348 and 111.0082). Further, MS-MS analyses showed the *m/z* 111 ion to be a fragment of the *m/z* 205 ion, and the same analysis showed that the *m/z* 205 ion fragmented to give *m/z* 173 and 143 ions. While no HR-MS was obtained from *m/z* 173, due to low intensity, *m/z* 143 appears to have a molecular formula of $C_6H_7O_4$ (calculated: 143.0344; found: 143.0350). Insufficient data are available, at this point, to postulate a possible chemical structure for biological evaluation.

Discussion

In species with wandering females who are laying down silk impregnated with pheromone (Rovner 1968; Tietjen 1977;

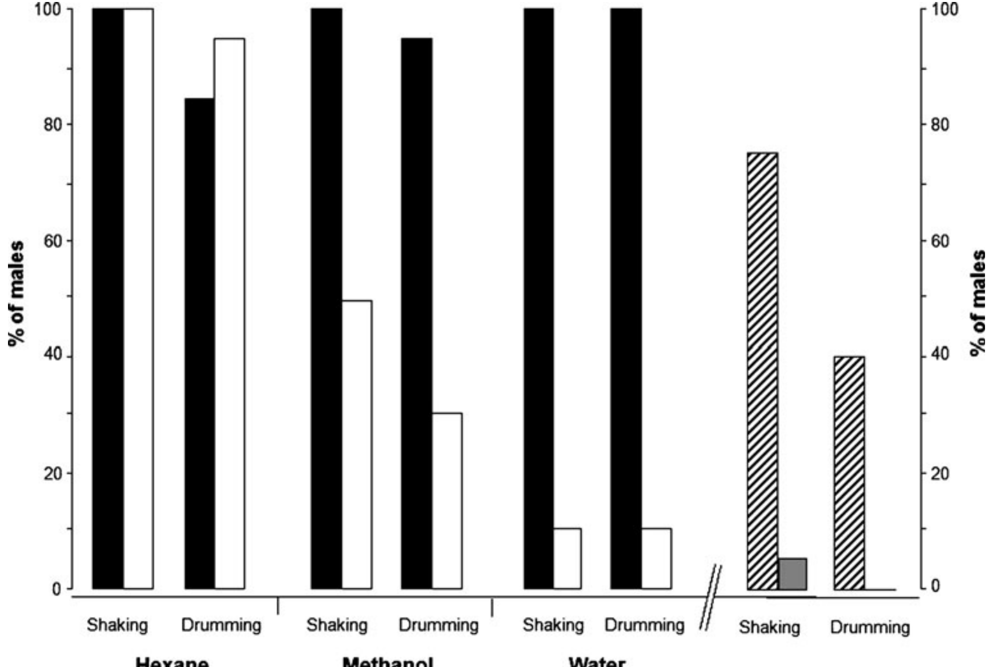


Fig. 2 Left: percent of *Schizocosa malitiosa* males responding to female silk before (*solid bars*) and after (*white bars*) solvent extraction. Right: percent of males responding to gauze treated with female silk water extract (*stripped bars*) or water control (gray bars)

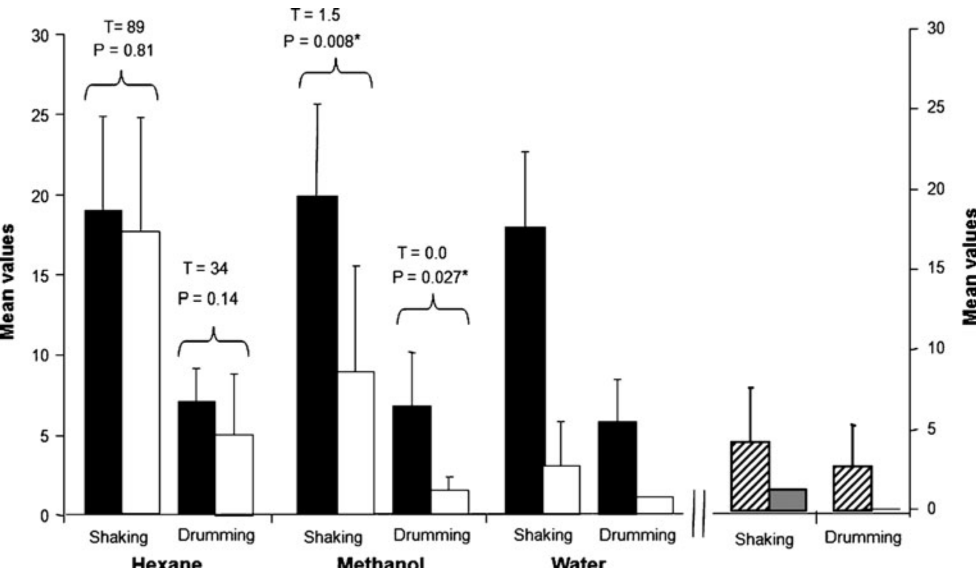


Fig. 3 Left: frequencies of leg shakes and palpal drumming bouts by *Schizocosa malitiosa* males before (*solid bars*) and after (*white bars*) solvent treatment of the silk (differences after extraction were tested with the Wilcoxon test; the effect of water extraction was not tested due to the low number of males that responded). Right: frequencies of leg shakes and palpal drumming bouts to gauze treated with female silk water extract (*stripped bars*) or water control (*gray bars*)

Tietjen and Rovner 1980; Lizotte and Rovner 1989), both conspecific males and the signaling female would benefit by quick degradation of the pheromone. The indication from active chemical signals that a female is nearby may facilitate the sexual meeting process and generate a higher mate encounter rate (Hegdekar and Dondale 1969). If the chemical cue in the silk lasts very long, males would increase their own predatory risks by spending energy and time unsuccessfully courting and following old silk remains of females that are distant in space and/or time (Andersson and Iwasa 1996).

In the silk left outdoors, the loss of attraction was due possibly to combined action of environmental factors such as sunlight, wind, and dew. Indeed, we found that both rain (Baruffaldi, unpublished data) and water extraction of the silk efficiently eliminated its attractiveness, which is consistent with the likely role of dew in our experimental results. The slower, although noticeable, decrease of attractiveness of the silk thread left indoors, suggests that the pheromone is chemically unstable or slowly evaporating, independently of such environmental factors. This observation is in agreement with previous results from Lizotte and Rovner (1989), who reported a decrease in the stimulatory efficacy produced by 1-day-old silk threads of the wolf spiders *Lycosa longitarsis* and *Lycosa tristani*.

As far as we know, no previous study has experimentally tested spider pheromone persistence under field conditions. An understanding of the reproductive strategies of male and female wolf spiders, specifically related to their wandering behavior, may benefit from these studies. Dondale and Hegdekar (1973) observed that silk from females of the wolf spider, *Pardosa lapidicina*, quickly lost the ability to attract males after it was washed with water or sprayed with water mist. These authors suggested that rain and/or dew could affect the persistence of chemical cues associated with the silk threads. Our findings support this notion, and specifically provide evidence for the important function of dew in modulating the activity of pheromone from *S. malitiosa* females. Rain is sporadic in Uruguay, but dew is common in late autumn and early winter, coinciding with the sexual period of this species (Costa 1991).

The treatment of silk threads with different solvents indicates that the active female-specific compound is polar, and, given the pheromone solubility in water and methanol, one may conclude that it is a highly polar molecule. All described female spider sex pheromones associated with silk are derivatives of short chain carboxylic acids, with free hydroxyl and carboxylic groups (Schulz 2004). Water-washed silk of females frequently released palpal investigation by males after leg contact, but elicited no sexual displays, suggesting that silk threads represent tactile unspecific stimuli that orient male perception, while the final cue is sensed after chemical examination with the palps. Male courtship responses when water extracts of silk from females were applied to clean gauze, indicates the presence of a water-soluble pheromone that triggers male sexual behavior.

The polarity of the sex pheromone in spiders may be related to characteristics of their habitat. Spiders that inhabit dry environments can develop water-labile compounds, whereas those associated with wet environments, such as the pisaurid, *Dolomedes triton*, and some lycosids, use water-resistant polar compounds (Roland and Rovner 1983; Lizotte and Rovner 1989). In this vein, *S. malitiosa* inhabits various terrestrial dry environments, being frequent in those modified by human action (Costa 1991), and appears to produce a water-labile polar sex pheromone.

Several related studies have reported male searching behavior and/or duration of male movement as an indication of the presence/absence of female cues, in particular when

Fig. 4 HR-MS spectra of aqueous silk extract of a *Schizocosa malitiosa* male (**a**), mated (**b**) and virgin females (old: **c**; newly molted: **d**). The two ions highlighted in **c** and **d** were observed consistently in silk extracts from 12 individual virgin females

the males were exposed to low intense stimuli such as extracts (Roberts and Uetz 2004; Stoltz et al. 2007; Perampaladas et al. 2008). In this study we did not include such data, since it is an ambiguous parameter in *S. malitiosa*, observed not only in the presence of female cues, but also when males are exposed to silk threads of adult males or juvenile individuals (Baruffaldi and Costa 2010). Instead, we obtained unambiguous differences by registering typical sexual behaviors such as leg shaking or palpal drumming.

The present mass spectral data provide a basis for speculation as to the pheromone structure. *M/z* 205 may be the ion M–H, which would result in a molecular formula of $C_7H_{10}O_7$ for the pheromone; this would be a highly oxygenated compound with three double bond equivalents, and possibly bearing at least one free carboxylic group. However, the loss of 32 mass units from *m/z* 205 in the MS-MS study suggests that it may be a methanol adduct of *m/z* 173, and this may also be the case with *m/z* 143 and 111, whose molecular formulae differ by CH_4O. The fragmentation of either parent ion to give the major *m/z* 111 suggests a labile compound, which is consistent with our results in the persistence experiments.

The ion at m/z 111 was the most abundant in all old virgin females, which are most attractive for males. In contrast, silk threads from newly-molted virgin and mated females, which trigger only low rates of palpal drumming and leg shaking (Baruffaldi and Costa 2010), showed low intensity of the m/z 111 in their water extracts, suggesting that this ion is, indeed, related to the chemical communication system. Interestingly, the low, but still detectable, occurrence of the putative pheromone in the silk of newly-molted virgin and mated females agrees with the hypothesis proposed by Baruffaldi and Costa (2010) that the release of chemical cues and sexual receptivity are not synchronous in these females, suggesting slow chemical and quick behavioral changes.

The final chemical characterization of *S. malitiosa* sex pheromone will be challenging due to the small amount in which it is found on the silk, and its apparent instability in water. A large sample will be needed for ^{1}H-NMR studies which, along with the mass spectral data, should provide the additional information required for absolute identification.

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Pheromone-based Mating and Aggregation in the Sorghum Chafer, *Pachnoda interrupta*

Jonas M. Bengtsson · Satya Prabhakar Chinta · Yitbarek Wolde-Hawariat · Merid Negash · Emiru Seyoum · Bill S. Hansson · Fredrik Schlyter · Stefan Schulz · Ylva Hillbur

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Abstract Adults of the sorghum chafer, *Pachnoda interrupta* Olivier (Coleoptera: Scarabaeidae: Cetoniinae), form aggregations during the mating period in July, but also in October. The beetles aggregate on food sources, e.g., *Acacia* spp. trees or sorghum with ripe seeds, to feed and mate. During the mating season, field trapping experiments with live beetles as bait demonstrated attraction of males to unmated females, but not to mated females or males, indicating the presence of a female-emitted sex pheromone. Unmated females combined with banana (food source) attracted significantly more males and females than did unmated females alone. Other combinations of beetles with banana were not more attractive than banana alone. Thus, aggregation behavior appears to be guided by a combination of pheromone and host volatiles. Females and males were extracted with hexane during the mating period, and the extracts were compared by using GC-MS. In a field trapping experiment, 19 compounds found only in females were tested, both singly and in a mixture. Traps baited with one of the female-associated compounds, phenylacetaldehyde, caught significantly more beetles than any other treatment. However, the sex ratio of beetles caught in these traps did not differ from that of control traps, and it is possible that other components may be involved in the sex pheromone signal. Furthermore, traps baited with a mixture of all 19 compounds attracted significantly fewer beetles than did phenylacetaldehyde alone.

Key Words Aggregation pheromone · Coleoptera · Field trapping · Gas chromatography-Mass spectrometry (GC-MS) · Olfaction · *Pachnoda interrupta* · Phenylacetaldehyde · Scarabaeidae · Semiochemicals · Sex pheromone · Sorghum · *Sorghum bicolor*

J. M. Bengtsson (✉) · Y. Wolde-Hawariat · B. S. Hansson · F. Schlyter · Y. Hillbur
Department of Plant Protection Biology,
Swedish University of Agricultural Sciences,
PO Box 102, 230 53 Alnarp, Sweden
e-mail: Jonas.Bengtsson@iasma.it

S. Prabhakar Chinta · S. Schulz
Institute of Organic Chemistry,
Technische Universität Braunschweig,
Hagenring 30,
Braunschweig, Germany

Y. Wolde-Hawariat · M. Negash · E. Seyoum
Department of Biology, Addis Ababa University,
PO Box 1176, Addis Ababa, Ethiopia

Y. Wolde-Hawariat
Department of Plant Sciences, Wollo University,
PO Box 1145, Dessie, Ethiopia

M. Negash · B. S. Hansson
Department of Evolutionary Neuroethology,
Max Planck Institute for Chemical Ecology,
Hans-Knoell-Strasse 8,
Jena, Germany

Present Address:
J. M. Bengtsson
IASMA Research and Innovation Center, Fondazione E. Mach,
Via E. Mach 1,
38010 San Michele all'Adige, Italy

Introduction

The sorghum chafer, *Pachnoda interrupta* Olivier (Coleoptera: Scarabaeidae: Cetoniinae), is present in semi-arid parts of Africa subject to strong seasonal variation in rainfall and temperature, mainly in the Sahel and Sudan ecoregions (Schmutterer 1969; Grunshaw 1992; Jago 1995; Sastawa and Lale 2000), but also parts of Ethiopia (Hiwot 2000). As

a reflection of the climatic conditions, the growth and abundance of plants is highly seasonal in these regions (White 1983), especially with regard to fruits and flowers, on which the polyphagous adults of *P. interrupta* feed (Clark and Crowe 1978; Grunshaw 1992; Hiwot 2000). During the dry period, which lasts approximately from November to May, adult *P. interrupta* estivate in the soil under trees and bushes (Wolde-Hawariat et al. 2007). After the short rains in June, adult beetles emerge to feed and mate, often forming dense aggregations on food sources, e.g., flowering *Acacia* spp. The mating season ends with the onset of the rainy season, when the females oviposit in the topsoil. The larvae develop in the soil, feeding on plant litter, and pupate towards the end of the rains, in August (Seneshaw and Mulugeta 2002). New adults emerge in September–October and feed intensively before going into estivation.

During the September–October feeding period, the sorghum chafer can be a devastating pest on sorghum, *Sorghum bicolor* (L.) Moench (Poaceae), causing mean yield loss of up to 70% in severely affected areas of Ethiopia (Yitbarek and Hiwot 2000). Efficient control methods are not available, but farmers trap adults with simple containers baited with fruit (Ministry of Agriculture and Ethiopian Agricultural Research Organization 1999). Traps baited with a combination of food (e.g., fruit) and pheromones have proved to be efficient for control of several beetle species that are pests on commercial crops (Alpizar et al. 2002; Oehlschlager et al. 2002). Field observations of mating and aggregation in adult *P. interrupta* led us to suspect that a pheromone could be involved in these behaviors. We performed field experiments and chemical analyses to investigate whether this was the case. The identification of pheromone compound(s) used in mating or aggregation could enable the development of highly attractive lures that could be employed in mass trapping of this pest.

Methods and Materials

General Methods in Field Experiments In all field experiments, Japanese beetle traps (Trécé, Palo Alto, CA, USA) were suspended from wooden poles ca. 3 m above ground. Except where otherwise noted, a randomized complete block design (N=10) was used, with 10 m between traps and at least 50 m between blocks. Traps were emptied and lures replaced each morning.

Field Activity Patterns of Males and Females Field experiments that investigated the activity patterns of male and female adults during the mating season were carried out in unused farmland with sparse vegetation near the village of Embuay Bad in Ethiopia (09°48′N, 40°00′E), 265 km northeast of Addis Ababa, 1206 m above sea level, 28 June–13 July 2008. Mean daily trap catch was used as an indicator for field activity. For these experiments, traps were baited with half a banana that was mashed and put in the collection chamber of the trap, where captured beetles could access it. This bait has been shown previously to be highly attractive to *P. interrupta* (Ministry of Agriculture and Ethiopian Agricultural Research Organization 1999; Hiwot 2000; Wolde-Hawariat et al. 2007). Traps were placed throughout the site, with a minimum distance of 50 m between them. For logistical reasons, we were not able to deploy 10 traps as originally planned throughout the active period (N=2 for 28 June and 3 July, N=4 for 29 June–2 July, N=10 for 4–9 July, and N=5 for 11–13 July). Data were not collected on 10 July.

Pheromone Communication in the Field Field experiments that investigated pheromone communication and host interactions were carried out at Rasa (09°55′N, 40°05′E), located 255 km northeast of Addis Ababa, Ethiopia. Experiments were performed 11–16 July and 7–13 October, 2005, in a grazing area characterized by scattered *Acacia* spp. trees. Live beetles and/or three 1 cm thick banana slices were placed in fine mesh metal net cages that were fitted into the top of the vanes of the traps. For treatments where beetles of one sex were used as part of the bait, 5 adults were placed in the mesh cage, whereas for treatments with both males and females as bait, 5 beetles of each sex were used. For experiments conducted during the mating period (July), beetles that were used as bait were excavated from estivation sites in Rasa during May, before mating had commenced. The beetles used in October were collected in the field during July. Beetles were separated according to sex and kept at the Department of Biology at Addis Ababa University, Ethiopia, under natural light and temperatures ranging between 20 and 25°C, and fed banana *ad libitum* until used in experiments. Unbaited traps were included as a negative control.

Identification of Compounds For investigation of female-specific compounds during the mating season, male and female beetles were excavated from estivation sites in the field, near Rasa, during May 2007, and were kept as described above. As mating takes place after the beetles emerge in June–July, in the early part of the rainy period, the beetles excavated were thus assumed to be unmated. As no distinct calling behavior was observed, beetles were selected for extraction by putting a male and female together in a box between 10 am and 2 pm, when beetles have been observed previously to mate (unpublished). When the males started clasping the females for mating, they were separated and put in 50 ml Erlenmeyer flasks.

Once 10 beetles had been added to a flask, redistilled hexane (HPLC grade, Tamro MedLab AB, Gothenburg, Sweden) was added, with just enough hexane to cover the beetles (approximately 5 ml). After 5 min with light agitation, the solvent was removed and stored for gas chromatography-mass spectrometry (GC-MS) analysis. These extracts were called "first extracts." Next, a similar amount of solvent was added to the beetles and left to stand overnight. The solvent was removed and labeled "second extract." Whole-body extractions as well as extractions of body parts [abdominal tip (outermost 3 mm), abdomen without the tip, and all body parts except abdomen] were performed.

GC-MS was used to compare extracts from males and females, and extracts from the different body parts. Analyses were carried out on an HP 6890 Series GC System connected to an HP 5973 Mass Selective Detector (Hewlett-Packard Company, Wilmington, DE, USA) fitted with a BPX5 fused-silica capillary column (25 m× 0.22 mm i.d., 0.25 µm film thickness, SGE Inc., Melbourne, Australia). A volume of 1 µl was injected in splitless mode (60 sec valve time), and inlet pressure was 77.1 kPa. The GC was programmed as follows: 5 min at 50°C, then with 5°C/min to 320°C, with helium as a carrier gas at a flow rate of 1 ml/min. The transfer line was set to 300°C. Identification of compounds was performed by comparison of mass spectra and gas chromatographic retention indices with those of synthetic reference compounds. Compounds were regarded as female specific when they occurred repeatedly in females and were absent or present in much lower doses in males. A semi-quantitative analysis of extract components was conducted by adding pure nonanal as an internal standard. No response factors were determined.

Field Testing of Female-Specific Compounds Compounds found by GC-MS to be specific to females during the mating season were tested in the field. Synthetic compounds (for purity and CAS number, see Table 1) were purchased (Sigma-

Table 1 Treatments for field tests of flight response of *Pachnoda interrupta* to female-produced compounds, Embuay Bad, Ethiopia, July 2008

No[a]	Treatment	Dispenser	Dose	Purity	CAS
1	Propyl acetate	capped vial, distal application	50 mg	>99.7%	109-60-4
2	1-Methylpropyl acetate	capped vial, distal application	50 mg	>99%	105-46-4
4	3-Methylbutyric acid	cotton roll in vial	50 mg	>98%	503-74-2
5	2-Methylbutyric acid	cotton roll in vial	50 mg	>98%	600-07-7
6	Hexanoic acid	cotton roll	100 mg	>99.5%	142-62-1
7	(+)-α-Pinene	capped vial, distal application	50 mg	98%	80-56-8
7	(−)-α-Pinene	capped vial, distal application	50 mg	99%	80-56-8
8	(1*S*)-(−)-β-Pinene	capped vial, distal application	50 mg	99%	18172-67-3
9	*p*-Cymene	capped vial, centered application	100 mg	99%	99-87-6
10	(*S*)-(−)-Limonene	capped vial, centered application	100 mg	95%	5989-54-8
10	(*R*)-(+)-Limonene	capped vial, centered application	100 mg	98%	5989-27-5
11	Phenylacetaldehyde	cotton roll	100 mg	95%	122-78-1
12	Benzoic acid[b]	cotton roll	65 mg	>99.5%	65-85-0
13	Octanoic acid	cotton roll	200 mg	>98%	124-07-2
14	(*E*)-2-Decenal	cotton roll	100 mg	>95%	3913-81-3
15	Indole[b]	cotton roll	65 mg	>99%	120-72-9
16	(*E*)-2-Undecenal	cotton roll	100 mg	>90%	53448-07-0
17	Geranylacetone	cotton roll	100 mg	>97%	689-67-8
18	(2*E*,4*E*)-2,4-Dodecadienal	cotton roll	50 mg	>90%	21662-16-8
19	4-Dodecanolide	cotton roll	200 mg	>99.5%	2305-05-7
–	Mixture[c]	plastic container / as above	as above	as above	as above
–	Unmated females	mesh cage	5 beetles	n / a	n / a
–	Males	mesh cage	5 beetles	n / a	n / a
–	Control - tetrahydrofuran	cotton roll	200 mg	>99%	109-99-9
–	Control - empty	none	none	n / a	n / a

[a] The numbers refer to Fig. 5

[b] 200 mg of 33% solution in tetrahydrofuran.

[c] All single compounds (including optical isomers) present in individual dispensers put together in a plastic container

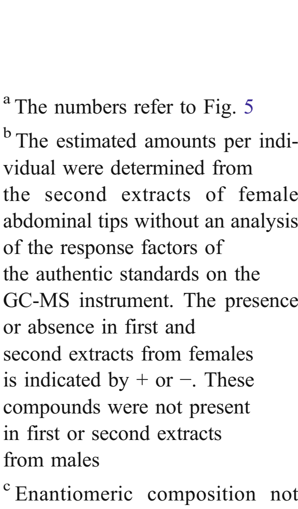
Aldrich Sweden AB, Stockholm, Sweden). Emission rates for dispensers were tested in a cylindrical wind tunnel (1 m long, 60 cm diam, 0.25 m/sec air flow, 25°C) in Alnarp, Sweden. Dispensers were replaced in the field each morning, just before the active period of adult *P. interrupta*, which are day-active (Grunshaw 1992), and release rates were calculated for the first 8 hr. Dispensers were weighed after loading, and then weighed again after 1, 2, 4, 6, and 8 hr in the wind tunnel. For comparison, we determined the release rates in this wind tunnel for plant compound lures that had been shown previously to be attractive in the field (Wolde-Hawariat et al. 2007). These were found to have a release rate of 0.5–1 mg/h during the 8 hr measurement period. Based on this analysis, we chose combinations of dose and dispenser for the female compounds that ensured a similar emission rate for each compound.

The least volatile compounds were put on cotton rolls (no. 2 dental cotton roll, Demedis GmbH, Langen, Germany) without any further modification ("cotton roll," Table 1). For more volatile compounds, cotton rolls were pushed into 4 ml glass vials (45×14.7 mm, clear, Skandinaviska GeneTec AB, Västra Frölunda, Sweden) until the cotton was level with the rim of the opening of the vial ("cotton roll in vial," Table 1). Cotton rolls were placed in vials before test compounds were applied. For the most volatile compounds, a cotton roll was put inside a vial closed with a cap (black, closed top, 13 mm, Skandinaviska GeneTec AB). A hole (2 mm diam) was made in the cap, and the cotton roll was placed so that it was in direct contact with the cap when it was screwed tightly to the vial. Two variants were made of this dispenser: 1) one for the most volatile compounds, where the chemical was applied towards the edge of the vial ("capped vial, distal application," Tables 1 and 2) one for compounds that were less volatile, where the chemical was applied just underneath the hole in the cap ("capped vial, centered application," Table 1). All compounds were applied neat, apart from indole and benzoic acid (solid at room temperature), which were dissolved in tetrahydrofuran (99+% by GC, Sigma-Aldrich Sweden AB) at a concentration of 33%.

Each compound was tested singly and in a mixture. In the mixture, all compounds were applied to individual dispensers, placed together in a round plastic container (diam 68 mm, height 30 mm, Semadeni, Ostermundingen, Switzerland) and secured with metal wire. For comparison, treatments also were included with live unmated male and female *P. interrupta* in wire mesh cages as bait. Beetles that were used as bait were excavated from estivation sites near Rasa in May 2008, separated by sex, and kept at the Department of Biology of Addis Ababa University as described above. Experiments were performed 10–12 July, 2008, at Embuay Bad (see above). The experimental design followed the general procedure outlined above, with two exceptions: $N=5$ was used and treatments were moved one position within blocks daily before replacing the dispensers, to decrease the influence of position effects. The tests were

Table 2 Volatile female-specific compounds identified from *Pachnoda interrupta*

No[a]	Compound	Estimated amount[b] (ng)	Extraction 1st	Extraction 2nd	Present in body part
1	Propyl acetate	1.3	–	+	Tip of abdomen only
2	1-Methylpropyl acetate	0.19	–	+	Tip of abdomen only
3	2-Methylpropyl acetate	0.42	–	+	Tip of abdomen only
4	3-Methylbutyric acid	0.18	–	+	Tip of abdomen only
5	2-Methylbutyric acid	0.18	–	+	Tip of abdomen only
6	Hexanoic acid	0.07	–	+	Tip of abdomen only
7	α-Pinene[c]	0.04	+	+	All body parts
8	β-Pinene[c]	0.07	+	+	All body parts
9	*p*-Cymene	0.05	+	+	All body parts
10	Limonene[c]	0.05	+	+	All body parts
11	Phenylacetaldehyde	0.17	–	+	Tip of abdomen only
12	Benzoic acid	0.11	–	+	Tip of abdomen only
13	Octanoic acid	0.10	–	+	Tip of abdomen only
14	(*E*)-2-Decenal	0.36	–	+	Tip of abdomen only
15	Indole	0.84	–	+	Tip of abdomen only
16	(*E*)-2-Undecenal	0.15	–	+	Tip of abdomen only
17	Geranylacetone	0.005	+	+	All body parts
18	(2*E*,4*E*)-2,4-Dodecadienal	0.15	–	+	Tip of abdomen only
19	4-Dodecanolide	0.02	–	+	Tip of abdomen only



[a] The numbers refer to Fig. 5

[b] The estimated amounts per individual were determined from the second extracts of female abdominal tips without an analysis of the response factors of the authentic standards on the GC-MS instrument. The presence or absence in first and second extracts from females is indicated by + or −. These compounds were not present in first or second extracts from males

[c] Enantiomeric composition not determined

conducted when the male bias in sex ratio of beetles caught by positive control traps got less pronounced, during the last 3 d of the active season in July 2008 (see also "Results", field activity patterns of male and female beetles).

Statistical Analyses For the statistical evaluation of data from the field experiments, cumulative trap catch over the field testing period was used. Male and female responses were treated separately. Treatments with a mean trap catch of females or males below 1 were excluded (no treatment had a mean trap catch below 1 for one sex, while having a mean trap catch above 1 for the other sex). For the remaining treatments, trap catch was $\log_{10}$-transformed before analysis with a General Linear Model (GLM), with treatment (type of lure) as a fixed effect, and block as a random effect (Minitab 14 for Windows). If there were significant effects in the GLM procedure, we compared treatment means by using a *Tukey's post hoc* test. Within each experiment, sex ratios of treatments (excluding those with a mean trap catch below 1) were compared to those of a positive control by using 95% binomial confidence intervals (Newcombe 1998), with the Bonferroni correction for multiple comparisons. The significance level used in all tests was $\alpha = 0.05$. For all experiments, data are presented in graphs as back-transformed means, with error bars denoting the standard error of the mean.

Results

Field Activity Patterns of Males and Females Male *P. interrupta* showed higher flight activity for most of the period during the mating season (Fig. 1); female flight was delayed. During the first 8 d of the trapping period, the mean trap catch was moderate (the highest mean daily catch was 29 beetles per trap), and there was a strong male bias in catch (>90%). From July 7, beetle activity increased, but the proportion of females remained low. Female activity drastically increased on 12 July, making the sex ratio approximately even. With the onset of heavy rains (13 July 2008), the beetles ceased flying and were not observed mating or feeding on hosts.

Pheromone Communication in the Field During the mating period in July, traps baited with unmated females caught a significantly higher proportion of males than the positive control traps (baited with mashed banana in the collection chamber; Figs. 2 and 3). The sex ratio caught by other treatments did not differ significantly from the positive control. Few beetles (1 or less of each sex) were attracted to

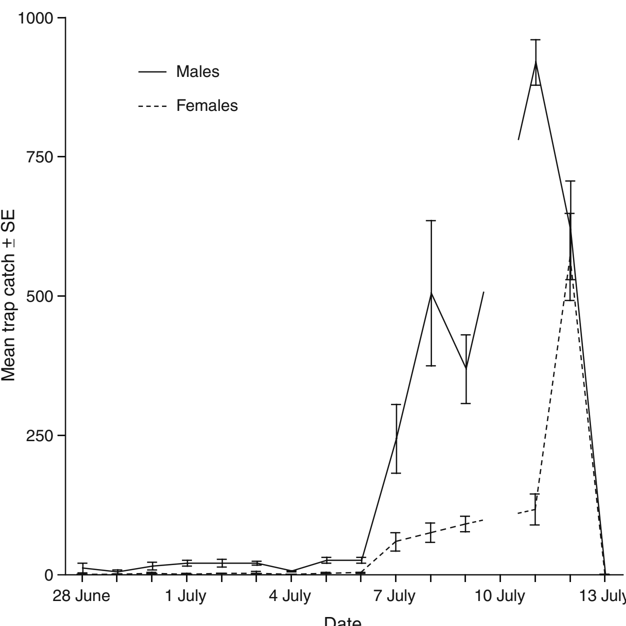


Fig. 1 Flight responses (trap catches) of male (solid line) and female (dashed line) *Pachnoda interrupta* to mashed banana, 28 June to 13 July 2008, Embuay Bad, Ethiopia. Error bars show standard error of the mean, and sample sizes were N=2 (28 June, 3 July), N=4 (29 June–2 July), N=10 (4–9 July), and N=5 (11–13 July). The break in the line denotes that no trap catch data were collected on 10 July

traps baited with males, males together with females, or unbaited traps (negative control). Unmated females combined with food (3 1 cm-thick slices of banana) caught significantly more males than any other treatment, but also attracted females to the extent that the sex ratio did not differ significantly from that of the positive control traps. Traps baited with males and food or both sexes and food did not catch significantly more than traps baited with banana slices alone. In October, the newly emerged adults were not attracted to traps baited with beetles alone, neither to males, females, nor both sexes together (Fig. 4). Traps baited with combinations of beetles and food, however, were significantly more attractive than traps baited with food alone. The highest catch was obtained in traps baited with both sexes and food, followed by traps baited with males and food.

Identification of Female-specific Compounds Extracts obtained during the mating season from male and female beetles were dominated by hydrocarbons and fatty acids, typical constituents of the insect cuticle and fat body. Small amounts of more volatile compounds also were identified (Fig. 5). The monoterpenes α-pinene, β-pinene, *p*-cymene, and limonene as well as geranylacetone were present in female first extracts from all parts of the body, but were absent in male extracts. The female second extracts also contained these compounds, and 14 additional female-

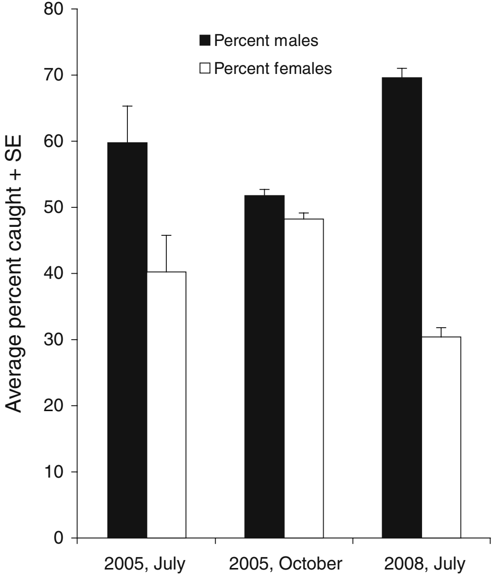


Fig. 2 Percentage male and female *Pachnoda interrupta* in positive control traps baited with mashed banana July 2005, October 2005, and July 2008 showing the differing sex ratios

specific compounds, which were identified in extracts from the abdominal tip (Table 2). The identified compounds were short chain carboxylic acids, esters, aromatic compounds, and aldehydes. Indole, propyl acetate, and 2-methylpropyl acetate were found in the largest estimated quantities.

Field Testing of Female Compounds The female-specific compounds were tested in the field. Two enantiomers of α-pinene, one enantiomer of β-pinene, and two enantiomers of limonene were included (Table 1), since the enantiomeric composition of these compounds in the natural product was unclear. The peak corresponding to 2-methylpropyl acetate was not clearly identified at the time of field testing, and this compound was not included. Traps baited with phenylacetaldehyde caught dramatically more beetles than all other treatments (Fig. 6), catching a mean of 1191 beetles per trap, compared to a mean of 64 beetles per trap for hexanoic acid, the second most attractive compound. The mixture of all compounds caught significantly less than did phenylacetaldehyde alone, whereas no treatment caught significantly less than unbaited traps or traps baited with solvent alone (negative controls). Sex ratios were male-biased for all treatments and did not differ significantly among treatments and the positive control (traps baited with mashed banana).

Discussion

Field trapping with live beetles as bait showed that during the mating period, unmated female *P. interrupta* were particularly attractive to males (Fig. 3), indicating that a female-emitted sex pheromone is present. However, females also were attracted, albeit to a far lower degree than males. With scarabs, the attraction of both sexes to a female-produced pheromone also has been observed in the Japanese beetle, *Popillia japonica* Newman (Potter and Held 2002), and opportunistic attraction of same-sex individuals to sex pheromones has been implied as a mechanism in the formation of aggregations in bark beetles (Schlyter and Birgersson 1999). The reason for the lack of male-biased attraction to unmated females in a later field study (Fig. 6) is unknown, but, as the mating period is brief, a possible explanation could be that the beetles had either passed or not yet reached mating maturity.

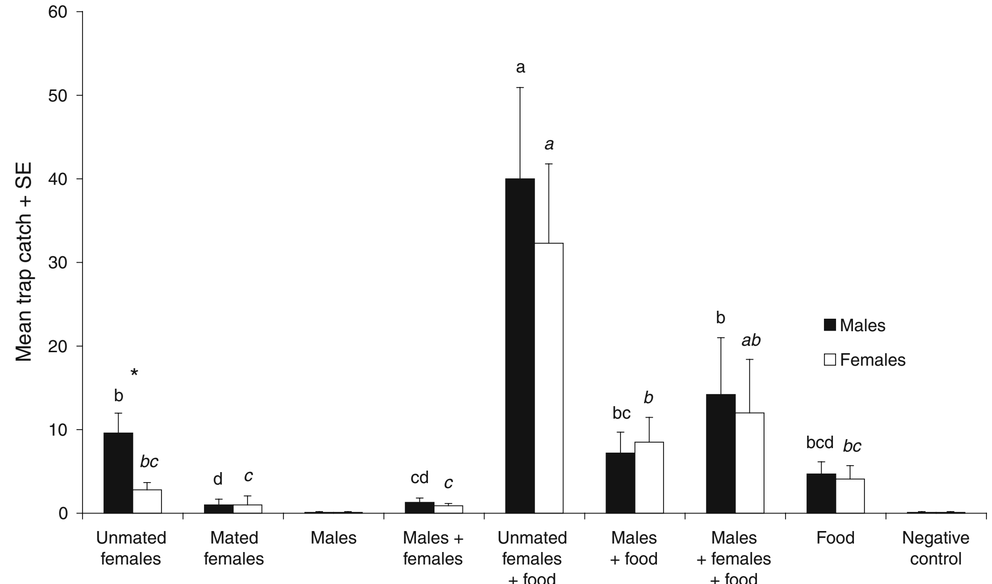


Fig. 3 Flight responses (trap catches) of both sexes of *Pachnoda interrupta* to various combinations of beetles and food (banana), 11–16 July 2005, Rasa, Ethiopia. Subgroups (males-normal font; females-italicized font) denoted by different letters are significantly different (α=0.05, Tukey's b on transformed data). Error bars show standard error of the mean and sample size was N=10. Treatments marked by asterisks differed significantly from the positive control (Fig. 2) in sex ratio. Data from treatments with a mean trap catch below 1 were not included in the statistical analysis

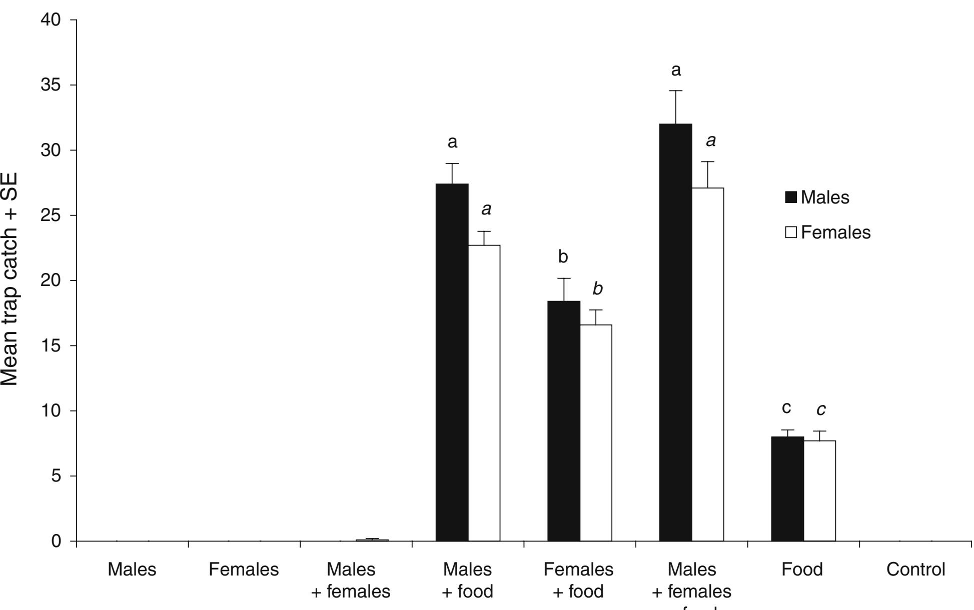


Fig. 4 Flight responses (trap catches) of both sexes of *Pachnoda interrupta* to various combinations of beetles and food (banana), 7–13 October 2005, Rasa, Ethiopia. Subgroups (males-normal font; females-italicized font) denoted by different letters are significantly different (α=0.05, Tukey's b on transformed data). Error bars show standard error of the mean and sample size was N=10. No treatments differed significantly from the positive control (Fig. 2) in sex ratio. Data from treatments with a mean trap catch below 1 were not included in the statistical analysis

In June–July, *P. interrupta* form large aggregations on *Acacia* spp. trees where they feed and mate, and the most obvious underlying reasons for the observed female attraction are facilitation of food and mate finding, as has been suggested for other insect species (Thornhill and Alcock 1983). The aggregations have been observed to increase in frequency and size later in the mating season. This could be linked to the observed delay in female flight activity (Fig. 1), since the aggregations are likely to be caused by an interaction between the female pheromone signaling system and the host, as evidenced by the strong attraction of both sexes to unmated females combined with food (banana) (Fig. 3). In other insects, increased attraction has been observed to combinations of pheromones with volatiles from hosts used for feeding (Klein et al. 1981; Seybold et al. 2006), oviposition (Dickens et al. 1993), or both (Bartelt et al. 1985), and shifts in sex ratio of attracted conspecifics when unmated females are combined with food volatiles have been observed in *P. japonica* (Klein et al. 1981) and the diamondback moth, *Plutella xylostella* (L.) (Reddy and Guerrero 2000). Access to food also could increase pheromone emission by females (McNeil and Delisle 1989), and aggregations might be influenced by pheromone release rate as well as the presence of host volatiles.

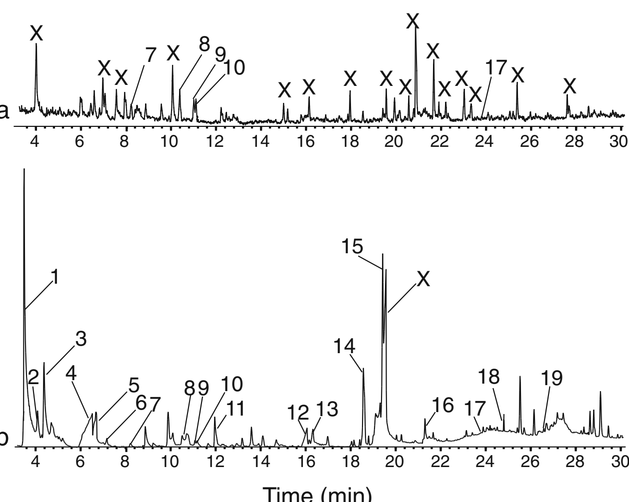


Fig. 5 Enlarged part of gas chromatograms of hexane extract of the abdominal tip of female *Pachnoda interrupta* during the mating season. **a** First extraction. **b** Second extraction. 1. Propyl acetate; 2.1-Methylpropyl acetate; 3. 2-Methylpropyl acetate; 4. 3-Methylbutyric acid; 5. 2-Methylbutyric acid; 6. Hexanoic acid; 7. α-Pinene; 8. β-Pinene; 9. *p*-Cymene; 10. Limonene; 11. Phenyacetaldehyde; 12. Benzoic acid; 13. Octanoic acid; 14. (*E*)-2-Decenal; 15. Indole; 16. (*E*)-2-Undecenal; 17. Geranylacetone; 18. (2*E*,4*E*)-2,4-Dodecadienal; and 19. 4-Dodecanolide (numbers as in Table 2). The symbol X indicates artifacts comprising silicone derivatives from the column and hydrocarbon impurities from the solvent

Insects also may alter the profile of volatile compounds emitted by their hosts by feeding damage or by inoculation with microorganisms (Zagatti et al. 1997; Macías-Sámano et al. 1998; Wertheim et al. 2002). In scarabs, adults feeding on plant hosts have been shown to induce emission of volatiles that facilitate aggregation (Harari et al. 1994), as well as mating behavior (Ruther et al. 2002). However, as we did not observe an increase in attraction during the mating season when we combined food with males, or with both sexes together (Fig. 3), it is unlikely that the synergy in attraction to unmated females with food during the mating season is due to a change in the volatile profile emitted by the food. During October, adult beetles are active above ground for 1–2 wk and feed (but do not mate) before going into estivation. Accordingly, unmated females

Fig. 6 Flight responses (trap catches) of both sexes of *Pachnoda interrupta* to various potential female-associated semiochemicals, 10–12 July 2008. Subgroups (males-normal font; females-italicized font) denoted by different letters are significantly different (α=0.05, Tukey's b on transformed data). Error bars show the standard error of the mean and the sample size was *N*=5. No treatments differed significantly from the positive control (Fig. 2) in sex ratio

were not attractive to males during this period (Fig. 4), whereas a combination of either or both sexes with food increased attraction above that to food alone. Possible mechanisms behind this increase in attraction are changes to the volatile profile of the host by feeding or inoculation with microorganisms, which however would contrast with the seeming lack of importance of such changes during the mating season.

Field testing of synthetic female-unique compounds established that phenylacetaldehyde (PAA), a common constituent of flower volatiles (reviewed in Knudsen et al. 2006), was highly attractive to males (Fig. 6). We hypothesize that PAA is a female-produced pheromone component for *P. interrupta,* but this will need to be proved through biochemical labeling or other approaches. No pheromones have been described for scarab species of the subfamily Cetoniinae, but other scarab species exhibit considerable diversity in pheromone semiochemistry, and several small aromatic compounds have been identified as pheromone components (e.g., phenol, anisole, and methyl benzoate) (reviewed in Leal 1998). On the other hand, some scarab attractants have been found in flowers (Knudsen et al. 2006), and the use of plant volatiles, including PAA (Honda 1980; Bellas and Hölldobler 1985), as attractants with pheromones has been observed in several insects (Boch and Shearer 1962; Baker 1989; Eller et al. 1994; Hassanali et al. 2005; Bartelt et al. 2009; Kim et al. 2009).

In agreement with the results of the field experiments where unmated females attracted both sexes, female *P. interrupta* also were attracted to PAA (Fig. 6). The strong attraction of both sexes to traps baited with PAA might in part be a dose effect, as suggested above concerning aggregations. We estimated that the release rate of PAA was 0.5–1 mg/h from the field lures. Although individual females only contained an estimated 0.2 ng of PAA, during the mating season, *P. interrupta* form aggregations with thousands of adults, and the effective emission of PAA from one of these aggregations may be quite high. High release rates of semiochemicals have been shown to be

necessary for attraction in the Coleoptera, e.g., to pheromone components in *Ips typographus* (L.) (Schlyter et al. 1987) or to host attractant monoterpenes in many other bark beetles (reviewed in Seybold et al. 2006). Furthermore, the involvement of additional components in the pheromone communication system of *P. interrupta* cannot be ruled out, and the inclusion of such compounds might enhance the specificity of the signal, and lower the threshold for response (Linn et al. 1986). Practical limitations forced us to forego extensive field testing of blends, and a treatment including all identified female-unique compounds proved to be less attractive than the treatment with PAA alone (Fig. 6).

The high level of attraction to the putative pheromone compound PAA is promising for future application. Both sexes are attracted, and traps catch adults, which is the life stage causing damage in this system. Trapping has proven efficient for control in other pest species where adults are the damaging stage (reviewed by Witzgall et al. 2010). PAA could be combined with previously identified host-related attractants, such as 2,3-butanediol (Bengtsson et al. 2009), eugenol, and methyl salicylate (Wolde-Hawariat et al. 2007) for evaluation of synergistic effects, and to further improve the attractiveness of lures. Such lures could be powerful instruments for monitoring, but also for mass trapping of *P. interrupta*.

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Sex Pheromone of the Spanish Population of the Beet Armyworm *Spodoptera exigua*

Patricia Acín · Gloria Rosell · Angel Guerrero · Carmen Quero

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Abstract The pheromone composition of the Spanish population of the beet armyworm (BAW), *Spodoptera exigua* (Lepidoptera: Noctuidae), was identified. Analysis of female gland extracts showed the presence of compounds *Z*9,*E*12–14:Ac (**1**), *Z*9–14:Ac (**2**), *Z*11–16:Ac (**3**), *Z*9,*E*12–14:OH (**4**), *Z*9–14:OH (**5**), and *Z*11–16:OH (**6**) in a ratio of 26:11:1:22:31:9. The amount of compound per gland ranged from 2.08 ng for **5** to 0.09 ng for **3**. However, analysis of female volatiles by SPME revealed only the presence of compounds, **1**, **2, 3,** and **5** in a 34:40:4:22 ratio. In electroantennogram assays, compound **1** elicited the highest response, and the C14 acetates evoked higher electrophysiological responses than the corresponding alcohols or C16 isomers. In a wind tunnel, no behavioral difference was observed between formulations based on the gland extracts and female volatiles. In both cases, males responded as when virgin females were used as the attractant source. Compound **1** alone elicited upwind flight by males, but required the presence of compound **5** in a 80:20 to 40:60 ratio for full activity. Ternary mixtures of **1**, **5** and the minor components did not improve the performance of the **1**+**5** blend in a 60:40 ratio. In the field, the mixture **1**+**5**+**3** in a 56:37:7 ratio was the most attractive formulation, and is expected to be useful in future pest control strategies.

Key Words Beet armyworm · *Spodoptera exigua* · Pest control · Field tests · Behavior · Pheromone composition · Lepidoptera · Noctuidae

P. Acín · A. Guerrero (✉) · C. Quero (✉)
Department of Biological Chemistry and Molecular Modelling, IQAC-CSIC,
Jordi Girona 18–26,
08034 Barcelona, Spain
e-mail: agpqob@iiqab.csic.es
e-mail: cqlqob@iiqab.csic.es

G. Rosell
Department of Pharmacology and Therapeutic Chemistry, Unity Associated to CSIC, Faculty of Pharmacy,
University of Barcelona,
Avda. Diagonal, s/n,
08028 Barcelona, Spain

Introduction

The beet armyworm (BAW), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest widely distributed in Africa, Asia, Australia, North America, and southern Europe that attacks numerous crops and ornamental plants in the field and greenhouses. In southern Spain, the damage caused by this moth has increased considerably, particularly in greenhouses, possibly due to pesticide resistance. One alternative proposed to control this pest has been the use of a synthetic pheromone as a specific and environmentally-friendly management tool. The pheromone composition of BAW has been studied by numerous researchers, but with a great disparity among all existing data. Initial studies on pheromone gland extracts from American strains identified (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14:Ac, compound **1**) as a pheromone compound, but the low efficiency of this acetate in field tests led to a reinvestigation of the pheromone complex (Brady and Ganyard 1972). Whereas Persoons et al. (1981) found compound **1**, (*Z*,*Z*)-9,12-tetradecadienyl acetate (*Z*9,*Z*12–14:Ac), (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac, compound **2**) and tetradecyl acetate (14:Ac) in pheromone gland extracts, Tumlinson et al. (1981, 1990) reported the

presence of compounds **1**, *Z*9,*Z*12–14:Ac, **2**, (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac, compound **3**) and (*Z*)-9-tetradecenol (*Z*9–14:OH, compound **5**) in a 40:48:6:2:4 ratio in volatile collections. By contrast, Rogers and Underhill (1981) established that the blend **1**+**5** in 1:10 ratio was highly attractive to males whereas Mitchell et al. (1983) found the mixture **1**+**5** in 10:1 ratio to be the most attractive. Analysis of the strains present in other countries has also shown a disparity in pheromone composition. Thus, in Taiwan Cheng et al. (1985) proved that the latter blend also was highly effective but, in Japan, Mochizuki et al. (1994) reported a pheromone blend composed of compounds **1**, **2**, (*Z*,*E*)-9,12-tetradecadienol (*Z*9,*E*12–14:OH, compound **4**), and **5** in a 98:60:86:100 ratio. In China, the pheromone composition of the insect was reported to be a mixture of these chemicals but in a 47:18:17:18 ratio (Dong and Du 2002). Therefore, it was not a surprise to discover that different commercial formulations of the pheromone were not sufficiently attractive in southern Spain to monitor and control the BAW in greenhouses. Consequently, the pheromone blend of the Spanish population was investigated.

Methods and Materials

Insects S. exigua moths were reared on an artificial diet based on wheat (T. Cabello, personal communication). The initial larvae were sent by T. Cabello (University of Almería, Spain), allowed to pupate, and sexed. The pupae then were placed in plastic containers until emergence and maintained on a reversed 16:8 L:D cycle at 24±1°C and 60% RH. The emerged adults were separated from pupae every day and fed with cotton balls soaked with a 10% sugar solution.

Chemicals Compound **1** was obtained from Sigma-Aldrich (St Louis, MO, USA), compound **2** from Acros-Organics (Geel, Belgium), and compounds **3, 4, 5,** and **6** were provided by SEDQ, S.A. (Barcelona, Spain). All compounds were >95% pure by GC or GC-MS analysis. Dimethyl disulfide was purchased from Sigma-Aldrich Química (Madrid, Spain). The solvents used (hexane, ether) were of analytical quality.

Pheromone Extracts Before proceeding to the gland excision, virgin females of different ages were observed to determine the moment of maximum calling activity. Groups of 10 virgin females were placed in plastic boxes under a red lamp in the dark, and their behavior was monitored every 30 min. During the calling period, the pheromone glands were excised and immersed in hexane. After 1 h of extraction, the supernatant was collected and stored at −80°C. A total of 28 extracts of 2–8 glands per extract were studied.

Volatiles Collection Five virgin females were selected and placed for 16 h (overnight) in a 40 ml vial (29×81 mm) containing a polydimethylsiloxane fiber (100 µm) for solid phase microextraction (SPME) (Matich et al. 1996). The fiber was inserted into the injection port of a GC-MS system for 5 min for analysis. A total of eight volatile collections were performed.

Derivatization with Dimethyl Disulfide (DMDS) Location of the double bonds in the monounsaturated compounds of the pheromone gland extracts was determined by DMDS derivatization, as previously described (Buser et al. 1983). Briefly, the pheromone gland extract (ca. 20 female equivalent) in 100 µl hexane was reacted with 100 µl DMDS and 5 µl iodine solution (60 mg/ml in ether) at 40°C for 24 h. The mixture was cooled, diluted with 200 µl hexane, and the iodine was removed by treatment with 100 µl of 5% aq. soln. of $Na_2S_2O_3$. The organic phase was concentrated to ca. 10 µl and kept at −80°C until GC-MS analysis.

Chemical Analysis Gland extracts and synthetic compounds were analyzed on a Fisons GC 8000 series coupled to a MD 800 mass spectrometer (ThermoFisher Scientific, Madrid, Spain) using helium (1 ml/min) as the carrier gas. The column used for analysis was a 30 m×0.25 mm i.d.×0.25 µm HP-5MS fused silica capillary column (Agilent Technologies, Madrid, Spain) under the following chromatographic conditions: injection at 70°C for 1 min, then program of 5°C/min up to 120°C, and 3°C/min up to 300°C, which was maintained for a further 10 min. Electron impact mass spectra were recorded at 70 eV in the range of m/z 40–400. The extracts were carefully concentrated to 1–2 µl, and then the entire extract was injected in splitless mode. For SPME analysis, a 30 m×0.25 mm i.d.×0.25 µm SPB-20 column (Supelco, Bellefonte, PA, USA) was used under the following conditions: injection at 100°C for 1 min, then program of 10°C/min up to 120°C, 3°C/min up to 250°C, and 5°C/min up to 260°C, which was kept for a further 10 min. Injections were made in splitless mode, and the mass range was also 40–400.

Electroantennogram (EAG) Assays and Coupled GC-EAD The EAG apparatus was commercially available from Syntech (Hilversum, The Netherlands). In brief, male antennae were excised, cut on both ends, and fixed to both electrodes with conducting gel Spectra 360 (Parker Lab. Inc., Hellendoorn, The Netherlands). A flow of humidified pure air (1000 ml/min) was directed continuously over the male antenna through the main branch of a glass tube (7 cm long×5 mm diam). Test stimulations were carried out by

giving puffs of air (300 ml/min) for 100 ms through a Pasteur pipette with the aid of a stimulus controller CS-01 (Syntech). The pipette contained a small piece of filter paper (1.5 cm diam) on which the gland extracts or the synthetic compounds **1–6** (10, 100, and 1000 ng diluted in hexane) had been deposited. The solvent was allowed to evaporate before the tests. Test compounds were applied at intervals of 60 sec on 10 antennae, and three times on each antenna. The antennae of at least 10 insects were used for the experiments. Control puffs with a piece of paper containing only solvent (hexane) were also intercalated between two consecutive stimuli to determine the baseline depolarization of the antennae. The signals were amplified (100 ×) and filtered (DC to 1 kHz) with an IDAC-2 interface (Syntech), digitized on a PC, and analyzed with the EAG Pro program. Depolarization means were compared for significance using analysis of variance (ANOVA) followed by LSD tests ($P<0.05$).

To establish the optimum conditions for performing the EAG experiments, the activity of different aged males (1–4 d-old) at different times of the photoperiod (3rd, 5th, 7th, and 8th h into the scotophase), 1 h before the onset of the scotophase and 1–2 h at the beginning of the photophase, was studied.

GC-EAD analysis was carried out on the pheromone glands of virgin females to which Hez-PBAN (pheromone biosynthesis-activating-neuropeptide, 50 pmol) had been applied 45 min before excision. Hexane extracts of the glands were analyzed on a Focus GC (Thermo Instruments, Barcelona, Spain) equipped with an FID detector, a split/splitless injector, and a second make up gas (nitrogen). Helium was the carrier gas (1–2 ml/min), and the column was a 30 m×0.25 mm i.d.×0.25 μm HP-5 fused silica capillary column (Agilent Technologies, Madrid, Spain). The effluent from the column was split 50:50, and branches to the FID and EAD were from a deactivated fused silica capillary column (35 cm long×0.25 mm i.d.). The transfer tube to the EAG preparation was heated to 230°C, and the GC conditions were the same as for the GC-MS.

Wind Tunnel Assays Assays were conducted in a glass tunnel of 180×50×50 cm as previously described (Quero et al. 1995). The wind was pushed through the tunnel by a 30 cm diam fan at 20 cm/s. The tunnel was illuminated with two red light fluorescent tubes dimmed to 1 lux. The temperature was maintained at 25±2°C, and the relative humidity was 43±10%. BAW males were acclimatized to the experimental conditions of the tunnel for 30 min, and individually released into the tunnel between the 6th and the 8th h of the scotophase. Before the tests, the insects were placed on filter paper in a Petri dish, then introduced into the tunnel at a height of 20 cm and a distance of 125 cm from the emission source. After a 30 sec acclimatization period, the behavior of the males was recorded for 5 min. For each responding insect, the following four types of behavior were recorded: taking flight; halfway: oriented upwind flight and arrival to the middle of the tunnel; final: arrival to the proximity of the lure; and contact: landing and contact with the source. In each treatment, a minimum of 40 virgin males of 1–3 d-old were used, and each insect was tested only once. The attractant source consisted of filter paper loaded with 1 μg of the most active EAG component (compound **1**) plus the corresponding amount of the other components to test the desired formulation. For statistical analyses, a χ^2 homogeneity test ($P<0.05$) was performed for every treatment.

Field Trials Field tests were conducted in greenhouses at two locations (Campo de Cartagena and Lorca) in the province of Murcia (southern Spain) from 6 July to 13 October 2006. In 2008, two more trials were conducted in the same province at the locations of Torre Pacheco from 1 July to 14 November, and Mazarrón from 21 July to 11 November. All tests were implemented by Kenogard, S.A. Three formulations were tested in 2006: F1: compounds **1+5** (91:9 ratio, 182 μg of **1**+18 μg of **5**); F2: compounds **1+5+3** (87:2.5:10.5 ratio, 174 μg of **1**+5 μg of **5**+21 μg of **3**); and F3: compounds **1+5+2+4** (31:31:16:22 ratio, 62 μg of **1**+ 62 μg of **5**, +32 μg of **2**+44 μg of **4**), along with a commercial formulation (C1=Pherocon® BAW, Trécé, Adair, OK, USA) (two replicates per formulation in each location). In 2008, five formulations were assayed: the same F2 as above; F4: **1+5** (60:40 ratio, 174 μg of **1**+116 μg of **5**); F5: **1+5** (80:20 ratio, 174 μg of **1**+43.5 μg of **5**); F6: **1+5+2** (35:23:42 ratio, 174 μg of **1**+116 μg of **5**+209 μg of **2**); and F7: **1+5+3** (56:37:7 ratio, 174 μg of **1**+116 μg of **5**+23 μg of **3**), along with two different commercial lures: C1 as above, and C2 (Econex, Econex S.L., Murcia, Spain) (two replicates per formulation in each location). The synthetic chemicals were dissolved in hexane, and the required amounts were deposited onto red rubber septa (Aldrich, Milwaukee, WI, USA). Delta traps containing the septa were suspended at a height of 120–150 cm, and the distance between traps was about 10–15 m. The number of males caught per trap was recorded every week, the traps were emptied, and the septa were replaced every 3 wk. For statistical analyses, the number of moths captured per trap was converted to $\sqrt{(x+0.5)}$ and subjected to ANOVA followed by LSD test ($P<0.05$).

Results

Pheromone Composition Females of all ages showed a maximum calling behavior during the last 3 h of the

scotophase (data not shown). Thus, young females (1st–3rd scotophase) started to call 6 h after the onset of the scotophase, with the number of calling females gradually increasing until the end of the dark period. Females of 3rd–5th scotophase reached peak calling activity during the 6–7 h into the scotophase.

Analysis of the pheromone glands excised at the maximum calling period showed the presence of the previously identified compounds **1**–**5** by their GC retention times on polar and non-polar columns, and mass spectra in comparison with those of authentic samples (Fig. 1). In addition, *Z*11–16:OH (**6**) also was detected in very low amounts (0.58 ng/gland).

To confirm the presence of this previously unidentified compound in any of the BAW strains studied, DMDS derivatization of the extract produced an adduct with a molecular ion of *m/z* (%) 334 (16) and diagnostic ions at *m/z* 117 (72) and 217 (100). The molecular ion corresponded to the addition of DMDS to one double bond and the other ions were assigned to fragments $[CH_3(CH_2)_3CH(SCH_3)^+]$ and $[(CH_3S)CH(CH_2)_{10}OH]^+$, confirming the structure of the new product. The presence of compounds **2**, **3,** and **5** was also verified by DMDS adducts. The final composition of the extract was a mixture of compounds **1**–**6** in a 26:11:1:22:31:9 ratio, and the amount of compound per gland ranged from 2.08 ng for **5** to 0.09 ng for **3** (Fig. 2). However, analysis of female volatiles using the SPME technique revealed only the presence of compounds **1**, **2**, **3**, and **5** in a 34:40:4:22 ratio (Fig. 2).

GC-EAD analysis of PBAN-treated pheromone gland extracts confirmed the presence of compounds **1, 2, 4, 5,** and **6** but in a different ratio from those found in non-treated glands (Fig. 3). Compound **1** elicited the highest response followed by the 14-carbon compounds **2, 4,** and **5**. Compound **6,** in turn, induced a very small EAG depolarization.

EAG Assays Males of 1st–2nd scotophase elicited higher electrophysiological responses (2.0 mV on average) than older males (1.6 mV of 2nd–3rd scotophase, 1.5 mV of 3rd–4th scotophase) (data not shown). In addition, males displayed increasing EAG responses with time, reaching a maximum at the end of the scotophase (values rose from 1.6 to 2.2 mV, and dropped to 1.6 mV after the scotophase). When the pheromone compounds **1–6** were tested at several doses (10, 100, and 1000 ng), the highest responses were displayed by compound **1** (Fig. 4). Depolarizations induced by compounds **2–6** were dose-dependent, with the responses induced by the C16 compounds being lowest (Fig. 4). The relative activity of the compounds followed the order **1** > **4** ≅ **2** > **5 > 3** ≅ **6**.

Wind Tunnel Assays Blends of synthetic compounds in a ratio similar to that found in gland extracts and volatile collections elicited similar responses by males as did five calling females (data not shown). No significant differences were observed at any stage of the courtship sequence, although the number of contacts elicited by virgin females was slightly higher (63%) than the formulations of gland extracts (43%) and volatiles (51%).

When tested alone, compound **1** evoked a remarkable long-range attraction (71% of males reached the proximity

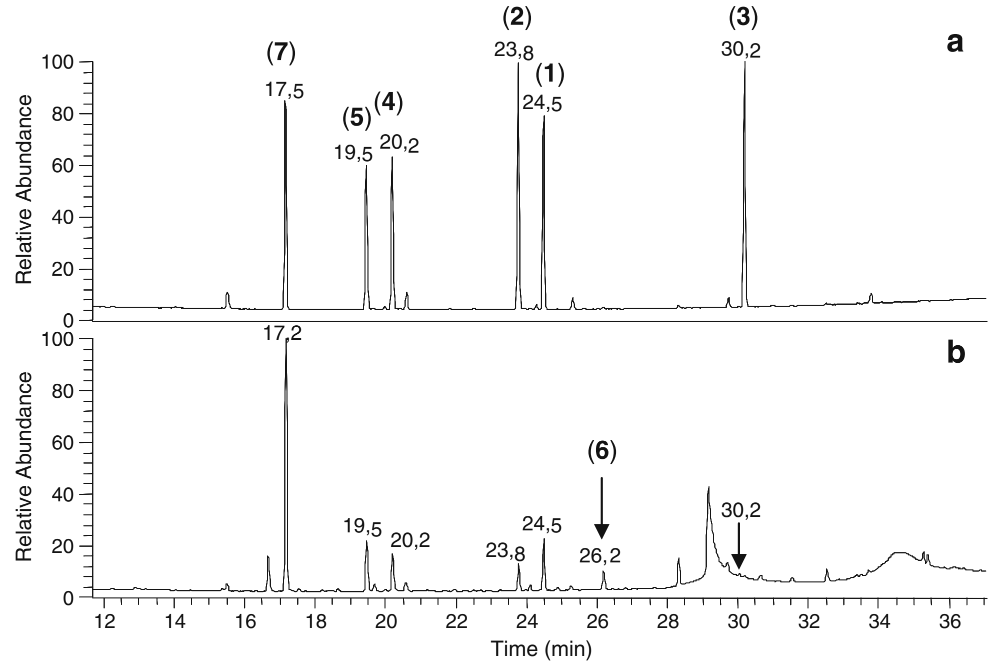


Fig. 1 Gas chromatography (GC) analyses of **a** blend of synthetic compounds **1-5** and internal standard (**7**), **b** gland extract of four *Spodoptera exigua* females. Identities of compounds are as follows: *Z*9,*E*12–14:Ac (**1**), *Z*9–14:Ac (**2**), *Z*11–16:Ac (**3**), *Z*9,*E*12–14:OH (**4**), *Z*9–14:OH (**5**) and 12:Ac (IS, **7**). Compound in **b** at retention time 26.2 min corresponds to *Z*11–16:OH (**6**) as shown independently by GC-MS analysis of the DMDS adduct

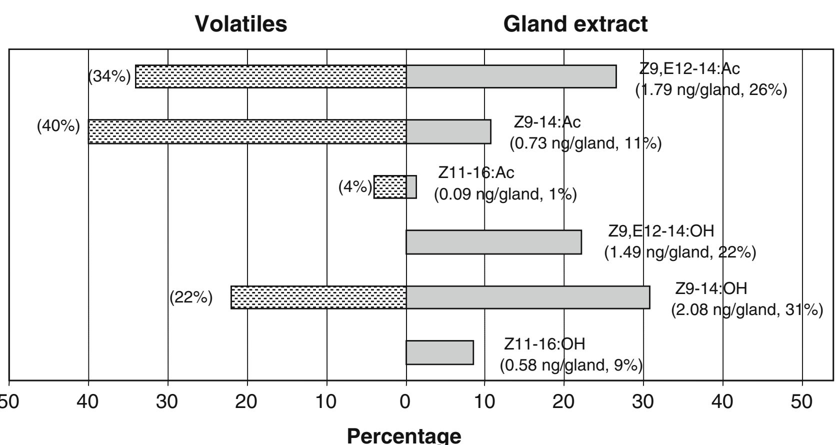


Fig. 2 Pheromone composition of *Spodoptera exigua* found in gland extracts during the calling period (*N*=28 extracts of 2–8 female glands), and volatiles from females (*N*=8 of 5 virgin females) by SPME. Percentage and estimated mean amounts of compounds are in parenthesis

of the lure) but no insect landed at the source (Fig. 5). Binary mixtures of **1** with compounds **2**, **3,** and **5** also induced upwind flight on males, but only blend A (**1**+**5** in a 60:40 ratio) induced males to significantly contact the source (71%). Other **1**+**5** binary mixtures ranging from 80:20 to 40:60 did not significantly differ in activity in comparison to the 60:40 blend, but higher relative amounts of **5,** such as 20:80 or 0:100 of the **1**+**5** blend dramatically affected the number of males arriving in the vicinity of the source (data not shown).

Binary blends of **1** with **2** or **3** induced close approach to the source in 45–55% of males but none of them made contact (Fig. 5). When both **2** and **3** were added to blend A, in the same proportion as in female volatiles, the resulting blends D (**1**+**5**+**2** in 35:23:42 ratio) and E (**1**+**5**+**3** in 56:37:7 ratio) also elicited the complete behavioral sequence in males. The slightly lower number of insects contacting the source in comparison to blend A was not significant (Fig. 5). Lowering the relative amount of compound **5** to 2.5% in the **1**+**5**+**3** blend (formulation F) resulted in a clearly less active formulation.

When compounds **4** and **6** were added to blend A, they evoked dissimilar effects. Compound **4** had no effect on the number of males contacting the source when added to blend A at two different doses (blend A+10% of **4**: 68% contacts, blend A+50% of **4**: 66% contacts). In contrast, the presence of compound **6** markedly decreased the number of males flying upwind and contacting the source (blend A: 71% of contacts, blend A+10% of **6:** 52%, blend A+50% of **6:** 40%) (Fig. 6).

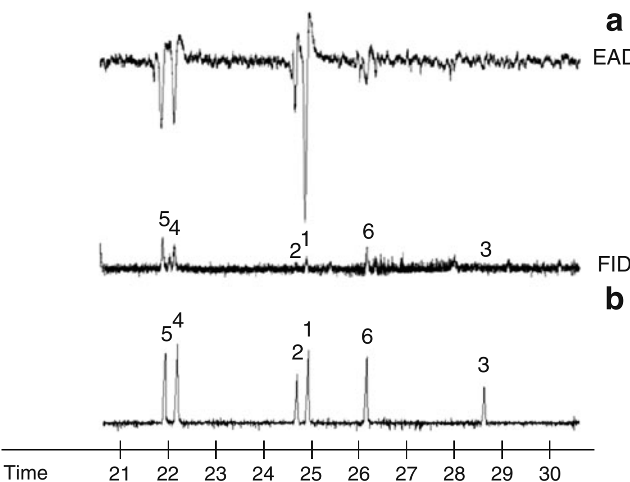


Fig. 3 **a** GC-EAD response of a *Spodoptera exigua* antenna from a male to a PBAN-treated pheromone extract. **b** GC of a mixture of synthetic compounds *Z*9,*E*12–14:Ac (**1**), *Z*9–14:Ac (**2**), *Z*11–16:Ac (**3**), *Z*9,*E*12–14:OH (**4**), *Z*9–14:OH (**5**), *Z*11-16:OH (**6**) (100 ng each)

Field Assays In the 2006 trials, a mixture of compounds **1**+**5** in a 91:9 ratio (formulation F1, Fig. 7) (Mitchell et al. 1983) resulted in a remarkably attractive lure (95.5±15.5 and 51±21 males per trap in each field). When compound **3** was added to this mixture to produce a similar composition to the one described by Tumlinson et al. (1990), the resulting formulation F2 attracted more males than F1, but the difference was significant in only one of the fields tested (149±55 males/trap caught in F2 vs. 51±21 in F1). By contrast, the mixture **1**+**5**+**2**+**4** in a 31:31:16:22 ratio (formulation F3), an initial pheromone composition found in gland extracts, displayed attractant activity similar to that of F1 (Fig. 7). In one field, all new formulations were superior to the commercial lure C1, whereas in the other field only F2 was more effective.

In 2008, formulation F7 (**1**+**5**+**3** in a 56:37:7 ratio) was the most attractive in the two fields (489±8 males/trap caught in field A and 620±95 in field B), being significantly more efficient than F2, F4, and F6 (Fig. 8).

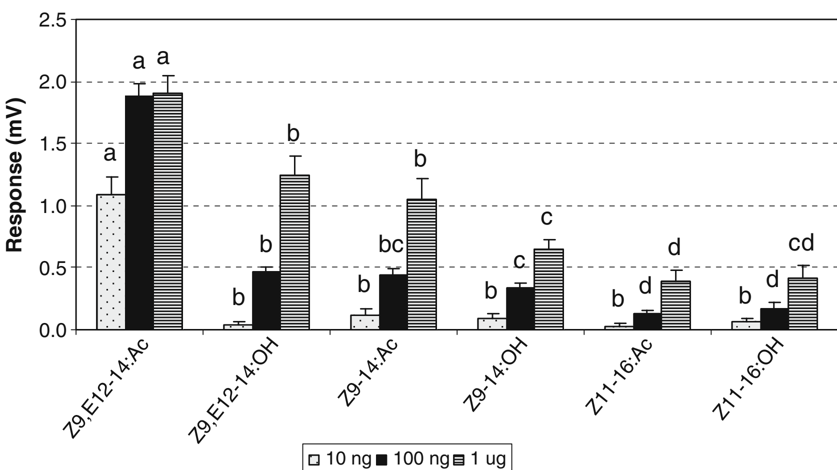


Fig. 4 EAG responses of antennae from male *Spodoptera exigua* (N=10) to different doses of compounds *Z*9,*E*12–14:Ac (**1**), *Z*9–14:Ac (**2**), *Z*11–16:Ac (**3**), *Z*9,*E*12–14:OH (**4**), *Z*9–14:OH (**5**), and *Z*11–16:OH (**6**) identified in female gland extracts. Bars (± SE) with the same letters within the same dose are not significantly different (ANOVA, LSD test, P<0.05)

F7 was also superior to the two commercial lures C1 and C2 in field B and to C1 in field A. By contrast, F6 was the least active formulation (Fig. 8).

Discussion

The sex pheromone of the Spanish population of the BAW, determined from female gland extracts, has been identified as a mixture of compounds **1–6** in a 26:11:1:22:31:9 ratio, although volatiles emitted by virgin females lack the presence of alcohols *Z*9,*E*12–14:OH (**4**) and *Z*11–16:OH (**6**). This is not surprising because it is known that the composition of the pheromone gland and volatiles released by females may differ remarkably (Cross et al. 1976; Hill et al. 1975). The composition of female volatiles was similar to that found by Tumlinson et al. (1990) with the exception of *Z*9,*Z*12–14:Ac, which was absent in our extracts. The relative differences in both strains could be due to the different origin of the strains or the different extraction methods used: volatile collections followed by entrainment on charcoal by the American team and SPME analysis of female effluvia in this work. The diene alcohol **4** was moderately EAG-active and had a neutral effect on male behavior. However, in the Japanese population, this chemical stimulated the receptor cells tuned to alcohol **5** (Mochizuki et al. 1993). Moreover, combining alcohol **4** with the corresponding acetate **1** resulted in an efficient formulation in the field (Rogers and Underhill 1981; Mochizuki et al. 1994). Alcohol **6**, in turn, was practically inactive by EAG, but inhibited the responses of males when mixed with the highly active formulation, **1+5** (60:40). Its presence in gland extracts suggests that it is likely a biosynthetic precursor of the corresponding *Z*11–16:Ac (**3**) (Bjostad et al. 1987). However, to our knowledge, no biosynthetic studies of the pheromone have been undertaken to prove this assumption.

In wind tunnel studies, the combination **1+5** in a 60:40 ratio (blend A) was the most active among all the binary and ternary mixtures tested, but this proportion is not critical because similar levels of activity were achieved by

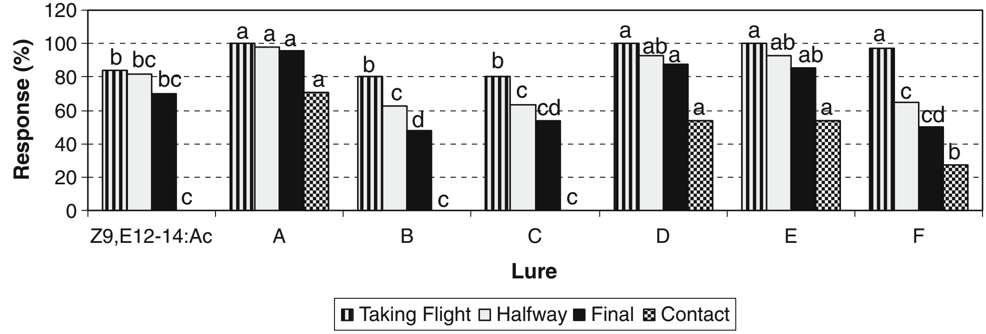


Fig. 5 Percentage of response of *Spodoptera exigua* males (N=40–48) in wind tunnel to 1 µg of *Z*9,*E*12–14:Ac (**1**) and to blends A: **1+5** (60:40), B: **1+2** (46:54), C: **1+3** (90:10), D: **1+5+2** (35:23:42), E: **1+5+3** (56:37:7), F: **1+5+3** (87:2.5:10.5). Bars with the same letters within the same behavior are not significantly different (*Chi square* test of homogeneity, P<0.05)

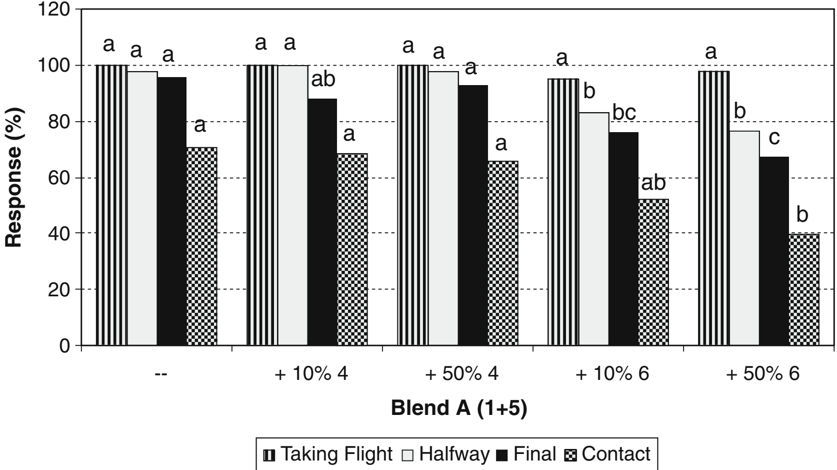


Fig. 6 Percentage of response of *Spodoptera exigua* males (N=41–69) in wind tunnel to blend A (**1**+**5**, 60:40) and mixtures with compounds **4** and **6** in different ratios (**1**: *Z*9,*E*12–14:Ac, **4**: *Z*9,*E*12–14:OH **5**: *Z*9–14:OH, **6**: *Z*11–16:OH). Bars with the same letters within the same behavior are not significantly different (*Chi square* test of homogeneity, P<0.05)

blends 80:20 to 40:60. This result is consistent with the relative amounts of both compounds found in gland extracts and female volatiles. Both chemicals have been found to be tuned to two different receptor neurons in male-specific sensilla trichodea (Dickens et al. 1993; Mochizuki et al. 1993), and, in the field, this formulation in a 10:1 ratio was reported to be active in Florida (Mitchell et al. 1983), Taiwan (Cheng et al. 1985), and in a 70:30 ratio in Japan (Wakamura 1987).

In field tests, initially we compared the most effective formulations found by Mitchell et al. (1983) (F1) and Tumlinson et al. (1990) (F2) with one commercially

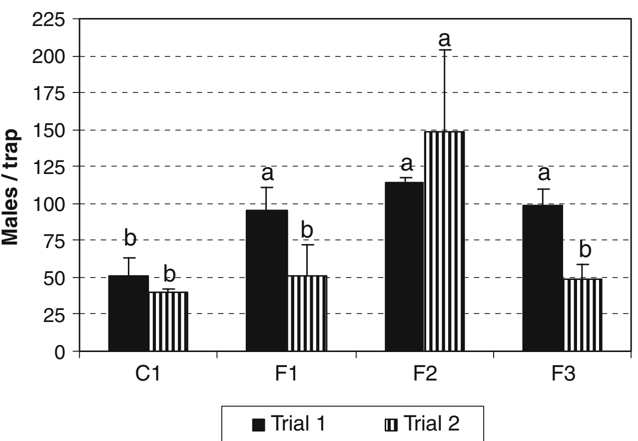
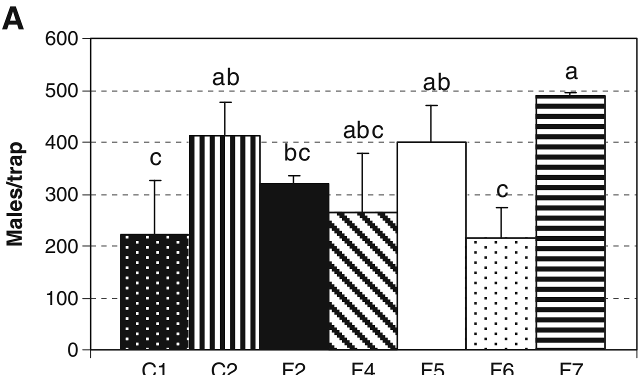


Fig. 7 Mean number of catches (± SE) of *Spodoptera exigua* males in traps baited with various pheromone formulations in two different fields in 2006: C1 (commercial bait), F1: **1**+**5** (91:9), F2: **1**+**5**+**3** (87:2.5:10.5), F3: **1**+**5**+**2**+**4** (31:31:16:22) (**1**: *Z*9,*E*12–14:Ac, **2**: *Z*9–14:Ac, **3**: *Z*11–16:Ac, **4**: *Z*9,*E*12–14:OH, **5**: *Z*9–14:OH). Bars with the same letter within the same trial are not significantly different (ANOVA, LSD test, P<0.05)



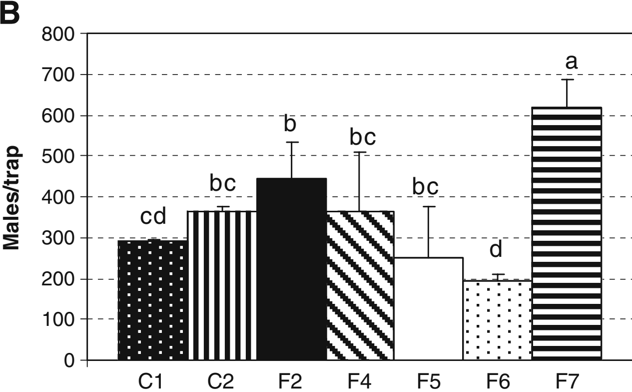


Fig. 8 Mean number (± SE) of *Spodoptera exigua* males caught in traps baited with various pheromone formulations in two different fields (A,B) in 2008: C1, C2 (commercial baits), F2: **1+5+3** (87:2.5:10.5), F4: **1+5** (60:40), F5: **1+5** (80:20), F6: **1+5+2** (35:23:42), F7: **1+5+3** (56:37:7) (**1**: *Z*9, *E*12–14:Ac, **2**: *Z*9–14:Ac, **3**: *Z*11–16:Ac, **4**: *Z*9,*E*12–14:OH, **5**: *Z*9–14:OH). Bars with the same letter within the same trial are not significantly different (ANOVA, LSD test, P<0.05)

available (C1), along with a new formulation (F3) of similar composition to the pheromone gland extract lacking compounds **3** and **6**. In the first trial, all formulations were significantly more attractive than the C1, although without a significant difference among them. In the second trial, F2 was the most efficient formulation. Based on these results, a new set of experiments was designed in 2008 to further improve formulation F2. Thus, addition of compound **3** (7%) to formulation F4 (blend A above) resulted in a more efficient blend (F7), not only in comparison to its parent F4 but also to F2. In fact, F7 was the most attractive formulation found for BAW males. Our results show that in the Spanish population the presence of the minor component **3** appears to be important for the development of new attractant formulations to catch BAW males.

Compound **2**, the major component of the pheromone released by females, deserves some comments. First, this compound was only moderately active on male antennae, activity similar to that of diene alcohol **4**, a component absent in female volatiles. Second, in a wind tunnel, compound **2** decreased the attractant activity of the major component **1**, and that of the highly active blend A as well. Third, in the field, addition of **2** to formulation F4 resulted in a less efficient lure, the resulting formulation F6 being the least efficient of all tested. These data are in agreement with those of Tumlinson et al. (1990) in which compound **2**, also the major component released by American BAW females, also reduced the number of catches when it was incorporated into the lure. It is surprising that this compound emitted by females as a major component reduces the attraction of conspecific males in the laboratory and in the field. Does the female use this compound to modulate the activity of the key components **1** and **5** under some specific but yet unknown conditions? It was also unexpected that occasionally compound **2** elicited in males spikes from neurons sensitive to the major compound **1** (Dickens et al. 1993), with the threshold of the cells responding to acetate **2** being ca. 1000 times higher than that of the cells responding to acetate **1** (Mochizuki et al. 1993). These data do not correspond to the relatively high emission of compound **2** by females relative to compounds **1** and **5**, the two components from which receptor cells have so far been identified (Mochizuki and Shibuya 1991; Mochizuki et al. 1993) (see above). These data indicate that more research is needed to clarify the role of compound **2** in the chemical communication system of the BAW.

In summary, we have identified the pheromone composition of the Spanish population of the BAW, and found that extracts of female glands exhibit a remarkably different composition relative to volatiles emitted by females. Electrophysiological and behavioral activity of the pheromone components, and mixtures thereof, have resulted in a new highly active formulation (F7) for the Spanish BAW population, which should be useful in future IPM strategies.

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Pheromone Binding to General Odorant-binding Proteins from the Navel Orangeworm

Zhao Liu · Diogo M. Vidal · Zainulabeuddin Syed · Yuko Ishida · Walter S. Leal

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Abstract General odorant-binding proteins (GOBPs) of moths are postulated to be involved in the reception of semiochemicals other than sex pheromones, the so-called "general odorants." We have expressed two GOBPs, AtraGOBP1 and AtraGOBP2, which were previously isolated from the antennae of the navel orangeworm, *Amyelois transitella*. Surprisingly, these two proteins did not bind compounds that are known to attract adult moths, particularly females. The proper folding and functionality of the recombinant proteins was inferred from circular dichroism analysis and demonstration that both GOBPs bound nonanal in a pH-dependent manner. EAG experiments demonstrated that female attractants (1-phenylethanol, propionic acid phenyl ester, and isobutyric acid phenyl ester) are detected with high sensitivity by the antennae of day-0 to day-4 adult females, with response declining in older moths. The same age-dependence was shown for male antennae responding to constituents of the sex pheromone. Interestingly, AtraGOBP2 bound the major constituent of the sex pheromone, Z11Z13-16Ald, with affinity comparable to that shown by a pheromone-binding protein, AtraPBP1. The related alcohol bound to AtraPBP1 with higher affinity than to AtraGOBP2. AtraGOBP1 bound both ligands with low but nearly the same affinity.

Key Words *Amyelois transitella* · AtraGOBP1 · AtraGOBP2 · AtraPBP1 · Circular dichroism · Electroantennogram recording · Female attractants · General odorant-binding proteins · Protein expression

Z. Liu · D. M. Vidal · Z. Syed · Y. Ishida · W. S. Leal (✉)
Department of Entomology, University of California-Davis,
1 Shields Ave,
Davis, CA 95616, USA
e-mail: wsleal@ucdavis.edu

Introduction

There is growing evidence in the literature suggesting that odorant-binding proteins (OBPs) are involved in the early events of odorant reception in insects. OBPs were first isolated from moth antennae (Vogt and Riddiford 1981), but they have now been identified from insects in all orders hitherto investigated [for a recent example, see (Gong et al. 2009a)]. RNA interference experiments in the southern house mosquito, *Culex quinquefasciatus* (Pelletier et al. 2010), and the malaria mosquito, *Anopheles gambiae* (Biessmann et al. 2010), showed that reduction in OBP expression leads to lower sensitivity for the detection of specific odorants, whereas a model has been proposed for the fruit fly, *Drosophila melanogaster*, in which an OBP, LUSH, activates the receptor while bound to a pheromone ligand (Laughlin et al. 2008). In contrast, moth pheromone receptors are activated directly by ligands, as demonstrated by expressing receptors in a heterologous system devoid of OBPs and stimulating with pheromones (Nakagawa et al. 2005). Previously, we demonstrated that an OBP from the silkworm moth, *Bombyx mori*, BmorPBP1, enhances sensitivity when the moth receptor BmorOR1 is expressed in the empty neuron system of the fruit fly (Syed et al. 2006).

The existence of at least two groups of OBPs became apparent when the first ones were isolated from moth species. Pheromone-binding proteins (PBPs), postulated to be functionally involved in pheromone reception, are enriched or expressed only in male antennae, and show poor conserved sequences between species (Vogt et al. 1991). In contrast, some highly conserved antennal proteins show expression in both males and females, thus suggesting that they are involved in the reception of "general" odorants (i.e., odorants other than pheromones) and, consequently,

they were dubbed general-odorant binding proteins (GOBPs) (Vogt et al. 1991). Immunocytochemical studies have further substantiated this hypothesis. PBPs have been expressed in the pheromone-detecting, long trichoid sensilla in the male antennae of the silkworm moth, *B. mori*, and the wild silkworm moth, *Antheraea polyphemus* (Steinbrecht et al. 1995). In contrast, GOBPs have been shown to be expressed in basiconic sensilla, which are sensitive to plant-derived compounds and other "general" odorants (Steinbrecht et al. 1995). Exceptionally, a GOBP from *Mamestra brassicae*, MbraGOBP2, has been expressed in long trichoid sensilla and has high affinity for a behavioral antagonist detected by neurons housed in these sensilla (Jacquin-Joly et al. 2000). Additionally, it has been shown that a female pheromone constituent of *A. polyphemus* binds to a GOBP expressed in male and female antenna (Ziegelberger 1995). In contrast to PBPs from *B. mori*, BmorGOBP2 was recently shown to bind bombykol, but not bombykal, thus suggesting a possible role in pheromone reception (Zhou et al. 2009).

Recently, we isolated and cloned the cDNAs that encode various olfactory proteins from the navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), a major pest of almonds, pistachios, and walnuts (Leal et al. 2009). Considering that two proteins, AtraGOBP1 and AtraGOBP2, were enriched in female antennae, we hypothesized that they may be involved in the reception of female attractants. Here, we report that recombinant AtraGOBP1 and AtraGOBP2 did not bind semiochemicals known to attract adult moths, particularly females, i.e., 1-phenylethanol, propionic acid phenyl ester (hereafter referred to as PAPE = phenyl propionate), and isobutyric acid phenyl ester (hereafter referred to as IBAPE = phenyl 2-methylpropionate) (Price et al. 1967), but rather showed higher affinity to nonanal, a component of the head space volatile from almonds (Beck et al. 2009). Electroantennogram (EAG) studies showed that all these semiochemicals are detected by the antennae, so reception of female attractants may involve other yet unknown OBPs. Interestingly, both AtraGOBP1 and AtraGOBP2 bound to and discriminate two constituents of the *A. transitella* sex pheromone (Leal et al. 2005b), suggesting a possible role in pheromone reception.

Methods and Materials

Protein Expression and Purification Each cDNA fragment was amplified by PCR with gene-specific primers and *PfuUltra* HotStart DNA polymerase (Stratagene, La Jolla, CA, USA) as *Taq* DNA polymerase, gel-purified by using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and subcloned into the recognition site of *Eco* RV in pBluescript SK (+) (Stratagene) as previously described (Leal et al. 2009). Each insert DNA was used as template for PCR-based insertion of recognition sites of restriction enzymes. The following primers were designed for the PCR: 5-KpnI-AtraGOBP1, 5′-CCGGGGTACCCGAC GTGGCCGTCATGAAGG-3′; 3-BamHI-AtraGOBP1, 5′-GCGCGGATCCTTATGTCTCGGCCTCCATGA-3′; 5-HaeIII-AtraGOBP2, 5′-TTTGCGGCCAGTGCTGAGG TGATGAGCCAT-3′; 3-XhoI-AtraGOBP2, 5′-CCGCTCGAGTCAATATTTCTCCATGACTG-3′. After incubation at 95°C for 2 min, we initiated forty cycles of stepwise PCR with the following amplification program (95°C for 30 sec, 40°C for 30 sec, and 72°C for 1 min). One microgram of pET-22b(+) plasmid DNA (Novagen, Gibbstown, NJ, USA) was double-digested with *Msc* I and *Bam* HI or *Xho* I (New England Biolabs, Ipswich, MA, USA), and gel-purified.

For preparation of pET-22b-AtraGOBP1, 1 μg of the PCR product amplified by 5-KpnI-AtraGOBP1 and 3-BamHI-AtraGOBP1 was treated with *Kpn* I (New England Biolabs) and subsequently re-purified by QIAquick PCR Purification Kit (Qiagen). Purified DNA was treated with T4 DNA polymerase (New England Biolabs) at 12°C for 20 min to remove 5′-protruding single strand DNA fragment. After re-purification of DNA by QIAquick PCR Purification Kit (Qiagen), DNA was digested with *Bam* HI. Five hundred bp of DNA fragment were gel-purified and ligated into pET-22b (+) digested previously. Construction of the vector was confirmed by sequencing.

For construction of pET-22b-AtraGOBP2, the PCR product amplified by 5-HaeIII-AtraGOBP2 and 3-XhoI-AtraGOBP2 was purified by QIAquick PCR purification kit and double-digested with *Hae* III and *Xho* I. The treated DNA fragment was gel-purified and ligated into pET-22b (+) vector as described above.

Expression was performed in LB medium with transformed BL21(DE3) cells (Novagen) following a previously reported protocol (Leal et al. 2009). Proteins in the periplasmic fraction were extracted with 10 mM Tris·HCl (pH 8) by using three cycles of freeze-and-thaw and centrifuging at 16,000×*g* to remove debris. The supernatant was collected and loaded on HiPrep DEAE FF column (GE Healthcare Biosciences, Piscataway, NJ, USA). Unless stated otherwise, all separations by ion-exchange chromatography were done with a linear gradient of 0–500 mM NaCl in 10 mM Tris·HCl (pH 8). Fractions containing the target proteins were further purified on HiPrep Q FF column (GE Healthcare Biosciences) and, subsequently, on Mono Q 10/100 GL column (GE Healthcare Biosciences). These fractions containing target proteins were concentrated by centrifuging in Centriprep YM-10 (Millipore, Billerica, MA, USA) at 3000 × *g* until sample volume was less than 5 ml. Then, the sample was loaded on HiLoad 26/60

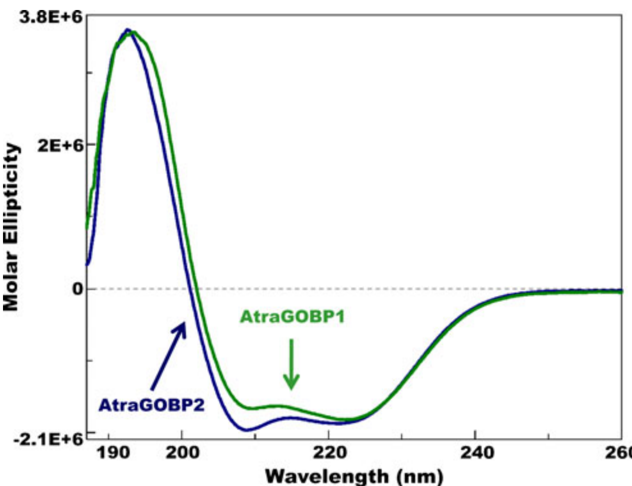
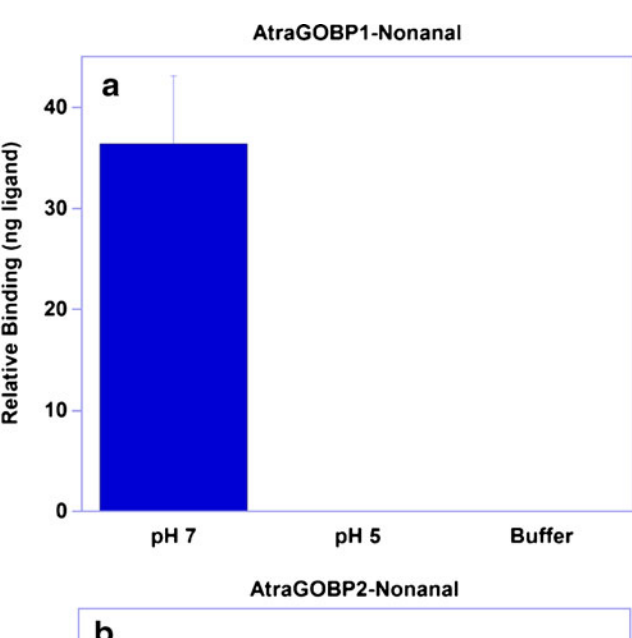


Fig. 1 Far-UV-CD spectra of two general-odorant binding proteins from the navel orangeworm, *Amyelois transitella*. AtraGOBP1, green trace; AtraGOBP2, blue trace. Note the lowest minima for AtraGOBP2 and AtraGOBP1 appeared at 209 nm and 223 nm, respectively

Superdex-75pg gel-filtration column (GE Healthcare Biosciences) preequilibrated with 150 mM NaCl and 20 mM Tris·HCl (pH 8). Fractions were analyzed by SDS-PAGE and LC-MS. LC-ESI-MS was performed with a LCMS-2010 (Shimadzu, Kyoto, Japan). HPLC separations were done on Zorbax 300SB-C8 Narrow Bore column (100×2.1 mm; 5 µm; Agilent Technologies, Palo Alto, CA, USA) with a gradient of water and acetonitrile plus 2% acetic acid as a modifier. The detector was operated with the nebulizer gas flow at 1.0 l/min and the curved desolvation line and heat block at 250°C. Fractions containing traces of nontarget proteins were purified further by Mono-Q column with a shallower gradient buffer. Highly purified protein fractions were concentrated by Centriprep YM-10, desalted on HiPrep 26/10 desalting column (GE Healthcare Biosciences) with water as mobile phase, analyzed by LC-ESI/MS, lyophilized, and stored at −80°C until use. The concentrations of AtraGOBPs were measured by UV radiation at 280 nm in 20 mM sodium phosphate (pH 6.5) and 6M guanidine HCl by using the theoretical extinction coefficients calculated with expasy software (http://us.expasy.org/tools/protparam.html). AtraPBP1 was prepared as previously reported (Leal et al. 2009). Circular dichroism (CD) spectra were recorded by using a J-810 spectropolarimeter (Jasco, Easton, MD, USA) with 0.2 mg/ml AtraGOBPs in either 20 mM ammonium acetate, pH 7 or 20 mM sodium acetate, pH 5.

Binding Assays Binding was measured by incubating AtraGOBPs or AtraPBP1 with test ligands, separating unbound and bound protein, extracting ligand from the latter sample, and analyzing by gas chromatography, according to a previously reported "cold binding assay" (Leal et al. 2005a). After lowering pH to release the ligand, bound protein fractions were extracted and analyzed by gas chromatography with flame ionization detection (GC-FID) for quantification and by GC-mass spectrometry (GC-MS) for confirmation of ligand identity. GC-FID and GC-MS were performed with a 6890 series GC and a 5973 Network Mass Selective Detector (Agilent Technologies), respectively. Both instruments were equipped with the same type of capillary column (HP-5MS, 25 m×0.25 mm; 0.25 µm; Agilent Technologies) operated under the same temperature program. For female attractants of low molecular weight, the oven was operated at 50°C for 1 min, increased to 180°C at a rate of 10°C/min, and held at the final temperature for 5 min, whereas for pheromones the



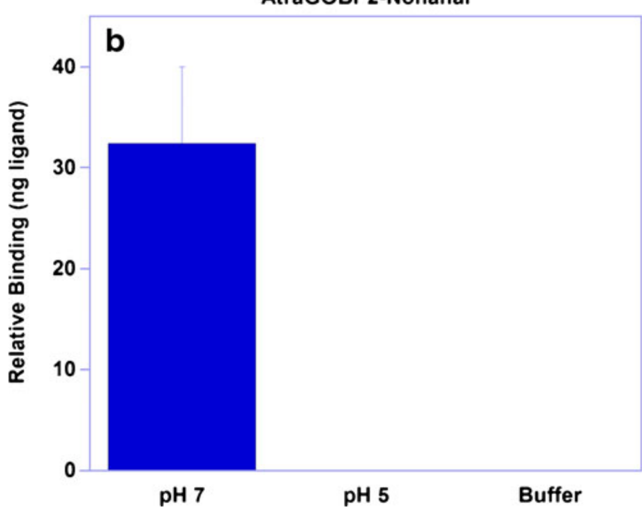


Fig. 2 Binding of nonanal to *Amyelois transitella* AtraGOBPs. This aldehyde showed high affinity to AtraGOBP1 (**a**) and AtraGOBP2 (**b**) at high pH, but no affinity at low pH. No traces of the ligand were detected in the buffer at pH 7. Histogram bars represent mean+SEM (*N*=5)

temperature program began at 70°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 10 min. Additionally, binding was measured by a competitive binding assay with *N*-phenyl-1-naphthylamine (NPN) as a fluorescent reporter (Ban et al. 2002). Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer.

Insects Details of *A. transitella* rearing have been previously described (Parra-Pedrazzoli and Leal 2006). Briefly, larvae were raised on roasted pistachio in growth chambers (Percival Scientific, Perry, IA, USA) under 28±2°C temperature, 75±10% relative humidity (RH), and a 16:8h (light:dark) photo regime. Emerging moths were collected daily, separated by sex, and transferred to plastic boxes (12×12×5 cm; 669 ml lunchbox, TakeAlong containers, Rubbermaid, Fairlawn, OH, USA) provided with up to ten layers of water soaked paper towels (Thirsty Ultra Absorbent, 27.9×27.9 cm; Safeway, Phoenix, AZ, USA). Box covers were perforated to allow air circulation. This arrangement provided ~100% RH.

Electroantennogram (EAG) Recordings We developed a method wherein an adult moth is restrained in a pipette tip (200 µl, USA Scientific Inc., Ocala, FL, USA) that was cut from the top to have a large (ca. 2 mm) tip diameter. The moth was pushed gently through the base of the pipette tip with a humidified tissue paper stub until the antennae and part of the head protruded from the tip. The head was immobilized with a non-drying clay (Claytoon™, Van Aken International, CA, USA) leaving a small part of one eye and two antenna exposed. The pipette tip holding the

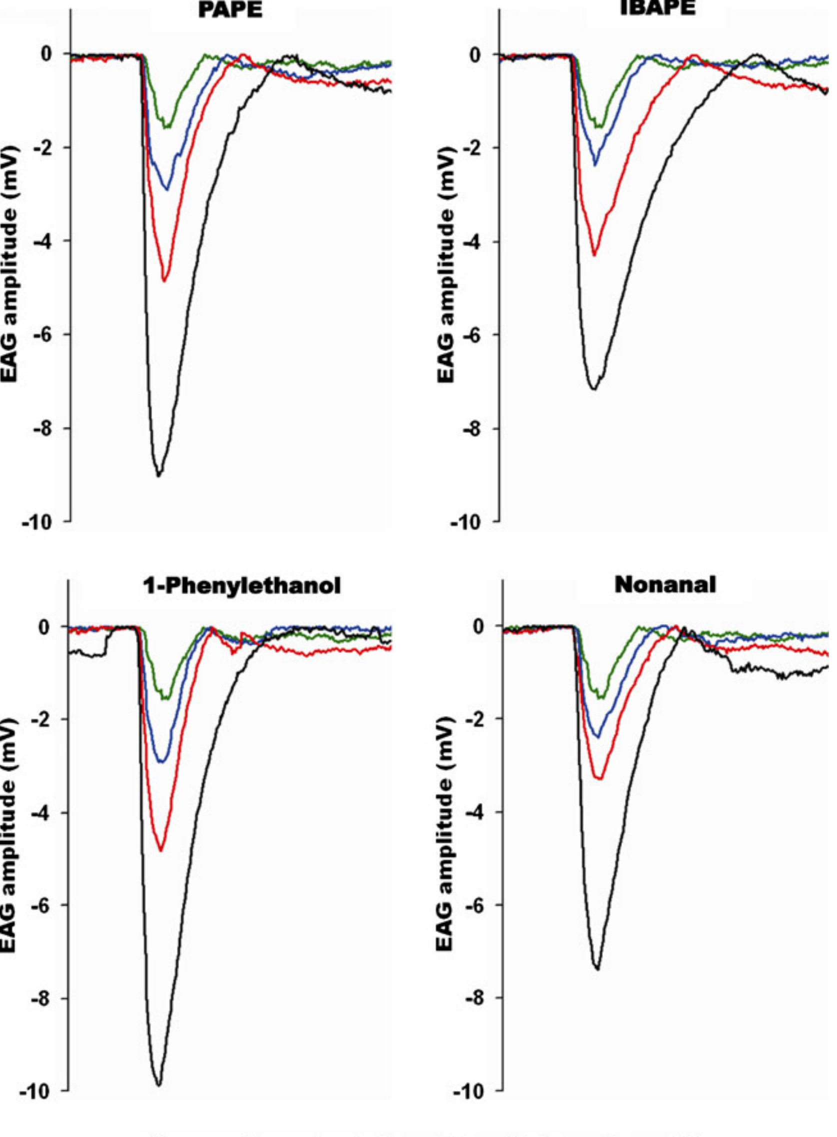


Fig. 3 EAG traces recorded from antennae of live female *Amyelois transitella* in response to PAPE, IBAPE, 1-phenylethanol, and nonanal. All traces from top to bottom show the responses to control, and increasing doses of the test compounds (from 0.1 to 10 µg/µl source dose)

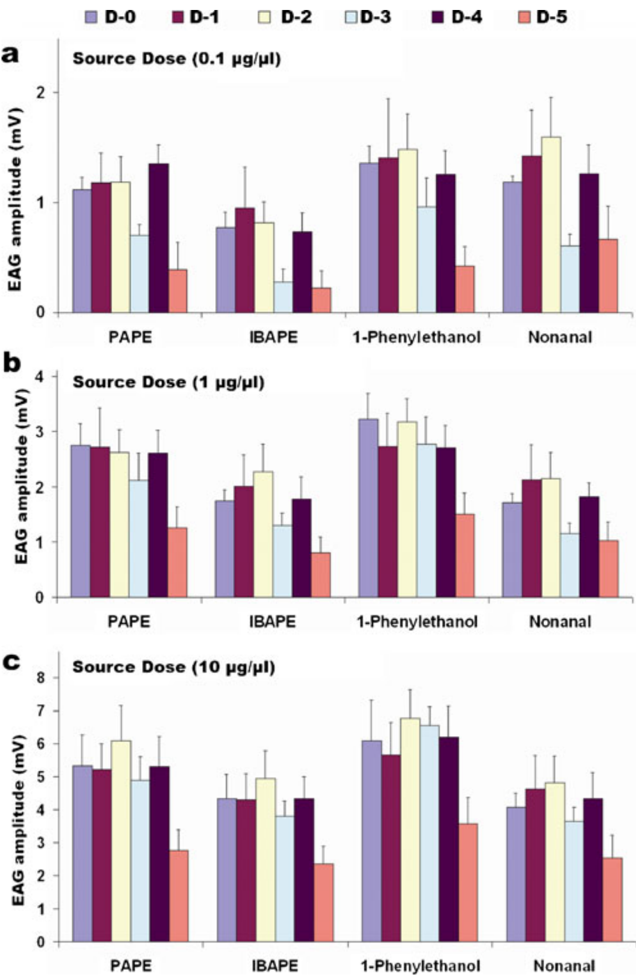


Fig. 4 Age-dependent responses of female *Amyelois transitella* antennae to attractants and nonanal. Responses displayed in (**a**), (**b**), and (**c**) were recorded with 0.1, 1, and 10 µg/µl source dose, respectively. Histogram bars represent mean+SEM (N=10)

restrained moth was placed on a platform of the EAG Micromanipulator MP-12 (Syntech, Kirchzarten, Germany) between the two electrode holders. A saline filled electrode impaled the exposed area of the eye under the microscope (Leica MZ75) that served as reference while the recording electrode contacted the two antennae whose distal segments were excised. Indifferent and recording electrodes were filled with 0.1M KCl solution with 0.5% polyvinylpyrrolidone. The EAG signals were amplified, monitored, and analyzed with software EAG 2000 (Syntech). The preparation was held in a humidified air stream delivered by the Syntech stimulus controller (CS-55 model; Syntech) at 26 ml/sec to which a stimulus pulse of 4 ml/s was added for 500 ms. Signals were recorded for 10 s**ec**, starting 2 sec before the onset of the stimulus pulse. The antennal preparation was stimulated with a 500 ms pulse during which ca. 2 ml of the purified air from a 5 ml polypropylene syringe containing the stimulus were added into the main air stream. Throughout this study, charcoal-filtered atmospheric air was used for the main airflow and stimulus delivery. To prevent changes in airflow during stimulation, a compensatory charcoal-filtered airflow of 2 ml/sec was delivered via another solenoid valve through a blank syringe into the glass tube, and at the same distance from the preparation. Following high responses, a gap of at least 1 min was allowed between stimulations.

Chemical Stimuli Nonanal and 1-phenylethanol (both >90% pure) were from Fluka (St. Louis, MO, USA). Propionic acid phenyl ester (PAPE) and isobutyric acid phenyl ester (IBAPE) (both >95% pure) were purchased from Tokyo Chemical Industry (Portland, OR, USA). (*Z,Z*)-11,13-Hexadecadienal (Z11Z13-16Ald), (*Z,Z*)-11,13-hexadecadienol (Z11Z13-16OH), and (*Z,Z,Z,Z,Z*)-3,6,9,12,15-tricosapentaene (Z3Z6Z9Z12Z15-23Hy) were

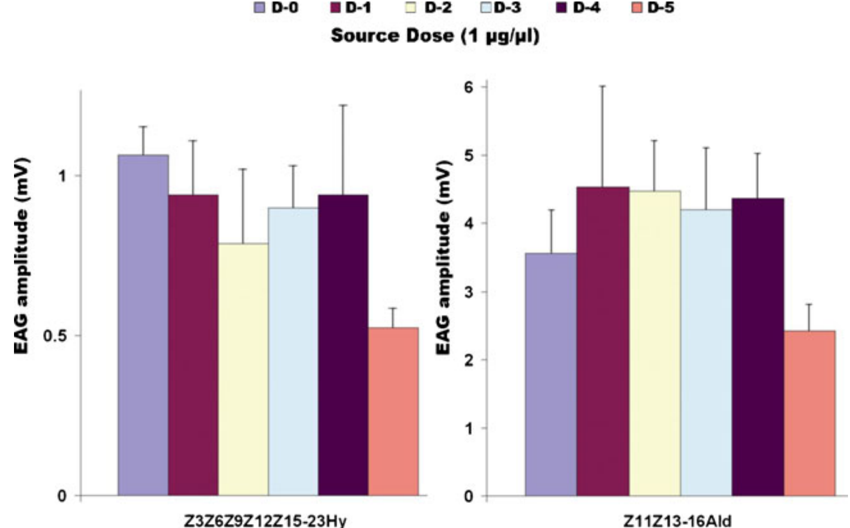


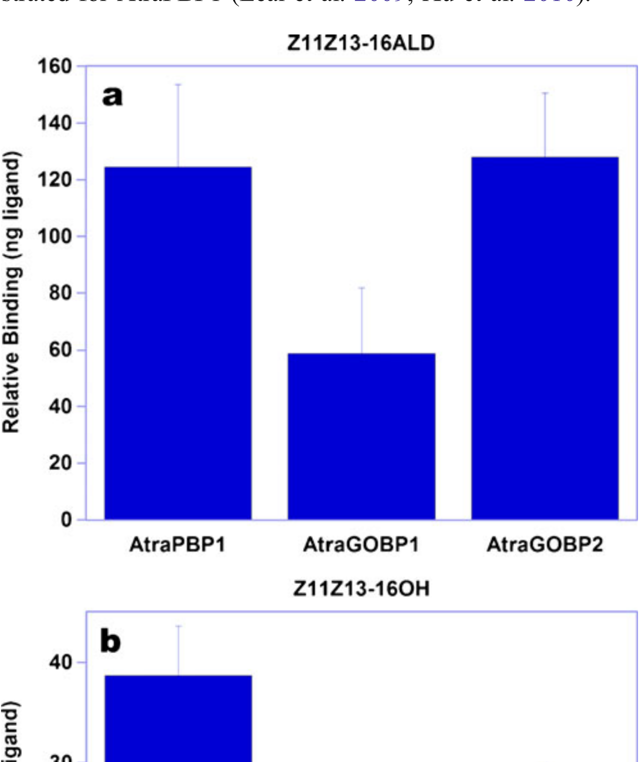
Fig. 5 Age-dependent responses of male *Amyelois transitella* antennae to the major constituent of the sex pheromone, Z11Z13-16Ald, and a key secondary constituent, Z3Z6Z9Z12Z15-23Hy. Histogram bars represent mean+SEM (N=10)

obtained from Bedoukian Research Inc. (Danbury, CT, USA). All chemicals were diluted, w/v, with hexane distilled in an all-glass apparatus to make a stock solution of 10 µg/µl, and decadic dilutions were made. An aliquot (10 µl) of a stimulus solution was loaded onto a filter paper strip (8×40 mm), hexane was evaporated by gently shaking for 10 sec under a fumehood, and the strip was placed in a 5 ml polypropylene syringe from which various volumes were ejected. Hexane alone and an empty syringe served as negative controls.

Results and Discussion

By using a previously developed protocol for functional expression of OBPs (Wojtasek and Leal 1999), we generated recombinant samples of AtraGOBP1 and AtraGOBP2 of high purity (>98%, by LC-MS). LC-ESI-MS analysis of AtraGOBP1 gave a molecular mass of 16,897 Da consistent with the calculated molecular mass of 16,903 Da (Leal et al. 2009) given the formation of three disulfide bridges (expected 16,897 Da). Deconvolution of the mass spectrum from AtraGOBP2 gave a molecular mass of 16,161 Da in close agreement with the calculated molecular mass of 16,166 Da (Leal et al. 2009) considering the formation of three disulfide linkages (expected, 16,160 Da). Far-ultraviolet (UV)-circular dichroism (CD) spectra from both proteins resembled that of the pheromone-binding protein AtraPBP1 (Leal et al. 2009), with maximum at ca. 193 nm and two minima at 209 nm and 223 nm (Fig. 1). Therefore, both AtraGOBP1 and AtraGOBP2 are α-helical-rich proteins, which is a common feature of moth OBPs (Wojtasek and Leal 1999). The spectra from AtraGOBP1 and AtraGOBP2 differed slightly in the maximum and in the intensity of the first of the two minima (Fig. 1).

Because gene expression analysis suggested that AtraGOBP1 and AtraGOBP2 are highly expressed in female antennae (Leal et al. 2009), we hypothesized that these proteins might be involved in the reception of attractants. We then tested by a cold binding assay (Leal et al. 2005a) whether AtraGOBP1 and/or AtraGOBP2 would bind semiochemicals like PAPE, IBAPE, and 1-phenylethanol, which are commonly referred to as female attractants (Price et al. 1967). Surprisingly, none of these ligands bound to the test proteins, but high background levels occurred even when these ligands were incubated with a buffer solution (devoid of protein), precluding an unambiguous conclusion. We then tested competitive binding by fluorescence by using a NPN reporter (Ban et al. 2002). None of the tested ligands displaced NPN, thus confirming that PAPE, IBAPE, and 1-phenylethanol do not bind to AtraGOBP1 or AtraGOBP2. To confirm that the recombinant proteins were functional, we searched the literature for other possible test compounds. Evidence that a GOBP from the Asiatic rice borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae), has high affinity for aldehydes (Gong et al. 2009b) led us to test nonanal, which has been identified in the headspace volatiles from almonds and demonstrated to stimulate antennae of female *A. transitella* in EAG studies (Beck et al. 2009). Nonanal bound with high affinity to both AtraGOBP1 (Fig. 2a) and AtraGOBP2 (Fig. 2b) at high pH. The fact that no binding was detected at low pH (Fig. 2) suggests that both AtraGOBP1 and AtraGOBP2 undergo pH-mediated conformational changes, as previously demonstrated for AtraPBP1 (Leal et al. 2009; Xu et al. 2010).



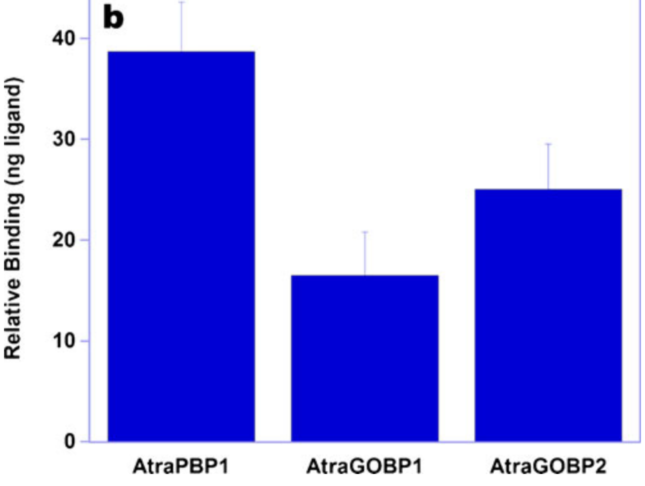


Fig. 6 Binding of the aldehyde and alcohol constituents of the *Amyelois transitella* sex pheromone system to male and female olfactory proteins. **a** test ligand, Z11Z13-16Ald and **b** test ligand, Z11Z13-16OH. The high affinity of the aldehyde to AtraPBP1 and AtraGOBP2 is comparable (**a**), whereas the alcohol showed higher affinity for AtraPBP1 than AtraGOBP2. AtraGOBP1 bound both aldehyde and alcohol with low but comparable affinity. Histogram bars represent mean+SEM (N=5)

The observation that both AtraGOBP1 and AtraGOBP2 were functional but do not bind known female attractants (Price et al. 1967) prompted us to test whether PAPE, IBAPE, and 1-phenylethanol indeed are detected by female antennae. EAG recordings from live moths showed that female antennae responded to these attractants as well as to nonanal in a dose-dependent manner (Fig. 3). With immobilized female moths we were able to record EAG response as high as 10 mV at the highest dose tested (Fig. 3). During these measurements, we noticed a small change in EAG responses with the age of the test moth. Therefore, we determined the effect of age on EAG response to female attractants. Regardless of the dose (0.1, 1, or 10 μg/μl source dose) and the compound tested, female antennal responses decreased significantly at day-5 (Fig. 4). At lower doses, we noticed a reduced response from day-3 moths in a few cases, but the fact that the responses of day-4 moths were always higher and that these discrepancies were not observed at a higher dose (10 μg/μl source dose) suggests the variations at day-3 with lower doses are not physiologically significant.

We then compared the age dependence on EAG responses of male antennae by stimulating with constituents of the *A. transitella* sex pheromone system (Leal et al. 2005b). With both the major component of the female-produced sex pheromone, Z11Z13-16Ald, and another key component, Z3Z6Z9Z12Z15-23Hy, we observed a similar trend (Fig. 5). EAG responses to these pheromones recorded from male moth antennae were almost unchanged from day-0 to day-4, with a significant decrease in day-5, thus mirroring female response to female attractants. Overall EAG responses to Z11Z13-16Ald were (≈4x) higher than those to Z3Z6Z9Z12Z15-Hy at the same concentration, in part because of the lower volatility of the hydrocarbon compared to the smaller aldehyde. Male and female antennae responded with comparable intensity to Z11Z13-16Ald and 1-phenylethanol, respectively, tested at the same dose (compare Figs. 4b and 5).

Finally, we tested whether AtraGOBP1 and/or AtraGOBP2 would bind and discriminate the aldehyde and alcohol constituents of the *A. transitella* sex pheromone as reported from a GOBP from *B. mori* (Zhou et al. 2009). Both AtraGOBP1 and AtraGOBP2 bound Z11Z13-16Ald and Z11Z13-16OH in a pH-dependent manner, i.e., high affinity at high pH and significantly lower (virtually no) binding affinity at low pH. Interestingly, AtraGOBP2 bound to the main constituent of the sex pheromone with affinity equivalent to that of AtraPBP1 (Fig. 6a). The secondary constituent, Z11Z13-16OH, bound to AtraGOBP2 with smaller apparent affinity than to AtraPBP1 (Fig. 6b). On the other hand, AtraGOBP1 bound both Z11Z13-16Ald and Z11Z13-16OH, but with lower affinity than that observed for both AtraGOBP2 and AtraPBP1 (Fig. 6).

Taken together, these data suggest that reception of PAPE, IBAPE, and 1-phenylethanol might involve other OBPs than AtraGOBP1 and AtraGOBP2. Since we employed a protein-based approach for the isolation and cloning of olfactory proteins, we were able to identify only proteins expressed at high levels. Therefore, we cannot exclude that other OBPs are involved in the reception of these female attractants in *A. transitella*. The genome of *B. mori*, for example, showed a much higher OBP diversity than previously envisioned from isolated OBPs (Gong et al. 2009a). As indicated by EAG data, these attractants are indeed perceived by female antennae, but their transport to the receptors is not likely to be mediated by either AtraGOBP1 or AtraGOBP2. Interestingly, these proteins bound constituents of the sex pheromone, with the affinity of AtraGOBP2 to the main component being comparable to that of a pheromone-binding protein, AtraPBP1. Given the previous examples of GOBPs binding pheromone components in *A. polyphemus* and *B. mori* (Ziegelberger 1995; Zhou et al. 2009) and our data, it is tempting to suggest that these AtraGOBPs, particularly AtraGOBP2, are involved in pheromone reception. The larger number of pheromone constituents (Leal et al. 2005b) compared to the number of known PBPs (Leal et al. 2009) and the affinity of GOBPs for pheromones favor this hypothesis. However, evidence for the expression of GOBPs in pheromone-detecting sensilla and how reducing their expression by RNA interference (Pelletier et al. 2010) may affect pheromone reception remain exciting topics for future research.

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J Chem Ecol (2010) 36:795–796
DOI 10.1007/s10886-010-9800-8

ERRATUM

Erratum to: Nestmate Recognition and the Role of Cuticular Hydrocarbons in the African Termite Raiding Ant *Pachycondyla analis*

Abdullahi A. Yusuf · Christian W. W. Pirk · Robin M. Crewe · Peter G. N. Njagi · Ian Gordon · Baldwyn Torto

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**Erratum to: J Chem Ecol (2010) 36:441–448
DOI 10.1007/s10886-010-9774-6**

In the original version of this article, some data on Table 1 were incorrect. The corrected Table is given below. The authors regret this error.

The online version of the original article can be found at http://dx.doi.org/10.1007/s10886-010-9774-6.

A. A. Yusuf · P. G. N. Njagi · I. Gordon · B. Torto
ICIPE,
P. O. Box 30772-00100, GPO, Nairobi, Kenya

A. A. Yusuf (✉) · C. W. W. Pirk · R. M. Crewe
Social Insects Research Group,
Department of Zoology and Entomology, University of Pretoria,
0002 Pretoria, South Africa
e-mail: ayusuf@icipe.org
e-mail: aayusuf@zoology.up.ac.za

Table 1 Compounds identified from the cuticular hydrocarbon profiles of *Pachycondyla analis*, along with retention indices and diagnostic ions

No[a]	Compound	Ri[b]	Diagnostic EI-MS ions (m/z)
1	*n*-Octane	800	114
2	*n*-Undecane	1100	156
3	5-Methylundecane	1154	43, 57, 71, 85, 99, 112
4	3-Methylundecane	1169	43, 57, 71, 85, 99, 112, 141, 170
5	Unidentified		
6	*n*-Tridecane	1300	184
7	*n*-Pentadecane	1500	212
8	3-Methylpentadecane	1572	43, 57, 71, 85, 99, 113, 127, 141, 155, 168, 197, 226
9	2-Methylheptadecane	1765	43, 57, 71,85, 99, 113, 127, 141, 155, 169, 183, 195, 211, 239, 254
10	1-Heptadecene	1679	83, 97, 111, 125, 196, 210, 239
11	8-Heptadecene	1679	41, 55, 69, 83, 97, 111, 125, 140, 238
12	5-Octadecene	1789	43, 55, 69, 83, 97, 111, 125, 139, 166, 180, 195, 224, 252
13	*n*-Octadecane	1800	254
14	9-Nonadecene	1875	43, 55, 69, 83, 97, 111, 125, 139, 153, 167, 238, 266
15	*n*-Nonadecane	1900	268
16	*n*-Eicosane	2000	282
17	*n*-Heneicosane	2100	296
18	1-Docosene	2195	43, 57, 69, 83, 97, 111, 125, 280, 308
19	*n*-Docosane	2200	310
20	(*Z*)-9-Tricosene	2270	43, 55, 69, 83, 97, 111, 125, 139, 153, 223, 237, 294, 322
21	*n*-Tricosane	2300	324
22	Unidentified		
23	1-Tetracosene	2396	43, 57, 69, 85, 97, 113, 309, 338
24	*n*-Tetracosane	2400	338
25	Cyclotetracosane	2445	43, 57, 69, 83, 97, 111, 125, 139, 153, 207, 392
26	9-Pentacosene	2465	43, 57, 69, 85, 97, 113, 141, 169, 197, 326, 350
27	(*Z*)-12-Pentacosene	2500	352
28	*n*-Pentacosane	2496	43, 57, 69, 83, 97, 125, 236, 257, 290, 322, 350
29	1-Hexacosene	2593	43, 57, 69, 83, 97, 111, 125, 139, 336, 364
30	*n*-Hexacosane	2600	366
31	*n*-Heptacosane	2700	380
32	*n*-Octacosane	2800	394
33	Squalene	2663	41, 55, 69, 81, 95, 109, 121, 136, 148, 341, 367, 410
34	*n*-Nonacosane	2900	408
35	*n*-Hentriacontane	3100	436

[a] No = Peak numbers referring to Fig. 2

[b] *RI* Retention Index

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RAPID COMMUNICATION

Odorant Receptor from the Southern House Mosquito Narrowly Tuned to the Oviposition Attractant Skatole

David T. Hughes · Julien Pelletier · Charles W. Luetje · Walter S. Leal

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Abstract Oviposition attractants are environmental cues that allow *Culex* gravid female mosquitoes to locate suitable sites for egg-laying and, therefore, may be exploited for environmentally friendly strategies for controlling mosquito populations. Naturally occurring skatole has been identified as an oviposition attractant for the Southern House mosquito, *Culex quinquefasciatus.* Previously, we identified in *Cx. quinquefasciatus* female antennae an olfactory receptor neuron (ORN) highly sensitive to skatole and an odorant-binding protein involved in the detection of this semiochemical. Here, we describe the characterization of an odorant receptor (OR), CquiOR10, which is narrowly tuned to skatole when expressed in the *Xenopus* oocyte system. Odorant-induced response profiles generated by heterologously expressed CquiOR10 suggest that this OR is expressed in the mosquito ORN sensitive to skatole. However, geranylacetone, which stimulates the antennal ORN, was not detected by CquiOR10-expressing oocytes, thus raising interesting questions about reception of oviposition attractants in mosquitoes.

Key Words Odorant receptor · CquiOR10 · *Culex quinquefasciatus* · *Xenopus* oocyte expression system · 3-Methylindole · 2-Methylphenol

D. T. Hughes · C. W. Luetje
Department of Molecular and Cellular Pharmacology,
University of Miami Miller School of Medicine,
Miami, FL, USA

J. Pelletier · W. S. Leal (✉)
Department of Entomology, University of California Davis,
1 Shields Ave,
Davis, CA 95616, USA
e-mail: wsleal@ucdavis.edu

Introduction

Culex mosquitoes are vectors of pathogens including the human filarial nematode, *Wuchereria bancrofti*, and encephalitis-causing viruses, such as St. Louis, Japanese, Venezuela equine, Western equine encephalitis, and West Nile virus (Nasci and Miller, 1996).[1] Given the resistance of *Culex* populations to modern insecticides, alternative methods of controls are sorely needed. Larval development is a particularly vulnerable phase in the life cycle of *Culex* mosquitoes, as eggs are laid in rafts from which hundreds of larvae emerge in confined areas—thus facilitating management. Gravid females rely on environmental oviposition attractants to locate oviposition sites. Skatole, a natural product found in animal excreta and also a product of fermentation of organic material, has been identified as an oviposition attractant for the Southern House mosquito, *Culex quinquefasciatus* (Millar et al., 1992). Field studies have demonstrated that traps baited with optimal doses of skatole collected significantly more eggs (Mboera et al., 2000) and gravid females (Leal et al., 2008) than control traps, thus suggesting that in combination with a biological agent, *Bacillus thuringiensis* var. *israelensis* (Barbosa et al., 2010) oviposition attractants may be used in "attract-and-kill" strategies. Chemical ecology and olfaction are the pillars of these semiochemical-based, environmentally friendly strategies. Therefore, identification of olfactory proteins involved in the reception of these semiochemicals may open the door for development of better oviposition attractants. Recently, we demonstrated by RNA interference that an odorant-binding protein (OBP) from *Cx. quinquefasciatus*, CquiOBP1, is involved directly in the

[1] We apologize for not being able to cite all the relevant literature due to reference limitations of rapid communication format.

reception of skatole and other oviposition attractants (Pelletier et al., 2010a). We also have characterized an odorant receptor (OR) from this mosquito species, CquiOR2, which is highly sensitive to indole and moderately sensitive to skatole (Pelletier et al., 2010b). Here, we characterize CquiOR10 and show this OR to be highly sensitive and narrowly tuned to skatole.

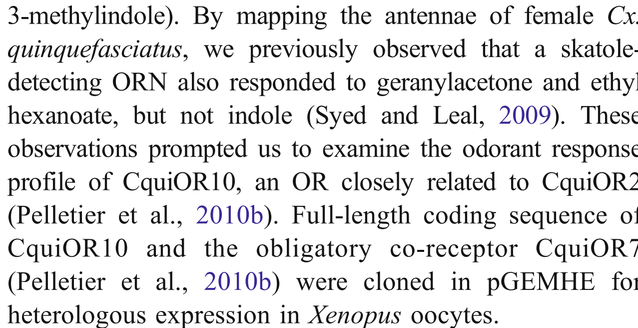
Methods and Materials

Expression of CquiOR10 in the Xenopus Oocyte System Oocytes were prepared as previously described (Pelletier et al., 2010b). CquiOR10 and CquiOR7, initially cloned into pBlueScript (Pelletier et al., 2010b), were transferred to pGEMHE for use as templates for synthesis of capped cRNA by using mMessage mMachine kits (Ambion). Twenty-five ng of cRNA encoding each OR subunit were injected into Stage V–VI Xenopus oocytes. Oocytes were incubated at 18°C in Barth's saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.3 CaNO3, 0.41 CaCl2, 0.82 MgSO4, 15 HEPES, pH 7.6, and 100 μg/ml amikacin) for 2–5 d prior to electrophysiological recording.

Electrophysiology and Data Analysis Odorant-induced currents were recorded under two-electrode voltage clamp from oocytes expressing ORs, by using an automated parallel electrophysiology system (OpusXpress 6000A; Molecular Devices). Oocytes were perfused with ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES, pH 7.5). Odorants were diluted in ND96 and applied for 20 sec at a flow rate of 1.65 ml/min with extensive washing in ND96 (5–20 min at 4.6 ml/min) between applications. Current responses approached a plateau during the 20 sec application (Pelletier et al., 2010b). Micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. The holding potential was −70 mV. Current responses were filtered (4-pole, Bessel, low pass) at 20 Hz (−3 db), sampled at 100 Hz, and were captured and stored with OpusXpress 1.1 software (Molecular Devices). Initial analysis of electrophysiological data was done with Clampfit 9.1 software (Molecular Devices). Curve fitting of concentration-response data was done with Prism 4 (Graphpad).

Results and Discussion

In our search for molecular targets that may be used in a reverse chemical ecology approach for the development of better oviposition attractants (Leal et al., 2008), we recently have mined the genome of *Cx. quinquefasciatus* and identified an OR sensitive to indole, CquiOR2, which also responded to methylindoles, including skatole (IUPAC name,



3-methylindole). By mapping the antennae of female *Cx. quinquefasciatus*, we previously observed that a skatole-detecting ORN also responded to geranylacetone and ethyl hexanoate, but not indole (Syed and Leal, 2009). These observations prompted us to examine the odorant response profile of CquiOR10, an OR closely related to CquiOR2 (Pelletier et al., 2010b). Full-length coding sequence of CquiOR10 and the obligatory co-receptor CquiOR7 (Pelletier et al., 2010b) were cloned in pGEMHE for heterologous expression in *Xenopus* oocytes.

To identify the best ligand for this receptor, oocytes expressing CquiOR10 + CquiOR7 were screened first with a panel of odorants (Fig. 1a), each applied for 20 sec at a concentration of 10 μM with extensive washing between applications. Skatole (3-methylindole) elicited the largest current responses, but the receptor also responded with lower sensitivity to indole, other methylindoles, and 2-methylphenol. Interestingly, CquiOR10 was unresponsive to many compounds in the test panel of 23 odorants, including other oviposition attractants such as trimethylamine, nonanal, and the mosquito oviposition pheromone (MOP) (Leal et al., 2008).

Next, we performed concentration-response analyses for skatole and two other ligands, indole and 2-methylphenol, which were identified as the best ligands among the indoles and phenols, respectively, for the related receptor CquiOR2 (Pelletier et al., 2010b). Skatole was the most potent of these compounds, activating the CquiOR10 + CquiOR7 receptor with an EC_{50} of 90 nM. Indole and 2-methylphenol were less potent, activating CquiOR10 + CquiOR7 with EC_{50} values of 2.4 μM and 41 μM, respectively. Interestingly, indole and 2-methylphenol also displayed lower efficacy (maximal response) than skatole (40±2% and 69±3% of skatole, respectively). In addition, the response threshold for skatole was two to three orders of magnitude higher than that observed for indole and 2-methylphenol (Fig. 1b, c). Thus, we found that heterologously expressed CquiOR10 is highly sensitive and narrowly tuned to the oviposition attractant skatole.

In female antennae of the Southern House mosquito, skatole is detected by a small-spike-amplitude ORN housed in A1 sensilla (Syed and Leal, 2009), which is also sensitive to a lower degree to geranylacetone and ethyl hexanoate, but does not respond to indole or 2-methylphenol [see Figs. S5, S6, supporting information in (Syed and Leal, 2009)]. In the *Xenopus* oocyte system, CquiOR10 was unresponsive to geranylacetone. Although expression of ORs in heterologous systems, such as the *Xenopus* oocyte system, is an invaluable tool for de-orphanizing and characterizing receptors, it does not completely mimic the insect olfactory system. Typically, these systems are devoid of OBPs, odorant-degrading enzymes, sensory neuron membrane proteins, and other

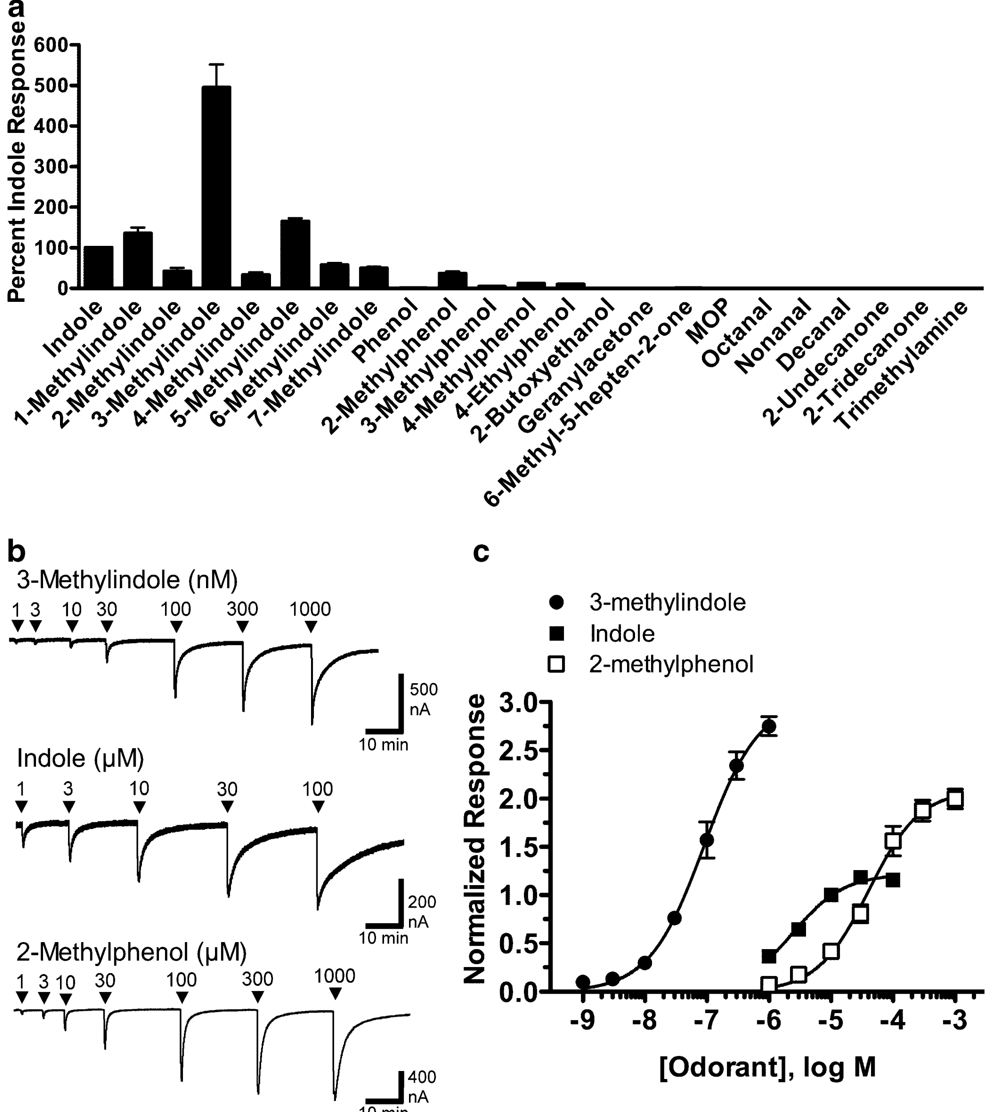


Fig. 1 Odorant receptor CquiOR10 is highly sensitive to skatole (3-methylindole). **a** Oocytes expressing CquiOR10 + CquiOR7 were challenged with a panel of odorant compounds. Each odorant was applied at a concentration of 10 μM for 20 sec with 10 min washes between applications. All responses are normalized to the response of the same oocyte to 10 μM indole (mean ± SEM, N=4–5). **b** Oocytes expressing CquiOR10 + CquiOR7 were challenged with a range of concentrations of 3-methylindole (*top trace*), indole (*middle trace*) or 2-methylphenol (*bottom trace*). Each odorant was applied for 20 sec with 5–20 min washes between applications. Note different scales: from top to bottom 500, 200 and 400 nA. **c** Oocytes expressing CquiOR10 + CquiOR7 were challenged with a range of concentrations of 3-methylindole, indole, and 2-methylphenol. Responses were normalized to the response of each oocyte to 10 μM indole and are presented as mean ± sem (N=3–5 for each odorant tested). Data were fit to the equation: $I = I_{max}/(1 + (EC_{50}/X)^n)$ where I represents the current response at a given concentration of odorant (X), I_{max} is the maximal response, EC_{50} is the concentration of odorant yielding a half maximal response, and n is the apparent Hill coefficient. Derived values are: 3-methylindole, EC_{50}=90±17 nM, N=1.0±0.1; indole, EC_{50}=2.4±0.3 μM, N=1.1±0.2; 2-methylphenol, EC_{50}=41±7 μM, N=1.0±0.1

olfactory proteins that may play a part in the selectivity and sensitivity of the olfactory system. Thus, it is conceivable that heterologously expressed CquiOR10 and the receptor in its native environment differ in the detection of geranylacetone because the former is devoid of OBPs. However, one cannot rule out the possibility that a separate ORN responding to geranylacetone has the same spike amplitude as the skatole-detecting ORN (Syed and Leal, 2009), thus rendering them indistinguishable by single unit recordings. Alternatively, the same small-spike neuron sensitive to skatole may express another OR along with CquiOR10. In marked contrast to the mammalian olfactory system, co-expression of ORs has been documented in *Drosophila melanogaster*. Co-expression of CquiOR10 and another OR would not be entirely surprising given the number of putative odorant receptors in the Southern House

mosquito genome (Pelletier et al., 2010b) and the number of ORNs in their sensory system (Syed and Leal, 2007, 2008). Future research aimed at testing these three hypotheses might lead to deeper understanding of odorant reception in mosquitoes.

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Binding Specificity of Recombinant Odorant-Binding Protein Isoforms is Driven by Phosphorylation

Fanny Brimau · Jean-Paul Cornard · Chrystelle Le Danvic · Philippe Lagant · Gerard Vergoten · Denise Grebert · Edith Pajot · Patricia Nagnan-Le Meillour

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Abstract Native porcine odorant-binding protein (OBP) bears eleven sites of phosphorylation, which are not always occupied in the molecular population, suggesting that different isoforms could co-exist in animal tissues. As phosphorylation is a dynamic process resulting in temporary conformational changes that regulate the function of target proteins, we investigated the possibility that OBP isoforms could display different binding affinities to biologically relevant ligands. The availability of recombinant proteins is of particular interest for the study of protein/ligand structure-function relationships, but prokaryotic expression systems do not perform eukaryotic post-translational modifications. To investigate the role of phosphorylation in the binding capacities of OBP isoforms, we produced recombinant porcine OBP in two eukaryotic systems, the yeast, *Pichia pastoris*, and the mammalian CHO cell line. Isoforms were separated by anion exchange HPLC, and their phosphorylation sites were mapped by MALDI-TOF mass spectrometry and compared to those of the native protein. Binding experiments with ligands of biological relevance in the pig, *Sus scrofa*, were performed by fluorescence spectroscopy on two isoforms of recombinant OBP expressed in the yeast. The two isoforms, differing only by their phosphorylation pattern, displayed different binding properties, suggesting that binding specificity is driven by phosphorylation.

Key Words Anion exchange HPLC · Fluorescence spectroscopy · Heterologous expression · Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry · Odorant-binding protein · Peptide mapping · Pheromone · Phosphorylation · Polymerase chain reaction (PCR) · Recombinant protein · Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) · *Sus scrofa* · Western blotting

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F. Brimau · C. Le Danvic · P. Nagnan-Le Meillour (✉)
INRA, UMR8576 CNRS/USTL, UGSF,
59655 Villeneuve d'Ascq, France
e-mail: patricia.le-meillour@univ-lille1.fr

J.-P. Cornard
UMR8516 CNRS/USTL, LASIR,
59655 Villeneuve d'Ascq, France

P. Lagant · G. Vergoten
CNRS, UMR8576 CNRS/USTL, UGSF,
59655 Villeneuve d'Ascq, France

D. Grebert · E. Pajot
INRA, UR1197 NOeMI,
78350 Jouy-en-Josas, France

Introduction

The pheromonal system in mammals is considered to be sensitive and specific (Tirindelli et al. 2009), requiring specific interactions between the molecular elements involved at every step of the detection and integration pathways (Brennan and Kendrick 2006). The first level of specificity is ensured by the nature of the pheromone itself: Its composition is stable within a species, both quantitatively and qualitatively. Integration of the signal from its reception by sensory organs to the data processing by the brain is highly specific (Brennan and Kendrick 2006). The olfactory receptors are also specific. They have only a small tolerance for changes in size or branching of ligand analogs (Buck 2004; Kato and Touhara 2009).

One step where the specificity is not clear is at the peripheral level of pheromone reception, in particular the

role of odorant-binding proteins (OBP) in olfactory transduction. These soluble proteins are secreted by specialized glands in the vicinity of olfactory neurons and function at least as vehicles of odors and pheromone component molecules, but a more specific role in their binding was suggested when they were discovered (Pelosi et al. 1982). Arguing against the specificity of OBP is their low diversity in each animal species, and their broad binding spectrum to molecules of different chemical structures (Pelosi 1996, 2001). In the last decade, structural studies [X-ray crystallography (Spinelli et al. 1998; Vincent et al. 2000), fluorescence spectroscopy (Paolini et al. 1999), and isothermal calorimetry (Burova et al. 1999)] were conducted on model OBPs (bovine, porcine), which were obtained by purification of the major form from nasal tissue. Alternatively, recombinant proteins were produced in high yields by overexpression in organisms such as bacteria and yeast (Parisi et al. 2005; Wei et al. 2008; Nagnan-Le Meillour et al. 2009a). These cells, however, display different metabolic pathways from mammalian cells, and the corresponding recombinant proteins do not always resemble their native counterparts. In particular, post-translational modifications (PTM) such as disulfide bridge formation, glycosylation, or phosphorylation, if present, can differ strongly from that obtained in mammalian cells (Narhi et al. 1991; Daly and Hearn 2005).

There is growing evidence that OBPs are involved in the first step of odorant discrimination by 1) enhancing receptor sensitivity and specificity (Ko and Park 2008; Vidic et al. 2008; Ko et al. 2010), and/or 2) selectively binding odorant molecules of various chemical structure by sub-types differing in their primary sequence (Loëbel et al. 1998; Briand et al. 2000; Tcatchoff et al. 2006). In addition, we demonstrated recently that a dynamic mechanism involving PTM could be responsible for OBP diversity and specificity (Le Danvic et al. 2009; Nagnan-Le Meillour et al. 2009b). Indeed, porcine OBP can be posttranslationally modified by phosphorylation, and we suggested that this PTM could generate a diversity of OBP isoforms with specific binding properties (Nagnan-Le Meillour et al. 2009b). PTM is known to regulate signalling pathways, and phosphorylation could be a mechanism of regulation of OBP specificity towards odorant ligands. To investigate further the binding specificity of potential phosphorylated isoforms, we undertook the production of recombinant OBP in two different eukaryotic systems, the yeast, *Pichia pastoris*, which successfully expresses high yields of secreted OBP, and a mammalian cell line, CHO (Chinese Hamster Ovary), the metabolic pathways of which could be close to those of pig, *Sus scrofa*, glandular cells. Our goal was to validate the use of recombinant proteins to study further the role of phosphorylation in OBP binding to pheromone components. We purified OBP isoforms by anion-exchange HPLC and we mapped their phosphorylation sites, which were compared with those previously characterized in the native form of OBP (Nagnan-Le Meillour et al. 2009b). Binding experiments with ligands of biological relevance to *S. scrofa* (male and female pheromones) were conducted by a fluorescent probe displacement assay on two isoforms of recombinant OBP expressed in the yeast. The two isoforms, differing only by their phosphorylation pattern, displayed different binding properties, suggesting that this binding specificity is indeed driven by phosphorylation.

Methods and Materials

Expression of Recombinant OBP in Yeast (OBP-Pichia) The recombinant porcine odorant binding protein (OBP) was expressed in the methylotrophic yeast, *Pichia pastoris* (GS 115 strain) and purified as previously described (Nagnan-Le Meillour et al. 2009a). After a ten-fold concentration (Vivaspin 20, 3000 MWCO, Sartorius, Palaiseau, France) and extensive dialysis against water, the culture supernatant was dried under vacuum (Eppendorf, Le Pecq, France) and stored at −20°C before purification.

Expression of Recombinant OBP in CHO cells (OBP-CHO) The cDNA sequence encoding OBP (GenBank accession number AF436848) was amplified by PCR from the *OBP/pPIC3.5K* plasmid used for expression in yeast, with the following primers: sense 5′-**CACC**ATGAAGAGTCTGCTGCTGAGT-3′ and antisense 5′-TCACTTGGCAGGACAGTCATCTCT-3′. The sense primer contains the CACC bases (in bold) allowing directional cloning into the *pcDNA™3.1D/V5-His-TOPO®* vector of the pcDNA™3.1 Directional TOPO Expression kit (Invitrogen, Paisley, UK), followed by codons of the natural signal peptide of the protein (underlined). PCR amplification was carried out on a Thermal Cycler (Mastercycler Gradient, Eppendorf) with 100 ng of cDNA template in a solution containing 1.25 U of Hot Start Proofreading DNA polymerase (Accu Prime™ *Pfx*, Invitrogen), 0.3 µM of each PCR primer, and 1 X of DNA polymerase manufacturer's buffer containing dNTP in a final volume of 50 µl. The reaction cycles were performed as follows: 95°C for 2 min, followed by 35 cycles of 15 sec at 95°C, 30 sec at 60°C, and 1 min at 68°C. The single PCR product obtained was ligated into the *pcDNA3.1d* expression vector according to the manufacturer's instructions. The plasmid DNA was amplified into One Shot® Top10 chemically competent cells (Invitrogen). After purification (QIAprep Spin Miniprep kit, QIAGEN, Courtaboeuf, France), the *OBP/pcDNA™3.1D* plasmid was sequenced in both senses (Genoscreen, Lille, France).

CHO-K1 cells (Chinese Hamster Ovary, clone K1) were grown in Dulbecco's Modified Eagle's Medium (DMEM F-12, Gibco BRL, Invitrogen) containing 10% non supplemented fetal bovine serum (FBS, Perbio, Thermo Fischer Scientific, Courtaboeuf, France) in a 5% (*v*:*v*) CO_2 atmosphere at 37°C. Cells were transfected with 3 µg of *OBP/pcDNA™3.1D* at a 50% confluence by using ExGen500 (Euromedex, Souffelweyersheim, France) in culture dishes. The supernatant medium was collected 48 hr after transfection.

Protein Purification Purification of recombinant proteins was achieved by high-resolution anion exchange liquid chromatography on a DIONEX HPLC device (Voisins-le-Bretonneux, France). Proteins were separated on a PROPAC PA1 column (4×250 mm), in 50 mM Tris/HCl, pH 6.0 (buffer A), by using a linear gradient of 0–1 M NaCl at a flow rate of 0.8 ml/min. Typically, samples of 500 µl in buffer A were injected, and resulting fractions were collected and extensively dialyzed against MilliQ water. OBP isoforms were identified in HPLC fractions by Western blot with anti-OBP antibodies (Nagnan-Le Meillour et al. 2009b). Their identity was confirmed by peptide mapping, followed by MALDI-TOF mass spectrometry as already described (Nagnan-Le Meillour et al. 2009b). A part of the recombinant OBP-Pichia was purified by FPLC (Nagnan-Le Meillour et al. 2009a), which does not separate the different isoforms (total OBP-Pichia). Recombinant proteins were quantified with the Micro BCA Protein Assay kit (PIERCE, Thermo Fischer Scientific).

Immunodetection of Phosphorylations with Specific Antibodies HPLC fractions (OBP-Pichia and OBP-CHO) and total OBP-Pichia were suspended in sample buffer, to be separated by SDS-PAGE (Nagnan-Le Meillour et al. 2009a). An aliquot of crude nasal tissue extract (Nagnan-Le Meillour et al. 2009b) was added as a migration control of OBP, together with molecular weight markers (Precision Plus Protein All Blue, Bio-Rad, Marnes-la-Coquette, France). Western blots with specific antibodies directed against phosphoserine, phosphotyrosine, and phosphothreonine (Invitrogen, Paysley, UK) were performed exactly as described in Nagnan-Le Meillour et al. (2009b). Detection of signal was carried out with an ECL Plus Western Blotting Detection kit (GE Healthcare, Orsay, France).

Mapping Phosphorylation Sites in Recombinant OBPs The sites of phosphorylation in recombinant OBP isoforms and total OBP-Pichia were determined by the BEMAD (Beta-Elimination followed by Michael Addition of DTT) method followed by MALDI-TOF MS analysis, fully described for total native OBP from pig nasal tissue in Nagnan-Le Meillour et al. (2009b). Briefly, total proteins and isoforms purified by HPLC were separated by SDS-PAGE and stained with Coomassie blue R solution. Band slices were treated with trypsin (T) and/or chymotrypsin (CT) without alkylation and reduction to generate specific peptides. Peptides then were incubated with alkaline phosphatase to remove phosphate groups prior to the linkage of DTT (dithiothreitol, Sigma-Aldrich, La Verpillière, France) by Michael addition. After a step of enrichment of DTT-modified peptides by using thiol columns, peptides were eluted directly into the matrix (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, La Verpillière, France) with 25 and 50% acetonitrile (Nagnan-Le Meillour et al. 2009b), and analyzed by MALDI-TOF MS. The theoretical masses of DTT-modified peptides were calculated from the OBP peptide map (NP_998961) by using the peptide-mass software at www://expasy.org/tools/peptide-mass.html.

Position of the Phosphate Groups in the Three-dimensional Structure of Porcine OBP Phosphate groups were added to the residues that could undergo phosphorylation in the OBP sequence. We used the complete set of atomic coordinates available for the non-phosphorylated OBP (PDB identifier 1E02) obtained after addition of the first eight N-terminal and the Lys158 C-terminal residues to the crystal structure obtained by Vincent et al. (2000) according to the method described in a previous work (Nagnan-Le Meillour et al. 2009a). We used the CHARMM program (McKerell et al. 1998) with the possibility of adding phosphated pre-residues, and by using a homemade program, the related Cartesian coordinates for the phosphate groups (mono-anionic) could be determined easily. Images were created with the MacPyMOL™ software.

Binding Assay Studies using Fluorescence Spectroscopy Androstenone (3-keto-5α-16-androstene), androstenol (3α-hydroxy-5α-androst-16-ene), testosterone (17β-hydroxy-3-oxo-4-androstene), palmitic acid (hexadecanoic acid), myristic acid (tetradecanoic acid), and AMA (1-aminoanthracene) were purchased from Sigma-Aldrich (La Verpillière, France).

UV-visible spectra were recorded on a double beam spectrometer (Cary-100 Varian, Les Ullis, France) with a cell of 0.5 cm path length. The protein concentration was calculated by UV-visible spectroscopy, by using the molar extinction coefficient of 11740/M•cm for OBP (calculated by the software "ProtParam tool" at www.expasy.org) at the maximum of absorption (280 nm). Fluorescence measurements were performed on a Fluoromax-3 (Jobin-Yvon, Longjumeau, France) spectrofluorimeter at 22°C with semi-micro quartz cell (0.5 cm light-path) with reflecting window. The steady-state fluorescence spectra were recorded at 0.5 nm intervals with an integration time of 1 sec. The solution absorbance was <0.1 at the excitation

wavelength, to avoid inner filter effects. The fluorescent probe AMA was dissolved in 100% ethanol as 1 mM stock solution. For protein titration, aliquots of AMA were successively added to the protein at 2.5 µM in 50 mM Tris buffer (pH 7.8), and emission spectra were acquired after a 15 min equilibration period. All solutions were excited at 295 nm, which corresponds to the protein absorption band, where the absorption of the Trp16 residue is much higher than that of tyrosines (Lakowitz 1999). Emission spectra were recorded without Glan-Thompson polarizers in the excitation and detection channels. The excitation and emission bandwidths were set to 3 nm. The spectra were recorded 3 times and averaged to increase the signal-to-noise ratio. Before data analysis, the spectra were normalized by taking the Raman line of water as reference. In order to obtain real intensities of the emission bands of free AMA and probe in interaction in the OBP pocket, a spectral decomposition of the fluorescence spectra was achieved with the LabSpec 5.21 software (Jobin Yvon). This decomposition was carried out after a conversion of the spectra into wave numbers.

The dissociation constant ($K_{d\text{-}AMA}$) of the OBP-AMA complex was calculated from the binding curve by fitting the experimental data with the use of the computer program Origin 7.5 (OriginLab Corporation, Northampton, MA, USA). The affinity of the five ligands was measured in competitive binding assays, using AMA as the fluorescent probe at a 3.75 µM concentration, whereas the competitor ligand concentration varied from 0 to 15 µM. The spectra were recorded 15 min after the ligand addition, and the competition was monitored by following the fluorescence intensity decrease of the band of AMA bound to the protein. The apparent K_d values of the ligand-protein systems were calculated according to $K_d = [IC_{50}]/(1 + [AMA])/K_{d-AMA}$ where $[IC_{50}]$ is the competitor concentration that yields a fluorescence decay of 50% of the protein-AMA complex.

Results and Discussion

Isoform Purification and Identification As phosphorylation could confer different global charges to isoforms, we used strong anion exchange chromatography with a linear gradient of NaCl for their purification. Isoforms of OBP-Pichia and OBP-CHO were obtained by HPLC purification (Fig. 1). The eluted fractions were collected manually and were extensively dialyzed against water. Fractions were dried under vacuum and suspended in sample buffer to be analyzed by SDS-PAGE (Fig. 2a) and by western blot with anti-OBP antibodies (Fig. 2b). For OBP-Pichia, four peaks (Fig. 1a) were immunodetected with anti-OBP antibodies (Fig. 2b), but only two (peak 2 = OBP-Pichia-iso2 and peak 3 = OBP-Pichia-iso3) were in sufficient quantity (135 µg per run) to perform further analysis. They were eluted at 0.31 M and 0.32 M NaCl respectively that reflects slightly different charges.

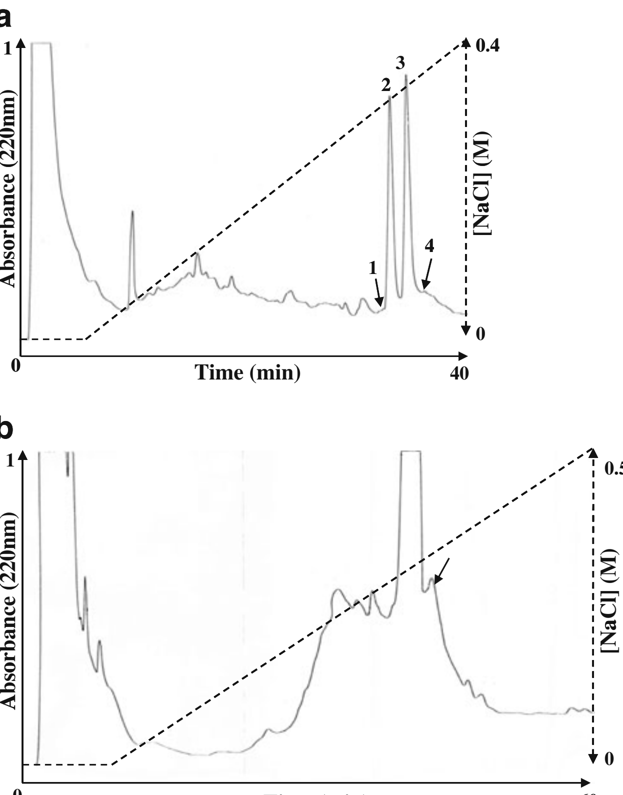


Fig. 1 Purification of recombinant *Sus scrofa* OBP isoforms by anion exchange HPLC. *Dotted lines* represent the linear gradient of NaCl. **a** Chromatogram of OBP-Pichia purification (peaks 1 to 4 contain protein immunoreactive to anti-OBP antibodies). **b** Chromatogram of OBP-CHO purification (the *arrow* indicates the protein that is immunoreactive to anti-OBP antibodies)

The purification of OBP-CHO allowed separating the protein from other elements of the DMEM medium (in particular BSA in large amounts in FBS), but only one peak eluted at 0.26 M NaCl was immunopositive to anti-OBP antibodies (Figs. 1b and 2b).

Peptide mapping analysis was performed on OBP-Pichia isoforms from SDS-PAGE gel (identical to the one in Fig. 2a), giving 70% of peptide recovery for OBP-Pichia-iso2 and 90% for OBP-Pichia-iso3 (data not shown). In comparison, the percentages were 80% for OBP-native and total OBP-Pichia (Nagnan-Le Meillour et al. 2009a). In both cases, the 1–15 peptide of theoretical mass 1711.7810 Da was retrieved, indicating that the signal peptide was properly removed through the secretion pathway, as it was previously observed for OBP-native and total OBP-Pichia (Nagnan-Le Meillour et al. 2009a). As the quantity of purified OBP-CHO isoform was scarce, the identity of the protein was confirmed by BEMAD,

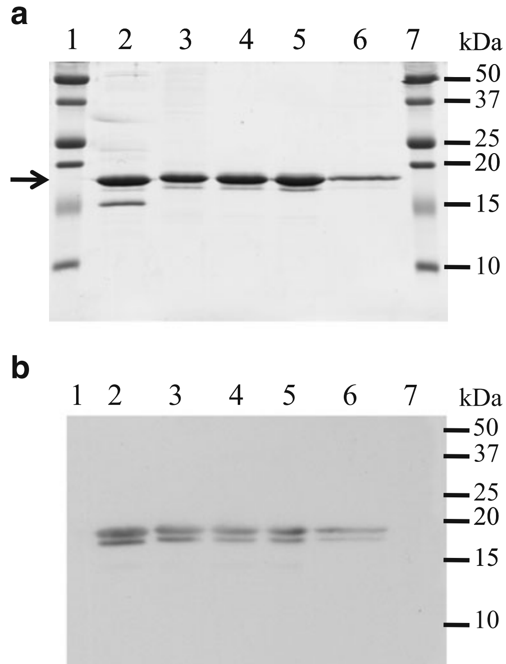
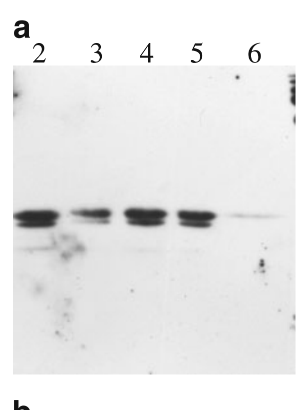


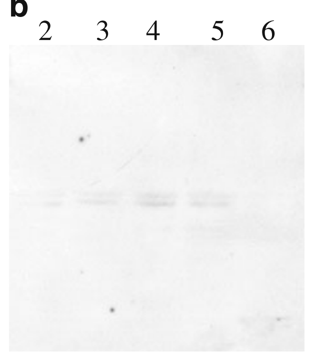
Fig. 2 Identification of recombinant *Sus scrofa* OBP isoforms. **a** SDS-PAGE, Coomassie blue staining. **b** Western blot with anti-OBP antibodies (1:20,000 dilution, ECL Plus detection, 15 sec exposure). (1 and 7) Molecular weight markers (BioRad), (2) crude extract of nasal mucus, (3) total OBP-Pichia, (4) OBP-Pichia-iso2, (5) OBP-Pichia-iso3, (6) OBP-CHO. Note that molecular weight markers are not labeled with specific antibodies (wells 1 and 7)

which gives information on both amino acid sequence and phosphorylation sites with a single sample.

Immunodetection of Phosphorylation Aliquots containing the same quantity of proteins as above were analyzed by western blot with specific antibodies raised against the three types of phosphorylation. A strong immunoreactivity of all recombinant proteins was observed with the anti-phosphoserine antibody (Fig. 3a, wells 3 to 6), identical to the one of native OBP (Fig. 3a, well 2). In contrast, the signal was weak with anti-phosphotyrosine antibody (Fig. 3b), and no signal was obtained with antiphospho-threonine antibody (Fig. 3c). These results are consistent with those already obtained with native OBP and could reflect the high number of modified serines compared to the two other residues (Nagnan-Le Meillour et al. 2009b).

Mapping of Phosphorylation Sites DTT addition confers a tag of defined molecular mass to peptides bearing phosphate groups: 136.2 Da for one DTT, 272.4 Da for 2 DTT and 408.6 Da for 3 DTT. The measured masses of peptides obtained after BEMAD treatment were compared to the theoretical list of potential DTT-modified peptides calculated from the OBP protein sequence (NP_998961). The BEMAD results are given in Table 1 for OBP-Pichia, Table 2 for OBP-CHO, Table 3 for OBP-Pichia-iso2, and Table 4 for OBP-Pichia-iso3. The spectra obtained for OBP-Pichia-iso2 and OBP-Pichia-iso3 are provided in Supplementary data. It should be noted that some peptides, unmodified by DTT also were found in the spectra (italicized in Tables), despite the use of thiol columns to enrich the samples in DTT-modified peptides. This was reported previously for the native protein (Nagnan-Le Meillour et al. 2009b) and reflects the fact that each phosphorylated isoform could be in minor quantity compared to non-phosphorylated forms. As phosphorylation is a dynamic process, only a small fraction of the pool of a protein is phosphorylated at a given time as a result of a stimulus. The consequence also is that phosphoproteins are heterogeneous and exist in several phosphorylated forms. Indeed, several forms of one peptide were observed for each recombinant protein or isoform.





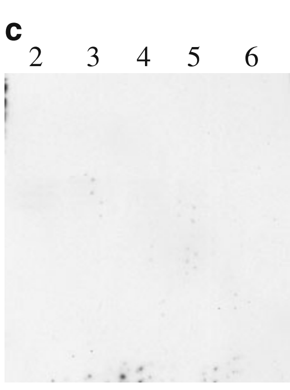


Fig. 3 Immunodetection of *Sus scrofa* phosphorylated OBP isoforms. **a** anti-phosphoserine antibodies (1:500 dilution). **b** anti-phosphotyrosine antibodies (1:2,000 dilution). **c** anti-phosphothreonine antibodies (1:500 dilution). Treatments in wells are as described in Fig. 2. Secondary antibodies at 1:40,000 dilution (rabbit IgG, HRP linked whole antibodies, GE Healthcare). ECL Plus detection, 1 min exposure. Molecular weight markers are not labeled with specific antibodies (wells 1 and 7)

Table 1 Beta-Elimination followed by Michael Addition of Dithiothreitol (BEMAD, DTT) performed on SDS-PAGE band containing *Sus scrofa* total OBP-Pichia to identify phosphorylation sites

Calculated mass (Da)				Measured mass[a]	Peptide	Peptide sequence
No DTT	1 DTT	2 DTT	3DTT	(M+H)+		
719.3722	855.5722			855.2951 (T+CT; 50%)	11–16	ELS([b])GKW
1950.9807		2223.3807		2223.2814 (CT; 50%)	21–38	IGS([b])S([b])DLEKIGENAPFQVF
2259.0750	2395.2750			2395.0388 (T; 50%)	29–47	IGENAPFQVFMRS([b])IEFDDK
721.3362	857.5362			857.9682 (T+CT; 25%)	45–50	DDKES([b])K
1096.5520		1368.9520		1368.8852 (CT; 50%)	45–53	DDKES([b])KVY([b])L
1017.5404		1289.9404		1289.7211 (T; 25%)	51–58	VY([b])LNFFS([b])K
755.4086	891.6086			891.3784 (T+CT; 25%)	53–58	LNFFS([b])K
1825.9000				1826.2253 (T+CT; 25%)	57–72	*SKENGICEEFSLIGTK*
1539.7359		1812.1359		1811.7640 (T+CT; 50%)	59–72	ENGICEEFS([b])LIGT([b])K
2232.0125				2231.9151 (T+CT; 25%)	59–78	*ENGICEEFSLIGTKQEGNTY*
618.3821		890.7821		890.6533 (T+CT; 50%)	67–72	S([b])LIGT([b])K
1310.6586			1719.2586	1719.1245 (T+CT; 50%)	67–78	S([c])LIGT([c])KQEGNT([c])Y([c])
711.2944		983.6944		983.5456 (T+CT; 25%)	73–78	QEGNT([b])Y([b])
994.4588	1130.6588			1130.5890 (T+CT; 50%)	79–87	DVNY([b])AGNNK
1098.5578				1098.6254 (CT; 25%)	83–92	*AGNNKFVVSY*
1039.5306			1448.1306	1448.2532 (CT; 50%)	89–98	VVS([c])Y([c])AS([c])ET([c])AL
1806.8789	1943.0789			1943.2798 (CT; 25%)	99–114	IIS([c])NINVDEEGDKT([c])IM
933.5437	1069.7437			1069.8632 (T; 50%)	112–120	T([c])IMT([c])GLLGK
949.5386		1221.9386		1221.7853 (T+CT; 50%)	112–120	T([b])IMT([b])GLLGK
2352.2517				2351.8611 (CT; 25%)	133–152	*KEVTRENGIPEENIVNIIER*
2853.4046	2989.6046			2989.6503 (T; 25%)	134–158	EVT([b])RENGIPEENIVNIIERDDCPAK

[a] Peptides derived from analysis after enrichment by thiol chromatography (Trypsin T, Chymotrypsin CT, T+CT) and elution at different percentages of acetonitrile

[b] Denotes mass addition at a Ser, Tyr, or Thr residue, indicating modification by dithiothreitol (DTT)

[c] Denotes potential phosphorylation at a Ser, Tyr, or Thr residue. Peptides unmodified by DTT addition are italicized

Table 2 Beta-Elimination followed by Michael Addition of Dithiothreitol (BEMAD, DTT) performed on SDS-PAGE band containing *Sus scrofa* OBP-CHO isoform to identify phosphorylation sites

Calculated mass (Da)		Measured mass[a] (M+H)+	Peptide	Peptide sequence
No DTT	1 DTT			
1711.7809	1847.9809	1847.9952 (T; 25%)	1–15	QEPQPEQDPFELS([b])GK
1197.5633	1333.7633	1333.9876 (T; 50%)	41–50	S([c])IEFDDKES([c])K
1226.5358	1362.7358	1362.8638 (T+CT; 50%)	57–66	S([b])KENGICEEF
2827.3917	2963.5917	2963.9894 (CT; 50%)	89–114	VVS([c])Y([c])AS([c])ET([c])ALIIS([c])NINVDEEGDKT([c])IM
1262.5746	1398.7746	1398.7400 (T; 25%)	121–131	GT([b])DIEDQDLEK
1537.7380		1538.0132 (T+CT; 25%)	121–133	*GTDIEDQDLEKFK*
2352.2517		2352.7411 (CT; 25%)	133–152	*KEVTRENGIPEENIVNIIER*
2224.1567		2224.2769 (T; 50%)	134–152	*EVTRENGIPEENIVNIIER*

[a] Peptides derived from analysis after enrichment by thiol chromatography (Trypsin T, Chymotrypsin CT, T+CT) and elution at different percentages of acetonitrile

[b] Denotes mass addition at a Ser, Tyr, or Thr residue, indicating modification by dithiothreitol (DTT)

[c] Denotes potential phosphorylation at a Ser, Tyr, or Thr residue. Peptides unmodified by DTT addition are italicized. Ser 13, Ser57, and Thr122 are phosphorylated, whereas Ser41, Ser49, Ser91, Tyr 92, Ser94, Thr96, Ser101, and Thr112 are potentially phosphorylated

Table 3 Beta-Elimination followed by Michael Addition of Dithiothreitol (BEMAD, DTT) performed on SDS-PAGE band containing *Sus scrofa* OBP-Pichia-iso2 to identify phosphorylation sites

Calculated mass			Measured mass[a] (M+H)$^+$	Peptide	Peptide sequence
No DTT	1 DTT	2 DTT			
1711.7809			1711.0458 (T; 50%)	1–15	*QEPQPEQDPFELSGK*
719.3722	855.5722		855.4926 (T+CT; 25%)	11–16	ELS([b])GKW
1950.9807		2223.3807	2223.2927 (CT; 50%)	21–38	IGS([b])S([b])DLEKIGENAPFQVF
1172.5768	1308.7768		1308.6959 (T+CT; 50%)	36–44	QVFMRS([b])IEF
1096.5520		1368.9520	1368.7275 (CT; 50%)	45–53	DDKES([b])KVY([b])L
1017.5404	1153.7404		1153.6753 (T; 50%)	51–58	VY([c])LNFFS([c])K
1017.5404		1289.9404	1289.6857 (T; 25%)	51–58	VY([b])LNFFS([b])K
1355.6147	1491.8147		1491.8855 (CT; 50%)	57–68	S([c])KENGICEEFS([c])L
2518.1766			2518.3683 (CT; 50%)	57–78	*SKENGICEEFSLIGTKQEGNTY*
618.3821		890.7821	890.4685 (T+CT; 50%)	67–72	S([b])LIGT([b])K
2233.0520	2369.2520		2369.3125 (CT; 50%)	69–88	IGT([c])KQEGNT([c])Y([c])DVNY([c])AGNNKF
994.4588			994.7456 (T+CT; 50%)	79–87	*DVNYAGNNK*
949.5386	1085.7386		1085.4845 (T; 25%)	112–120	T([c])IMT([c])GLLGK
632.3726	768.5726		768.0517 (CT; 25%)	133–137	KEVT([b])R
2352.2517			2352.2819 (CT; 25%)	133–152	*KEVTRENGIPEENIVNIIER*
2981.4996			2981.6791 (CT; 50%)	133–158	*KEVTRENGIPEENIVNIIERDDCPAK*

[a] Peptides derived from analysis after enrichment by thiol chromatography (Trypsin T, Chymotrypsin CT, T+CT) and elution at different percentages of acetonitrile. The corresponding spectra are shown in Supplementary Figs. 1, 2, and 3

[b] Denotes mass addition at a Ser, Tyr, or Thr residue, indicating modification by dithiothreitol (DTT)

[c] Denotes potential phosphorylation at a Ser, Tyr, or Thr residue. Peptides unmodified by DTT addition are italicized

The results obtained for the total recombinant OBPs were compared to those obtained for the native protein in the same experimental conditions (Nagnan-Le Meillour et al. 2009b; Table 5). Ser13, Ser41, Ser49, and Ser57 are phosphorylated in the three proteins (OBP-native, OBP-Pichia and OBP-CHO). The phosphorylation of Ser23, Ser24, Tyr52, Ser67, Thr71, Tyr82 is common to the OBP-native and OBP-Pichia, while Ser91, Tyr92, Ser94, Thr96, Ser101, Thr112, and Thr115 are possibly phosphorylated in the recombinant OBP-Pichia and OBP-CHO, but never in the native OBP. These special features shared by recombinant proteins could indicate differences between the phosphorylation processes in the three eukaryotic cells.

The OBP-CHO isoform is less affected globally by phosphorylation (Tables 2 and 5). This could mean that the protein is indeed less phosphorylated in CHO cells, or that the quantity of recombinant OBP-CHO is too small to provide a representative phosphorylation pattern of the protein through the single isoform that could be obtained by purification, even after a ten-fold concentration of the culture supernatant. Other isoforms could exist but in too small quantity to be detected and analyzed. Whatever the case, it was not possible to perform binding assays with such small quantities, and we did not consider further the recombinant OBP from CHO in this work.

Despite the combined use of T and CT to obtain the best assignment of phosphorylation sites, some ambiguous positions remained, in particular in peptides 89–98 and 99–114 from OBP-Pichia (Table 1), and in peptide 89–114 from OBP-CHO (Table 2). For example, three DTT are linked to the peptide 89–98 bearing 4 potential phosphorylation sites (Ser91, Tyr92, Ser94, Thr96), and peptide 99–114 bears only one DTT, but 2 sites could be phosphorylated, Ser 101 and Thr112. Besides these ambiguities in the sites location, several masses observed for one given peptide suggest the presence of more heterogeneity: peptide 112–120 of total OBP-Pichia (Table 1) could be phosphorylated on either Thr112 or Thr115 (mass 1069.7437 Da for one DTT), or on both amino acids (mass 1221.9386 Da for 2 DTT). In OBP-Pichia-iso2, this peptide is modified by only one DTT (mass of 1085.4845 Da, Table 3), so that either Thr112 or Thr115 is phosphorylated. In OBP-Pichia-iso3, the same peptide bears 2 DTT, indicating that both residues are phosphorylated. The presence of several forms of different peptides increases the number of possible combinations that could be estimated as 114 for total OBP-Pichia, 15 for OBP-Pichia-iso2, and 44 for OBP-Pichia-iso3 (data not shown). Obviously, all these isoforms do not exist, but the presence of the same peptides with different phosphorylation status suggests a much higher

Table 4 Beta-Elimination followed by Michael Addition of Dithiothreitol (BEMAD, DTT) performed on SDS-PAGE band containing *Sus scrofa* OBP-Pichia-iso3 to identify phosphorylation sites

Calculated mass (Da)				Measured mass[a] (M+H)$^+$	Peptide	Peptide sequence
No DTT	1 DTT	2 DTT	3DTT			
533.2929	669.4929			669.2454 (T+CT; 25%)	11–15	ELS(^{b})GK
2259.0750	2395.2750			2395.2038 (T; 50%)	29–47	IGENAPFQVFMRS(^{b})IEFDDK
2243.0801				2243.4810 (T; 50%)	29–47	*IGENAPFQVFMRSIEFDDK*
655.3450	791.5450			791.0705 (T+CT; 50%)	51–55	VY(^{b})LNF
1155.4986	1291.6986			1291.1699 (T+CT; 25%)	57–66	S(^{b})KENGICEEF
2518.1766				2518.1952 (CT; 25%)	57–78	*SKENGICEEFSLIGTKQEGNTY*
1539.7359		1812.1359		1811.7640 (T+CT; 50%)	59–72	ENGICEEFS(^{b})LIGT(^{b})K
1310.6586			1719.2586	1719.2498 (T+CT; 50%)	67–78	S(^{c})LIGT(^{c})KQEGNT(^{c})Y(^{c})
711.2944		983.6944		983.0770 (T+CT; 25%)	73–78	QEGNT(^{b})Y(^{b})
994.4588	1130.6588			1130.3285 (T+CT; 50%)	79–87	DVNY(^{b})AGNNK
1098.5578				1098.6248 (T+CT; 25%)	83–92	*AGNNKFVVSY*
1039.5306			1448.1306	1448.5877 (CT; 25%)	89–98	VVS(^{c})Y(^{c})AS(^{c})ET(^{c})AL
2078.0321		2350.4321		2350.5974 (CT; 50%)	99–117	IIS(^{c})NINVDEEGDKT(^{c})IMT(^{c})GL
949.5386		1221.9386		1221.6399 (T+CT; 50%)	112–120	T(^{b})IMT(^{b})GLLGK
2352.2517				2352.6148 (CT; 50%)	133–152	*KEVTRENGIPEENIVNIIER*
2981.4996				2981.8129 (CT; 50%)	133–158	*KEVTRENGIPEENIVNIIERDDCPAK*
2224.1567				2224.4837 (T; 50%)	134–152	*EVTRENGIPEENIVNIIER*

[a] Peptides derived from analysis after enrichment by thiol chromatography (Trypsin T, Chymotrypsin CT, T+CT) and elution at different percentages of ACN. The corresponding spectra are shown in Supplementary Figs. 4, 5, and 6

[b] Denotes mass addition at a Ser, Tyr, or Thr residue, indicating modification by dithiothreitol (DTT)

[c] Denotes potential phosphorylation at a Ser, Tyr, or Thr residue. Peptides unmodified by DTT addition are italicized

Table 5 Phosphorylation patterns of recombinant *Sus scrofa* OBPs

OBP-native[a]	OBP-Pichia	OBP-Pichia-iso2	OBP-Pichia-iso3	OBP-CHO
Ser13	Ser13	Ser13	Ser13	Ser13
Ser23	Ser23	Ser23		
Ser24	Ser24	Ser24		
Ser41	Ser41	Ser41	Ser41	*Ser41*
Ser49	Ser49	Ser49		*Ser49*
Tyr52	Tyr52	Tyr52	Tyr52	
Ser57	Ser57	Ser57	Ser57	Ser57
Ser67[b]	Ser67	Ser67	Ser67	
Thr71	Thr71	Thr71	Thr71	
	Thr77	*Thr77*	Thr77	
	Tyr78	*Tyr78*	Tyr78	
Tyr82	Tyr82	*Tyr82*	Tyr82	
	Ser91		*Ser91*	*Ser91*
	Tyr92		*Tyr92*	*Tyr92*
	Ser94		*Ser94*	*Ser94*
	Thr96		*Thr96*	*Thr96*
	Ser101		*Ser101*	*Ser101*
	Thr112	*Thr112*	Thr112	*Thr112*
	Thr115	*Thr115*	Thr115	
Thr122				Thr122
	Thr136	Thr136		

[a] Phosphorylation sites of native OBP were determined in Nagnan-Le Meillour et al. (2009b)

[b] Potentially phosphorylated amino acids are italicized

number of isoforms than the two obtained by HPLC purification. Indeed, it is visible in SDS-PAGE (Fig. 2a) that each protein is composed of two bands, even after strong anion exchange purification. This means that each OBP-Pichia isoform should be purified further by a second different HPLC system.

Ligand Binding Monitored by Fluorescence Spectroscopy Recombinant proteins obtained by expression in CHO cells were in too small quantities to perform ligand binding studies, despite adaptation of the method of fluorescence spectroscopy to the use of purified isoforms. We, thus, measured the affinity of the two isoforms expressed in the yeast (OBP-Pichia-iso2 and OBP-Pichia-iso3) for five selected ligands, all of biological relevance in the porcine species (androstenol, androstenone, testosterone, palmitic acid, and myristic acid). The two isoforms were first titrated with the fluorescent probe, AMA, and displayed comparable affinity to total OBP-Pichia. However, OBP-Pichia-iso3 presents a slightly better affinity for AMA than OBP-Pichia-iso2. The $K_{d\text{-}AMA}$ were estimated to 0.56±0.04 μM, 0.46±0.03 μM and 0.55±0.04 μM, for OBP-Pichia-iso2, OBP-Pichia-iso3 and total OBP-Pichia (Nagnan-Le Meillour et al. 2009a), respectively. Competitive binding experiments then were carried out with proteins saturated with 3.75 μM AMA. The displacement curves are reported in Fig. 4 and the calculated binding constants are listed in Table 6. Recombinant OBPs from *P. pastoris*, isoforms or total protein, did not bind testosterone. This result was expected from the in-gel binding assay with native OBP that did not bind testosterone (Le Danvic et al. 2009), which is the natural ligand of VEG (Von Ebner's Gland protein), another protein of the nasal mucus. Whatever the ligand, OBP-Pichia-iso2 displayed

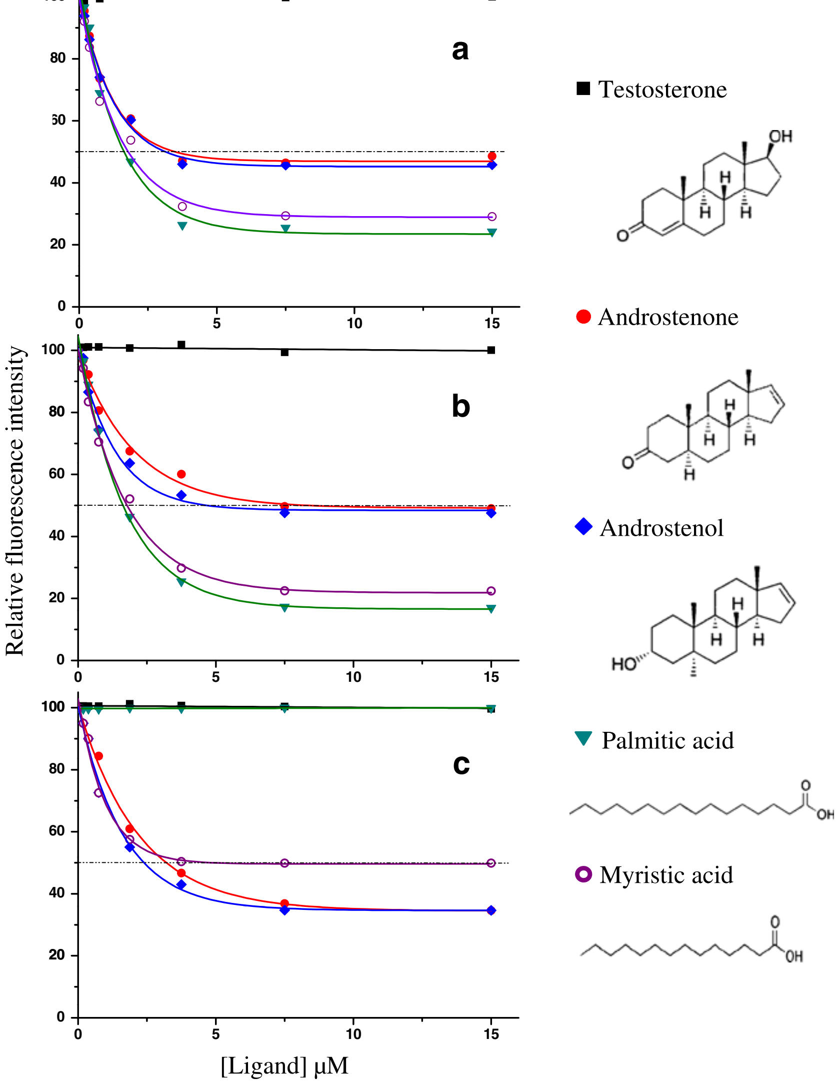


Fig. 4 Competitive binding assays of five selected ligands of biological relevance to recombinant *Sus scrofa* OBPs expressed by the yeast. Proteins were assayed at 2.5 μM and saturated with 3.75 μM of the fluorescent probe 1-AMA. Competitors were added at increasing concentration from 0 to 15 μM. **a** Total OBP-Pichia; **b** OBP-Pichia-iso2; and **c** OBP-Pichia-iso3

Table 6 Values of $[IC_{50}]$ and calculated dissociation constants relative to the binding of ligands (testosterone, androstenone, androstenol, palmitic acid and myristic acid) to total *Sus scrofa* OBP-Pichia, OBP-Pichia-iso2 and OBP-Pichia-iso3

Ligand	Total OBP-Pichia		OBP-Pichia-iso2		OBP-Pichia-iso3	
	IC_{50} (μM)	Kd (μM)	IC_{50} (μM)	Kd (μM)	IC_{50} (μM)	Kd (μM)
Testosterone	–	–	–	–	–	–
Androstenone	3.5	0.5	7.7	1.0	3.2	0.3
Androstenol	3.2	0.4	4.5	0.6	2.4	0.2
Palmitic acid	2.1	0.3	1.6	0.2	–	–
Myristic acid	2.2	0.3	1.8	0.2	4.3	0.5

the same behavior as total OBP-Pichia; that could be explained by the fact that it is the closest to total OBP-Pichia, in terms of phosphorylation. However, when compared to total OBP-Pichia, OBP-Pichia-iso2 presents a lesser affinity for androstenone and androstenol and a higher affinity towards the two fatty acids studied.

OBP-Pichia-iso3 presents very different behaviors according to the ligand. It has a high affinity for the pig sex steroids (androstenone: $K_d = 0.3$ and androstenol $K_d = 0.2$) but less affinity for myristic acid ($K_d = 0.5$), and it displays no binding to palmitic acid, these two fatty acids differing only in the chain length, C_{14} and C_{16}, respectively. This isoform seems to be more likely tuned to steroids. This binding specificity could be relied on the absence of Thr136, Ser23, Ser24, Ser49, and Ser91 to Ser101, and the phosphorylation of the three residues Thr77, Tyr78, and Tyr82 (Table 5). It is surprising to note the important difference of affinity between the two OBP isoforms for palmitic acid, which is illustrated by the spectral data (Fig. 5). A marked decrease in the fluorescence intensity of

Fig. 5 Fluorescence spectra set for the competitive binding assay. **a** OBP-pichia-iso2-AMA/palmitic acid; **b** OBP-pichia-iso3-AMA/palmitic acid; **c** OBP-pichia-iso2-AMA/ androstenone; and **d** OBP-pichia-iso3-AMA/androstenone. [protein] = 2.5 μM, [AMA] = 3.75 μM, and [ligand] from 0 to 15 μM (excitation wavelength: 295 nm). *Arrows* indicate the fluorescence decrease according to increasing ligand concentration

the AMA band (484 nm) is observed with successive additions of palmitic acid for OBP-Pichia-iso2 (Fig. 5a) while this ligand does not compete with AMA bound to OBP-Pichia-iso3 (Fig. 5b). This behavior is reversed to a lesser extent for androstenone where the fluorescence decrease of the probe is more pronounced for OBP-Pichia-iso3 (Fig. 5c) than for OBP-Pichia-iso2 (Fig. 5d). It also may be observed on spectra set of Fig. 5, normalized with the water Raman line (328 nm), that the protein fluorescence band (342 nm) is not affected by the substitution of AMA by ligands. Thus, the two isoforms selectivity are quite complementary, OBP-Pichia-iso2 presents a good affinity for fatty acids (components of the female appeasing pheromone, Guiraudie et al. 2003), while OBP-Pichia-iso3 has marked affinity for the male sex steroids (Katkov et al. 1972).

Location of Phosphate Groups on the 3-D Structure of Porcine OBP The phosphate groups were added to the OBP three-dimensional structure to visualize their position and predict their possible involvement into OBP-ligand interactions. As a starting point, the phosphorylated native OBP was constructed (Fig. 6a), as it bears residues that are always phosphorylated in the recombinant proteins (Table 5), except Thr122, non-phosphorylated in *Pichia* proteins. Phe35 was added, as we have shown that this residue participates in the reorientation of the ligand towards the exit of the protein (Nagnan-Le Meillour et al. 2009a), together with Tyr 82 that has a key-role in the binding and release processes (Golebiowski et al. 2006, 2007; Nagnan-Le Meillour et al. 2009a). In Fig. 6, these two residues are in the open position, for a better view of the binding pocket. In the closed position, they display hydrophobic interactions, certainly reinforced by negative charges of the phosphate group born by Tyr82. Ser 23 could have interactions with ligands, as its side chain and phosphate group point to the internal cavity. Side chains and phosphates of the other phosphorylated residues clearly are positioned outside the binding pocket, pointing out to the surface of the protein. Interestingly, most of these residues are located on β-strands determined by X-ray crystallography (Spinelli et al. 1998): Ser23, Ser24 on β1, Ser41 in β2, Tyr52 and Ser57 in β3, and Ser67 in β4. Ser13 is located on the N-terminal flexible part, Ser49 on the loop between β2 and β3, and Thr122 on the loop near the α-helix.

In a second step, we highlighted on the 3-D structure the differences in phosphorylation patterns of the two *Pichia* isoforms studied for their binding properties (Fig. 6b, c). Contrary to the mapping of phosphorylation sites of native OBP, several ambiguities remained in the location of these sites in the two *Pichia* isoforms. We made the choice to represent all the possibly phosphorylated residues for each isoform. For OBP-Pichia-iso2 (Fig. 6b), either Thr77, or Tyr78, or Tyr82 is phosphorylated, but phosphate groups were added on the three because they have different positions in the structure: Thr77 is out of the binding pocket, Tyr78 is in the bottom of the binding pocket, and Tyr82 lines the gate. The alternative phosphorylation of these residues could influence strongly the binding, in particular if Tyr82 is not phosphorylated. These residues are phosphorylated in OBP-Pichia-iso3 (Fig. 6c), while Ser23, Ser24, and Ser49 are not. In addition, among Ser91, Tyr92, Ser94, Thr96, and Ser101, only one is phosphorylated in OBP-Pichia-iso3, but they are all represented with a phosphate group on the structure (Fig. 6c). They are located

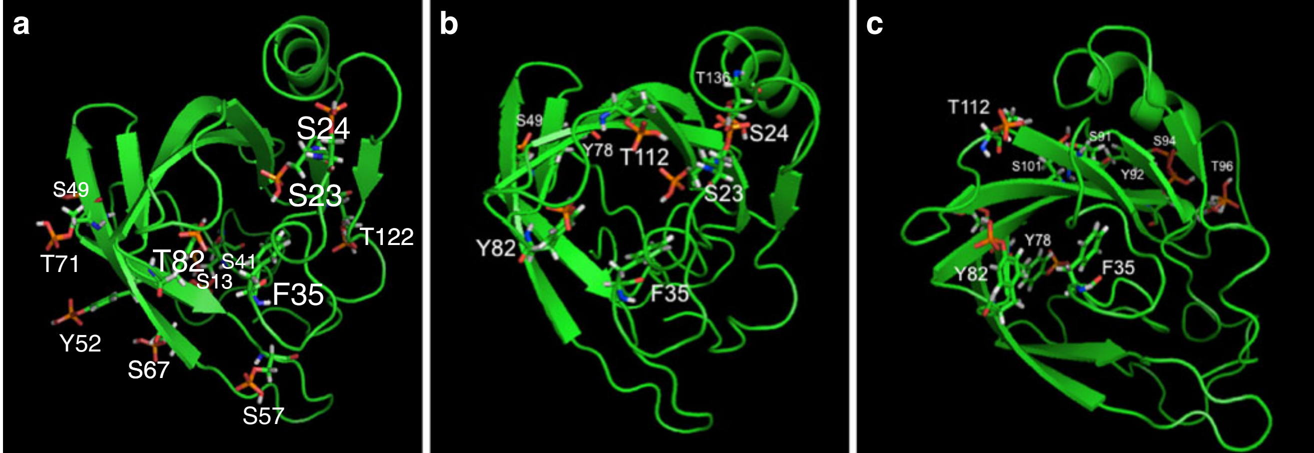


Fig. 6 Three-dimensional representation of phosphorylated porcine OBP. Images were generated from the modified PDB file (1E02) with MacPyMOL. The OBP backbone is represented as a ribbon (*green*). The side chains of the selected residues and their phosphates (*orange*) are in stick representation. The identities of the residues are indicated (*white*). The protein is oriented with the face displaying the binding pocket. **a** Native OBP; **b** OBP-Pichia-iso2; and **c** OBP-Pichia-iso3

at the protein surface, so their involvement in the binding with ligand is less probable. Phosphate groups located at the protein surface could more likely be involved in OBP-OBP and/or OBP-receptor interactions. Moreover, the binding selectivity could result from different positions of the ligand in the binding cavity, constrained by changes in the structure upon phosphorylation/dephosphorylation of both internal and external residues. The location of phosphates in the structure helps to visualize the results, but docking experiments and molecular dynamics studies will be required to understand the electrostatic interactions that they could establish with the ligands.

In summary, OBP is secreted by Bowman's gland into the extracellular mucus lining the nasal cavity of *S. scrofa*. This secreted protein is not supposed to undergo phosphorylation in the rough endoplasmic reticulum compartment, as kinases and phosphatases are localized in the cytoplasm of the cell. However, there is evidence for the presence of ecto-enzymes at the cell surface in a variety of cell types, in particular ecto-kinases and ecto-phosphatases that have a potential role in intercellular regulation and reception and transduction of external stimuli (Nath et al. 2008). Our study indicates that the phosphorylation of secreted proteins is a mechanism that is common to yeast and mammalian cells. As phosphorylation is a dynamic mechanism, the results observed reflect the phosphorylation pattern of OBP at a given time, corresponding to a given physiological status of the cell (Mann et al. 2002). Differences in phosphorylation patterns of OBP produced by the three types of cells could arise from different ratio in the kinases (e.g., more Ser/Thr kinase in *Pichia* than in other cells), or from different responses to culture conditions (or the physiological state for the secreting cells). Taking into account the heterogeneity of recombinant proteins from different cells, an exhaustive study of ligand binding capacities now will be conducted with isoforms purified from pig nasal tissue. Fortunately, the large amount of OBP produced by an animal (5–15 mg) will allow structure function-relationship studies on native proteins.

In this perspective, the present ligand binding studies already have a biological meaning. Even though the two *Pichia* isoforms differ slightly from native isoforms, they display properties of binding to pheromonal ligands that are physiologically relevant. The ligands used in this study are pheromone components in the pig species. The two fatty acids are part of a maternal pheromone that has an appeasing effect on piglets (McGlone and Anderson 2002). Androstenol and androstenone are secreted by the sub maxillary glands into male saliva (Katkov et al. 1972), but only androstenone has been identified as a pheromone component that facilitates expression of both attraction to the male and a receptive mating stance in estrous females (Dorries et al. 1995). For adult females, androstenone is a sex pheromone, but for piglets, the perception of androstenone reduces agonistic behaviors and is considered as a submissive pheromone (McGlone 1985; McGlone and Morrow 1988). Testosterone, the natural ligand of VEG (a pheromone binding protein) (Le Danvic et al. 2009), is never bound by any of the OBP isoforms. Conversely, both OBP isoforms bind a closely related steroid, androstenone, differing from testosterone by a single alcohol. Our results lead us to conclude that OBP is not a passive carrier of odorant or pheromonal molecules. Rather, OBP is a pool of isoforms with different binding properties, capable of distinguishing between two fatty acids differing by 2 CH_2 or between two steroids differing by an alcohol, ensuring a fine coding of molecules with a high significance in the mating or the maintaining of hierarchy inside the porcine species.

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Positions and Stereochemistry of Methyl Branches in the Novel Sex Pheromone Components Produced by a Lichen Moth, *Lyclene dharma dharma*

Yasushi Adachi · Nguyen Duc Do · Masakatsu Kinjo · Saori Makisako · Rei Yamakawa · Kenji Mori · Tetsu Ando

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Abstract Female moths of *Lyclene dharma dharma* (Arctiidae, Lithosiinae) produce three sex pheromone components (**I**–**III**), for which we assigned the following novel chemical structures; 6-methyl-2-octadecanone (**1**) for **I**, 14-methyl-2-octadecanone (**2**) for **II**, and 6,14-dimethyl-2-octadecanone (**3**) for **III**. In the Iriomote Islands where the insects were collected, a lure including racemic **1** and **2** attracted the male moths without mixing **3**. In this study for further confirmation of the plane structures, the positional isomers with a methyl branch at the 4-, 5-, 7-, 13-, or 15-position (**4**–**8**, respectively) were synthesized. The GC-MS analyses revealed that natural components **I** and **II** were best fitted with those of **1** and **2**, respectively, among the methyl-2-octadecanones examined, indicating the usefulness of this analytical instrument and authentic standards for the determination of the positions of methyl branches. In field trapping tests, **4**–**8** could not substitute for **1** or **2**, nor did these compounds inhibit the active binary lure of **1** and **2**, indicating that the males strictly recognized the 2-ketones with a methyl branch at the 6- or 14-positions. Next, the absolute configurations of **I** and **II** were determined by HPLC with a normal-phased chiral column (Chiralpak AD-H), which could separate the enantiomers of both **1** and **2**. The chiral HPLC analysis of a crude pheromone extract indicated that the females exclusively produced (*S*)**-1** and (*S*)**-2**. Furthermore, a field evaluation of each enantiomer revealed that (*S*)**-1** and (*S*)**-2** were bioactive but (*R*)**-1** and (*R*)**-2** were not.

Key Words Female sex pheromone · Lepidoptera · Methyl-branched 2-ketones · Positional isomers · Absolute configuration · Enantiomers · Resolution by chiral HPLC · Field evaluation of synthetic isomers

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Y. Adachi · N. D. Do · S. Makisako · R. Yamakawa · T. Ando (✉)
Graduate School of Bio-Applications and Systems Engineering,
Tokyo University of Agriculture and Technology,
Koganei,
Tokyo 184-8588, Japan
e-mail: antetsu@cc.tuat.ac.jp

M. Kinjo
Iriomote Station, Tropical Biosphere Research Center,
University of the Ryukyus,
Uehara, Taketomi-cho,
Okinawa 907-1541, Japan

K. Mori
Photosensitive Materials Research Center, Toyo Gosei Co., Ltd,
Wakahagi 4-2-1, Inba-mura, Inba-gun,
Chiba 270-1609, Japan

Introduction

To date, lepidopteran sex pheromones have been identified from more than 600 species (El-Sayed 2009; Ando 2010). These pheromones mainly are composed of unsaturated C_{10}–C_{18} fatty alcohols and their derivatives (Type I) and C_{17}–C_{23} unbranched polyenyl hydrocarbons and their epoxides (Type II) (Ando et al. 2004). The Type II pheromones have been identified from 17 species in the family of Arctiidae, but no pheromones of species in the arctiid subfamily Lithosiinae have been reported. Larvae of the lithosiine species, which feed mainly on lichen in a forest, are not agricultural pests, but contribute to the ecosystem of the forest. Recently, we found novel pheromone components (**I**–**III**) from females of a lichen moth,

Lyclene dharma dharma Moore (Arctiidae, Lithosiinae), and their chemical structures were assigned as follows: 6-methyl-2-octadecanone (**1**) for **I**, 14-methyl-2-octadecanone (**2**) for **II**, and 6,14-dimethyl-2-octadecanone (**3**) for **III** (Yamamoto et al. 2007). The assignment was accomplished mainly by GC-MS analyses of the pheromone extract before and after the Wolff-Kishner reduction, which converted the ketones to methyl-branched hydrocarbons. In addition to the analyses, an optically inactive sample of each component was synthesized (Do et al. 2009; Taguri et al. 2010). The GC-MS data of the synthetic **1**–**3** coincided with those of the natural components, and the male moths were attracted by a mixture of **1** and **2** in the field, whereas the biological activity of **3** was unclear (Do et al. 2009). While these experiments suggested the accuracy of our structure assignments, at least for **I** and **II**, we were also interested in obtaining GC-MS data and data on the biological activity of structural analogs of **1** and **2** as this information would further confirm the structural assignments. This identification is the first case of the methyl-branched 2-ketones produced by Lithosiinae species. They are the first such compounds reported for moths and, thus, chemical data of the compounds in this group is limited. Since Lithosiinae includes a large number of species, it is expected that some species utilize other methyl-branched ketones as a pheromone. For these reasons, we synthesized positional isomers of **1** and **2** as follows: 4-methyl-, 5-methyl-, 7-methyl-, 13-methyl-, and 15-methyl-2-octadecanones (**4**–**8**, respectively). The GC-MS data were compared to those of the natural components **I** and **II**, and the male attraction activities were evaluated in the field, along with 6-methyl-2-nonadecanone (**9**). The stereochemistry of the natural pheromone components had not been studied previously. Furthermore, although males had been caught in traps with racemic synthetic compounds (Do et al. 2009), the complete structures of the components produced by females, including their absolute configurations, remained to be determined. Therefore, we analyzed the *L. d. dharma* pheromone by utilizing chiral chromatography, and tested responses of males to the chiral compounds in field tests by using each of the synthetic enantiomers of **1** and **2** (Mori 2009).

(Scheme I) **10** → a,b (37%) → **11** → c,d (40%) → **12** → e (64%) → **4**

(Scheme II) **13** → f (50%) → **14** → e (68%) → **15** → g (94%) → **5**

(Scheme III) **16** → h (84%) → **17** → i, j (46%) → **18** → e (64%) → **19** → g (94%) → **6**

(Scheme IV) **20** → k, l (60%) → **21** → d, m (63%) → **22** → h, i, n (43%) → **23** → e, g (57%) → **7**

(Scheme V) **24** → k, l (65%) → **25** → o, m (68%) → **26** → h, i, p (39%) → **27** → e, g (64%) → **8**

Fig. 1 Synthetic schemes for the positional isomers of the sex pheromone components secreted by *Lyclene dharma dharma* females. Scheme I) 4-methyl-2-octadecanone (**4**), Scheme II) 5-methyl-2-octadecanone (**5**), Scheme III) 7-methyl-2-octadecanone (**6**), Scheme IV) 13-methyl-2-octadecanone (**7**), and Scheme V) 15-methyl-2-octadecanone (**8**). a, $(COCl)_2$, DMSO, Et_3N/CH_2Cl_2; b, MeLi/THF; c, TsCl/DMAP/pyridine; d, $CH_2{=}CHCH_2MgCl/Li_2CuCl_4$/THF; e, O_2/$PdCl_2$, CuCl/DMF, H_2O; f, $CH_3(CH_2)_{11}CH{=}PPh_3$/THF; g, H_2/Pd-C/hexane; h, I_2-Ph_3P/imidazole/THF; i, PPh_3/Δ; j, 1) $NaN(SiMe_3)_2$, 2) 2-tridecanone; k, HBr/toluene; l, 3,4-dihydropyran; m, *p*-TsOH/EtOH; n, 1) $NaN(SiMe_3)_2$, 2) 2-heptanone; o, $CH_2{=}CH(CH_2)_2MgBr$/$LiCuCl_4$/THF; p, 1) $NaN(SiMe_3)_2$, 2) 2-pentanone/THF

Table 1 ^{13}C-NMR assignments for methyl-branched 2-octadecanones (**1** and **4**–**6**)

Position	Chemical shift (δ ppm)			
	4-Methyl (**4**)	5-Methyl (**5**)	6-Methyl (**1**)	7-Methyl (**6**)
1	29.8	29.8	29.8	29.9
2	209.3	209.5	209.4	209.4
3	51.3	41.6	44.2	43.9
4	30.4	30.8	21.5	24.2
5	36.9	32.4	36.5	26.7
6	27.0	36.8	32.7	36.8
7	~30	27.0	36.9	32.6
8	~30	~30	27.0	37.1
9	~30	~30	~30	27.1
10–15	~30	~30	~30	~30[a]
16	31.9	32.0	31.9	32.0
17	22.7	22.7	22.7	22.7
18	14.1	14.2	14.1	14.1
Methyl branch	19.8	19.4	19.6	19.6

6-Methyl-2-octadecanone (**1**), 4-methyl-2-octadecanone (**4**), 5-methyl-2-octadecanone (**5**), 7-methyl-2-octadecanone (**6**). NMR data for **1** have been reported in our previous paper (Do et al. 2009)

[a] 29.39, 29.68, 29.73 (×2), 29.86, and 30.04

Methods and Materials

Instuments ^{1}H and ^{13}C NMR spectra were recorded by a Jeol Delta 2 Fourier transform spectrometer (JEOL Ltd., Tokyo, Japan) at 399.8 and 100.5 MHz, respectively, for $CDCl_3$ solutions containing TMS as an internal standard. GC-MS was conducted in EI mode (70 eV) with an HP5973 mass spectrometer system (Hewlett-Packard) equipped with a split/splitless injector and a DB-23 column (0.25 mm ID×30 m, 0.25 µm film, J & W Scientific, Folsom, CA, USA). The column temperature program was 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C. The carrier gas was helium. HPLC employed a system composed of a pump (PU-980, Jasco), a refractive index (RI) detector (RI-98SCOPE, Labo System, Tokyo, Japan), and an integrator (807-IT, Jasco). Resolution of the enantiomers was accomplished with a chiral column (Chiralpak AD-H, 4.6 mm ID×25 cm; Daicel Chemical Industry, Osaka, Japan), which was eluted with 0.05% 2-propanol in hexane at a flow rate of 0.5 ml/min.

Chemicals The syntheses of optically inactive **1**–**3** were reported previously (Yamamoto et al. 2007; Do et al. 2009; Taguri et al. 2010). Those optically active compounds (97% ee) were synthesized by a different route (Mori 2009). Other positional isomers, **4**–**8**, were prepared by the following procedures (see Fig. 1). NMR data of synthetic intermediates are shown in supplements. 6-Methyl-2-nonadecanone (**9**) was synthesized by the same route as **1** (Yamamoto et al. 2007) by using dodecylmagnesium bromide instead of undecylmagnesium bromide.

*4-Methyl-2-octadecanone (**4**, Scheme I)* By Swern oxidation, 1-pentadecanol (**10**) was converted to pentadecanal, which was coupled with methyllithium to yield 2-hexadecanol (**11**). The alcohol (**11**) was converted to tosylate by the treatment of tosyl chloride, and the tosylate was coupled with allylmagnesium chloride by employing the Schlosser's copper-catalyzed Grignard reaction (Fouquet and Schlosser 1974) to yield 4-methyl-1-octadecene (**12**). The 2-ketone (**4**) was obtained by Wacker oxidation of **11** (Tsuji 1984; Taguri et al. 2010). ^{1}H NMR δ: 0.88 (3H, t, J=6.8 Hz), 0.89 (3H, d, J=6.6 Hz), ~1.3 (26H, m), 1.98 (1H, m), 2.13 (3H, s), 2.21 (1H, dd, J=15.7, 8.1 Hz), 2.41 (1H, dd, J=15.7, 5.7 Hz).

*5-Methyl-2-octadecanone (**5**, Scheme II)* A phosphonium salt was prepared from 1-bromotridecane and triphenylphosphine (PPh_3) and converted to an ylide by treatment with butyllithium. 5-Hexen-2-one (**13**) was coupled with the ylide to synthesize a 1:1 mixture of (Z)- and (E)-5-methyl-1,5-octadecadiene (**14**). A selective reaction at the terminal double bond of **14** by Wacker oxidation produced 5-methyl-5-octadecen-2-one (**15**), which was reduced by catalytic hydrogenation to yield 5-methyl-2-octadecanone (**5**). ^{1}H NMR δ: 0.86 (3H, d, J=6.2 Hz), 0.88 (3H, t, J=6.6 Hz), ~1.3 (24H, m), ~1.4 (2H, m), ~1.60 (1H, m), 2.14 (3H, s), 2.42 (2H, m).

*7-Methyl-2-octadecanone (**6**, Scheme III)* 5-Hexen-1-ol (**16**) was converted to iodide (**17**) by reaction with a mixture of I_2, PPh_3, and imidazole. The phosphonium salt, which was prepared from **17** and PPh_3, was treated with $NaN(SiMe_3)_2$ to obtain an ylide, and then a 1:1 mixture of

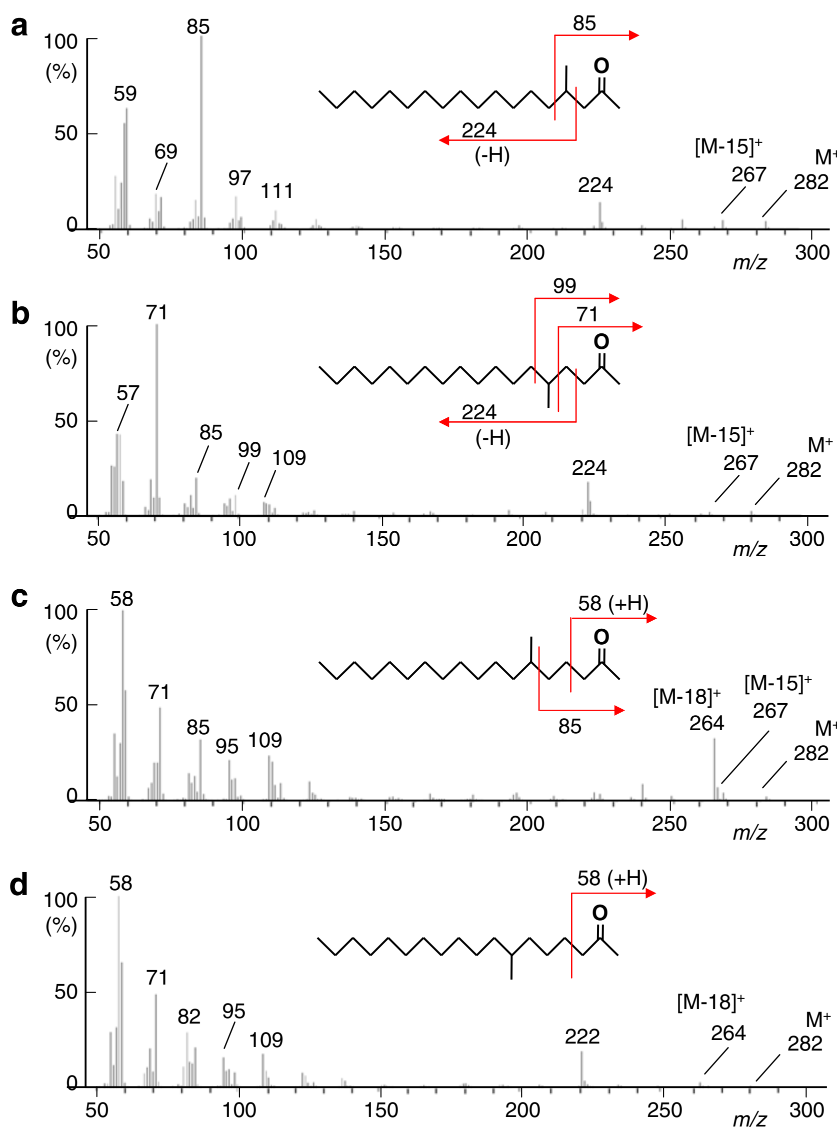


Fig. 2 Mass spectra of synthetic 2-ketones: a, 4-methyl-2-octadecanone (**4**); b, 5-methyl-2-octadecanone (**5**); c, 6-methyl-2-octadecanone (**1**); and d, 7-methyl-2-octadecanone (**6**)

(*Z*)- and (*E*)-7-methyl-1,6-octadecadiene (**18**) was synthesized by Wittig coupling with the ylide and 2-tridecanone. Wacker oxidation at the terminal double bond of **18** produced 7-methyl-6-octadecen-2-one (**19**), which was reduced by catalytic hydrogenation to yield 7-methyl-2-octadecanone (**6**). ^{1}H NMR δ: 0.83 (3H, d, *J*=6.5 Hz), 0.88 (3H, t, *J*=6.7 Hz), ~1.1 (2H, m), ~1.3 (23H, m), ~1.55 (2H, m), 2.13 (3H, s), 2.42 (2H, t, *J*=7.4 Hz).

*13-Methyl-2-octadecanone (***7***, Scheme IV)* Starting from 1,9-nonanediol (**20**), tetrahydropranyl (THP) ether of 9-bromononan-1-ol (**21**) was prepared by mono-bromination with HBr and protection of the remaining hydroxyl group. The bromide **21** was coupled with allylmagnesium chloride under the copper-catalyzed condition, and 11-dodecen-1-ol (**22**) was obtained after cleavage of the THP ether. Using a similar procedure to that in Scheme III, the alcohol **22** was converted to a 1:1 mixture of (*Z*)- and (*E*)-13-methyl-1,12-octadecadiene (**23**) by iodination and Wittig coupling with 2-heptanone, and 13-methyl-2-octadecanone (**7**) was synthesized by Wacker oxidation and catalytic hydrogenation of the diene **23**. ^{1}H NMR δ: 0.84 (3H, d, *J*=6.5 Hz), 0.88 (3H, t, *J*=6.8 Hz), ~1.1 (2H, m), ~1.3 (23H, m), ~1.55 (2H, m), 2.13 (3H, s), 2.41 (2H, t, *J*=7.5 Hz).

*15-Methyl-2-octadecanone (***8***, Scheme V)* With a similar procedure to that in Scheme IV, 1,10-decanediol (**24**) was introduced to tetrahydropranyl (THP) ether of 10-bromodecan-1-ol (**25**), which was coupled with 3-butenylmagnesium bromide under the copper-catalyzed condition and deprotected to yield 13-tetradecen-1-ol (**26**). The alcohol **26** was converted to a 1:1 mixture of (*Z*)- and (*E*)-15-methyl-1,14-octadecadiene (**27**) by iodination and Wittig coupling with 2-pentanone, and 15-methyl-2-octadeca-

Table 2 Field evaluation of synthetic pheromone analogs (**4**–**6**) for attraction of the *Lyclene dharma dharma* males

Lure components (μg/rubber septum)					Captured males	
Natural component		Positional isomer				
1	**2**	**4**	**5**	**6**	/trap/night[a]	Total
400	200	0	0	0	2.31±1.27 a	249
0	200	400	0	0	0.01±0.01 b	1
0	200	0	400	0	0.00±0	0
0	200	0	0	400	0.00±0	0
400	200	400	0	0	2.22±0.36 a	240
400	200	0	400	0	1.48±0.71 a	160
400	200	0	0	400	2.96±0.40 a	320
0	0	0	0	0	0.02±0.01 b	2

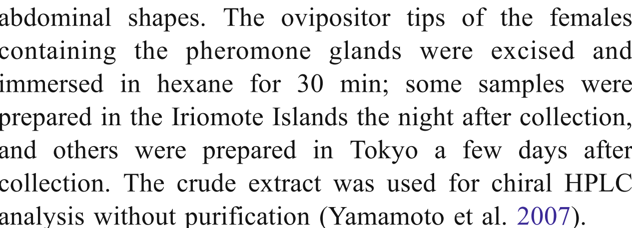
Tested at a copse in the Iriomote Islands from July 1 to August 6, 2009, using three traps for each lure. All chemicals are racemates; **1** = 6-methyl-2-octadecanone, **2** = 14-methyl-2-octadecanone, **4** = 4-methyl-2-octadecanone, **5** = 5-methyl-2-octadecanone, **6** = 7-methyl-2-octadecanone

[a] Values within each column followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

none (**8**) was synthesized by Wacker oxidation and catalytic hydrogenation of the diene **27**. ^{1}H NMR δ: 0.84 (3H, d, J=6.5 Hz), 0.87 (3H, t, J=6.8 Hz), ~1.1 (2H, m), ~1.3 (23H, m), ~1.55 (2H, m), 2.13 (3H, s), 2.41 (2H, t, J=7.5 Hz).

Field Trapping of Male Moths The attraction of *L. d. dharma* males by synthetic lures was examined in the Iriomote Islands, located in a subtropical zone (24.29°N, 123.86°E), from 2008 to 2010. Rubber septa (white rubber, OD 8 mm; Sigma-Aldrich, St. Louis, MO, USA) were used as dispensers, and synthetic components (> 97% purities on GC-MS analyses) dissolved in hexane (100 μl) were applied to them. Each lure was placed at the center of a sticky board trap (a 30×27 cm bottom plate with a roof; Sumitomo Chem. Co., Tokyo, Japan), which was set separately from the others by at least 10 m at about 1.5 m above the ground in a mixed forest area including many evergreen brood-leaved trees, such as *Castanopsis sieboldii* and *Ficus variegata*. Three traps were deployed for each synthetic lure, and three control traps with septa treated only with hexane (100 μl) also were tested. The number of captured males was counted at least every 2 weeks.

Pheromone Extract The adults of *L. d. dharma* were collected with a black-light in the Iriomote Islands in 2008 and 2009. The collection was carried out during 17:00–23:00 on several days without strong wind or moonlight. Moths attracted to the black-light were kept singly in a small glass vials, and separated by sex based on their


abdominal shapes. The ovipositor tips of the females containing the pheromone glands were excised and immersed in hexane for 30 min; some samples were prepared in the Iriomote Islands the night after collection, and others were prepared in Tokyo a few days after collection. The crude extract was used for chiral HPLC analysis without purification (Yamamoto et al. 2007).

Results

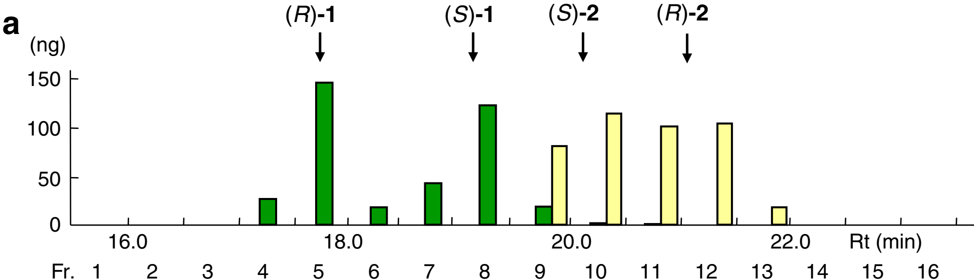
*Synthesis of Methyl-branched 2-Octadecanones (***4**–**8***)* As shown in Fig. 1, 4-methyl-, 5-methyl-, 6-methyl-, 13-methyl-, and 15-methyl-2-octadecanones (**4**–**8**, positional isomers of **1** and **2**) were prepared by Wacker oxidation of the corresponding methyl-branched olefins, which were synthesized in moderate yields starting from an alcohol (**10**, **16**, **20**, or **24**) or a ketone (**13**). The olefin (**12**) is a monoenyl compound, and others are dienyl compounds. Since the Wacker oxidation preferentially proceeds at a terminal position (Tsuji 1984), the dienes (**14**, **18**, **23**, and **27**) were successfully converted to mono-unsaturated 2-ketones (**15**, **19**, and two other octadecen-2-ones), which were hydrogenated over Pd-C to yield expected saturated 2-ketones (**4**–**8**). Their chemical structures were confirmed by NMR analyses; in particular, the ^{13}C-NMR spectra reflected positions of the keto and branched methyl groups. Table 1 shows the ^{13}C-NMR assignments of **4**–**6** in addition to the published data for **1**. The methyl branch approaches the 2-

Table 3 Field evaluation of synthetic pheromone analogs (**7**–**9**) for attraction of the *Lyclene dharma dharma* males

Lure components (μg/rubber septum)					Captured males	
Natural component		Pheromone analog				
1	**2**	**7**	**8**	**9**	/trap/night[a]	Total
400	200	0	0	0	2.69±1.39 a	234
400	0	200	0	0	0.00±0	0
400	0	0	200	0	0.00±0	0
0	200	0	0	400	0.07±0.10 c	6
400	200	200	0	0	3.18±0.40 a	277
400	200	0	200	0	3.47±1.31 a	302
400	200	0	0	400	1.08±0.19 b	94
0	0	0	0	0	0.00±0	0

Tested at a copse in the Iriomote Islands from January 14 to February 12, 2010, using three traps for each lure. All chemicals are racemates; **1** = 6-methyl-2-octadecanone, **2** = 14-methyl-2-octadecanone, **7** = 13-methyl-2-octadecanone, **8** = 15-methyl-2-octadecanone, **9** = 6-methyl-2-nonadecanone

[a] Values within each column followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test



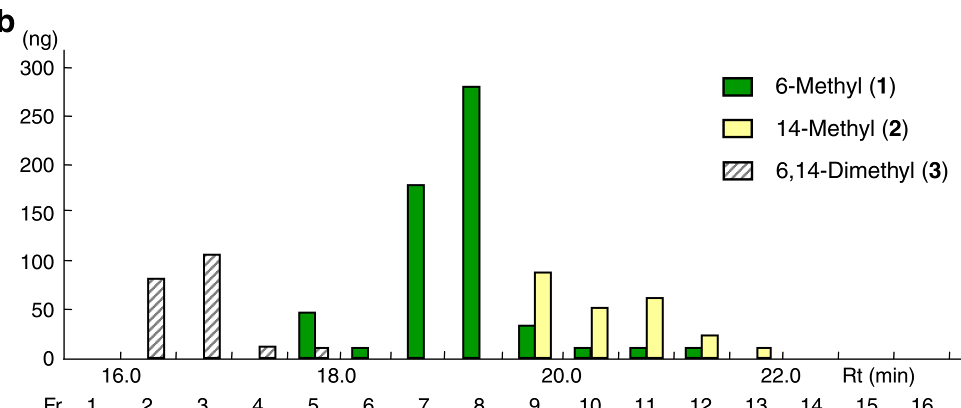


Fig. 3 Stereochemistry of pheromone components produced by the *Lyclene dharma dharma* females. A sample was injected into a chiral HPLC column (Chiralpak AD-H), and eluted with 0.05% 2-propanol in hexane at 0.5 ml/min. The eluted materials were collected every 30 s and quantitatively analyzed by GC-MS. a, Synthetic compounds (a mixture of racemic **1** and **2**, 500 ng each); and b, a pheromone extract (80 FE)

keto group from the 7-position of **6** to the 4-position of **4**. Chemical shifts of the methylene carbons between the two groups were strongly affected by their accession, and used to characterize the structures of these positional isomers. The signals of each carbon at a higher position than the methine carbon resonated at almost the same frequency in the four isomers, while the total numbers of the signals of the methylene carbons were different as a matter of course. On the other hand, **7** and **8** possessing the methyl branch at a terminal side showed a signal pattern around the carbonyl group common to **2,** but these three positional isomers could be differentiated by the patterns at the terminal side (see supplemental Table 1).

GC-MS Analysis of Synthetic Methyl-2-octadecanones Figure 2 shows the EI mass spectra of **1** and **4**–**6**. Among these synthetic methyl-2-octadecanones, the mass spectrum of the natural pheromone component **I** (Yamamoto et al. 2007) best coincides with that of **1**. Since ionization dominantly occurred at the 2-keto group, each positional isomer exhibited a distinguishable spectrum. The 6-methyl-2-ketone **1** and the 7-methyl isomer **6** showed the same base peak at *m/z* 58, which was produced by a McLafferty rearrangement, and characteristic ions derived from cleavage around the methyl branches could not be clearly recorded. However, abundant $[M–H_2O]^+$ was characteristically detected from **1**. In the case of 4-methyl isomer **4** and 5-methyl isomer **5**, base peaks were recorded at *m/z* 85 and 71, respectively. These ions are expected to be produced by a cleavage of the bond between the 4- and 5-positions, namely, the β- and γ-positions from the 2-keto group. The *m/z* values are different by fourteen mass units reflecting the positions of their methyl branches. These four isomers showed the following retention times (RTs) on a polar capillary column (DB-23): **1**, 19.61 min; **4**, 19.25 min; **5**, 19.78 min; and **6**, 19.60 min. On the other hand, the mass spectra of **7** and **8** are similar to that of **2**, with a base peak at *m/z* 58 (see supplemental Fig. 1). Detailed examination of the spectra revealed diagnostic ions as follows; $[MeCH(CH_2)_9]^+$ at *m/z* 153 for the 13-methyl isomer **7**, $[MeC(CH_2)_{10}]^+$ at *m/z* 167 for the 14-methyl isomer **2**, and $[MeC(CH_2)_{11}]^+$ at *m/z* 181 for the 15-methyl isomer **8**. These fragment ions might be produced by a two-step procedure. Acetone (*m/z* 58) might be first expelled from the molecule and then the produced alkene might be cleaved, losing the short alkyl chain next to the branching point. While their relative intensities are small (< 3%), these three positional isomers could be differentiated. The mass spectrum of the natural pheromone component **II** (Yamamoto et al. 2007) best coincides with that of **2**. These three isomers showed different RTs on the DB-23 column as follows: **2**, 19.81 min; **7**, 19.72 min; and **8**, 19.94 min.

Field Evaluation of the Synthetic Pheromone Analogs Since lures baited with racemic **1** and **2** in a ratio of 2:1 successfully attracted the *L. d. dharma* males in our previous field tests (Do et al. 2009), the biological activities of the positional isomers **4**–**8** and a long-chain analog **9**

also were examined as racemic mixtures. Table 2 shows the results of the field evaluation of **4**–**6**, which were mixed with **2** in place of **1** or with the binary lure of **1** and **2**. Their binary mixtures with **2** were completely unattractive to male *L. d. dharma* moths, indicating that pheromone analogs **4**–**6** could not substitute for **1**. In the case of the ternary lures, all the mixtures of **1** and **2** with one of the analogs attracted males the same as did the binary lure of **1** and **2**, indicating that no analogs were inhibitory. Table 3 shows results of the field evaluation of **7**–**9**. The octadecanones **7** and **8** were mixed with **1** in place of **2**, and the nonadecanone **9** was mixed with **2** in place of **1**. The positional isomers **7** and **8** could not substitute for **2** nor did they interfere with the **1** and **2** binary lure activity. The C_{19} analog **9** could not replace **1** for the male attraction, and inhibited the activity of the lure with **1** and **2**.

Chiral HPLC Analysis of the Natural Pheromone Each racemic mixture of methylketones, **1** and **2**, showed two peaks. Although the resolution of **2** was not perfect, the two enantiomers of **1** were almost separated completely. The chiral HPLC analysis of optically pure samples showed the following RTs: (*S*)-**1**, 19.2 min; (*R*)-**1**, 17.7 min; (*S*)-**2**, 20.2 min; and (*R*)-**2**, 21.1 min. In the case of dimethylketone **3,** including four stereoisomers, the optically inactive mixture showed one broad peak. Since the sensitivity of the RI detector is low, a small amount of the synthetic and natural methylketones could not be detected. Thus, after their injection onto the chiral HPLC column, the eluted materials were collected every 30 s and analyzed quantitatively by GC-MS. Even if **1** and **2** were included by the same fraction, GC-MS could quantify them separately. Figure 3a and b shows the chiral HPLC analyses of a synthetic pheromone (a mixture of racemic **1** and **2**, 500 ng each), and a pheromone extract (80 female equivalent, FE), respectively. This analysis indicated that two enantiomers of each component were equivalently included in the synthetic sample but (*S*)-**1** and (*S*)-**2** were dominant in the extract. The histogram of Fig. 3b shows that optical purity of the natural 6-methyl component is higher than that of the 14-methyl component.

Table 4 Field evaluation of the lures including optically active 6-methyl component (**1**) for attraction of the *Lyclene dharma dharma* males

Lure components (μg/rubber septum)				Captured males	
(*S*)-**1**	(*R*)-**1**	**2**	**3**	/trap/night[a]	Total
200	200	200	200	13.29±5.75 a	638
400	0	200	200	10.48±3.45 a	503
0	400	200	200	1.54±1.38 b	74
0	0	0	0	0.00±0	0

Tested at a copse in the Iriomote Islands from June 4 to 20, 2008, using three traps for each lure. **1**-6-methyl-2-octadecanone, **2** = 14-methyl-2-octadecanone (a racemic mixture), **3** = 6,14-dimethyl-2-octadecanone (a mixture of four stereoisomers)

[a] Number of Males captured by one trap during one night (mean ± SE). Values within each column followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

Table 5 Field evaluation of the lures including optically active 14-methyl component (**2**) for attraction of the *Lyclene dharma dharma* males

Lure components (μg/rubber septum)				Captured males	
1	(*S*)-**2**	(*R*)-**2**	**3**	/trap/night[a]	Total
400	100	100	200	0.91±0.57 a	98
400	200	0	200	1.16±0.27 a	125
400	0	200	200	0.19±0.10 b	21
0	0	0	0	0.00±0	0

Tested at a copse in the Iriomote Islands from December 8, 2008 to January 13, 2009, using three traps for each lure. **1** = 6-methyl-2-octadecanone (a racemic mixture), **2** = 14-methyl-2-octadecanone, **3** = 6,14-dimethyl-2-octadecanone (a mixture of four stereoisomers)

[a] Number of Males captured by one trap during one night (mean ± SE). Values within each column followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

Field Evaluation of Optically Active **1**–**3** The biological activities of optically active samples of the pheromone components were evaluated in the field. In the first experiment, each enantiomer of **1** was mixed with **2** and **3**, and male attraction to the combined lure was compared with that of the lure containing racemic **1**. Optically inactive **2** and **3** were used in this test. The lure including (*S*)-**1** captured as many male moths, as did the lure with racemic **1** (Table 4). The lure including (*R*)-**1** attracted some moths, but the total number was about seven times smaller than the lure with (*S*)-**1**, indicating that the males exclusively responded to the (*S*)-isomer and that the (*R*)-isomer did not interfere. Next, each enantiomer of **2** was mixed with optically inactive **1** and **3**, and the activity was compared with that of racemic **2**. The lure including (*S*)-**2** captured as many male moths as did the lure with racemic **2** (Table 5). The activity of the lure with (*R*)-**2** was very weak, also indicating the exclusive male response to the (*S*)-isomer and no disturbance by the (*R*)-isomer. Finally, the influences of (6*S*,14*S*)-**3** and (6*R*,14*R*)-**3** on the male attraction by a mixture of (*S*)-**1** and (*S*)-**2** were examined. The female moths produce three pheromone components, **I**–**III**, in a ratio of 2:1:1, but the role of component **III** was not be clarified in our previous field tests, which used optically inactive **3** as component **III**. Therefore, these three optically active compounds were mixed in a 2:1:1 ratio, and two other ratios, 2:1:3 and 2:1:10. Table 6 shows

Table 6 Attraction of *Lyclene dharma dharma* males by lures baited with optically active synthetic pheromone components (**1**–**3**)

Lure components (μg/rubber septum)				Captured males (/trap/night[a])	
(*S*)-**1**	(*S*)-**2**	(6*S*,14*S*)-**3**	(6*R*,14*R*)-**3**	Jan. 19 to Feb. 9	May 20 to June 17
100	50	0	0	0.73±0.59 a	1.82±0.66 a
100	50	50	0	0.23±0.10 a	2.76±1.27 a
100	50	150	0	0.43±0.14 a	1.82±0.44 a
100	50	500	0	0.05±0.07 b	0.68±0.35 b
100	50	0	50	0.40±0.29 a	2.10±0.52 a
100	50	0	150	0.30±0.17 a	2.60±0.85 a
100	50	0	500	0.37±0.24 a	1.73±0.60 a
0	0	0	0	0.00±0	0.00±0

Tested at a copse in the Iriomote Islands using three traps for each lure in 2009. **1** = 6-methyl-2-octadecanone, **2** = 14-methyl-2-octadecanone, **3** = 6,14-dimethyl-2-octadecanone

[a] *L. d. dharma* males captured by one trap during one night (mean ± SE). Values within each column followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

the results. Similar to the result of optically inactive **3** (Do et al. 2009), inclusion of (6*S*,14*S*)-**3** or (6*R*,14*R*)-**3** as the third component in a naturally occurring ratio did not affect male attraction to the binary lure of (*S*)-**1** and (*S*)-**2**. However, the number of captured males was significantly reduced when the dose of (6*S*,14*S*)-**3** was increased up to five times that of (*S*)-**1**. This reduction was not brought about by (6*R*,14*R*)-**3**. While this result did not elucidate the function and stereochemistry of **III**, it suggests that males might recognize (6*S*,14*S*)-**3** but not (6*R*,14*R*)-**3**. The former stereoisomer (6*S*,14*S*)-**3** bears two chiral centers, as does the bioactive combination of (*S*)-**1** and (*S*)-**2**.

Discussion

The *L. d. dharma* females produce a novel sex pheromone composed of methyl-branched 2-octadecanones. In this study, the methyl branches at the 6- and 14-positions, which had been determined by comparing the mass spectra of natural components and synthetic candidates **1**–**3**, were supported by GC-MS analyses of several positional isomers (**4**–**8**; Fig. 1). The mass spectra of **4**–**8** were different from those of the natural pheromone components (Yamamoto et al. 2007) to a greater or lesser extent. Methyl-branched hydrocarbons produced diagnostic secondary ions cleaved at a methyl branch (Ando et al. 2004). In the case of methyl-branched ketones, ionization is expected to occur mainly at the keto group, and the intensity of the secondary ions is extremely diminished. However, the mass spectra of 4-, 5-, and 6-methyl 2-ketones, **4**, **5**, and **1**, are quite different because each methyl branch affects the McLafferty rearrangement. While the spectrum of 7-methyl-2-ketone **6** had a pattern similar to that of **1**, **6** showed an abundant fragment ion at *m/z* 222 (18%) and could be differentiated from **1**. The relative intensities of the ion at *m/z* 222 in the spectra of 13-, 14-, and 15-methyl 2-ketones, **7**, **2**, and **8**, were also low (<5%, see supplemental Fig. 1). Unfortunately, a fragmentation pathway reflecting the methyl branch at the 7-position cannot be proposed for the *m/z* 222 ion; thus, diagnostic ions of **6** could be identified only after measurement of the mass spectra of some other positional isomers, especially 8-methyl-2-ketone. The mass spectra of three 2-ketones, which included a methyl branch at the position far from the keto group, were similar, as expected from their structures, but detailed analysis of the spectra identified diagnostic ions which characterized each from the others. These results indicate the usefulness of GC-MS analysis to determine the positions of both the keto group and the methyl branch in methyl-branched ketones.

This is the first identification of a pheromone from the Lithosiinae, a large group in the family Arctiidae, including 78 species inhabiting Japan. Thus, similar compounds might be identified from related species. Several Lithosiinae species, such as *Bizone hamata* and *Nipponasura sanguinea*, have been recorded in the Iriomote Islands; however, traps baited with synthetic 2-ketones attracted no male moths except for *L. d. dharma* in our field tests. While the larvae feeding on lichen contribute to the ecosystem of the forest, information on the ecological aspects of the lithosiine species is limited, and the pheromone could be utilized as a monitoring tool for the adults of *L. d. dharma*.

No branched ketones have been characterized from any other female moths, but 6-methyl-5-hepten-2-one (sulcatone) was found in brushes of a male moth (Aplin and Birch 1970) and wings of a male butterfly (Honda 1980).

Furthermore, structurally related pheromones have been identified from insects in other orders, such as 3,11-dimethyl-2-heptacosanone and its C_{29} derivative in a cockroach, *Blattella germanica* (Nishida et al. 1974; Schal et al. 1990), and 10-methyl-2-tridecanone (Guss et al. 1983) and 6,12-dimethyl-2-pentadecanone (Chuman et al. 1987) in *Diabrotica* spp. beetles.

Pheromone components of *L. d. dharma* include chiral centers and, while chiral GC did not resolve these methyl-branched compounds (Mori 2009), normal-phase HPLC equipped with a chiral column (Chiralpak AD-H) was successful in the enantiomeric separation of **1** and **2**. The chiral HPLC analysis of the pheromone extract revealed that natural mono-methyl components were not optically pure, but females dominantly produced (*S*)-**1** and (*S*)-**2** (Fig. 3). In our pheromone research, the chiral HPLC has been used for the resolution of several epoxy compounds in lepidopteran Type II pheromones (Qin et al. 1997; Yamamoto et al. 1999; Ando et al. 2004), an acetate of secondary alcohol produced by a midge (Hall et al. 2009), and a ketol and a diol produced by a long-horned beetle (Kiyota et al. 2009). This study is the first successful application of chiral HPLC to methyl-branched pheromones, and it is noteworthy that enantiomers of less polar volatiles showed different chromatographic behaviors on a chiral HPLC column. Several sex pheromones with a chiral center at a methyl-branched position have been identified from lepidopteran species, and their stereochemistry has been reported as follows: (*R*)-10-methyldodecyl acetate in *Adoxophyes honma* (Tamaki et al. 1983), (*S*)-14-methyl-1-octadecene in *Leucoptera clerkella* (Sato et al. 1986), (5*S*,9*S*)-5,9-dimethylheptadecane in *L. scitella* (Tóth et al. 1989), (5*R*,11*S*)-5,11-dimethylheptadecane in *Lambdina fiscellaria fiscellaria* (Li et al. 1993a), (*S*)-2,5-dimethylheptadecane in *Lambdina fiscellaria lugubrosa* (Li et al. 1993b), (*S*)-7-methylheptadecane and *meso*-7,11-dimethylheptadecane in *L. athasaria* and *L. pellucidaria* (Duff et al. 2001), and (3*S*,13*R*)-3,13-dimethylheptadecane in *Nepytia freemani* (King et al. 1995). All of the absolute configurations were presumed by bioassays with synthetic stereoisomers, but direct instrumental analyses have never been accomplished. It is important to know whether or not chiral HPLC can be applied to these chiral compounds, particularly, the hydrocarbons produced by Lyonetiidae and Geometridae species. In this study, the absolute configurations of the dimethyl component **III** could not be determined by the chiral HPLC analysis because the separation of four stereoisomers was not achieved. In order to utilize chiral HPLC for a wide range of pheromone studies, the property and applicability of some chiral columns should be examined in detail.

The field evaluation of synthetic methylketones **1**–**9** indicated that the *L. d. dharma* male moths recognized only the 2-ketones with a methyl branch at the 6- or 14-positions on a C_{18} chain. This result corroborated the assignments of **1** and **2** to the pheromone components **I** and **II**. The positional isomers **4**–**8** could not play a role as a substitution of **1** or **2** or as an inhibitor against the binary lure baited with **1** and **2**. These isomers could not compete with the natural pheromone components. Moreover, the field tests with stereoisomers showed that the bioactive enantiomers were (*S*)**-1** and (*S*)**-2**, which were dominant in the pheromone glands. This result confirms the mating communication of *L. d. dharma* triggered by (*S*)-**1** and (*S*)-**2**, and attraction by the mixture of (*S*)-**1** and (*S*)-**2** was not inhibited by the antipodes. While GC-MS data of the synthetic 6,14-dimethy-2-ketone **3** coincided well with that of the natural component **III**, the assignment of the 6,14-dimethyl structure was not confirmed by the biological activity of the four stereoisomers of **3** (Do et al. 2009). As for the optically inactive sample of **3**, field tests with the stereoisomers of **3** did not elucidate a behavioral role for this compound although the pheromone extract included as much **III** as **II**. One possibility is that **III** has some effects, such as a repellent effect, on males of related species. We are analyzing the pheromones of other Lithosiinae species, and expect that the role of **III** will be clarified through those studies.

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2,3-Dihydrohomofarnesal: Female Sex Attractant Pheromone Component of *Callosobruchus rhodesianus* (Pic)

Kenji Shimomura · Hiroyuki Koshino · Arata Yajima · Noriko Matsumoto · Yuuma Kagohara · Koichi Kamada · Shunsuke Yajima · Kanju Ohsawa

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Abstract *Callosobruchus rhodesianus* (Pic) (Coleoptera: Chrysomelidae: Bruchinae) is a pest of stored legumes through the Afro-tropical region. In laboratory bioassays, males of *C. rhodesianus* were attracted to volatiles collected from virgin females. Collections were purified by various chromatographic techniques, and the biologically active component isolated using gas chromatographic-electroantennographic detection analysis. Gas chromatography-mass spectrometry and NMR analyses suggested that the active compound was 2,3-dihydrohomofarnesal, i.e., 7-ethyl-3,11-dimethyl-6,10-dodecadienal. The structure was confirmed by non-stereoselective and enantioselective total synthesis. Using chiral gas chromatography, the absolute configuration of the natural compound was confirmed as (3*S*,6*E*)-7-ethyl-3,11-dimethyl-6,10-dodecadienal. Y-tube olfactomter assays showed that only the (*S*)-enantiomer attracted males of *C. rhodesianus*. The (*R*)-enantiomer and racemate did not attract males, suggesting that the (*R*)-enantiomer inhibits the activity of the natural compound. In combination with previous reports about sex attractant pheromones of congeners, we suggest that a saltational shift of the pheromone structure arose within the genus *Callosobruchus*.

Key Words *Callosobruchus rhodesianus* · 2,3-Dihydrohomofarnesal · (3*S*,6*E*)-7-Ethyl-3,11-dimethyl-6,10-dodecadienal · GC-EAD · Seed beetle · Sex attractant pheromone · Saltational shift

K. Shimomura (✉) · N. Matsumoto · K. Kamada · S. Yajima · K. Ohsawa
Department of Biosciences, Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan
e-mail: k3shimom@nodai.ac.jp

H. Koshino
Chemical Biology Core Facility, Advanced Science Institute, RIKEN, Saitama 351-0198, Japan

A. Yajima · Y. Kagohara
Department of Fermentation Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan

Introduction

Pheromones are an important component of communication systems of many taxa, especially in insects. The composition of pheromones can be diverse, even in closely related species (Symonds and Elgar, 2008). In insects, closely related species commonly use the same, or similar, compounds as pheromones, with species specificity achieved by using a unique blend in a multi-component pheromone system. This phenomenon has been studied extensively in moths. Closely related taxa often use the same major component, while the pheromones of related species are different, in terms of the minor components of the blend or in ratios of components (specific ratios of *cis*/*trans* and/or enantiomeric, isomers) (Cardé and Haynes, 2004).

There are two ways in which pheromone composition is thought to evolve. The first involves a gradual process of small changes in components, or a change in relative proportions of components over time, such that related species share closely related, or identical, components (Roelofs and Brown, 1982). In the second way, major changes, leading to saltational shifts, generate a new phenotype that is greatly or completely different from the antecedent (Baker, 2002).

Seed beetles of the genus *Callosobruchus* (Coleoptera: Chrysomelidae: Bruchinae) are serious pests of stored pulses, cowpeas, pigeon peas, and other legumes (Rees, 1996, 2004). To date, the genus *Callosobruchus* is comprised of some 20 species (Borowiec, 1987), and is distributed worldwide, particularly across the Old World. Female-produced sex attractant pheromones have been identified from four species: *Callosobruchus analis* (Fabricius) (Cork et al., 1991), *C. maculatus* (Fabricius) (Phillips et al., 1996), *C. subinnotatus* (Pic) (Shu et al., 1999), and *C. chinensis* (L.) (Shimomura et al., 2008). The species, *C. analis*, *C. maculatus*, and *C. subinnotatus* use methyl branched short-chain fatty acids as pheromones (Cork et al., 1991; Phillips et al., 1996; Shu et al., 1999), while *C. chinensis* uses homosesquiterpene aldehydes (Shimomura et al., 2008). In light of this, we initiated a project to clarify two matters: first, whether the structural shifts of the sex attractant pheromone were restricted only to *C. chinensis*, and second, to determine if there is a correlation between pheromone structure and molecular-based phylogenies previously reported (Tuda et al., 2006).

Callosobruchus rhodesianus (Pic) is found mainly in southern Africa, but has spread around the equator where it is sporadically reported (Southgate, 1979; Giga and Smith, 1983). In terms of morphological similarity, *C. rhodesianus* had been regarded as synonymous with *C. chinensis* (e.g., Skaife, 1926). However, Southgate (1958) concluded that they were separate species, based on morphological characteristics of antennae and genitalia. In a comparative study of four *Callosobruchus* species, *C. rhodesianus* was found to be more successful in a greater range of climatic conditions than the other species (Giga and Smith, 1983). Therefore, it was suggested that *C. rhodesianus* had potential to spread further, especially to subtropical regions, although there are no reports of this occurring to date.

Because of its narrow distribution and limited damage to legumes (Booker, 1967), the biology of *C. rhodesianus* rarely has been studied. In this paper, we report the isolation and identification of the female sex attractant pheromone of *C. rhodesianus*, and discuss differences in the pheromone compared to previously identified pheromones of congeners.

Methods and Materials

Insects A laboratory colony of *C. rhodesianus* was used. The beetles were reared on *Vigna angularis* in a dark incubator, at 28°C, in ambient humidity. Newly emerged adults from beans were separated, by sex, until used.

Pheromone Collection Charcoal-filtered air was passed over approximately 1,000–1,500 virgin females, in a group, in a 1-l glass chamber. The headspace volatiles were drawn by an air pump through a glass column (12 mm i.d., 90 mm length) filled with Tenax TA (1.5 g, 80/100 mesh, GL Science, Tokyo, Japan). The airborne collections were made for 10–14 d and eluted from the adsorbent with 10 ml of pentane. The extracts were concentrated in a nitrogen stream in an ice bath and kept in a freezer (−30°C) until use.

Column Chromatography Crude volatile collections were subjected to column chromatography (1 g of silica gel; Wakogel C-200, Wako Pure Chemical Industries, Osaka, Japan). The column was eluted sequentially with 10 ml each of pentane–diethyl ether in ratios of 10:0 (v/v), 9.5:0.5, 9:1, 8:2, 5:5, and 0:10.

High Performance Liquid Chromatography (HPLC) Active fractions were further purified by HPLC using a LC-10A (Shimadzu, Kyoto, Japan) equipped with a silica gel column (Inertsil SIL-100A, 3 μm, 4.6×250 mm, GL Science), and with 3% diethyl ether in pentane (1 ml.min^{-1}) as the mobile phase. The eluent was monitored with a photodiode array detector (SPD-M10Avp, Shimadzu), and fractions were collected every 1 min.

Coupled Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analysis The GC-EAD technique was performed as previously described (Shimomura et al., 2008), using a DB-5 or DB-23 capillary column (30 m× 0.25 mm i.d., 0.25 μm film thickness; J&W scientific, Folsom, CA, USA) for the analyses. Nitrogen was used as the carrier gas at a head pressure of 135 kPa and a flow rate of 2 ml.min^{-1}. Samples were injected in splitless mode, with the purge valve off for 2 min. The oven temperature was set initially at 60°C for 3 min, increased at 10°C.min^{-1} to 220°C (for DB-23) or 280°C (for DB-5), and then held for 10 min. The injector temperature was set at 250°C, and the flame ionization (FID) and EAD outlet temperatures at 280°C. The column eluent was combined with nitrogen make-up gas (30 ml.min^{-1}) and then split 1:1 to the FID and EAD.

Gas Chromatographic-Mass Spectrometric (GC-MS) Analyses Gas chromatography-electron ionization mass spectrometry (GC-EI-MS) was carried out with a Shimadzu GC 17A, equipped with a DB-5 capillary column (30 m× 0.25 mm i.d., 0.25 μm film thickness) and splitless injection, coupled to a Shimadzu QP-5000 quadrupole mass spectrometer in EI mode (70 eV). Helium was the carrier gas, at a head pressure of 100 kPa and a flow rate of 1.6 ml.min^{-1}. The oven temperature was set initially at 60°C for 3 min, increased at 10°C.min^{-1} to 280°C, and held for 10 min. The injector and interface temperatures were set at

250°C and 280°C, respectively. Gas chromatography-chemical ionization mass spectrometry (GC-CI-MS) was performed on a Shimadzu GCMS-QP2010. Iso-butane, at 50 kPa, was used as reagent gas, with the other analytical conditions the same as in the GC-EI-MS analysis.

Preparative Gas Chromatography GC-EAD-active compounds were isolated by a micro-preparative GC system (Nojima et al., 2008), with modification. An HP 5890 series II GC (Palo Alto, CA, USA), equipped with a ZB-1 wide-bore capillary column (30 m×0.53 mm i.d., 1.50 μm film thickness; Phenomenex, Torrance, CA, USA) was operated in splitless mode. Nitrogen was used as carrier gas, at a head pressure of 30 kPa and a flow rate of 5 ml.min^{-1}. The oven temperature was set initially at 60°C for 3 min, increased at 10°C.min^{-1} to 250°C, and held for 10 min. The temperatures of the injection and collection (modified from an injection port) ports were set at 250°C. To collect an active component, a pre-cleaned collection trap, consisting of a 40-cm-long, deactivated wide-bore capillary tube (Agilent, Palo Alto, CA, USA), was connected to the end of the column, with a direct injection glass liner (1 mm Uniliner®, 1 mm ID, 6.3 mm OD×78.5 mm length; Restek, Bellefonte, PA, USA) set on the collection port just prior to the retention time of a target component. A preconditioned cooling sheath (Nojima et al., 2004), containing dry ice, was set on the exposed collection trap. Retention times of active components were checked previously by FID under the same analytical conditions. After sample collection, the sheath was removed and the collection trap pulled out to detach it from the injection liner. The collection trap was set up immediately for NMR sample preparation and eluted with C_6D_6 (99.96%, Merck, Darmstadt, Germany) directly into a 3 mm OD NMR tube (Shigemi, Tokyo, Japan).

NMR Analysis ^{1}H-NMR analysis of isolated active compound was performed on a JNM-ECA 600 spectrometer (JEOL, Tokyo, Japan). Chemical shifts were expressed in ppm relative to the residual solvent signal.

Enantioselective Gas Chromatography An HP 5890 series II GC, equipped with two β-DEX 225 capillary columns [each 30 m (60 m)×0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA], connected with a press fit connector (Agilent), was operated in splitless mode. Helium was used as carrier gas at a head pressure of 200 kPa and a flow rate of 1.2 ml.min^{-1}. The oven temperature was set at 120°C and held for 360 min. The injector temperature was set at 250°C, and the FID temperature at 280°C.

Synthesis NMR spectra (400 MHz for ^{1}H and 100 MHz for ^{13}C) were recorded on a JNM-ECA 400 spectrometer (JEOL). Chemical shifts are expressed in ppm relative to the residual solvent signal. IR spectra were recorded on an IR-4100 spectrometer (Jasco, Tokyo, Japan). Optical rotation was measured on a P-2100 polarimeter (Jasco). Elemental compositions (ECs) were analyzed on a Micro-corder JM10 (J-Science, Kyoto, Japan).

*Synthesis of Stereoisomeric Mixture of 2,3-Dihydrohomofarnesal (**1**)* A mixture of geometrical isomers at C-6 of 2,3-dihydrohomofarnesal (**1**) was prepared from homofarnesal **2** (Shimomura et al., 2008) by chemo-selective reduction of the α,β-unsaturated aldehyde using Stryker's reagent (Brestensky and Stryker, 1989) (Scheme 1).

To a stirred solution of a stereoisomeric mixture of **2** (10 mg, 0.043 mmol) and *tert*-butyldimethylchlorosilane (TBSCl) (16 mg, 0.107 mmol), in dry, deoxygenated benzene (0.5 ml), was added a solution of $[(Ph_3P)CuH]_6$ (50 mg, 0.026 mmol) in dry, deoxygenated benzene (2 ml). The mixture was stirred for 1 h, filtered through a Celite pad, and the filter cake washed with benzene. The combined filtrate and washings were concentrated under reduced pressure. The residue was dissolved in tetrahydrofuran (THF, 0.5 ml) and 70 mM phosphate buffer (pH 7.0, 0.2 ml). To this solution was added 1 M tetrabutylammonium fluoride (TBAF, 120 ml, 120 mmol). The mixture was stirred for 30 min and then poured into water. The aqueous layer was extracted with Et_2O, and the combined organic layers were washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=80:1) to afford a stereoisomeric mixture of **1** (7 mg, 70%) as a colorless oil. EI-MS *m/z* (relative intensity): (6*Z*)-**1**: 236 (M^+, 1), 207 (6), 193 (21), 175 (9), 137 (24), 123 (26), 107 (16), 95 (29), 81 (46), 69 (100), 55 (59), 41 (68). EI-MS: (6*E*)-**1**: 236 (M^+, 1), 207 (6), 193 (22), 175 (10), 137 (30), 123 (27), 107 (18), 95 (29), 81 (54), 69 (100), 55 (66), 41 (71).

*Stereoselective Synthesis of 2,3-Dihydrohomofarnesal (**1**)* Both enantiomers of 2,3-dihydrohomofarnesal were synthesized from (*R*)- and (*S*)-citronellol (>97% e.e.) (Scheme 2). Briefly, known **3**, prepared from citronellol (Pichlmair et al., 2006), was epoxidized with *m*-chloroperbenzoic acid, and the resulting epoxide treated with periodic acid to give aldehyde **4**. Addition of Grignard reagent, prepared from 2-bromo-1-butene to **4**, afforded allylic alcohol **5**, which was subjected to Johnson-Claisen rearrangement to provide ester **6**. The geometry of the

Scheme 1 Synthesis of a stereoisomeric mixture of 2,3-dihydrohomofarnesal. **a** TBSCl, $[(Ph_3P)CuH]_6$, benzene, then TBAF, pH 7 phosphate buffer, THF (70%)

Scheme 2 Synthesis of both enantiomers of 2,3-dihydrohomofarnesal. **a** *m*-CPBA, CH_2Cl_2; **b** HIO_4, THF, H_2O (90%); **c** 1-buten-2-yl magnesium bromide, THF (77%); **d** triethyl orthoacetate, propanoic acid, toluene (96%); **e** LAH, Et_2O (95%); **f** PCC, MS4A, CH_2Cl_2 (73%); **g** isopropylidene triphenyl phosphorane, THF (89%); **h** Li, liq. NH_3, THF, EtOH (96%); **i** Dess-Martin periodinane, $NaHCO_3$, CH_2Cl_2 (85%)

resulting double bond was confirmed to be exclusively *E* by ^{1}H NMR analysis of **6**. Ester **6** was converted into the corresponding alcohol **7** by LAH reduction, and oxidation of the resulting alcohol with PCC afforded aldehyde **8**. Wittig condensation of **8** with isopropylidene triphenyl phosphorane (generated from isopropyl triphenylphosphonium iodide and *n*-BuLi) gave **9**, and then the *p*-methoxybenzyl protecting group of **9** was removed under Birch conditions to give alcohol **10**. Finally, oxidation of the hydroxyl group of **10** with Dess-Martin periodinane (Dess and Martin, 1983) afforded (3*S* or *R*,6*E*)-2,3-dihydrohomofarnesal **1**. The details of the syntheses were as follow.

(S)-6-(4-Methoxybenzyloxy)-4-methylheptanal (**4**) To a stirred and cooled (0°C) solution of (*S*)-**3** (469 mg, 1.70 mmol) in CH_2Cl_2 (10 ml) was added *m*-chloroperbenzoic acid (*m*-CPBA) (77%, 528 mg, 3.06 mmol). The mixture was stirred for 1 h and quenched with 1 M NaOH. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were washed with water and brine. The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure to give the corresponding epoxide (500 mg). To a stirred and cooled (0°C) solution of the epoxide in THF/water (10:1, 13 ml) was added periodic acid dihydrate (974 mg, 4.27 mmol). The mixture was stirred for 1 h and quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with Et_2O, and the combined organic layers were washed with water and brine. The organic layer was dried with $MgSO_4$, concentrated under reduced pressure, and purified by SiO_2 column chromatography (hexane/EtOAc=30:1) to afford **4** (381 mg, 90%) as a colorless oil. $[\alpha]_D^{25}$=−3.4 (*c*=1.3, $CHCl_3$). IR (film): ν=1,724 cm^{-1} (C = O). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.89 (d, *J*=6.4 Hz, 3H), 1.44 (m, 2H), 1.65 (m, 3H), 2.43 (m, 2H), 3.49 (m, 2H), 3.80 (s, 3H), 4.41 (d, *J*=11.8 Hz, 1H), 4.43 (d, *J*=11.8 Hz, 1H), 6.88 (d, *J*=8.7 Hz, 2H), 7.25 (d, *J*=8.7 Hz, 2H), 9.76 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=19.2, 28.7, 29.4, 36.4, 41.5, 55.1, 67.8, 72.5, 113.6, 113.7, 129.1, 130.5, 159.0, 202.8. EC calculated for $C_{15}H_{22}O_3$: C, 73.00; H, 8.80. Found: C, 72.86; H, 8.59.

(4RS,7S)-9-(4-Methoxybenzyloxy)-7-methyl-3-methylene-nonan-4-ol (**5**) A Grignard reagent was prepared from 2-bromo-1-butene (466 mg, 3.45 mmol), Mg (91 mg, 3.73 mmol), and a catalytic amount of I_2 in dry THF (6 ml) under Ar atmosphere at 75°C. The resulting solution was added into a stirred and cooled (−78°C) solution of **4** (345 mg, 1.38 mmol) in dry THF (1 ml). The mixture was stirred for 15 h at room temperature and then quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with Et_2O. The combined organic layers were washed with brine, dried with $MgSO_4$, and concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=12:1) to afford **5** (326 mg, 77%) as a colorless oil. $[\alpha]_D^{24}$=−3.9 (*c*=1.0, $CHCl_3$). IR (film): ν=3,427 cm^{-1} (O–H). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.879 and 0.883 (d, *J*=6.3 Hz, 3H), 1.04 (t, *J*=7.1 Hz, 3H), 1.19–1.65 (m, 8H), 1.99 (m, 1H), 2.08 (m, 1H), 3.47 (m, 2H), 3.80 (s, 3H), 4.04 (t, *J*=6.3 Hz, 1H), 4.41 (d, *J*=12.0 Hz, 1H), 4.42 (d, *J*=12.0 Hz, 1H), 4.83 (s, 1H), 4.99 (m, 1H), 6.87 (d, *J*=8.8 Hz, 2H), 7.25 (d, *J*=8.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.2, 15.3, 19.6, 19.7, 23.7, 23.9, 29.8, 29.9, 32.7, 32.78, 32.83, 36.6, 36.7, 55.3, 68.26, 68.28, 72.53, 72.55, 75.8,

76.0, 108.3, 108.5, 113.7, 129.2, 130.7, 153.5, 153.6, 159.1. EC calculated for $C_{19}H_{30}O_3$: C, 74.47; H, 9.87. Found: C, 74.20; H, 9.82.

*(4E,8S)-Ethyl 4-ethyl-10-(4-methoxybenzyloxy)-8-methyl-4-decenoate (***6***)* A mixture of **5** (55 mg, 0.18 mmol) and freshly distilled triethyl orthoacetate (156 mg, 0.96 mmol), with a few drops of propanoic acid in toluene (2 ml), was stirred and heated at reflux under conditions for the distillation of ethanol. Heating was continued for 24 h, followed by concentration of the reaction mixture under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=30:1) to afford **6** (66 mg, 96%) as a colorless oil. $[\alpha]_D^{23}=-4.0$ (c=1.0, $CHCl_3$). IR (film): ν=1,736 cm^{-1} (C = O). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.87 (d, J=6.7 Hz, 3H), 0.96 (t, J=7.5 Hz, 3H), 1.10–1.68 (m, 5H), 1.23 (t, J=7.1 Hz, 3H), 1.98 (m, 2H), 2.01 (q, J=6.7 Hz, 2H), 2.31 (m, 2H), 2.38 (m, 2H), 3.46 (m, 2H), 3.80 (s, 3H), 4.12 (q, J=7.5 Hz, 2H), 4.41 (d, J=12.0 Hz, 1H), 4.42 (d, J=12.0 Hz, 1H), 5.08 (t, J=7.1 Hz, 1H), 6.87 (d, J=8.8 Hz, 2H), 7.25 (d, J=8.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.2, 14.2, 19.5, 23.1, 25.0, 29.6, 31.5, 33.4, 36.7, 37.4, 55.3, 60.2, 68.4, 72.6, 113.7, 124.9, 129.2, 130.8, 139.1, 159.1, 173.6. EC calculated for $C_{23}H_{36}O_4$: C, 73.37; H, 9.64. Found: C, 73.28; H, 9.37.

*(4E,8S)-4-Ethyl-10-(4-methoxybenzyloxy)-8-methyl-4-decen-1-ol (***7***)* To a stirred and cooled (0°C) suspension of lithium tetrahydridoaluminate (LAH, 93 mg, 2.44 mmol) in dry Et_2O (2 ml) was added a solution of **6** (92 mg, 0.24 mmol) in dry Et_2O (1 ml). The mixture was stirred for 30 min at room temperature. The stirred mixture was cooled to 0°C, and excess reagent destroyed by successive additions of water (0.1 ml), 15% NaOH (0.1 ml) and water (0.3 ml). After stirring for 45 min at room temperature, the mixture was filtered through a Celite pad, and the filter cake was washed with Et_2O. The combined filtrate and washings were concentrated under reduced pressure, and the residue purified by SiO_2 column chromatography (hexane/EtOAc=20:1) to afford **7** (78 mg, 95%) as a colorless oil. $[\alpha]_D^{23}=-2.5$ (c=1.0, $CHCl_3$). IR (film): ν=3,390 cm^{-1} (O–H). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.88 (d, J=6.3 Hz, 3H), 0.96 (t, J=7.5 Hz, 3H), 1.10–1.69 (m, 8H), 1.92–2.08 (m, 6H), 3.47 (m, 2H), 3.63 (t, J=6.3 Hz, 2H), 3.80 (s, 3H), 4.41 (d, J=12.0 Hz, 1H), 4.42 (d, J=12.0 Hz, 1H), 5.10 (t, J=7.1 Hz, 1H), 6.87 (d, J=8.8 Hz, 2H), 7.25 (d, J=8.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.2, 19.5, 22.9, 25.0, 29.6, 31.0, 32.7, 36.7, 37.5, 55.2, 62.8, 68.4, 72.5, 113.7, 124.8, 129.2, 130.7, 140.3, 159.0. EC calculated for $C_{21}H_{34}O_3$: C, 75.41; H, 10.25. Found: C, 75.23; H, 10.42.

*(4E,8S)-4-Ethyl-10-(4-methoxybenzyloxy)-8-methyl-4-decenal (***8***)* To a stirred suspension of pyridinium chlorochromate (PCC, 495 mg, 2.30 mmol) and powdered 4A molecular sieves (MS4A, 250 mg) in CH_2Cl_2 (8 ml) was added a solution of **7** (386 mg, 1.15 mmol) in CH_2Cl_2 (1 ml). The mixture was stirred for 3 h, and then diluted with Et_2O. The mixture was filtered through a Celite pad, and the filter cake was washed with Et_2O. The combined filtrate and washings were concentrated under reduced pressure, and the residue was purified by SiO_2 column chromatography (hexane/EtOAc=30:1) to afford **8** (278 mg, 73%) as a colorless oil. $[\alpha]_D^{21}=-1.7$ (c=0.98, $CHCl_3$). IR (film): ν=1,725 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.88 (d, J=6.3 Hz, 3H), 0.96 (t, J=7.5 Hz, 3H), 1.00–1.69 (m, 5H), 1.97 (m, 2H), 2.02 (q, J=7.5 Hz, 2H), 2.32 (m, 2H), 2.50 (m, 2H), 3.47 (m, 2H), 3.80 (s, 3H), 4.41 (d, J=12.0 Hz, 1H), 4.42 (d, J=12.0 Hz, 1H), 5.07 (t, J=7.1 Hz, 1H), 6.87 (d, J=8.8 Hz, 2H), 7.25 (d, J=8.8 Hz, 2H), 9.75 (t, J=1.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.1, 19.5, 23.2, 25.0, 28.6, 29.6, 36.7, 37.4, 42.3, 55.3, 68.4, 72.6, 113.7, 125.3, 129.2, 130.7, 138.7, 159.1, 202.8. EC calculated for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 75.77; H, 9.92.

*(3S,6E)-7-Ethyl-3,11-dimethyl-6,10-dodecadienyl 4-methoxybenzyl ether (***9***)* To a stirred suspension of isopropyl triphenylphosphonium iodide (692 mg, 1.90 mmol) in dry THF (10 ml) at −78°C was added dropwise 1.6 M n-BuLi in hexane (890 µl, 1.40 mmol) under Ar atmosphere. After stirring for 30 min at room temperature, the mixture was added dropwise to a solution of **8** (50 mg, 0.15 mmol) in dry THF (1 ml) at −78°C under Ar. After stirring for 1 h, the mixture was poured into water. The aqueous phase was extracted with hexane, and the combined extracts were washed with water, brine, and dried with Na_2SO_4. The organic layer was concentrated under reduced pressure, and the residue was purified by SiO_2 column chromatography (hexane/EtOAc=100:1) to afford **9** (48 mg, 89%) as a colorless oil. $[\alpha]_D^{25}=-4.0$ (c=1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.88 (d, J=6.3 Hz, 3H), 0.95 (t, J=7.5 Hz, 3H), 1.11–1.62 (m, 5H), 1.60 (s, 3H), 1.68 (s, 3H), 2.01 (m, 8H), 3.47 (m, 2H), 3.80 (s, 3H), 4.41 (d, J=12.0 Hz, 1H), 4.42 (d, J=12.0 Hz, 1H), 5.06 (t, J=7.1 Hz, 1H), 5.10 (m, 1H), 6.87 (d, J=8.8 Hz, 2H), 7.26 (d, J=8.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.3, 17.7, 19.6, 23.1, 25.0, 25.7, 27.0, 29.6, 36.5, 36.7, 37.6, 55.3, 68.4, 72.5, 113.7, 124.2, 124.5, 129.2, 130.8, 131.2, 140.7, 159.1. EC calculated for $C_{24}H_{38}O_2$: C, 80.39; H, 10.68. Found: C, 80.44; H, 10.84.

*(3S,6E)-7-Ethyl-3,11-dimethyl-6,10-dodecadien-1-ol (***10***)* Under Ar atmosphere, lithium (19 mg, 2.7 mmol) was added to liquid NH_3 (10 ml) at −78°C. After stirring for 30 min below −40°C, a solution of **9** (48 mg, 0.13 mmol) in THF (20 ml) and ethanol (1.5 ml) was added dropwise. Stirring

was continued for 1.5 h at −40°C and, during this time, lithium (19 mg, 2.7 mmol) was added to the reaction mixture. The reaction was quenched with NH_4Cl. Subsequently, NH_3 was removed by gentle warming, and the residue was diluted with water and extracted with hexane. The combined organic layers were washed with brine and dried with $MgSO_4$. After concentration, the residue was purified by SiO_2 column chromatography (hexane/EtOAc= 30:1) to afford **10** (30 mg, 96%) as a colorless oil. $[\alpha]_D^{23}$= −2.4 (c=0.68, $CHCl_3$). IR (film): ν=3,352 cm^{-1} (O–H). ^1H NMR (400 MHz, $CDCl_3$): δ=0.91 (d, J=6.7 Hz, 3H), 0.96 (t, J=7.5 Hz, 3H), 1.14–1.67 (m, 6H), 1.60 (s, 3H), 1.68 (d, J=0.7 Hz, 3H), 2.03 (m, 8H), 3.68 (m, 2H), 5.06 (t, J= 6.7 Hz, 1H), 5.10 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.3, 17.7, 19.6, 23.1, 25.0, 25.7, 26.9, 29.2, 36.5, 37.5, 39.9, 61.2, 124.1, 124.5, 131.3, 140.8. EC calculated for $C_{16}H_{30}O$: C, 80.61; H, 12.68. Found: C, 80.34; H, 12.89.

*(3S,6E)-2,3-Dihydrohomofarnesal (***1***)* To a stirred and cooled (0°C) solution of **10** (19 mg, 0.079 mmol) in CH_2Cl_2 (1 ml) was added $NaHCO_3$ (31 mg) and Dess-Martin periodinane (158 mg, 0.37 mmol). After stirring for 1 h at room temperature, the reaction was quenched with saturated aqueous $NaHCO_3$. The aqueous phase was extracted with Et_2O, and the organic extract was washed with saturated aqueous $NaHCO_3$, water, and brine, and then dried with Na_2SO_4. After concentration, the residue was purified by SiO_2 column chromatography (hexane/ EtOAc=40:1) to afford (S)-**1** (16 mg, 85%) as a colorless oil. $[\alpha]_D^{22}$=−10.0 (c=0.66, $CHCl_3$). IR (film): ν= 2,712 cm^{-1} (C–H), 1728 (C = O). ^1H NMR (400 MHz, C_6D_6): δ=0.78 (d, J=6.3 Hz, 3H), 1.02 (t, J=7.7 Hz, 3H), 1.05–1.27 (m, 2H), 1.62 (s, 3H), 1.74 (s, 3H), 1.75–2.01 (m, 5H), 2.07 (q, J=7.5 Hz, 2H), 2.15 (m, 2H), 2.23 (m, 2H), 5.16 (t, J=7.1 Hz, 1H), 5.29 (m, 1H), 9.40 (t, J= 2.0 Hz, 1H). ^{13}C NMR (100 MHz, C_6D_6): δ=13.4, 17.7, 19.8, 23.4, 25.3, 25.8, 27.3, 27.7, 36.9, 37.4, 50.9, 124.2, 124.9, 131.2, 141.1, 200.8.

*(3R,6E)-2,3-Dihydrohomofarnesal (***1***)* (R)-**1** was prepared from (R)-**3** in the same manner as described above. $[\alpha]_D^{23}$=+9.8 (c=0.55, $CHCl_3$). IR, ^1H and ^{13}C NMR spectra were identical with that of the (S)-isomer.

Behavioral Assays A Y-tube olfactometer (15-mm i.d. glass Y-tube, 70-mm long arms connected to a 100-mm long stem at 120° angle) was used for the behavioral assays. The assays were carried out at 28°C and 60% relative humidity. Newly emerged beetles were kept singly in a glass tube (8-mm i.d.×30-mm long), both ends of which were covered with cotton. Males were acclimated in an assay room overnight. To test the biological activity of crude extracts, fractions of extracts, and synthetic compounds, the test sample was applied to a filter paper disc (6 mm). The disc was inserted in the end of one arm of the olfactometer after the solvent had evaporated, while a control disc (treated with pentane or hexane) was inserted in the end of the other arm. The position of the discs was randomly assigned in every assay. Subsequently, the cotton covers of the glass tube containing the beetle were removed, and the tube introduced into the end of the Y-tube olfactometer. Immediately, a vacuum pump was connected to the stem, and air, filtered through activated charcoal, was drawn through the Y-tube at approximately 200 ml.min^{-1}. Which disc a beetle reached (or not) within 10 min was recorded. Each beetle was tested only once and exchanged every time. Thirty beetles were tested to each treatment. The distribution of beetles in the two arms was subjected to a binomial test, against a test proportion of 0.5, in a two-tailed test (Siegel, 1956). Beetles not reaching either disc were not included in the data sets used in the binominal tests. The number of beetles that reached the discs was compared between fractions using Fisher's exact probability test.

Results

In a preliminary Y-tube olfactometer assay, all males that entered an arm chose the female volatiles over the control (Table 1). GC-EAD analyses of the female volatiles consistently revealed two EAD-active compounds, with retention times of 16.3 min and 17.6 min on DB-5 (Fig. 1). GC-MS analysis of the headspace collection suggested that these compounds were unknown. Therefore, a bulk crude collection (from ca 30,000 virgin females) was made and fractionated by silica gel column chromatography. The presence of active fractions/compounds was monitored by the Y-tube olfactometer assay and GC-EAD, respectively. Only the 5% ether fraction was significantly attractive (16 of 17 males chose the sample disc, P<0.001); both EAD-active compounds were found in this fraction. The fraction

Table 1 Y-tube olfactometer responses of adult male and female *Callosobruchus rhodesianus* to volatiles collected from virgins of each sex

Sample[a]	Target[b]	Sample	Control	No choice	P
Female volatile	Female	2	1	27	ns
	Male	17	0	13	<0.001
Male volatile	Female	1	0	28	ns
	Male	5	0	24	ns

[a] Tested at 1.5 female equivalents

[b] Thirty virgin beetles were tested to each sample

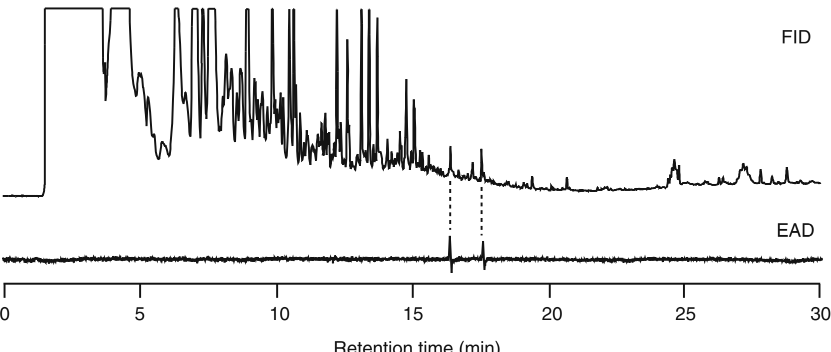

Fig. 1 Coupled gas chromatographic (flame ionization detection; FID)-electroantennographic detection (EAD) analysis of antenna of male *Callosobruchus rhodesianus* to volatile collections from virgin female *C. rhodesianus* (DB-5 column)

was purified further by normal-phase HPLC. The two EAD-active compounds were found in the 8 min to 9 min. (first compound; 16.3 min on DB-5) and 11 min to 12 min. (second compound; 17.6 min on DB-5) fractions. Y-tube olfactometer assays of the two fractions, showed only the second fraction (11 min to 12 min. fraction) to be attractive (13 of 15 males chose the sample disc, $P<0.01$). When both fractions were combined, 16 of 17 males chose the disc treated with the combined fraction (not different from the activity of the second compound fraction alone; Fisher's test; $P=0.588$) over the control. Therefore, we determined only the structure of the second compound. The active HPLC fraction was purified further by preparative GC, and the isolated compound (approximately 25,000 female equivalents, 20 μg/C_6D_6 0.2 ml) was subjected to GC-MS and NMR analyses.

GC-EI-MS analysis of the compound showed a base peak at *m/z* 69 and fragments at *m/z* (% intensity relative to the base peak) 207 (3), 193 (11), 175 (5), 163 (1), 149 (3), 137 (18), 123 (17), 107 (12), 95 (21), 81 (33), 55 (25) and 41 (60) (Fig. 2). The fragmentation pattern was similar to that reported previously for homofarnesal, the female sex attractant pheromone of *C. chinensis* (Shimomura et al., 2008). GC-CI-MS analysis showed characteristic ions at *m/z* 219 $[M + 1\text{-}H_2O]^+$ (100) and 237 $[M + 1]^+$ (14), while no peaks were detected in the high-mass region between *m/z* 238 and 300, indicating a molecular weight of 236. The high-resolution EI-mass spectrum of the compound was consistent with a molecular formula of $C_{16}H_{28}O$ [observed *m/z*: 236.2129 (3% intensity relative to the base peak); calculated *m/z*: 236.2140]. This molecular formula indicated three degrees of unsaturation.

In the ^{1}H-NMR spectrum of the compound, the presence of an aldehyde group adjacent to a methylene group (CHO—CH_2) was evident from a signal at 9.35 ppm (t, 1H, $J=2$ Hz). Two distinct olefinic protons at 5.24 (1H) and 5.11 (1H) ppm were detected. Two methylene groups at allylic positions (CH_2—C═C) were detected at 2.18 (m, 2H) and 2.11 (m,

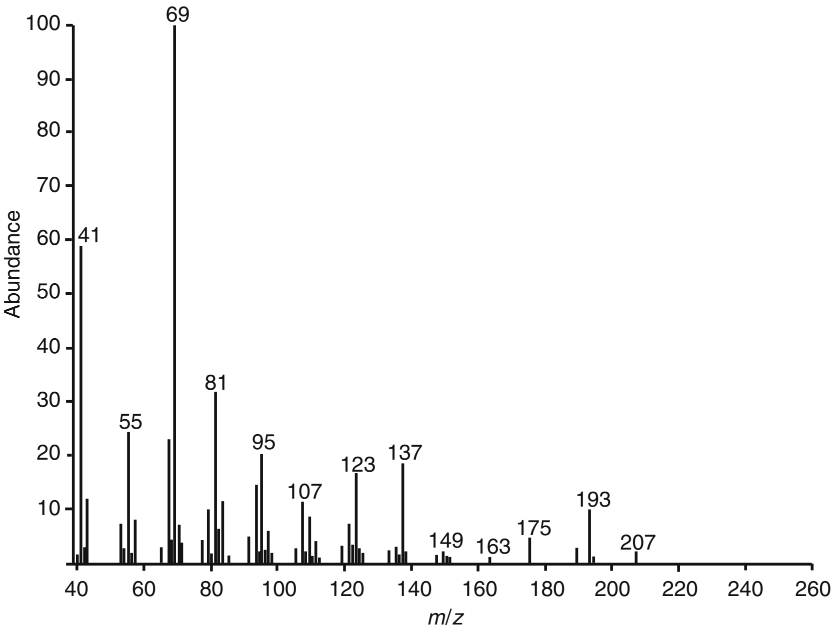

Fig. 2 Electron ionization mass spectrum of the active pheromone compound from female *Callosobruchus rhodesianus*

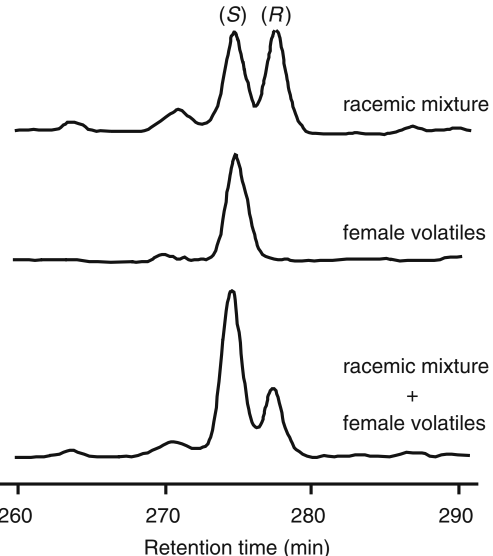

Fig. 3 Chiral gas chromatographic (β-DEX 225) analysis of the active compound from female *Callosobruchus rhodesianus* and synthetic racemic (*E*)-7-ethyl-3,11-dimethyl-6,10-dodecadienal

2H) ppm. Obscure signals also were detected at 1.72–1.96 (5H) ppm, which could be assigned to two methylene groups at another allylic position and an α-position of an aldehyde group, along with one methine group. Three distinct methyl signals were clearly observed: two signals at 1.68 (s, 3H) and 1.57 (s, 3H) ppm, derived from two methyl groups on the double bond (CH_3)—C═C, and one signal adjacent to the methine group (CH_3—CH) at 0.73 ppm (d, 3H, J=6.9 Hz). The presence of one ethyl group on the double bond (CH_3—CH_2—C═C) was evident from signals at 0.97 (t, 3H, J= 7.6 Hz) and 2.02 (q, 2H, J=7.6 Hz) ppm for a terminal methyl and an allylic methylene group, respectively, with an appropriate coupling constant.

These data, plus the similarity of the MS fragmentation pattern with homofarnesal, suggested the compound was a 2,3-dihydrohomofarnesal, i.e., 7-ethyl-3,11-dimethyl-6,10-dodecadienal. GC-EAD analysis of the synthetic mixture of the geometric isomers showed that one of the two geometrical isomers elicited responses in male antennae. The retention times and mass spectrum of the isomer matched that of the natural compound.

2,3-Dihydrohomofarnesal is a homolog of 2,3-dihydrofarnesal (3,7,11-trimethyl-6,10-dodecadienal), in which a C-7 ethyl group is substituted for a methyl group. Given the elution order on GC columns of the two *cis*/*trans* isomers of 2,3-dihydrofarnesal (Bartschat et al., 1997), the compound was proposed to be (*E*)-7-ethyl-3,11-dimethyl-6,10-dodecadienal. Synthetic (*E*)-2,3-dihydrohomofarnesal (see below) had retention times and spectroscopic data identical to the natural compound.

To confirm the absolute configuration of (*E*)-2,3-dihydrohomofarnesal, both enantiomers of (*E*)-2,3-dihydrohomofarnesal were synthesized, and the absolute configuration of natural (*E*)-2,3-dihydrohomofarnesal was investigated by chiral capillary column GC analysis. Natural (*E*)-2,3-dihydrohomofarnesal collected from female *C. rhodesianus* showed only one peak at the same retention time of (*S*)-**1** (Fig. 3).

The pheromonal activity of both synthetic enantiomers was confirmed in Y-tube olfactometer assays. 50% of males were significantly attracted to 10 ng of (3*S*,6*E*)-2,3-dihydrohomofarnesal (Fig. 4). Neither (3*R*,6*E*)-2,3-dihydrohomofarnesal nor the racemate of (*E*)-2,3-dihydrohomofarnesal was more attractive than solvent controls (Fig. 4).

Discussion

In laboratory bioassays, we confirmed that only male *C. rhodesianus* beetles were attracted to odors from live virgin females, suggesting the presence of a female-produced sex attractant pheromone. GC-EAD analysis, using male antennae, of the volatiles of virgin females showed two compounds to be EAD-active. Chromatographic fractionation and bioassay showed only the later-eluting compound to be attractive, and this was identified as (*E*)-2,3-dihydrohomofarnesal. In insect pheromonal communication, bioactivity often depends on chirality (reviewed in Mori, 2007). By using chiral GC analysis, we confirmed

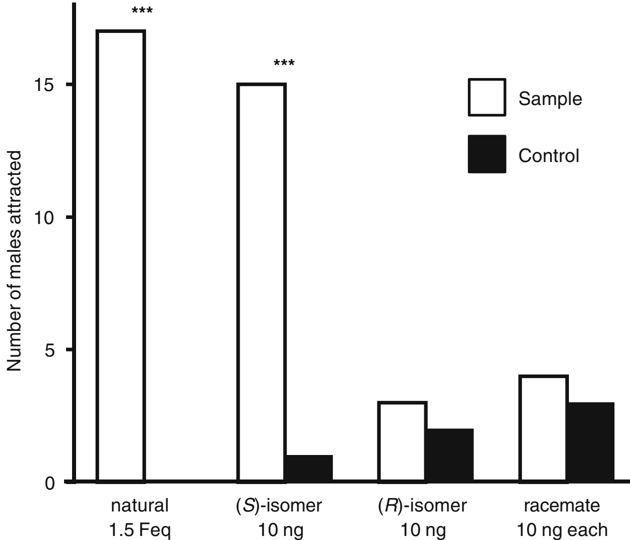

Fig. 4 Y-tube olfactometer responses of male *Callosobruchus rhodesianus* to volatile collections from virgin females, synthetic (3*S*,6*E*)- and (3*R*,6*E*)-7-ethyl-3,11-dimethyl-6,10-dodecadienal, and racemate. Thirty males were tested in each assay. The distribution of males between sample and control in each treatment was subjected to a binominal test (two-tailed test with the test proportion set at 0.5). *Asterisks* above bars indicate significant differences (***; P<0.001) from the test proportion. Males that did not reach either disc were not included in the data analysis. Feq = female equivalent

that female *C. rhodesianus* produced only the (*S*)-enantiomer. The olfactometer assay confirmed that the (*S*)-isomer was attractive to male beetles, whereas the (*R*)-enantiomer or the racemate were not. Thus, (3*S*,6*E*)-2,3-dihydrohomofarnesal is a natural pheromone component of *C. rhodesianus*, and the (*R*)-isomer inhibits its activity.

2,3-Dihydrohomofarnesal is the second example of a homosesquiterpene aldehyde being a sex attractant pheromone in the genus *Callosobruchus*. The first, homofarnesal, (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal, was identified in *C. chinensis* (Shimomura et al., 2008). In both pheromone examples, an extra carbon unit is added to the C-7 methyl group of the sesquiterpenoid skeleton. Other homosesquiterpenes have been reported from insects (e.g., juvenile hormone), and these are synthesized *via* the mevalonate pathway from mevalonate and homomevalonate (Morgan, 1999). The pheromone components of *C. rhodesianus* are likely to be biosynthesized *via* a similar pathway, followed by enantioselective reduction of the double bond at position 2-3, but this remains to be clarified.

The genus *Callosobruchus* is comprised of some 20 species (Borowiec, 1987). In this genus, female sex attractant pheromones now have been identified from five species. While the pheromones of *C. rhodesianus* and *C. chinensis* are homosesquiterpene aldehydes, the pheromones of the other species consist of short chain fatty acids, including (*Z*)-3-methyl-2-heptenoic acid for *C. analis* (Cork et al., 1991), a mixture of (*Z*)-3-methyl-2-heptenoic acid and (*E*)-3-methyl-2-heptenoic acid for *C. subinnotatus* (Shu et al., 1999), and a mixture of (*Z*)-3-methyl-2-heptenoic acid, (*E*)-3-methyl-2-heptenoic acid, (*Z*)-3-methyl-3-heptenoic acid, (*E*)-3-methyl-3-heptenoic acid, and 3-methyleneheptanoic acid for *C. maculatus* (Phillips et al., 1996). Although new pheromone compounds can arise by simple, gradual modification of a biosynthetic pathway (Symonds and Elgar, 2008), the very different structures of the homosesquiterpene aldehydes and short-chain fatty acids in the genus *Callosobruchus* suggest more saltational changes in biosynthetic pathway usage for pheromone biosynthesis.

A molecular-based phylogenic study that used mitochondrial DNA of the genus *Callosobruchus* separated it into three distinct clades: one clade containing *C. rhodesianus, C. analis*, *C. subinnotatus*, and *C. maculatus*, designated the *maculatus* clade; a second containing *C. chinensis*, designated the *chinensis* clade; and a third paraphyletic group of species (Tuda et al., 2006). In the *maculatus* clade, *C. rhodesianus* has a different sex attractant pheromone structure from the other species. Moreover, *C. rhodesianus* is phylogenetically distant from *C. chinensis*, yet the sex attractant pheromones of both species are structurally comparable. This is somewhat similar to the biosynthesis of aggregation pheromones of bark beetles in the genera *Dendroctonus* and *Ips*, in which the most closely related species within each genus have the greatest differences in pheromone composition (Symonds and Elgar, 2004), yet in both genera the same mevalonate pathway is used (Seybold et al., 2000). We suggest that both gradual and saltational shifts in pheromone evolution occur in the genus *Callosobruchus.* Biosynthetic studies of these compounds are required to clarify the evolution of pheromones in the genus.

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RAPID COMMUNICATION

Sexually Mature Cuttlefish are Attracted to the Eggs of Conspecifics

Jean G. Boal · Krista N. Prosser · Johanna B. Holm · Tiffany L. Simmons · Robert E. Haas · Gregg T. Nagle

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Abstract Cuttlefish typically are solitary, but form aggregations to spawn. We tested the hypothesis that bioactive factors in the eggs of conspecifics may facilitate the formation of spawning groups of *Sepia officinalis*. Cuttlefish detected odors from cuttlefish eggs, resulting in an increased ventilation rate. Extracts from female ovaries induced the largest increase in ventilation rate, suggesting that this organ could be a potential source of the bioactive chemicals. In y-maze assays, sexually mature, but not subadult, cuttlefish, were attracted to odors of cuttlefish eggs. These data suggest that *Sepia* eggs could be a source of reproductive pheromones.

Key Words Chemoattraction · Reproductive behavior · Spatial orientation · Invertebrate · Mollusk

Introduction

Many species of decapod cephalopods (cuttlefishes, squids) form large, near-shore aggregations for spawning, and lay their eggs in large, communal masses (Hanlon and Messenger 1996). The mechanisms that facilitate this behavior are largely unknown. Cuttlefishes typically are solitary or semi-solitary and semelparous; spawning occurs inshore when they are 1 year old to 2 years old.

Chemical attractants facilitate reproduction in many mollusks (Susswein and Nagle 2004), and cuttlefish are capable of chemoreception over a distance (Budelmann et al. 1997). Here, we tested the hypothesis that *Sepia officinalis* eggs could provide a source of chemoattractants (Zatylny et al. 2000). First, we evaluated whether cuttlefish detect odors from freshly laid eggs, measuring ventilation rate in a bioassay (Boal and Golden 1999). This method has been used previously to show that cuttlefish detect a wide range of odors, including those of food (Boal and Golden 1999). Second, we recorded ventilation responses to eggs laid ~36–48 h previously, and to extracts of female reproductive organs, to determine the potential source(s) of bioactive factor(s). Third, we investigated whether cuttlefish were attracted to egg odors when presented in a simple y-maze.

Methods and Materials

Subjects were first generation *S. officinalis* (National Resource Center for Cephalopods, Galveston, Texas). Sexual maturity was determined behaviorally; sex was confirmed by necropsy. Housing and experimental tanks were interconnected within a 57,000-l marine system of recirculating artificial seawater (ASW; salinity 34±2 ppt, temperature 17.5±1.5°C; Hvorecny et al. 2007).

Ventilation rate Trials were conducted in Plexiglas tanks (38.1×19.6×26.7 cm, 18.5 l) with flow rates of 91±3.8 ml/sec (Boal and Golden 1999). In dye tests, red food coloring almost instantly spread evenly throughout the tank, with no color remaining after ~10 min. Subjects were sub-adults (5–7 mo post-hatching, approx. 9.0–13.0 cm mantle length) for

J. G. Boal (✉) · K. N. Prosser · J. B. Holm · T. L. Simmons · R. E. Haas
Department of Biology, Millersville University,
50 East Frederick Street,
Millersville, PA 17551-0302, USA
e-mail: jean.boal@millersville.edu

G. T. Nagle
Department of Neuroscience and Cell Biology,
University of Texas Medical Branch,
Galveston, TX 77555, USA

tests of newly laid eggs. In the remaining trials, these same subjects were sexually mature adults (eight female, six male; 8–10 mo post-hatching).

A single cuttlefish was placed into an experimental tank and allowed to settle (typically 20–30 min). After this, ventilations were counted for 20 sec. in each minute. If the number of ventilations was steady for 5 consecutive min., the sampling period was increased to 30 sec. If the number of ventilations remained steady for a further 5 min., an odor sample was added to the water supply line and ventilations were counted (30 sec/min) for 10 min. Changes in ventilation rate (VR: ventilations/min) were calculated by subtracting the average VR, in the 5-min. period before addition of odor, from peak VR in the 5 min. after addition of odor. Changes in VR among treatments were compared by using a single Friedman two-way analysis of variance by ranks, followed by multiple comparisons tests.

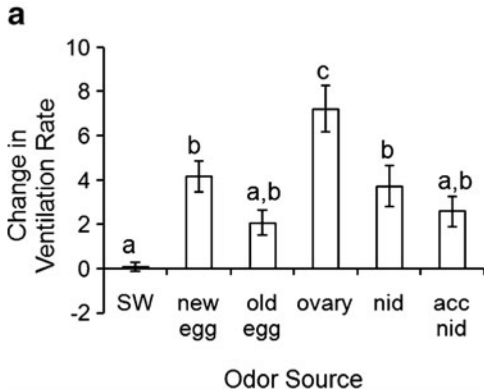
Y-Maze The y-maze base compartment (49×63×20 cm) was connected to two arms (each 24.5×70×20 cm), separated by a 60-degree angle, with a perforated gate between the base and arms (Boal and Marsh 1998). During trials, the sides and top of the maze were covered with dark towels. Cuttlefish were viewed in mirrors beneath the transparent maze floor. Cuttlefish rarely looked down toward the mirrors.

The y-maze was filled to a depth of 15 cm; seawater entered the distal ends of the arms and drained at the opposite end of the maze. During trials, odor solutions were dripped into the seawater inflow (1 drop/sec) using intravenous solution bags. It took 1.5 min (early trials) or 1 min (later trials) for control dye to travel from the inflow to the drain. Trials typically were separated by many hours, and the location (left/right) of odors added to the maze was randomized.

At the trial start, one cuttlefish was placed in the base compartment. After a 30-sec delay, the odor drips were switched on. After a further delay (1.5 min, early trials; 1 min, later trials), behavioral monitoring began. Once the cuttlefish reached the behavioral criteria (see below), an overhead light was switched on and the gate raised, allowing it access to the y-maze arms. A cuttlefish was considered to have made a choice when its entire body entered one maze arm. Two criteria were used to ensure that a cuttlefish was exposed to odors from each maze arm before it gained access to the arms: it had to travel to both sides (left and right) of the base compartment, and then be positioned at least half-way back in the base compartment so that it would not move simply into the nearest maze arm once the gate was raised.

The first group of cuttlefish (N=15; eight females, seven males) was tested at 7- and 9-mo post-hatching; the second group (N=16; six females, ten males) was tested at 11-mo post-hatching. Not all cuttlefish responded by choosing an arm.

Odor preparation Sepia officinalis eggs (fresh <1 h, N=10; old=36–48 h, N=16) and reproductive tract organs from two anesthetized individuals were frozen on dry ice. Eggs (individually), ovaries (30 g, 90 g), nidamental glands (50 g, 46 g), and accessory nidamental glands (3.6 g, 7.0 g) were extracted and purified on C18 Sep-Pak Plus cartridges, as previously described (Buresch et al. 2003). Peptides and proteins were eluted with 5 ml (eggs) or 15 ml (reproductive organs) of 50% acetonitrile/0.1% heptafluorobutyric acid. The eluates (new eggs: 50 ml; old eggs: 80 ml; ovaries: 45 ml or 135 ml; nidamental glands: 60 ml or 75 ml; accessory nidamental glands: 15 ml or 30 ml) were pooled, and divided into 1-ml aliquots (eggs) or ten equal aliquots (organs), and lyophilized.



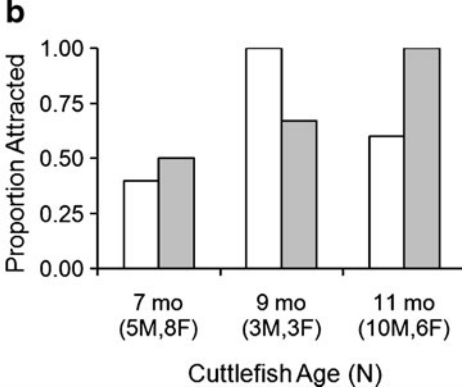


Fig. 1 *Sepia officinalis* responses to various extracts of eggs and reproductive tissue. **a** Ventilation responses (±SEM) to purified extracts of freshly laid eggs (new egg), 36–48 h-old eggs (old egg), ovary, nidamental glands (nid), and accessory nidamental glands (acc nid), or seawater controls (SW). **b** Attraction of males (*open bars*) and females (*shaded bars*), of different ages, to purified extracts of 36–48 h-old eggs. Different letters atop bars indicate means that were significantly different (P<0.05; Friedman analysis of variance by ranks)

In all experiments, fresh ASW was a control. ASW (100 ml) containing a mashed shrimp, then filtered to remove particulates, was the food control. Odor samples were prepared by re-suspending 1 aliquot of gland or egg extract in 15 ml ASW (VR experiments) or 20 ml ASW (attraction experiment). In each trial, 5 ml (VR experiments) or 20 ml (attraction experiment) of the resulting suspension were used.

Results and Discussion

Cuttlefish increased their ventilation rate in response to odors from recently laid eggs, extracts of ovary, and nidamental glands, but not to odors from older eggs or extracts from accessory nidamental glands (Friedman analysis of variance by ranks, $Fr=31.43$, $N=14$, $k=6$, $P<0.001$; Fig. 1a). Responses to extracts of ovaries were significantly greater than responses to all other odors tested, suggesting that the stimulatory chemicals in eggs might originate there.

In the y-maze, more sexually mature cuttlefish were attracted to the arm containing purified extracts of freshly laid eggs than to the arm containing only ASW (16 of 22 attracted, $X^2=4.54$, $df=1$, $P<0.05$; Fig. 1b; c.f., food control, four of 13 attracted; ASW control, 7 to left arm, 6 to right arm), indicating their attraction to odors from eggs. Previously, Zatylny et al. (2000) isolated a peptide from eggs of S. *officinalis* that stimulated contractions of the female genital tract. Although we do not know whether this compound is the same or related to the compound(s) that mediate VR rate and attraction in our study, it is likely that eggs contain a number of bioactive factors (Susswein and Nagle 2004) that stimulate different responses from cuttlefish.

Our results indicate that in *Sepia*, as in *Loligo* (Buresch et al. 2003) and *Aplysia* (Painter et al. 1998), eggs are a source of pheromones that play a role in coordinating essential reproductive behavior.

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Lack of Evidence for HLA-Linked Patterns of Odorous Carboxylic Acids Released from Glutamine Conjugates Secreted in the Human Axilla

Andreas Natsch · Fabian Kuhn · Jean-Marie Tiercy

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Abstract Genetic factors within the major histocompatibility locus (MHC) have been shown to influence body odors in mice. MHC-dependent preferences for body odors also have been reported in humans. The axillary glands are a key odor-forming organ in humans, and it is assumed that they provide behaviorally relevant odors. Volatile carboxylic acids are the most diverse class of known axillary odorants, and the pattern of these acids is genetically determined. These acids are released by an N_α-acyl-glutamine-aminoacylase present in skin bacteria. We tested a hypothesis concerning whether or not the inherited individual-specific patterns of odorous acids are strongly influenced by polymorphic genes in the MHC. Axilla secretions were collected in 12 families, comprising 3 to 6 siblings, who had been typed for HLA-A, B, and DRB1 loci. The samples were treated with N_α-acyl-glutamine-aminoacylase, and the methyl esters of the released acids were analyzed with comprehensive two-dimensional gas chromatography (GC × GC) and time-of-flight mass spectrometry (ToF MS). The patterns of the analytes were compared by distance analysis. The distance was lowest between samples taken from the same individual, confirming the presence of donor-specific odor-prints. A much higher distance was observed between siblings, but there were no differences among siblings sharing none, one, or both HLA-A,B,DRB1 haplotypes. By applying principal component analysis, a clear clustering of samples taken from one individual was confirmed, but no clustering was observed for siblings sharing identical HLA-A,B,DRB1 alleles. Thus, the genetically determined pattern of N-acyl-glutamine conjugates of volatile carboxylic acids, secreted in the human axilla, appears not to be determined by genes residing in the HLA complex.

Key Words Human body odor · Odortype · Volatile carboxylic acids · Major histocompatibility locus · Human leucocyte antigen complex · HLA-dependent mate selection · Comprehensive two-dimensional gas chromatography · Time-of-flight mass spectrometry

Abreviations

MHC	Major histocompatibility locus
HLA	human leucocyte antigen
GC	gas chromatography
ToF MS	time-of-flight mass spectrometry
N-AGA	N_α-acyl-glutamine-aminoacylase
PCA	principal component analysis

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A. Natsch
Biosciences, Givaudan Schweiz AG, Ueberlandstrasse, 138,
Duebendorf 8600, Switzerland

F. Kuhn
Analytical Chemistry, Givaudan Schweiz AG, Ueberlandstrasse, 138,
Duebendorf 8600, Switzerland

J.-M. Tiercy
National Reference Laboratory for Histocompatibility,
Geneva University Hospitals and University of Geneva,
1211 Geneva 14, Switzerland

A. Natsch (✉)
Givaudan Schweiz AG, Ueberlandstrasse, 138,
Duebendorf 8600, Switzerland
e-mail: andreas.natsch@givaudan.com

Introduction

A large body of literature describes the fascinating phenomenon of mice being able to discriminate odors of urine originating from congenic mice that differ solely in their major histocompatibility complex (MHC) loci (Yamaguchi et al., 1981). This MHC-dependent odor discrimination forms the basis of a number of well-established behavioral effects, such as odor-dependent mate selection, in which mice prefer to mate with MHC-dissimilar partners (Yamazaki et al., 1976), and parent-progeny recognition (Yamazaki et al., 2000). MHC-dependent mating preferences also have been reported in a variety of other species, including non-mammals (Reusch et al., 2001; Olsson et al., 2003). MHC-dependent mating preferences may help mice to maximize the frequency of MHC-heterozygotes in their offspring and prevent inbreeding (Penn and Potts, 1999).

The MHC in humans is better known as the HLA-region (Human leucocyte antigen). Studies in which humans evaluated the pleasantness of body odors on worn T-shirts found a preference for body odors of HLA-dissimilar persons (Wedekind et al., 1995; Wedekind and Furi, 1997). Interestingly, in females, this preference was reversed if they used contraceptive pills. Later, Thornhill et al. (2003) found similar effects when men rated female odors, but not when women rated male odors. Santos et al. (2005), on the other hand, found significant correlations between the frequency of shared HLA-alleles and the rating of body odors as 'indifferent' when women rated male odors but not *vice versa*. The preference for odors from HLA-different donors was not confirmed in a recent study (Roberts et al., 2008), although this study found an MHC-correlated shift in pleasantness when women started to use oral contraceptives. Evidence for higher than random MHC-dissimilarity within married couples also comes from a recent genomic study (Chaix et al., 2008). HLA-associated odor choices were reported to be influenced by paternally inherited HLA alleles, with a preference for odors of male donors with more HLA matches to a woman's paternally inherited alleles (Jacob et al., 2002). These studies suggested that in humans, as in mice, individuals may have a distinct body odortype, which is (a) partially genetically determined, and (b) more specifically determined, at least partly, by MHC or MHC-linked alleles, although the actual underlying mechanism is unknown and the chemicals involved have not been identified.

In most of the human studies, worn T-shirts served as an odor source, whereas in mice the odortype was the urine (Yamaguchi et al., 1981). Singer et al. (1997) showed that various carboxylic acids (e.g., phenyl-acetic acid) were important discriminators in mouse urine, as their relative abundances were associated with different MHC-types. They concluded that the body odortype resulted from a 'compound odor' of several acids, with the relative abundance of different acids, rather than presence or absence of specific compounds, generating the odor differences in mice.

Several studies have addressed the chemical nature of human body odorants secreted in the axilla. Early studies found the two odoriferous steroidal pig pheromones, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, in human axillary secretions (Brooksbank et al. 1974; Claus and Alsing 1976). Zeng et al. (1991) reported (*E*)-3-methyl-2-hexenoic acid as a key odor component, along with several other carboxylic acids. In our previous work (Natsch et al., 2006), we identified a series of 26 carboxylic acids in axillary secretions. These molecules are secreted as N_{α}-acyl-glutamine-conjugates, with the free acids released from the conjugates by the enzyme N_{α}-acyl-glutamine-aminoacylase (N-AGA), produced by the commensal skin bacteria inhabiting human axilla (Natsch et al., 2003, 2006). In a recent population study on pairs of monozygotic twins, we showed that the pattern of odorous acids is similar between different sampling days of the same individual, and also similar within pairs of homozygotic twins, but differs between unrelated individuals (Kuhn and Natsch, 2009). Four sulfanyl-alkanols have been reported as further odor components in axillary secretions (Hasegawa et al., 2004; Natsch et al., 2004; Troccaz et al., 2004). These compounds are released from secreted Cys-Gly conjugates by the sequential action of a dipeptidase and a β-lyase, present in the commensal bacteria of the human axilla (Emter and Natsch, 2008).

The studies above were all performed on human 'odorants' that could be perceived by the human nose (i.e., the studies always had a specific focus to identify only odorous compounds, generally determined with a gas chromatograph equipped with a sniff port). Other studies have looked at volatile chemicals on human skin, not discriminating odorous from non-odorous ones. Penn et al. (2007) studied all compounds detectable by gas chromatography (GC) in samples from axilla skin in a large population in the European Alps and identified 373 consistent peaks, based on mass spectrometric (MS) evidence. However it proved difficult to separate endogenous from exogenous volatiles and no information on the odorous nature of the chemicals was given.

Only a few studies have addressed HLA-associated chemosignals in humans, and only with basic analytical methodology. Eggert et al. (1998) used GC to assess volatiles in human urine, and concluded that the patterns were linked to the HLA, without actual identification of the chemicals Savelev et al. (2008) found variation in the levels of 3-methylbutanal on human panelists. No HLA-typing of the panelists was performed, but the bacterial flora from two 3-methylbutanal-emitting panelists was shown to be able to

metabolize a peptide, derived from a HLA-class 1 protein, with a terminal leucine. These data show that leucine-containing peptides are degraded to 3-methylbutanal by the microflora of some panelists, but still give no clear link to a chemical basis of HLA determined odortypes.

Thus, the key published studies on HLA-correlated odor preferences have been performed at a behavioral level and did not identify the chemicals involved. Here, we try to fill this gap by using a targeted analytical approach to address a specific hypothesis: do the genetically determined patterns of Gln-bound odorant carboxylic acids reveal part of an HLA-dependent body-odor type in humans?

Because the HLA complex is a multigenic system, comprising highly polymorphic loci forming an even larger number of different HLA-haplotypes, a general population study is unlikely to find many instances of HLA-identical pairs suitable for a statistically relevant comparison. On the other hand, two siblings from the same family have a 25% chance of being HLA-identical. Therefore, we determined the HLA-A,B,DRB1 haplotypes of 48 individuals in 12 families, and analyzed patterns of carboxylic acids in three repeated samplings with a GCxGC–Time-of-Flight (ToF) MS method. Individuals from the same family identical at none, one, or both of their HLA-haplotypes were studied to elucidate the effect of identity in the HLA complex on the similarity of the carboxylic acid pattern.

Methods and Materials

General All reagents and solvents were purchased from Fluka (Buchs, Switzerland), if not noted otherwise. The synthesis of reference compounds has been described previously (Natsch et al., 2003, 2006).

Subjects and Study Setup Twelve families comprising 3–7 siblings of identical sex were recruited (1 family with 3 individuals; 9 families with four individuals, 1 family with five individuals and 1 family with seven individuals; two individuals decided to withdraw from the study resulting in a total study population of N=48; see supplementary Table S1). Participation was voluntary, with subjects informed in detail about the goals of the study. After they gave their written consent, subjects were compensated for their participation with a voucher (100 Swiss francs). Sweat from each subject was sampled three times on different days (with the exceptions of four panelists sending back only two samples, see Table S1), with at least a 1wk interval between sampling days. The samples from the right and left axilla were pooled for analysis, as our previous analytical study that used identical methods had shown high similarity between right and left samples obtained on the same day (Kuhn and Natsch, 2009). On two sample days, volunteers donated DNA in the form of mouth swabs (Gentra/Qiagen Puregene Buccal cell Core kit A, Qiagen, Hombrechtikon, Switzerland).

DNA Extraction and HLA-Typing For each donor, one DNA-sample was extracted from buccal swabs by the high salt procedure, and a parallel sample was extracted with the Gentra/Qiagen Puregene Buccal cell Core kit A, according to manufacturers instructions (Qiagen, Hombrechtikon, Switzerland). The donors were typed for HLA-A, B (intermediate resolution), and DRB1 (high resolution) by reverse polymerase chain reaction-sequence-specific oligonucleotides (PCR-SSO) hybridization on microbeads (luminex technology), using the LabType reagents (OneLambda, Ingen, Chilly-Mazarin, France) (Rahal et al., 2008). A total of 11 HLA-A, 18 HLA-B, and 19 HLA-DRB1 alleles were identified in this group of donors.

Collection of Axillary Secretions and Treatment with Recombinant N-AGA Axillary secretions of individual donors were sampled on cotton pads fixed in the axilla during 45–60 min of physical exercise, as physical exercise is a reliable method for secretion of significant amounts of odor precursors (Starkenmann et al., 2005; Kuhn and Natsch, 2009). Immediately after collection, individual pads were suspended in 25 ml of 30% EtOH in H_2O to avoid any bacterial metabolism, and sent to the laboratory where they were frozen at -80°C until analysis. Pads were removed from the sampling solution, and the liquid phase was extracted once with 5 ml hexane. The aqueous phase, containing the glutamine conjugates of the carboxylic acids of the left and right axilla sampled from the same day, were pooled and lyophilized for 16 h and re-suspended in 1 ml H_2O. A 0.6 ml sample of this concentrated aqueous extract was amended with the Gln-conjugate 5-amino-2-(2-methylundecanamido)-5-oxopentanoic acid as internal standard (final concentration 0.166 μM). The sample then was treated with 5 μg of the recombinant enzyme N-AGA [produced and purified as described before (Natsch et al., 2003); this is sufficient to ensure complete hydrolysis of Gln-conjugates even if present at a total concentration of >100 mM]. In addition, the two enzymes (5 μg of each) that release sulfanyl-alcohols from Cys-Gly conjugates were added, (Emter and Natsch, 2008). After 4 h incubation at 36°C, 50 μl 1 M HCl and 80 mg NaCl were added, and the sample extracted with 140 μl methyl-*t*-butyl-ether. The organic phase (100 μl) was transferred to a GC vial, 7 μl of a 2 M solution of (trimethylsilyl)-diazomethane and 18 μl of methanol containing 0.025 mM ethyl-caprinate as a second internal standard, were added, and the sample was heated to 40°C for 30 min to form the methyl esters of the enzymatically released acids.

GCxGC-ToF MS Analysis of Axillary Secretions A LECO Pegasus 4D GCxGC-ToF MS system (LECO, St. Joseph, MI, USA) was used, consisting of a 6890 N gas chromatograph equipped with split/splitless injector (Agilent Technologies, Palo Alto, CA, USA), a dual stage, four-jet cryogenic modulator (licensed from Zoex), a second-dimension column oven, and a Pegasus III time-of-flight mass spectrometer (LECO). The analytical method has been described in detail recently (Kuhn and Natsch, 2009). The following modifications, from the previously used method, were applied. Samples were injected in the pulsed splitless mode, with the inlet pulse pressure set to 65.3 psi for 4.6 min. A capillary column of 30 m length×0.25 mm i.d., coated with 0.25 µm of VF-5MS (5% phenyl- 95% dimethyl-polysiloxane), was used for the first-dimension (Varian, Palo Alto, CA, USA), with a temperature program of: 35°C for 5 min, 10°C.min^{-1} to 120°C, 5°C.min^{-1} to 210°C, then 30°C. min^{-1} to 260°C, and held for 8 min. The modulation time was 5 sec. The separate, second-dimension oven was kept at the temperature as the first-dimension oven, but the modulator housing was kept 15°C above the first-dimension oven temperature.

As the sweat samples from different donors, and even from the same donors on different days, varied greatly in concentrations of key analytes, each sample was analyzed first by GC (using flame ionization detection) to determine the level of the methyl-ester of 3-methyl-2-hexenoic acid. This dominant compound is the first to reach the non-linear range of the analytical method if samples are too concentrated. Based on preliminary analysis, the samples were diluted with MTBE/methanol (85/15, v/v), and the injection volume was adjusted (0.2 – 3.0 µl) so that 5-8 ng of this dominant analyte was injected on to the column in all samples. With this approach, data distortion, due to measuring concentrated samples outside of the linear range, was avoided.

The study design required that 9453 sub-peaks be identified, correctly combined, and quantified (137 samples×23 analytes, with an assumed three modulations per ^{1}D peak; 21 endogenous analytes and two internal standards). This was done with the data processing, peak identification, and peak quantification approach described in detail elsewhere (Kuhn and Natsch, 2009), with all the parameters adjusted for the changed column lengths used in this study. The chemical names and structures of the analytes are given Table 1 and supplementary Table S2, respectively. The precision of the GCxGC-MS method was determined by three times injecting the same sample (07C2);

Table 1 The 21 analytes investigated and methods of identification

Analyte	Chemical name	Identification method[a]
A2	3-Hydroxy-3-methylhexanoic acid methyl ester	Reference
A3	3-Hydroxy-3-methylheptanoic acid methyl ester	Homologue
A4	3-Hydroxy-3-methyloctanoic acid methyl ester	Homologue
B1	3-Hydroxy-4-methylhexanoic acid methyl ester	Library & Homologue
B2	3-Hydroxy-4-methylheptanoic acid methyl ester	Reference
B3	3-Hydroxy-4-methyloctanoic acid methyl ester	Reference
B4	3-Hydroxy-4-methylnonanoic acid methyl ester	Homologue
B5	3-Hydroxy-4-methyldecanoic acid methyl ester	Homologue
B6	Unknown	Unknown
C1	3-Hydroxyhexanoic acid methyl ester	Homologue
C2	3-Hydroxyheptanoic acid methyl ester	Homologue
C3	3-Hydroxyoctanoic acid methyl ester	Reference
C4	3-Hydroxydecanoic acid methyl ester	Library & Homologue
E1	(E)-3-Methylhex-2-enoic acid methyl ester	Reference
E2	(Z)-3-Methylhex-2-enoic acid methyl ester	Reference
F1	(E)-4-Methyloct-3-enoic acid methyl ester	Reference
F2	(Z)-4-Methyloct-3-enoic acid methyl ester	Reference
F4	(E)-4-Methylnon-3-enoic acid methyl ester	Reference
Ph	Phenylacetic acid methyl ester	Library
Z1	4-Ethyloctanoic acid methyl ester	Reference
Z3	4-Methyl octanoic acid methyl ester; hypothetical structure not verified by synthetic sample	Unknown

[a] Identification method was either (1) synthetic reference compound injection, (2) library database search, (3) homologues series position on the separation plane and interpretation of its mass spectrum

the relative standard deviation of the peak area for each of the 21 analytes was between 0.1 and 6.0 per cent. Attempts also were made to quantify the sulfanyl-alkanols, but these were below the limit of quantification in the majority of the samples and, thus, were not evaluated further.

Evaluation of Results The consolidated GCxGC-ToF MS analyses resulted in a matrix of 134 samples of sufficient concentration and quality (see supplementary Table S1), with peak areas for 21 endogenous analytes per sample. First, a normalization step (Xu et al., 2007) was performed, as the amount of sweat in the different samples is difficult to control (different intensity of physical exercise, different transfer of sweat from skin to the pad). For each sample, each analyte was divided by the sum of all evaluated analytes, thus normalizing the sum of all evaluated analytes in a sample to 1. The data analysis then was performed with these normalized data.

The distance between two samples was calculated with the normalized Euclidean distance according to Eq. 1:

$$\text{Normalized Euclidean distance} = \sqrt{\sum_{n=1}^{m}\left(\frac{(X_n - Y_n)}{s_n}\right)^2} \quad (1)$$

Where X and Y are the two samples, m is the number of the analytes, X_n and Y_n are the normalized peak areas of analyte n in the two samples, and s_n is the measured standard deviation for the peak n over all samples.

This normalized distance measure corrects for the fact that the different analytes have different relative abundances and different scales (i.e., different *m/z* were used for their integration). Also this distance measure is symmetrical (e.g., distance of sample X to Y is identical to the distance of Y to X). Based on this, a distance matrix with all possible comparisons between individual samples within a particular family was calculated. The distances between samples (a) from the same individual on different days, (b) from two siblings, (c) from two siblings sharing none, (d) siblings sharing one, or (e) siblings sharing two HLA-haplotypes, then were compared. In addition, the distance matrix was transformed to ranks, assigning to each sample a rank based on the distance it has to each of the other samples of the same family. Finally, the averages for each normalized peak area per individual were also calculated. With these averages, the similarity matrix (also according to Eq. 1) was calculated again to compare individuals between families.

Principal component analysis (PCA) was performed based on the correlation matrix using Minitab statistical software (Minitab Inc., version 15.1.1.0., Coventry, UK).

Results

HLA-Typing of the Study Families The segregation of HLA haplotypes within siblings of each family could be inferred based on HLA-A, B, and DRB1 alleles. In 8 families, we were able to define all four possible individual HLA-A, B, DRB1 haplotypes (referred to as a, b, c, and d), whereas in 4 families only 3 haplotypes (a, b, c) were present. In the few instances in which HLA-A or HLA-B typing could not be performed, due to low amplification efficiency, high resolution HLA-DRB1 typing confirmed the distribution of haplotypes. The HLA alleles detected in this study group are all common in Caucasoid populations. Table 2 summarizes the HLA-haplotyping in the study group. In all families, at least two individuals shared both HLA-haplotypes. As expected, the frequency was highest for two siblings being haplo-identical (i.e., sharing 1 haplotype), whereas non-identical comparisons (i.e., with 2 different haplotypes) within families were slightly under-represented in the study population.

Distance Analysis of Odor Samples Within One Family Figure 1 shows a box-plot for the averages of the five distance values over the 12 families. In addition, the rank of each sample relative to others in the same family was calculated (closest = rank 1 or most distant = rank 11 in a family with 12 samples). Table 3 summarizes the averages

Table 2 Study population and segregation of hla haplotypes

	Individual						
Family	A	B	C	D	E	F	G
01	a/b	a/b	a/b	b/c	a/b		
02	a/b	c/d	a/c	a/c			
03	a/b	a/c	a/b	a/b			
04	a/b	a/b	NT	a/c			
05	a/b	b/c	a/d	a/d			
06	a/b	a/c	c/d	c/d			
07	a/b	c/d	a/b				
08	a/b	a/c	b/d	NT	a/c	a/b	c/d
09	a/b	a/c	b/d	a/c			
10	a/b	a/c	a/c	a/c			
11	a/b	a/c	a/c	c/d			
12	a/b	b/c	a/d	a/d			

NT not tested, individual withdrew from study

HLA-A,B,DRB1 haplotypes in each family were designated with the letters a,b,c, and d

For example in family 07, individuals A and C share the same 2 HLA haplotypes (a/b) whereas individual B has inherited 2 different haplotypes (c/d). In each family, haplotypes are formed by different combination of HLA antigens, with the exception of the common haplotype A1,B8,DR3 that was identified in 2 families

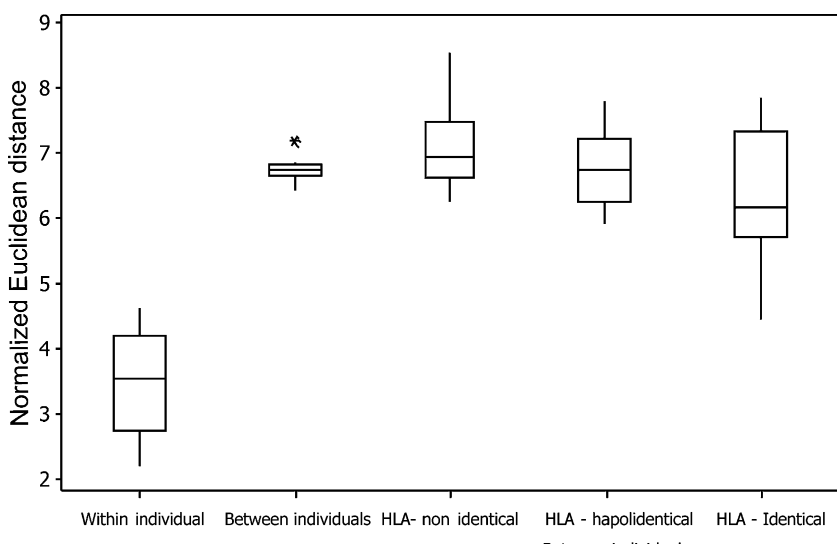

Fig. 1 Distance analysis for the comparisons among individual samples. Within each family the distance matrix between all individual samples was calculated. These distances were separated according to the following: (1) within one subject among samples taken on different sampling days, (2) between subjects in the same family, (3) between samples originating from two siblings sharing none, one or both HLA-alleles. For each family, the average of these comparisons was calculated. The boxplots show the distribution of all these averages in the twelve families

of the ranks for the individual families. Both these evaluations clearly indicate that samples from the same individual are closest to each other; when comparing different individuals within families, the distance is consistently higher. However, the distance of the acid patterns are, on average, similar, regardless of whether the individuals share none, one, or two HLA-haplotypes (Fig. 1). Also, the ranks of the comparisons are only slightly lower for the HLA-identical siblings compared to the haplo-identical or non-identical comparisons (Table 3), thus excluding HLA-linked genes as a key influence on the pattern of odorous acids.

Principal Component Analysis for Individual Families PCA was performed on all individual samples from within one family. The PCA results for four families are shown in Fig. 2 (see Supplementary information Figure S1 for the PCA plots of all 12 families). Inspection of these PCA plots showed no indication that HLA-identical siblings, overall, have an acid pattern that is closer to each other compared with haplo-identical or non-identical pairs. Thus, for example, in Fig. 2a, the three HLA-identical siblings A, C and D span the whole range of PC1 and are equally distant from their haplo-identical brother B. In Fig. 2b, the HLA-identical siblings C and D are at a similar distance to each

Table 3 The average ranks of the comparisons between samples within the twelve families

	Within individual	Between individuals	Between individuals		
			HLA-non identical	HLA-haploidentical	HLA-identical
Family 1	2.7	6.1		6.3	6.0
Family 2	1.5	5.8	5.5	5.8	5.8
Family 3	1.5	6.4		6.6	6.2
Family 4	2.4	5.2		4.6	6.3
Family 5	1.7	6.3	8.4	5.6	5.6
Family 6	1.5	7.0	7.4	7.6	4.3
Family 7	1.5	5.5	5.3		6.0
Family 8	1.6	10.0	10.6	10.1	7.5
Family 9	1.6	6.4	6.0	6.2	7.4
Family 10	2.5	6.8		6.8	6.7
Family 11	2.8	6.7	8.4	6.7	5.1
Family 12	1.8	6.3	5.9	7.4	5.1
AVERAGE	1.9[a]	6.5	7.2	6.7	6.0

[a] The theoretical average rank for samples from the same individual would be 1.5 with 3 samples per individual, if samples from one individual are always closest to each other; the observed average value of 1.9 is quite close to this value

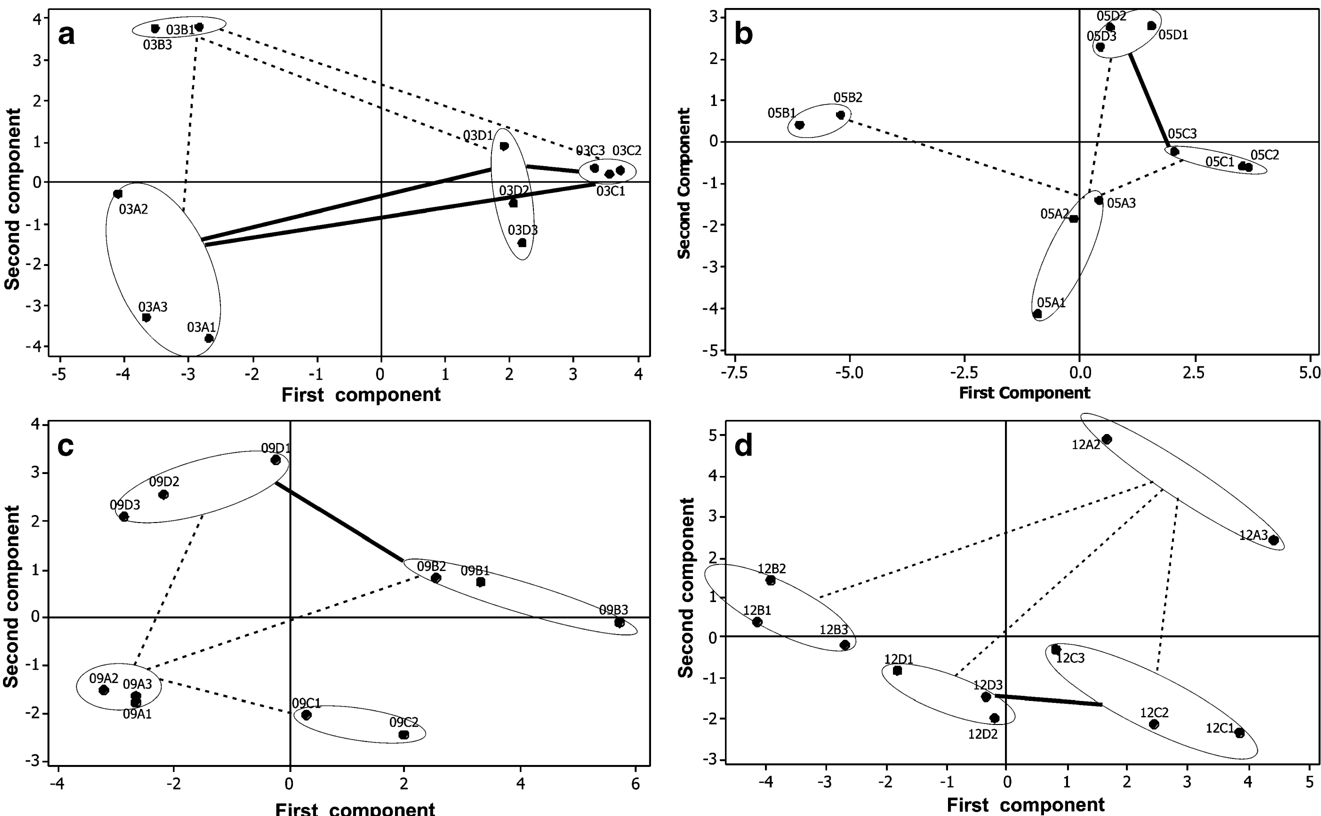

Fig. 2 Principal component analysis based on the correlation matrix of all the samples from families 3 **a**, 5 **b**, 9 **c**, and 12 **d**. The samples from different individuals (marked with capital letters) are connected with a solid line if HLA-identical, with a dotted line if HLA-haploidentical, or not connected if they are HLA-dissimilar at both loci

other, as is their haplo-identical sister A. In Fig. 2c, the non-identical brother C is separated from B at the same distance as is his HLA-identical brother D. The same also is true in Fig. 2d for the HLA-identical pair D and C, being at the same distance as the non-identical pair D and B. For more comparisons see Figure S1.

Analysis of the Average Values for each Individual: Comparing Different Families A final evaluation was made based on average normalized peak areas of each individual in order to look for differences between individuals within one family, compared to differences between individuals from different families. Overall, the average Euclidean distance between individuals from the same family is 5.07, whereas it is slightly higher (5.94) between individuals from different families ($P<0.001$) (data not shown). The average rank for samples from the same family is 17.2 compared to 24.5 for individuals from different families ($P<0.001$; 48 individuals evaluated, ranks go from 1 to 47, with a theoretical average of 24). This indicates that, overall, siblings (sharing on average 50% of the genome) are closer in their odorous acid profile than are unrelated individuals, although the effect is weak.

Discussion

The subject of HLA-linked body odors in humans has received much attention since the seminal paper of Wedekind et al. (1995). This topic mainly has been addressed in behavioral work, with a variety of studies on HLA-typed odor donors and HLA-typed odor sniffers rating the attractiveness of odor samples on T-shirts (Havlicek and Roberts, 2009). So far, no detailed chemical analysis has revealed specific classes of odorous compounds that are linked to HLA-genes or HLA-associated loci. Here, we addressed a specific hypothesis: is the composition of odorous acids, which can be released from glutamine-conjugates secreted in the human axilla, strongly affected by genes in the HLA complex, thus leading to similar odorous acid-patterns in HLA-identical siblings as compared to siblings with differing HLA loci? Our results indicated that HLA-linked genes are not the major determinants for the genetic influence on patterns of these odorous compounds. Both similarity analysis and PCA indicated that these acids form part of a stable odortype of individuals, confirming our earlier results of a twin study (Kuhn and Natsch, 2009), yet the patterns in HLA-identical

siblings were not significantly closer than were the patterns from HLA-disparate odor donors.

We can think of four possible explanations why the HLA-loci appear to influence human body odors, but that this effect is not apparent in the odorous compounds we analyzed. First, our targeted analytical approach may have been focused too narrowly. The odorous carboxylic acids investigated in this study were considered candidate molecules to form part of an HLA-determined odor for several reasons: (a) they are the most diverse group of known human body odorants, and therefore have potential to carry sufficient information to reveal individuality and individual-specific information, (b) their pattern is genetically determined to a significant extent (Kuhn and Natsch, 2009), (c) they are strongly perceived by the human nose at low concentrations (Gautschi et al., 2007), and (d) they are absent in a low-odor population in the Far East (carrying a specific loss-of-function mutation in the transport protein ABCC11; see below), confirming their contribution to human body odor (Martin et al., 2010). Nevertheless, we cannot exclude other groups of compounds as candidates for HLA-determined odorants. One diverse group of compounds in human axillary secretions is the sulfanyl-alkanols (Hasegawa et al., 2004; Natsch et al., 2004; Troccaz et al., 2004). These compounds appear to share the same biosynthetic route as the acids (the most abundant compound, 3-methyl-3-sulfanyl-hexan-1-ol, shares the same unusual carbon skeleton as the two dominant acids; additionally, a similar methylbranching is found in other compounds). Given their potentially similar biosynthesis and physiology, we consider it unlikely that sulfanyl-alkanols specifically, but not the carboxylic acids, are linked to the HLA. Another class of molecules reported from axillary secretions is amino-acid degradation products, such as isovaleric acid, 2-methyl-butyric acid, and iso-butyric acid, and their corresponding aldehydes. Such molecules could be derived from bacterial catabolism of HLA fragments or HLA-bound peptides, as previously suggested (Savelev et al., 2008), which would give a direct functional link to the HLA. As the HLA-proteins are not the main proteins available for non-specific bacterial catabolism in the axilla, and because all these compounds are derived from essential amino acids, it seems unlikely to us that they are key determinants of a genetically determined body odor.

A second reason for a lack of an HLA-influence in our study is the exclusion of the effect of skin micro-flora by our experimental setup. We mimicked the effect of bacterial hydrolysis by addition of an excess of a recombinant enzyme to each sample to ensure complete hydrolysis of the freshly secreted Gln-conjugates in the axillary secretions. Consequently, we effectively measured the 'maximal formation of the carboxylic acids', rather then the levels of acids actually present on axilla, and thus excluded any effect of different bacterial populations on the skin of the subjects, which could be confounded by personal grooming and lifestyle. In mice, addition of the single enzyme pronase to serum liberated the MHC-determined odorants (Yamazaki et al., 1999), although germ-free mice also had MHC-determined odorants in the urine (Yamazaki et al., 1990). If the same were true in humans, microflora would not affect the HLA-determined odortype, and hence our experimental approach would not affect odortype. However, the (yet unproven) 'micro-flora hypothesis' (Penn and Potts, 1998) suggests that MHC-genes influence the composition of the commensal microflora, which, in turn, determine odor, rather than genes in the MHC-locus directly determining odorants or precursors. If this were the case in humans, our approach would miss such effects. However, for the odorous acids we studied, we consider it unlikely to be the case. All these molecules are linked by the same peptide bound to glutamine, and can be released by one single enzyme with broad substrate specificity (Natsch et al., 2006). We consider it unlikely that subtle differences in the specificity of an aminoacylase in different bacterial strains would confer specific odor patterns to individuals.

Third, we cannot be certain that the HLA-linked body odorants are secreted by axillary glands, as has been assumed in the literature (see Havlicek and Roberts, 2009). However, there are several reasons to consider the axilla the most likely source of HLA-related body odors: (a) the onset of axillary odors is triggered by hormonal changes at puberty, suggesting that these odors are linked to sexual behavior and partner selection, (b) in terms of intensity, odors from the axilla are the strongest emitted by human skin, and (c) the axilla is a specific 'scent organ' (Stoddart, 1990), present only in humans, and is thought to have evolved with upright walking to present odors to other humans. Based on the results of the current study, future analytical studies on HLA-dependent odorants should focus on other body regions (e.g., the face or neck) as sources of odors with a relevant biological meaning.

Finally, the HLA-effect on human odors could be inherently weak, and therefore not detected in our approach. A recent study (Roberts et al., 2008) found no significant general preference for MHC-dissimilarity across normal menstrual-cycle women, nor a significant preference for MHC-similarity associated with contraceptive-pill use.

Kwak et al. (2010) recently reviewed why it had also proven difficult to find a chemical basis of MHC-odortypes in mice, and concluded that studies had largely focused on key volatiles associated with the MHC, rather then looking for patterns of odorants. They proposed that only the latter approach may move the field ahead. Yet, our study used this approach and failed to reveal a chemical basis for

human HLA-linked odortypes. Given that axillary odors have been investigated in great detail (Zeng et al. 1991; Gautschi et al., 2007), with different groups confirming or independently finding the same key odorous compounds (Hasegawa et al., 2004; Troccaz et al., 2004; Starkenmann et al., 2005), it seems fairly unlikely that new odorous compounds that are strongly HLA-linked will be found in axillary secretions. We suggest it may be more productive to search for HLA-linked odorants in other skin regions. It has been established recently, that a large fraction of the Asian population lacks axillary odor, and notably the ability to produce the acids investigated in this study, due to a single nucleotide polymorphism (SNP) in the ABCC11 transport protein (Nakano et al., 2009; Martin et al., 2010). In individuals carrying this SNP on both chromosomes, body odors that are not secreted by apocrine glands can be investigated. If a preference for body odors of HLA-dissimilar persons exists in this population, a detailed analysis could find new human odor components, outside of the classical axillary odors, which carry HLA-linked odor information.

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Family Scents: Developmental Changes in the Perception of Kin Body Odor?

Camille Ferdenzi · Benoist Schaal · S. Craig Roberts

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Abstract There is increasing evidence that human body odors are involved in adaptive behaviors, such as parental attachment in infants or partner choice in adults. The aim of the present study was to investigate changes in body-odor perception around puberty, a period largely ignored for odor-mediated behavioral changes, despite major changes in social needs and in odor emission and perception. Nine families with two children (8 pre-pubertal, aged 7–10, and 10 pubertal, aged 11–18) evaluated body odors of family members and unfamiliar individuals for pleasantness, intensity, and masculinity, and performed a recognition task. The hypothesized emergence of a parent–child mutual aversion for the odor of opposite-sex family members at puberty was not found, contradicting one of the few studies on the topic (Weisfeld et al., J. Exp. Child Psychol. 85:279-295, 2003). However, some developmental changes were observed, including reduced aversion for odor of the same-sex parent, and increased ability of adults, compared to children, to recognize odor of family members. Sex and personality (depressive and aggressive traits) also significantly influenced odor judgments. Further research with larger samples is needed to investigate the poorly explored issue of how olfactory perception of self and family members develops, and how it could correlate with normal reorganizations in social interactions at adolescence.

Key Words Body odor · Preferences · Kin recognition · Mate choice · Attachment · Axilla · Puberty · Personality

C. Ferdenzi · S. C. Roberts
Evolutionary Psychology and Behavioral Ecology Research Group, University of Liverpool,
Biosciences Building, Crown Street,
Liverpool L69 7ZB, UK

B. Schaal
Developmental Ethology and Cognitive Psychology Group,
Centre des Sciences du Goût, CNRS (UMR 6265),
Université de Bourgogne,
15 rue Picardet,
21000 Dijon, France

C. Ferdenzi (✉)
Swiss Center for Affective Sciences (CISA),
University of Geneva,
7 rue des Battoirs,
1205 Geneva, Switzerland
e-mail: cferdenzi@hotmail.com

Introduction

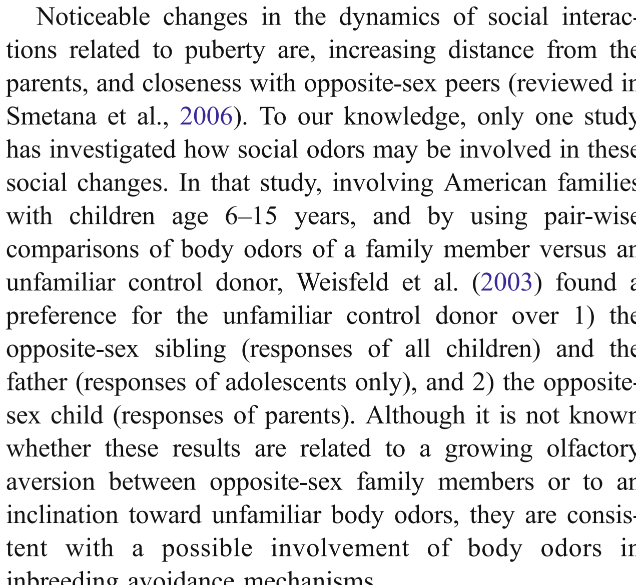
The extent to which human body odors influence interpersonal relationships generates considerable interest in western societies, within the scientific community and the general public. Although the function of social odors is well-documented in other animals (e.g., Brown and Macdonald, 1985; Wyatt, 2005), it has, until recently, been underestimated in humans, a species improperly considered to have a poor sense of smell (see Schaal and Porter, 1991). In the past few decades, however, research interest in the perception of social odors, notably in infant and adult populations, has increased considerably.

Mothers can discriminate the odor of their own child (e.g., Schaal et al., 1980; Kaitz et al., 1987), and infants recognize and prefer the body odor of their mother over that of another woman (e.g., Macfarlane, 1975). This maternal odor appears to guide infants toward the breast and to have a calming effect (Schaal et al., 1980; Doucet et al., 2007). In older children, a preference for maternal odor is present (e.g., Montagner, 1974), and the comforting effect of parental odors is reported by some children (Ferdenzi et al., 2008). Therefore, it has been hypothesized that body

odor is involved in the development of infant–mother attachment (Winberg and Porter, 1998), although such a mechanism still needs to be documented more rigorously. The feeling of security brought by affective bonds with the attachment figure is essential to a child's social and emotional development (Bowlby, 1988). Because familiar parental body odors contribute to create reassurance, they could have a significant contribution to attachment processes.

In adults, research has focused mainly on body-odor perception in the context of sexual-partner choice. Human odors convey information about factors involved in mate choice, such as a woman's fertility state (Singh and Bronstad, 2001), a man's personality (Havlicek et al., 2005), age, sex, and individual identity (Schleidt et al., 1981; Chen and Haviland-Jones, 1999; Olsson et al., 2006), and levels of genetic similarity (reviewed in Havlicek and Roberts, 2009). Disassortative preferences [i.e., oriented toward the odor of people having fewer alleles in common at the major histocompatibility complex (MHC)], have been reported in several studies (e.g., Wedekind et al., 1995; Thornhill et al., 2003; see Havlicek and Roberts, 2009, for a discussion). Such a tendency might have evolved in mammals, thus increasing resistance to pathogens by favoring MHC-heterozygosity in offspring and/or by avoiding general inbreeding (Potts et al., 1991; Penn et al., 2002). As body odors appear to be more genetically than environmentally determined (Roberts et al., 2005), and as there seems to be a familial component to body odor (Porter et al., 1986), it is likely that adult raters prefer odors of individuals who are genetically different, not only from themselves, but also from one or both of their parents (see also, Jacob et al., 2002).

In view of the studies on children and adults, we hypothesized that preference for odors of kin over non-kin change adaptively during development. Specifically, the preference for maternal body odor during infancy and childhood (serving attachment functions) may shift in sexually-mature adults toward a preference for body odors that differ from family odor, thus serving a mate-choice function. There is precedent for the idea that odor preferences can shift according to context. For example, the use of hormonal contraception (mimicking hormonal levels experienced during pregnancy) appears to disrupt adaptive disassortative preferences for a man's body odor (Wedekind et al., 1995) toward individuals who are more genetically similar to themselves (Roberts et al., 2008). This has been interpreted as an adaptive behavioral shift that normally would occur during pregnancy to promote kin interaction and elicit additional offspring care. Similarly, we suggest that olfactory perception of parental odors may shift around puberty because of major changes in reproductive physiology and social consequences (emergent sexual behavior, fluctuation in attachment to parents).



Noticeable changes in the dynamics of social interactions related to puberty are, increasing distance from the parents, and closeness with opposite-sex peers (reviewed in Smetana et al., 2006). To our knowledge, only one study has investigated how social odors may be involved in these social changes. In that study, involving American families with children age 6–15 years, and by using pair-wise comparisons of body odors of a family member versus an unfamiliar control donor, Weisfeld et al. (2003) found a preference for the unfamiliar control donor over 1) the opposite-sex sibling (responses of all children) and the father (responses of adolescents only), and 2) the opposite-sex child (responses of parents). Although it is not known whether these results are related to a growing olfactory aversion between opposite-sex family members or to an inclination toward unfamiliar body odors, they are consistent with a possible involvement of body odors in inbreeding avoidance mechanisms.

The aim of this study was to investigate possible changes in the perception of kin body odor around puberty. Involving entire families, we investigated age-related changes in recognition and pleasantness, intensity, and masculinity evaluations of the body odor of parents, siblings, self, and unfamiliar individuals, as well as the impact of other potentially confounding variables (children's body self-esteem and temperament). To perform an absolute, rather than comparative, approach and to test the existence of aversions, we measured each sample's pleasantness by using rating scales rather than paired comparisons between a familiar and a control odor, such as those used by Weisfeld et al. (2003). We hypothesized that we would find further evidence of a bidirectional aversion (child–parent and parent–child) for the odor of opposite-sex individuals, in pubertal children only.

Materials and Methods

Participants Nine families, each with two children, took part in the experiment. The sample consisted of $N=9$ mothers (age, 39–52 yr), $N=7$ fathers (44–54 yr) and $N=18$ children (8 pre-pubertal, including 6 boys, age 7–10 yr, and 10 pubertal, including 7 boys, age 11–18). The children were reported as related biologically to both tested parents. Families were characterized by relatively high income and education level of the parents (>£50,000/year, degree/diploma), and of Caucasian origin. Age groups were completed following the children's answers to the Pubertal Development Scale (PDS; Petersen et al., 1988), the 7–10-yr-olds being classified almost exclusively in the pre-pubertal/early pubertal stages, and the 11–18-yr-olds in the mid-pubertal/late pubertal/post-pubertal stages. The families were recruited through press releases and word-of-mouth among

University of Liverpool staff. For their participation, they received £20 and a free family ticket to a local botanical garden. The study was approved by the Committee on Research Ethics of the University of Liverpool and complied with the Declaration of Helsinki guidelines on human experimentation.

Odor-Collection Procedure Instructions on how to collect body odors were given both in person (CF) and through use of an illustrated instruction sheet. The participants also received reminders by email shortly before each key step of the 5-day testing period. Participants were provided with 100% cotton t-shirts (Fruit of the Loom Inc., Bowling Green, KY, USA), previously washed with an unscented detergent (Surecare Sensitive™, Robert McBride Ltd, Manchester, UK). The t-shirts were worn for three consecutive nights, simultaneously by all family members. Starting 2 d before wearing the shirts, participants were asked to refrain from drinking alcohol and eating strong foods (e.g., curry, chili and other spices, garlic, onion, pepperoni, blue cheese, cabbage, asparagus; see Roberts et al., 2005). On the evening that odor collection started, before putting the t-shirt on, all participants were required to shower with a non-perfumed soap (Simple™, Accantia Health & Beauty Ltd, Solihull, UK) and were instructed not to use any scented products, such as antiperspirants, deodorants, perfumes, or colognes. Participants also were instructed to avoid sexual intercourse and odor contamination (from partner, cooking, other odorous clothes or pets) during the time they wore the t-shirt. No obvious contamination (by perfume, coffee, or tobacco, for example) was recorded by the experimenter (CF), who smelled every sample once. On the morning of each collection night, participants were instructed to store their t-shirt in a closed and identified zip-lock bag. Parents returned the samples of the whole family to the laboratory on the morning after the third collection night. No major infringement (food, smoking, drinking, etc.) to the instructions was noted, according to the answers of each participant to a questionnaire concerning their behaviors during the testing period. Each t-shirt was cut into four halves (from navel to collar and along the side seams), combined into two samples (front left + back right, and front right + back left), and stored at −80°C for a maximum length of 46 d. This procedure was used to duplicate the number of available samples while limiting possible side-related differences in odor quality (Ferdenzi et al., 2009). Previous studies showed that freezing for this length of time (and longer) does not influence body odor quality (Roberts et al., 2008; Lenochova et al., 2009).

Personality Questionnaires Children completed the Body Esteem Scale for Adolescents and Adults (BESAA; Mendelson et al., 2001), that measures how they feel about their appearance and weight. Both parents evaluated the personality of their children by means of the Early Adolescent Temperament Questionnaire Revised (EATQ-R; Capaldi and Rothbart, 1992; Ellis and Rothbart, 2001). Only four dimensions were considered in the present study, either because they were related with some aspects of social interactions (Affiliation, 6 items; Aggression, 7 items) or because they were shown previously to be influential on odor perception (Depressive Mood, 5 items; Shyness, 5 items; Herberner et al., 1989; Pause et al., 1998).

Evaluation Procedure T-shirt halves were placed in zip-lock plastic bags (30×30 cm) and left at ambient temperature 2.5–3 hr before beginning the session. None of the participants reported nasal congestion or olfactory dysfunction. Participants were instructed to avoid wearing perfume on the testing day, and not to smoke or eat/drink in the 30 min preceding the session.

Perceptual Ratings of Familiar and Unfamiliar Individuals During the first olfactory task, participants rated all samples from the family (including their own t-shirt), the same number of unfamiliar samples, and a blank (unworn t-shirt). That is, 7–9 samples in total, according to whether the father took part or not. Unfamiliar samples were provided by members of other families and matched for sex and approximate age with the samples of the target family (age variation: 0.00±1.61 yr in children, 0.00±3.70 yr in parents). Families were unacquainted with each other. Participants evaluated each sample in a random order, on 9-point scales, for *pleasantness* [ranging from −4 (not pleasant at all) to +4 (very pleasant)], *intensity*, and *masculinity* [ranging from 1 (not intense/masculine at all) to 9 (very intense/masculine)]. They were instructed to take 15-sec breaks between samples. Special care was given to the children to ensure that they understood the rating labels.

Recognition of Familiar vs. Unfamiliar Individuals The second olfactory task was a recognition task involving pairs of odors, i.e., a target sample (from a family member) and the paired control (unfamiliar). As an example, a child smelled his father's odor and the odor from an unknown man of approximately the same age and had to answer the question: "which one belongs to your dad?" Once samples of all family members were evaluated, separated by 15-sec breaks, the same task was repeated twice. In total, 9–12 pairs of samples (according to whether the father took part or not) were evaluated. Across the three repetitions, pairs and members of the pairs were presented in random order, with sample coding altered. The number of correct answers was computed for each target odor (from 0 to 3). To limit fatigue and odor adaptation, the two olfactory tasks were

separated by a break of several minutes and completion of the questionnaire.

Data Analysis Most variables were distributed normally (Kolmogorov-Smirnov tests, α=0.05) but, because of the small size of the sample and the nature of some variables (e.g., recognition scores, with a small number of categories), we used non-parametric statistics.

First, the ability to distinguish the odor of a family member from the odor of an unknown individual was tested with a *Chi-square* goodness-of-fit test. This test compares the observed distribution of participants falling into each score modality (0, 1, 2, or 3 correct answers over the 3 trials) and the distribution expected if participants answered by chance (e.g., 0, 9, 9, and 0 participants in the categories 0, 1, 2, and 3, respectively, if there were 18 participants in total, answering by chance would give 50% correct answers and thus a score of 1 or 2). An odor is recognized when the observed distribution is significantly different from the distribution expected by chance, because of a high number of participants having three correct answers (for example, 0, 3, 4, and 11 participants giving 0, 1, 2, and 3 correct answers, respectively). Note, that a significant difference between the distributions could also be because of a high number of participants having 0 correct answers (for example 11, 4, 3, and 0 participants giving 0, 1, 2, and 3 correct answers, respectively).

To test our main hypothesis, that an aversion for the odor of opposite-sex family members would appear with advancing puberty, we computed the differences between the pleasantness score of the odor of each family member minus the pleasantness score of the unfamiliar odor it was paired with. We used Mann-Whitney *U*-tests to compare pre-pubertal and pubertal children for this variable. As the number of girls was limited in this experiment, because of recruitment difficulties, we did not perform analyses on separate sexes, but considered olfactory evaluations between opposite-sex individuals and between same-sex individuals. Although pooling evaluations of female and male odors could have been problematic, it is acceptable here, as there was no significant difference of pleasantness between the odor of mothers and fathers, unknown adult females and males (Wilcoxon matched pairs tests), and girls and boys (Mann-Whitney *U*-tests on the rating of the mother and the father). Note, that an exploratory analysis of sex differences showed that girls rated the odor of their father (but not the unfamiliar man) as less pleasant than did boys (Mann-Whitney *U*-test, $P<0.05$).

Finally, we tested the influence of personality variables by using a Principal Component Analysis performed on the Kendall tau correlation matrix. For higher intelligibility, only results for recognition and pleasantness were linked to personality dimensions (that is, average of the mother's and the father's evaluation on each personality dimension of the EATQ-R, and children evaluations on the body esteem scale BESAA).

Results

Recognition of Familiar vs. Unfamiliar Individuals

By Children When children were asked to distinguish the odor of self from the odor of an unfamiliar child on three occasions, their answers did not differ from chance levels ($\chi^2(3)=4.44$, $P>0.05$). They also answered at chance levels for the odors of their mother and sibling ($\chi^2(3)=5.00$ and 4.44, respectively, $P>0.05$), but not for their father ($\chi^2(3)=8.29$, $P<0.05$. However, this effect was not from more frequent right answers but from more frequent wrong answers).

By Adults Parents tended to recognize their own smell, compared to the odor of an unfamiliar donor, better than by chance ($\chi^2(3)=7.62$, $P=0.054$). Mothers recognized the odor of their child better than by chance ($\chi^2(3)=11.78$, $P<0.01$), whereas fathers did not ($\chi^2(3)=5.86$, $P>0.05$. Parents also identified the odor of their spouse at above-chance levels ($\chi^2(3)=8.71$, $P<0.05$).

Hedonic Ratings of Familiar vs. Unfamiliar Individuals

By Children Contrary to our expectations, analysis of the pleasantness difference, between the odor of familiar and unfamiliar adults, revealed no significant pubertal age-group difference for the ratings of opposite-sex adults (Fig. 1). However, a marginal difference for the ratings of same-sex adult was found (Mann-Whitney *U*-test, $P=0.074$). Pre-pubertal children preferred the odor of an unfamiliar same-sex adult (parent = -1.33 ± 2.07 vs. unfamiliar = 0.33 ± 3.39) whereas, pubertal children preferred the familiar adult (parent = 0.70 ± 1.95 vs. unfamiliar = -0.50 ± 1.96). No significant difference between pubertal age groups appeared for the intensity and masculinity ratings of the stimuli. Likewise, these groups did not differ for the pleasantness, intensity, or masculinity rating differences between self and an unfamiliar child, and between the sibling and an unfamiliar child.

By Parents The analysis of the pleasantness difference between the odors of own child, vs. an unfamiliar child, revealed no significant difference between pre-pubertal and pubertal odors, either when parents evaluated a child from the opposite sex (pre-pubertal children: own = $-0.14\pm.69$

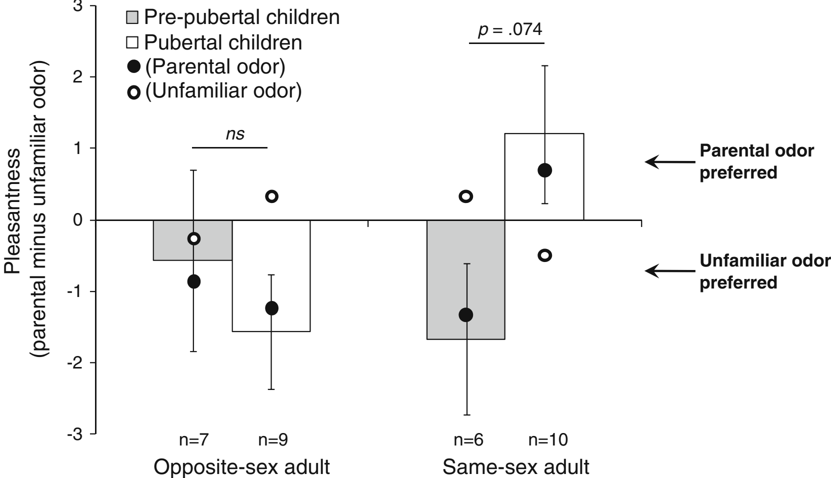


Fig. 1 Ratings by children of the body odors of familiar and unfamiliar adults. The *bars* represent the difference of pleasantness between the odors of familiar and unfamiliar adults (parent minus unfamiliar control adult, matched in sex and age), as a function of the age group of the rater (*grey* = pre-pubertal; *white* = pubertal), and for adults of the opposite and same sex as the rater (Mean ± SEM). A positive difference indicates that parental odor is preferred over unfamiliar odor. Age groups marginally differ (at $P<0.10$) on the rating of the same-sex adult (*ns*=not significant, $P>0.10$). The average ratings are also presented (*black circle*: odor of the parent; *white circle*: odor of the unfamiliar individual)

vs. unfamiliar = 0.29±1.80; pubertal children: own = 0.78± 1.48 vs. unfamiliar = −0.33±2.35) or the same sex as their own (pre-pubertal children: own = 1.33±1.63 vs. unfamiliar = −0.67±2.80; pubertal children: own = 0.00±1.33 vs. unfamiliar = 0.67±1.50). The body odors of children were evaluated positively, on average, and there was no aversion appearing at adolescence for the opposite-sex child (Fig. 2), under the conditions of the study.

Link with Personality Variables The factor analysis on the personality and pleasantness/recognition data are presented in Table 1. Six factors had eigen values higher than 1.00 and were, therefore, considered for interpretation. The first factor, explaining 18% of the variance, is interpretable in terms of the link between the level of the child's depressive mood and his/her ability to recognize the odor of his/her mother. Aggression and body esteem were negatively

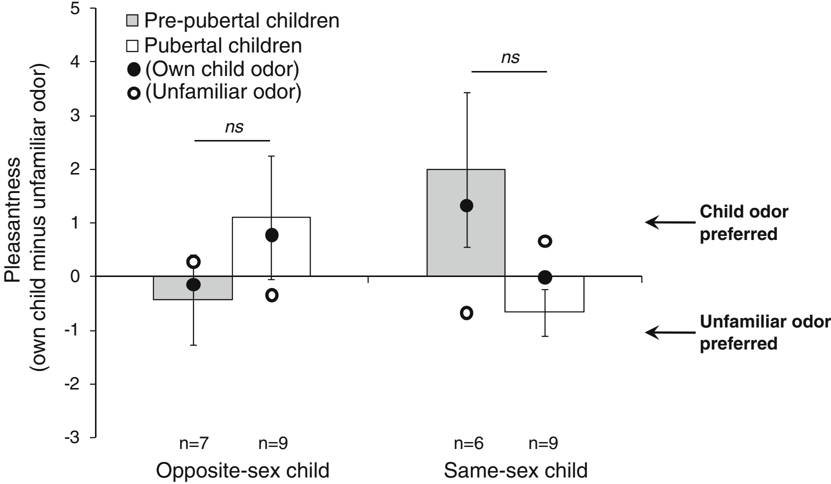


Fig. 2 Ratings by parents of the body odors of familiar and unfamiliar children. The *bars* represent the difference of pleasantness between the odors of familiar and unfamiliar children (own child minus unfamiliar control child, matched in sex and age), as a function of the age group of the rated child (*grey* = pre-pubertal; *white* = pubertal), and for children of the opposite and the same sex as the adult rater (Mean ± SEM). A positive difference indicates that the odor of a parent's child's is preferred over that of an unfamiliar one. Age group differences were not significant (*ns* = not significant, $P>0.10$). The average ratings are presented (*black circle*: odor of the own child; *white circle*: odor of the unfamiliar child)

Table 1 Results of the Principal Component Analysis on the personality variables (Affiliation, Aggression, Depressive Mood and Shyness dimensions of the EATQ-R questionnaire, and the BESAA body esteem score) and the olfactory evaluations of the children (pleasantness and recognition of the odor of self, the mother, the father and the sibling), based on the Kendall tau correlation matrix. Only loadings superior to 0.50 on Factors F1 to F6 are visible

	F1	F2	F3	F4	F5	F6
Depressive Mood	0.66					
Recognition—Mother	0.92					
Pleasantness—Self		0.77				
Pleasantness—Mother		−0.82				
Recognition—Father		0.64	−0.55			
Recognition—Self			0.64			
Recognition—Sibling			−0.81			
Body Esteem				−0.58		
Aggression				−0.50		
Pleasantness—Sibling				0.91		
Pleasantness—Father					0.91	
Affiliation						0.73
Shyness						−0.81
Explained variance	18%	16%	12%	11%	9%	8%
Eigenvalue	2.30	2.05	1.57	1.47	1.22	1.01
Total explained variance	74%					

linked to pleasantness of the sibling's odor, as shown by the fourth factor (11% of the variance). The sixth factor shows that affiliation and shyness are inversely correlated. Finally, and of minor interest to our questions, the second and third factors represent the inverse relationship firstly, between the pleasantness of the odors of self and of mother, and secondly, between the ability to recognize the odor of self from the odor of father and sibling.

Discussion

Developmental Effects The main aim of this study was to investigate the possibility of a pubertal shift in preferences for family body odors, characterized by the development of a mutual aversion between opposite-sex children and parents. The rationale for such a hypothesis is the transition from child–parent attachment to mate-choice necessity, both mechanisms potentially involving body-odor perception and evaluation. Initial results by Weisfeld et al. (2003) suggested such a phenomenon. However, here we did not find any such shift in children or parents. Specifically, no aversion occurred in parents who gave positive ratings of the odors of children. The only developmental difference that we found was a marginally significant effect on children's perception of odor of the same-sex parent. Pre-pubertal children tended to display an aversion for odor of the same-sex parent (the unfamiliar adult was rated more positively), while pubertal children rated the same-sex parent more positively than the unfamiliar adult, with overall ratings less negative than those of pre-pubertal children. As same-sex pairs were mostly boy–father (11 vs. only 5 girl-mother pairs), a possible explanation to this effect could be that perceptions become less negative after puberty, because of a specific reduction in sensitivity to major odorous compounds of male axillary sweat (androstenone and androstadienone: Dorries et al., 1989; Hummel et al., 2005) by boys. Adolescent males' greater tolerance for androstenes, and other unpleasant odorants with less biological significance (Chopra et al., 2008), is believed to be caused by pubertal changes in the levels of gonadal steroids. Our study also revealed that, whereas parents, who might have developed this ability through years of experience, recognized the odors of self, of their child (mothers only), and of their spouse, children (pre-pubertal and pubertal) were unable to recognize their own odor or the odors of other family members. Furthermore, in contrast to the results of another study (Weisfeld et al., 2003), children tended to pick out the odor of the unfamiliar man as being the odor of their father. This intriguing result needs further investigation.

In summary, these results suggest that there may be modifications in social odor perception at adolescence, but that these seem related to general phenomena (e.g., global sensitivity changes, experiential factors) rather than to specific aversions to opposite-sex family members. However, our limited sample size precludes a firm conclusion that such aversions do not exist. Further research with larger samples is needed to test developmental shifts in the perception of familiar body odor on each sex separately.

Sex Differences Although sex could not be taken into account in the present study, because of the small sample of girls, a preliminary analysis revealed that girls rated the odor of their father (but not those of an

unfamiliar adult male) as more unpleasant than boys did. Many studies have documented sex differences in social olfaction and reported a significant advantage for females. Females appear to be more sensitive than males to volatile chemicals present in axillary secretions, namely androstenone and androstadienone, at least from puberty onwards (Dorries et al., 1989; Hummel et al., 2005). Female sensitivity seems to be particularly focused on odors with social significance (Lundström et al., 2006), and even more so during the fertile period of the menstrual cycle (Doty et al., 1981; Grillo et al., 2001; Ferdenzi et al., 2009). In addition, females rely on body odors during social interactions related to partner choice significantly more often than do males (Herz and Inzlicht, 2002; Havlicek et al., 2008). Females also recognize the odor of familiar individuals more readily than do males (Schleidt et al., 1981), which was also true for adults in the present study; mothers significantly recognized the odor of their child, whereas fathers did not. This male–female difference is probably linked to a greater physical proximity between mothers and offspring, particularly in child care (Geary, 1998) and through adolescence (Smetana et al., 2006). Because of this female advantage in social olfaction, and because of differential changes in olfactory sensitivities in adolescent boys and girls (see above), sex is a factor to be considered in future research on the function of social odors during human development.

Personality and Body Odor Perception In the present study, the depressive trait of a child's temperament was positively related to its ability to recognize its mother's odor. Although previous studies found no link between personality and perception of the body-related compound androstadienone (Filsinger et al., 1987), our results corroborate studies that have shown that neuroticism in adults (depression loads heavily on the neuroticism dimension; Eysenck, 1990) is associated with higher olfactory sensitivity and better identification abilities, perhaps because of higher emotional activation (Pause et al., 1998; Chen and Dalton, 2005). Biologically significant odors, such as mother's odor, might thus be more salient to children with depressive tendencies than to others. We also found that the pleasantness of a sibling's odor was negatively linked to personality traits such as aggression. To interpret this particular link, it would be useful to determine if aggression is linked with higher levels of sibling rivalry, which could be linked to more negative evaluation of a sibling's odor. The roles of odors in the interactive behavior of siblings (Porter and Moore, 1981; Weisfeld et al., 2003) has been even less studied than odors in child–parent interactions, and is deserving of greater attention.

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Host Specific Social Parasites (*Psithyrus*) Indicate Chemical Recognition System in Bumblebees

Stephen J. Martin · Jonathan M. Carruthers · Paul H. Williams · Falko P. Drijfhout

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Abstract Semiochemicals influence many aspects of insect behavior, including interactions between parasites and their hosts. We studied the chemical recognition system of bumblebees (*Bombus*) by examining the cuticular hydrocarbon cues of 14 species, including five species of social parasites, known as cuckoo bees (subgenus *Psithyrus*). We found that bumblebees possess species-specific alkene positional isomer profiles that are stable over large geographical regions and are mimicked by three host-specific cuckoo parasites. In three host-cuckoo associations where mimicry is poor, possibly due to recent host shifts, these cuckoos produce dodecyl acetate a known chemical repellent that allows the cuckoos to invade their host colonies. Our findings indicate cuckoos use two chemical mechanisms, mimicry and repellents, to invade their hosts, and this may reflect different stages of an ongoing dynamic arms race.

Key Words Recognition · Cuticular hydrocarbons · Isomers · *Bombus* · Cuckoos

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S. J. Martin (✉) · J. M. Carruthers
Department of Animal and Plant Sciences,
University of Sheffield,
Sheffield S10 2TN, UK
e-mail: s.j.martin@sheffield.ac.uk

P. H. Williams
Department of Entomology, The Natural History Museum,
Cromwell Road,
London SW7 5BD, UK

F. P. Drijfhout
Chemical Ecology Group, School of Physical and Geographical Sciences, Lennard-Jones Laboratory, Keele University,
Keele ST5 5BG, UK

Introduction

The first line of defence in an insect society is its sophisticated cuticular hydrocarbon recognition system. Colonies usually can recognize their nest-mates and detect intruders, whom they often attack vigorously. Many social parasites, however, are able to break into the fortresses of their hosts and take advantage of them for extended periods without being harmed. Parasites have evolved some remarkable strategies, such as chemical mimicry (Lenoir et al. 2001) that enable them to evade the colony's recognition system. These adaptations provide a way of studying the chemical recognition systems of social insects.

Recent studies have begun to reveal the groups of hydrocarbons (Greene and Gordon 2007; Martin et al. 2008a) and mechanisms (Ozaki et al. 2005; Martin et al. 2008b) involved in the recognition systems used by social insects. These studies show the importance of looking at relationships within and between groups of hydrocarbons, rather than assuming that all hydrocarbons form part of the signal. By using this new approach, rather than the standard multivariate statistical methods that compare all components simultaneously (Martin and Drijfhout 2009a), we studied the host specific *Psithyrus* cuckoo bees as a tool to search for the bumblebee species recognition system. Hydrocarbons are synthesized in the oenocytes and transported to the cuticle and Dufour's gland where they are secreted. These hydrocarbons are present as a thin oily layer that covers the insects' entire surface and that acts primarily as an anti-desiccation agent. In bumblebees, these hydrocarbons are dominated by *n*-alkanes and alkenes. The alkenes are unusual in that they are rich in (*Z*) positional isomers, which include all positions from 5 to 15 (Lanne et al. 1987; Tengö et al. 1991; Ayasse et al. 1995, 1999). This unique richness is likely to have a functional role, since it

should be costly to maintain biochemical mechanisms to produce a variety of isomers (Morgan 2004), and bees are able to detect and recognize different isomers (Châline et al. 2005; Blažytė-Čereškienė and Būda 2007).

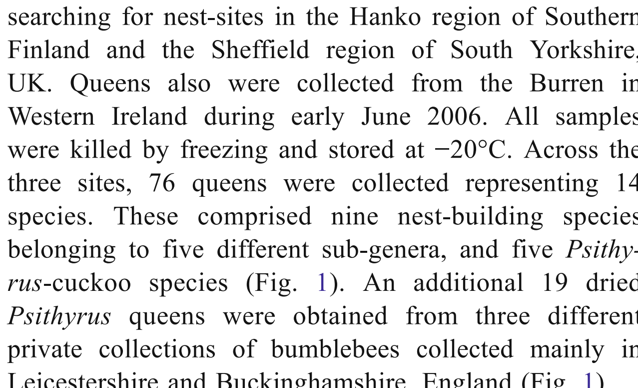
There are over 250 species of bumblebees, *Bombus*, of which 30 have evolved into social parasites known as cuckoo bumblebees (Williams 1998). For simplicity, all cuckoo bumblebees are referred to as *Psithyrus,* although they are now considered a subgenus within *Bombus* (Williams 1998), while all remaining non-parasitic bumblebee species are referred to as *Bombus*. The *Psithyrus* female (queen) invades an established nest of its host bumblebee species and lays eggs that are reared by host workers into new *Psithyrus* sexuals, since all *Psithyrus* species lack a worker caste. Typically, *Psithyrus* females of a given species only parasitize nests of one, or a few, host species (Richards 1927; Reinig 1935; Alford 1975; Fisher 1987; Williams 2008), but their host invasion behavior can be highly variable (Kupper and Schwammberger 1995; Frehn and Schwammberger 2001).

Psithyrus females are well adapted to their parasitic life style, having thicker cuticles, longer stings, tougher inter-segmental membranes, sharper more powerful, mandibles, and a larger venom sac and Dufour's gland than their hosts (Richards 1927; Free and Butler 1959; Alford 1975). These features facilitate the successful usurpation of the host nest (Fisher and Sampson 1992) and are found also in socially parasitic wasps (Edwards 1980) and ants (Tsuneoka and Akino 2009), both distantly related but socially similar taxa. The *Psithyrus* females often visually mimic their host color patterns, especially in Europe (Williams 2008), and employ a variety of chemical mechanisms that facilitate nest invasion. For example, *B. (Ps.) norvegicus* secrete dodecyl acetate from their enlarged Dufour's gland, which repels host workers (Zimma et al. 2003). *Psithyrus* females are thought to locate their host colony by species-specific olfactory cues (Sladen 1912; Fisher 1983), since *Psithyrus* species can distinguish host from non-host species based on their Dufour's gland extract (Fisher et al. 1993).

Free and Butler (1959) suggested that, to aid in nest invasion, *Psithyrus* species may have evolved similar recognition cues to their host species that could explain their high host-specificity. Here, we tested the hypothesis that *Psithyrus* species mimic the chemical profiles of their host species. In addition, we investigated whether the presence of the repellent dodecyl acetate is a general feature of *Psithyrus* bumblebees, since the production of a repellent appears counter-productive for species thought to use chemical mimicry.

Methods and Materials

Sample Collection Between April and June 2007, bumblebee queens were caught while feeding on flowers or



searching for nest-sites in the Hanko region of Southern Finland and the Sheffield region of South Yorkshire, UK. Queens also were collected from the Burren in Western Ireland during early June 2006. All samples were killed by freezing and stored at −20°C. Across the three sites, 76 queens were collected representing 14 species. These comprised nine nest-building species belonging to five different sub-genera, and five *Psithyrus*-cuckoo species (Fig. 1). An additional 19 dried *Psithyrus* queens were obtained from three different private collections of bumblebees collected mainly in Leicestershire and Buckinghamshire, England (Fig. 1).

Chemical Analyses Cuticular hydrocarbon extracts from each bumblebee were prepared by removing one pair of wings and immersing them in a vial containing 80 µl of HPLC grade hexane at room temperature for 15 min. Wings were used in order to minimize potential contamination from glandular secretions. The extracts were evaporated and stored at 5°C. A Dufour's gland extract of each bumblebee (excluding the 19 dried *Psithyrus* samples) also was obtained following the same procedure used for the wings. The Dufour's gland contains only internally produced hydrocarbons. The remaining pairs of wings then were pooled for each species providing a stronger extract that allowed better resolution of the positional isomers after dimethyl disulfide (DMDS) derivatization (see below). The cuticular hydrocarbon extracts from the 19 dried *Psithyrus* samples were obtained by immersing the entire body and wings in a small glass dish containing 1 ml of hexane for 5 min before transferring the hexane to a vial where it was evaporated. This ensured that sufficient extract was available for DMDS derivatization. Just prior to analysis, 30 µl of hexane were added to the vials, and the samples were analyzed on an HP6890 gas chromatograph (GC) equipped with an HP-5MS column (length: 30 m; internal diam: 0.25 mm; film thickness: 0.25 µm) connected to an HP5973 quadrupole mass spectrometer (MS) with 70 eV electron impact ionization. Samples were injected in the splitless mode, and the oven was programmed from 70°C to 200°C at 40°C/min, and then from 200°C to 320°C at 15°C/min, and held for 2 min at 320°C. Helium was used as carrier gas, at a constant flow rate of 1.0 ml/min. Compounds were characterized by the use of standard MS databases, diagnostic ions, and their Kovats indices. Pooled wing extracts, individual body washes, and individual Dufour's gland samples were subject to DMDS derivatization in order to determine the alkene double bond positions (Carlson et al. 1989) and re-analyzed on the GC-MS under the same conditions as the non-derivatizated samples.

Data Analyses The analysis of the three main groups of compounds (acetates, *n*-alkanes, and alkenes) were con-

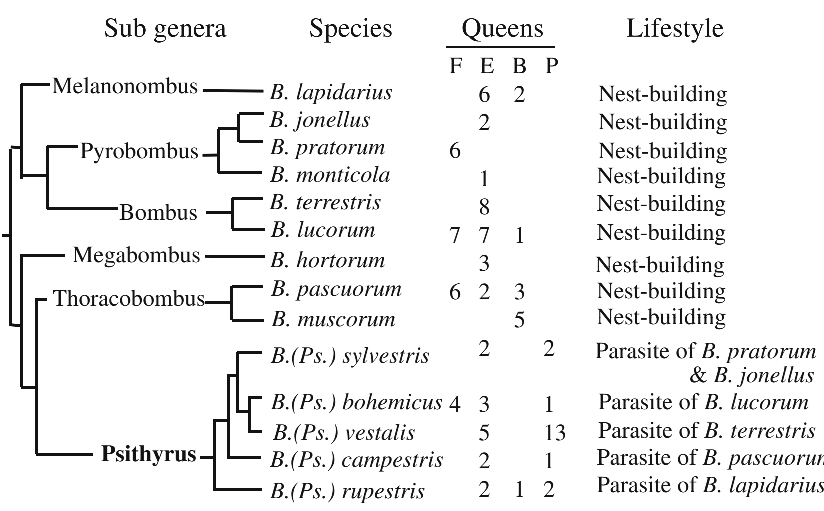


Fig. 1 The simplified sub-generic classification of the bumblebees studied adapted from Williams et al. (2008) showing the phylogenetic relationships among the 14 species studied. The number of *Bombus* queens and *Psithyrus* females of each species collected from Finland (*F*), England (*E*), the Burren in Ireland (*B*) or from private English collections (*P*). Their lifestyle also is presented

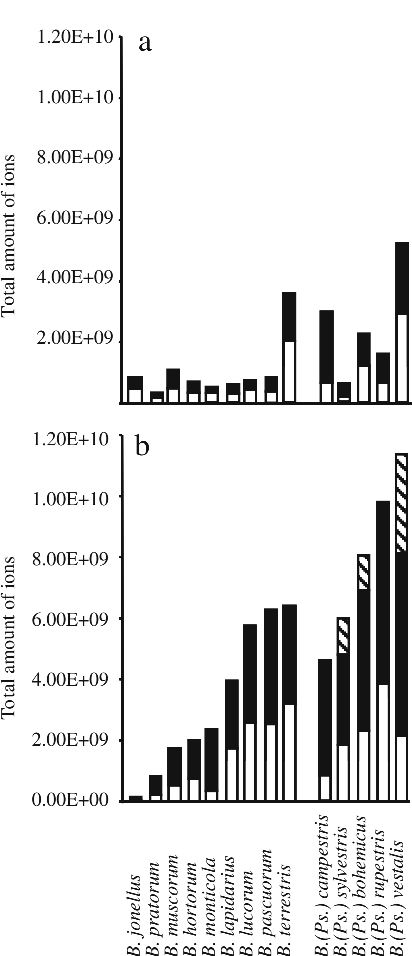


Fig. 2 The average relative amount of *n*-alkanes (white), alkenes (black), and dodecyl acetate (stripes) extracted from **a** wings and **b** the Dufour's gland of 14 bumblebee species. Error bars are not shown for clarity

ducted separately, as there is growing evidence (Châline et al. 2005; Greene and Gordon 2007; Martin and Drijfhout 2009a) that the profile perceived by the insect and that produced by the GC-MS are not congruent. For acetates and *n*-alkanes, peak areas were integrated from the original (i.e., non-derivatized) individual Dufour's gland and wing gas chromatograms. Data were log-transformed in order to meet the assumption of equal variances before conducting *t*-tests in SPSS v14. For the alkenes, the presence of several isomers, often with overlapping retention times, meant accurate integration from the original gas chromatograms was not possible. Therefore, the proportion of each alkene isomer present was calculated from the derivatized sample chromatograms as follows. By using the characteristic ions for each isomer at each chain length, the amounts of these ions were individually integrated using the 'extract ion' function in MSD Chem-Station. This produced a table of ion counts for each isomer at each chain-length for each individual. This was converted to a percentage of the overall count, and any compound (i.e., an isomer at a specific chain length) that represented less than 0.5% was excluded. The percentage of the remaining isomers was re-calculated.

We then investigated whether any patterns existed that connected the positional isomers to chain-length before calculating the proportion of each isomer irrespective of its chain-length for each individual. Initially, the proportion of each isomer was calculated in the normal way, i.e., total (*Z*)-10 = (*Z*)-10C_{23} + (*Z*)-10C_{25} + (*Z*)-10C_{27}. This produced an unusual isomer pattern in *B. hortorum* (see "Results"). However, the apparent anomaly might be explained better from a functional viewpoint since isomers potentially can be read from either end of the molecule. Therefore, we calculated the proportion of each isomer so that only *odd* isomers were present e.g., (*Z*)-13 = (*Z*)-10C_{23} + (*Z*)-12C_{25} + (*Z*)-13C_{27}. This approach is supported by the facts that even positional isomers (e.g., [*Z*]-8 and [*Z*]-10) are a biochemical rarity,

since (*Z*)-9-oleic acid is the key precursor, and in insects Δ desaturases are normally odd-numbered (e.g., 5, 9, 11, and 11) (Byers 2006).

Results

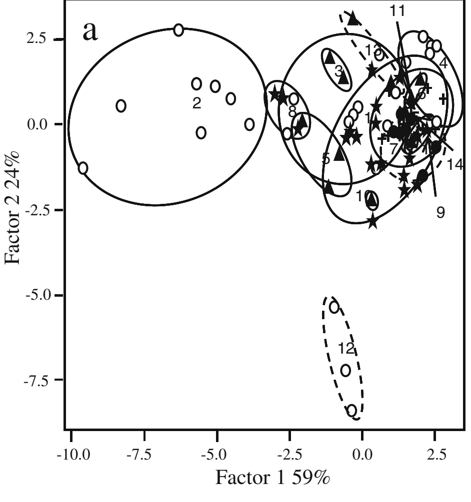
General Findings Figure 2 shows that non-polar compounds extracted from the bumblebee wings and their Dufour's glands are almost exclusively (>99%) composed of *n*-alkanes (C_{21}–C_{31}) and alkenes (various isomers) except in *B. (Ps.) campestris,* in which it is >90% due the presence of methylated compounds that are rare (<1%) in all other species. In addition to the hydrocarbons, dodecyl acetate, a volatile compound, was detected in the Dufour's gland of three of the five *Psithyrus* species (Fig. 2). The hydrocarbon ion counts (*n*-alkanes and alkenes) extracted from the wings (Fig. 2a) and the Dufour's gland (Fig. 2b) are significantly higher among *Psithyrus* than the non-parasitic *Bombus* (wings $P=0.018$, $t=-2.729$ $df=12$; Dufour's gland $P=0.018$, $t=-2.735$ $df=12$).

n-Alkanes For all species, the *n*-alkanes account for 23–54% of the hydrocarbons in the wing extracts and 14–50% of the Dufour's gland extracts (Figs. 2a, b). Comparison of the *n*-alkane profiles shows high variability between individuals. Consequently, no distinct differences between species, and no clear associations between the *n*-alkane profiles of *Psithyrus* and their hosts were apparent in either the wing or the Dufour's gland samples (Supplemental Fig. 1). This is further supported by Discriminant Analysis (DA) in which the *n*-alkanes only separate one of the non-parasitic *Bombus* species, whereas the alkenes separate all of the non-parasitic *Bombus* species (Fig. 3).

Alkenes The alkenes are the most abundant group of compounds detected in the wing, Dufour's gland, and whole body extracts with a wide range of isomers detected, even within the profile of a single species. These include all isomers from (*Z*)-5 to (*Z*)-14. All three *B. hortorum* queens possessed the same rich and highly unusual isomer pattern, with respect to which isomer was prevalent at each chain-length (Fig. 4). However, when double bond positions are viewed differently see ("Methods"), the apparently unusual isomer pattern of *B. hortorum* becomes clear; i.e., the production of mainly the (*Z*)-13 isomer (Fig. 4).

Although we detected odd and even isomers from (*Z*)-5 to (*Z*)-14, our re-interpretation of the results is that odd isomers from (*Z*)-5 to (*Z*)-21 were detected (as shown in subsequent figures). The same overall conclusions are reached, irrespective of the method used to calculate the isomer profiles, but fewer odd isomers are implied.

We were able to separate all of the non-parasitic *Bombus* species by using only the observed variation between species in their alkene isomer profile (Fig. 3). The only exceptions were three samples from the *B. terrestris*-group that all had distinctive isomer profiles (Fig. 5). This chemo-group (*B. terrestris II*) was composed of a Finnish *B. lucorum*, and two *B. terrestris* from Sheffield that were morphologically indistinguishable from their conspecifics.



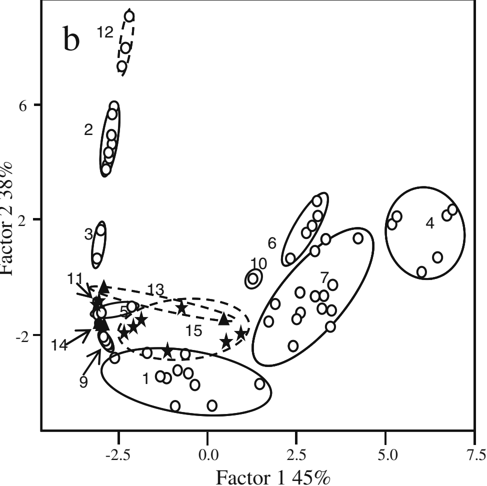


Fig. 3 Comparison of the discriminant analysis using SPSS based on the transformed proportions (for method see Martin and Drijfhout 2009a) for **a** alkanes and **b** alkene isomers from the Dufour's gland of each bumblebee queen. Different symbols are used where ellipses that enclose all individuals of that species overlap. Ellipses for the cuckoo species are dashed. The two factors indicate the amount of variation explained by each discriminate component. The isomer profile of *Bombus hortorum* (No. 8) was so different from all other species that it was removed from the analysis otherwise it was difficult to visualize the data. 1.*B. pascuorum*; 2. *B. lapidarius*; 3. *B. jonellus*; 4. *B. pratorum*; 5.*B. terrestris II*; 6. *B. terrestris*; 7. *B. lucorum*; 8.*B. hortorum*; 9. *B. muscorum*; 10. *B. monticola*; 11. *B. (Ps.) campestris*; 12. *B. (Ps.) rupestris*; 13. *B. (Ps.) sylvestris*;14. *B. (Ps.) vestalis*; 15. *B. (Ps.) bohemicus*

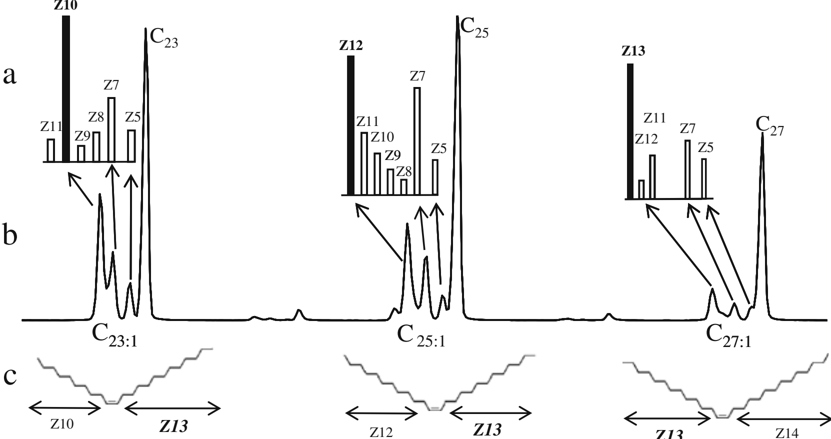


Fig. 4 **b** The gas chromatogram of *Bombus hortorum* showing at each chain length three alkene peaks that contain up to seven different co-eluting isomers and a single *n*-alkane peak. **a** The proportion of each isomer after derivatization that revealed an unusual isomer pattern. **c** However, the most abundant isomer at each chain length (*Z*)-10-$C_{23:1}$, (*Z*)-12-$C_{25:1}$, and (*Z*)-13-$C_{27:1}$ are in fact all (*Z*)-13 if position is determined from either end of the molecule

In two species (*B. pascuorum* and *B. lucorum*), conspecific queens were collected from three different countries (Fig. 1). Despite this, all 11 *B. pascuorum* queens had similar alkene isomer profiles, as shown by the small amount of variation (Fig. 5) and forming a single cluster (Fig. 3b). Although the 15 *B. lucorum* queens also form a single cluster (Fig. 3b), there were small but consistent differences in the isomer profiles between the Sheffield and Finnish populations (Fig. 5).

Mimicry of Hosts' Species Specific Alkene Isomer Patterns by Psithyrus The nine non-parasitic *Bombus* species all had species-specific alkene isomer patterns in their Dufour's gland extracts (Fig. 5) that mirrored their pooled wing extracts (Fig. 5). The (*Z*)-7 and (*Z*)-9 alkene isomer ratios of several *Psithyrus* species showed a remarkable resemblance to those of their hosts (Figs. 5, 6), irrespective of whether wing, entire body, or Dufour's gland extracts were compared (Fig. 5). For example, the (*Z*)-7: (*Z*)-9 ratio is dominated by (*Z*)-9 in both *B. pascuorum* and its cuckoo *B. (Ps.) campestris*, while it is dominated by the (*Z*)-7 isomer in *B. lapidarius* and its cuckoo *B. (Ps.) rupestris*. This chemical similarity between the cuckoos and their hosts is even more remarkable considering that the hosts are spread across four sub-genera (Fig. 1) with greatly differing (*Z*)-7: (*Z*)-9 ratios (Fig. 6). *Bombus (Ps.) vestalis* is a close mimic of a rare chemo-type detected within the *terrestris*-group, while all other *B. terrestris* possess a very different isomer ratio. Furthermore, the match between host and cuckoo in the case of *B. lucorum* and *B. (Ps.) bohemicus* is closer in the UK population than the Finnish population, as the UK *B. (Ps.) bohemicus* queens are the only *Psithyrus* species to produce isomers other than (*Z*)-7 and (*Z*)-9 in any appreciable amounts (Fig. 5).

Presence of Dodecyl Acetate The presence of volatile repellent dodecyl acetate was not detected on any of the wing samples (N=76) (Fig. 2a) nor the Dufour's gland extracts of any non-parasitic *Bombus* species studied (Fig. 2b). However, dodecyl acetate was a major compound detected in the Dufour's gland of all *B. (Ps.) bohemicus* (N=6), *B. (Ps.) sylvestris* (N=3) and *B. (Ps.) vestalis* (N=2) females (Fig. 2b). These are all species that invade hosts with slightly different alkene isomer patterns from their own (Fig. 5). In these species, dodecyl acetate represented an average of 21% of the non-polar compounds extracted from the Dufour's gland. Dodecyl acetate was absent from the Dufour's glands of all *B. (Ps.) campestris* (N=2) and *B. (Ps.) rupestris* (N=3) (Fig. 2b), both species that mimic closely the isomer profiles of their hosts (Fig. 5).

Discussion

This study has revealed an additional level of detail contained within an insect's cuticular hydrocarbon profile that is a potential candidate for encoding the recognition cues used by bumblebees. Among the insects, bumblebees are unusual in the variety of isomers they produce, as shown by this and previous studies (Lanne et al. 1987; Tengö et al. 1991; Ayasse et al. 1995, 1999; Urbanová et al. 2004). The nine non-parasitic *Bombus* species in this study and four additional species (*B. ignitus, B. diversus, B. hypocrita,* and *B. deuteronymus*; authors' unpublished data) all have species-specific alkene isomer patterns. The high isomer diversity and stable species-specific patterns are consistent with a functional role in recognition, which is supported by the mimicry of these isomer profiles by the three corresponding

Fig. 5 Species-specific alkene isomer patterns determined from the pooled wing, Dufour's gland and entire body extracts for 14 bumblebee species. The top two graphs represent the non-parasitic *Bombus* species, while the three lower inverted graphs present the data for the corresponding *Psithyrus*-cuckoo species. Arrows indicate alternative hosts. The error bars represent one SD. No error bars are present for the pooled wing samples as sample sizes are one. The asterisk indicates species that produce the repellent dodecyl acetate

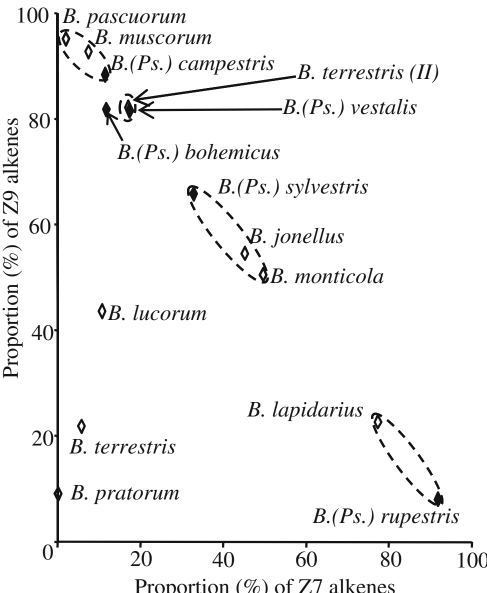


Fig. 6 The matching of a wide range of host (*Z*)-7: (*Z*)-9 alkene isomer profiles occurring across four sub-genera by five *Psithyrus* species that all belong to the same sub-genus (see Fig. 1). The dotted ellipses link the host and cuckoo species

species of social parasite (i.e., cuckoos). In contrast, in those cases where the host and cuckoo isomer profiles do not match, the cuckoos produce a known worker repellent, dodecyl acetate, that represents an alternative strategy for invading colonies. Within each species, the variability of alkene isomers remained small, even where specimens were collected from three countries and had different color forms e.g., *B. pascuorum*. Species-specific alkene isomer profiles found in this study also can be seen in *B. terrestris* collected from Sweden, Israel, and Germany (Tengö et al. 1991) and *B. lapidarius* collected from Sweden (Tengö et al. 1991). This stability of discrimination signals within a species across coarse geographical scales also has been found previously in *Drosophila* flies (Rouault et al. 2001), bark beetles (Symonds and Elgar 2004), and *Formica* ants (Martin et al. 2008c). Conversely the *n*-alkanes show high variability both among individuals within species and among species, resulting in no species-specific profiles. Alkane production is influenced by environmental factors, such as temperature, humidity, and task (Wagner et al. 2001; Martin and Drijfhout 2009b), which may explain this variation.

Hydrocarbons identified in the Dufour's gland of bumblebees also are present on their eggs (Ayasse et al. 1999) as well as on their cuticles (Oldham et al. 1994; Ayasse et al. 1995; this study). This may help "fool" host workers into rearing *Psithyrus* eggs. The recent finding that *Psithyrus* females have retained their ability to produce wax also may help explain their chemical integration into the colony (Sramkova and Ayasse 2008).

The possibility that the cuckoos' profiles have been acquired from either the hosts that raised them or that they will invade is small, because 82 out of the 95 *Psithyrus* females were caught searching for nests rather than being removed from host colonies. As there is a close match between the external (entire body or wing extracts) and internal (Dufour's gland extract) hydrocarbon profiles, *Psithyrus* females may be examples of mimics that biosynthesize their chemical disguise prior to invasion rather than acquire it from their hosts after invasion (Lenoir et al. 2001).

The production of dodecyl acetate by three of the five *Psithyrus* species appears inconsistent, but it is produced only by *Psithyrus* species that fail to closely mimic their host in terms of alkenes. Dodecyl acetate is a known bumblebee worker repellent (Zimma et al. 2003) and represents an alternative way of invading colonies. Limited behavioral observations show that host-cuckoo interactions that involve the two cuckoo species lacking dodecyl acetate (*B. (Ps.) rupestris* and *B. (Ps.) campestris*) are non-aggressive (Hoffer 1888; Sladen 1912; Fisher 1988), whereas aggressive and non-aggressive interactions have been observed against cuckoos that produce dodecyl acetate (Sladen 1912; Van Honk et al. 1981; Küpper and Schwammberger 1995). Similar compounds (e.g., decyl-acetate and decyl butyrate) also are found in the enlarged Dufour's glands of slave-making *Formica* and *Polyergus* ants, where they act as repellents during raids (Regnier and Wilson 1971; Graham et al. 1979; D'Ettorre et al. 2000; Tsuneoka and Akino 2009).

Co-evolution is a dynamic process that may be at different stages for different cuckoo-host associations. For example, *B. pascuorum* and *B. lapidarius* are both common species, and their cuckoos (*B. (Ps.) campestris* and *B. (Ps.) rupestris*) are close color and chemical mimics. *Bombus (Ps.) sylvestris* is a close chemical mimic of *B. jonellus* and *B. monticola,* both uncommon species in Britain, but its other host there is *B. pratorum,* a species with a similar ecology (e.g., early nesting, pollen-stores, small colony size (Sladen 1912)) and that is common and widespread (Williams 2007). This may indicate that *B. (Ps.) sylvestris* has undergone a host shift. This is supported by the facts that *B. (Ps.) sylvestris* produces the worker repellent dodecyl acetate and remains a much closer color mimic of *B. jonellus* than *B. pratorum.* The UK *B. (Ps.) bohemicus* population is unique in its ability to produce other isomers besides (*Z*)-7 and (*Z*)-9 in substantial amounts (Fig. 5). This may result from the cuckoo tracking the profiles of different species within the *lucorum*-complex, and could be evidence of an ongoing "arms race".

The presence of two distinct chemo-types within the *terrestris*-group (Fig. 5) is supported by Pamilo et al. (1984) who also suggested the presence of two sibling species of *B. lucorum* with a wide distribution in

Fennoscandia based on phosphoglucomutase (PGM) gene variation. German laboratory colonies of *B. lucorum* also had isomer profiles similar to our *B. terrestris* II group (Tengö et al. 1991), whereas, field caught *B. terrestris* (around Bonn) have isomers similar to UK *B. terrestris* and *B. lucorum* (Sramkova and Ayasse 2009). The *B. lucorum-terrestris* species complex is poorly resolved taxonomically, and additional morphological, chemical, and genetic data are needed before clear species boundaries can be defined.

The invasion behavior of *Psithyrus* females ranges from highly aggressive to passive (Dronnet et al. 2005), as an invading queen may usurp the host queen (Wilson 1971) or live in peaceful cohabitation with her (Fisher 1987, 1988; Carvell et al. 2008). This might arise from different chemical mechanisms being employed (i.e., mimicry or repellents) by different *Psithyrus* species.

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New Types of Flavonol Oligoglycosides Accumulate in the Hemolymph of Birch-Feeding Sawfly Larvae

Matti Antero Vihakas · Lauri Kapari · Juha-Pekka Salminen

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Abstract Larvae of nine species of sawflies (*Symphyta*) were fed with the foliage of three birch species, after which the larval hemolymph composition was studied by HPLC–DAD and HPLC–ESI–MS. The hemolymph of sawfly larvae contained high concentrations of flavonol oligoglycosides (tri-, tetra-, penta-, and hexaglycosides) that could not be found in the larval foliar diet. In addition, there were significant between-sawfly species differences in both flavonoid composition and concentration (from 0.6 to 12.3 mg/ml) of the hemolymph. This suggested that the studied species have different biosynthetic activities for the synthesis of flavonoid oligoglycosides. Variation in the foliar diets did not cause differences in the hemolymph composition. Our hypothesis is that sawflies use foliar flavonoid monoglycosides rather than flavonoid aglycones to produce these new types of oligoglycosides. These findings open up new possibilities for understanding the more holistic role of flavonoids in insect biochemistry and complex interactions between plants and herbivores.

Key Words Sawfly larvae · Hemolymph · Flavonol oligoglycosides · Mass spectrometry

M. A. Vihakas (✉) · J.-P. Salminen
Laboratory of Organic Chemistry and Chemical Biology,
Department of Chemistry, University of Turku,
Turku FI-20014, Finland
e-mail: maviha@utu.fi

L. Kapari
Department of Arctic and Marine Biology,
University of Tromsø,
Tromsø N-9037, Norway

Introduction

Flavonoids are a well-known group of phenolic compounds due to their ubiquitous presence in nature and potential health–promoting effects on humans (Iwashina, 2000; Ross and Kasum, 2002). The role of flavonoids in plant-herbivore interactions is complex, as they can function both as feeding stimulants and anti-feedants (Simmonds, 2001, 2003; Cipollini et al., 2008). In addition, some insects have developed methods to utilize flavonoids to increase their fitness. Some butterfly species can e.g., sequester flavonoids into their wing pigments that can help in mate recognition (Geuder et al., 1997; Burghardt et al., 2000).

Earlier studies with birch-feeding insect herbivores have shown that the lipophilic flavonoid aglycones of the epidermal layer of birch leaves act as defensive compounds e.g., against the larvae of autumnal moth (*Epirrita autumnata*) (Lahtinen et al., 2004). However, insects also have developed methods to avoid the harmful effects of these defenses. *Epirrita autumnata* and other birch-feeding insects—such as sawfly larvae—were shown to partially detoxify flavonoid aglycones by glycosylation (Salminen et al., 2004; Lahtinen et al., 2005); addition of a single sugar unit into the lipophilic flavonoid aglycone enables the excretion of the formed water-soluble flavonoid monoglycoside into the feces of larvae. However, not all flavonoid aglycones were glycosylated. Only 22% of the ingested flavonoid aglycones were glycosylated by *E. autumnata* larvae (Salminen et al., 2004), and with sawfly larvae the glycosylation percentages varied from 35 to 44%, depending on the species (Lahtinen et al., 2005). It has remained unclear what the metabolic fate for the remaining portion of ingested flavonoid aglycones is, since only traces were found excreted as such in the feces, and the new fecal monoglycosides did not represent even one half of the ingested amounts of aglycones.

The purpose of this study was to clarify whether any of the missing portions of flavonoid aglycones could be found in larval hemolymph. We had not studied larval hemolymph composition earlier. Therefore we first did some preliminary HPLC-analyses of hemolymph with several species of insect herbivores. We found no flavonoids in the hemolymph of lepidopteran larvae (*E. autumnata* and *Agriopis aurantiaria*), possibly because of the high polyphenol oxidase activities in their hemolymph (Ruuhola and Salminen, unpublished data). On the contrary, hemolymph of sawfly larvae contained high levels of flavonoids. To study this phenomenon further, we collected a diverse set of larvae from nine species of birch-feeding sawflies that grew in the northern part of Finland. Each larval species was fed with the foliage of three birch species. These birches were known to differ in their flavonoid aglycone composition and content (Valkama et al., 2003, 2004). Our hypothesis was that different flavonoid aglycone compositions of the three types of foliar diets would be seen directly as differential flavonoid composition of the hemolymph samples.

Methods and Materials

Insect Bioassay and Collection of the Hemolymph Field experiments were conducted at the Kevo Subarctic Research Station in Utsjoki, northern Finland (69°45'N, 27°00'E). The forest of the study area is dominated by mountain birch (*Betula pubescens* subsp. *Czerepanovi* (Orlova) Hämet-Ahti) that is a tree line forming species in northern Fennoscandia. The larvae used in the experiment originated from a field grown 'laboratory stock' replenished by yearly collections of wild larvae. Each year pairs of adult males and females were enclosed in mesh bags on mountain birch branches, and the hatching larvae were allowed to feed on naturally growing leaves until the end of the last instar. Prepupating larvae were placed in plastic vials containing moist sphagnum moss where the larvae formed cocoon for overwintering. The species selected for the experiment, from the phenologically earliest to the latest, were: *Amauronematus amplus* Konow, *Pristiphora alpestris* Konow, *Nematus viridescens* Cameron, *Nematus brevivalvis* Thomson, *Arge* sp. Schrank, *Nematus pravus* Konow, *Trichiosoma scalesii* Leach, *Nematus viridis* Stephens, and *Dineura pullior* Schmidt. *Arge* sp. belongs to the family Argidae, *Trichiosoma scalesii* to the family Cimbicidae, while the rest are members of the subfamily Nematinae in the family Tenthredinidae.

During the summer of 2004 emerging adult females were allowed to feed on honey water, after which they were enclosed in mesh bags on the branches of haphazardly chosen mountain birch trees. Larvae were allowed to feed in the mesh bags until 2nd–3rd instar, after which the bags were cut down and the larvae were taken into the laboratory. The larvae were divided into three groups to be fed with leaves from either mountain birch, dwarf birch (*Betula nana* L.), or silver birch (*Betula pendula* Roth). We used the same three mountain and silver birch individuals throughout the experiment. Since dwarf birches are so small in size, we used haphazardly chosen tree individuals for feeding until the last 4 d of the experiment when the larvae were fed with leaves from the chosen three experimental trees. Larvae were grown singly in 48 ml plastic vials and fed with new leaves every 3rd d. When the larvae had reached their last instar, they were preserved in liquid nitrogen and transferred to the University of Turku in southern Finland (Table 1). Hemolymph sampling was done by piercing a small hole with a needle into the integument of larva and then gently pressing out a small droplet of hemolymph.

HPLC and MS Analyses Hemolymph was analyzed immediately after its collection by HPLC–DAD without any purification steps. Samples were manually injected (5 or 20 μl) because of the small volumes. The HPLC column was LiChroCART Superspher 100 RP-18 (75×4 mm I.D., 4 μm, Merck, Germany). The HPLC–DAD system (Merck-Hitachi, Tokyo, Japan) consisted of a manual Rheodyne injector, an L-7100 pump, an L-7455 diode array detector, and a D-7000 interface module. Two solvents were used: 0.05 M H_3PO_4 (A) and acetonitrile (B). The elution profile was: 0–3 min, 100% A; 3–20 min, 0–25% B in A (linear gradient); 20–23 min, 25–70% B in A (linear gradient); 23–47 min, column wash and stabilization. The flow rate was 1 ml/min. The UV spectra of hemolymph components were acquired between 195 and 450 nm, and detection wavelength was 341 nm. Additional HPLC–ESI–MS analyses were done by a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) that has ion spray interface and Analyst Software 1.1 for data handling. The column used and chromatographic conditions were the same as in Salminen et al. (1999).

Table 1 Insect bioassay information

	Dates for the final feeding day in 2004	Number of larvae per tree species
Amauronematus amplus	5.7.	33
Pristiphora alpestris	7.7.	33
Nematus viridescens	15.7.	33
Nematus brevivalvis	16.7.	33
Arge sp.	28.7.	33
Nematus pravus	10.8.	33
Trichiosoma scalesii	11.8.	18
Nematus viridis	14.8.	33
Dineura pullior	24.8.	39

The mass spectrometer was used in the positive ion mode, and mass spectra were obtained between 100 to 1600 atomic mass units.

Flavonoid Quantification The total flavonoid contents of hemolymph samples were quantified from the HPLC–DAD analyses at 341 nm. First, we detected all such HPLC peaks that had UV spectra characteristic of flavonoids. Then the HPLC peak areas of all the flavonoids were summed up to obtain the total flavonoid content of each sample. Flavonoids were quantified as quercetin equivalents using quercetin (Sigma Chemical Co, St. Louis, MO, USA) as an external standard.

Results

At first, hemolymph samples of nine sawfly species were analyzed by HPLC–DAD. In the HPLC–DAD chromatograms most of the peaks eluted shortly after 12 min, and many of the peaks co-eluted and produced a hump (Fig. 1). The short elution time of peaks suggested that the compounds were hydrophilic and they might contain sugars. The unknown compounds could not be simple flavonoid aglycones or monoglycosides that were known to elute much later in the chromatogram (Ossipov et al., 1995, 1996; Valkama et al., 2003). These data suggested that the unknown compounds might contain at least two sugar units. The UV spectra of the main compounds were characteristic of flavonols (Fig. 1). In flavonol aglycones such as kaempferol, quercetin, and myricetin, the band I UV absorptions vary between 359–370 nm. However, the band I UV absorptions of unknown hemolymph compounds were mainly between 341–349 nm. This kind of hypsochromic shift in band I maxima has been seen associated with the glycosylation of flavonols (Markham and Mabry, 1975).

Hemolymph samples then were analyzed by HPLC–ESI–MS, but there was enough hemolymph material for only seven of the nine sawfly species: *Amauronematus amplus*, *Arge* sp., *Nematus brevivalvis*, *Nematus pravus*, *Nematus viridis*, *Pristiphora alpestris,* and *Trichiosoma scalesii*. Time range 11–19 min in the HPLC-chromatograms was named as a

Fig. 1 HPLC-chromatograms of hemolymph samples of nine sawfly species. Arrows show UV spectra of some the main peaks (obtained between 195–450 nm)

"flavonoid part" of the chromatogram, and mass spectra were first acquired from this area (Fig. 2). Most of the mass spectra from the "flavonoid part" had an ion at *m/z* 303, which fit with the flavonol aglycone quercetin (Kite et al., 2007; Veitch et al., 2008). In addition, a clear pattern of ions could be seen in the mass spectra of different sawfly species: quercetin was followed by ions differing by 162 Da from each other: 465, 627, 789, 951, 1113, and 1275. Value 162 Da indicated a glycosidic bond between quercetin and carbohydrate units (glycohexopyranose e.g., glucose or galactose). The ions 465, 627, 789, 951, 1113, or 1275 could derive from molecular ions ($[M+H]^+$) of flavonol monoglycosides or oligoglycosides, or they could be fragments of these ions. For this reason, each peak in the HPLC chromatograms was analyzed separately for its composition using single ion monitoring. This analysis revealed that all main peaks in the chromatograms belonged to individual flavonol oligoglycosides i.e., compounds where several carbohydrates were attached to a flavonol nucleus. The molecular ion of flavonol oligoglycoside was always preceded by its mass fragments, and each fragment represented a loss of one carbohydrate unit from the original flavonol oligoglycoside structure. The largest oligoglycoside was a quercetin hexaglycoside (*m/z* 1275), but quercetin tri- (*m/z* 789), tetra- (*m/z* 951), and pentaglycosides (*m/z* 1113) also were present (Fig. 3). There were only traces of quercetin mono- (*m/z* 465) and diglycosides (*m/z* 627) in the samples. Interestingly, each sawfly species had its own selection of flavonol oligoglycosides in the hemolymph. Quercetin hexa- and pentaglycosides were found in three sawfly species: *Arge* sp., *Nematus viridis,* and *Nematus brevivalvis,* and the other species contained different compositions of quercetin tri- and tetraglycosides (Table 2).

Many of the mass spectra had ions from the following ion pattern: *m/z* 597, 759, 921 and 1083. These ions were 30 Da less than the quercetin oligoglycoside ions next to them (represented by *m/z* 627, 789, 951, and 1113). This indicated that one of the glycohexopyranosides in quercetin oligoglycosides was replaced by a glycopentofuranoside e.g., arabinose sugar that has a molecular mass 30 Da

Fig. 2 Positive ion mass spectra of an HPLC–ESI–MS run of seven sawfly species. Mass spectra were recorded from the flavonoid part (11–19 min) of the chromatogram

Fig. 3 Positive ion mass spectra of flavonol oligoglycosides in the sawfly hemolymph. A: quercetin triglycoside, B: quercetin tetraglycoside, C: quercetin pentaglycoside, D: quercetin hexaglycoside E: quercetin tetraglycoside with one pentose sugar (*m/z* 921) co-eluting with a quercetin tetraglycoside (*m/z* 951), F: kaempferol pentaglycoside (*m/z* 1097) co-eluting with a quercetin hexaglycoside (*m/z* 1275). Interpretation of the ions: *m/z* 303=quercetin; *m/z* 465=quercetin with one hexose sugar; *m/z* 597=quercetin with one hexose sugar and one pentose sugar; *m/z* 627=quercetin with two hexose sugars; *m/z* 759=quercetin with two hexose sugars and one pentose sugar; *m/z* 773=kaempferol with three hexose sugars; *m/z* 789=quercetin with three hexose sugars; *m/z* 921=quercetin with three hexose sugars and one pentose sugar; *m/z* 935=kaempferol with four hexose sugars; *m/z* 951=quercetin with four hexose sugars; *m/z* 1097=kaempferol with five hexose sugars; *m/z* 1113=quercetin with five hexose sugars; *m/z* 1275=quercetin with six hexose sugars; *m/z* 1577=$[2M+H]^+$ ion of a quercetin triglycoside. A hexose sugar refers to a glycohexopyranoside and a pentose sugar to a glycopentofuranoside

Table 2 Detected flavonol glycosides in the hemolymph of different sawfly species

	$[M+H]^+$	*Amauronematus amplus*	*Arge* sp.	*Nematus brevivalvis*	*Nematus pravus*	*Nematus viridis*	*Pristiphora alpestris*	*Trichiosoma scalesii*
Quercetin triglycoside	759				x		x	x
Kaempferol triglycoside	773				x		x	x
Quercetin triglycoside	789	x		x	x		x	x
Quercetin tetraglycoside	921	x	x			x		
Kaempferol tetraglycoside	935	x	x	x		x	x	
Quercetin tetraglycoside	951	x	x	x	x	x	x	
Quercetin pentaglycoside	1083			x		x		
Kaempferol pentaglycoside	1097		x					
Quercetin pentaglycoside	1113		x	x		x		
Quercetin hexaglycoside	1275		x	x		x		

less than glucose or galactose. However, only one of the glycohexopyranosides in flavonol oligoglycosides was replaced by a glycopentofuranoside since there was no mass spectral evidence of flavonol oligoglycosides with two or more glycopentofuranosides. The largest quercetin oligoglycoside with a glycopentofuranoside sugar was a quercetin pentaglycoside in *Nematus brevivalvis* and *Nematus viridis* (*m/z* 1083). This compound had four glycohexopyranosides and one glycopentofuranoside attached to the quercetin backbone (Table 2).

Some of the mass spectra showed yet another ion pattern: *m/z* 287, 449, 611, 773, 935, and 1097. The ions in the pattern were divided from each other by 162 Da. Again, this indicated a glycosidic bond between a flavonoid and a glycohexopyranoside. The peak at *m/z* 287 was interpreted as kaempferol (Veitch et al., 2008), and the following ions represented several glycohexopyranosides attached to the kaempferol nucleus. The largest kaempferol oligoglycoside was a kaempferol pentaglycoside (*m/z* 1097) in *Arge* sp. (Table 2). Kaempferol oligoglycosides always were less common than quercetin oligoglycosides.

During the feeding experiments, each species of sawfly larvae were divided into three groups that were fed with different birch diets (*B. nana*, *B. pendula,* and *B. pubescens* subsp. *Czerepanovi)*. Different birch diets had no significant effect on the hemolymph composition within the same sawfly species (data not shown). This means that, for instance, quercetin triglycosides were always the main metabolites in the hemolymph of *Trichiosoma scalesii* although the larvae had eaten different birch diets. The total flavonoid contents of hemolymph samples were quantified from the HPLC–DAD chromatograms (Fig. 4). Total flavonoid contents varied markedly among different sawfly species (from 0.6 to 12.3 mg/ml). Different birch diets did not have significant effects on the total flavonoid contents within the same sawfly species.

Discussion

Hemolymph of sawfly larvae was found to contain high concentrations of flavonol oligoglycosides. These compounds seem to be *de novo* metabolites i.e., produced by the sawfly larvae themselves since the diet of larvae, birch leaves, are known to contain only flavonol aglycones and monoglygosides (Ossipov et al., 1996; Keinänen and Julkunen-Tiitto, 1998; Lahtinen et al., 2005). Ability to glycosylate flavonoids also has been reported in insects other than sawfly larvae: Larvae of *Epirrita autumnata* detoxify flavonoid aglycones into flavonoid monoglycosides (Salminen et al., 2004); *Dissoteira carolina* grasshoppers glycosylate quercetin (Hopkins and Ahmad, 1991); *Polyommatus icarus* butterflies produce kaempherol 3-*O*-glucoside from dietary kaempherol (Wiesen et al., 1994); and *Bombyx mori* silkworms produce quercetin mono-, di-, and triglucosides that are not originally present in their diet (Tamura et al., 2002; Hirayama et al., 2008). Ferreres et al. (2007) studied the larvae of *Pieris brassicae* L. and found

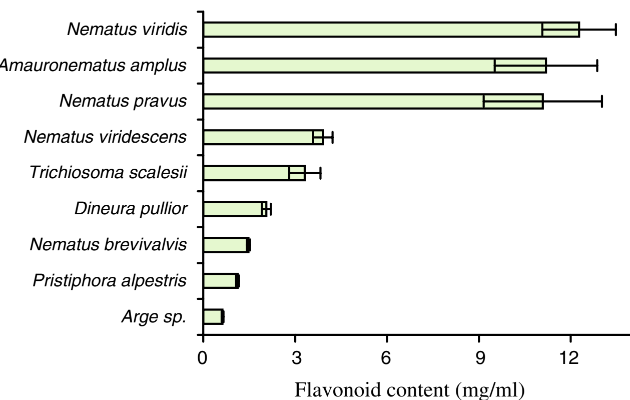

Fig. 4 Total flavonoid contents in the hemolymph of sawfly species. Bars show standard error of the mean

several quercetin and kaempherol derivatives that could be either sequestration or metabolism products of the insect.

This study raises the question of how flavonol oligoglycosides are metabolized by sawfly larvae. The studied birch species (*B. nana, B. pendula, and B. pubescens* subsp. *czerepanovii*) have different compositions and concentrations of epidermal flavonoid aglycones (Valkama et al., 2003, 2004), and our original hypothesis stated that different flavonoid aglycone compositions of foliar diets would be seen as differential flavonoid compositions of the hemolymph samples. It could also be possible that sawfly larvae use these epidermal flavonoid aglycones as a starting material for the flavonol oligoglycoside synthesis. However, our results showed that different birch diets did not have significant effects on the compositions of hemolymph samples or concentrations of flavonol oligoglycosides. Also, no trace of epidermal flavonoid aglycones (except kaempferol in kaempherol oligoglycosides) was found in the hemolymph. The main flavonol aglycone part of flavonol oligoglycosides in the sawfly hemolymph was quercetin but this compound seems to be absent from birch leaf epidermal flavonoid aglycones (Valkama et al., 2003; Lahtinen et al., 2006). These findings oppose the idea that sawfly larvae use epidermal flavonoid aglycones for flavonol oligoglycoside synthesis.

Additionally, this study could not answer our original question about the metabolic fate of birch-derived flavonoid aglycones in sawfly larvae. It seems likely that the larvae use birch leaf flavonol monoglycosides rather than flavonol aglycones for flavonol oligoglycoside synthesis. First, all the birch species in this study contain many vacuolic quercetin monoglycosides (Ossipov et al., 1996; Keinänen and Julkunen-Tiitto, 1998; Graglia et al., 2001; Riipi et al., 2002; Salminen and Lempa, 2002). The concentrations of vacuolic quercetin monoglycosides in birch leaves also are generally higher than the concentrations of other flavonol monoglycosides (kaempherol or myricetin). This would give a partial explanation of why the majority of flavonol oligoglycosides in sawfly hemolymph were quercetin oligoglycosides and why kaempferol oligoglycosides were less common. Birch leaves contain also myricetin monoglycosides, but the hemolymph samples did not show clear evidence of myricetin oligoglycosides. This can mean that the sawfly species of this study may not have the ability to synthesize myricetin oligoglycosides.

The exact structures of flavonol oligoglycosides are unknown. At least one of the sugar units has to be attached directly to a flavonol nucleus, and the remaining sugars will be attached to other hydroxyl groups in flavonol nucleus, or the sugar units may chain to each other. Examples of plant derived flavonol oligoglycosides (e.g., Kite et al., 2007; Taylor et al., 2007; Veitch et al., 2008) give insight about the possible conformations of sawfly flavonol oligoglycosides. To fully solve the structures, one needs to isolate individual flavonol oligoglycosides and determine their structures, e.g., by NMR techniques. However, one sawfly larva contains only a few microliters of hemolymph, and it would require substantial amounts of larval material (several hundreds of individuals) to produce enough hemolymph for the isolation process. With the sparsely populated birch-feeding sawfly larvae, it was beyond the scope of this study to collect such large amounts of hemolymph.

Flavonoid compositions and concentrations within one sawfly species were approximately the same among feeding experiments with different birch species. Small differences could be detected in some of the replicates: e.g., the larvae of *Nematus viridis* with one birch diet contained quercetin hexaglycosides but the same larvae with another birch diet contained only quercetin pentaglycosides. Species to species differences in total flavonoid concentrations and flavonol oligoglycoside compositions were more obvious (Fig. 4. and Table 2). Some sawfly species contained a series of quercetin oligoglycosides up to hexaglycosides, while other species produced only quercetin triglycosides. This suggested that the sawfly species have different biosynthetic abilities to produce flavonol oligoglycosides.

The total flavonoid contents of hemolymph samples were estimated with quercetin as an external standard. However, the use of quercetin as a standard for flavonol oligoglycosides poses a problem. With quercetin, the whole molecule exhibits UV absorption but the situation is different with flavonol oligoglycosides: in this case, only the flavonol part of the molecule shows UV absorption, and the sugar stays “invisible” to UV detection. This is due to different chemical structural properties of sugars and flavonols. The UV-absorbing chromophore of quercetin oligoglycosides is quercetin (302 Da). With quercetin triglycoside (788 Da), for instance, 302/788=38% of molecular mass of the molecule belongs to the UV-absorbing quercetin while 62% of the molecule stays invisible to UV detection. This means that the total flavonoid contents of hemolymph samples (0.6 to 12.3 mg/ml, Fig. 4) are underestimated when only a quercetin standard is used. To get more accurate results one needs an authentic flavonol oligoglycoside standard that is isolated from sawfly larvae. However, it was beyond the scope of this study to collect such large quantities of birch sawfly larvae. Our goal in future studies is to isolate flavonol oligoglycoside standards from larval hemolymph, and these standards will allow us to make more accurate estimations of the flavonol oligoglycoside concentrations in hemolymph samples.

The biological significance of flavonol oligoglycosides for sawfly larvae remains unknown. Glycosylation of flavonoid aglycones has been seen as a detoxification mechanism

for the larvae of *Epirrita autumnata* (autumnal moth) (Salminen et al., 2004). Another study showed that sawfly larvae are capable of glycosylating birch leaf flavonoid aglycones and excreting them as flavonoid monoglycosides into their feces (Lahtinen et al., 2005). A simple hypothesis could propose flavonol oligoglycosides as detoxification products of sawfly metabolism. However, as shown by Salminen et al. (2004), mere flavonoid monoglycosides are hydrophilic enough to be excreted into feces. Why would sawfly larvae need to spend extra sugar units and energy for this oligoglycosylation process? Perhaps flavonol oligoglycosides have some other function for the sawfly larvae. One possibility is that larvae utilize plant secondary chemicals to promote their own defense strategies againts their natural enemies e.g., ants, parasites, or birds. Many Tenthredinidae (Hymenoptera) sawfly species exhibit a defensive strategy termed "easy bleeding", which means the release of hemolymph through the integument as a result of even slight mechanical pressure or disturbance (Müller et al., 2001; Boevé and Schaffner, 2003; Prieto et al., 2007). We have observed easy bleeding in many of our study species too, but the role of flavonoid metabolism in this respect remains hypothetical. The biological role of flavonol oligoglycosides in sawfly hemolymph needs to be examined in more detail in future studies.

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Intraspecific Geographic Variation of Fragrances Acquired by Orchid Bees in Native and Introduced Populations

Santiago R. Ramírez · Thomas Eltz · Falko Fritzsch · Robert Pemberton · Elizabeth G. Pringle · Neil D. Tsutsui

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Abstract Male orchid bees collect volatiles, from both floral and non-floral sources, that they expose as pheromone analogues (perfumes) during courtship display. The chemical profile of these perfumes, which includes terpenes and aromatic compounds, is both species-specific and divergent among closely related lineages. Thus, fragrance composition is thought to play an important role in prezygotic reproductive isolation in euglossine bees. However, because orchid bees acquire fragrances entirely from exogenous sources, the chemical composition of male perfumes is prone to variation due to environmental heterogeneity across habitats. We used Gas Chromatography/Mass Spectrometry (GC/MS) to characterize the perfumes of 114 individuals of the green orchid bee (*Euglossa* aff. *viridissima*) sampled from five native populations in Mesoamerica and two naturalized populations in the southeastern United States. We recorded a total of 292 fragrance compounds from hind-leg extracts, and found that overall perfume composition was different for each population. We detected a pronounced chemical dissimilarity between native (Mesoamerica) and naturalized (U.S.) populations that was driven both by proportional differences of common compounds as well as the presence of a few chemicals unique to each population group. Despite these differences, our data also revealed remarkable qualitative consistency in the presence of several major fragrance compounds across distant populations from dissimilar habitats. In addition, we demonstrate that naturalized bees are attracted to and collect large quantities of triclopyr 2-butoxyethyl ester, the active ingredient of several commercially available herbicides. By comparing incidence values and consistency indices across populations, we identify putative functional compounds that may play an important role in courtship signaling in this species of orchid bee.

Key Words Hymenoptera · Monoterpenes · Sesquiterpenes · Pheromone evolution · Euglossini · Invasive species · Orchids · Bees · Triclopyr BEE

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S. R. Ramírez (✉) · N. D. Tsutsui
Environmental Science, Policy & Management,
University of California Berkeley,
137 Mulford Hall #3114,
Berkeley, CA 94720, USA
e-mail: sramirez77@berkeley.edu

T. Eltz · F. Fritzsch
Department of Neurobiology,
Sensory Ecology Group, University of Düsseldorf,
Universitätstr. 1,
40225 Düsseldorf, Germany

R. Pemberton
Invasive Plant Research Laboratory,
United States Department of Agricultural Research Service,
3225 College Avenue,
Fort Lauderdale, FL 33314, USA

E. G. Pringle
Department of Biology, Stanford University,
Stanford, CA 94305, USA

Introduction

Most insects rely on chemical compounds (pheromones) to identify and attract conspecific mates (Wyatt, 2003). The chemical composition of mating pheromones may range from single molecules to diverse, complex mixtures (Symonds and Elgar, 2004, 2008; Billeter et al., 2009). Because mating pheromones usually consist of species-

specific blends, they often mediate prezygotic reproductive isolation among closely related lineages (Cardé et al., 1977; Roelofs et al., 2002). In insects, mating pheromones are synthesized *de novo* or from sequestered precursors. In either case, both biotic and abiotic factors influence pheromonal chemical composition (Stennett and Etges, 1997; Groot et al., 2009). Hence, an important question is how insect populations that colonize novel environments respond to environmental changes that influence mating pheromones. In addition, to understand the underlying mechanisms of pheromone evolution in insects, we must examine intraspecific phenotypic variation in pheromone chemistry across habitats, geographic regions, and genetic gradients.

Orchid (or euglossine) bees constitute a diverse lineage of Neotropical insect pollinators (Ramírez, 2009; Ramírez et al., 2010) that are best known for their fragrance-collecting behavior (Darwin, 1862; Dressler, 1968; Dodson et al., 1969). Male bees collect volatile compounds from multiple floral and non-floral sources that they use subsequently during courtship display (Dressler, 1982; Eltz et al., 2005b). Males of all species of orchid bees (~200) have enlarged hind-tibial organs in which they deposit perfumes that consist primarily of terpenoids and aromatic compounds (Williams and Whitten, 1983; Eltz et al., 1999; Zimmermann et al., 2009a). Male orchid bees land on the surface of fragrance sources and secrete large quantities of long-chain lipids from cephalic labial glands that dissolve the volatile compounds to be collected (Whitten et al., 1989; Eltz et al., 2007). Although it has not been confirmed directly via behavioral experiments, it is likely that perfume chemistry enables females to choose mates and/or discriminate non-specific males (Eltz et al., 1999, 2003, 2005a; Bembé, 2004; Zimmermann et al., 2006). In a recent study, Zimmermann et al. (2009a) analyzed the chemical composition of the fragrances acquired by male orchid bees from 15 closely related sympatric species in the genus *Euglossa* from central Panama. They showed that related lineages of bees accumulate species-specific fragrance blends that have differentiated rapidly during lineage diversification, potentially in response to strong selection against hybridization or mating interference between species. Previous studies also have shown that perfume variation tends to be lower within lineages than between lineages (Eltz et al., 1999, 2005a, 2008; Zimmermann et al., 2006), even when individuals co-occur in the same habitat (Eltz et al., 2005a, 2008). However, the extent of intraspecific perfume variation across geographic regions and genetic gradients has not yet been examined.

Male orchid bees may accidentally accumulate a considerable diversity of inactive (noisy) compounds during the process of perfume collection (Eltz et al., 2005a), as they often collect volatiles from multiple sources, even at local scales (Pemberton and Wheeler, 2006). In addition, variation in the availability and abundance of specific chemicals among habitats may result in disparate fragrance phenotypes (Eltz et al., 2005a). Thus, orchid bees must deal with environmentally induced variability while maintaining a reliable chemical communication system. What proportion of fragrance compounds is biologically active, and thus required for mating? Do conspecific male orchid bees collect similar compounds across disparate habitats? Can different chemical compounds serve homologous functions in distant populations? We designed a comprehensive analysis of fragrance variation within and between interbreeding populations of a single species, *Euglossa* aff. *viridissima*, to answer some of these questions. In particular, we focused on elucidating phenotypic change in the perfumes of *E.* aff. *viridissima* upon the invasion of a novel environment.

The green orchid bee *Euglossa viridissima* is distributed throughout lowland dry forests in northern Mesoamerica (Hinojosa-Diaz et al., 2009), ranging in distribution from southern Costa Rica to Northern Mexico (Fig. 1). Previous taxonomic treatments recognized *E. viridissima* as a single distinct species (e.g., Roubik and Hanson, 2004). However, a recent study of populations from the Yucatan peninsula (Mexico) showed instead that this lineage consists of two cryptic sister species (Eltz et al., 2008). The most prominent morphological difference between the two lineages is in the number of teeth on the mandible of males: two or three dentitions. Additionally, whereas males with three dentitions (hereafter *E.* aff. *viridissima*) collect large quantities of the compound 2-hydroxy-6-nona-1,3-dienyl-benzaldehyde (abbreviated HNDB), males with two dentitions (*E. viridissima*) lack HNDB in their perfumes (Eltz et al., 2008). A detailed systematic analysis of these lineages based on morphology, chemistry, and genetic data is in preparation (Eltz et al., unpublished).

As early as 2003, bees of *E.* aff. *viridissima* were introduced accidentally and naturalized in southeastern United States (Skov and Wiley, 2005). Since then, vigorous populations have become established throughout most of the southern coastal peninsula of Florida (Pemberton and Liu, 2008; Liu and Pemberton, 2009; Pemberton and Ramírez, personal observation). A chemical analysis of the perfumes of naturalized bees from Florida showed that male bees collect multiple fragrance compounds from several plant species, including native, naturalized, and horticultural plants (Pemberton and Wheeler, 2006). However, the composition of these fragrances has not been compared with that of native *E.* aff. *viridissima* populations.

In the present study, we analyzed the chemical composition of the fragrances acquired by *E.* aff. *viridissima* across its native range in Mesoamerica and its introduced range in Florida (U.S.). We estimated the magnitude of

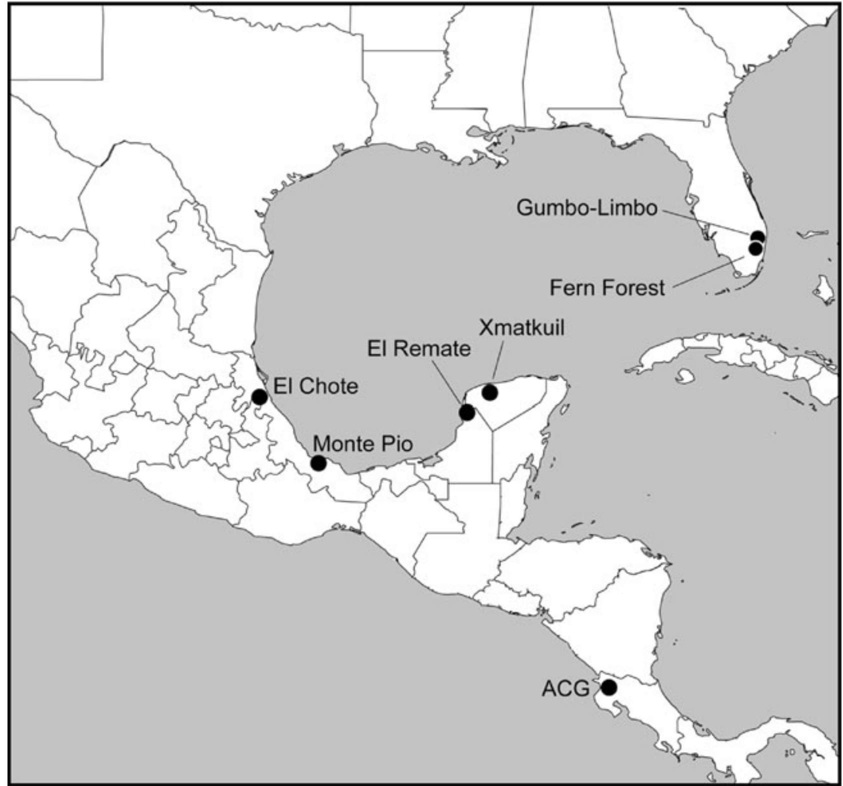


Fig. 1 Map of Mesoamerica and southern United States indicating sampling localities where male *Euglossa* aff. *viridissima* were collected. Native range populations in Mesoamerica were El Chote, Monte Pio, El Remate, Xmatkuil and ACG. Naturalized populations in the United States were Gumbo-Limbo and Fern Forest

intraspecific geographical variation in fragrance composition and identified volatile compounds that vary within and between bee populations. In addition, because the recent introduction of *E.* aff. *viridissima* to the United States may have resulted in both a genetic bottleneck and a habitat shift, we asked whether changes in perfume chemistry have accompanied the naturalization of this lineage of bees.

Methods and Materials

Sampling A total of 114 male orchid bees belonging to the *Euglossa* aff. *viridissima* lineage were collected from five localities across the native range in Costa Rica and Mexico, and two localities in the introduced range in the U.S. (Florida) in 2008 and 2009 (Fig. 1; Supplementary Material Appendix 1). Male bees were lured with synthetic chemical baits consisting of squares of blotter paper impregnated with *p*-dimethoxybenzene, methyl cinnamate, or eugenol (Sigma-Aldrich, St. Louis, MO, USA; Eltz et al., 2008). The impregnated paper squares were suspended 1.5 m aboveground and covered with screen mesh to prevent male bees from accessing the bait. Baiting stations were monitored every 15 min from 9AM until 1PM. Males were captured with hand nets and kept in the shade inside screen cages until subsequent dissection, typically conducted the same day.

Florida populations were sampled at two natural area preserves. Fern Forest (Broward County) is primarily a tropical hardwood forest grading into freshwater cypress swamp and upland pine habitats. Gumbo-Limbo Nature Center (Palm Beach County) is located on a barrier island with a tropical hardwood forest and mangroves. Mesoamerican populations were sampled from various habitats, including secondary seasonally dry tropical forest (ACG), secondary seasonally dry scrubland (Xmatkuil), farmland surrounded by stretches of tropical perennial forest (El Chote), settlement adjacent to tropical perennial forest (Monte Pio), and coastal mangroves (El Remate).

Fragrance Extraction and Chemical Analysis Male bees were cold-anaesthetized either on ice or inside a freezer (−20°C) for 5 min immediately prior to dissection. Right hind tibiae were removed with clean dissecting scissors and deposited in 2 ml screw-cap autosampler vials (Agilent Technologies, Santa Clara, CA, USA), which contained 500 µl of hexane to extract the fragrances. To distinguish between exogenous volatiles and endogenous male-produced lipids, acetates, and straight-chain hydrocarbon compounds, labial glands were dissected, extracted, and analyzed from a subset of individuals (N=43) using the same protocol described above. All compounds present in both leg extracts and labial glands were excluded from the analysis (Fig. 2). Extracted samples were stored at −20°C

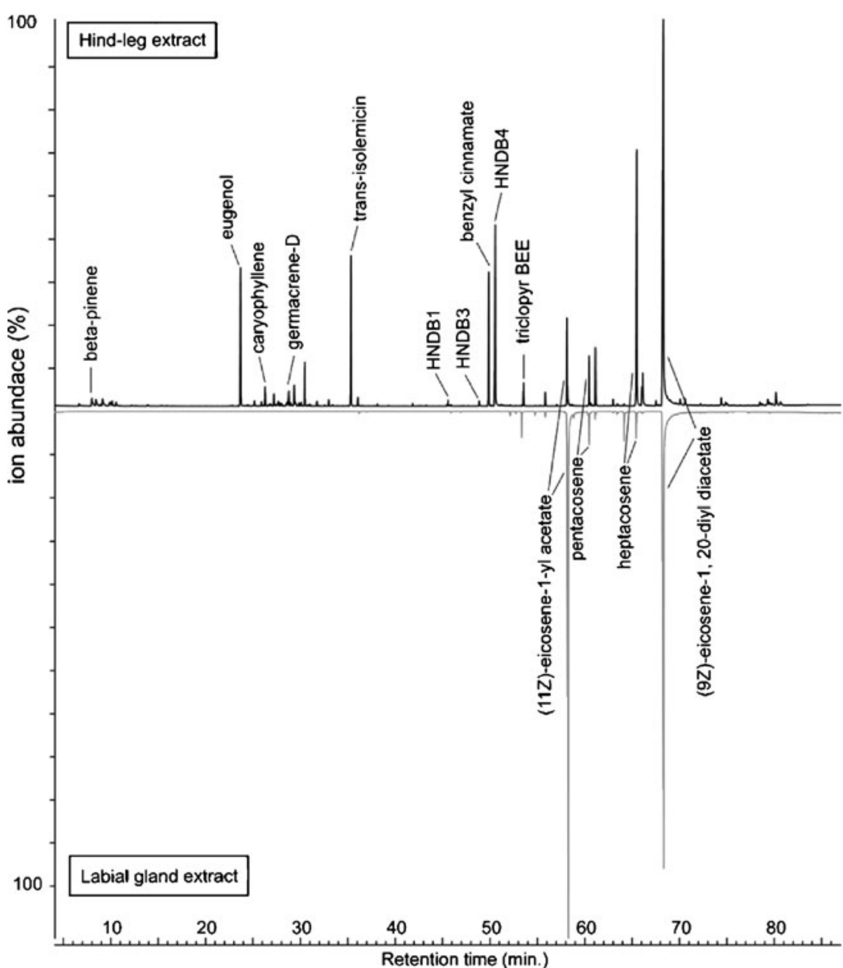


Fig. 2 Overlaid total ion current chromatograms corresponding to hind-leg (black) and labial gland (grey) extracts from the same individual bee. Compounds present in both hind legs and labial glands were considered endogenous in origin and thus were excluded from the analysis. The Y axis indicates ion abundance

until subsequent GC/MS analysis. Voucher specimens of all samples were either pinned or deposited in 200 proof ethanol.

Gas Chromatography/Mass Spectrometry (GC/MS) was conducted at the Department of Environmental Sciences, Policy and Management at the University of California Berkeley, using a 7890A Agilent GC coupled with a 5975C Agilent Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). The GC was fitted with a 30 m long, 0.25 mm internal diam, non-polar Agilent HP-5MS capillary column (cat. # 19091S-433). Sample aliquots of 1 µl were injected by using an Agilent 7683B automatic injector operated in splitless mode. The oven temperature was programmed from 60 to 300°C at 3°C/min, using helium as carrier gas with a constant flow rate set to 0.7 ml/min. Mass Selective Detector (MSD) scanning parameters ranged from 50 to 550 amu, with a sampling rate of 2.91 scans/sec and a threshold detection of 150 counts.

Chemical analyses revealed the presence of triclopyr 2-butoxyethyl ester (hereafter triclopyr BEE), in Florida populations only. Triclopyr BEE is the active ingredient of a variety of commercial herbicides. To determine whether male *E.* aff. *viridissima* were actively attracted to this compound, we performed attraction bioassays with both captive and wild bees. Captive bees were nest-trapped in Florida (August 2009) and reared at UC Berkeley in an insectary facility maintained at 25°C, 40–60% humidity, and kept under a 12/12 h light/dark cycle. Male bees were not exposed to any fragrances prior to the bioassay, in which we presented male bees with hind leg extracts that contained large quantities of triclopyr BEE and as little as possible of other volatile compounds. We applied 100 µl of the hexane extract to clean blotter paper squares placed inside a flight cage where the bees were reared. As an experimental control, we presented male bees with labial gland extracts from the same individuals for which fragrance extracts were selected in the experimental treatment. The number of bees displaying fragrance gathering behavior within the first 2 min was recorded.

In addition, to test whether male *E.* aff. *viridissima* from native-range populations also were attracted to triclopyr BEE, we purified this compound from leg extracts using a preparative GC system similar to that of Nojima et al. (2008). A megabore DB-5 capillary column (30 m,

0.53 mm ID, 0.5 um film thickness) was used to separate compounds in an HP 5890 II gas chromatograph (50 to 300°C at 10°/min). Injection was on column, with a 30 m retention gap preceding the actual analytical column. The peak of triclopyr BEE was captured by using a 40 cm piece column trap (HP-1, 0.53 ID) that was connected to the preparative outlet at the time of elution. The substance retained in the trap was washed out with *n*-hexane. Repeated runs yielded approximately 75 μg of purified triclopyr BEE diluted in 1 ml of *n*-hexane. Subsequently, we presented male *E.* aff. *viridissima* with purified extracts of triclopyr BEE. The bioassay was conducted in a disturbed dry forest near Xmatkuil (Merida, Mexico) during the morning of 14 March, 2010. We applied 200 μl of isolated triclopyr BEE in *n*-hexane (~15 μg) to filter paper (Whatman 1, 2 cm) pinned to a small tree at breast height. In the vicinity (>4 m away), we also exposed two *p*-dimethoxybenzene baits, which were visited by dozens of male *E.* aff. *viridissima* during the same morning. Bees that landed on the triclopyr BEE filter paper were observed briefly to verify volatile collection.

Compound Characterization We used the software Chemstation *vE.02.00* (Agilent Technologies) to register chromatogram peaks and save their corresponding spectra in user-built mass-spectral libraries (Ramírez and Eltz, unpublished) which we use to cross-reference additional chromatograms. Libraries were recursively updated as new compounds were found. We further characterized individual compounds by comparing spectra against several published libraries (Adams, 2001; pal600K, Palisade Corporation, USA). Authentic standards of monoterpene and sesquiterpene compounds (Sigma-Aldrich, St. Louis, MO, USA), injected and analyzed under the same conditions and instrument described above (UC Berkeley), were also used to corroborate compound identities (Table 1). Uncharacterized compounds were named based on retention times and EI-mass spectrum ions (Table 1). Automatic peak integration was conducted using the RTE integrator in the software Chemstation *vE.02.00* (Agilent Technologies) set to a minimum-area detection threshold equivalent to 0.5% of the area of the largest peak.

Statistical Methods We built a square matrix containing the absolute quantities (total ion currents) for each compound, containing all samples from all localities. Total ion currents were transformed to relative amounts (percentage) per individual. We compared individual chemical profiles within and between populations via non-metric Multidimensional Scaling (MDS), an ordination technique where a predetermined number of axes of variation are chosen, and non-metric distances are fitted to those dimensions. Because ordination via MDS makes few assumptions about the nature of the data, any distance measure can be applied. We calculated a triangular distance matrix between samples (individuals) using the Bray-Curtis index of dissimilarity, which has the advantage of being insensitive to compounds jointly absent in sample pairs (i.e., pairwise dissimilarities are fixed). We computed 2- and 3-dimensional MDS plots (50 iterations per run) using the software package *ecodist v1.2.2* (written in R). We ran each analysis 10 times; convergence between solutions was visually inspected. To assess statistically whether orchid bee fragrances exhibit greater dissimilarity between populations than within populations, we conducted an Analysis of Similarity (ANOSIM) test, implemented in the software package *vegan v1.15-4* (written in R). Additional descriptive statistics, tests, and plots were produced using R basic packages, freely available at www.cran.r-project.org. We estimated the relative contribution of individual compounds to the observed ordinal dissimilarities using the Similarity Percentage (SIMPER) method, implemented in the software package *primer v6* (Clarke and Gorley, 2006). All MDS calculations were verified using the software *primer*.

Results

We registered a total of 333 compounds in hind leg extracts of 114 males sampled from seven populations (Fig. 1). By comparing the chemical profiles of hind leg extracts against those from labial glands (Fig. 2) we determined that 41 compounds were produced in the bees' labial glands (endogenous origin), and included straight chain hydrocarbons, acetates, diacetates, and alcohols (Supplementary Material Appendix 2). The remaining 292 compounds are thus exogenous in origin, and included monoterpenes, sequiterpenes, bicyclic sesquiterpenes, triterpenes, and other compounds (Table 1). The per-capita number of exogenous compounds across all populations ranged from 1 to 65 chemicals (average 21.50±12.66) with marginally similar means in all seven populations (ANOVA F=3.2118, P=0.075). On average, male bees from Fern Forest (U.S.) exhibited the lowest number of compounds per capita (15.83±10.08), whereas El Remate (Mexico) exhibited the highest number (29.23±6.24; Fig. 3). When the two U.S. populations were combined, they had significantly fewer compounds per individual (17.46±10.78) than all Mesoamerican populations combined (24.14±13.17; ANOVA F= 8.0419, P=0.005).

Our non-metric Multidimensional Scaling (MDS) analysis included exogenous compounds only. When all volatile compounds in the data matrix were included (coded by relative abundances), a strong clustering by population was found (Fig. 4a). This observation was corroborated by the

Table 1 Most common exogenous fragrance compounds collected by male *Euglossa* aff. *viridissima* ranked by their incidence (%) across populations

Compound name	Compound class	Retention time (min)	Entry #	Incidence (%)	Contribution to dissimilarity (%) USA vs. Mesoamerica[a]
eugenol	phenylpropanoid	23.679	164	79	**8.54**
2-hydroxy-6-nona-1,3-dienyl-benzaldehyde 4 (HNDB4)	aromate	50.698	54	74	**19.04**
caryophyllene	sesquiterpene	26.288	173	68	**2.60**
isolemecin, trans-	NA	35.388	61	50	**2.25**
ocimene, beta-	monoterpene	10.547	199	44	**3.44**
benzyl benzoate	aromate	39.306	218	42	**3.00**
2-hydroxy-6-nona-1,3-dienyl-benzaldehyde 1 (HNDB1)	aromate	45.628	51	41	**1.65**
m/z:55,69,81,95,107,119,135,147,161,175,189,207,218,426	triterpene	80.675	59	40	**1.25**
2-hydroxy-6-nona-1,3-dienyl-benzaldehyde 3 (HNDB3)	aromate	48.890	53	38	**2.27**
m/z:53,65,74,92,120,155	unknown	16.572	373	36	**1.17**
humulene, alpha-	sesquiterpene	27.673	165	36	**1.22**
cadinene, delta-	sesquiterpene	30.465	110	35	0.07
benzyl cinnamate	NA	49.863	208	34	**3.48**
m/z:55,68,81,93,107,121,133,147,161,175,189,204	sesquiterpene	25.149	245	32	**1.26**
pinene, alpha-	monoterpene	6.639	174	28	0.61
m/z:55,69,81,95,107,119,135,147,161,175,189,203,218	unknown	79.337	127	26	0.25
3-cyclohexane-1ol, 4-methyl-1m ethylethyl	monoterpene	15.926	180	25	0.23
2-hydroxy-6-nona-1,3-dienyl-benzaldehyde 2 (HNDB2)	aromate	47.231	52	25	**1.18**
triclopyr 2-butoxyethyl ester	aromate	53.553	76	25	**17.37**
germacrene, D	sesquiterpene	28.794	359	25	0.69
similar to elemicin	aromate	31.781	413	25	0.48
similar to amorphene, alpha-	sesquiterpene	30.105	45	23	0.29
similar to elemicin	unknown	31.753	49	23	0.33
similar to amyrin, alpha-	triterpene	79.800	424	23	0.55
1,4-dimethoxybenzene,	aromate	15.308	179	19	0.14
cubene, beta-	sesquiterpene	28.789	194	19	**1.03**
epizonarene	sesquiterpene	29.504	43	18	0.45
m/z:55,115,127,173,183,201,215,228,244,269,283,293,311,326,344	unknown	64.722	333	18	0.28
cineole, 1,8-	monoterpene	9.906	255	18	0.23
copaene, alpha-	sesquiterpene	24.486	182	18	0.20
methyl ester	NA	36.067	235	18	**1.79**
myrcene, beta-	monoterpene	8.424	254	17	0.25
bisabolene, beta-	sesquiterpene	29.922	378	16	0.82
pinene, beta-	monoterpene	7.989	175	15	0.30
barbatene, beta-	sesquiterpene	27.227	188	15	0.17
m/z:55,67,77,81,91,105,119,133,161,204	sesquiterpene	28.068	193	15	0.13
m/z:65,77,91,115,129,147,175,244,260	unknown	51.766	72	15	0.26
m/z:55,71,99,115,128,141,157,171,186,200,213,228,245,301,326	unknown	62.691	311	15	0.10
m/z:55,69,83,95,108,115,127,145,159,173,183,201,215,225,244,253,285,299,324,342	unknown	66.279	334	15	0.16
similar to amorphene, alpha-	sesquiterpene	28.611	161	14	0.15

[a] Similarity Percentage (SIMPER) was used to calculate the relative contribution of each compound to the observed dissimilarity between US and Mesoamerican populations. Values >1% are indicated in bold

ANOSIM results, which indicated that fragrance dissimilarity within populations was lower than among populations ($R=0.433$, $P<0.001$). We found a similar pattern when we excluded the two compounds with the highest contribution to the dissimilarity between U.S. and Mesoamerica (Fig. 4b; see SIMPER analysis below), namely HNDB4 (one of four stereoisomers) and triclopyr BEE. Likewise, MDS ordinations based only on the 50 compounds with the

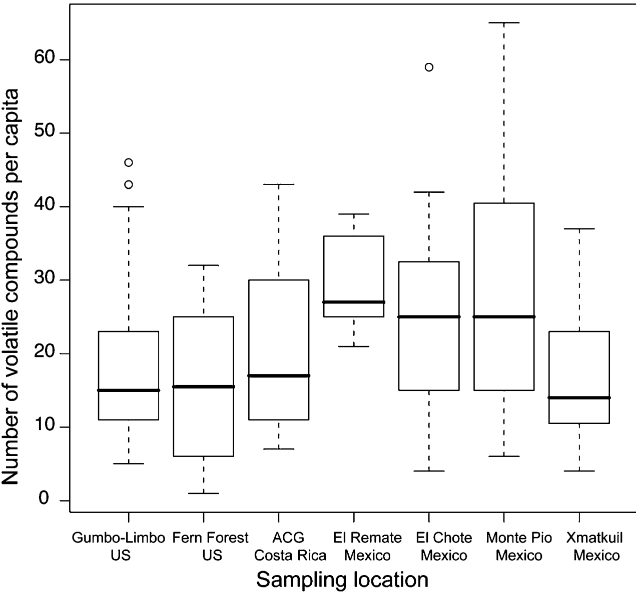


Fig. 3 Boxplots of the number of volatile exogenous compounds per capita in fragrances of male *Euglossa* aff. *viridissima* in native and introduced populations

highest incidence ranks across all populations (Fig. 4c), and the entire dataset transformed into discrete binary (presence/absence) characters (Fig. 4d), also supported a strong differentiation between all populations. In addition, regardless of which data partition we used, we found that introduced (U.S.) and native (Mesoamerica) populations were strongly differentiated (Fig. 4; ANOSIM populations not pooled: R=0.483, P<0.001). To explore the possibility that per-capita compound diversity (number of compounds) affected population differences, we overlaid a bubble-plot on an MDS ordination (based on all fragrance data) with circle diameters proportional to the number of compounds per capita (Fig. 5). Although mean per capita fragrance richness was marginally different among populations (Fig. 3), populations did not appear to cluster based on fragrance diversity alone (Fig. 5).

Our SIMPER analysis revealed that five compounds jointly contributed to >50% of the observed chemical dissimilarity between populations from U.S. and Mesoamerica (Table 1). These included HNDB4 (19.04%), triclopyr BEE (17.37%), eugenol (8.54%), benzyl cinnamate (3.48%), and beta-ocimene (3.41%). With the exception of triclopyr BEE, which was present only in U.S.

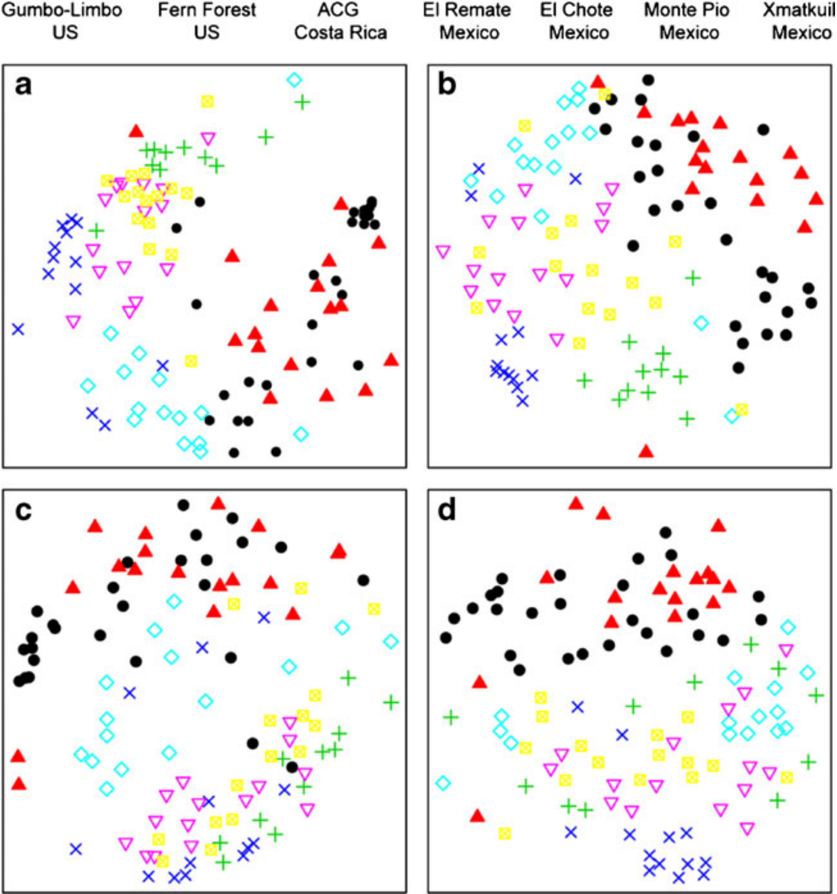


Fig. 4 Non-metric multidimensional scaling (nMDS) plots based on the chemical composition of exogenous compounds present in hind legs of male *Euglossa* aff. *viridissima*. Ordination plots were computed based on **a** all volatile compounds, **b** all volatile compounds except the two most dissimilar between U.S. and Mesoamerican populations (i.e., HNDB4, triclopyr BEE), **c** the 50 compounds with highest incidence across all populations, and **d** all volatile compounds coded as binary characters (presence/absence). Filled circles and triangles correspond to individuals from naturalized populations

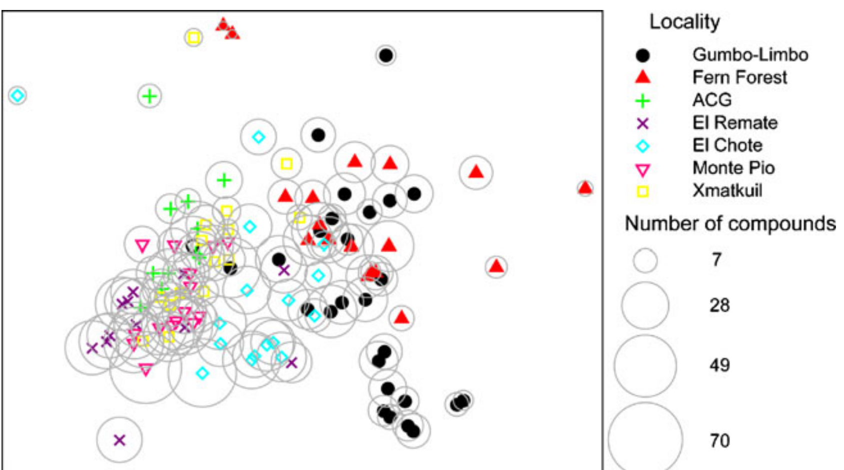


Fig. 5 Bubble plot overlaid on an MDS ordination based on all exogenous fragrance compounds. Circle diameters correspond to the number of volatile exogenous compounds present in the fragrances of each individual. Analyses were performed using the software *primer* (Clarke and Gorley 2006). Filled circles and triangles correspond to individuals from naturalized populations

populations, these compounds exhibited high incidence values across all populations (Table 1). The SIMPER analysis also revealed that of the ten most dissimilar compounds between U.S. and Mesoamerica, eight were present in both population sets (Table 1; Fig. 6 except panels f, j). Of these, six were present in more individuals and had greater relative quantities in Mesoamerican than in U.S. populations (Fig. 6a,c,d,e,g,h). The two remaining compounds (eugenol and caryophyllene) were present in more individuals and had greater relative amounts in U.S. than Mesoamerican populations (Fig. 6b,i). Triclopyr BEE and benzyl benzoate were present only in U.S. and Mesoamerica, respectively (Fig. 6f,j). Triclopyr BEE contributed up to 69% of total fragrance composition in U.S. bees, and it was detected in 63% of individuals (N= 43). Benzyl benzoate contributed up to 17.84% of the fragrance composition in individual bees, and it was detected in 18% of Mesoamerican individuals (N=71).

We also conducted a SIMPER analysis among all seven populations. In 15 of the 21 possible pairwise comparisons, five compounds jointly explained >50% of the observed fragrance dissimilarity (Supplementary Material Appendix 3). In all but two pairwise comparisons, El Remate (Mexico) v. ACG (Costa Rica) and El Remate v. Monte Pio (Mexico), the compounds HNDB4 and eugenol ranked among the top five compounds with highest dissimilarity between populations (Supplementary Material Appendix 3). Sesquiterpene compounds, followed by monoterpenes, contributed the most to the observed dissimilarity between populations (Table 1; Online Appendix 3). Although diverse, triterpenes contributed little or nothing to population structuring.

As described earlier for *E.* aff. *viridissima* (Eltz et al., 2008), four stereoisomers of the compound HNDB were found in male perfumes (Supplementary Material Fig. 1). The compound HNDB4 was by far the most abundant stereoisomer in the majority of populations, except in El Remate (Mexico). The fragrances of all individuals in El Remate had greater relative amounts of the stereoisomers HNDB1, HNDB2, and HNDB3 than all other populations. In some cases, other stereoisomers were more abundant than HNDB4 itself (Supplmentary material Fig. 1). Because our spectral searches against published libraries did not return any significant matches for triclopyr BEE, the identification of this compound was conducted by comparison against analytical standards provided by Dow Agro-Sciences (Indianapolis, IN, US). Both the retention time and EI-spectrum of the analytical standard of triclopyr 2-butoxyethyl ester (triclopyr BEE) perfectly matched peaks in our samples (Fig. 7). Male *E.* aff. *viridissima* were observed displaying fragrance collecting behavior on areas where the herbicide Garlon had been applied in Fern Forest (Pemberton, Pers. Obs.). Triclopyr BEE is one of three available active ingredients present in commercially available herbicides (including Garlon) used for broadleaf weed control. The other active ingredients are triclopyr acid and triclopyr triethylamine salt (TEA).

In our laboratory bioassay, we presented 20 male bees with three hexane leg extracts containing large amounts of triclopyr BEE and trace amounts of other volatile compounds. Within the first two minutes of exposure, six, five, and six individual bees exhibited obvious collecting behavior in the three trials, respectively. Controls (labial lipid extracts) did not elicit fragrance-collecting behavior in any of the trials.

Our bioassay using purified aliquots of triclopyr BEE with native bee populations in the field revealed that male *E.* aff. *viridissima* are attracted to, and collect, this volatile compound. A total of five male *E.* aff. *viridissima* approached the filter paper with triclopyr BEE, landed on it, and performed typical collection behavior. Other males circled the filter paper but did not land on it.

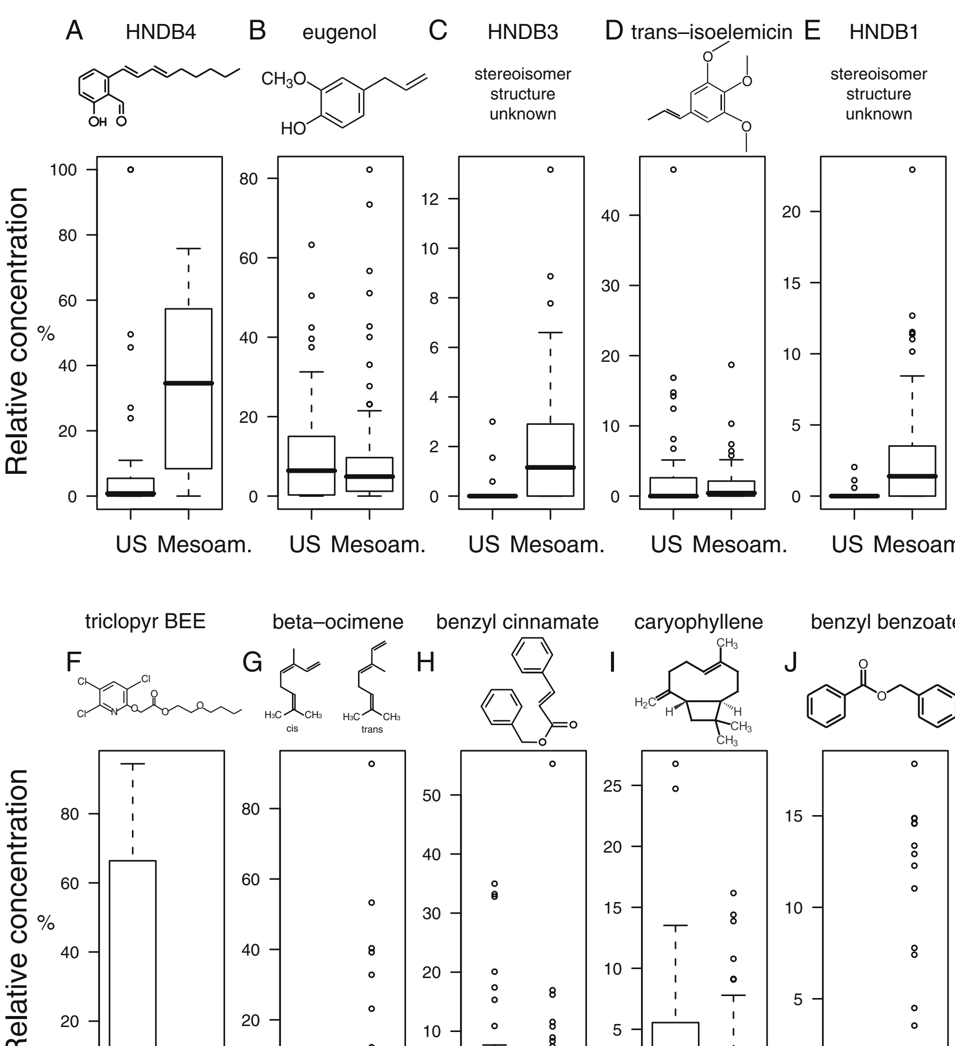


Fig. 6 Boxplots of per-capita relative concentration (relative amount of each compound over all compounds) of ten chemicals detected in bees sampled from native (Mesoamerica) and naturalized (U.S.) populations. These ten compounds had the greatest contributions to dissimilarity between native and naturalized populations

Discussion

Euglossine bees acquire species-specific fragrance bouquets from a great diversity of floral and non-floral sources (Williams, 1982; Whitten et al., 1993; Ramírez et al., 2002; Pemberton and Wheeler, 2006; Zimmermann et al., 2009a). As a result, individual perfume phenotypes may not be attributable to single sources, but instead, emerge from multiple visits to numerous hosts (Eltz et al., 2005a; Pemberton and Wheeler, 2006). Although sympatric species of orchid bees, and particularly closely related lineages (Eltz et al., 1999; Zimmermann et al., 2006, 2009a), exhibit divergent perfume phenotypes, it has been unclear to what extent fragrances vary across geographic regions within lineages. Our results indicate that male *E.* aff. *viridissima* maintain most of the individual compounds of their fragrance phenotypes across distant populations in disparate habitats, but a few major (abundant) compounds can be present or absent from perfume bouquets. Moreover, since populations of *E.* aff. *viridissima* in Florida have been stable for at least seven years, our data suggest that the observed phenotypic changes have negligible effects on population viability. This observation is congruent with the previous report that male *Euglossa tridentata* and *E. erythrochlora* sampled in Isla del Caño, an island 17 km off the coast of Costa Rica, collect substantially fewer compounds per capita than in mainland populations (Eltz et al., 2005a, Pers. Obs.).

The remarkable qualitative consistency we found in fragrance phenotypes is at odds with the considerable habitat variability among the seven sites sampled, but supports the hypothesis that orchid bee perfume preferences are under strong selection (Zimmermann et al., 2009a). The differences in perfume composition that we observed between native and naturalized populations could be

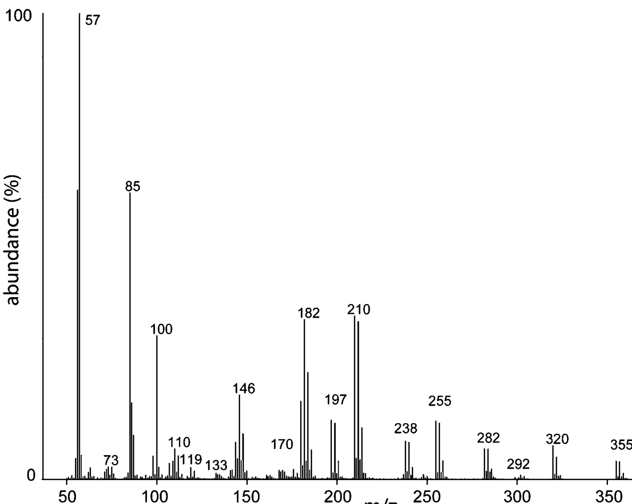
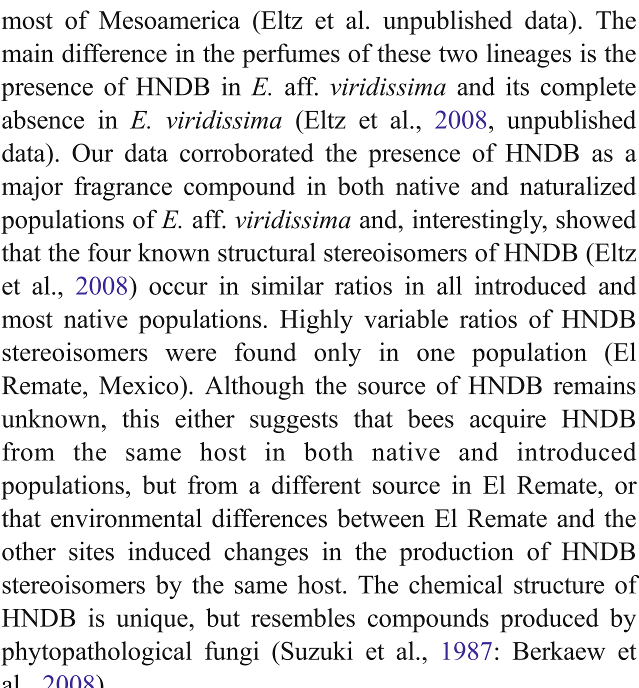


Fig. 7 Mass spectrum of triclopyr 2-butoxyethyl ester, a prevalent compound found in leg extracts of males from naturalized populations of *Euglossa* aff. *viridissima* in Florida (U.S.) and absent in native (Mesoamerican) populations

attributed to environmental factors, to differences in chemical preference between populations, or to both. Several factors may influence intraspecific fragrance variation. First, species turnover (beta diversity) of fragrance hosts across habitats may force phenotypic changes via chemical abundance and availability. In fact, a recent survey of volatile compounds present in resins of Amazonian trees revealed not only a vast diversity of monoterpenes and sesquiterpenes, but also pronounced chemical turnover across tree species from different habitats (Courtois et al., 2009). Second, strong female preference for certain fragrance phenotypes may restrict the amount of perfume phenotypic plasticity. Although direct evidence for female choice of fragrance phenotypes is still missing for orchid bees, perfume phenotypes are likely to be under strong sexual selection (Zimmermann et al., 2009a, b). Hence, it is also conceivable that, at evolutionary timescales, new male fragrance phenotypes may emerge through novel female preferences for perfumes that indicate mate genotypic quality or simply exploit a sensory bias (Andersson, 1994).

Genetic data indicate that naturalized populations of *E.* aff. *viridissima* have gone through a severe population bottleneck (Zimmermann et al. unpublished data), and therefore it is possible that population differences in perfume phenotypes have an underlying genetic basis. Rapid evolutionary change in pheromone phenotypes has been demonstrated in several species of insects (Lofstedt, 1993; Takanashi et al., 2005; Groot et al., 2009), and could be tested in orchid bees via common garden experiments.

The orchid bee lineage *E.* aff. *viridissima* is broadly sympatric with its sister taxon (*E. viridissima*) throughout



most of Mesoamerica (Eltz et al. unpublished data). The main difference in the perfumes of these two lineages is the presence of HNDB in *E.* aff. *viridissima* and its complete absence in *E. viridissima* (Eltz et al., 2008, unpublished data). Our data corroborated the presence of HNDB as a major fragrance compound in both native and naturalized populations of *E.* aff. *viridissima* and, interestingly, showed that the four known structural stereoisomers of HNDB (Eltz et al., 2008) occur in similar ratios in all introduced and most native populations. Highly variable ratios of HNDB stereoisomers were found only in one population (El Remate, Mexico). Although the source of HNDB remains unknown, this either suggests that bees acquire HNDB from the same host in both native and introduced populations, but from a different source in El Remate, or that environmental differences between El Remate and the other sites induced changes in the production of HNDB stereoisomers by the same host. The chemical structure of HNDB is unique, but resembles compounds produced by phytopathological fungi (Suzuki et al., 1987: Berkaew et al., 2008).

The presence of large quantities of triclopyr BEE in leg extracts of naturalized bee populations, together with our field and laboratory bioassays, demonstrates that male bees are attracted to and actively collect this herbicide active ingredient. Indeed, previous studies have shown that males of the orchid bee *Eufriesea purpurata* from Brazil collect large quantities of the synthetic pesticide DDT (Roberts et al., 1982; Walter and Roberts, 2007). Although we did not detect triclopyr BEE in leg extracts from native populations, male bees from Mesoamerica clearly were attracted to this compound. This result suggests that a pre-existing sensory bias, rather than rapid adaptation among introduced populations, may explain the collection of organohalogen synthetic chemicals by male orchid bees. Both behavioral and neurophysiological approaches are needed to elucidate the sensory basis of this unusual behavior.

Our results illustrate that male *E.* aff. *viridissima* collect a broad diversity of molecules in native and introduced populations. As shown previously for orchid bees (e.g., Williams and Whitten, 1983; Eltz et al., 1999, 2005a; Zimmermann et al., 2009a), fragrance bouquets were composed mainly of monoterpenes, bicyclic sesquiterpenes, and aromates. We found that perfume compounds that differed the most in abundance among populations also tended to be common to all populations. This was the case for several monoterpene, sesquiterpene, and aromate compounds. For example, whereas eugenol and HNDB were present in >73% of the sampled individuals and contributed significantly to population dissimilarity, most rare compounds contributed little to population dissimilarity. We confirmed this pattern by plotting the contribution to the

dissimilarity between U.S. and Mesoamerica as a function of compound average abundance (data not shown).

Despite contributing little to overall population differences, rare compounds were diverse in our dataset. For instance, we detected numerous triterpenes in fragrances of *E.* aff. *viridissima*, but most of them were present in few individuals and in low concentrations. Because triterpenes exhibit high molecular weights (>400), and thus may not volatilize at ambient temperature, they may constitute poor airborne signaling molecules. We speculate that orchid bees actually collect triterpenes accidentally since they are common components of tree resins (e.g., Burseraceae), which are known to contain numerous volatile compounds (De La Cruz-Cañizares et al., 2005; Courtois et al., 2009). Hence, most of the compounds that were abundant in our dataset, such as sesquiterpenes, monoterpenes, and aromates, contributed the most to population differences, and also are likely to constitute the main courtship signaling molecules in orchid bees.

In summary, the preference for most fragrance compounds acquired by orchid bees appears to be under strong selection. Evidence for this comes from the fact that male bees managed to collect similar compounds across disparate habitats ranging from tropical rain forests to tropical dry forests, to mangroves, to disturbed habitats. However, we also found evidence for pronounced quantitative and qualitative changes across geographic (and possibly genetic) gradients. Previous studies have suggested that saltational changes in fragrance preferences may constitute an important mechanism of evolution of perfume phenotypes as well as speciation in orchid bees (Eltz et al., 2008; Zimmermann et al., 2009a). Because mutations that affect the olfactory system of orchid bees can induce the acquisition or loss of specific chemicals, perfume phenotypes have the potential to evolve fast enough to facilitate lineage differentiation (Eltz et al., 2008; Jackson, 2008; Zimmermann et al., 2009a). Our analysis highlights the opportunistic capacity that orchid bees have to incorporate novel compounds in their perfumes, as illustrated by the gain and loss of several compounds in naturalized populations. Whether population-level changes in perfume phenotypes have underlying genetic components, and are thus subject to selection, should be a fruitful area of future research.

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Survey of a Salivary Effector in Caterpillars: Glucose Oxidase Variation and Correlation with Host Range

Herb Eichenseer · M. C. Mathews · Jaimie S. Powell · Gary W. Felton

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Abstract Salivary glucose oxidase (GOX) has been reported in a few insect species where it plays a role in protection against infectious disease. Our recent research has focused on the role of this salivary enzyme in the noctuid *Helicoverpa zea*, where it functions as an effector to suppress the induced defenses of the host plant *Nicotiana tabacum*. In this study, we examined the labial gland GOX activities in 23 families of Lepidoptera (85 species) and two families of plant-feeding Hymenoptera (three species). We analyzed the relationship between host breadth and GOX activities, and we found a significant relationship, where highly polyphagous species were more likely to possess relatively high levels of GOX compared to species with more limited host range. We also examined the effect of diet on GOX activity and found that the host plant had a significant effect on enzyme activity. The significance of these findings is discussed in relation to caterpillar host breadth.

Key Words Elicitor · Effector · Saliva · Labial gland · Silk gland · Caterpillar · Lepidoptera · Hymenoptera · *Helicoverpa zea* · Heliothines · Induced resistance · Plant defense · Herbivore offense

H. Eichenseer
Pioneeer Hi-Bred International Crop Genetics
Research and Development,
Johnston, IA 50131, USA

M. C. Mathews
Department of Entomology, University of Arkansas,
321 Agriculture Building,
Fayetteville, AR 72701, USA

J. S. Powell
Department of Biology, Portland State University,
Portland, OR 97202, USA

G. W. Felton (✉)
Department of Entomology, Center for Chemical Ecology,
Penn State University,
University Park, PA 16802, USA
e-mail: gwf10@psu.edu

Introduction

Caterpillar saliva and regurgitant (=oral secretions) contain chemical cues that may be recognized by plants, thus providing the basis for eliciting unique plant responses to herbivory (Felton and Eichenseer, 1999; Musser et al., 2002; Felton, 2008). Whereas regurgitant arises from the digestive system, caterpillars secrete saliva from their labial and mandibular glands (Felton and Eichenseer, 1999; Felton, 2008; Peiffer and Felton, 2009). Saliva is purported to aid in antimicrobial defense (Liu et al., 2004; Musser et al., 2005b), digestion (Lenz et al., 1991), detoxification (Ahmad and Hopkins, 1993; Mathews et al., 1997), and suppression of host plant defenses (Musser et al., 2002, 2005a). Additional functions may be revealed when proteomic and transcriptomic studies are conducted with lepidopteran salivary glands. This approach has proved successful in identifying key components of saliva from other arthropods (Valenzuela et al., 2003; Harmel et al., 2008).

We reported a salivary glucose oxidase (GOX) from the labial and mandibular glands of *Helicoverpa zea* (Eichenseer et al., 1999), and proposed several functions for the enzyme including mediation of plant defenses. In some cases, GOX may contribute to the initial oxidative burst of H_2O_2 observed in leaves damaged by herbivores

(Bi and Felton, 1995; Bi et al., 1997a; Orozco-Cardenas and Ryan, 1999; Musser et al., 2006). GOX belongs to the family of GMC-oxidoreductases (Iida et al., 2007) and catalyzes the following reaction:

$$\mathrm{D-glucose} + O_2 \rightarrow H_2O_2 + \mathrm{D-gluconic\ acid}.$$

The rapid increase in reactive oxygen species, such as H_2O_2, serves as an initial signal that leads to the production of proteins and other natural products that are involved in induced resistance to herbivores and/or pathogens (Orozco-Cardenas et al., 2001).

GOX has been reported sporadically in other insect species including honey bees (Iida et al., 2007). The highest GOX activity in *H. zea* was found in labial glands of actively feeding larvae (1.2 μmol/min/mg protein or 2.1 nmol/min/labial gland) (Eichenseer et al., 1999). This is among the highest activities recorded for any insect, although only a few caterpillar species (Zong and Wang, 2004; Merkx-Jacques and Bede, 2005; Bede et al., 2006; Babic et al., 2008; Yong-Hong et al., 2008), honey bees (Yang and Cox-Foster, 2005), and one grasshopper species have been assayed for GOX activity (Candy, 1979). While GOX is commonly thought of as an enzyme produced by fungi (Wong et al., 2008), these findings suggest that caterpillars are a rich and novel source of GOX. We currently, however, do not have any evidence for how widespread the enzyme is among Lepidoptera.

Following our early report of GOX in caterpillar saliva (Eichenseer et al., 1999), subsequent studies have revealed that caterpillar saliva functions in suppressing both direct and indirect plant defenses (Musser et al., 2002, 2005a; Zong and Wang, 2004; Bede et al., 2006; Delphia et al., 2006; Weech et al., 2008; Diezel et al., 2009). In several cases, GOX is implicated as the primary salivary effector responsible for suppression (Musser et al., 2002; Zong and Wang, 2004; Bede et al., 2006). GOX may suppress defensive responses by eliciting a salicylic acid burst, which then attenuates jasmonate (JA) and ethylene levels (Felton and Eichenseer, 1999; Musser et al., 2005a; Diezel et al., 2009). The accumulation of salicylic acid may antagonize JA-mediated herbivore defenses in some instances (Bi et al., 1997c; Felton et al., 1999; Stout et al., 1999). JA and/or ethylene (Howe and Jander, 2008; Ankala et al., 2009) regulate many of the induced defenses targeted against chewing herbivores. Moreover, GOX may mask the eliciting action of fatty acid conjugates, such as volicitin on JA and ethylene signaling (Diezel et al., 2009).

To date, GOX suppression of host defenses has been reported from generalist or polyphagous caterpillars—those species feeding on host plants from multiple plant families. We hypothesize that the high expression of salivary GOX may be a strategy used by polyphagous species to evade the inducible plant defenses of their hosts. Due to the potentially broad importance of this enzyme to caterpillar fitness, we conducted this study to: 1) survey for GOX among different caterpillar species; 2) determine if GOX activity is associated with any particular life history traits such as host breadth; and 3) explore how the diet contributes to variation in GOX activities.

Methods and Materials

Source of Insects and Dissection Caterpillars were collected from various host plants in Fayetteville, AR, USA and surrounding areas, or were provided to us from various sources. Table 1 indicates whether insects were from laboratory colonies or were collected from a particular host plant species. Caterpillars were reared on plant material or artificial diet until the last instar, or when were large enough to dissect easily. Some caterpillars were reared from eggs. In all cases, caterpillars were kept at 25°C with a 16:8 h L:D photoperiod. Prior to dissection, healthy, actively feeding caterpillars were anesthetized by crushed ice and water. Entire labial glands were removed from dissected caterpillars with forceps and placed into 1.5 ml microcentrifuge tubes containing phosphate-buffered saline (pH 7.0, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM $Na_2HPO_4 \times 7H_2O$) and 1 μM phenylmethylsulfonylfluoride (PMSF) protease inhibitor, and were frozen at −20°C for no more than 3 mo. Previous results with *H. zea* labial glands indicated no significant loss in activity occurred within this period. Depending on the size of the species, samples contained from one to 20 pairs of labial glands from actively feeding larvae, indicated by midguts full of food. Voucher specimens were retained for identification and deposited in the University of Arkansas Arthropod Museum. Unidentifiable larvae were reared to adulthood and then identified. We consulted multiple sources for larval and adult identification (Peterson, 1962; Covell, 1984; Scott, 1986; Stehr, 1987; Wagner, 2005; Powell and Opler, 2009). These references also were consulted to determine diet breadths. For ease of reference, we use the nomenclature of Stehr (1987). In the cases where we had significant representation of species from a given super-family (i.e., Noctuiodea and Papillionoidea), distribution of GOX activities was presented graphically to illustrate relationships to phylogeny.

Artificial Diet A wheat germ meridic diet (Broadway and Duffey, 1986) was used for all experiments not utilizing foliage. Diet ingredients were purchased from BioServ (Frenchtown, NJ, USA). Soy protein concentrate was the source of protein. This regular feeding diet contained 2.1%

Table 1 Glucose oxidase activities in the labial glands of collected caterpillars

Species	Mean nmol/min/mg	Mean nmol/min/gland	Median nmol/min/mg	Diet/plant
Incurvariidae				
Tegeticula yuccasella (Riley)	100.2	1.3		*Yucca* sp. fruit
Psychidae				
Thyridopteryx ephemeraeformis (Haworth)	1.6±1.1	0.1±0.1	1.2	Dwarf *Juniperus* sp.
Oecophoridae				
Depressaria pastinacella (Duponchel)	36.7±11.1	2.8±0.5	37.3	Artificial diet
Plutellidae				
Homadaula anisocentra Meyrick	26.1±8.5	0.1	26.1	*Mimosa strigullosa*
Yponomeutidae				
Atteva punctella (Cramer)	ND[a]	ND	ND	*Alianthus altissima*
Tortricidae				
Ancylis plantana Clemens	95.6±60.5	0.9±0.5	95.6	*Platanus occidentalis*
Cydia pomonella (L.)	19.9±6.6	0.3±0.1	16.5	*Malus* fruit
Hesperiidae				
Epargyreus clarus (Cramer)	17.2±5.1	0.6±0.2	18.7	*Pueraria lobata*
Papilionidae				
Papilio troilus L.	29.9±15.1	0.8±0.4	41.0	*Sassafrass albidium*
Papilio glaucus L.	9.1±1.8	0.7±0.2	9.6	*Prunus serotina/Fraxinus sp.*
Eurytides marcellus (Cramer)	1.3±1.3	0.0±0.0	0.0	*Asimina triloba*
Papilio polyxenes Stoll	ND	ND	ND	*Foeniculum vulgare*
Pieridae				
Colias eurytheme Boisduval	59.4±32.8	1.0±0.6	58.0	*Chamaecrista fasciculata*
Phoebis agarithe Boisduval	52.2±34.9	1.2±0.9	25.4	*Chamaecrista fasciculata*
Pontia protodice (Boisduval and LeConte)	46.7±40.7	0.9±0.8	9.4	*Brassica juncea*
Pieris rapae (L.)	35.1±13.4	0.2±0.1	23.3	*Brasscia oleracea*
Nymphalidae				
Polygonia interragationis (F.)	271.4±26.8	2.7±0.9	303.9	*Ulmus alata*
Asterocampa clyton (Boisduval and LeConte)	51.0±16.2	1.1±0.4	40.6	*Celtis occientalis*
Chlosyne nycteis (Doubleday and Hewitson)	42.8±25.4	0.2±0.1	40.4	*Verbesina sp.*
Precis coenia (Hübner)	26.9±15.1	0.4±0.3	18.7	*Plantago lanceolata*
Eupoieta claudia (Cramer)	11.6±11.2	0.3±0.3	11.6	*Passiflora lutea*
Vanessa carduii (L.)	6.3±2.7	0.3±0.1	8.9	*Artificial diet*
Danaus plexippus (L.)	4.4±3.1	0.1±0.1	2.2	*Asclepias incarnata*
Zygaenidae				
Harrisina americana (Guerin)	2.9±2.9	0.02±0.02	2.9	*Parthenocissus quinquefolia*
Megalopygidae				
Norape ovina (Sepp)	8.2±6.8	0.2±0.2	2.9	*Cercis canadensis*
Pyralidae				
Tetralopha expandens (Walker)	62.9±35.5	0.7±0.4	29.6	*Quercus/Carya*
Saucrobotys futilialis (Lederer)	27.6±14.9	2.4±1.2	31.6	*Apocynum cannabinum*
Desmia funeralis (Hübner)	25.5±23.5	0.3±0.3	4.1	*Amelopsis cordata*
Cadra cautella (Walker)	9.4±9.4	0.1±0.1	9.4	*Artificial diet*
Galleria mellonella (L.)	1.3±0.2	0.2±0.1	1.5	*Apis mellifera comb*
Diaphania nitidalis (Stoll)	0.9±0.9	0.0	0.0	*Artificial diet*
Thyatiridae				
Euthyatira pubens (Guenée)	114.7	2.3		*Quercus/Carya*

Table 1 (continued)

Species	Mean nmol/min/mg	Mean nmol/min/gland	Median nmol/min/mg	Diet/plant
Apatelodidae				
Apatalodes torrefacta (J.E. Smith)	378.3±195.7	21.4±13.7	378.4	*Fraxinus sp.*
Lasiocampidae				
Malacosoma americanum (F.)	8.0±5.8	0.1±0.1	3.3	*Prunus serotina*
Malacosoma disstria Hübner	ND	ND	ND	*Quercus palustris*
Saturniidae				
Actias luna (L.)	133.1±45.2	7.8±2.0	121.8	*Liquidambar styraciflua*
Anisota stigma (F.)	58.4±25.9	4.0±1.8	84.0	*Quercus alba*
Sphingidae				
Ceratomia catalpae (Boisduval)	1858.1±111.8	66.8±48.4	1802.1	*Catalpa bignonioides*
Hyles lineata (F.)	197.9±117.6	0.7±0.3	160.6	*Malus sp.*
Manduca sexta (L.)	101.2±19.7	22.8±6.4	100.8	*Physalis heterophylla*
Manduca sexta (L.)(source = Carolina)	0.2±0.1	0.4±0.3		Artificial diet
Smerinthus jamaicenis (Drury)	12.8	3.6		*Salix nigra*
Sphinx chersis (Hübner)	2.5±1.4	0.2±0.1	3.0	*Fraxinus sp.*
Notodontidae				
Nerice bidentata (Walker)	728.9±451.8	29.5±22.2	728.9	*Ulmus sp.*
Heterocampa biundata Walker	326.3	12.2		*Diospyros virginiana*
Clostera inclusa (Hübner)	306.1±112.8	37.8±10.3	*238.0*	*Salix nigra*
Lochmaeus manteo Doubleday	292.9±64.2	10.7±4.1	324.9	*Quercus alba*
Misogada unicolor (Packard)	194.2	134.7		*Platanus occidentalis*
Lochmaeus bilineata (Packard)	188.6±118.4	15.5±5.8	72.7	*Ulmus sp.*
Datana integerimma (Grote and Robinson)	166.3±20.0	15.5±1.9	174.8	*Juglans nigra*
Datana ministra (Drury)	122.7±20.3	17.6±2.5	123.1	*Quercus marilandica*
Schizura unicornis (J.E. Smith)	107.0±53.9	22.2±8.0	85.8	*Salix nigra*
Cerura scitiscripta Walker	66.0±19.4	20.5±16.9	66.1	Salix nigra
Arctiidae				
Halysidota tessellaris (J.E. Smith)	847.9±362.2	26.0±15.7	586.4	*Quercus/Carya*
Grammia arge (Drury)	105.2			Artificial diet
Spilosoma virginiana (F.)	37.9±32.9	1.2±0.5	37.9	*Physalis angulata*
Estigmene acrea (Drury)	18.7			Artificial diet
Hyphantria cunea (Drury)	16.8±8.8	0.2±0.1	40.1	*Juglans nigra*
Halysidota harrissi Walsh	1.3±1.3	0.1±0.1	0.0	*Platanus occientalis*
Grammia geneura Strecker	0.6±0.6	0.4±0.01	0.0	Artificial diet
Lymantriidae				
Orygia leucostigma (J.E. Smith)	57.2±13.9	0.7±0.1	48.6	Artificial diet
Lymantria dispar (L.)	46.7±22.2	5.8±3.1	62.5	Artificial diet
Lymantria monacha L.	77.0±33.4	4.0±1.6	68.2	Artificial diet
Nolidae				
Nola sorghiella (Riley)	14.8±14.8	0.0±0.0	0.0	*Sorghum bicolor*
Noctuidae				
Hypeninae				
Plathypena scabra (F.)	14.9±5.3	0.1±0.06	17.2	*Glycine max*
Acronictinae				
Acronicta americana (Harris)	221.7±131.3	95.0±93.0	221.7	*Cercis canadensis*
Acronicta oblinita (J.E. Smith)	36.4±6.2	5.8±1.7	33.6	*Salix nigra*

Table 1 (continued)

Species	Mean nmol/min/mg	Mean nmol/min/gland	Median nmol/min/mg	Diet/plant
Agaristinae				
Psychomorpha epimenis (Drury)	713.8±252.2	43.8±26.4	795.7	*Vitis* sp.
Eudryas unio (Hübner)	535.5	15.2		NA[b]
Heliothinae				
Helicoverpa zea (Boddie)	2680.6±678.0	62.3±10.0	2331.4	Artificial diet
Heliothis virescens (F.)	599.9±92.6	12.4±2.0	581.2	Artificial diet
Heliothis subflexa Guenée	231.4±54.6	4.8±1.2	258.0	Artificial diet
Plusinae				
Psuedoplusia includens (Walker)	74.8±26.7	1.4±0.6	55.2	Artificial diet
Trichoplusia ni (Hübner)	73.3±23.6	1.7±0.4	79.6	Artificial diet
Hadeninae				
Orthosia hibisci (Gueneé)	84.0±14.6	0.6±0.3	69.8	*Prunus serotina*
Psuedaletia unipuncta (Haworth)	6.0±2.2	0.4±0.2	7.1	Artificial diet
Noctuinae				
Xestia c-nigrum (L.)	386.9±349.7	7.2±6.7	736.6	*Taraxicum officiniale*
Choephora fungorum Grote and Robinson	196.6±36.1	6.0±1.1	133.2	Artifical diet
Peridroma saucia (Hübner)	149.7	7.6		NR
Agrotis ipsilon (Hufnagel)	26.2±12.8	0.7±0.4	23.7	Artificial diet
Macronoctua onusta Grote	25.2±12.8	0.4±0.2	20.0	*Daucus carota* root
Agrotis gladiaria Morrison	ND	ND	ND	NR
Amphipyrinae				
Spodoptera ornithogalli (Guenée)	373.8±136.3	5.3±1.9	293.2	*Nicotiana /Lycopersicon*
Spodoptera frugiperda (J.E. Smith)	245.9±74.3	6.6±2.1	192.6	*Zea mays*
Spodoptera exigua (Hübner)	90.5±25.2	1.6±0.5	110.2	Artificial diet
Amphipyra pyrimidoides (Guenée)	42.4	4.7		*Parthenocissus quinquefolia*
Catacolinae				
Anticarsia gemmatalis Hübner	150.6±28.4	4.7±0.3	176.2	*Glycine max*
Anomis erosa Hübner	14.5±7.1	0.5±0.4	6.5	*Arbutilon theophrasti*
Hymenoptera-				
Tenthridinidae				
Nematus ventralis Say	3.2±3.2	0.1±0.1		*Salix nigra*
Caliroa quercuscoccinae (Dyar)	ND	ND	ND	*Quercus palustris*
Cimbicidae				
Cimbex americana Leach	35.0±11.6	5.6±1.8		*Betula* sp.

[a] ND, no detectable glucose oxidase activity in sample

[b] NR, host plant/diet not recorded

dextrose (D-glucose) on a wet weight basis and 36% D-glucose on a dry weight basis. In one experiment, L-glucose was substituted for D-glucose, and in another experiment sucrose or amylose was added in place of dextrose. In both these experiments, three samples of 5 pairs of glands from 2-d-old fifth instars were collected.

Enzyme Assay and Protein Determinations We measured GOX activities as described (Eichenseer et al., 1999). The assay measures the rate of dianisidine oxidation by H_2O_2 produced by GOX. Oxidized dianisidine was measured with a SLM Aminco (Rochester, NY, USA) 3000 diode array spectrophotometer with rate analysis software at 460 nm, using an extinction coefficient of 8.3 $mM^{-1} cm^{-1}$ for 3 min. Ten µl of labial gland homogenate were added to a reaction mixture containing 0.21 mM dianisidine-HCl in 0.1 M phosphate buffer at pH 7.0 (vigorously stirred before adding to cuvettes), 0.14 mmol D-glucose, and 21 U

horseradish peroxidase to make a final volume of 1.56 ml. The amount of protein in each sample was determined by using bovine serum albumin as a standard (Bradford, 1976). Three GOX and protein determinations were made for each sample. By storing a known number of labial glands in a constant volume of saline, we could calculate the relative GOX activity from specific activities. A minimum of three samples containing glands were tested for each species unless otherwise noted.

Glucose Oxidase and Host Specialization To determine if there was a relationship between host specialization and GOX activities, we grouped species into six median GOX activity categories (0–10, 11–50, 51–99, 100–200, 201–400, and >400 nmol/min/mg protein). We tested if there was a significant trend of GOX activity with polyphagous caterpillars (defined here as greater than 10 recorded host genera in more than three plant families) to have higher GOX than oligophagous caterpillars (recorded hosts from one or two host plant families or a narrow range of plant genera) using the Wilcoxon rank sum test.

Diet and Variation in Glucose Oxidase Activities Foliage for experiments with *H. zea*, *H. virescens*, and *M. sexta* were cut from plants grown in pots filled with Terra-Lite®Redi-Earth®Peat-Lite® Mix (Scotts-Sierra Horticultural Products, Marysville, OH, USA) in a greenhouse with temperatures set at 27°C (range 23°–31°), under high pressure sodium lights (100 W) and a 15-h photophase. Pots were watered regularly and supplied with Peters (Scotts-Sierra Horticultural products) fertilizer (N-P-K = 20:20:20) weekly. Pre-flowering plants were selected for all assays. For tobacco plants, leaves were collected from plants between the rosette and bolting stage but before plants produced flowers. In each experiment below, at least three samples of 5 larvae were collected unless noted otherwise.

Neonate *H. zea* larvae were placed on foliage of ‘Hutcheson’ soybeans (*Glycine max*), ‘DeltaPine 50’ cotton (*Gossypium hirsutum*, Delta Pine and Land, Scott, MS), ‘Ozark Pink’ tomato (*Lycopersicum esculentum*), Carolina geranium (*Geranium carolinianum*) grown from field-collected seeds and the soy-protein based artificial diet. Larvae reared on the foliage were placed into 30 ml plastic cups filled with approximately 5 ml solidified agar to provide moisture. Fresh leaves/leaflets were provided every other day. The date when the larva molted to the last instar was recorded and one day later, the glands were dissected and stored for subsequent GOX assays.

For *Manduca sexta* larvae, two populations were assayed: a colony from Carolina Biological Supply (Burlington, NC, USA) and larvae originating from adults reared and collected near Fayetteville, AR, USA. The larvae originating from Carolina Biological were reared on the soy-protein based diet, ‘Ozark Pink’ tomato leaflets, ‘Xanthi-nc’ tobacco (*Nicotiana tabacum*) and *Nicotiana attenuata* grown from seeds collected near Flournoy, CA, USA. Larvae collected from the Arkansas population were reared on ‘Ozark Pink’ tomato foliage, ‘Xanthi-nc’ tobacco, and *Physalis heterophylla* (grown from field-collected seeds) foliage. All caterpillars were reared on their foliage treatments from neonates and dissected as actively feeding fifth instars, 2–3 d after molting. Each of 5 samples per diet treatment consisted of one pair of glands.

For *H. virescens*, larvae were obtained from the University of Arkansas insect rearing facility and reared on the soy protein-based artificial diet, ‘Xanthi-nc’ tobacco leaves, and a transgenic tobacco expressing a bacterial salicylate hydroxylase gene, *nahG*. The hydroxylase metabolizes salicylic acid and compromises the systemic acquired resistance response to various phytopathogens (Felton et al., 1999). More details on the tobacco plants selected are described elsewhere (Bi et al., 1997b; Felton et al., 1999). Two-day old fifth instars were dissected for labial glands.

An outbreak of fall webworm, *Hyphantria cunea*, in Fayetteville, AR enabled us to test how different tree species could affect caterpillar GOX activities. Larvae were collected from webs that were defoliating mulberry, *Morus alba* (Moracaeae), sweetgum, *Liquidambar styraciflua* (Hammalidaceae), persimmon, *Diospyros virginiana* (Ebenaceae), and black walnut, *Juglans nigra* (Juglandaceae) trees. Three samples containing glands from 5 to 7 larvae were collected from one web nest collected off 3 (2 for sweetgum) separate trees. The larvae were all actively feeding (midguts full with leaf material) late instars. Insects were dissected no more than 5 d after collection.

Summary statistics, one-way, two-way analyses of variance, and non-parametric statistics were calculated using JMP 4.0 software (SAS institute, Cary, NC, USA). To understand sources of variation of GOX activities in *H. cunea,* the main effects of tree species, nest, sample, and their interactions were tested with two-way analysis of variance. Significant differences between tree species were tested with Tukey’s HSD test. The remaining experiments designed to understand how diet influences caterpillar GOX activities were tested by one-way analysis of variance, with diet and sample tested as significant sources of variation. If diet was a significant source of variation, Tukey’s HSD test was used to separate means between the dietary treatments.

Results

Glucose Oxidase Variation Among Caterpillar Species Eighty-five species of Lepidoptera in 23 families were examined for GOX activity, as well as three species of

phytophagous Hymenoptera (Table 1). Most caterpillars were leaf feeders, with a few species representing more specialized feeding habits (e.g., *Galleria, Macronoctua, Cadra*). Differences between families were apparent, especially and even between some subfamilies of Noctuidae (Fig. 1). For example, all the Notodontids assayed had detectable GOX activities compared to the variation detected in some of the Noctuid subfamilies (e.g., compare Noctuinae with Heliothinae). With the exception of *P. interragationis*, the Papillionoidea tended to have low GOX activities (Fig. 2). Little activity was found in the labial glands of phytophagous Hymenopterans (Table 1).

GOX activities were distributed unevenly among species of caterpillars (Fig. 3). More than half of the species surveyed possessed GOX activities of less than 50 nmol/min/mg, while 18% had high GOX activities greater than 200 nmol/min/mg, and the activities of the remaining 29% ranged between 51 and 200 nmol/min/mg (Fig. 3).

There was a statistically significant trend for polyphagous caterpillars to have higher GOX than oligophagous

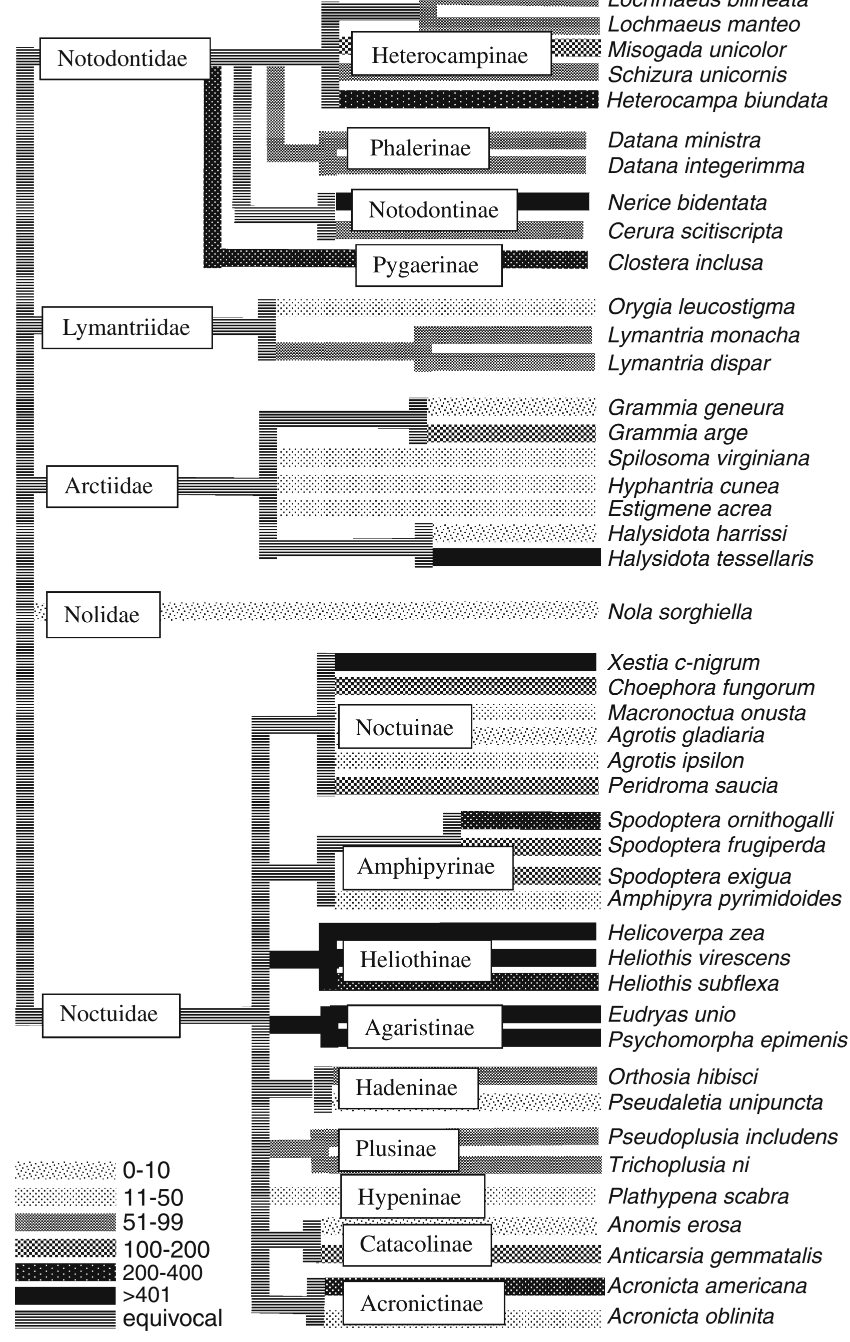


Fig. 1 Distribution of median labial gland glucose oxidase activities based on Noctuoidea phylogeny. Different line patterns represent classes of GOX activities delineated in Fig. 3. Equivocal data indicates uncertainty about the ancestral state of glucose oxidase activity for each taxon. Families and subfamily names are labeled

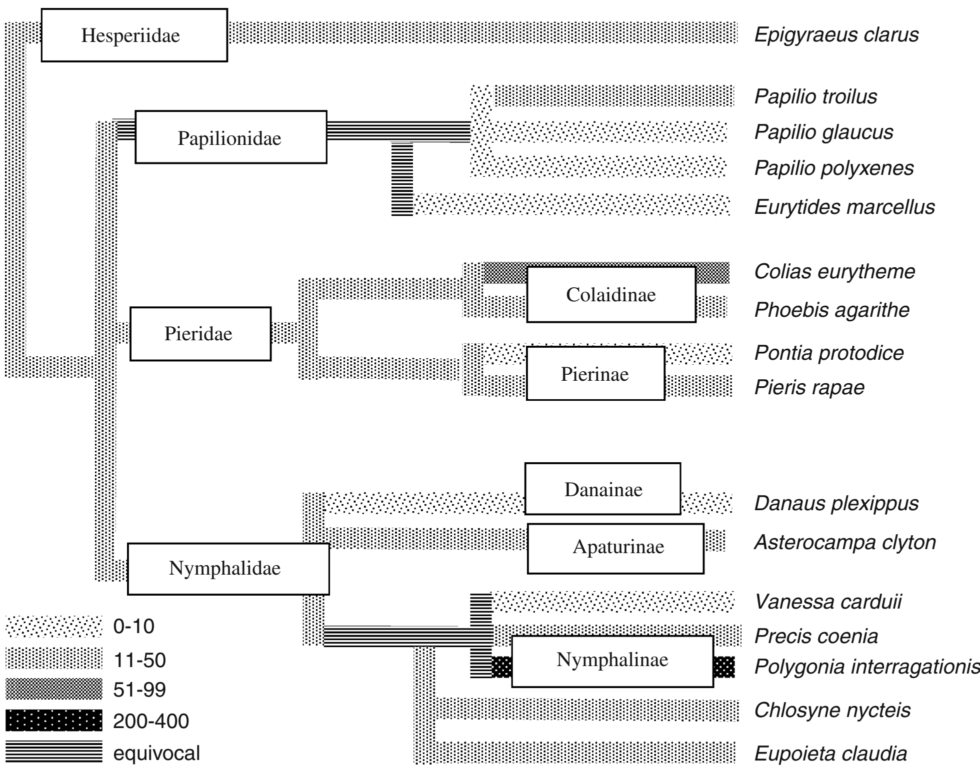


Fig. 2 Distribution of median labial gland glucose oxidase activities based on Papillionoidea phylogeny. Different line patterns represent classes of GOX activities delineated in Fig. 3. Equivocal data indicates uncertainty about the ancestral state of glucose oxidase activity for each taxon. Families and subfamily names are labeled

caterpillars (Wilcoxon rank sum test, $Z=-2.98$, $P=0.003$) (Fig. 4). Sixty-two % of the oligophagous caterpillar species (median activity = 25.4) had GOX activities of less than 50 nmol/min/mg protein, whereas 53% of the polyphagous species (median activity = 107.7) had activities greater than 100 nmol/min/mg. Forty-seven % of the oligophagous species and 53% of the polyphagous species were collected from woody species, but no trend for high or low GOX activities was found for caterpillars collected from woody or herbaceous host plants. A comparison of GOX activities among species collected from the same or closely related host plants or diets also did not reveal any pattern. For example, caterpillars reared on artificial diet had activities that ranged from 0.2 nmol/min/mg (*Manduca sexta*) to the highest activity of 2,680 nmol/min/mg (*H. zea*).

Glucose Oxidase Variation Among Caterpillars Fed Foliage from Different Hosts *Hyphantria cunea* collected on different tree species from four different plant families had seven-fold variation in GOX activities. Caterpillars collected on mulberry, *Morus alba*, and persimmon, *Diospyros virginiana,* had significantly higher activities ($P<0.05$) than caterpillars fed sweet gum, *Liquidambar styraciflua,* and black walnut*, Juglans nigra* foliage (Fig. 5). Two-way analysis of variance detected no significant differences between nests collected from of each

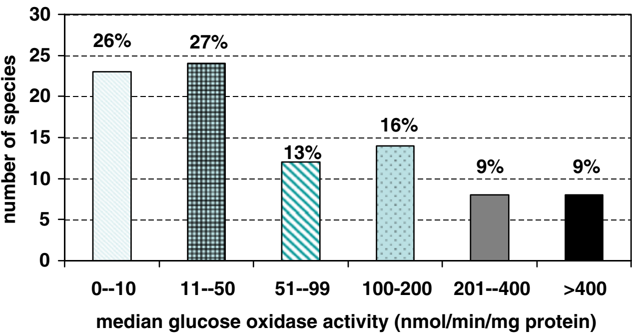


Fig. 3 Distribution of median glucose oxidase activities among caterpillar species. Activities were grouped into six categories (0–10, 11–50, 51–99, 100–200, 201–400, >400). Percentage of species is shown above each bar

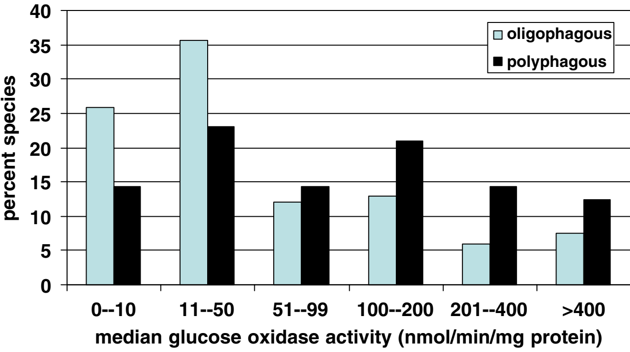


Fig. 4 Distribution of median glucose oxidase activities among polyphagous (≥3 host plant families) and oligophagous families (<3 host plant families). Activities were grouped into six categories (0–10, 11–50, 51–99, 100–200, 201–400, >400)

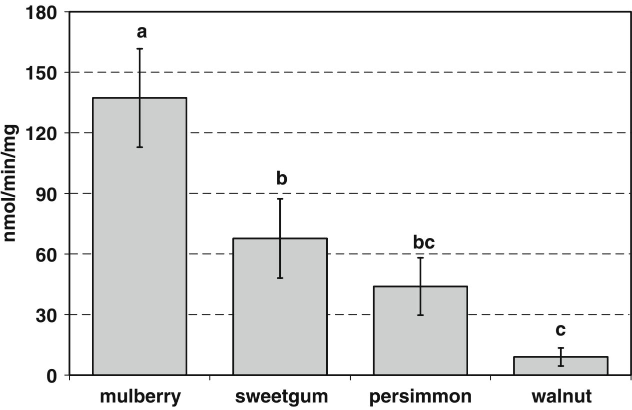


Fig. 5 Glucose oxidase activities of *Hyphantrea cunea* labial glands collected from four different tree species. Bars with different letters are significantly different (Tukey's HSD $P<0.05$)

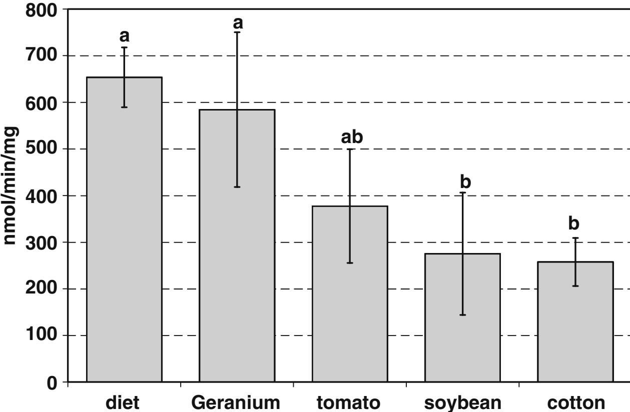
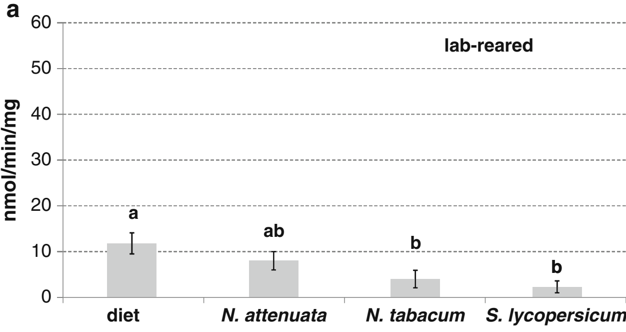


Fig. 7 Effect of diet on glucose oxidase activities in *Helicoverpa zea*. Bars with different letters are significantly different (Tukey's HSD $P<0.05$)

tree species ($F=0.60$, $P=0.45$, $df=2$) but there was significant variation caused by the tree species ($F=8.02$, $P<0.005$, $df=3$) and a significant tree × nest interaction ($F=3.72$, $P=0.03$, $df=6$). The GOX activities in *Manduca sexta* (Carolina colony) were not affected by the various solanaceous hosts tested, but when compared to larvae fed an artificial diet, GOX activities were somewhat higher than those larvae fed foliage (Fig. 6a). *Manduca sexta* collected near Fayetteville, AR however, had significantly higher GOX activities than the laboratory colony (Fig. 6b). *Helicoverpa zea* also showed significant variation in GOX activities when reared on different diets (Fig. 7). Larvae feeding on cotton and soybean showed lower activity than diet-fed or geranium-fed larvae. Larval *H. virescens* fed tobacco showed lower activity than diet-fed, but there was no statistical difference between larvae on wild-type or nahG tobacco (Fig. 8).

Glucose Oxidase Variation in Artificial Diets Supplemented with Different Ingredients The stereochemistry of dietary glucose did not significantly affect GOX activities. Both D- and L-glucose rations resulted in similar GOX activities in the labial glands of 2-day old sixth instar *H. zea* (Fig. 9a). In the same experiment, when no supplemental glucose was added to the diet, lower GOX activities were measured in the labial glands (Fig. 9a). Adding the disaccharides amylose and sucrose to test diets resulted in GOX activities less than when larvae were fed 2% glucose (Fig. 9b).



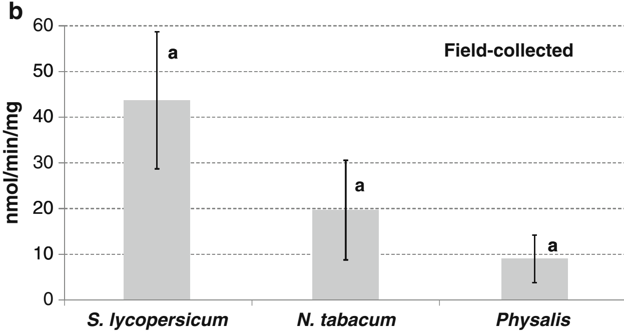


Fig. 6 Effect of diet on glucose oxidase activities in *Manduca sexta*. **a** Glucose oxidase activity in laboratory-reared caterpillars fed different diets. **b** Glucose activity in field-collected caterpillars fed different diets. Bars with different letters are significantly different (Tukey's HSD $P<0.05$)

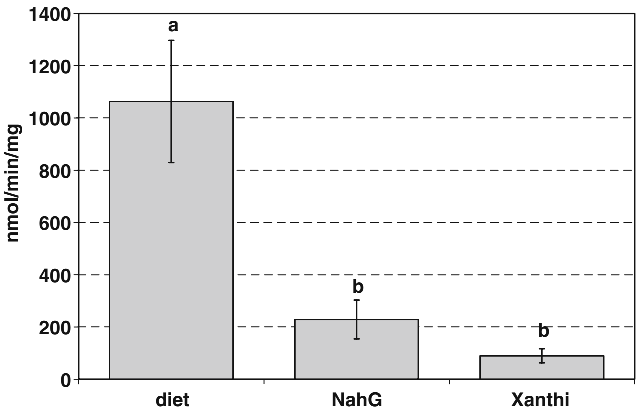
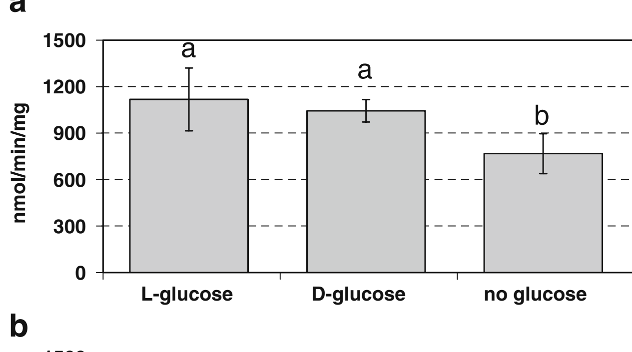


Fig. 8 Effect of diet on glucose oxidase activities in *Heliothis virescens*. Bars with different letters are significantly different (Tukey's HSD $P<0.05$). nahG = transgenic line with reduced salicylic acid levels, Xanthi = wild type



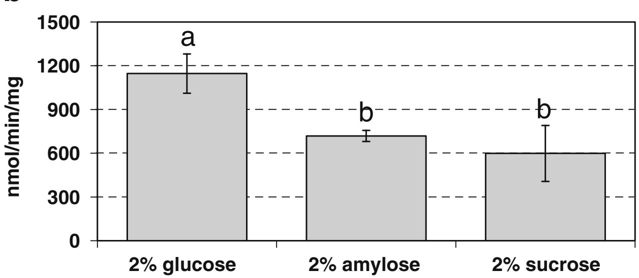


Fig. 9 Effect of carbohydrate on glucose oxidase activity in *Helicoverpa zea*. Larvae were fed artificial diets supplemented with different types of carbohydrate. **a** Larvae fed diets with D-glucose, L-glucose and no glucose were supplemented at 2%. **b** Larvae fed diets with 2% D-glucose, 2% amylose or 2% sucrose. Bars with different letters are significantly different (Tukey's HSD $P<0.05$)

Discussion

While GOX has been studied extensively in certain fungi such as *Aspergillus niger* (Wong et al., 2008), GOX has been identified sporadically in insects such as the honey bee *Apis mellifera* (Iida et al., 2007; Li et al., 2008), the aphid *Myzus persicae* (Harmel et al., 2008), lepidopterans including *Helicoverpa zea* (Eichenseer et al., 1999), *Helicoverpa armigera* (Zong and Wang, 2004), *Helicoverpa assulta* (Zong and Wang, 2004), *Spodoptera exigua* (Babic et al., 2008), *Mamestra configurata* (Merkx-Jacques and Bede, 2004), and the grasshopper *Schistocerca americana* (Candy, 1979). This current study is the most comprehensive survey of GOX yet conducted in insects, and greatly extends the number of species that possess quantifiable salivary GOX activities.

Interspecific comparisons were confounded by different diets and the specific time during development when glands were dissected; nevertheless, we believe that species differences do exist. For example, in the heliothine caterpillars significant variation exists among the three species assayed, as they were 1) reared on the same artificial diet, and 2) the glands were collected at similar points during larval development, two days into the last stadium. In another comparison, four noctuid species (*P. scabra*, *A. gemmatalis*, *H. zea*, and *P. includens*) collected on soybeans in the same field late in the growing season within several days of each other also showed at least 10-fold differences in GOX activities.

Because of its multifunctional roles in immunity (Eichenseer et al., 1999; Musser et al., 2005b; Yang and Cox-Foster, 2005), suppression of host defenses, and detoxification, it is not surprising that our findings indicate that GOX is widespread among caterpillar species. However, there were at least three orders of magnitude range in the activities of GOX in the labial glands of caterpillars. This variability is due to interspecific and intraspecific variation in activity. The host plant contributes to significant variation in GOX activities. In some cases, GOX activity was much lower when larvae feed on artificial diet (Merkx-Jacques and Bede, 2005; Yong-Hong et al., 2008), but in the case of *H. zea*, we found that although the particular species of host plant affects activity, there is still substantial activity compared to artificial diet-fed larvae (Peiffer and Felton, 2005). The nutritional or phytochemical factors that may contribute to GOX differences observed in larvae feeding on various host plants are not known. In studies with artificial diets or leaf supplementation, both protein and carbohydrate levels appear to be important in regulating GOX activity. Dietary protein was positively correlated with GOX activity in *S. exigua* (Babic et al., 2008). In *Helicoverpa armigera*, carbohydrates, including sucrose and glucose, were found to increase GOX activities, whereas several plant phenolics such as chlorogenic acid, rutin, and quercetin had no effect (Yong-Hong et al., 2008). In our studies, we have found that dietary glucose similarly affects GOX activities, but that labial GOX activity remains high even during starvation regimes (data not shown). It appears that development has a greater influence on variation than the presence or absence of diet. The host plant or diet not only affects the GOX synthesis within the glands, it also affects the secretion onto the plant surface (Peiffer and Felton, 2005). Consequently, the amount of GOX activity measured in the labial glands does not necessarily indicate how much a given caterpillar species may deposit on the plant surface. At present, we do not know if this variation in GOX synthesis and secretion plays an adaptive role in fitness on respective host plants or if it is merely a consequence of dietary factors and developmental stage.

We observed not only the wide occurrence of GOX among caterpillar species, but also its association with dietary host plant breadth. The majority of herbivorous insects are host specialists, and the mechanisms contributing to host specialization have been comparatively well-studied (Bernays and Graham, 1988; Futuyma and Moreno, 1988; Dyer, 1995). Unfortunately, our understanding of the behavioral/physiological traits that facilitate the expansion of host-range in polyphagous herbivores are, by comparison, poorly understood. Traits such as broad substrate specificity of detoxification enzymes (Mao et al.,

2007), canal cutting behaviors to deactivate defensive plant canals (Dussourd, 2009), and avoidance or suppression of induced defenses (Agrawal, 2000) have been studied as potential mechanisms underpinning host expansion. Our finding that GOX activities are generally higher in polyphagous species suggests a potential mechanism that could contribute to host-range expansion in caterpillar species. Karban and Agrawal (2002) argued that phenotypic plastic traits often are employed by generalist herbivores to maximize their fitness in variable environments. There is now ample data indicating that GOX is subject to considerable phenotypic plasticity due to diet. The relationship of GOX activity and host plant breadth warrants further research.

It is unknown if the effect of GOX on suppression of host-induced defenses is a widespread phenomenon or if it may be restricted to a limited number of host plant species. Nevertheless, GOX suppression of jasmonate-regulated, induced defenses is becoming increasingly recognized as more plant-insect systems are investigated (Musser et al., 2002; Zong and Wang, 2004; Bede et al., 2006; Delphia et al., 2006; Weech et al., 2008). To date, GOX is the one component of caterpillar saliva that has been identified in the suppression of these defenses (Musser et al., 2002; Zong and Wang, 2004; Bede et al., 2006; Diezel et al., 2009).

In addition to suppressing induced defenses, the breadth of potential GOX actions that can circumvent plant defenses is striking. For instance, fungal GOX inhibits the activity of several tomato oxidative enzymes (e.g., polyphenol oxidase, peroxidase, and lipoxygenase) (Begliomini et al., 1995), all of which have been implicated in defense against lepidopteran larvae (Felton et al., 1989, 1994; Felton and Duffey, 1991; Bi and Felton, 1995; Bi et al., 1997a). Because insect GOX has similar substrate specificity and catalytic activity compared to the fungal enzyme, it is not surprising that *H. zea* salivary GOX also inhibits these enzymes (unpublished data). GOX is a potent O_2 scavenger (Wong et al., 2008), and its enzymatic product, gluconic acid, is known to scavenge free radicals (Kataria et al., 1997) and inhibit polyphenol oxidases (Lopez-Caballero et al., 2006). In the presence of low O_2 levels, GOX enzymes may utilize quinone substrates as electron acceptors, thus functioning as a quinone reductase (Leskovac et al., 2005). This may be particularly significant when saliva is ingested in the nearly anaerobic guts of some lepidopteran larvae (Johnson and Barbehenn, 2000). We have found that GOX is stable in the digestive tract of *H. zea* (Eichenseer et al., 1999) and even retains activity in the feces (unpublished data). Consequently, GOX may be a factor in mitigating the oxidation of ingested plant compounds/nutrients formed at the plant-insect interface and throughout the digestive system.

Further molecular characterization of GOX and the analysis of the salivary proteome of *H. zea* is underway in our laboratory. We anticipate that the roles of caterpillar saliva in circumventing plant defense, detoxifying plant products, and providing immunity to disease will be greater appreciated as additional salivary proteins are characterized from *H. zea* and other lepidoptera. Our findings to date, suggest that GOX may be one factor contributing to the expansion of host range among lepidopteran species.

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Response of *Epilachna paenulata* to Two Flavonoids, Pinocembrin and Quercetin, in a Comparative Study

Georgina N. Diaz Napal · Maria T. Defagó · Graciela R. Valladares · Sara M. Palacios

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Abstract We examined the effects of the flavonoids pinocembrin and quercetin on the feeding behavior, survival, and development of the Cucurbitaceae pest *Epilachna paenulata* (Coleoptera: Coccinellidae). In no-choice experiments, 48 hr-consumption of *Cucurbita maxima* Duch. leaves treated with pinocembrin at 1, 5, and 50 μg/cm^2 was less than one third of that for leaves treated with 0.1 μg/cm^2 of pinocembrin or untreated leaves. Larvae stopped feeding after 9 days of high doses of pinocembrin (5 and 50 μg/cm^2), and larval weight and survival were negatively affected by pinocembrin at 1–50 μg/cm^2. Delayed mortality in comparison to food-deprived larvae suggests that the mechanism of action for pinocembrin is chronic intoxication, rather than simple starvation from antifeedant effects. In contrast, leaf consumption and larval weight were not significantly affected by quercetin (at 0.1, 1, 5, and 50 μg/cm^2) while mortality rates were only slightly increased. The response of *E. paenulata* larvae in a choice-test to combinations of pinocembrin at antifeedant doses (5 and 50 μg/cm^2) and quercetin at phagostimulant doses (0.01 and 0.1 μg/cm^2) indicated that the feeding deterrent activity of the former completely overshadowed the stimulant activity of the latter. These results demonstrate the different responses of one insect species to two widely distributed plant flavonoids. Pinocembrin strongly affected survival of *E. paenulata* while quercetin had only a weak effect without major consequences on the insect life-cycle.

Key Words Flavonoids · Pinocembrin · Quercetin · *Epilachna paenulata* · Antifeedant · *Coleoptera coccinellidae*

G. N. Diaz Napal · S. M. Palacios
Laboratorio de Química Fina y Productos Naturales,
Universidad Católica de Córdoba,
Camino a Alta Gracia Km 10,
(5000) Córdoba, Argentina

M. T. Defagó · G. R. Valladares
Centro de Investigaciones Entomológicas de Córdoba,
Facultad de Ciencias Exactas, Físicas y Naturales,
Universidad Nacional de Córdoba,
Av. Vélez Sarsfield 1611,
(5016) Córdoba, Argentina

S. M. Palacios (✉)
Facultad de Ciencias Químicas, Universidad Católica de Córdoba,
Camino a Alta Gracia Km 10,
(5000) Córdoba, Argentina
e-mail: sarapalacios@ucc.edu.ar

Introduction

Flavonoids are widely distributed in the plant kingdom and are involved in various aspects of plant interactions with other organisms. Increasing evidence suggests a role of flavonoids in plant defenses by contributing to disease resistance and protection against insect herbivory (Harborne and Williams, 2000; Treutter, 2006). The role of flavonoids in insect-plant interactions is complex, with some compounds apparently involved in host recognition, while others may deter or stimulate either feeding or oviposition (Simmonds, 2001).

Recently, we reported (Diaz Napal et al., 2009) on the differential effects of two flavonoids that are widely distributed in nature, pinocembrin (**1**), a flavanone which is found in conifers and Asteraceae species (Dillon and Mabry, 1977; Tewtrakul et al., 2003; Adelmann et al., 2007; Lin et al., 2007), and quercetin (**2**), a ubiquitous, abundant and intensively studied flavonol (Harnly et al., 2006; Harwood et al., 2007) (Fig. 1). When choice-tested at

different doses on *Epilachna paenulata*, **1** demonstrated a clear antifeedant activity, whereas **2** elicited variable behavioral responses (phagostimulant or antifeedant) in the same insect species, depending on the dosage (Diaz Napal et al., 2009). *Epilachna paenulata* (Germar) (Coleoptera: Coccinellidae) is a folivorous South American pest of the Cucurbitaceae species (Camarano et al., 2009), which we have used extensively as a model insect for natural insecticide activity studies (Carpinella et al., 2002, 2003; Maggi et al., 2005; Palacios et al., 2007).

We asked whether the antifeedant (**1** and **2**) and phagostimulant (**2**) effects have consequences on the survival of insects. In order to answer these questions we carried out a comparative study on the effects of these compounds on feeding behavior and survival of *E. paenulata*.

Behavioral experiments have shown that insect responses to a particular compound can be modulated by the presence of other compounds (Simmonds, 2001, 2003). We examined how insects react to the simultaneous presence of both compounds. The system *E. paenulata*-Cucurbitaceae, in which the insect is sensitive to both compounds, and neither compound is found in the plant, provides a neutral study system.

Methods and Materials

General Experimental Procedures and Apparatus ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker NMR with a Bruker AC 200 console (Bruker, Germany). Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H and ^{13}C NMR spectra measured in DMSO-d_6. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC–MS on a Hewlett–Packard 5970 Series mass spectrometer interfaced with a Hewlett–Packard 5890 gas chromatograph (Hewlett-Packard, Santa Clara, CA, USA) fitted with an HP-5MS, 15 m×0.25 mm i.d. column. The temperature was increased from 100 to 200°C at 10°C/min. The optical rotation angle was measured in a JASCO DIP-370 spectropolarimeter (JASCO Co., Tokyo, Japan).

Fig. 1 Chemical structures of pinocembrin (**1**) and quercetin. (**2**)

Chemicals and Chromatographic Media Pinocembrin, used as a reference sample, quercetin, used in the bioassays, and SiO_2 for column chromatography were purchased from Sigma Chemical Co. Inc. (St. Louis, MO, USA). Analytical TLC was performed on silica gel 60 F_{254} Merck plates (Darmstadt, Germany). All other solvents were purchased from Merck (Darmstadt, Germany) and Fisher Scientific (Jersey City, NJ, USA).

Isolation of Pinocembrin **1** was isolated from aerial parts of *Flourensia oolepis* S.F. Blake (Asteraceae) as reported previously (Diaz Napal et al., 2009). In brief, air-dried aerial parts of *F. oolepis* (97 g) were extracted with ethanol for 24 hr at room temperature. After solvent removal under reduced pressure, the extract (1.8 g) was fractionated by silica gel column chromatography until a crystalline solid was isolated (258 mg, 1.14% yield with respect to plant material) and identified as pinocembrin by ^{1}H NMR, ^{13}C NMR and MS.

Pinocembrin (**1**): white powder; mp 194–195°C; $[\alpha]_D^{20}$= −22.81 (*c* 0.86, EtOH); EI-MS: *m/z* (relative intensity, %) 256 (100 M^+) , 179 (82), 152 (67), 124(52), 96 (31), 69 (34); ^{1}H NMR (DMSO-d_6), 20.7°C, δ 2.77 (1H, dd, *J*=17.2, 3.2 Hz, H-3a), 3.06 (1H, dd, *J*=12.8, 17.2 Hz, H-3b), 5.44 (1H, dd, *J*=3.2, 12.8 Hz, H-2), 5.52 (1H, d, *J*=2.2 Hz, H-6), 6.01 (1H, d, *J*=2.2 Hz, H-8), 7.41 (5H, m, H-2′-6′); ^{13}C NMR 25°C, δ 40.45 (C-3), 80.17 (C-2), 95.94 (C-8), 96.84 (C-6), 102.69 (C-10), 127.47 (C-2′/6′), 129.39 (C-4′) 129.46 (C-3′/5′), 139.59 (C-1′), 163.59 (C-9), 164.41 (C-5), 167.62 (C-7), 196.75 (C-4). The spectral data were identical to previously published data of **1** (Bick et al., 1972; Neacsu et al., 2007; Adelmann et al., 2007).

Insects Epilachna paenulata larvae were obtained from a laboratory colony, reared on a natural diet of *Cucurbita maxima* Duch. leaves and maintained in a growth chamber at 24±1°C and 70–75% relative humidity, with a photoperiod of 16/8 hr light-dark cycle, and periodically renewed with field specimens (Maggi et al., 2005).

Leaf Treatments For all bioassays, cotyledon leaves of *C. maxima* seedlings were treated with acetone, which was allowed to evaporate before the insect trial began. The compounds were dissolved at sufficient concentration to achieve the desired doses, ranging from 0.01 to 50 μg/cm^2, in a single 10 μl aliquot. Control leaves were treated with pure acetone. The compounds were applied, and acetone was allowed to evaporate before starting the assay.

Feeding Choice Assay The antifeedant experiments were carried out by the leaf-disk choice test (Carpinella et al., 2002). The dosages applied for **1** and **2** were 1, 5 and 50 and 0.1 and 0.01 μg/cm^2, respectively. Two cotyledon leaves

from a *C. maxima* seedling were placed in a Petri dish; a glass disk with two 1 cm^2 diam holes was placed on top. A third-instar *E. paenulata* was placed equidistant from both a treated (with 10 µl of test solution) and an untreated (10 µl of acetone, solvent control) leaf disk and was allowed to feed for 24 hr. Ten replicates were run for each binary combination tested. Relative amounts (recorded in percentages from 0 to 100) of the treated and untreated substrate area eaten in each test were estimated visually by dividing the food area into imaginary quarters. A feeding index (FI%) was calculated as $[(C-T)/(C+T)] \times 100$ (e.g., Mazoir et al., 2008), where T and C represent consumption on treated and untreated foods, respectively. Effective dose 50 (ED_{50}) values for **1** were determined from the FI% data, using Probit analysis.

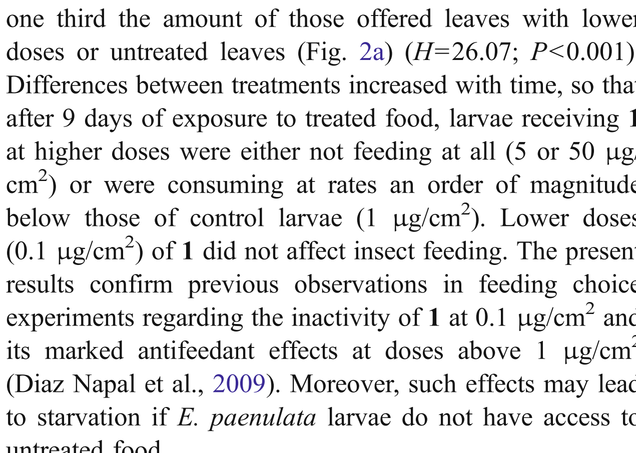
No-choice Feeding Assay One *E. paenulata* larva (first instar) was placed in a Petri dish containing *C. maxima* leaves that had been treated with acetone solutions of either pure **1** or pure **2,** at dosages of 0.1, 1, 5, or 50 µg/cm^2. Leaves were replaced every 24 hr. Ten replicates were used for each treatment. Leaf consumption and body weight were recorded every 24 hr and 48 hr, respectively (Carpinella et al., 2003).

Mortality Assay A group of 10 larvae of *E. paenulata* (first instar) was fed continuously with leaves treated either with 0.1, 1, 5, and 50 µg/cm^2 of pure **1**, pure **2** or acetone (control). A similar set of larvae was not fed at all and acted as food-deprived controls. Three replicates were performed for each treatment. Mortality was recorded every 24 hr. From mortality data, lethal dose 50 (LD_{50}) and lethal time 50 (LT_{50}) values for **1** and **2** were determined by Probit analysis. LD_{50} and LT_{50} were calculated at doses or times at which **1** provoked mortality values below as well as above 50%, thus enabling the Probit calculation.

Statistical Analysis Data did not conform to requirements of normal distribution and homocedasticity, therefore non-parametric tests were used. Results from choice tests were analyzed by the Wilcoxon Signed Rank Test. For each compound, results from no-choice tests on average larval body weight, accumulated consumption values, and average mortality rates at each date, were compared among concentrations by the Kruskal Wallis non-parametric analysis of variance, followed by the Dunn test. Statistical analyses were performed using the SPSS statistical package. Differences were considered significant at $P \leq 0.05$.

Results and Discussion

After 48 hr of exposure to leaves treated with **1** at 1, 5, or 50 µg/cm^2, larvae of *E. paenulata* had consumed less than



one third the amount of those offered leaves with lower doses or untreated leaves (Fig. 2a) ($H=26.07$; $P<0.001$). Differences between treatments increased with time, so that after 9 days of exposure to treated food, larvae receiving **1** at higher doses were either not feeding at all (5 or 50 µg/cm^2) or were consuming at rates an order of magnitude below those of control larvae (1 µg/cm^2). Lower doses (0.1 µg/cm^2) of **1** did not affect insect feeding. The present results confirm previous observations in feeding choice experiments regarding the inactivity of **1** at 0.1 µg/cm^2 and its marked antifeedant effects at doses above 1 µg/cm^2 (Diaz Napal et al., 2009). Moreover, such effects may lead to starvation if *E. paenulata* larvae do not have access to untreated food.

In contrast, the food consumption by larvae fed with **2** did not differ significantly from that of the controls at any dosage or at any time during the experiment (Fig. 3a). In the 12-day experiment, larvae faced with leaves treated with 0.1, 1, 5, and 50 µg/cm^2 of **2** ate as much food as the controls ($H=6.50$; $P=0.066$). Thus, neither the antifeedant nor the dose-dependent phagostimulant activity previously

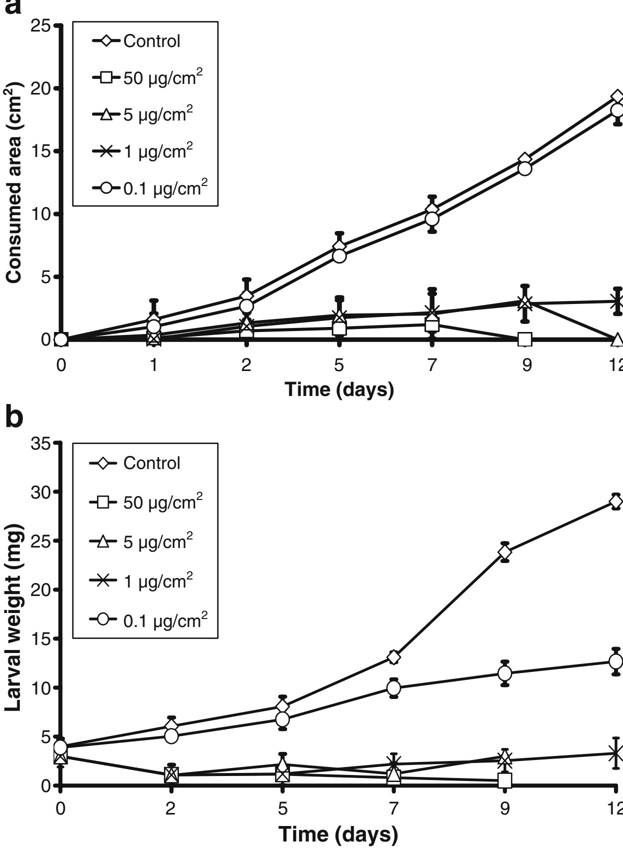


Fig. 2 Average leaf area consumed (**a**) and average body weight (**b**) of *Epilachna paenulata* larvae fed on leaves treated with **1** in no-choice assay. Error bars show standard deviation, where error is not shown it is smaller than the symbol

observed in choice assays with **2** (Diaz Napal et al., 2009), were detected in this system where insects could not switch to untreated food. These results confirm the necessity of comparing different methodologies when studying insect response to plant allelochemicals (Stuart and Polavarapu, 1998).

Larval weight data (Fig. 2b) demonstrated that whereas control larvae body weight steadily increased, larvae treated with **1** at 1 μg/cm^2 or higher doses either did not gain weight or lost weight. From 48 hr onward, significant differences in body weight (H=38.62; P<0.001) were observed between control larvae and those receiving food treated with **1** at doses of 1 μg/cm^2 or higher. After 7 days of receiving food treated with 5 or 50 μg/cm^2 of **1**, larvae had lost about half their original weight (Fig. 2b). Larvae fed on leaves treated with 0.1 μg/cm^2 of **1** gained some weight, but less weight (H=4.20; P<0.001) than control larvae starting at day 9. Compound **2** did not affect the body weight of *E. paenulata* larvae (Fig. 3b) (H=5.51; P=0.2379).

Treatment of leaves with **1** increased larval mortality (Fig. 4a), particularly at the highest dose (50 μg/cm^2), which induced 100% mortality after 12 days. At lower doses of **1**, mortality reached between 20 and 60%. After 5 days of receiving treated food, mortality rates were significantly higher than those observed in the controls (H=30.20; P<0.001) for all concentrations higher than 0.1 μg/cm^2. Food-deprived larvae did not survive longer than 5 days. These results indicate that the presence of **1** on the food of *E. paenulata* larvae strongly affected their survival through feeding inhibition, consistent with the well-established role of flavonoids in the defense of plants against herbivory (Harborne and Williams, 2000).

Larvae offered food treated with **2** at any of the doses tested died at the same rate as the controls (P>0.05) (Fig. 4b). All treatments were significantly different (P< 0.001) from the food-deprived group. According to these results, **1** strongly affected survival of *E. paenulata* larvae, whereas **2** had no major effects on the insect life-cycle.

The LD_{50} of **1** at 124 hr was 18.4 (6.0–56.6) μg/cm^2. A comparison of this value with those reported for the same insect for meliartenin (LD_{50}=0.76 μg/cm^2) and azadirachtin (LD_{50}=1.24 μg/cm^2) (Carpinella et al., 2003), indicates that pinocembrin is 24 and 15 times less active than those compounds, respectively. In molar units, LD_{50} for pinocembrin (70 nmol/cm^2) was 50 and 41 times higher than the

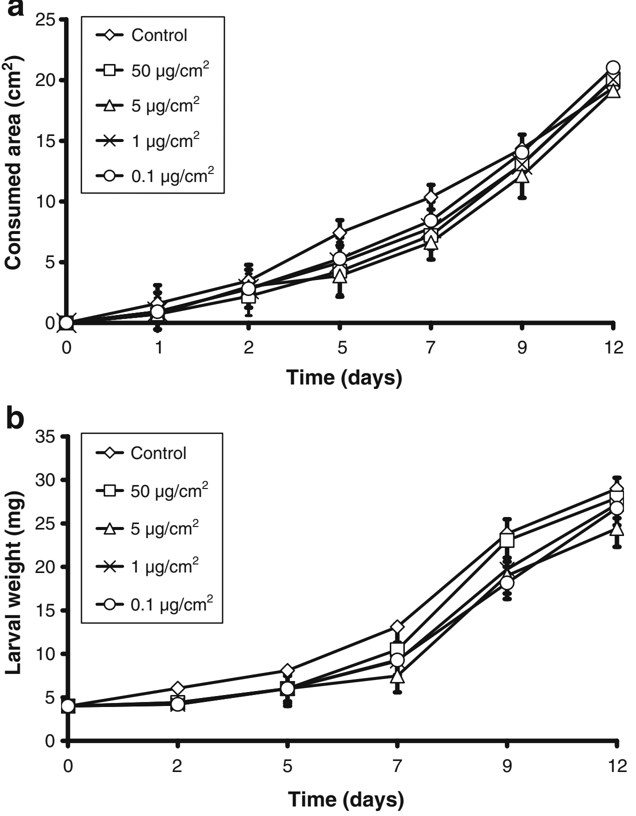


Fig. 3 Average leaf area consumed (**a**) and average body weight (**b**) of *Epilachna paenulata* larvae fed on leaves treated with **2** in no-choice assay. Error bars show standard deviation, where error is not shown it is smaller than the symbol

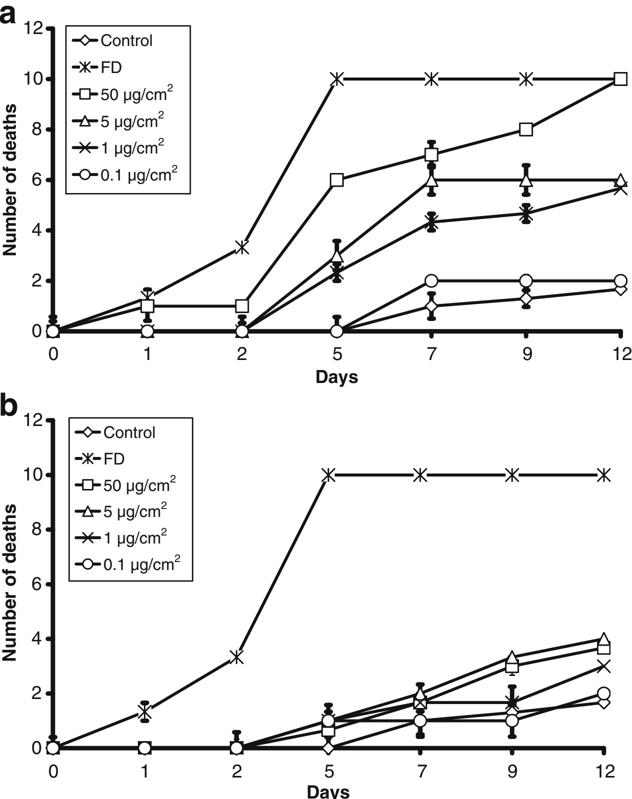


Fig. 4 Cumulative mortality rates (%) of *E. paenulata* larvae fed with leaves treated with **1** (**a**) and **2** (**b**). FD represents food-deprived treatment. Error bars show standard deviation, where error is not shown it is smaller than the symbol

Table 1 Lethal time (LT_{50}) for compound **1** and for food-deprived control in larvae of *Epilachna paenulata* in no-choice feeding assay

Dosage (μg/cm^2)	LT_{50}[a] in days (values and 95% confidence interval)	
	1	Food-deprived larvae
50	5 (4.73–5.87)	
5	8 (6.47–9.47)	
1	10 (8.10–11.85)	
		4 (3.07–4.23)

[a] LT_{50} is the time required to obtain 50% mortality

value for meliartenin (1.4 nmol/cm^2) and azadirachtin (1.7 nmol/cm^2), respectively.

Insects consuming leaves treated with **1** died later than food-deprived larvae, suggesting that the mechanism of activity for **1** is chronic intoxication rather than simple starvation (Defagó et al., 2009). Lethal times 50 (LT_{50}) for **1** (Table 1) were 5, 8, and 10 days for 50, 5 and 1 μg/cm^2, respectively. The LT_{50} for food-deprived larvae was 4 days. Comparing the LT_{50} at 1 μg/cm^2 with the values obtained for meliartenin and azadirachtin on the same insect (Carpinella et al., 2003), **1** acted approximately 4 and 2 times more slowly than meliartenin and azadirachtin, respectively.

It was not possible to calculate LD_{50} and LT_{50} for **2,** since mortality rates did not reach 50% at any point of the experiment (Fig. 4b).

Responses to plant secondary metabolites often play a crucial role in the feeding behavior of herbivore insects. Such responses result from the integration of information provided by taste and olfactory cells (van Loon, 1990) as well as from other extrinsic and intrinsic factors operating on and in the insect (Bernays and Chapman, 2001). Responses of chemical receptors to a specific stimulus may be modified by the presence of other plant constituents, so it may be impossible to predict the response to complex stimuli on the basis of single compound responses (Schoonhoven and van Loon, 2002). Combinations of **1** and **2** were assayed in a choice test, at doses at which **2** was expected to act as phagostimulant and **1** as an antifeedant. Choice assays of these mixtures with *E. paenulata* revealed that the ED_{50} of **1** was approximately the same when offered with or without **2** (Table 2). This insect recognized **1** as a feeding deterrent, and this effect was not modified by the presence of **2** at phagostimulant levels. Increased concentration of a deterrent compound can not only increase activity of the deterrent-sensitive cells, but may also suppress the activity of cells responding to phagostimulants (Bernays and Chapman, 2000), and therefore, negative inputs may dominate insect response to feeding stimulation (Chapman, 2003).

It is not possible to predict the effect of different flavonoids on insects from structural features of the compounds, although empirical evidence suggests that the structure can be important (Simmonds, 2001). According to Morimoto et al. (2000), hydroxyl groups at any position favor antifeedant activity against *Spodoptera litura*, but this

Table 2 Feeding choice assay for mixtures of **1** and **2** against *Epilachna paenulata*

Dosage (μg/cm^2)		FI%[a]	ED_{50} of **1** in μg/cm^2 (95% confidence interval)
1	**2**		
1		6	
5		12	10.08 (2.42–41.9)
50		98**	
	0.01	−48.72**	
	0.1	−16.22*	
1	0.01	5	
5	0.01	15.4	13.55 (2.71–67.74)
50	0.01	87**	
1	0.1	2	
5	0.1	20	13.18 (2.29–75.62)
50	0.1	82**	

[a] Feeding index for pinocembrin, calculated as $(FI\%) = [(C - T)/(C + T)] \times 100$, values are means of 10 replicates. Significant differences between consumption on treated and control leaves (Wilcoxon signed rank test *$P<0.05$, **$P<0.01$)

idea was not supported by our results since **2** has three more hydroxyl groups than **1**. A further complication arises from the variability in the responses of different insects to the same flavonoid (Simmonds, 2001, 2003). Our results support this observation and suggest that such effects might also depend on compound dosage. The growth of the corn borer *Ostrinia nubilalis* Hubner was reduced by 10 mg/g of **1** added to an artificial diet (Abou-Zaid et al., 1993). In contrast, **1** was reported as attractant and phagostimulant to *Scolytus mediterraneus* (Levy et al., 1974). Insect response to **2** varies greatly. This flavonoid may act as a phagostimulant for some insects, may be either a phagostimulant or a deterrent depending on the concentration tested for other insects (Lattanzio et al., 2000; Green et al., 2003; Simmonds, 2003), and may be inactive in other cases (Morimoto et al., 2000).

In summary, we have shown that compound **1** inhibited feeding and negatively affected survival of the cucurbit insect pest *E. paenulata*. We also found that **1** masked the phagostimulant effects of **2** in a choice test. According to the present results, **1** is less active than other well-known antifeedants such as azadirachtin or meliartenin, but its availability in a variety of plant species and its molecular simplicity, which could allow inexpensive chemical synthesis, suggest that **1** could be applied as an feeding deterrent or could be a target for plant improvement programs to increase natural crop resistance to herbivory.

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Differential Effects of Indole and Aliphatic Glucosinolates on Lepidopteran Herbivores

René Müller · Martin de Vos · Joel Y. Sun · Ida E. Sønderby · Barbara A. Halkier · Ute Wittstock · Georg Jander

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Abstract Glucosinolates are a diverse group of defensive secondary metabolites that is characteristic of the Brassicales. *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) lines with mutations that greatly reduce abundance of indole glucosinolates (*cyp79B2 cyp79B3*), aliphatic glucosinolates (*myb28 myb29*), or both (*cyp79B2 cyp79B3 myb28 myb29*) make it possible to test the *in vivo* defensive function of these two major glucosinolate classes. In experiments with Lepidoptera that are not crucifer-feeding specialists, aliphatic and indole glucosinolates had an additive effect on *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) larval growth, whereas *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) and *Manduca sexta* (L.) (Lepidoptera: Sphingidae) were affected only by the absence of aliphatic glucosinolates. In the case of two crucifer-feeding specialists, *Pieris rapae* (L.) (Lepidoptera: Pieridae) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), there were no major changes in larval performance due to decreased aliphatic and/or indole glucosinolate content. Nevertheless, choice tests show that aliphatic and indole glucosinolates act in an additive manner to promote larval feeding of both species and *P. rapae* oviposition. Together, these results support the hypothesis that a diversity of glucosinolates is required to limit the growth of multiple insect herbivores.

Key Words Glucosinolates · Lepidoptera · Oviposition · Feeding · *Arabidopsis thaliana* · *Manduca sexta* · *Trichoplusia ni* · *Pieris rapae* · *Plutella xylostella* · *Spodoptera exigua*

R. Müller · U. Wittstock
Institut für Pharmazeutische Biologie,
Technische Universität Braunschweig,
Braunschweig, Germany

M. de Vos · J. Y. Sun · G. Jander (✉)
Boyce Thompson Institute for Plant Research,
1 Tower Road,
Ithaca, NY 14853, USA
e-mail: gj32@cornell.edu

I. E. Sønderby · B. A. Halkier
VKR-Research Centre for Pro-Active Plants,
Department of Plant Biology and Biotechnology,
Faculty of Life Sciences, University of Copenhagen,
Copenhagen, Denmark

Present Address:
M. de Vos
Keygene N.V.,
Agro Business Park 90,
6708 PW Wageningen, The Netherlands

Present Address:
I. E. Sønderby
Department of Medical Genetics,
Oslo University Hospital Ullevaal,
Postboks 4956 Nydalen,
0424 Oslo, Norway

Introduction

The glucosinolate-myrosinase system is a characteristic defense of the Brassicaceae and related plant families (Halkier and Gershenzon, 2006). In the absence of herbivore attack, glucosinolates, β-thioglucoside-*N*-hydroxyiminosulfates with diverse, amino acid-derived side chains, are stored separately from the activating enzyme, myrosinase (β-thioglucoside glucohydrolase, EC 3.2.1.147). Tissue disruption during herbivory brings glucosinolates into contact with myrosinase, resulting in release of the glucose moiety and formation of biologically active breakdown products, including nitriles, isothiocyanates, thiocyanates, oxazolidine-2-thiones, and epithionitriles (Wittstock and Burow, 2010).

More than 120 different glucosinolate side chains have been identified (Fahey et al., 2001), and about forty of these are found in natural isolates of the model plant *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) (Kliebenstein et al., 2001; Reichelt et al., 2002). The metabolic diversity of glucosinolates, combined with variation in the breakdown pathways (Wittstock and Burow, 2010), likely results in several hundred defensive metabolites that can be formed during herbivory. Genetic modification of the glucosinolate breakdown pathway shows that qualitative changes affect herbivore resistance (Jander et al., 2001; Lambrix et al., 2001; Burow et al., 2006). Less is known about the specific defensive function of different glucosinolate classes, e.g., those derived from tryptophan, methionine, or phenylalanine. Increased production of both tryptophan-derived (indole) and methionine-derived (aliphatic) glucosinolates through overexpression of the MYB51 and MYB28 transcription factors, respectively, reduced herbivory by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) on *A. thaliana* (Gigolashvili et al., 2007a, b). Both total glucosinolate content and, more specifically, methylsulfinylalkylglucosinolate levels were negatively correlated with *S. exigua* weight gain (Mewis et al., 2005; Rohr et al., 2006). The related *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) showed improved growth in the absence of indole glucosinolates (Schlaeppi et al., 2008). Similarly, reduced aliphatic glucosinolate content increased the growth of the generalist lepidopteran herbivore *Mamestra brassicae* (L) (Lepidoptera: Noctuidae) (Beekwilder et al., 2008).

Some crucifer-specialist insects, including *Pieris rapae* (L.) (Lepidoptera: Pieridae) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), not only circumvent the release of toxic breakdown products from glucosinolates (Ratzka et al., 2002; Wittstock et al., 2004) but also co-opt these defensive metabolites as attractive signals. Glucosinolates elicit specific responses in maxillary chemoreceptors and stimulate feeding in both *P. rapae* and *P. xylostella* larvae (Verschaffelt, 1910; Thorsteinson, 1953; Van Loon et al., 2002; Miles et al., 2005). Among ten glucosinolates with side chains derived from tryptophan, methionine, or phenylalanine, indol-3-ylmethylglucosinolate was identified as the strongest oviposition stimulant for *P. rapae* oviposition *in vitro* (Renwick et al., 1992; Städler et al., 1995). Although most experiments show that glucosinolates have positive effects on *P. xylostella* feeding and oviposition (Sarfraz et al., 2006), individual glucosinolates that were increased in transgenic lines did not have unequivocal effects (Sarosh et al., 2010), and 3-butenylglucosinolate levels in *A. thaliana* field studies were negatively correlated with female adult weight (Bidart-Bouzat et al., 2005).

In many *A. thaliana* accessions, the side chains of the most abundant foliar glucosinolates are derived from methionine or tryptophan (Wittstock and Halkier, 2002). Whereas plants with knockout mutations of both *CYP79B2* and *CYP79B3*, two cytochrome P450s that lead to the production of indole-3-acetaldoxime, are blocked in the production of indole glucosinolates (Zhao et al., 2002), double mutants of the *MYB28* and *MYB29* transcription factors have very low levels of aliphatic glucosinolates (Sønderby et al., 2007; Beekwilder et al., 2008). Quadruple mutants (*cyp79B2 cyp79B3 myb28 myb29*) are nearly devoid of both major classes of foliar glucosinolates found in *A. thaliana* Columbia-0 (Col-0; Sun et al., 2009). Together, these mutant lines provide a unique opportunity to investigate the relative *in vivo* function of aliphatic and indole glucosinolates in plant defense against different herbivores.

Methods and Materials

Plants and Growth Conditions *A. thaliana* Col-0 wildtype seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org). The *cyp79B2 cyp79B3* mutant (Zhao et al., 2002) was kindly supplied by J. Celenza (Boston University, Boston, MA, USA). Creation of *myb28 myb29* and *cyp79B2 cyp79B3 myb28 myb29* mutants has been described previously (Sønderby et al., 2007; Sun et al., 2009). All mutations are in the Col-0 genetic background.

A. thaliana plants for *S. exigua, T. ni, P. xylostella,* and *Manduca sexta* (L.) (Lepidoptera: Sphingidae) experiments were grown in a Conviron (Winnipeg, Canada) chamber in 12×12 cm pots using Cornell Mix [by weight 56% Peatmoss, 35% Vermiculite, 4% Lime, 4% Osmocoat slow-release fertilizer (Scotts, Marysville, OH, USA), and 1% Unimix (Peters, Allentown, PA, USA)] at 23°C, 60% relative humidity, with a light intensity of 180 $\mu mol\, m^{-2} s^{-1}$ photosynthetic photon flux density and a 16:8 h light:dark photocycle. *Arabidopsis thaliana* for *P. rapae* experiments were grown on soil composed of 80% potting soil (Compo, Münster, Germany), 10% sand, 10% Perligran (Knauf Perlite, Dortmund, Germany), and Triabon (Compo, Münster, Germany) and Sierrablen (Scotts, Heerlen, The Netherlands) as fertilizer at 22°C, 60–70% relative humidity, 300 $\mu mol\, m^{-2} s^{-1}$ photosynthetic photon flux density, and a 10:14 h light:dark photocycle.

Insects and Growth Conditions *P. xylostella, T. ni,* and *S. exigua* eggs were purchased from Benzon Research (Carlisle, PA, USA). *M. sexta* eggs were kindly supplied by M. del Campo (Cornell University) and J. Beal (Boyce Thompson Institute). *P. xylostella* for feeding choice experiments were reared in paper cups (0.5 L, International Paper, Memphis, TN, USA) on artificial diet (Southland Products, Lake Village, AK, USA) at 23°C. *P. rapae* were

maintained on Brussels sprouts (*Brassica oleracea var. gemmifera*) in a chamber at 24°C, 65% relative humidity and a 16:8 h light:dark photocycle.

Larval Feeding no Choice Tests Lepidopteran eggs (*T. ni, M. sexta,* and *S. exigua*, and *P. xylostella*) were hatched at 23°C in paper cups (0.5 l, International Paper, Memphis, TN, USA) with a moist paper towel (C-fold white, Kimberly-Clark, Dallas, TX, USA). Individual neonate larvae were placed onto paired 18-d-old mutant and wildtype plants growing together in the same pot, and each individual plant was covered with a mesh cup. Larvae were harvested at 9 d (*T. ni*), 8 d (*M. sexta*), or 10 d (*S. exigua*). The length of the experiment for each species was chosen based on the differing intrinsic growth and feeding rates of the larvae. In each case, even the largest larvae had not consumed the entire *A. thaliana* plant at the end of the experiment. *Plutella xylostella* were harvested at pupation (8–10 d). Larvae and pupae were frozen on dry ice and were lyophilized overnight (~12 h), and weighed. Each experiment was conducted two to four times, giving similar results.

For *P. rapae* larval growth experiments, one neonate larva was placed in a pot containing one 6-wk-old *A. thaliana* Col-0 wildtype or mutant plant, respectively, covered with a perforated plastic bag and placed at 24°C and 65% relative humidity. The weight of the larvae was recorded after 10 d of feeding. For *P. rapae* pupation experiments, groups of ten neonate larvae were placed in a pot containing four 6-wk-old *A. thaliana* Col-0 wildtype or mutant plants, respectively, covered with a perforated plastic bag and placed at 24°C and 65% relative humidity. Additional food plants were provided on demand. Each experiment was run twice independently.

Larval Feeding Choice Tests For *P. xylostella* choice tests, leaves of similar age and size were harvested from paired 20-d-old mutant and wildtype *A. thaliana* plants growing together in the same pot. Leaf areas were scanned, and paired leaves were placed in Petri dishes (9 cm diam, ThermoFisher, Waltham, MA, USA), with their petioles embedded in 0.8% Phytagar (Invitrogen, Carlsbad, CA, USA). Larvae for experiments were raised for 3 d on artificial diet. A single larva was placed with each leaf pair and, after 3 d, leaves were collected and scanned again. Leaf area consumed was calculated using ImageJ (Rasband, 1997–2007). Leaf plug choice experiments were conducted in a similar manner. A single 3-d-old larva was placed in a Petri dish (4.5 cm diam, Thermo-Fisher, Waltham, MA, USA) with paired equal-sized leaf disks of each genotype (0.6 cm^2) on moist filter paper. After 24 h, leaves were scanned and leaf areas were calculated with ImageJ (Rasband, 1997–2007). Intact leaf and leaf plug experiments were conducted twice independently.

For *P. rapae* feeding choice tests, one third-instar *P. rapae* larva was allowed to feed on a pot containing two 6-wk-old plants of two *A. thaliana* lines that were being compared. After 24 h, the plant rosettes were harvested and scanned using a desktop scanner. The removed leaf area was completed and calculated with ImageJ (Rasband, 1997–2007). These experiments were conducted twice independently.

Oviposition Choice Tests For *P. rapae* oviposition experiments, adults were kept without plants for 6 d after eclosion to ensure mating and maximize egg production. A single *P. rapae* female then was introduced to a 39×28×28 cm plastic box containing two 7-wk-old plants, each from a different plant genotype. After 24 h, the eggs laid per plant were counted. These experiments were conducted twice independently.

Glucosinolate Analysis Glucosinolate extraction and HPLC analysis of desulphoglucosinolates was performed as previously described (Kliebenstein et al., 2001; Burow et al., 2006; Hansen et al., 2007), using half rosettes of 6-wk-old plants.

Data Analysis Statistical analyses were conducted with JMP (SAS Institute, Cary, NC, USA) and SigmaStat 3.1 (Systat Software, San José, CA, USA).

Results

Under the growth conditions used for insect experiments, *cyp79B2 cyp79B3* mutants contain little or no indole glucosinolates, *myb28 myb29* mutants have greatly decreased aliphatic glucosinolates, and *cyp79B2 cyp79B3 myb28 myb29* quadruple mutants are deficient in both indole and aliphatic glucosinolates (Table 1; Sun et al., 2009). To determine the relative effects of indole and aliphatic glucosinolate decreases on non-crucifer-specialist Lepidoptera, neonate *S. exigua*, *M. sexta,* and *T. ni* were caged on individual mutant and wildtype Col-0 *A. thaliana* plants. *Spodoptera exigua* larval weight was increased (Fig. 1a) on both *cyp79B2 cyp79B3* and *myb28 myb29* mutants compared to wildtype Col-0. An additive effect was observed in that the larvae grew better on the *cyp79B2 cyp79B3 myb28 myb29* quadruple mutant than on either of the double mutants (Fig. 1a). In contrast, *M. sexta* and *T. ni* weight gain was increased relative to controls only on lines deficient in aliphatic glucosinolates (*myb28 myb29* and the quadruple mutant; Fig. 1b, c). Although the effect was not

Table 1 Foliar glucosinolates of Col-0 wildtype and mutants

glucosinolate	Col-0[a]		*cyp79B2 cyp79B3*[a]		*myb28 myb29*[a]		QKO[a]	
	Mean[b]	SE	Mean[b]	SE	Mean[b]	SE	Mean[b]	SE
3MSP	1.41	0.23	1,44	0.09	**0.07**	**0.06**	**0.01**	**0.00**
4MTB	3.52	0.55	2.36	0.24	**0.29**	**0.29**	**0.00**	**0.00**
4MSB	8.52	1.34	9.55	0.71	**0.48**	**0.48**	**0.01**	**0.00**
5MSP	0.30	0.05	0.35	0.02	**ND**		**ND**	
7MTH	0.27	0.10	0.13	0.01	**0.02**	**0.02**	**0.00**	**0.00**
7MSH	0.18	0.04	0.20	0.01	**ND**		**ND**	
8MTO	0.36	0.09	0.20	0.02	**0.03**	**0.03**	**0.00**	**0.00**
8MSO	0.89	0.16	0.80	0.04	**0.10**	**0.07**	**ND**	
Total aliphatic	15.45	2.56	15.04	1.15	**0.98**	**0.95**	**0.02**	**0.01**
I3M	1.30	0.14	**0.02**	**0.00**	1.60	0.28	**0.00**	**0.00**
4MOI3M	0.02	0.01	0.01	0.00	0.02	0.01	**ND**	
NMOI3M	0.41	0.11	**0.01**	**0.00**	0.62	0.16	**ND**	
4OHI3M	0.57	0.12	**0.03**	**0.00**	0.58	0.06	**0.00**	**0.00**
Total indole	2.29	0.37	**0.06**	**0.01**	2.82	0.50	**0.00**	**0.00**

[a] nmol/mg fresh weight, [b] significant differences relative to wildtype Col-0 are marked in bold ($P<0.05$, Mann-Whitney *U-test*, $N=5$); QKO=*cyp79B2 cyp79B3 myb28-1 myb29-1* quadruple knockout; SE=standard error; ND=not detected; glucosinolate side chain abbreviations: 3MSP=3-methylsulfinylpropyl, 4MTB=4-methylthiobutyl, 4MSB=4-methylsulfinylbutyl, 5MSP=5-methylsulfinylpentyl, 7MTH=7-methylthioheptyl, 7MSH=7-methylsulfinylheptyl, 8MTO=8-methylthiooctyl, 8-MSO=8-methylsulfinyloctyl, I3M=indol-3-ylmethyl, 4MOI3M=4-methoxyindol-3-ylmethyl, and NMOI3M=*N*-methoxyindol-3-ylmethyl, 4OHI3M=4-hydroxyindol-3-ylmethyl.

quantified, smaller larvae of all three species consumed visibly less plant material than larger ones.

Exogenous addition of glucosinolates is known to result in feeding stimulation for *P. xylostella* (Verschaffelt, 1910; Thorsteinson, 1953; Van Loon et al., 2002). However, we did not observe significant differences in pupal dry weight (Fig. 2a) or time to pupation (Fig. 2b) for *P. xylostella* larvae feeding on mutant and wildtype *A. thaliana.* Although choice tests with intact leaves did not show significant feeding preferences (Fig. 2c), larvae consumed significantly

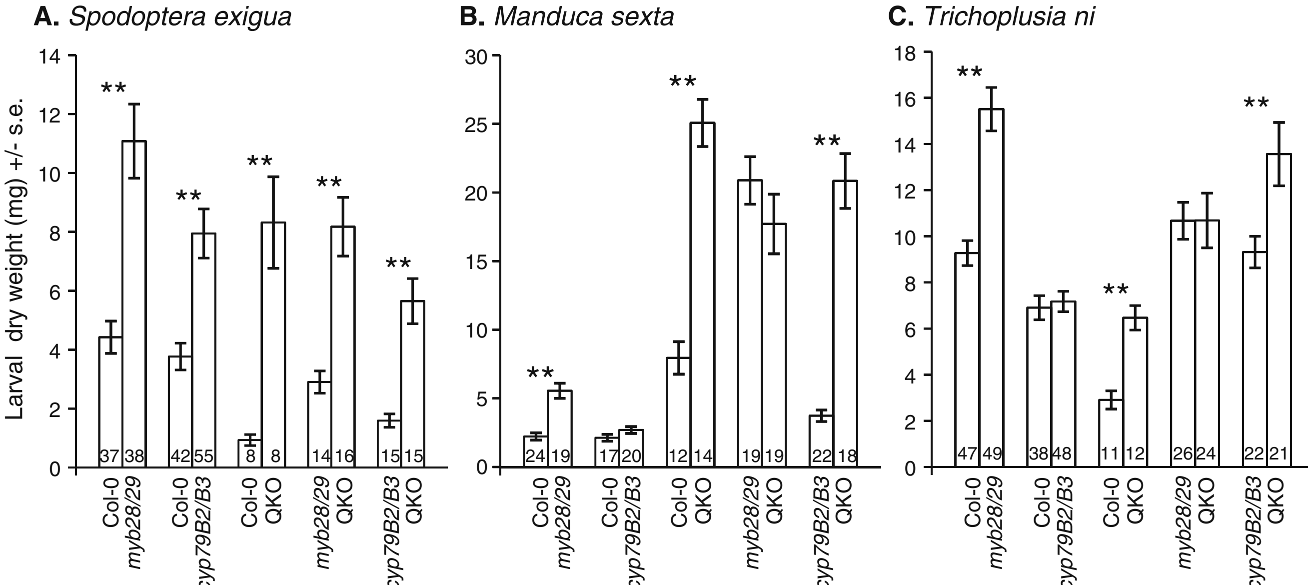

Fig. 1 Dry weight of non-crucifer-specialist Lepidoptera feeding on *Arabidopsis thaliana* Col-0 wildtype and mutants deficient in aliphatic glucosinolates (*myb28/29*), indole glucosinolates (*cyp79B2/B3*), or both (QKO, quadruple knockout) in no-choice tests. **a** *Spodoptera exigua* after 10 d **b** *Manduca sexta* after 8 d **c** *Trichoplusia ni* after 9 d. Bars are mean +/− standard error. Numbers in the bars represent sample sizes. $^{**}P<0.001$, Mann-Whitney *U-test*, in each case comparing two sets of plants growing at the same time in the same flats. Additional replicates of these experiments showed similar results

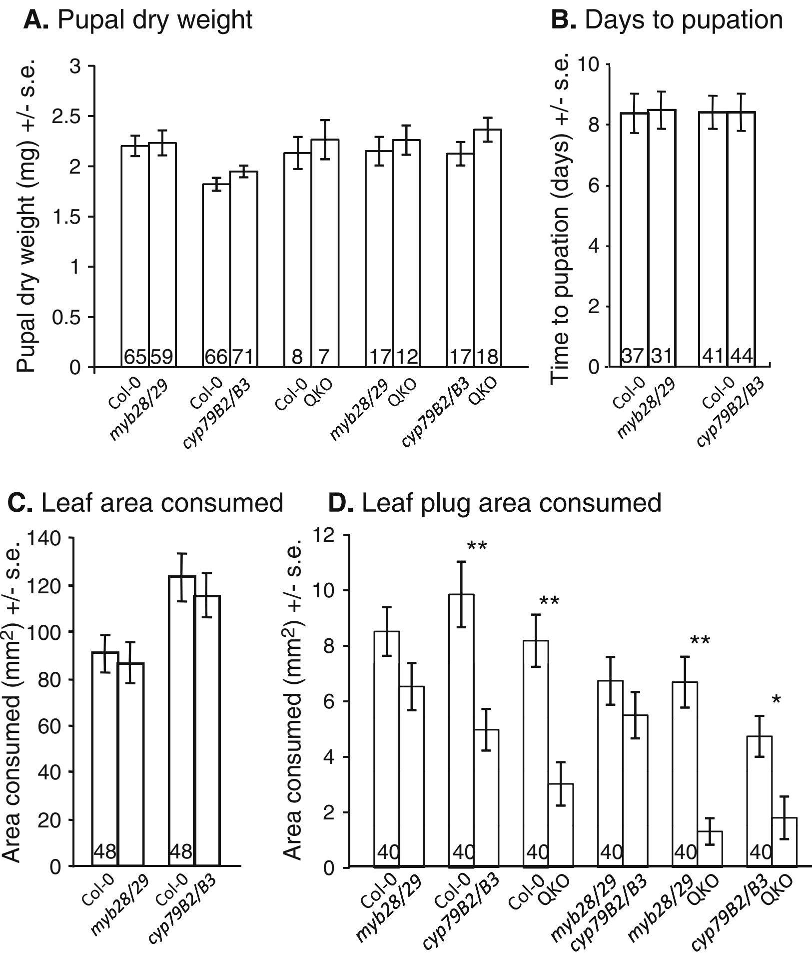

Fig. 2 Growth and feeding of *Plutella xylostella* on *Arabidopsis thaliana* Col-0 wildtype and mutants deficient in aliphatic glucosinolates (*myb28/29*), indole glucosinolates (*cyp79B2/B3*), or both (QKO, quadruple knockout). **a** Dry weight of pupae from larvae grown on whole plants in no-choice tests. **b** Age of larvae at pupation in no-choice tests. **c** Area consumed in detached-leaf choice tests. **d** Area consumed in leaf plug choice tests. All comparisons are with pairs of genotypes grown at the same time in the same flats. Bars are mean +/− standard error. Numbers in the bars represent sample sizes. *$P<0.01$, **$P<0.001$, pair-wise comparisons with Mann-Whitney *U-tests* (A–C) or Wilcoxon signed rank tests (D)

less material from the quadruple knockout mutant than the other genotypes in leaf plug choice tests (Fig. 2d).

When *P. rapae*, another crucifer-feeding specialist, fed on *A. thaliana* deficient in aliphatic and/or indole glucosinolates, there was no effect on larval weight compared to larvae feeding on wildtype plants. Only slight effects were seen in *P. rapae* pupation, with pupae being slightly heavier on *cyp79B2 cyp79B3* mutants (Fig. 3a) and a little longer time to pupation on *myb28 myb29* mutants (Fig. 3c). In contrast, larval choice tests show that Col-0 tissue is highly preferred over all other genotypes in choice tests (Fig. 3d). Experiments comparing double and quadruple mutants show that aliphatic and indole glucosinolates have an additive effect in stimulating larval feeding (Fig. 3d). Direct comparison of the *cyp79B2 cyp79B3* and *myb28 myb29* mutants also show that indole glucosinolates are more important than aliphatic glucosinolates in attracting *P. rapae* feeding.

Choice tests were conducted to determine the relative importance of aliphatic and indole glucosinolates for eliciting *P. rapae* oviposition on intact *A. thaliana.* In these experiments, reduction in aliphatic or indole glucosinolates individually did not affect oviposition in comparisons to wildtype Col-0 ($P>0.05$; Fig. 4). However, the quadruple knockout mutant received fewer eggs when compared to Col-0 wildtype, *myb28 myb20*, or *cyp79B2 cyp79B3* plants (Fig. 4), suggesting that there are additive effects of the aliphatic and indole glucosinolate classes in eliciting *P. rapae* oviposition.

Discussion

The experiments described here support the hypothesis that the glucosinolate diversity found in *A. thaliana* and other crucifers is necessary to limit growth of multiple insect herbivores. Consistent with the negative effects of glucosinolate overproduction (Gigolashvili et al., 2007a, b), decreases in indole and aliphatic glucosinolates cause additive increases in larval growth of the generalist *S. exigua* (Fig. 1a). In contrast, two other non-crucifer specialists, *M. sexta* and *T. ni*, are sensitive only to changes

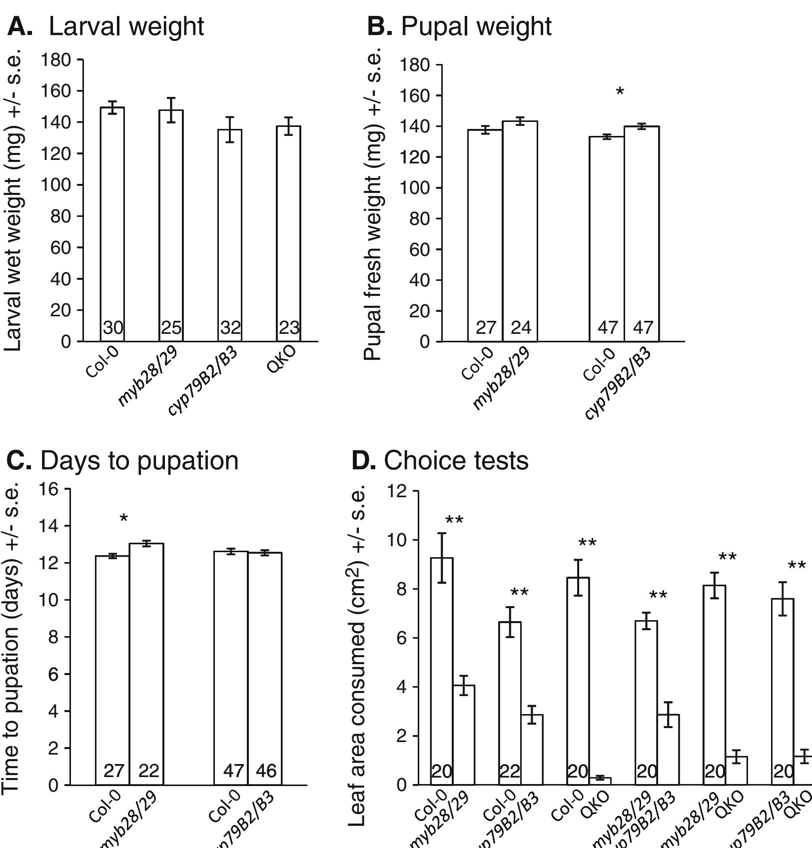

Fig. 3 *Pieris rapae* larval feeding on *Arabidopsis thaliana* Col-0 wildtype and mutants deficient in aliphatic glucosinolates (*myb28/29*), indole glucosinolates (*cyp79B2/B3*), or both (QKO, quadruple knockout) in no-choice (A–C) and dual choice tests (D). **a** Larval weight at day 10 in no-choice tests (no significant differences by ANOVA). **b** Pupal fresh weight. **c** Age of larvae at pupation in no-choice tests. **d** Leaf area consumed in dual choice tests. All data are mean +/− standard error. Numbers in the bars represent sample sizes. **P*<0.01, ***P*<0.001, unpaired Student's *t-tests* (B) or Mann-Whitney *U-tests* (C, D). Shown are data from single experiments that have been repeated with similar results except in B where the difference between Col-0 and *cyp79B2/B3* was not significant in a second experiment

in aliphatic glucosinolate content and are unaffected by the presence or absence of indole glucosinolates (Fig. 1b, c). Conversely, other research shows that indole glucosinolates are particularly important for *A. thaliana* defense against generalist aphids (Kim and Jander, 2007; Kim et al., 2008; De Vos and Jander, 2009; Pfalz et al., 2009). Therefore, *A. thaliana* plants in nature would require both aliphatic and indole glucosinolates for improved protection against a range of different herbivore species. Loss of either glucosinolate class would allow some generalist herbivores to feed more readily (Fig. 5).

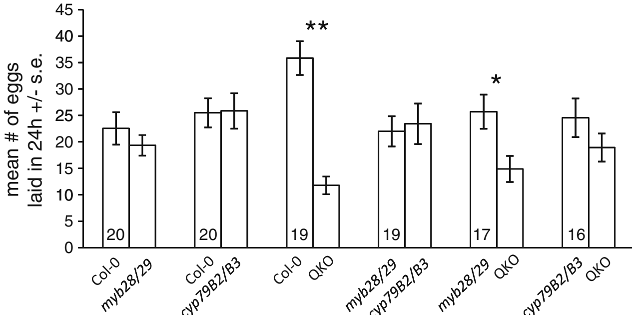

Fig. 4 *Pieris rapae* oviposition on whole *Arabidopsis thaliana* plants in 24-h choice tests comparing Col-0 wildtype and mutants deficient in aliphatic glucosinolates (*myb28/29*), indole glucosinolates (*cyp79B2/B3*), or both (QKO, quadruple knockout). All data are mean +/− standard error. Numbers in the bars represent sample sizes. **P*<0.05, ***P*<0.001, Mann-Whitney *U-test*. Shown are data from a single experiment. Repetition of this experiment showed the same oviposition trends and analysis of the combined data sets showed a significant reduction in oviposition on the QKO relative to Col-0, *myb28/29,* and *cyp79B2/B3*

It is possible that the observed differences in larval growth (Fig. 1) result from variation in plant responses to the three herbivores. However, other experiments have shown similar *A. thaliana* transcriptional responses to different Lepidoptera (Reymond et al., 2004). Additionally, compared to the effects of *myb28 myb29* and *cyp79B2 cyp79B3*, herbivore-induced glucosinolate changes in *A. thaliana* are not large, generally less than two-fold increases in total glucosinolate content (Schlaeppi et al., 2008). Although the *myb28 myb29* and *cyp79B2 cyp79B3* mutations almost completely eliminate aliphatic and indole glucosinolates, respectively, we cannot rule out the possibility that other, as yet uninvestigated *A. thaliana* defense mechanisms also influence the outcome of these experiments. For instance, the MYB28 and MYB29 transcription factors may regulate other pathways that play a role in insect defense, and additional indole compounds are metabolically downstream from CYP79B2 and CYP79B3 (Zhao et al., 2002). CYP79B2 and CYP79B3 are also

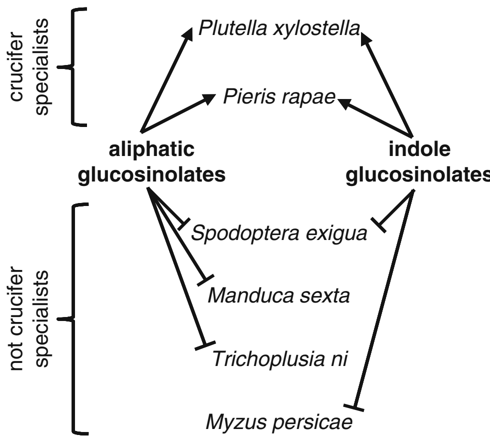

Fig. 5 Indole and aliphatic glucosinolates have differential effects on two crucifer-specialist insect herbivores and four non-specialist herbivores. As indicated by the arrows, both aliphatic and indole glucosinolates act as feeding stimulants for *Pieris rapae* and *Plutella xylostella*. However, growth of these crucifer-feeding specialists is largely unaffected by glucosinolate content. As indicated by the bars, growth of insects that are not crucifer-feeding specialists is reduced in the presence of glucosinolates. Specifically, *Manduca sexta* and *Trichoplusia ni* are affected primarily by aliphatic glucosinolates, *Myzus persicae* (green peach aphid) is affected primarily by indole glucosinolates (Kim et al., 2008), and the two glucosinolate classes have additive effects on *Spodoptera exigua*

required for the biosynthesis of camalexin (Glawischnig et al., 2004). However, unlike glucosinolates, production of this indole phytoalexin is not induced much by the jasmonate-mediated defense pathway, nor is it known to affect insect feeding (Brader et al., 2001; Pegadaraju et al., 2005).

So far, the actual toxic or antifeedant effects of glucosinolate breakdown products on insect herbivores have not yet been identified. However, the observed differences in glucosinolate sensitivity among insects with similar feeding styles (Fig. 1) may provide an inroad into such research. The three tested Lepidoptera might differ in their target site sensitivity, taste receptors, or detoxification mechanisms. However, given that *M. sexta* does not normally consume crucifers, it is unlikely to have specific detoxification enzymes for glucosinolates or their breakdown products.

Consistent with experiments showing that glucosinolates are *P. xylostella* feeding stimulants *in vitro* (Thorsteinson, 1953; Van Loon et al., 2002), aliphatic and indole glucosinolates have additive effects in a leaf plug choice experiments (Fig. 2d). However, in experiments with detached leaves, larvae show no preference when comparing Col-0 and plants lacking either indole or aliphatic glucosinolates (Fig. 2c). One notable difference between the two experiments is the exposed cut leaf surface of the leaf plugs, which might cause the release of volatile glucosinolate-derived stimuli that attract *P. xylostella* larvae. Despite the feeding preferences, even the quadruple knockout mutant did not affect pupal size nor time to pupation (Fig. 2a, b). Glucosinolates are not an absolute requirement for food acceptance, as *P. xylostella* can be raised over several generations on artificial diets or *P. sativum* (sweet pea; Gupta and Thorsteinson, 1960), which do not contain glucosinolates.

Consistent with their efficient glucosinolate detoxification mechanisms (Wittstock et al., 2004), *P. rapae* growth was not affected much by variation in glucosinolate content (Fig. 3). Nevertheless, *P. rapae* exhibited slightly higher pupal weight (Fig. 3b) and later pupation (Fig. 3c) on *cyp79B2 cyp79B3* and *myb28 myb29* mutant lines, respectively. Slower development in nature could be deleterious because larvae would be exposed to predators for a longer period of time, and pupal size has been associated with egg production and overall adult fitness in Lepidoptera (del Campo et al., 2005). Even though the observed glucosinolate effects on the development of individual larvae in this laboratory experiment are quite small (Figs. 3b and c), such fitness changes could lead to more dramatic effects over several generation in a natural setting.

Similar to *P. xylostella* (Fig. 2d), aliphatic and indole glucosinolates have additive effects in stimulating *P. rapae* larvae to feed on *A. thaliana* plants (Fig. 3d). In other research, larvae grew more rapidly on wildtype *A. thaliana* than on *tgg1 tgg2* myrosinase-deficient mutants (Barth and Jander, 2006). Since these myrosinase mutations do not alter glucosinolate content, breakdown products, rather than intact glucosinolates may be the primary *P. rapae* feeding stimulants. *Arabidopsis thaliana* mutants defective in both myrosinase and glucosinolate production could be used to determine whether this is the case. Alternatively, glucosinolate breakdown to simple nitriles upon ingestion (Wittstock et al., 2004) may promote growth due to the release of sulfate and likely elemental sulfur that may be valuable for nutrition.

Although in prior research (De Vos et al., 2008), *P. rapae* laid fewer eggs on *cyp79B2 cyp79B3* mutants compared to wildtype plants, this was not the case in the current experiments (Fig. 4). These dissimilar results have several possible causes: (i) different growth conditions of the *A. thaliana* plants could affect oviposition; (ii) there may be strain-specific differences in the *P. rapae* that were studied; and (iii) different diets consumed by larvae could affect the oviposition preferences of adult butterflies in these experiments. Decreased oviposition on the quadruple knockout line compared to the *myb28 myb29* mutant (Fig. 4) suggests that the role of indole glucosinolates as oviposition stimulants may depend on the relative abundance of aliphatic glucosinolates or their breakdown products. Similarly, aliphatic glucosinolates were important for stimulating *P. xylostella* oviposition on intact *A.*

thaliana plants only in the absence of indole glucosinolates (Sun et al., 2009).

The importance of *A. thaliana* glucosinolate diversity for defense against herbivores in nature remains relatively uninvestigated. (but see Mauricio, 1998; Mauricio and Rausher, 1997; Bidart-Bouzat and Kliebenstein, 2008) Taken together, our results of *in vivo* experiments with *A. thaliana* mutants show that producing more than one class of glucosinolates is advantageous for limiting the growth of generalist herbivores, which are not universally sensitive to all glucosinolate classes (Fig. 5). At the same time, both aliphatic and indole glucosinolates make the plant more susceptible to attack by specialist herbivores. Therefore, a diversity of glucosinolates, as well as tight regulation of their biosynthesis and breakdown, is likely necessary for *A. thaliana* to survive and adapt in a complex natural environment with both specialist and generalist herbivores.

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Enhancing Sorgoleone Levels in Grain Sorghum Root Exudates

Md. Romij Uddin · Kee Woong Park ·
Yong Kyoung Kim · Sang Un Park · Jong Yeong Pyon

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Abstract Sorgoleone, found in the root exudates of sorghum [(*Sorghum bicolor* (L.) Moench], has been a subject of continued research. Sorgoleone production in grain sorghum roots was investigated under different growth conditions. Methanol was the most effective solvent for extracting sorgoleone from grain sorghum roots. Sorgoleone production is high in young developing plants. The maximum concentration (μg mg^{-1} root dry weight) was produced in 5-d-old seedlings; beyond this age, production declined. However, considering both root weight and sorgoleone content per seedling, 10-d-old seedlings had the highest total amounts (μg). Compared with the control, sorgoleone content increased 6.1, 8.6, and 14.2 times when sorghum seeds were treated with auxins, Hoagland solution, and a combination of auxins and Hoagland solution, respectively. Among the innate immunity response elicitors, cellulose (an elicitor of plant origin) stimulated higher sorgoleone production than the others, and it produced 6.2 times more sorgoleone than the control. Combined treatment of sorghum seeds with half strength Hoagland solution and 5 μg ml^{-1} of IBA significantly increased both root growth and sorgoleone content in sorghum seedlings.

M. R. Uddin · Y. K. Kim · S. U. Park · J. Y. Pyon (✉)
Department of Agronomy, Chungnam National University,
220 Gung-Dong, Yuseong-Gu,
Daejeon 305-764, Korea
e-mail: jypyon@cnu.ac.kr

K. W. Park
Bio-Evaluation Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB),
Cheongwon-gun 363-883, Korea

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Introduction

The allelopathic effect of sorghum was first noticed in crops grown in rotation with sorghum (Breazeale, 1924) and later supported by several studies (Putnam et al., 1983; Forney et al., 1985; Einhellig and Rasmussen, 1989). Several workers have examined the chemical composition of root exudates of sorghum (Guenzi et al., 1967; Lehle and Putnam, 1983; Alsaadawi et al., 1986; Panasiuk et al. 1986). Netzly and Butler (1986) isolated hydrophobic *p*-benzoquinones from grain sorghum roots exudates, which were ultimately identified and named sorgoleone (Netzly et al., 1988). The root hairs of sorghum produce an oily exudate containing the lipid benzoquinone sorgoleone (2-hydroxy-5-methoxy-3-[(8′*Z*, 11′*Z*)-8′, 11′, 14′-pentadecatriene]-*p*-benzoquinone), which is a potent allelochemical (Netzly and Butler, 1986; Inderjit and Duke, 2003). Sorgoleone and its 1 4-hydroquinone form together account for ~50% of the oily exudates from sorghum root hairs (Erickson et al., 2001; Dayan et al., 2009). The remaining percentage consists primarily of alkyl resorcinol analogs, along with small amounts of several sorgoleone congeners that vary in the substitutions in the aromatic ring (Fate and Lynn, 1996; Rimando et al., 1998; Kagan et al., 2003). All these variants of sorgoleone appear to contribute to the overall allelopathic potential of sorghum (Kagan et al., 2003; Rimando et al., 2003). Sorgoleone is phytotoxic to broadleaf and grass weeds at concentrations as low as 10 μM in hydroponic assays (Einhellig and Souza, 1992; Nimbal et al., 1996), and broadleaf weed species are more susceptible than grass

weed species (Uddin et al., 2009). Sorgoleone exerts both pre-emergence and post-emergence effects on different weeds (Czarnota et al., 2001; Weston and Czarnota, 2001).

Factors that affect root hair production and sorgoleone biosynthesis are not well understood. However, sorgoleone biosynthesis is linked intrinsically to the presence of living root hairs (Czarnota et al., 2001; Yang et al., 2004). Furthermore, root hair production is inhibited by excess water (Hess et al., 1992; Yang et al., 2004; Dayan et al., 2007). Sorgoleone levels are sensitive to light; they decreased by nearly 50% upon exposure to blue light (470 nm) and by 23% upon exposure to red light (670 nm) (Dayan, 2006). Sorgoleone levels increase in plants treated with a crude extract of velvetleaf (*Abutilon theophrasti* Medik.) root (Dayan, 2006).

Although the cellular localization and the biosynthetic steps involved in sorgoleone production have been determined (Dayan et al., 2003; Pan et al., 2007; Baerson et al., 2008), limited information is available regarding the factors that affect its biosynthesis. Therefore, this research was conducted to determine the factors that enhance both root growth and sorgoleone content in sorghum roots. Such knowledge may be used to develop a bioherbicide.

Methods and Materials

Plant Material and Growth Conditions Seeds of sorghum (cultivar Chalsusu) were collected from the College of Agriculture & Life Sciences, Gangwon National University, Korea. They were treated with benomyl (a fungicide used for seed disinfection) for 4 h and then rinsed several times in distilled water. For most experiments, 25 seeds along with the treatment material were placed in sterile Petri dishes (100×40 mm) on the surface of sterile Whatman #1 filter paper (diam, 90 mm). The dishes then were placed in a growth chamber at 30°C under standard cool-white fluorescent tubes with a flux rate of 550 μmol s^{-1} m^{-2} and a 16-h photoperiod for 3 wk. However, the seedlings were allowed to grow for 40 d for the experiment to determine the age of maximum seedling growth. Each experiment had four replications and was repeated two to three times.

Extraction Procedure and Sorgoleone Analysis by HPLC Sorgoleone was extracted according to the procedures described by Netzly and Butler (1986); Netzly et al. (1988), and Czarnota et al. (2003a), except that methanol was used as a solvent instead of methylene chloride. Seedling roots were excised and immersed in methanol (1:20 w/v) for 30 sec to extract. The crude extract was filtered and evaporated under vacuum. The dried extract was dissolved in methanol (1 mg ml^{-1}), and the solution was filtered through a poly filter (pore size, 0.45 μm). The filtrate was diluted 4-fold with methanol prior to HPLC analysis. HPLC quantification of sorgoleone was performed using the Futecs NS-4000 HPLC systems (Futecs Co. Ltd.,Daejeon, Korea) with a C_{18} column (250× 4.6 mm, particle size 5 μm; RStech, Daejeon, Korea). The mobile phase was 75% acetonitrile + 25% acidified water. Water was acidified with glacial acetic acid (97.5:2.5 v/v). Sorgoleone was detected at 280 nm with a Waters tunable absorbance detector after injection of 20 μl of the methanol solubilized crude extract sample. The column flow rate was 1 ml min^{-1} with a 40 min total run time for each sample. All samples were run in triplicate. The amount of sorgoleone was calculated on the basis of a standard curve obtained from a purified sample. The sorgoleone standard was provided by Franck Dayan, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Natural Products Utilization Research Unit.

Optimization of Sorgoleone Extraction Different organic solvents were used, namely methanol, methylene chloride, chloroform, ethanol, butanol, ethyl acetate, hexane, and water, were used to extract sorgoleone from 5-d-old sorghum seedling roots to determine the best solvent for obtaining maximum amounts. The extraction procedure was the same as described above.

Age of Seedling for Maximum Root Growth To determine the optimum age of seedlings for measuring maximum root growth and sorgoleone content, eight sets of Petri dishes (100×40 mm) with four replications were arranged to grow sorghum seedlings. In each dish, 25 seeds were grown. At each 5-d interval, one set of Petri dishes (four dishes) was removed from the growth chamber for measuring root

Table 1 Effect of different organic solvents on sorgoleone extraction from sorghum roots

Organic solvent	Amount of sorgoleone (μg mg^{-1} RDW[a])
Methanol	40.1 a[b]
Methylene chloride	15.7 b
Chloroform	14.0 b
Ethanol	9.8 c
Butanol	0 d
Ethyl acetate	0 d
Hexane	0 d
Water	0 d

[a] *RDW* Root dry weight

[b] Mean values (mean of four replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Tukey's studentized range test)

Table 2 Effect of the age of seedlings on root growth and sorgoleone content in sorghum roots

Age of seedlings (d)	RDW[a] (mg)	Sorgoleone concentration (μg mg^{-1} RDW)	Total sorgoleone content (μg)
5	4.0 d[b]	40.1 a	160.2 ab
10	5.9 c	30.1 b	177.3 a
15	6.8 bc	18.8 c	127.7 cd
20	7.7 b	17.3 d	133.4 cd
25	8.8 a	17.0 d	149.4 bc
30	9.6 a	11.9 e	113.9 de
35	9.1 a	11.0 e	100.0 e
40	7.6 b	9.2 f	70.3 f

[a] *RDW* Root dry weight

[b] Mean values (for RDW, mean of 4 replicates with an average of ten seedlings for each replicate; for sorgoleone, mean of four replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Tukey's studentized range test)

growth and sorgoleone content, until 40 d after seeding. Water was applied during the entire experimental period.

Auxin Treatment To determine the effect of auxins on root growth and sorgoleone content in roots, three different auxins, namely, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneaceti acid (NAA) were tested at different concentrations (0 μg ml^{-1}, 0.1 μg ml^{-1}, 0.5 μg ml^{-1}, 0.75 μg ml^{-1}, 1, 5 μg ml^{-1}, 10 μg ml^{-1}, 25 μg ml^{-1}, and 50 μg ml^{-1}).

Hoagland Solution Treatment Seedlings were grown in Petri dishes as described above, in different strengths Hoagland solution. After seed placement in the dishes, three strengths, i.e., quarter, half, and full Hoagland solution (Hoagland and Arnon, 1950) were applied at three different time points during growth (0 d, 4 d, and 8 d).

Combined Treatment with Auxins and Hoagland Solution Seedlings were grown in Petri dishes in combined solutions of auxins and Hoagland solution (50:50 v/v). Seeds were applied with, quarter, half, or full strengths Hoagland solution along with the optimal concentrations of IAA (1 μg ml^{-1}), IBA (5 μg ml^{-1}), and NAA (1 μg ml^{-1}).

Effect of Elicitors To evaluate the effect of elicitors on growth and sorgoleone content, two elicitors of plant origin (pectin and cellulose) and two of microorganism origin (chitin and chitosan) were tested at different concentrations (0 μg ml^{-1}, 0.1 μg ml^{-1}, 0.5 μg ml^{-1}, 0.75 μg ml^{-1}, 1 μg ml^{-1}, 5 μg ml^{-1}, 10 μg ml^{-1}, and 100 μg ml^{-1}).

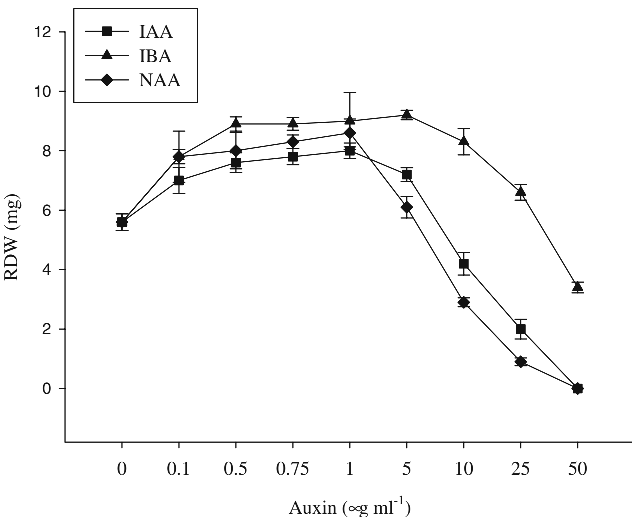


Fig. 1 Effect of different growth hormones on root dry weight of sorghum. Values are presented as mean (SD) (each point is the mean of four replicates with an average of ten seedlings for each replicate). IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, NAA: 1-naphthaleneacetic acid, SD: Standard deviation

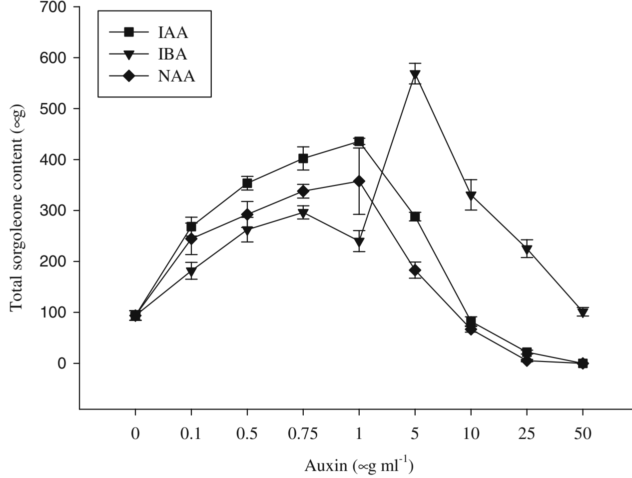


Fig. 2 Effect of different growth hormones on sorgoleone content in sorghum roots. Values are presented as mean (SD) (each point is the mean of four replicates with three samples from each replicate). IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, NAA: 1-naphthaleneacetic acid, SD: Standard deviation

Table 3 Effect of different strengths of Hoagland solution, applied at different time points, on root growth and sorgoleone production in sorghum roots

Strength of Hoagland solution	Time of application (DAS)[a]	RDW[b] (mg)	Sorgoleone concentration (μg mg^{-1} RDW)	Total sorgoleone content (μg)
Quarter	0	8.2 cd[c]	47.2 cd	387.3 cd
	4	7.3 e	34.3 e	249.6 ef
	8	6.8 e	25.4 f	173.5 fgh
Half	0	11.1 a	76.2 a	844.3 a
	4	9.4 b	60.9 b	569.6 b
	8	8.3 c	48.7 c	401.8 c
Full	0	9.5 b	12.1 g	115.0 gh
	4	8.0 cd	23.7 f	188.8 fg
	8	7.2 de	41.4 d	299.9 de
Control	–	6.5 e	15.1 g	98.3 h

[a] *DAS* Days after seed placement

[b] *RDW* Root dry weight

[c] Mean values (for RDW, mean of four replicates with an average of ten seedlings for each replicate; for sorgoleone, mean of four replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Tukey's studentized range test)

Another experiment was performed using the optimal concentration of each elicitor (chitin, pectin, and cellulose: 1 μg ml^{-1}; chitosan: 5 μg ml^{-1}) applied at 0 d, 3 d, 6 d, and 9 d after seed placement in Petri dish. Distilled water was used before elicitor treatment. Thereafter, seedlings were allowed to grow for 3 wk.

Statistical Analysis All data were analyzed using the SAS 9.1 Software (released in 2006; SAS Institute Inc., Cary, NC, USA). Analysis of variance was performed for each compound concentration, and mean differences were calculated using Tukey's studentized range test. Standard deviations were also provided to indicate the variations associated with the particular mean values.

Results

Optimization of Sorgoleone Extraction The highest amount of sorgoleone was extracted with methanol, followed by methylene chloride and chloroform (Table 1). The amount

Table 4 Combined effect of auxins and Hoagland solution on root growth and sorgoleone production in sorghum roots

Auxin concentration (μg ml^{-1})	Strength of Hoagland solution	RDW[a] (mg)	Sorgoleone concentration (μg mg^{-1} RDW)	Total sorgoleone content (μg)
IAA (1)	Quarter	10.6 cd[b]	46.7 e	495.1 d
	Half	11.9 ab	60.4 c	721.4 b
	Full	11.1 bc	32.5 g	360.3 e
IBA (5)	Quarter	10.5 cd	60.3 c	631.5 c
	Half	13.2 a	84.6 a	1120.5 a
	Full	12.2 ab	40.4 f	492.7 d
NAA (1)	Quarter	9.5 de	53.7 d	511.3 d
	Half	10.5 cd	66.4 b	700.1 bc
	Full	9.1 e	36.0 fg	327.5 e
Control	–	5.0 f	15.8 h	78.1 f

IAA Indole-3-acetic acid, *IBA* Indole-3-butyric acid, *NAA* 1-naphthaleneacetic acid

[a] RDW: Root dry weight

[b] Mean values(for RDW, mean of four replicates with an average of ten seedlings for each replicate; for sorgoleone, mean of four replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Tukey's studentized range test)

of sorgoleone in methanol was 2.6 and 2.9 times higher than that in methylene chloride and chloroform, respectively. No sorgoleone was detected upon extraction with butanol, ethyl acetate, hexane, or water.

Change in Sorgoleone Content with Seedling Age Root weight increased with seedling age for up to 30 d, but sorgoleone production decreased with age. Sorgoleone concentration was 40.1 μg mg^{-1} and 30.1 μg mg^{-1} root dry weight (RDW) in 5-and 10-d-old seedlings, respectively (Table 2). The RDW was 4.0 mg and 5.9 mg in 5-and 10-d-old seedling, respectively, and it increased up to 9.6 mg at 30 d. Although sorgoleone concentration decreased with seedling age, root weight increased with time; thus, the total amount of sorgoleone was highest in 10-d-old seedlings. The trend of increase in root growth persisted for up to 30 d, after which root growth declined.

Effect of Auxins on Sorgoleone Production Root growth and sorgoleone content varied widely with different concentrations of auxins (Figs. 1, 2). Considerable RDW and amounts of sorgoleone were observed at auxin concentrations of 0.1–5 μg ml^{-1}, after which both RDW and sorgoleone content decreased drastically. The maximum root growth and sorgoleone were observed by treatment with IBA at 5 μg ml^{-1}, followed by treatment with IAA and NAA at 1 μg ml^{-1}. Compared with the control, 5 μg ml^{-1} IBA, 1 μg ml^{-1} IAA, and 1 μg ml^{-1} NAA produced 6.1, 4.6, and 3.8 times more sorgoleone, respectively.

Effect of Hoagland Solution on Sorgoleone Production Root dry weight was higher when Hoagland solution was applied at 0 d than when the solution was applied at 4 or 8 d after seed placement (delayed application), for all strengths (Table 3). Sorgoleone content showed the same trend as RDW, except when full strength solution was applied. It decreased with delay in the application of both quarter- and half-strength. In the case of the application of full-strength Hoagland solution, sorgoleone content increased with delayed application. Half-strength solution produced maximum root growth and sorgoleone content. Compared with the control, total sorgoleone content and root biomass were 8.6 and 1.7 times higher when half-strength solution was applied at 0 d. Treatment of sorghum seeds with half-strength at 0 d produced 2.8 and 2.2 times more sorgoleone based on RDW, than treatments with full-strength and quarter-strength solution, respectively.

Combined Effect of Auxins and Hoagland Solution on Sorgoleone Production Different strengths of Hoagland solution along with the most optimal concentrations of IAA (1 μg ml^{-1}), IBA (5 μg ml^{-1}), and NAA (1 μg ml^{-1}) were applied to sorghum seeds to increase sorgoleone production. Combined treatment dramatically increased root growth and sorgoleone production (Table 4). In particular, combined application of 5 μg ml^{-1} IBA with half-strength Hoagland solution produced 14.2 times more sorgoleone than the control. Further, this combination increased sorgoleone content by 1.6 and 1.5 times as compared with the combination of half-strength Hoagland

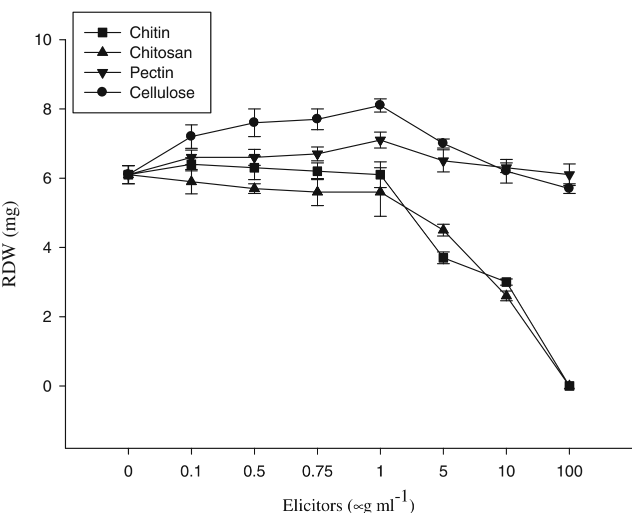


Fig. 3 Effect of elicitors on root growth of sorghum seedlings. Values are presented as mean (SD) (each point is the mean of four replicates with an average of ten seedlings for each replicate). SD: Standard deviation

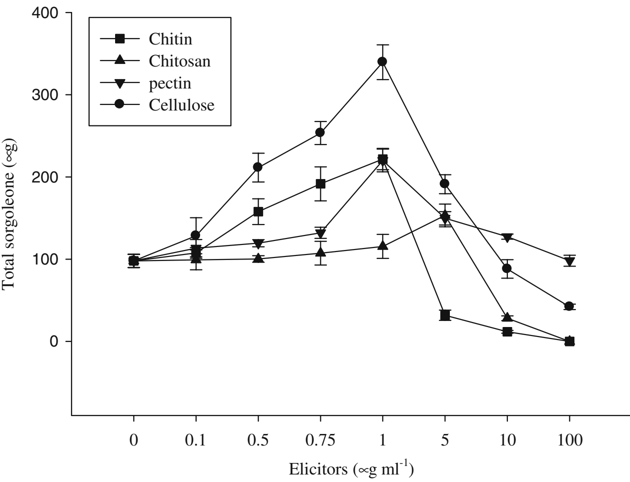


Fig. 4 Effect of different concentrations of elicitors on sorgoleone production in sorghum roots. Values are presented as mean (SD) (each point is the mean of four replicates with three samples from each replicate). SD: Standard deviation

solution with 1 μg ml^{-1} NAA and 1 μg ml^{-1} IAA, respectively.

Effect of Elicitors on Root Growth and Sorgoleone Production Four elicitors—2 of microorganism origin (chitin and chitosan) and 2 of plant origin (pectin and cellulose)—were applied at different concentrations to sorghum seeds. Chitin, pectin and cellulose produced more sorgoleone at 1 μg ml^{-1} than at other concentrations (Figs. 3, 4), while chitosan produced a higher amount at 5 μg ml^{-1} than at other concentrations. With the increase in the concentration of elicitors, sorgoleone content decreased drastically, and in the case of chitin and chitosan, the plants did not survive even at 100 μg ml^{-1} (Fig. 3). Cellulose, an elicitor of plant origin produced the highest root biomass and sorgoleone content at 1 μg ml^{-1}, followed by chitin at 1 μg ml^{-1}. Compared with the control, cellulose at 1 μg ml^{-1} produced 3.5 and 1.3 times more sorgoleone and root biomass, respectively.

Effect of Elicitors, Applied at Different Time Points During Growth, on Sorgoleone Production After seed placement, the optimal concentrations of different elicitors were applied at different time points during growth. Sorghum roots contained more sorgoleone when an elicitor was applied a few days later than when applied in the initial days of growth (Table 5). The highest amount of sorgoleone was obtained when cellulose was applied 6 d after seed placement, followed by chitin, chitosan, and pectin. Compared with the control, cellulose, chitin, chitosan, and pectin produced 6.2, 4.2, 3.3, and 3.2 times more sorgoleone, respectively.

Discussion

Methylene chloride is the most commonly used solvent (Netzly and Butler, 1986; Netzly et al., 1988; Czarnota et al., 2001; Yang et al., 2004) to extract sorgoleone from roots, although chloroform also has been used (Dayan, 2006). In this study, methanol proved to be the best solvent and extracted the maximum amount of sorgoleone, followed by methylene chloride and chloroform. Methanol extracted 2.6 times more sorgoleone than methylene chloride (Table 1).

While root weight increased, sorgoleone production decreased with the age of seedlings. Considering both root weight and sorgoleone content, 10-d-old seedlings were the best, producing the maximum amount of sorgoleone. The trend of increase in root growth persisted until 30 d after which the root growth decreased since the seedlings were

Table 5 Effect of elicitors, applied at different time points, on root growth and sorgoleone production in sorghum roots

Elicitor concentration (μg ml^{-1})	Time of application (DAS)[a]	RDW[b] (mg)	Sorgoleone concentration (μg mg^{-1} RDW)	Total sorgoleone content (μg)
Chitin (1)	0	6.1 e	36.5 g	222.7 hi
	3	6.3 cde	59.7 bc	376.1 cd
	6	6.4 cde	63.7 b	407.7 bc
	9	6.6 cde	19.8 i	130.7 k
Chitosan (5)	0	4.5 f	34.2 gh	153.9 jk
	3	5.9 e	55.2 d	325.7 def
	6	6.1 de	51.7 de	315.4 ef
	9	6.3 cde	31.4 h	197.8 ij
Pectin (1)	0	7.1 bc	30.9 h	219.4 hi
	3	7.0 bcd	42.3 f	296.1 efg
	6	6.4 cde	48.5 e	310.4 ef
	9	6.3 cde	38.3 fg	241.3 ghi
Cellulose (1)	0	8.1 a	41.9 f	339.4 de
	3	8.3 a	55.2 cd	458.2 b
	6	8.7 a	69.2 a	602.0 a
	9	7.9 ab	34.9 gh	275.7 fgh
Control		6.1 e	16.2 i	98.8 k

[a] DAS: Days after seed placement

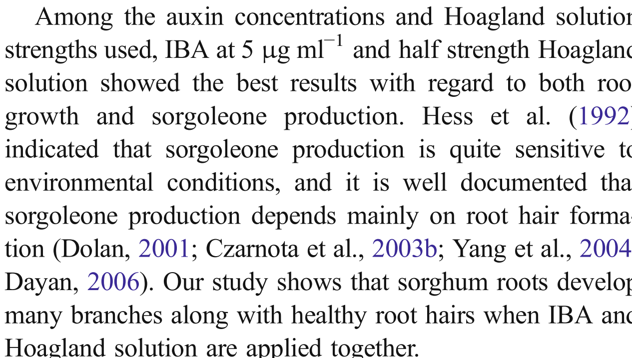
[b] RDW: Root dry weight

[c] Mean values (for RDW, mean of four replicates with an average of ten seedlings for each replicate; for sorgoleone, mean of four replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Tukey's studentized range test)

grown under hypoxic conditions. Hypoxic conditions did not impede root growth until 30 d but growth decreased thereafter; this finding was consistent with that of Yang et al. (2004) that sorghum root development is reduced under hypoxic conditions.

In our study, auxins exerted a positive effect on both root growth and sorgoleone production. Auxins govern many biological processes in plants, such as cell enlargement and division, differentiation of vascular tissue, apical dominance, root initiation, and signaling (Teale et al., 2006). Studies conducted using differentiated tissues to investigate the biochemical relationship between exogenous and endogenous auxin levels have provided interesting findings regarding root-derived biologically active compounds. Researchers investigating the physiological role of exogenously applied auxins in root growth and secondary metabolite production have established that signaling molecules can affect plant tissue stability and secondary product accumulation either individually or through interactions with phytohormones. With regard to the effects of auxins on secondary metabolite production, Bais et al. (2001) noted that high levels of exogenous auxins, specifically IAA and NAA, in the presence of low cytokinin levels, decrease the ability of root cultures of *Cichorium. intybus* to produce coumarin. Lin et al. (2003) showed that coniferin content in *Linum flavum* is increased significantly in the presence of auxins. However, Arroo et al. (1995) showed that IAA addition inhibits secondary metabolite accumulation in the hairy roots of *Tagetes patula*. In contrast, addition of either IBA or NAA stimulates ajmalicine and ajmaline production in *Rauvolfia micrantha* hairy root cultures in a hormone-free medium (Sudha et al., 2003), whereas Rhodes et al. (1994) observed a decrease in nicotine content in the hairy roots of *Nicotiana rustica* when the roots were supplied with auxins together with cytokinins. Luczkiewicz et al. (2002) discovered that the production of the sesquiterpene lactone pulchelin E is enhanced in hairy roots of *Rudbeckia hirta*, compared with that in callus and suspensions cultures, in the presence of auxins.

Czarnota et al. (2003b) confirmed that sorghum root hairs are physiologically active with a complex network of smooth endoplasmic reticulum and possibly Golgi bodies. Small globules of cytoplasmic exudates also were observed to deposit an oily material between the cell wall and the plasma membrane near the root hair tips. Our study confirms that sorgoleone production is related directly to the development of sorghum root hairs. Our positive results from using different auxins for enhanced sorgoleone production, suggests that sorghum roots benefit from developing more sorgoleone-rich root hairs. Sorgoleone production also was stimulated by Hoagland solution treatment, suggesting that sorghum roots benefit from Hoagland solution to develop healthy and vigorous roots.



Among the auxin concentrations and Hoagland solution strengths used, IBA at 5 μg ml^{-1} and half strength Hoagland solution showed the best results with regard to both root growth and sorgoleone production. Hess et al. (1992) indicated that sorgoleone production is quite sensitive to environmental conditions, and it is well documented that sorgoleone production depends mainly on root hair formation (Dolan, 2001; Czarnota et al., 2003b; Yang et al., 2004; Dayan, 2006). Our study shows that sorghum roots develop many branches along with healthy root hairs when IBA and Hoagland solution are applied together.

Stimulation of secondary metabolites by elicitation is one of the few strategies that currently is finding commercial application. Elicitors, compounds of biotic or abiotic origin, upon contact with the cells of higher plants trigger increased production of pigments, flavones, phytoalexins, and other defense related compounds (Flores and Curtis, 1992; Sim et al., 1994; Bhagyalakshmi and Bopanna, 1998; Singh, 1999). Sorgoleone production may be affected by pathogenic infections. It has antifungal properties (Suzuki et al., 1998). Initiation of sorgoleone biosynthesis by eliciting plant defense mechanisms have yielded varying results. Cellulose at 1 μg ml^{-1} significantly increased sorgoleone production, followed by chitin at 1 μg ml^{-1} (Table 5). Treatment with chitin, which is known to induce the expression of systemic acquired resistance (SAR) genes (Hahn, 1996; Zhang et al., 2002), exerted a significant effect on sorgoleone production. Treatment with chitosan and pectin also increased production. Moreover, Savitha et al. (2006) observed a positive effect of different biotic and abiotic elicitors on the production of betalain in the hairy root cultures of *Beta vulgari.*

In summary, sorgoleone production is constitutive to the physiology of mature root hairs of sorghum. Great differences in sorgoleone levels are observed during the early stages of seedling development, and sorgoleone biosynthesis is positively affected by most of the stimuli used in this study. In particular, sorghum seeds treated with half strength Hoagland solution and 5 μg ml^{-1} of IBA significantly increase root growth and sorgoleone content in grain sorghum roots.

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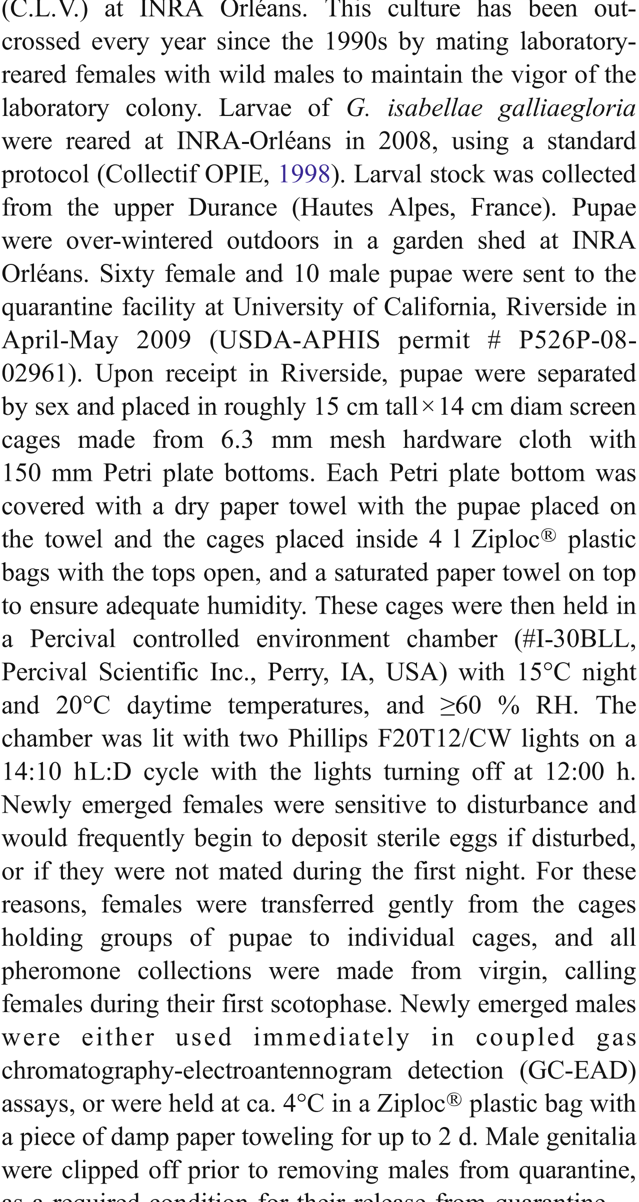
patterns, and restricted geographical distribution, it has become a symbol for several entomological and conservation organizations. It is univoltine, and adults fly at dusk from March to July in pine forests in the eastern part of Spain and the French Alps (Ylla Ullastre, 1997). *Graellsia isabellae* is protected by the Habitats Directive (the European Community initiative for an ecological network of special protected areas, known as "Natura 2000"), Bern Convention, Red Book of Lepidoptera, and Spanish and French red lists (Ministerio de Medio Ambiente, 2006; Ministère de l'Écologie et du Développement Durable, 2008). Despite this protective legislation, the latest Red list of the IUCN considered *G. isabellae* as "data deficient" in terms of its current range and population density (IUCN, 2007). The natural history and ecology of *G. isabellae* has been studied (Ylla Ullastre, 1997, Chefaoui and Lobo, 2007, 2008, Vila et al., 2009), but conservation genetic studies have just started (Vila et al., 2010), and the current distribution and conservation status of the species needs to be updated because new populations have been discovered well beyond the previously known limits of its range (Ibáñez Gázquez et al., 2008).

Previous attempts at identifying the sex pheromone of *G. isabellae* were not successful, due in part to the very small amount of pheromone produced by the insects, and exacerbated by the relatively small number of individuals that were available for analysis of the pheromone gland contents (Zagatti and Malosse, 1998). A mass rearing program produced 245 females, but the amount of pheromone in a composite extract of the dissected glands from those females still proved to be below the threshold for detection by coupled gas chromatography-mass spectrometry (GC-MS). One fragment of information obtained from these efforts was that the antennae of male moths were extremely responsive to C_{16} aldehydes (Zagatti and Malosse, 1998), indicating that the pheromone was probably an aldehyde similar to those reported from some other saturniid species (Witzgall et al., 2004, El-Sayed, 2009).

As part of a larger effort to more effectively assess population sizes and delineate the range of *G. isabellae*, we describe here the identification and synthesis of the female-produced sex attractant pheromone of this insect. The results of preliminary field trials with the pheromone have shown that it is highly attractive to male moths from a number of widely separated populations, suggesting that pheromone-based methods will be effective for detection and sampling of this protected species, and will allow detailed assessments of its conservation status.

Methods and Materials

Insects Pupae of *G. isabellae galliaegloria* Oberthür were obtained from a culture maintained by one of the authors (C.L.V.) at INRA Orléans. This culture has been outcrossed every year since the 1990s by mating laboratory-reared females with wild males to maintain the vigor of the laboratory colony. Larvae of *G. isabellae galliaegloria* were reared at INRA-Orléans in 2008, using a standard protocol (Collectif OPIE, 1998). Larval stock was collected from the upper Durance (Hautes Alpes, France). Pupae were over-wintered outdoors in a garden shed at INRA Orléans. Sixty female and 10 male pupae were sent to the quarantine facility at University of California, Riverside in April-May 2009 (USDA-APHIS permit # P526P-08-02961). Upon receipt in Riverside, pupae were separated by sex and placed in roughly 15 cm tall × 14 cm diam screen cages made from 6.3 mm mesh hardware cloth with 150 mm Petri plate bottoms. Each Petri plate bottom was covered with a dry paper towel with the pupae placed on the towel and the cages placed inside 4 l Ziploc® plastic bags with the tops open, and a saturated paper towel on top to ensure adequate humidity. These cages were then held in a Percival controlled environment chamber (#I-30BLL, Percival Scientific Inc., Perry, IA, USA) with 15°C night and 20°C daytime temperatures, and ≥60 % RH. The chamber was lit with two Phillips F20T12/CW lights on a 14:10 hL:D cycle with the lights turning off at 12:00 h. Newly emerged females were sensitive to disturbance and would frequently begin to deposit sterile eggs if disturbed, or if they were not mated during the first night. For these reasons, females were transferred gently from the cages holding groups of pupae to individual cages, and all pheromone collections were made from virgin, calling females during their first scotophase. Newly emerged males were either used immediately in coupled gas chromatography-electroantennogram detection (GC-EAD) assays, or were held at ca. 4°C in a Ziploc® plastic bag with a piece of damp paper toweling for up to 2 d. Male genitalia were clipped off prior to removing males from quarantine, as a required condition for their release from quarantine.



Solid Phase Micro-Extraction (SPME) Sampling of Sex Pheromone Glands Crude extracts of pheromone were prepared from calling females by wipe sampling of extruded glands with 100 µm PDMS SPME fibers (Supelco, Bellefonte, PA, USA). Females were held in a controlled environment chamber under the same conditions as those for emergence (see above). Approximately 1–2 h after the start of scotophase, calling females were removed from the chamber into the darkened room. The SPME device was taped to the bench top so that both hands could be used to manipulate the insect, and approximately 5–7 mm of the SPME fiber was extruded. A microscope light source controlled with a rheostat was used to illuminate the fiber, and a small flashlight with red film covering the lens was used to confirm that females were indeed calling

immediately prior to pheromone collection. Thus, a female was grasped firmly by the abdomen, with gentle pressure towards the tip of the abdomen to evert the sex pheromone gland (the scale-free segment just anterior to the ovipositor). Once exposed, this area of the abdomen was wiped gently over the SPME fiber, with all surfaces being wiped at least twice. Females then were placed individually into glassine envelopes and held in a refrigerator because they would usually begin to oviposit after this procedure. The loaded SPME fiber was analyzed by GC-EAD or GC-MS.

Solvent Extraction of Sex Pheromone Glands Two composite solvent extracts of abdomen tips of calling females were made by removing glands and rinsing them in pentane. For the first composite extract, all 6 females were extracted on the same day, whereas the second extract from 7 females was made over a 4 d period, again using females during their first scotophase. Sex pheromone glands were removed approximately 1.5–2 h into the scotophase by gently forcing eversion of the sex pheromone gland, clamping the abdomen just anterior to the gland with forceps to maintain the pressure in the gland, then slicing the gland off with a razor blade such that the gland remained inflated in the forceps. The gland then was rinsed with gentle agitation in ca. 1 ml of clean pentane for 30 sec. These extracts were analyzed by coupled GC-EAD without concentration, and by GC-MS after concentration under a gentle stream of nitrogen.

GC-EAD and GC-MS Analyses GC-EAD analyses were performed using DB-5 and DB-Wax columns (both 30 m× 0.25 mm i.d., 0.25 µm film; J&W Scientific, Folsom CA, USA). Helium was used for both the carrier and makeup gas. Temperature programs were 100°C for 1 min, 10°C per min to 275°C for DB-5 and 250°C for DB-Wax, hold for 60 min. SPME injections were made by desorbing the fiber in splitless mode for 60 sec prior to starting the run (injector temperature 250ºC). Solvent extracts (1 µl aliquots) were analyzed in splitless mode. The effluent from the columns was split using an 'X' cross, with half of the sample going to the FID detector and the other half to the EAD. The portion directed to the EAD was diluted in a humidified air stream prior to exposure to the antenna. Males used in GC-EAD analyses were removed from quarantine after the genitalia had been removed, allowed to warm to room temperature, and then a single antenna was removed by pulling it off at the scape. The terminal rami were removed with a razor blade, and the tip was excised to ensure good contact with the saline solution (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 l Milli-Q purified water) in the glass electrodes, which were fitted with an internal gold wire for connection to the amplifier. A single antennal preparation was used for as many as four runs. Kovat's indices were calculated for unknowns and standards relative to blends of straight-chain hydrocarbons.

Syntheses of Authentic Standards The syntheses of mixtures of (4*E*/*Z*, 6*E*,11*E*)- and (4*E*/*Z*,6*E*,11*Z*)-hexadecatrienals are described in the supporting online information.

Tetrahydrofuran was purified by distillation from sodium-benzophenone ketyl under argon. Unless otherwise specified, solutions of crude products were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by flash or vacuum flash chromatography on 230–400 mesh silica gel. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-400 spectrometer (Palo Alto, CA, USA) (400 and 100.5 MHz, respectively), as $CDCl_3$ solutions unless otherwise stated. Solvent extracts of reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Integer resolution mass spectra were obtained with a Hewlett-Packard (HP) 6890 GC (Avondale PA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium carrier gas. The GC was equipped with a DB5-MS column (25 m×0.20 mm ID×0.25 µ film).

4-Nonyn-1-ol (**3**) A solution of THP-protected 4-pentyn-1-ol **1** (5.04 g, 30 mmol) and 25 mg triphenylmethane as an indicator in 50 ml THF was cooled to 0ºC, and treated dropwise with n-BuLi (2.56 M in hexanes, ~12.5 ml) until the solution began to turn orange-red, indicative of excess *n*-BuLi. The solution was stirred 20 min, the ice bath was removed, and butyl iodide (6.44 g, 35 mmol) was added by syringe over 5 min. The solution was refluxed 44 h under Ar, then quenched by pouring into cold 1 M NH_4Cl. The mixture was extracted with hexane, and the hexane layer was washed with saturated brine, then dried and concentrated. The residue was purified by Kugelrohr distillation (oven temp ~105ºC, 0.05 mm Hg), yielding the protected alkynol **2** as a colorless oil (5.6 g, 83%). The product was dissolved in 20 ml MeOH, 0.25 g of *para*-toluenesulphonic acid (PTSA) was added, and the solution was stirred overnight at room temperature. The solution then was poured into 100 ml of 0.5 M aqueous NaOH, and the mixture was extracted 3 times with hexane. The combined hexane extracts were washed with brine, dried, and concentrated. The residue was purified by Kugelrohr distillation, taking off a preliminary fraction containing THP-protected MeOH (bp<40ºC at 1 mm Hg), followed by distillation of the remaining 4-nonyn-1-ol **3** (3.13 g, 94%; oven temp<100ºC at 1 mm Hg). ^{1}H NMR: δ 3.76 (t, 2H, J=6.2 Hz), 2.26–2.31 (tt, 2H, J=7.2, 2.4 Hz), 2.12–2.17 (m, 2H), 1.74 (overlapped tt, 2H, J~7, 7 Hz), 1.67 (br. s, OH), 1.35–1.5 (m, 4H), 0.91 (t, 3H, J=7.2 Hz). ^{13}C NMR: δ 13.8, 15.7, 18.6, 22.2, 31.3, 31.8, 62.3, 79.4, 81.3 ppm.

The ^{13}C spectrum matched that previously reported (Poleschner and Heidenreich, 1995).

(4Z)-Nonen-1-ol (**4**) A 1 M solution of $NaBH_4$ was prepared by dissolving 0.4 g $NaBH_4$ in a mixture of 9.5 ml EtOH and 0.5 ml 2 M aqueous NaOH, and the slightly cloudy solution was filtered. In a separate flask, a solution of $NiOAc_2$•4 H_2O (1.0 g, 4 mmol) was prepared in 50 ml of degassed 95% EtOH under Ar, giving a clear green solution. A balloon of hydrogen gas was attached to the flask, the Ar line was removed, and with vigorous stirring, 1 ml of the $NaBH_4$ solution (1 mmol) was added dropwise, producing a black suspension with the evolution of H_2. The mixture was stirred 10 min, then 0.8 ml of ethylenediamine was added. After stirring an additional 10 min, 4-nonyn-1-ol **3** (2.90 g, 21.4 mmol) was added by syringe. The mixture was stirred until all the starting material had been consumed (3 h), then flushed with Ar, and filtered with suction through a pad of Celite®, rinsing well with EtOH. The violet-colored solution was concentrated to ~10 ml, then poured into 100 ml 1 M HCl and extracted 3 times with hexane. The combined hexane layers were washed with saturated aqueous $NaHCO_3$ and brine, then dried and concentrated. The residue was purified by Kugelrohr distillation (oven temp to 75ºC, 0.5 mm Hg), yielding (4*Z*)-nonen-1-ol **4** (2.76 g, 91%) as a colorless oil. ^1H NMR: δ 5.39 (m, 2H), 3.66 (t, 2H, J=6.4 Hz), 2.13 (m, 2H), 2.06 (m, 2H), 1.64 (overlapped dt, 2H, J~6.5 Hz), 1.49 (br. s, OH), 1.28–1.36 (m, 4H), 0.90 (t, 3H, J=7.2 Hz). ^{13}C NMR: δ 14.2, 22.5, 23.8, 27.1, 32.1, 32.9, 62.9, 129.0, 121.0 ppm. The ^1H NMR data agreed with that previously reported (Joshi et al., 1984).

(4Z)-1-Bromononene (5) Methanesulphonyl chloride (1.89 g, 16.5 mmol) was added dropwise to a solution of (4*Z*)-nonen-1-ol **4** (2.13 g, 15 mmol) and Et_3N (2.02 g, 20 mmol) in 50 ml CH_2Cl_2 at 0ºC. The resulting slurry was stirred at 0ºC for 2.5 h, then poured into ice-water. The organic layer was separated and washed with 1 M HCl, saturated aqueous $NaHCO_3$, and brine, then dried and concentrated. The residue was taken up in 40 ml acetone, LiBr (3.9 g, 45 mmol) was added, and the mixture was stirred at 50ºC overnight, by which time all the mesylate had been consumed. The slurry was cooled and poured into 250 ml water, and the product was extracted with hexane. The hexane layer was washed with brine, dried, and concentrated, and the residue was purified by Kugelrohr distillation (oven temp ≤75ºC at 5.5 mm Hg), yielding the bromide **5** (2.76 g, 74%) as a colorless oil. ^1H NMR: δ 5.45 (dtt, 1H, J=10.2, 7.2, 1.4 Hz), 5.31 (dtt, 1H, J= 10.8, 7.6, 1.6 Hz), 3.42 (t, 2H, J=6.6 Hz), 2.20 (m, 2H), 2.06 (m, 2H), 1.92 (overlapped tt, 2H, J~7 Hz), 1.31–1.37 (m, 4H), 0.91 (t, 3H, J=6.8 Hz). ^{13}C NMR: δ 14.2, 22.5, 25.9, 27.2, 32.1, 32.9, 33.6, 127.6, 132.0 ppm. The ^1H and ^{13}C NMR data were in agreement with those previously reported (Ducoux et al., 1992).

(6Z)-Undecen-1-yne (**6**) A dry flask under Ar was loaded with lithium acetylide-ethylenediamine complex (0.92 g, 10 mmol), and dry DMSO (10 ml) and NaI (150 mg, 1 mmol) were added, followed by dropwise addition of (4*Z*)-1-bromononene **5** (1.44 g, 7 mmol). The mixture was stirred 1 h at room temperature, then quenched by pouring into 100 ml water. The mixture was extracted twice with hexane, and the combined hexane layers were washed with brine, dried, and concentrated. The residue was purified by Kugelrohr distillation (oven temp ~80ºC, 12 mm Hg), giving 0.87 g (83%) of (6*Z*)-undecen-1-yne **6** as a colorless oil. ^1H NMR: δ 5.42 (dtt, 1H, J=10.8, 7.2, 1.2 Hz), 5.33 (dtt, 1H, J=10.8, 7.2, 1.2 Hz), 2.20 (td, 2H, J=7.0, 2.4 Hz), 2.16 (overlapped td, 2H, J~7.2 Hz), 2.05 (m, 2H), 1.96 (t, 1H, J=2.4 Hz), 1.59 (overlapped tt, 2H, J~7.4 Hz), 1.34 (m, 2H), 0.91 (t, 3H, J=7.0 Hz). ^{13}C NMR: δ 14.2, 18.1, 22.5, 26.4, 27.1, 28.7, 32.1, 68.4, 84.7, 128.5, 131.3 ppm. The ^1H and ^{13}C NMR spectra agreed with those previously reported (Asao et al., 2005).

(4E,6E,11Z)-Hexadecatrien-1-ol (**11**) A dry flask was charged with bis(cyclopentadienyl)-zirconium hydridochloride (1.2 g, 4.65 mmol; Alfa Aesar, Ward Hill MA, USA) and flushed thoroughly with Ar. The flask was cooled in an ice-bath, THF was added, and the slurry was stirred 15 min, followed by dropwise addition of (6*Z*)-undecen-1-yne **6** (0.60 g, 4 mmol). The mixture was shielded from light, and stirred at 0ºC for 20 min, then warmed to room temperature and stirred for 3.5 h. The resulting solution was then cooled to 0ºC again, and a solution of iodine (1.07 g, 4.2 mmol) in 5 ml THF was added dropwise until the brown color of excess iodine persisted. The solution was stirred at 0ºC for 45 min, then warmed to room temperature and quenched by pouring into 50 ml of 2 M sodium thiosulphate solution. Hexane (100 ml) was added, and the slurry was mixed thoroughly, then filtered with suction through a pad of Celite®. The organic layer was separated and washed sequentially with sodium thiosulphate solution and brine, then dried and concentrated. The residue was purified by vacuum flash chromatography on silica gel, eluting with pentane. The purified (1*Z*,6*Z*)-1-iodoundecadiene **7**, contaminated with (6*Z*)-1,6-undecadiene (from reduction instead of iodination of the zirconium intermediate), was concentrated and used immediately in the next step. ^1H NMR: δ 6.52 (dt, 1H, J= 14.4, 7.2 Hz), 5.99 (dt, 1H, J=14.4, 1.4 Hz), 5.36 (m, 2H), 1.99–2.1 (m, 6H), 1.46 (overlapped tt, 2H, J~7.4 Hz), 1.30–1.37 (m, 4H), 0.91 (t, 3H, J=7.2 Hz).

A dry flask was charged with bis(cyclopentadienyl) zirconium hydridochloride (1.7 g, 6.6 mmol) and evacuated

and refilled with Ar 5 times. The flask was cooled to~ −15ºC in an ice-salt bath, and 7.5 ml of dry CH_2Cl_2 were added. The mixture was stirred 15 min, then *t*-butyldimethylsilyl-protected 4-pentyn-1-ol **8** (1.19 g, 6 mmol) was added dropwise. The mixture was stirred 30 min, warmed to 0ºC and stirred 30 min, then warmed to room temperature and stirred 30 min. The solvent then was removed under vacuum, and the alkenylzirconium intermediate **9** was taken up in 15 ml THF. A solution of anhydrous $ZnCl_2$ (0.94 g, 7 mmol) in 10 ml THF was added, and the solution was cooled to −78ºC and degassed under vacuum, then warmed to 0ºC, followed by addition of a solution of (1*Z*,6*Z*)-1-iodoundecadiene **7** (0.83 g, ~3 mmol) and tetrakis(triphenylphosphine)palladium in 10 ml THF. The mixture was stirred for 1 h, warmed to room temperature, and stirred until the iodide had been completed consumed (~2 h). The reaction was quenched by pouring into 50 ml saturated NH_4Cl, and the resulting mixture was extracted twice with hexane. The combined hexane extracts were washed with brine, and treated with anhydrous Na_2SO_4 and decolorizing charcoal for 1 h with stirring. The mixture was filtered with suction through a pad of Celite®, then concentrated. The residue was purified by vacuum flash chromatography on silica gel, eluting with 5% EtOAc in hexane. The purified product **10** was concentrated, and the residue was stirred in a mixture of 6 ml THF, 6 ml AcOH, and 3 ml H_2O at room temperature overnight to hydrolyze the silyl protecting group. The mixture then was poured into 100 ml water and extracted with 75 ml hexane. The hexane layer was washed sequentially with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue was purified by vacuum flash chromatography, eluting with 15% EtOAc in hexane, yielding (4*E*,6*E*,11*Z*)-hexadecatrien-1-ol **11** (0.57 g, 80%, 96% isomerically pure by GC). Attempted recrystallization from hexane at −20ºC was not successful, and so the product was Kugelrohr distilled (0.55 g, 78%; bp~110ºC, 0.1 mm Hg) to remove traces of silica gel. ^{1}H NMR: δ 6.04 (m, 2H), 5.58 (m, 2H), 5.36 (m, 2H), 3.67 (t, 2H, J=6.8 Hz), 2.16 (m, 2H), 2.0–2.1 (m, 6H), 1.67 (overlapped tt, 2H, J~7 Hz), 1.4–1.48 (m, 2H), 1.29–1.36 (m, 4H), 0.90 (t, 3H, J=7 Hz). ^{13}C NMR: δ 14.2, 22.6, 26.9, 27.1, 29.1, 29.6, 32.2, 32.3, 32.5, 62.7, 129.6, 130.5 (2C), 131.2, 131.4, 132.8 ppm. The spectra agreed with those previously reported (Tomida and Fuse, 1993).

(4E,6E,11Z)-Hexadecatrienal (**12**) Oxalyl chloride (0.13 ml, 1.5 mmol) was added by syringe to 3 ml of dry CH_2Cl_2 cooled under Ar to −78ºC in a dry-ice acetone bath. A solution of dry dimethylsulfoxide in CH_2Cl_2 (0.225 ml, 3.36 mmol in 1 ml) was added dropwise over 15 min. The mixture was stirred 20 min, then (4*E*,6*E*,11*Z*)-hexadecatrien-1-ol **11** (0.236 g, 1 mmol) in 1 ml of CH_2Cl_2 was added over 5 min, and the mixture was slowly warmed to~ −45ºC over 30 min, and stirred at that temperature for 30 min. The mixture was cooled to −78ºC again, 1 ml of Et_3N was added dropwise, and the resulting mixture was slowly warmed to room temperature over ~1 h. The mixture then was poured into ice-water and extracted with hexane. The hexane layer was washed successively with saturated $NaHCO_3$ and brine, then applied directly to a column of silica gel (20 ml), eluting the column with 10% EtOAc in hexane. Fractions containing the aldehyde were combined and purified further by Kugelrohr distillation, yielding the aldehyde as a pale yellow liquid (171 mg, 73%). The aldehyde was diluted immediately with hexane to a 5% solution, ~5 mg BHT were added as stabilizer, and the solution was sealed in brown glass ampoules flushed with nitrogen to minimize degradation. NMR spectra were run in deuteroacetone to minimize problems with acid-catalyzed trimerization of the long-chain aldehyde that might result from traces of DCl in the typical NMR solvent, $CDCl_3$. ^{1}H NMR (deuteroacetone): δ 9.71 (t, 3H, J=1.6 Hz), 5.95–6.08 (m, 2H), 5.53–5.62 (m, 2H), 5.29–5.39 (m, 2H), 2.51 (br. t, 2H, J~6.8 Hz), 2.34 (br. quart., 2H, J ~ 6.9 Hz), 2.0–2.1 (m, 6H, partially obscured by deuteroacetone peak), 1.38–1.45 (m, 2H), 1.24–1.34 (m, 4H), 0.87 (t, 3H, J=7.2 Hz). ^{13}C NMR: δ 13.56, 22.28, 25.06, 26.59, 26.85, 31.99, 32.04, 43.10, 129.47, 130.17 (2C), 130.73, 131.52, 132.69, 201.63 ppm. One carbon signal appeared to be obscured by the deuteroacetone signals from 28.6–29.7 ppm. The NMR spectra were in general agreement with those previously reported in $CDCl_3$ solvent (Tomida and Fuse, 1993). MS (EI, 70 EV): 234 (M^+, 2), 216 (1), 190 (7), 177 (6), 163 (7), 159 (9), 150 (23), 134 (38), 121 (38), 119 (47), 107 (26), 95 (51), 93 (53), 91 (48), 81 (59), 79 (100), 67 (86), 55 (55), 41 (57).

Field Trials

Lures were prepared at INRA Orléans from 11-mm red rubber septum lures (Wheaton Scientific, Millville, NJ, USA) loaded with heptane solutions (100 µl, 1 mg/ml) of the synthesized pheromone that had been shipped by courier to INRA from UC Riverside. Loaded septa were placed in a 20-ml glass vial and kept in a freezer or an ice-chest when not in use. Five different experiments were set up to test the efficiency of the lures and compare the attraction of male moths to pheromone lures, light traps, or calling females:

Experiment 1

Lures were tested on 19 May 2009 in the Spanish Pyrenees (San Juan de la Pena, Huesca province, 42.51675°N, 0.69056°W, 1100 m above sea level [a.s.l.]). Two

pheromone-treated or untreated control septa were placed in one mesh cage [16 cm H×32 cmW×16 cm D]. In total, 4 control and 4 treatment cages (a total of 16 septa) were suspended from branches of *Pinus sylvestris,* one of the moth's main host plants, ~0.5 m above ground. Cages were spaced approximately 2 m apart, and were monitored from 21:30 till 23:00 (males mostly fly at dusk). The temperature ranged from 25.4°C at the beginning of the experiment to 11.4°C at the end. Responding males were collected with a net, tissues were sampled non-lethally for DNA analyses, as part of a conservation genetics study, and adults were then released (Vila et al., 2010).

At the same time, we set up a light trap consisting of a night collecting sheet (Bioquip, CA, USA) with a 175 W mercury vapor light mounted on a tripod on one side of the sheet. The light trap was placed 280 m away from the lures (42.51824°N, 0.69341°W, 952 ma.s.l.).

Experiment 2

On 20 May 2009, lures were tested in the Spanish Central Sierra of Guadarrama (Dehesas de Cercedilla 40.76768°N, 4.07006°W, 1375 ma.s.l.). The experimental design was the same as described above, but temperature ranged from 21.9°C to 15.5°C. At the same time, we set up a light trap and 2 calling females (2 d old) in one cage one km away from the lures (40.75798°N, 4.07148°W, 1300 ma.s.l.).

Experiment 3

On 27 and 28 May 2009, lures were tested in the Swiss Alps (Schallberg, Canton du Valais, 46° 29.594′N, 8° 02.581′E, 1364 ma.s.l.). A single pheromone treated septum was nailed to the trunk of a *P. sylvestris* tree 1.5 m above the ground. We placed a total of 7 unmated females a few days old in 5 cages at approximately 15 m from the lure. The experiment started at 21:30 and finished at 23:00. On May 27, temperature was 11ºC when the experiment started and 4ºC at the end. On May 28, temperature was 11ºC at the beginning, and 6ºC when the experiment was terminated.

Experiment 4

On 3 June 2009, we set up lures at the Spanish Natural Park of Puebla de San Miguel (Valencia province, 40.04325°N, 1.06284°W, 1717 ma.s.l.). A single pheromone treated septum was nailed to the trunk of a *P. sylvestris* tree, 1.5 m from the ground. We set a light trap (one 175 W lamp) approximately 140 m from the lure. We monitored both lures and light trap from 21:30 till 23:00. Males were collected with a net, tissues were non-lethally sampled for DNA analyses, and adults were then released.

Experiment 5

On 11 June 2009, we nailed two pheromone treated septa to two pine trees 800 m apart, in the same area as experiment 4 (40.07914°N 1.10293°W and 40.07209°N 1.10473°W). In addition, we set a sticky trap baited with an untreated septum (control), nailed to a pine trunk on the same pine tree used for experiment 4. We simultaneously placed the lures and the control 1.5 m above the ground at 21:30. Different observers monitored the septa until 23:00.

Results

Identification of the Pheromone Analyses of SPME wipe samples and solvent extracts of dissected pheromone glands by GC-EAD showed that only a single trace component in the extracts elicited responses from antennae of male moths (Fig. 1). This compound had a Kovats index (KI) of 1861 on a relatively nonpolar DB-5 column, and 2383 on a polar DB-WAX column. In GC-MS analyses, the active component showed a small molecular ion at *m/z* 234 (1% of base peak), and a base peak at *m/z* 79, for a possible molecular formula of $C_{16}H_{26}O$, consistent with a C_{16} trienal. Comparison of its KI values with those of model compounds suggested that the compound might contain a conjugated diene and an isolated carbon-carbon double bond. Specifically, the KI of the model compound (6*E*,11*Z*)-hexadecadienal was 80 KI units less than that of the unknown on the DB-5 column, suggesting that at least one of the double bonds was conjugated either with another carbon-carbon double bond or with the aldehyde. Furthermore, the KI values of the model compound (5*Z*,7*Z*,11*Z*)-

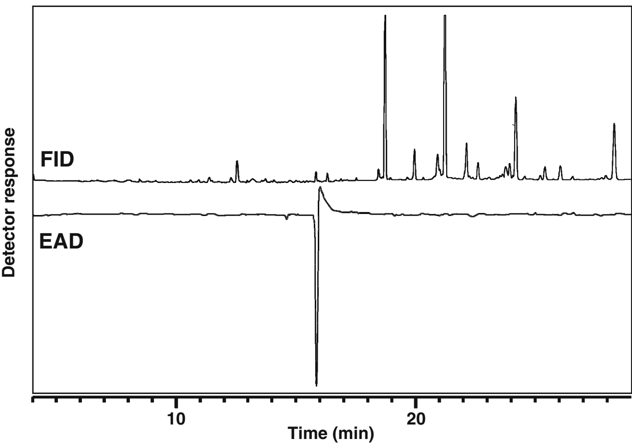


Fig. 1 Coupled gas chromatography-electroantennogram analysis of a SPME wipe sample of everted pheromone gland of a female *Graellsia isabellae*. Top trace is the GC trace; bottom, inverted trace is the response from the male moth antenna. DB-5 column, 100ºC/1 min, 10ºC/min to 275ºC for 60 min

hexadecatrienal (1855 on DB-5 and 2383 on DB-WAX) were similar to those of the unknown, providing support for a C_{16} trienal with a conjugated diene structure. An attempt to determine the position of the diene by derivatization with MTAD failed due to the limited amount of pheromone gland extract available, and the trace amount of pheromone in the extracts. Furthermore, the much stronger responses of antennae of male moths to model compounds with aldehyde vs. alcohol functional groups provided corroborating evidence for the presence of a C_{16} trienal rather than the corresponding tetraenol that would be required by the molecular weight of *m/z* 234.

From the limited number of pheromones known from moths in the same subfamily (Saturniinae), two species (*Antheraea polyphemus* and *A. pernyi*) had been shown to produce (6*E*,11*Z*)-hexadecadienal as a pheromone component (Kochansky et al., 1975, Bestmann et al., 1987, respectively), and a third species, the Ailanthus silkmoth *Samia cynthia ricini*, produced (4*E*,6*E*,11*Z*)-hexadecatrienal (Bestmann et al., 1989). Thus, with no other hard information as to the positions and geometries of the double bonds in the unknown, we synthesized four of the eight possible isomers of this compound. The retention indices of three of these four isomers on the DB-5 column did not match that of the unknown, excluding them from further consideration (KI values: 4*E*,6*E*,11*E*: 1840; 4*Z*,6*E*,11*E*, 1871; 4*Z*,6*E*,11*Z*, 1837). However, the retention time of synthetic (4*E*,6*E*,11*Z*)-hexadecatrienal exactly matched that of the unknown on both the DB-5 and the DB-WAX columns, and the mass spectra of the synthetic and natural compounds were also a good match, providing a tentative identification of the pheromone as (4*E*,6*E*,11*Z*)-hexadecatrienal. Further confirmation of this identification was obtained from field bioassays (see below).

Synthesis of (4E,6E,11Z)-hexadecatrienal Four of the eight possible isomers of 4,6,11-hexadecatrienal were synthesized as isomeric pairs, using Wittig reactions to assemble the conjugated diene structures (see Supplementary Online Information). Having determined that the (4*E*,6*E*,11*Z*)-isomer matched the insect-produced compound in all respects, we then developed a route to that specific isomer (Fig. 2). A key starting material, 4-nonyn-1-ol, was not commercially available, and so it was assembled by alkylation of THP-protected 4-pentyn-1-ol with butyl iodide (83%) (Buck and Chong, 2001). After deprotection (94%), the resulting alkynol **3** was stereoselectively reduced to (*Z*)-4-nonen-1-ol with P-2 nickel and hydrogen, and then converted to (*Z*)-4-nonenyl bromide **5** by treatment with methanesulphonyl chloride and pyridine followed by LiBr in acetone (67% over 2 steps). Reaction of bromide 5 with lithium acetylide-ethylene diamine complex in DMSO (Sonnet and Heath, 1980) then produced enyne **6** (83%). Treatment with bis (dicyclopentadienyl)zirconium hydridochloride followed by iodine (Zeng et al. 2004) stereospecifically gave the key (*E*)-vinyl iodide **7**. A second application of hydridozirconation to *t*-butyldimethylsilyl-protected 4-pentyn-1-ol **8** gave the alkenylzirconium intermediate **9**. This was converted *in situ* to the corresponding zinc organometallic, and palladium-catalyzed coupling of this intermediate with iodide **9** completed the assembly of the *E*,*E*-conjugated diene system (Ribe et al., 2000). Removal of the alcohol's *t*-butyldimethylsilyl protecting group in aqueous acid gave trienol **11** in 78% yield from enyne **6**. Swern oxidation of alcohol **11** completed the synthesis, and after purification, the resulting aldehyde was immediately diluted in hexane and sealed in brown ampoules to minimize degradation.

Fig. 2 Stereoselective synthesis of (4*E*,6*E*,11*Z*)-hexadecatrienal

Field Bioassays

Experiment 1

A total of 20 males were attracted to the treatment cages, whereas no males were attracted to cages containing untreated control septa. Cages with pheromone-treated septa attracted males from 21:48 till 22:30. No males were attracted with the light trap.

Experiment 2

A total of 11 males were attracted by the treated lures. No males were attracted by the controls, by the light trap, or by caged calling females.

Experiment 3

During the course of this experiment conducted over two successive evenings, no males flew towards the caged females; all males observed flew rapidly to the pheromone lures. On May 27, we collected 10 males (between 21:36 and 22:30), all of which were marked by writing a number in the upper-side right forewing, then released at 23:00. On May 28, we collected 34 males, seven of which were recaptures from the previous evening. The first male (non-recaptured) arrived only one min after setting out the pheromone lure.

Experiment 4

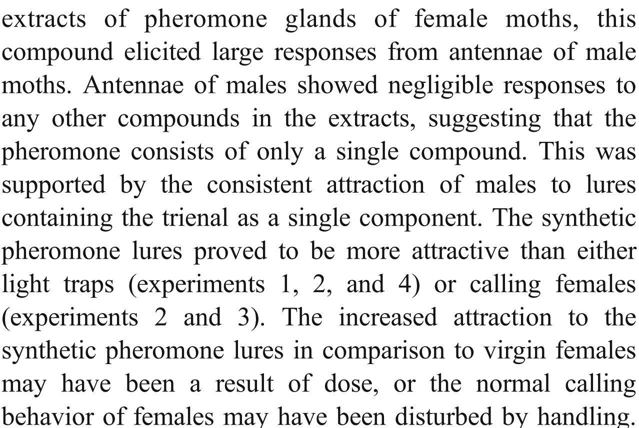
We collected 48 males attracted by the pheromone lure, whereas only one male was attracted by the light trap. Males showed no hesitation in approaching the lure, indicating that the single component was both necessary and sufficient to obtain attraction.

Experiment 5

Four males were attracted by the lure set at site 40.07209°N 1.10473°W, and seventeen males were attracted to the lure deployed at site 40.07914°N 1.10293°W. Interestingly, individuals flew off after a few minutes. No males came to the control lure.

Discussion

As suggested by the strong EAG responses elicited by aldehyde standards in preliminary work by Zagatti and Malosse (1998), the pheromone of *G. isabellae* proved to be a triunsaturated aldehyde, (4*E*,6*E*,11*Z*)-hexadecatrienal. In GC-EAD analyses of SPME wipe samples or solvent extracts of pheromone glands of female moths, this compound elicited large responses from antennae of male moths. Antennae of males showed negligible responses to any other compounds in the extracts, suggesting that the pheromone consists of only a single compound. This was supported by the consistent attraction of males to lures containing the trienal as a single component. The synthetic pheromone lures proved to be more attractive than either light traps (experiments 1, 2, and 4) or calling females (experiments 2 and 3). The increased attraction to the synthetic pheromone lures in comparison to virgin females may have been a result of dose, or the normal calling behavior of females may have been disturbed by handling.



We often observed the first male/s flying readily to the lures a few minutes (1–5) after deploying the septa. In experiments where the septa were nailed to the trunk of a tree, males stayed on the septum itself most of the time, or walked and flew close by or on the tree trunk before flying off a few minutes later. The fact that males that were initially attracted to lures abandoned their search for a female and flew off after a few minutes suggests that other, short-range signals such as contact sex pheromones may be required in order to elicit the full sequence of mating behaviors and/or that males that cannot quickly find the pheromone source (i.e., a female) allocate further efforts to searching for a more accessible mate.

(4*E*,6*E*,11*Z*)-Hexadecatrienal had been previously reported as a pheromone component for another saturniid species, the Ailanthus silkmoth *Samia cynthia ricini* (Bestmann et al., 1989), and the related dienal, (6*E*,11*Z*)-hexadecadienal had been found in other members of the family (*Antherea polyphemus*, Kochansky et al., 1975; *Antherea pernyi* Bestmann et al., 1987). Although (4*E*,6*E*,11*Z*)-hexadecatrienal had been synthesized previously, all reported syntheses used reactions with low stereoselectivity, such as Wittig reactions (Bestmann et al., 1989, Tomida and Fuse, 1993) or Claisen orthoester rearrangements (Singh et al., 1992). Thus, we developed an improved synthesis based on zirconium and zinc organometallics, with a key step being the palladium-catalyzed coupling reaction between alkenylzirconium intermediate **9** and vinyl iodide **7**. In our hands, this proved to be highly stereoselective, giving the desired product in 96% isomeric purity.

Although the pheromone was identified from females belonging to subspecies *G. isabellae galliaegloria* (French Alps), the synthesized pheromone attracted males from a total of three subspecies: *G. isabella paradisea* Marten (experiment 1: Central Spanish Pyrenees), *G. i. isabellae* Graells (experiment 2: Sierra Guadarrama; experiments 4&5: Valencia), and *G. i. galliaegloria* (experiment 3: Swiss Alps).

Previous to this work, field surveys for this species had been carried out using light traps and calling female moths. The identification and testing of the pheromone described

here has demonstrated how the pheromone can be used to dramatically increase the sensitivity of survey methods while simultaneously decreasing the time and effort required to carry out surveys. Having the pheromone in hand will allow us to survey areas where the species is potentially present (Chefaoui and Lobo, 2007, 2008), demonstrate absence with far greater certainty than previously possible, and identify isolated populations of potentially high conservation value.

The success of this project also has shown that problems associated with working with the pheromones of rare or endangered lepidopteran species are manageable, for several reasons. First, the extensive literature on lepidopteran pheromones allows predictions as to likely pheromone structures based on the taxonomic placement of species. Second, with care, it is possible to collect pheromone samples by SPME wipe sampling with no damage to calling females, so that the females can be used subsequently in captive breeding programs. Alternatively, if it is necessary to make solvent extracts of pheromone glands, the sensitivity of current analytical instruments requires that only a few females be sacrificed. Third, field surveys can be carried out with observers or video cameras simply watching pheromone lures, with no insects being caught, so surveys should have minimal impact on moth populations. If responding males are indeed caught with nets, if done carefully, the males sustain minimal damage, as evidenced by our being able to use males captured in this way for mark-recapture studies. For all these reasons, the identification and use of pheromones would seem to be a practical and efficient method of detecting and monitoring rare and endangered species.

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Male-Specific Cuticular Compounds of the Six *Drosophila paulistorum* Semispecies: Structural Identification and Mating Effect

Taina H. Chao · Lee Ehrman · Adrianna Permaul · Rachel Vincent · Lana Sattaur · Dan Brandt

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Abstract We have identified cuticular pheromones that sustain the integrity of the six *Drosophila paulistorum* semispecies. Hexane extracts of male and female cuticles were separated on a silica gel column and analyzed using gas chromatography/mass spectrometry. Both sexes of each of the six semispecies have the same fifteen major cuticular components, all hydrocarbons ranging from C31 to C37. However, all males have four additional ester compounds. Bioassay observations showed that this four-component ester complex imparts a strong anti-aphrodisiac effect on intra-semispecific mating behavior, thus confirming its pheromonal role. The three major ester components are methyl (Z)-9-tetradecenoate ($C_{15}H_{28}O_2$), 11-docosenyl acetate ($C_{24}H_{46}O_2$), and 19-triacontenyl acetate ($C_{32}H_{62}O_2$). The fourth ester is a di-unsaturated acetate with molecular formula $C_{32}H_{60}O_2$, but the positions of unsaturation have not been determined. Bioassays indicate that the male-specific complex of the transitional semispecies, the relict ancestor, imparts anti-aphrodisiac effects on the other semispecies as well, but effectiveness decreases with phylogenetic distances. Across the six semispecies, the male-specific compounds are the same, but vary quantitatively. Apparently, the quantitative differences among these incipient species act efficiently to preclude hybridization in nature. Because *Drosophila paulistorum* is a cluster of incipient species, this opportunity to observe pheromonal influences on speciation is unique.

T. H. Chao (✉) · L. Ehrman (✉) · A. Permaul · R. Vincent · L. Sattaur · D. Brandt
School of Natural and Social Sciences,
Purchase College, State University of New York,
Purchase, NY 10577, USA
e-mail: Taina.Chao@purchase.edu
e-mail: Lee.Ehrman@purchase.edu

Key Words *Drosophila paulistorum* · Male pheromone · Cuticular hydrocarbon · Methyl (Z)-9-tetradecenoate · 11-docosenyl acetate · 19-triacontenyl acetate

Introduction

Extensive literature exists concerning the biology of the *Drosophila paulistorum* superspecies (Ehrman, 1965; Ehrman and Powell, 1982), because this complex represents unique evolutionary material caught in *statu nascendi* (Dobzhansky and Spassky, 1959). *Drosophila paulistorum* is a species complex consisting of six semispecies, native to South and Central America. These units do not interbreed in nature, where they are partially separated by their geographical distribution, as their names imply: Andean-Brazilian (AB), Amazonian (AM), Centroamerican (CA), Interior (IN), Orinocan (OR), and Transitional (TR). The TR semispecies is thought to be the relict ancestral one, and is confined to Colombia. The semispecies are morphologically indistinguishable, but can be identified by allozymes, chromosomes, courtship behaviors, and hybridization only in the laboratory. The phylogenetic order of the six semispecies is: TR, IN, CA, OR, AB, AM, as proposed by Gleason et al. (1998) based upon gene sequencing. The semispecies coexist sympatrically over parts of their different geographic distributions in nature, and many degrees of social isolation among them have been detected. In addition to geographical isolation, there are three intrinsic isolating mechanisms: sexual isolation, hybrid inviability, and hybrid male sterility owing to the presence of endosymbiotic microbes (Ehrman et al., 1995). Sexual isolation is the most important of all three, and operates efficiently in nature. Here, we concentrated on the chemicals that foster intra-semispecific, fertile matings via contact/olfactory discrimi-

nation afforded by pheromonal compounds found on insect cuticles.

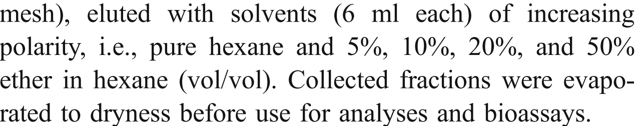
In our earlier brief communication concerning *D. paulistorum* cuticular compounds (Chao et al., 2001), we noted the presence of two male-specific cuticular compounds, methyl (Z)-9-tetradecenoate ($C_{15}H_{28}O_2$) and 11-docosenyl acetate ($C_{24}H_{46}O_2$), and their anti-aphrodisiac properties. These male-specific pheromonal compounds increase in quantity as flies age, and this increase is greatest between the ages 2 days to 3 days, the age of sexual maturity. Kim et al. (2004) studied the influences of social experience on the production of two major cuticular compounds by *D. paulistorum* males, 11-docosenyl acetate, one of the male-specific compounds, and 2-methyltriacontane, a compound common in both male and female species, concluding that socially-isolated flies possessed higher quantities of both compounds compared to communally raised ones. Neither of the above two articles reported the detailed chemical structure characterization of cuticular compounds. More recently, using different GC conditions, we discovered two additional male-specific components, as well as several clusters of higher molecular weight hydrocarbons, which were not revealed in the two earlier studies. In this paper we report the chemical characterizations and quantitative comparisons of these male-specific components, as well as their effects on inter- (sterile) and intra- (fertile) semispecific mating behavior. We also tentatively identify the cuticular hydrocarbons common to both males and females.

Methods and Materials

Fly Strains For full descriptions of origins and maintenance of all *Drosophila paulistorum* strains, see Ehrman and Powell (1982) and references therein.

Cuticular Compound Extraction Three-day-old, sexually mature male and female virgin flies were examined. In a typical experiment, approximately 100 3-d-old virgin flies per sex were killed by freezing at −70°C. These were later thawed to room temperature, dried on filter papers, and then placed in vials. After 1 ml hexane (HPLC grade) was added, the vial was left standing for 5 min, mixed on a Vortex mixer for 2 min, and left standing for another 5 min before hexane wash was removed. The extraction process was repeated. Combined hexane washes were filtered, stored, evaporated to 0.50 ml, and quantitatively transferred to 1-ml conical vials with Teflon-lined screw caps. These extracts were evaporated to dryness under nitrogen.

Column Chromatography Separation Extract samples were reconstituted in 0.50 ml hexane, applied to a 15×1 cm mini-glass column packed with silica gel (Merck, 60–200 mesh), eluted with solvents (6 ml each) of increasing polarity, i.e., pure hexane and 5%, 10%, 20%, and 50% ether in hexane (vol/vol). Collected fractions were evaporated to dryness before use for analyses and bioassays.



Chemical Derivatization In order to locate positions of C=C bonds, dimethyl disulfide (DMDS; 99.0 + %, Aldrich) derivatives of cuticular compounds were synthesized (Buser et al., 1983; Carlson et al., 1989). Experiments were carried out on the hexane fraction and the 5% ether/hexane fraction. Each pre-dried fraction was dissolved first in 100 μl hexane and then treated with 0.2 ml DMDS and 0.10 ml of iodine/diethyl ether solution (60 mg/ml). The reaction mixture was left standing with mixing at room temperature for 24 h. Hexane (4 ml) was added to the mixture, followed by 0.2 ml sodium thiosulfate aqueous solution (10%), reducing the remaining iodine. The organic layer was evaporated to dryness under nitrogen.

Gas Chromatography/Mass Spectrometry (GC/MS) Analyses Dried samples containing whole extracts or column chromatography fractions from approximately 100 flies were reconstituted in 5 μl hexane immediately before analyses. Electron impact (EI) analyses of fly extracts were carried out on an HP 5890A/5971A GC/MS instrument (Hewlett Packard Corporation, Palo Alto, CA, USA equipped with a 30 m× 0.25 mm×0.25 μm AT-1 column (non-polar), programmed between 150°C and 320°C at 6°C/min, and held at 320°C for 10 min. Injection port temperature was 275°C. Peak areas were calculated using an "auto-integrate" method provided by HP ChemStation software.

Dimethyl disulfide derivatization reaction mixtures were analyzed on a Varian Saturn 2200 ion trap mass spectrometer/ 3800 gas chromatograph instrument; GC conditions were identical to those used with the HP instrument; ion trap temperature was 200°C.

Positive CH_4 chemical ionization (CI) experiments were carried out on an HP 5890/5988A, equipped with a 30 m× 0.25 mm×0.25 μm DB-1 column (non-polar), programmed from 50°C (1 min) to 320°C at 6°C/min.

Mating Observations Bioassay of male cuticular extracts involved direct mating observations. All details concerning our bioassay experimental protocols have been itemized by Ehrman (1965, 1975), and reviewed and adjudicated by Knoppien (1985). Briefly: All replicas were conducted mornings at room temperature in daylight facing north. Beforehand, virgin flies, unexposed to anything male, were aged 2 d to 3 d after light ether anesthetization during which they were sexed (there are no sex differences in abdominal banding in this superspecies), and half were marked via minute distal wing clips (used as control in bioassays). Such marked abrasions have tested neutral

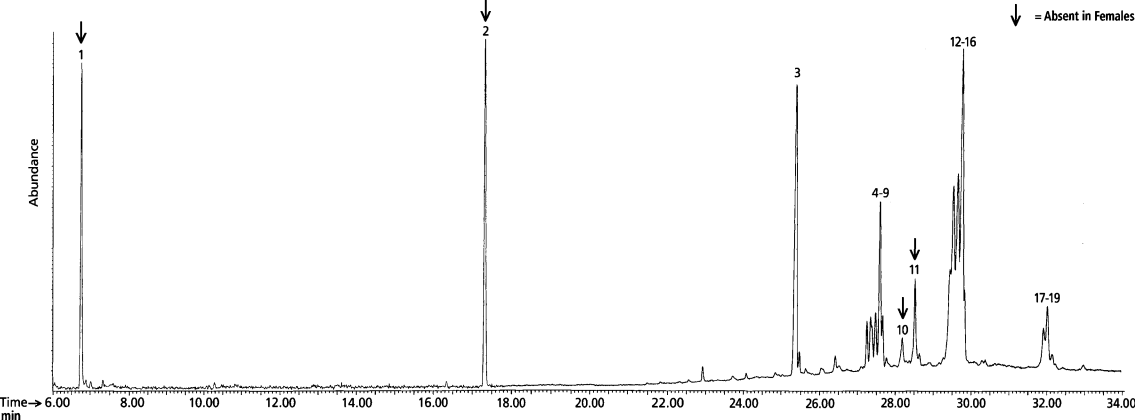


Fig. 1 Gas chromatogram of hexane cuticular extract from 3-day-old Centroamerican (CA) males. Compounds 1, 2, 10 and 11 are not found in females

regarding behavioral influences (Leonard and Ehrman, 1983) in this superspecies.

Samples of the hexane extract or column chromatographic fractions obtained from 20 flies were dissolved in 20 μl of hexane and applied to strips of filter paper that were allowed to stand until the solvent completely evaporated. Twenty virgin females, without repeated anesthetization at any time, walked on the solvent-free filter paper impregnated with samples for 30 sec. Exposed females then were placed, with no further anesthetization, in a glass mating chamber (Elens and Wattiaux, 1964; Ehrman, 1965) together with 20 unexposed, clean females (wings clipped to facilitate identification), plus 40 males. A four-power hand lens was employed to score the following until all flies copulated in approximately 30–40 min: the time (from beginning of observations) each mating takes place; its sequence among other copulae that occur; where in the chamber the mating pair is located (for this purpose a grid constitutes the floor); the kind of female involved; and the kind of male involved. Each copulation lasts approximately 17 min, and females mate only once in these arenas. Recording location of copula, even upside down, prevented scoring a copula more than once. Only the first 20 matings became part of our data, scored as mating preferences, because after the first half of all 40 possible matings, male flies try to mate with any fly still available of any sex indiscriminately (Ehrman and Kim, 1998). (Note that any single male can mate more than once, likely within the second half of all matings. Females never mate more than once under these experimental conditions, nor apparently in nature, as noted by Richmond and Ehrman, 1974.)

Results

Cuticular hexane extracts from male flies of all six semispecies (TR, IN, CA, OR, AB, AM) have the same set of nineteen major compounds, with small variations in intensity patterns between different semispecies (Fig. 1). Samples of males and females, however, have clear differences; i.e., peaks 1, 2, 10, and 11 are absent in all females. Both males and females shared the same remaining fifteen compounds, and each corresponding compound has the same retention time and mass spectrum. Male-specific compounds responsible for peaks 1, 2, 10, and 11 are the focus of this study.

Column Chromatography Fractions of the Male Cuticular Extracts Silica gel column chromatography fractions collected were analyzed by GC/MS. The non-polar hexane fraction gave GC peaks 3, 4–9, and 12–19 (Fig. 1); the slightly polar 5% ether-in-hexane fraction gave only peaks 1, 2, 10, and 11 (GC data not shown). The more polar

Table 1 Numbers of intraspecific matings observed between virgin females (n=20) previously exposed to TR male cuticular compounds and males

	TR × TR	IN × IN	CA × CA	OR × OR	AB × AB	AM × AM
Whole extract	3.0±1.1	5.3±1.0	6.0±3.6	6.0±1.4	6.6±0.3	10.6±2.6
Hexane fraction	10.2±1.2	10.5±1.1	9.5±1.2	10.0±1.0	10.1±1.4	9.8±0.5
5% Ether fraction	2.6±2.0	1.6±1.3	4.6±1.7	4.6±1.2	4.0±0.2	6.6±1.2

Observations were made of 40 males plus 20 untreated females and 20 treated females. A score of ten corresponds to a neutral (no mating preference) response. See Ehrman (1975) for further details and about statistics

TR Transitional, *IN* Interior, *CA* Cetroamerican, *OR* Orinocan, *AB* Andean-Brazilian, *AM* Amazonian

Table 2 Percent distribution of male specific compounds across the six semispecies (average ± standard deviation)

Peak no.	TR	IN	CA	OR	AB	AM
1	40.8±0.3	24.3±1.0.4	24.9±0.8	13.0±0.2	25.7±2.2	16.5±4.5
2	31.6±1.6	32.4±2.5	30.1±1.5	35.7±1.1	24.0±3.0	23.2±1.6
10	5.9±1.3	11.0±1.9	14.8±2.1	12.8±1.8	18.1±2.3	20.5±3.6
11	21.7±0.3	32.3±0.8	30.2±1.4	38.5±1.5	32.2±0.9	39.8±4.6

TR Transitional, *IN* Interior, *CA* Cetroamerican, *OR* Orinocan, *AB* Andean-Brazilian, *AM* Amazonian

fractions (>10% ether) only gave trace amounts of components corresponding to some of the very weak, unlabeled compounds in Fig. 1. A similar study of extracts of females exhibited only compounds found in the hexane fraction of males. Males and females, therefore, share the same set of non-polar compounds; male-specific compounds, on the other hand, are all slightly polar.

Effects of Male Cuticular Extracts on Intraspecific Mating Behaviors Direct mating observations were carried out to determine the sex pheromonal roles of the compounds present in various fractions from silica gel column separation. Results from the AM semispecies are presented here. This experimental design was planned to determine whether the presence of these compounds on virgin females significantly reduced male courtship. Numbers of copulae were recorded for unexposed, clean virgin females vs. virgin females exposed to the whole extract or column chromatographic fractions. A hexane blank also was included in this survey. Number of matings (average ± standard deviation) for the initial halves of the first 20 females exposed to matings were 10.0±1.0, 3.0±1.1, 9.3±2.2, 2.2±1.0, 12.4±2.7, and 11.3±1.5 for the hexane blank, whole extract, hexane fraction, 5%, 10%, and combined 20–50% ether in hexane fractions, respectively. The numbers represent the average of 5 replicas. Hexane blanks elicited a neutral response (number of matings=10) as anticipated. Only the whole extract (number of matings=3.0±1.1) and the 5% ether-in-hexane fraction (number of matings=2.2±1.0) had a "negative" effect on subsequent mating behavior. All others were inactive or neutral in courtship, and all fractions and controls were statistically tested vs. expectations of random mating, which did not occur when biologically-active, anti-aphrodisiacal fractions were surveyed. The mixture found in the 5% ether-in-hexane fraction has an anti-aphrodisiac mating effect.

Bioassay Results of Male TR Cuticular Extract on Mating Behavior of All D. paulistorum Semispecies Following the same bioassay experimental design outlined above, whole extracts and column chromatographic fractions of the male ancestral TR cuticular compounds were tested on all six semispecies. For each semispecies, twenty females were exposed to extracts from twenty male TR (an average of 1 fly-equivalent exposure per female fly), and the mating behaviors of these exposed females vs. twenty clean females were recorded. Only the first twenty matings were tabulated as indicators of mate preferences. Results listed in Table 1 itemize averaged numbers of intraspecific matings of three replicas. The TR hexane fraction, containing a mixture of non-polar compounds, resulted in approximately random matings, a neutral response, for all. The 5% ether fraction elicited a strong anti-aphrodisiac effect, with AM matings affected minimally. The effect of whole extracts resembles that of the 5% ether fraction, though slightly weaker.

Percent Distribution Comparison of the Male-Specific Compounds Across the Six Semispecies Using integrated areas under each GC peak of the whole extract, percent distributions of the four male-specific compounds for each species were calculated. Table 2 summarizes the results showing the quantitative variation of the male-specific pheromonal components across the six semispecies. Values were calculated from three samples of 100 flies each.

Structural Characterization of Male-Specific Compounds EI-MS of the four male-specific compounds (GC peaks 1, 2, 10, 11) share the general characteristics of straight, long chain hydrocarbons with gradually diminishing ion intensities spaced 14 atomic units (au) (CH_2) apart. Definitive assignment of the molecular ion $[M]^+$ was difficult based on EI data alone because of the lack of the $[M]^+$ peak intensity. Molecular weight (MW), therefore, was deduced from the strong $[M+1]^+$ ion of the compound's positive CH_4-CI-MS. Positions of the C=C bonds were determined via MS analyses of the DMDS (CH_3S-SCH_3, MW 94) derivatives. For compounds containing one C=C, DMDS adds across the C=C bond of the compound RCH = CHR', giving the addition product $R(SCH_3)CH$–$CH(SCH_3)R'$ which is 94 au heavier than the underivatized compound. Cleavage of the CH−CH bond results in two diagnostic MS ions due to $[R(SCH_3)CH]^+$ and $[CH(SCH_3)R']^+$ fragments (Carlson et al., 1989). Table 3 summarizes the analysis results.

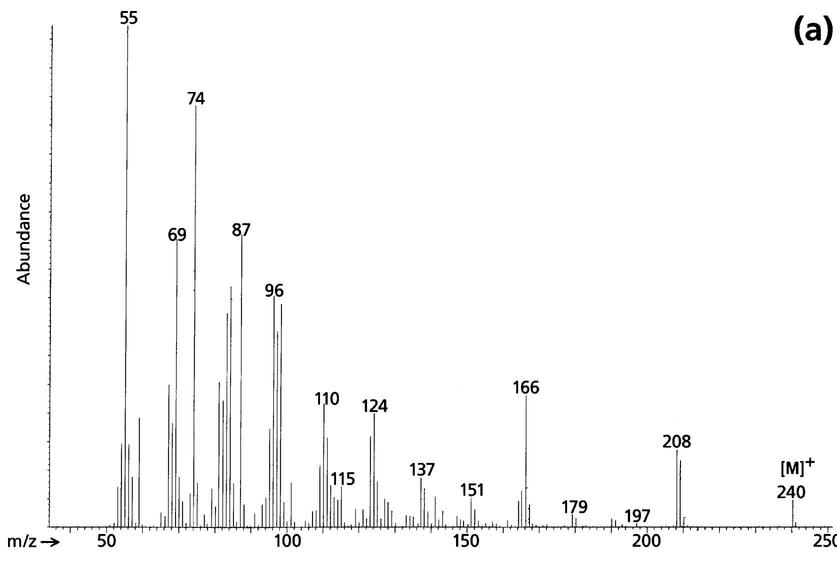
Compound 1 was identified as methyl (Z)-9-tetradecenoate. EI-MS and CI-MS of compound 1 are shown in Fig. 2a and b, respectively. The strong EI ion at *m/z* 74

Table 3 Structural information of the male-specific compounds and important MS peaks

Peak no.	EI- MS m/z (abundance[a])	CI –MS m/z(abundance[a])	Molecular formula	Compound
1	74 (83), 240 (6.2)	209 (78), 241(100), 269 (25)	$C_{15}H_{28}O_2$	(Z)-methyl 9-tetradecenoate
2	61(18), 306 (15)	61(32),305(28),367(25),395(8.4)	$C_{24}H_{46}O_2$	11-docosenyl acetate
10	61(13), 476 (10)	415(23), 475 (16)	$C_{32}H_{60}O_2$	a di-unsaturated acetate
11	61 (16), 418 (19), 478 (1.1)	61(6.2), 417(29), 479(17)	$C_{32}H_{62}O_2$	19-triacontenyl acetate

[a] Abundance is relative to the base peak (100%)

indicates a methyl ester (McLafferty and Turecek, 1993). The weak EI *m/z* 240 $[M]^+$ ion has a strong CI counterpart at *m/z* 241 $[M+1]^+$, confirming a MW of 240. The *m/z* 209 $[M-31(CH_3O)]^+$ ion is present in both EI and CI. The *m/z* 269 $[M+(C_2H_5)]^+$ CI ion together with the *m/z* 166 [M-74] EI ion further supports the assigned MW (Harrison, 1992). EI-MS of the DMDS adduct gave a molecular ion at a *m/z* 334, $[334-47(SCH_3)]^+$ ion at 287 and a $[334-94(SCH_3-SCH_3)]^+$ ion at 240. Two diagnostic ions at *m/z* 117 $[CH(SCH_3)(CH_2)_3CH_3]^+$ and *m/z* 217 $[CH_3OOC(CH_2)_7(SCH_3)CH)]^+$ point to C-9 as the location of the C=C bond. An increase of retention time from 6.69 min of the compound to 15.33 min for the DMDS adduct is consistent with the increase of 94 au, under our GC conditions.



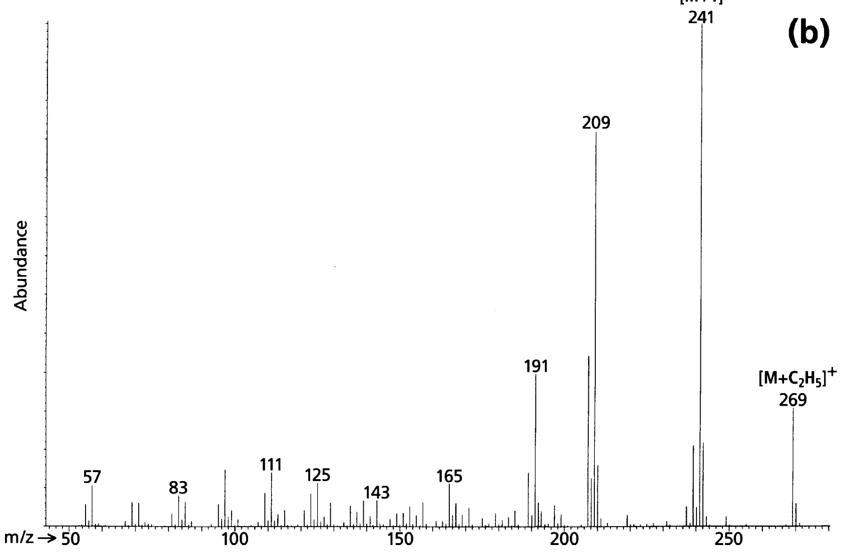
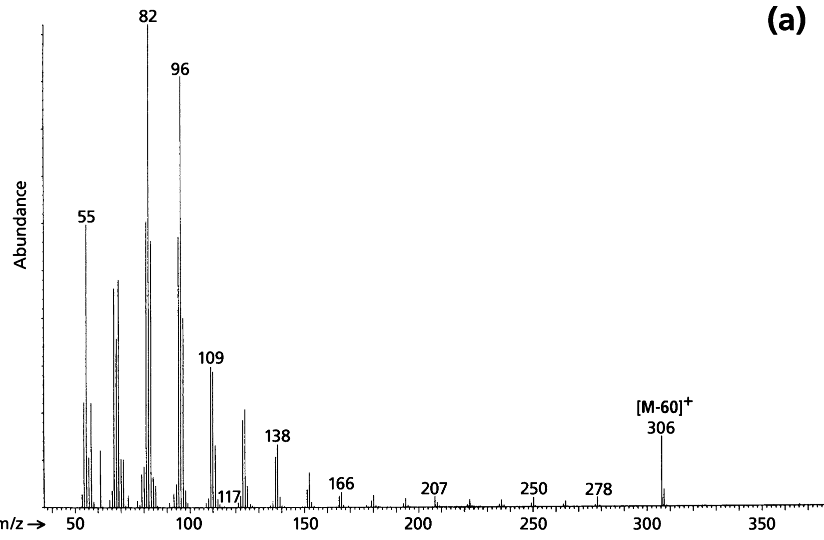


Fig. 2 Mass spectra of GC compound peak 1. Methyl (Z)-9-tetradecenoate ($C_{15}H_{28}O_2$, MW 240. **a** Electron impact MS. Weak molecular ion at *m/z* 240. **b** Methane chemical ionization MS. Strong $[M+H]^+$ ion at *m/z* 241, $[M+H+28]^+$ at *m/z* 269, and $[M+H-32]^+$ at *m/z* 209



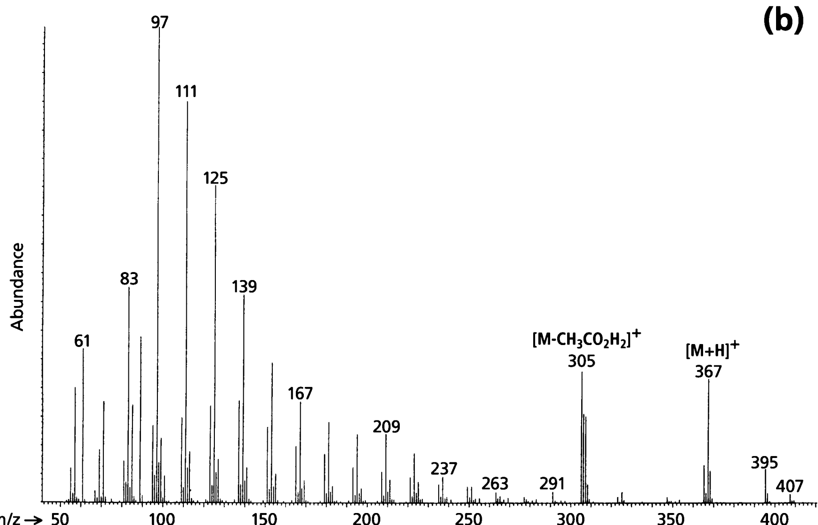


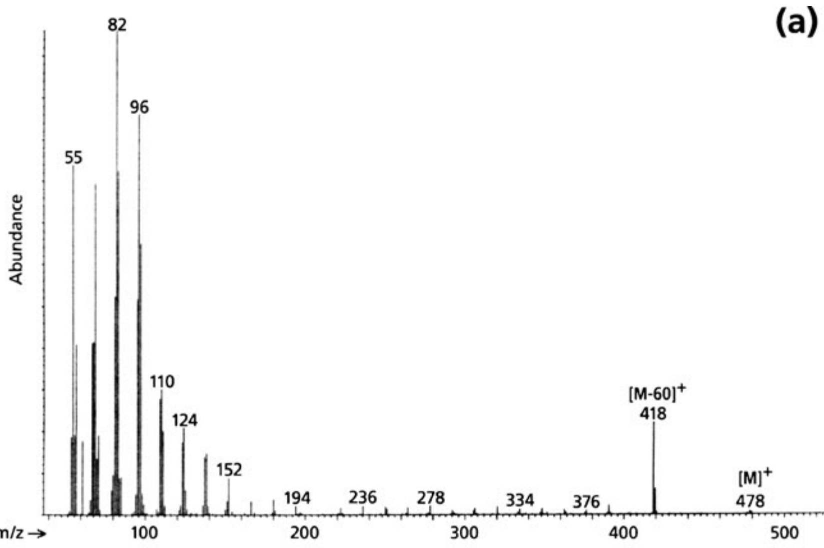
Fig. 3 Mass spectra of GC compound peak 2. 11-Docosenyl acetate ($C_{24}H_{46}O_{2,}$ MW 366). **a** Electron impact MS. A strong $[M\text{-}60)]^+$ (*m/z* 306) ion. Molecular ion (*m/z* 366) absent. **b** Methane chemical ionization MS. $[M+H]^+$ ion at *m/z* 367, $[M\text{-}61]^+$ at *m/z* 305, and $[M+H+28]^+$ at *m/z* 395

Structural identification including the "Z" configuration of the double bond was confirmed by GC-MS data of the synthetic methyl (Z)-9-tetradecenoate standard ($C_{15}H_{28}O_2$, MW 240), purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

The compound responsible for GC peak 2 is the most abundant of the four male-specific compounds (Chao et al., 2001; Kim et al., 2004). Both EI-MS and CI-MS (Fig. 3) gave the characteristic *m/z* 61 ion for acetate due to the protonated $CH_3CO_2H_2^+$ species (Harrison, 1992). Although the EI (Fig. 3a) had a strong *m/z* 306 ion, based on our CI data, this is not due to the molecular ion; rather it is due to the $[M\text{-}60]^+$ fragment after the neutral loss of an acetic acid (MW 60) moiety. The CI data indicated a 366 MW. Strong CI (Fig. 3) ions at *m/z* 367, 305, and 395, can be assigned to $[M+H]_,^+$ $[M\text{-}CH_3CO_2H_2\ ]^+$, and $[M+C_2H_5]^+$. Based on the overall smooth varying MS intensity pattern, chain branching is not indicated. Its DMDS derivative yielded EI-MS molecular ion $[M]^+$ at *m/z* 460 plus the two diagnostic ions at *m/z* 201 and 259, corresponding to DMDS addition at the C-11 position. Compound 2 is therefore 11-docosenyl acetate ($C_{24}H_{46}O_{2,}$ MW 460). Its stereochemistry is not yet determined.

Compound 11 is due to 19-triacontenyl acetate ($C_{32}H_{62}O_2$, MW 478). Its EI-MS (Fig. 4a) had a weak molecular ion at *m/z* 478 and an intense $[M\text{-}60(C_2H_3O_2)]^+$ ion at 418. Its CI-MS (Fig. 4b) had a prominent ion at *m/z* 479, $[M+H]^+$, giving a MW of 478. Again, both CI and EI had the characteristic *m/z* 61 ion for acetate. The EI-MS of its DMDS adduct exhibited an $[M+H]^+$ ion at *m/z* 573, $[M+H\text{-}SCH_3]^+$ at 526, two diagnostic ions at *m/z* 201 and 372, and an ion at *m/z* 312 (372- 60), indicating a C=C (unknown stereochemistry) at the C19 position in the underivatized acetate.

We were not able to fully characterize the compound responsible for GC peak 10. Its EI-MS (Fig. 5) has a weak



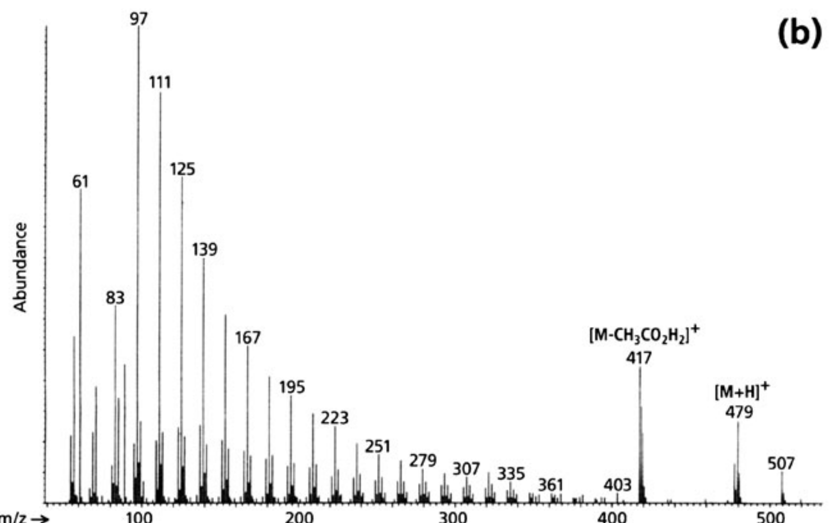


Fig. 4 Mass spectra of GC compound peak 11. 19-Triacontenyl acetate ($C_{32}H_{62}O_2$, MW 478). **a** Electron impact MS. Molecular ion at *m/z* 478, and an intense $[M\text{-}60]^+$ peak at 418. **b** Methane chemical ionization MS. Prominent ions at *m/z* 479 ($[M+H]^+$), *m/z* 417 ($[M\text{-}61]^+$), and *m/z* 507 ($[M+H+28]^+$)

$[M]^+$ ion at *m/z* 476, an *m/z* 61 ion for acetate; its CI has a 475 $[M\text{-}H]^+$ ion and a 415 $[M\text{-}61]^+$ ion. Therefore, this compound is a di-unsaturated acetate ($C_{32}H_{60}O_2$, MW 476). GC-MS analysis of the DMDS derivatization reaction product did not give GC peaks that can be attributed to the DMDS adduct of this fourth component, despite numerous efforts.

Structural Information about the Non-polar Hydrocarbons Both male and female extracts yielded hydrocarbons (GC peaks 3–9 and 12–19 in Fig. 1) in the hexane fraction eluted from the silica gel column. Although their exact chemical structures are not central to this investigation, they warrant preliminary reporting here.

As we have noted previously, Kim et al. (2004) studied the effect of rearing conditions on the quantity of the compound responsible for GC peak 3. Here, we present the MS data analyses for this peak. Its EI-MS (Fig. 6) gave a molecular ion at *m/z* 436. Branch point at C-2 is indicated since the smooth decline of MS peak abundance is disrupted by ions at *m/z* 421 $[M\text{-}CH_3]^+$ and 393 $[M\text{-}C_3H_7]^+$ due to elimination of a terminal methyl group and an isopropyl group, respectively. Its CI-MS (not shown) has ions at *m/z* 435 $[M\text{-}H]^+$, 421 $[M\text{-}CH_3]^+$, and 407 $[M\text{-}C_2H_5]^+$, consistent with the EI data. This compound is tentatively identified as 2-methyltriacontane ($C_{31}H_{64}$), the only saturated hydrocarbon of all 19 cuticular compounds detected.

The rest of the compounds occur by GC in three clusters of peaks (peaks 4–9, 12–16, and 17–19; Fig. 1). Each cluster consists of several slightly overlapping peaks. Figure 7 shows the gas chromatograms of AM and OR females in this region. Although both sexes of all six semispecies gave the similar pattern of three clusters, the detailed quantitative variations differed as illustrated in Fig. 7. Mass spectra of peaks in this region all have the

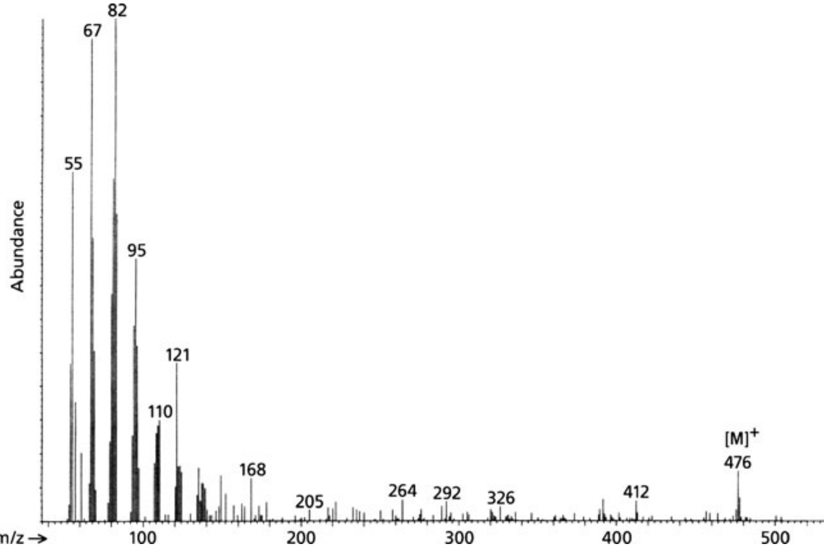


Fig. 5 Electron impact MS of GC compound peak 10. A di-unsaturated acetate with formula $C_{32}H_{60}O_2$ and MW 476

characteristic fragmentation pattern of a long chain linear hydrocarbon. Each weak EI-MS molecular ion $[M]^+$ has a corresponding strong CI ion at *m/z* M-1. Compounds 4 and 5, both with molecular ions at *m/z* 462, are likely due to two positional isomers of a $C_{33}H_{66}$ monoene. Similarly, compounds 6–9 all have molecular ions at *m/z* 460, probably due to four positional isomers of $C_{33}H_{64}$. Compounds 12–16, an overlapping cluster of 5 discernable peaks, share the same EI- and CI-MS. An intense CI-MS ion at *m/z* 487 $[M\text{-}H]^+$, and the corresponding EI-MS ion at *m/z* 488, suggest isomers of $C_{35}H_{68}$ (MW 488). Finally, the last cluster of at least three overlapping peaks, numbered 17–19, based on their MS data, can be assigned to isomers of $C_{37}H_{72}$ (MW 516). In conclusion, cuticular compounds common to both males and females, though not fully characterized, are odd-carbon hydrocarbons, including one terminally branched alkane, 2-methyl triacontane ($C_{31}H_{64}$), plus isomers of unsaturated hydrocarbons $C_{33}H_{66}$, $C_{33}H_{64}$, $C_{35}H_{68}$, and $C_{37}H_{72}$.

Discussion

Cuticular lipid profiles of all six *D. paulistorum* semi-species were found to be similar. Males and females share the same 15 major hydrocarbons. Males possess four additional compounds, all long-chain esters: one methyl ester, methyl (Z)-9-tetradecenoate ($C_{15}H_{28}O_2$); and three acetates—$C_{24}H_{46}O_2$ (11-docosenyl acetate), $C_{32}H_{60}O_2$ (an unsaturated acetate), and $C_{32}H_{62}O_2$ (19-triacontenyl acetate). These three acetates have not been confirmed against their authentic compounds, and organic syntheses are currently underway. Bioassays of the various chromatographic fractions confirmed the pheromonal role of the

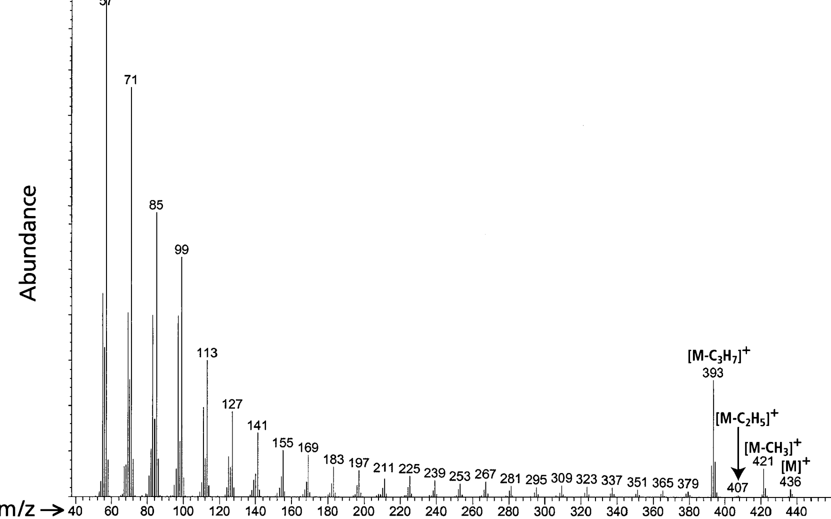


Fig. 6 Electron impact MS of GC compound 3. 2-Methyltriacontane ($C_{31}H_{64}$, MW 436). Molecular ion peak at *m/z* 436

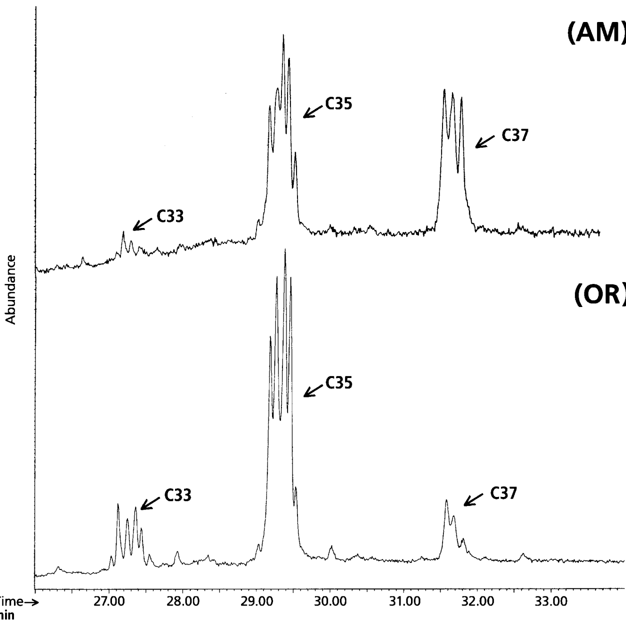


Fig. 7 Gas chromatograms of Amazonian (AM) and Orinocan (OR) females, showing only the region where all unsaturated hydrocarbons, C33 (peaks 4–9), C35 (peaks 12–16) and C37 (peaks 17–19), are eluted

male ester mixture. Preliminary structural data on the fifteen hydrocarbons, ranging from C31 to C37, are reported, but detailed analyses will be the topic of future studies.

Our findings bear close resemblance to the literature on the most studied drosophilid, *D. melanogaster*. For example, in *D. melanogaster*, almost identical chemical compositions of cuticular hydrocarbons were found among members of individual populations, and quantitative variations have been noted between geographic populations (Jallon and Penchine, 1989). It is known that contact during mating may result in mutual exchanges of cuticular hydrocarbons from one sex to the other, subsequently decreasing the attractiveness of both males and females to members of the other sex (Ferveur, 1996). In *D. melanogaster*, a male pheromone compound, (*Z*)-vaccenyl acetate, was found to inhibit mating behavior (Jallon, 1984). More recently, by observing the mating behavior of decapitated flies and by disrupting a single *D. melanogaster* gene, Marcillac et al. (2005) were able to partition production and discrimination of cuticular hydrocarbons functioning as sex pheromones, reflecting *desat1* locus pleiotropy. In *D. paulistorum*, we found a four-component ester blendthat elicited strong anti-aphrodisiac effects on mating behavior. These male-specific pheromones from different semispecies differ only quantitatively, suggesting that both chemical identities as well as quantitative proportions are critical factors that determine effects on mating behaviors.

Gleason et al. (1998), based on gene sequencing, have proposed the phylogenetic order of the six semispecies as: TR (the relict ancestor), IN, CA, OR, AB, AM. These researchers also proposed that because of geographic separation from siblings, Amazonian is approaching the status of a distinct species. Our bioassay results regarding male TR cuticular extracts on mating behaviors of all other semispecies demonstrated that the TR male pheromones impart anti-aphrodisiac effects on the other five semispecies too, but the effect decreases as phylogenetic distances of each semispecies from TR increases. AM, the one most remote from TR, is least affected. This suggests that the male-produced pheromone is at least partially responsible for reproductive isolation by preventing inter-semispecific mating and, therefore, production of sterile offspring. Ehrman et al. (1995) have observed that hybrids of the different semispecies have never been reported in nature, nor has a trapped sperm-storing gravid female ever borne alien sperm. This is so even in regions of geographic sympatry, demonstrating the startling efficiency of pheromones as sensory cues.

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sweep out comparatively large areas as they patrol tree trunks and other substrates for females, so that they can locate mates efficiently (Hanks et al., 1996; Hanks, 1999). To our knowledge, contact pheromones have not yet been identified for beetles in the more ancestral subfamilies that lack elongate antennae, including the Prioninae, Parandrinae, and Aseminae (*sensu* Monné and Hovore, 2005). Although males of the prionine, *Prionus californicus* Motschulsky, recognize females via contact chemoreception (Cervantes et al., 2006; Barbour et al., 2007), the compounds that mediate mate recognition have not yet been identified. A greater understanding of the use of contact pheromones in ancestral subfamilies will lend insight into the evolution of mating systems in the Cerambycidae.

Mallodon dasystomus, the hardwood stump borer, is a widely distributed prionine that is native to the southern U.S. The elongate robust adults range in length from 23–47 mm and vary in color from reddish brown to black (Solomon, 1995). Females oviposit near the base of their hosts at sites where the wood is exposed, such as at wounds or areas of previous infestation. Larvae feed in the boles of trees, typically complete their development in 3–4 yr, and emerge as adults from May through July leaving large ovoid emergence holes in the bark (Linsley, 1962; Solomon, 1995). Preferred hosts *of M. dasystomus* include sugarberry, hackberry, oak, sycamore, hickory, willow, boxelder, sweetgum, and in some instances, conifers (Solomon, 1995; Yanega, 1996).

In this study, we explored the chemically-mediated mating behavior of *M. dasystomus*, and tested the hypothesis that males recognize females by contact chemoreception. We characterized the mating behavior of males, and report the identification and synthesis of the bioactive components. Additionally, we sampled cuticular hydrocarbons of males and females using both whole-body solvent extraction and solid phase microextraction (SPME). SPME is a solventless sampling technique that was used in conjunction with solvent extraction to identify the contact pheromones of the cerambycine species *Megacyllene robiniae* (Förster) (Ginzel et al., 2003b), *Megacyllene caryae* (Gahan) (Ginzel et al., 2006), and *Neoclytus acuminatus acuminatus* (F.) (Lacey et al., 2008). Wipe-sampling yields hydrocarbon profiles that are quantitatively different from whole-body solvent extraction (Ginzel et al., 2006). For example, the contact pheromone of *M. caryae,* (*Z*)-9-nonacosene, comprised ~16% of the total hydrocarbons in the hexane extracts of females, but represented ~34–36% of the hydrocarbons in SPME wipe samples of the elytra (Ginzel et al., 2006). These findings suggest that SPME samples more accurately represent those compounds that are accessible to the antennae of males and can be used to predict hydrocarbons that may act as contact pheromones. In this study we also test the hypothesis that contact pheromones are more abundant on the surface of the wax layer and are present in greater abundance in SPME samples when compared to whole-body extracts.

Methods and Materials

Source of Beetles Beetles used in experiments were reared from infested willow and sweetgum at the USDA Forest Service Center for Bottomland Hardwoods Research, Stoneville, MS, from late May to early August 2007 through 2009. As adult beetles emerged, they were shipped to Purdue University, West Lafayette, IN (USDA—APHIS permit No. P526P-09-01631). Upon arrival, beetles were housed individually in cylindrical cages of aluminum window screen (300 cm^3) with 9-cm glass Petri dishes covering top and bottom, and provided feeder vials of 10% sucrose solution (glass vial with a cotton dental roll; Patterson Dental Supply, South Edina, MN, USA). Beetles were kept in an environmental chamber (Mod. No. 1-30BLL, Percival Scientific, Boone IN, USA) on a 16 L –29°C:8D – 25°C cycle that was 12 hr out of phase with natural daylight, and maintained at 80% humidity. Reared beetles may have mated before they were individually caged. Beetles were isolated in these cages for at least 24 hr before being used in bioassays. Beetles used in bioassays were vigorous and active, and were used no more than once per day.

Characterization of Mating Behavior To characterize the mate recognition behavior of *M. dasystomus* in the laboratory, we observed individual males paired with individual females in Petri dish arenas (9 cm diam × 2 cm tall) lined with filter paper (No. 1, Whatman, Maidstone, England). Observations were made in the dark under a red light and ambient laboratory conditions for a minimum of 2 min or until beetles mated. The behaviors of 17 pairs of female and male beetles in these arenas were recorded digitally using a Sony Handy-Cam, model DCR-SR42. All Petri dishes were cleaned, washed with acetone, and air dried between trials. We analyzed videotapes to determine whether one sex displayed directed movement toward the other, a behavior consistent with the use of either vision or volatile pheromones as mate location signals. We also observed whether males responded to females only after contacting them with their antennae, behavior that would suggest the use of contact pheromones.

Role of Contact Chemoreception To illustrate that male *M. dasystomus* recognize females via a contact pheromone, we used the following bioassay adapted from Ginzel *et al.* (2003b).

1. *Freeze-killed female.* An individual female was freeze-killed (−4°C for 20 min), allowed to warm to room temperature (~15 min), and presented to a vigorous male in a Petri dish arena to demonstrate that mate recognition signals were intact. The male was allowed to attempt to mate the female, but separated before copulation occurred.
2. *Solvent-extracted female carcass.* Non-polar compounds were removed from the dead female by immersing her in two successive 10-ml aliquots of analytical grade hexane for 5 min each. The aliquots then were combined and concentrated to 3 ml under nitrogen. The solvent-washed female was presented to the male to test whether he displayed mating behavior. Lack of a response by the male was taken as evidence that chemical recognition signals had been removed.
3. *Reconstituted female.* To test the bioactivity of the extract, we gradually pipetted 0.1 female equivalent (FE) of extract back onto the female carcass, coating the body with the extract. The solvent then was allowed to evaporate, and the reconstituted female was presented again to the same male to confirm that the recognition signal had been restored. If the male did not respond, we incrementally added 0.1 FE to the female, up to a maximum of 1 FE, and retested the female against the same male.

We conducted this videotaped bioassay with six dead females each paired with three different males ($N=18$). Once a male touched a female with his antennae, a clear progression of behavioral steps lead to copulation (arrestment of male, body alignment with female, mounting, and copulation; see Results for details). A trial was scored as a "response" if the male, after making antennal contact with the female, displayed any of these behavioral steps. Bioassays were conducted in glass Petri dish arenas (see above), and numbers of males responding to reconstituted females were compared with those responding to freeze-killed females with a Fisher's exact test (Sokal and Rohlf, 1995).

Identification of Cuticular Hydrocarbons We sampled cuticular components of five female beetles by wiping the length of a SPME fiber (100 µm, polydimethylsiloxane; Supelco Inc., Cat. No. 57300-U, Bellefonte, PA, USA) across the elytra ten times, rotating the fiber between wipes. Samples were analyzed at Purdue University by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard (HP) 6890N gas chromatograph (Hewlett-Packard, Sunnyvale, CA, USA) equipped with a DB-5 capillary column (30 m×0.25 mm×0.25 µm film, J&W Scientific, Folsom, CA, USA) in splitless mode, and interfaced to an HP 5973 mass selective detector (MSD), with helium as the carrier gas. The SPME fiber was thermally desorbed in the heated GC injection port for 1 min and the oven temperature was held at 50°C for 1 min and then ramped at $10°C\,min^{-1}$ to 280°C, with a hold at 280°C for 20 min. Injector and transfer line temperatures were 250°C.

Five female beetles and five males also were sampled by whole-body hexane extraction, allowing us to compare the hydrocarbon profiles of males and females, and the profiles of females produced by the two sampling methods. The cuticular chemicals were extracted from individual freeze-killed beetles by immersing each in two 10-ml aliquots of hexane for 5 min. The aliquots were combined and concentrated to 3 ml under nitrogen. Extracts were analyzed initially at Purdue University by coupled GC-MS as described above. The percentage that each peak contributed to the total hydrocarbons was calculated by integrating the areas under peaks of all hydrocarbons that were consistently present in the total ion chromatograms (Chemstation, Version D.05.01; Hewlett Packard Corp.). Quantitative data presented in Table 1 and Figs. 1 and 2 were produced by these analyses. Differences in mean relative abundance of compounds that comprise the contact pheromone (area under peak/total area of all peaks) between whole-body hexane extraction and SPME of females were compared by analysis of variance (StatSoft, Inc., 2005).

Compounds in whole-body extracts were identified at the University of California, Riverside by GC-MS using the same model of GC-MS instrument and the same type of column as described above. The column was programmed from 100°C for 1 min, $10°C\,min^{-1}$ to 280°C, with a final hold for 20 min at this temperature. Injector and transfer line temperatures were 280°C. Linear and branched chain hydrocarbons were identified by comparing their retention times and mass spectra with those of standards, or by interpretation of the mass spectra in combination with Kovats retention indices. Retention times of methylalkanes relative to straight-chain compounds, and characteristic mass spectral fragments (M^+ ions in combination with diagnostic fragments from cleavage on either side of branch points) can be used to completely and unequivocally identify saturated hydrocarbons found in insect cuticular extracts, as described in detail by Nelson (1993), Nelson and Blomquist (1995), and Carlson et al. (1998).

Synthesis of 2-Methylhexacosane and 2-Methyloctacosane Solvents were Optima grade (Fisher Scientific, Pittsburgh, PA, USA). Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under Ar. Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under Ar. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-400 (400 and 100.5 MHz,

Table 1 Cuticular hydrocarbons of male and female *Mallodon dasystomus*

		% of total hydrocarbons± SE[a]			
		Hexane Extracts		SPME	
Peak No.[b]	Hydrocarbon	Male	Female	Female	Diagnostic Ions[c]
1	C_{23} monoene	0.14±0.03	ND	ND	**322** (M^+)
2	C_{23}	2.6±0.28	4.52±0.39	1.69±0.51	**324** (M^+)
3	11Me-C_{23}	ND	0.5±0.17	0.15±0.05	338 (M^+), 168/196
4	C_{24} monoene	0.64±0.21	ND	ND	**336** (M^+)
5	C_{24}	2.44±0.14	0.45±0.06	1.65±1.13	**338** (M^+)
6	2Me-C_{24}	0.33±0.07	ND	ND	352 (M^+), 337, 309
7	C_{25} monoene	18.6±5.59	1.09±0.32	0.42±0.12	**350** (M^+)
8	C_{25} monoene	ND	0.34±0.18	0.31±0.09	**350** (M^+)
9	C_{25}	41.7±3.79	8.64±1.08	10.2±2.66	**352** (M^+)
10	Diunsaturated C_{25}	1.59±0.22	0.18±0.04	0.56±0.33	**348** (M^+)
11	11Me-C_{25}	3.83±0.52	1.63±0.34	1.8±0.32	**366** (M^+), 168/224
	13Me-C_{25}				**366** (M^+), 196
12	9,13-DiMe-C_{25}	1.27±0.23	0.4±0.11	ND	380 (M^+), 140/267, 196/211, 365
13	Diunsaturated C_{25}	ND	0.13±0.04	0.2±0.04	**348** (M^+)
14	C_{26}	0.72±0.06	0.98±0.12	1.61±0.63	**366** (M^+)
15	2Me-C_{26}	0.47±0.09	4.58±0.85	7.58±0.39	**380** (M^+), 365, 337
16	C_{27} monoene	0.92±0.21	7.13±1.07	3.95±1.03	**378** (M^+)
17	C_{27} monoene	ND	0.57±0.12	0.35±0.11	**378** (M^+)
18	C_{27} monoene (front shoulder)	7.01±0.72	18.3±1.78	17.6±4.94	**378** (M^+)
	C_{27}				**380** (M^+)
19	Diunsaturated C_{27}	ND	0.44±0.06	ND	**376** (M^+)
20	11Me-C_{27}	0.36±0.05	4.14±0.59	3.87±0.65	394 (M^+), 168/252
	13Me-C_{27}				394 (M^+), 196/224
21	Diunsaturated C_{27}	ND	0.24±0.03	0.17±0.05	**376** (M^+)
22	11,15-DiMe-C_{27}	ND	3.22±0.25	3.8±0.61	**408** (M^+), 168/267, 196/239
23	3Me-C_{27}	ND	0.82±0.16	1.37±0.19	394 (M^+), 365
24	C_{28}	1.37±0.92	1.02±0.12	1.65±0.79	**394** (M^+)
25	12Me-C_{28}	ND	1.1±0.21	1.15±0.25	**408** (M^+), 182/252
	13Me-C_{28}				**408** (M^+), 196/238
	14Me-C_{28}				**408** (M^+), 210/224
26	2Me-C_{28}	ND	13±2.77	12.5±2.68	**408** (M^+), 365, 393
27	C_{29} monoene	ND	7.22±1.51	3.5±1.03	**406** (M^+)
28	C_{29}	5.57±0.43	6.05±0.87	7.87±1.61	**408** (M^+)
29	11Me-C_{29}	ND	4.03±0.73	4.51±1.04	422 (M^+), 168/280
	13Me-C_{29}				422 (M^+), 196/252
	15Me-C_{29}				422 (M^+), 224
30	13,17-DiMe-C_{29}	ND	2.92±0.22	4.15±0.73	436 (M^+), 196/267
31	2Me-C_{30}	5.49±1.52	0.97±0.06	2.05±1.03	436 (M^+), 393, 421
32	Diunsaturated C_{31}	ND	3.58±1.67	1.89±0.46	**432** (M^+)
	C_{31} monoene	0.58±0.09		ND	**434** (M^+)
33	C_{31}	1.63±0.26	ND	ND	**436** (M^+)
34	11Me-C_{31}	0.85±0.15	1.33±0.23	1.91±0.31	450 (M^+), 168/308
	13Me-C_{31}				450 (M^+), 196/280
35	2Me-C_{32}	0.49±0.15	ND	ND	464 (M^+), 421, 449
36	13Me-C_{33}	1.46±0.34	0.69±0.11	1.51±0.53	478 (M^+), 196/308

[a] Percent of total hydrocarbons represents means for five individuals. ND, not detected, SPME, solid-phase microextraction

[b] Peaks are numbered in order of elution from a DB-5 capillary column (see Methods and Materials) and correspond to those in Fig. 1

[c] Molecular ions in bold were observed. Molecular ions in normal font were not observed but could be inferred from the diagnostic ions

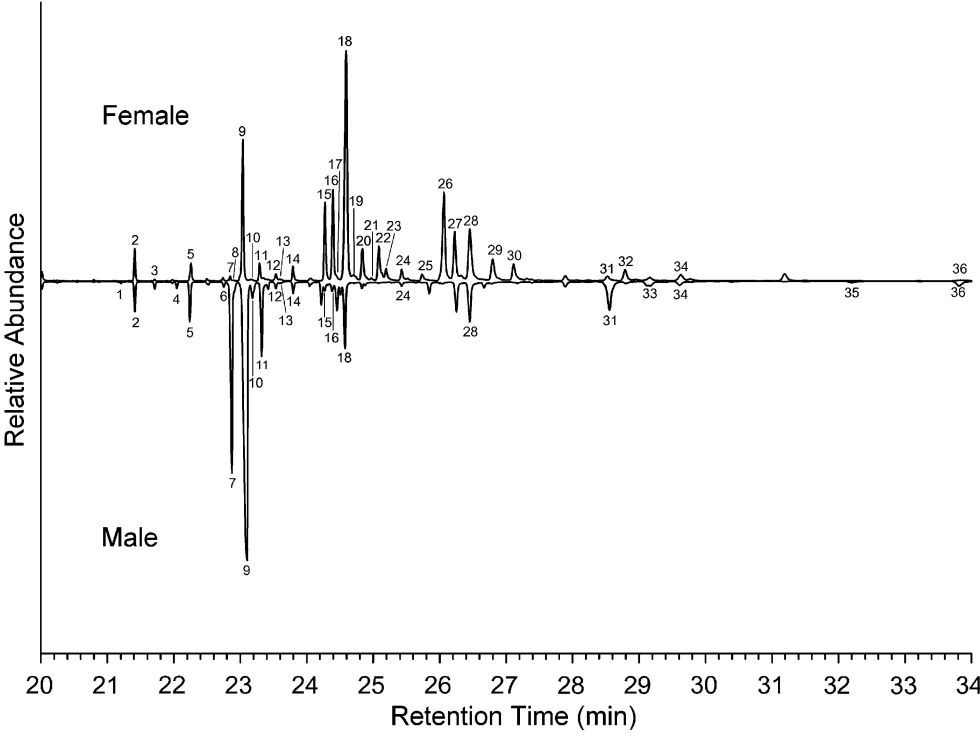

Fig. 1 Representative chromatograms of solvent extracts of female (*top*) and male (*bottom*, *inverted*) *Mallodon dasystomus*

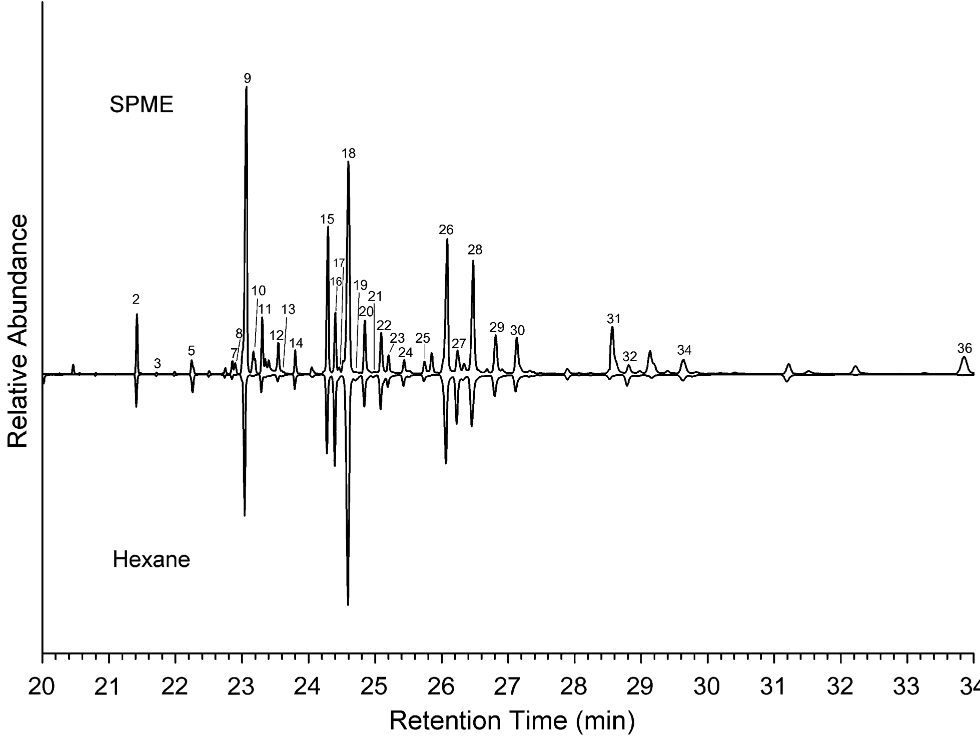

Fig. 2 Representative chromatograms of SPME (*top*) and solvent extracts (*bottom*, *inverted*) of female *Mallodon dasystomus* cuticular hydrocarbons

respectively) spectrometer (Palo Alto, CA, USA), as $CDCl_3$ solutions. ^{1}H NMR chemical shifts are expressed in ppm relative to residual $CHCl_3$ (7.27 ppm) and ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ (77.16 ppm). Unless otherwise stated, solvent extracts of reaction mixtures were dried by treatment with anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by vacuum flash chromatography (VFC) on silica gel (230–400 mesh; Fisher Scientific). Integer resolution mass spectra were obtained with a Hewlett-Packard (HP) 6890 GC (Avondale, PA, USA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium carrier gas. The GC was equipped with a DB5-MS column (25 m×0.20 mm ID×0.25 µm film, J&W Scientific, Folsom, CA, USA). Electrospray ionization exact mass data were obtained with an Agilent 6210 LCTOF instrument, introducing the sample via flow injection into a stream of 95% MeOH and 5% water (0.1% formic acid). The vaporizer and gas temperatures in the ESI interface were 200°C. Fragmenter voltage was 125 V. The instrument was operated in the multisource mode. GC-MS exact mass data were obtained on a Waters GCT Premier instrument operated in EI mode (70 eV) with ion source temperature 200°C and injector temperature 240°C. The GC was programmed from 50°C for 1 min, then 10°C min^{-1} to 300°C, using a DB-5 column as described above.

Synthesis of 2-methylhexacosane

12-(Tetrahydropyran-2-yloxy)-dodecan-1-ol **(2)** This compound was made by a modification of the method of Nishiguchi et al. (2000). Thus, 1,12-dodecanediol **1** (50.0 g, 247 mmol) was dissolved in a mixture of hexane (580 ml), dihydropyran (62.40 g, 741 mmol) and dimethylsulfoxide (21 ml). The mixture was stirred vigorously at 50–55°C while a solution of HCl (0.2 M, 49 ml) was slowly added, and the resulting mixture was stirred 14 h. After cooling, the aqueous phase was separated and extracted with hexane (3×50 ml). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (3×50 ml) and brine (2×100 ml), dried, and concentrated. The crude product was purified by vacuum flash chromatography (VFC) on silica gel affording 57.10 g (81%) of the pure mono-protected product **2** (plus 15.4 g ~55% pure by GC). ^{1}H NMR: δ 1.20–1.40 (m, 16H), 1.46–1.64 (m, 8H), 1.66–1.75 (m, 1H), 1.76–1.88 (m, 1H), 3.37 (dt, 1H, J=6.8 and 9.6 Hz), 3.45–3.54 (m, 1H), 3.62 (t, 2H, J=6.8 Hz), 3.72 (dt, 1H, J=6.8 and 9.6 Hz), 3.82–3.90 (m, 1H), 4.56 (dd, 1H, J= 2.5 and 4.3 Hz). ^{13}C NMR: δ 19.92, 25.72, 25.96, 26.45, 29.65, 29.70, 29.78, 29.79 (3C), 29.81, 29.97, 31.00, 62.58, 63.28, 67.93, 99.09. MS: m/z 285 (M-1, 1), 201 (1), 125 (1), 111 (3), 101 (27, 85 (100), 69 (17), 55 (35), 41 (33).

12-(Tetrahydropyran-2-yloxy)-dodecyl-1-p-toluenesulfonate **(3)** Tosyl chloride (40.92 g, 215 mmol) was added in small portions over 2 hr to a solution of 12-(tetrahydropyran-2-yloxy)-dodecan-1-ol **2** (55.90 g, 195 mmol) in dry pyridine (30.86 g, 390 mmol) and chloroform (390 ml), while stirring at 0°C. The reaction was allowed to warm to room temperature, stirred for 1 d, and then *N,N*-dimethylamino-pyridine (0.10 g) was added. After stirring 2 d, the mixture was concentrated under vacuum. The residue was taken up in ethyl ether (300 ml), and the solution was thoroughly washed with aqueous HCl (10%) (50 ml), saturated aqueous $NaHCO_3$ (2×50 ml), and brine (2×100 ml), then dried and concentrated. The resulting oil was purified by VFC (hexane: ethyl acetate, 9:1) giving the tosylate **3** as a white solid (64.31 g, 74.8% yield). ^{1}H NMR: δ 1.18–1.40 (m, 16H), 1.46–1.65 (m, 8H), 1.66–1.75 (m, 1H), 1.77–1.88 (m, 1H), 2.44 (s, 3H), 3.37 (dt, 1H, J=6.6 and 9.6 Hz), 3.46–3.53 (m, 1H), 3.62 (t, 2H, J=6.8 Hz), 3.72 (dt, 1H, J=6.8 and 9.6 Hz), 3.83–3.90 (m, 1H), 4.56 (dd, 1H, J=2.5 and 4.3 Hz), 7.31–7.35 (m, 2H), 7.76–7.80 (m, 2H). ^{13}C NMR: δ 19.95, 21.87, 25.54, 25.73, 26.46, 29.03, 29.15, 29.60, 29.70 (2C), 29.76, 29.78, 29.98, 31.02, 62.61, 67.92, 70.94, 99.12, 128.12 (2C), 130.02 (2C), 133.45, 144.83. HRMS (ESI): calc. m/z=463.2489 $(M+Na)^+$; found: m/z=463.2486.

15-Methyl-1-(tetrahydropyran-2-yloxy)-hexadecane **(4)** A solution of 1-bromo-3-methylbutane (5.14 g, 34.0 mmol) in dry THF (15 ml) was added dropwise (ca 3 hr) to a suspension of Mg turnings (1.24 g, 51 mmol) in dry THF (19 ml) at room temperature under Ar. After 5 hr, the resulting Grignard reagent solution was diluted with dry THF (30 ml) and added dropwise at –60°C to a previously prepared solution of tosylate **4** (5.00 g, 11.3 mmol) in dry THF (85 ml) under Ar, followed by addition of a solution of Li_2CuCl_4 in THF (0.1 M, 5.7 ml, 0.57 mmol; Aldrich Chemical Co., Milwaukee, WI, USA). The mixture was warmed to room temperature over 2 hr and stirred overnight. The reaction was quenched with saturated aqueous NH_4Cl (50 ml), then extracted with ethyl acetate (4×30 ml). The organic layer was washed with saturated aqueous $NaHCO_3$ (50 ml) and brine (50 ml), dried, and concentrated. The residue was purified by VFC (hexane: ethyl acetate, 95:5) affording compound **4** (3.65 g) in 94.5% yield. ^{1}H NMR: δ 0.85 (d, 6H, J=6.6 Hz), 1.10–1.20 (m, 2H), 1.20–1.40 (m, 21H), 1.44–1.62 (m, 8H), 1.66–1.75 (m, 1H), 1.78–1.88 (m, 1H), 3.37 (dt, 1H, J=6.8 and 9.6 Hz), 3.45–3.53 (m, 1H), 3.72 (dt, 1H, J=6.8 and 9.6 Hz), 3.83–3.91 (m, 1H), 4.57 (dd, 1H, J=2.7 and 4.3 Hz). ^{13}C NMR: δ 19.92, 22.88 (2C), 25.73, 26.47, 27.65, 28.19, 29.72, 29.83, 29.84, 29.89, 29.91, 29.92 (2C), 29.95, 29.99, 30.17, 31.01, 39.28, 62.55, 67.92, 99.06. MS: m/z 339 (M – H, 1), 267 (1), 115 (2), 101 (7), 85 (100), 71 (10), 57 (25), 43 (31) 41 (25).

15-Methylhexadecan-1-ol **(5)** *p*-Toluenesulphonic acid (100 mg) was added to a solution of 15-methyl-1-(tetrahydropyran-2-yloxy)-hexadecane **4** (3.60 g, 10.6 mmol) in methanol (20 ml) and the mixture was stirred at room temperature overnight. Most of the methanol was removed under reduced pressure, water (20 ml) was added to the residue, and the mixture was extracted with hexane (3× 20 ml). The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue was purified by VFC (hexane: ethyl acetate, 95:5) affording alcohol **5** (2.53 g) in 93.2% yield. ^{1}H NMR: δ 0.85 (d, 6H, *J*=6.6 Hz), 1.10–1.20 (m, 2H), 1.20–1.40 (m, 22H), 1.51 (non, 1H, *J*=6.6 Hz) 1.53–1.60 (m, 2H), 3.63 (t, 2H, *J*=6.7 Hz). ^{13}C NMR: δ 22.89 (2C), 25.96, 27.65, 28.20, 29.66, 29.83, 29.85, 28.89, 29.91, 29.92 (2C), 29.96, 30.18, 33.04, 39.29, 63.33. MS: *m/z* 238 (M – 18, 1), 223 (1), 210 (3), 182 (3), 168 (1), 153 (2), 140 (2), 125 (8), 111 (21), 97 (36), 83 (55), 71 (26), 57 (96), 43 (100) 41 (87).

1-Iodo-15-methylhexadecane **(6)** Iodine (1.34 g, 5.3 mmol) was added in small portions to a mixture of 15-methylhexadecan-1-ol **5** (0.90 g, 3.5 mmol), triphenylphosphine (1.38 g, 5.3 mmol), and imidazole (0.36 g, 5.3 mmol) in ethyl ether-acetonitrile (3:1–12 ml), and the resulting slurry was stirred at room temperature for 1 hr. The mixture then was diluted with ethyl ether (50 ml), washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue was purified by VFC (hexane) affording 1-iodo-15-methylhexadecane **6** (1.21 g) in 94.1% yield. ^{1}H NMR: δ 0.86 (d, 6H, *J*=6.6 Hz), 1.10–1.20 (m, 2H), 1.20–1.34 (m, 20H), 1.34–1.44 (m, 2H), 1.51 (non, 1H, *J*=6.6 Hz), 1.77–1.86 (m, 2H), 3.18 (t, 2H, *J*=7.0 Hz). ^{13}C NMR: δ 7.56, 22.90 (2C), 27.66, 28.20, 28.79, 29.66, 29.79, 29.85, 29.89, 29.92, 29.93, 29.96, 30.18, 30.75, 33.82, 39.30. MS: *m/z* 239 (M – I, 5), 183 (2), 169 (3), 155 (6), 141 (4), 127 (6), 113 (8), 99 (14), 85 (43, 71 (63, 57 (100), 43 (80).

25-Methylhexacos-9-yne **(8)** *n*-BuLi (1.6 M in hexanes, 4.30 ml, 6.9 mmol) was added dropwise to a solution of 1-decyne **7** (0.91 g, 6.6 mmol) in dry THF (7.0 ml) under Ar at –10°C, and the mixture was stirred for 2 hr, forming a suspension. Dry THF (6 ml) and DMPU (3.36 g, 26 mmol) were added, the mixture was stirred for 1 hr at 0°C, then recooled to –10°C, and a solution of 1-iodo-15-methylhexadecane **6** (1.20 g, 3.3 mmol) in dry THF (2.0 ml) was added dropwise. The reaction was quenched with saturated aqueous NH_4Cl (10 ml) and extracted with hexanes (4× 10 ml). The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue was purified by VFC (hexane), affording alkyne **8** contaminated with excess 1-decyne **7**. The impure product was used directly in the next step. ^{1}H NMR: δ 0.86 (d, 6H, *J*=6.6 Hz), 0.88 (t, 3H, *J*=7.0 Hz), 1.15–1.19 (m, 2H), 1.22–1.31 (m, 27H), 1.31–1.42 (m, 4H), 1.42–1.56 (m, 4H), 2.10–2.17 (m, 4H). ^{13}C NMR: δ 14.33, 18.99, 22.89, 27.65, 28.19, 29.10, 29.37, 29.40, 29.46, 29.80, 29.87, 29.91, 29.93, 29.96, 30.18, 32.08, 39.29, 77.55, 80.47. MS: *m/z* 376 (M^+, 12), 281 (3), 193 (8), 152 (7), 137 (18), 124 (24), 109 (36), 95 (70), 81 (100), 67 (92), 43 (98), 41 (68). HRMS (ESI): calc. *m/z*=375.3985 $(M\text{-}H)^+$; found: *m/z*=375.3991.

2-Methylhexacosane **(9)** A mixture of 25-methylhexacos-9-yne **8** (+ 1-decyne, 0.90 g) and 5% Pd/C (0.090 g,) in hexane (15 ml) was stirred at room temperature under hydrogen for 16 hr. The mixture was filtered through Celite, and the filtrate was concentrated. The resulting mixture of hydrocarbons was subjected to Kugelrohr distillation (oven temp to 140°C, 0.05 mm Hg) to remove decane, affording 0.75 g (60% over 2 steps) of pure 2-methylhexacosane **9**. ^{1}H NMR: δ 0.86 (d, 6H, *J*=6.8 Hz), 0.88 (t, 3H, *J*=6.8 Hz), 1.11–1.18 (m, 2H), 1.20–1.34 (m, 44H), 1.45–1.57 (m, 1H). ^{13}C NMR: δ 14.35, 22.89, 27.66, 28.20, 29.59, 29.89, 29.93, 29.96, 30.18, 32.16, 39.29. MS: *m/z* 380 (M^+, 1), 365 (3), 337 (16), 239 (3), 225 (2), 211 (3), 197 (4), 183 (4), 169 (5), 155 (6), 141 (8), 127 (9), 113 (12), 99 (19), 85 (42), 71 (63), 57 (100), 43 (93).

Synthesis of 2-methyloctacosane

2-Methyltetradec-5-yne **(11)** *n*-BuLi (2.88 M, 35 ml, 100 mmol) was added dropwise under Ar to a solution of 1-decyne **7** (13.8 g, 100 mmol) in 200 ml THF cooled in a dry ice-acetone bath. The resulting slurry was warmed to room temperature, and NaI (1.5 g, 10 mmol) and 1-bromo-3-methylbutane **10** (14.3 g, 95 mmol) were added. The resulting mixture was refluxed under Ar for 40 hr, then cooled and quenched with saturated aqueous NH_4Cl. The mixture was extracted with hexane, and the organic layer was washed with brine, dried, and concentrated. The residue was Kugelrohr distilled, collecting a forerun (oven temp <50°C, 0.1 mm Hg) consisting mostly of unreacted 1-decyne **7**. The desired product **11** then was collected in a clean bulb (15.33 g, oven temp 70–80°C, 0.1 mm Hg) in 73% overall yield (86% based on recovered 1-decyne). ^{1}H NMR: δ 0.89 (t, 3H, *J*= 6.8 Hz), 0.89 (d, 6H, *J*=6.8 Hz), 1.25–1.35 (m, 10H), 1.35–1.52 (m, 4H), 1.63–1.72 (m, 1H), 2.12–2.22 (m, 4H). ^{13}C NMR: δ 14.30, 16.95, 18.95, 22.39, 22.86, 27.37, 29.05, 29.32, 29.36, 29.41, 32.04, 38.37, 80.30, 80.40. MS: *m/z* 208 (M^+, 1), 193 (18), 166 (1), 152 (2), 137 (3), 123 (7), 109 (26), 95 (100), 81 (76), 69 (26), 67 (46), 55 (36), 43 (23). HRMS (ESI): calc. *m/z*=208.2186 (M^+); found: *m/z*=208.2193.

13-Methyltetradec-1-yne **(12)** A dry 1 liter 3-neck flask was flushed with Ar and charged with 225 ml dry 1,3-diaminopropane, followed by Li wire (3.2 g, 460 mmol). The mixture was stirred 1 hr at room temperature during which time the Li partially dissolved to give a blue solution. The mixture was heated to 70°C, and stirred for 2 hr, by which time all the Li had dissolved and the blue solution changed to a white suspension. The mixture was cooled to room temperature, and potassium *t*-butoxide (33 g, 295 mmol) was added in one portion with stirring, giving a yellow suspension. After stirring 30 min, 2-methyltetradec-5-yne **11** (15.2 g, 73 mmol) was added over 10 min, and the mixture became warm and turned red-brown. The progress of the isomerization was followed by GC, and after ~1 hr, there was no further change. The mixture was poured into 1 L of a slurry of crushed ice and water, and the mixture was extracted with hexane (3× 250 ml). The combined hexane extracts were washed with water and brine, dried, concentrated, and purified by Kugelrohr distillation (forerun, oven temp <60°C at 0.5 mm Hg; product, oven temp≤100°C, 0.2 mm Hg), yielding 12.76 g of alkyne **12** (83%). ^{1}H NMR: δ 0.87 (d, 6H, J=6.8 Hz), 1.11–1.18 (m, 2H), 1.22–1.34 (m, 12H), 1.35–1.44 (m, 2H), 1.47–1.57 (m, 3H), 1.94 (t, 1H, J= 2.5 Hz), 2.19 (td, 2H, J=7.0, 2.5 Hz). ^{13}C NMR: δ 18.6, 22.9 (2C), 27.6, 28.2, 28.7, 29.0, 29.3, 29.7, 29.8, 29.9, 30.1, 39.2, 68.2, 85.0. MS: *m/z* 193 (M$^+$-15, trace), 179 (trace), 137 (1), 123 (4), 109 (18), 95 (48), 81 (100), 69 (36), 67 (64), 57 (33), 55 (48), 43 (59). HRMS (ESI): calc. *m/z*=207.2107 (M-H)$^+$; found: *m/z*=207.2103.

2-Methyloctacos-13-yne **(14)** A solution of tetradecanol (2.14 g, 10 mmol) and pyridine (0.85 g, 10.5 mmol) in CH_2Cl_2 was cooled to <-10°C in an ice-salt bath, and triflic anhydride (2.2 ml, 13 mmol) was added dropwise. The resulting solution was slowly warmed to room temperature, by which time all the starting alcohol had been consumed. The solution was diluted with 100 ml hexane, and filtered through a 2 cm pad of Celite. The colorless filtrate was concentrated and pumped under vacuum for a few minutes to remove traces of solvent, then the crude triflate **13** was taken up in 10 ml THF and used immediately.

In a separate flask, a solution of 13-methyltetradec-1-yne **12** (2.6 g, 12.5 mmol) in 25 ml THF under Ar was cooled in an ice bath and n-BuLi (2.8 M, 4.8 ml, 13.4 mmol) was added dropwise. After stirring for 15 min, the mixture was cooled to –20°C, the solution of freshly prepared triflate was added dropwise, and the resulting slurry was slowly warmed to room temperature and then stirred for 4 hr. The mixture was quenched with ice water and extracted with hexane. After washing with brine, the hexane solution was dried and concentrated, and the residue was purified by VFC (hexane), followed by Kugelrohr distillation (oven temp up to 100°C, 0.2 mm Hg) to remove low-boiling impurities. The pot residue consisting of essentially pure alkyne **14** was used directly in the next step. ^{1}H NMR: δ 0.84–0.91 (m, 9H), 1.12–1.41 (m, 43H), 2.15 (m, 4H). ^{13}C NMR: δ 14.31, 18.56, 22.86, 27.62, 28.17, 29.07, 29.38, 29.56, 29.78, 29.89, 30.14, 31.79, 32.13, 39.26, 80.44. MS: *m/z* 404 (M$^+$,6), 292 (1), 291 (1), 278 (5), 277 (5), 264 (5), 263 (5), 236 (3), 221 (8), 207 (11), 194 (7), 180 (4), 166 (4), 151 (8), 137 (19), 123 (33), 109 (54), 97 (55), 96 (89), 95 (97), 83 (60), 82 (91), 81 (100), 69 (46), 67 (65), 57 (38), 55 (43), 43 (47). HRMS (ESI): calc. *m/z*=403.4298 (M-H)$^+$; found: *m/z*= 403.4291.

2-Methyloctacosane **(15)** Alkyne **14** was taken up in 50 ml hexane, and 5% Pd on activated charcoal (0.5 g) was added. The flask was fitted with a balloon filled with H_2, and the headspace was flushed with H_2 before sealing and stirring under H_2 until hydrogenation was complete (3 hr). The resulting mixture was filtered through a pad of Celite, rinsing with 20 ml hexane, and the combined filtrate (~70 ml) was cooled overnight at –20°C. The resulting white crystals were filtered with suction in a cold room, yielding 1.38 g of 2-methyloctacosane **15** (>99.5% pure), and 2 g of impure product from the remaining liquor after concentration. ^{1}H NMR: δ 0.86 (d, 6H, J=6.8 Hz), 0.88 (t, 3H, J=6.8 Hz), 1.11–1.18 (m, 2H), 1.20–1.32 (m, 48H), 1.45–1.57 (m, 1H). ^{13}C NMR: δ 14.33, 22.86, 27.64, 28.17, 29.58, 29.90, 30.17, 32.13, 39.29. MS: *m/z* 408 (M$^+$,1), 393 (9), 365 (30), 351 (2), 337 (2), 323 (3), 309 (3), 295 (4), 281 (4), 267 (4), 253 (5), 239 (5), 225 (6), 211 (7), 197 (8), 183 (8), 169 (10), 155 (12), 141 (15), 127 (19), 113 (25), 99 (36), 85 (77), 71 (88), 57 (100), 43 (55).

Bioassays of Hydrocarbon Standards We tested the bioactivity of female-specific compounds in bioassays similar to those described above. The bioactivity of standards of 2Me-C_{26} and 2Me-C_{28} were tested individually and in combination at 1 FE doses in ratios similar to those of SPME samples (28 and 43 ug, respectively). Compounds were quantified by comparing their peak areas in total ion chromatograms with those of an external standard (*n*-docosane). Numbers of males responding to treatments were compared with those responding to freeze-killed females using a Fisher's exact test (Sokal and Rohlf, 1995). Bioassays were conducted in the dark under a red light between 12.00 and 17.00 hr during late June through July, 2009. The remaining female specific compounds were not synthesized and tested because these two synthetic compounds elicited a response in males equal to that of freeze-killed females (see Results).

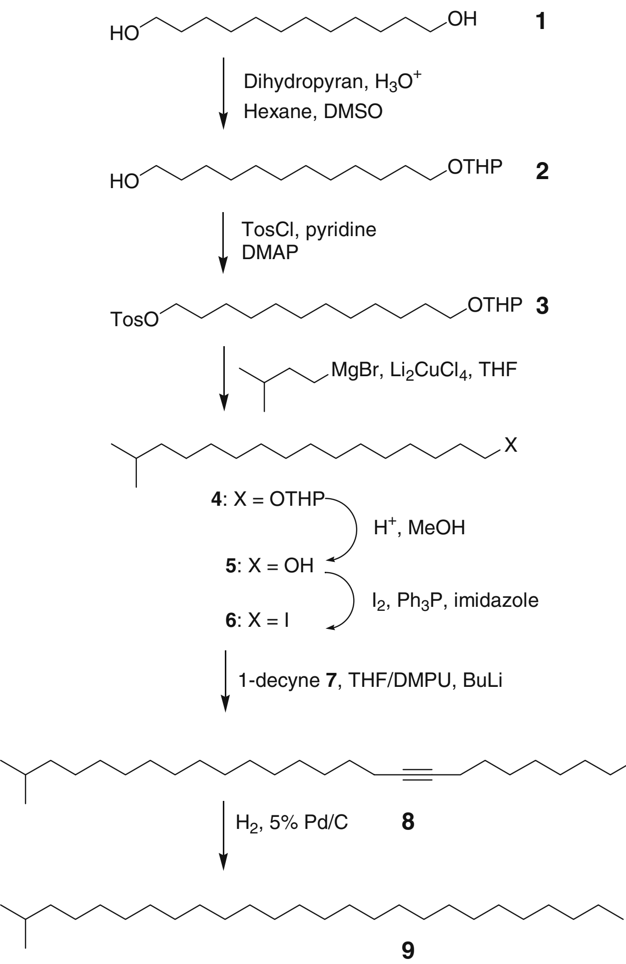

Fig. 3 Synthesis of 2-methylhexacosane

Results

Characterization of Mating Behavior Once a male touched a female with his antennae ($N=17$), a clear progression of cumulative behavioral steps led to copulation: Step 1) male stopped walking and antennated the female; Step 2) the male aligned his body with the female; Step 3) the male mounted the female; Step 4) the male bent his abdomen to connect his genitalia to those of the female. Out of 17 males tested, 15 responded to live females with the full range of behavioral steps after making antennal contact. The remaining two males either ignored the female or responded aggressively (e.g., head raised, biting female's elytra and/or legs) upon encountering the female. In preliminary assays, males were also paired with other males ($N=10$) and, after brief antennal contact, responded aggressively to each other (e.g., biting and kicking) and made no attempt to mate.

Role of Contact Chemoreception All males that were tested displayed a Step 4 behavioral response to freeze-killed females, confirming that signals mediating mate recognition were intact and behavioral signals were not important in mate recognition. None of the males responded to the solvent-washed females, demonstrating that the compounds that mediate mate recognition had been removed by solvent extractions. Of the 18 males tested, 16 displayed Step 4 behavior toward female carcasses to which solvent extracts had been reapplied (response not different from response to freeze-killed females; Fisher's exact test, $F=14.06$, $P>0.05$). Males displayed mating behavior to female carcasses after application of an average (±SE) of 0.15±0.03 FE of crude extract. Two males did not display any mating behaviors to females reconstituted with as much as 0.9 FE of crude extract.

Identification of Cuticular Hydrocarbons Thirty-one compounds were identified in hexane extracts of females, including a series of straight chain compounds from C_{23} to C_{29}. Thirteen compounds were essentially specific to females (Table 1, Fig. 1). Although there were many qualitative differences between hydrocarbon profiles of extracts of females and males, those of females contained two dominant compounds (2Me-C_{26} and 2Me-C_{28}) that were virtually absent in extracts of males. Of these two compounds, the relative abundance of 2Me-C_{26} in SPME wipe samples increased by 65% over solvent extracts (peak 15; Table 1, Fig. 2, means for two sampling methods significantly different, ANOVA: $F_{(1,8)}=10.28$, $P=0.01$),

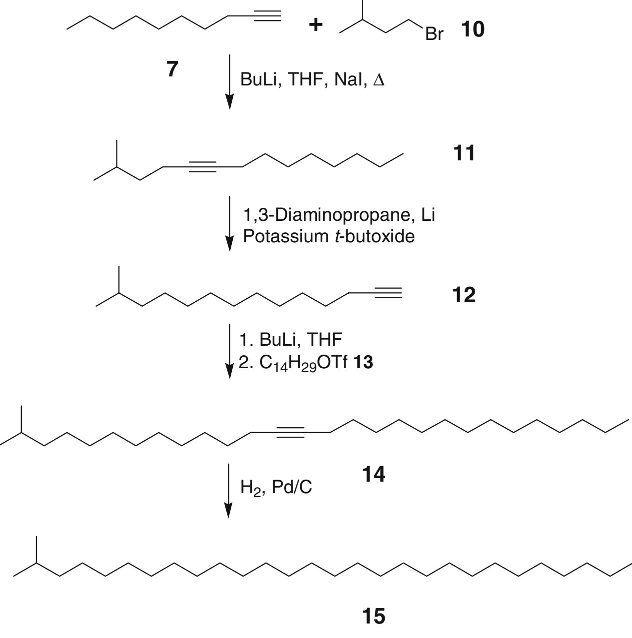

Fig. 4 Synthesis of 2-methyloctacosane

Table 2 Responses of male *Mallodon dasystomus* to freeze-killed females (controls) and to solvent-washed females that had been treated with one female equivalent of hydrocarbon standards

Compound(s)	No. of males tested	Percent of males responding per step in behavioral sequence[a]			
		Step 1	Step 2	Step 3	Step 4[b]
2Me-C_{26}	21	90 (100)	71 (100)	52 (100)	38 (86)*
2Me-C_{28}	24	96 (100)	79 (100)	67 (100)	58 (100)**
2Me-C_{26}+2Me-C_{28}	27	100 (100)	100 (100)	100 (100)	81 (100)

[a] Response of males to treatments *versus* controls (values in parentheses) were tested with Fisher's exact test (*$P<0.05$, **$P<0.001$)

[b] Numbers of males reaching behavioral step 4 were compared to the response to controls (H_o=no difference in bioactivity between treatment and freeze-killed female)

suggesting that it may be an important component of the contact pheromone. Other less abundant, female-specific compounds included: a C_{25} diene, 11-methyl- and 13-methyl-C_{27}, 11,15-dimethyl-C_{27}, 12-methyl-, 13-methyl-, and 14-methyl-C_{28}, 11-methyl-, 13-methyl-, and 15-methyl-C_{29}, and 13,17-dimethyl-C_{29} (Table 1, Fig. 1). Most of the compounds present in the solvent extracts also were present in the SPME samples of females.

Synthesis of 2Me-C_{26} and 2Me-C_{28} Two different routes for making 2-methylalkanes were explored. In the first (Fig. 3), 1,12-dodecanediol **1** was mono-protected with 3,4-dihydropyran (DHP) to give **2** in 81% isolated yield (Nishiguchi et al., 2000), and the remaining hydroxyl group then was converted to the tosylate **3** (75%). Coupling of tosylate **3** with 3-methylbutyl magnesium bromide with Li_2CuCl_4 catalysis installed the methyl branch, giving 15-methyl-1-(tetrahydropyran-2-yloxy)-hexadecane **4** (95%), which was deprotected in acidic MeOH to give 15-methyl-hexadecan-1-ol **5**. The alcohol was converted to iodide **6** in 94% yield (Corey et al., 1983), giving a synthon that could be converted to any desired long-chain 2-methylalkane. Thus, treatment of 1-decyne **7** in DMPU/THF with *n*-butyllithium followed by addition of iodide **6** gave crude 25-methylhexadec-9-yne **8** contaminated with unreacted 1-decyne. This mixture was reduced with H_2 and 5% Pd/C catalyst, giving 2-methylhexacosane **9** in 60% isolated yield over 2 steps after purification.

2-Methyloctacosane was made by a shorter route (Fig. 4). Thus, reaction of 1-bromo-3-methylbutane **10** with 1-decynyllithium in refluxing THF with NaI catalysis (Buck and Chong, 2001) gave 2-methyltetradec-5-yne **11** in 73% yield (86% based on recovered starting material). Isomerization of the alkyne to the terminal position with the acetylene zipper reaction (Abrams and Shaw, 1988) then gave 13-methyltetradec-1-yne **12**, an alternate synthon for synthesis of a variety of long-chain 2-methylalkanes. Deprotonation of 13-methyltetradec-1-yne **12** with butyllithium followed by reaction with the triflate **13** derived from tetradecan-1-ol in THF gave 2-methyloctacos-13-yne **14** in good yield (Wang and Zhang, 2007), which was then hydrogenated with H_2 and 5% Pd/C catalyst to give the desired 2-methyloctacosane **15**. This route was shorter and more efficient overall than the route used to make 2-methylhexacosane.

Bioassays of Hydrocarbon Standards In bioassays, 2Me-C_{26} and 2Me-C_{28} as individual components elicited some mating responses in males (Step 4 behavior). However, males responded at a rate similar to that elicited by freeze-killed females only when the two compounds were presented as a blend (Table 2).

Discussion

Our results support the hypothesis that male *M. dasystomus* recognize females by a cuticular contact pheromone blend. Males attempted to mate with females only after contacting them with their antennae. In bioassays with freeze-killed females, all males displayed a full progression of mating behaviors and attempted to connect the genitalia, excluding behavioral signals as a component of mate recognition. Additionally, no males attempted to mate with a solvent washed female, suggesting that the mate recognition signals had been removed by the solvent, and that visual signals were not involved. When paired with extracted female carcasses that had been treated with the cuticular extract, 15 of 17 males attempted to mate only after making antennal contact, providing further evidence for an extractable contact pheromone.

Because of the marked differences in the profiles of cuticular lipids between the sexes, our strategy for identifying the contact pheromone components was to synthesize and test the bioactivity of female specific compounds, starting with the most abundant. This approach was effective in the identification of the contact pheromones of *Xylotrechus colonus* F. (Ginzel et al., 2003a) and,

more recently, *N. a. acuminatus* (Lacey et al., 2008). The first two compounds that were synthesized (2Me-C_{26} and 2Me-C_{28}) comprised over 17% of the total hydrocarbons in the cuticular extracts of females. Moreover, there was a significant increase in the proportion of 2Me-C_{26} in the SPME samples when compared to the whole-body hexane extracts, suggesting that it may be an important component of the contact pheromone.

Male *M. dasystomus* responded to both 2Me-C_{26} and 2Me-C_{28} alone in bioassays. However, a blend of the two compounds elicited the greatest response in males, and the response was similar to that of the crude extract, suggesting that these compounds together are the most important components of the contact pheromone. Other cerambycids also have contact pheromones that are multicomponent blends. For example, the contact pheromone of *X. colonus* is comprised of at least three compounds: *n*-C_{25}, 9Me-C_{25}, and 3Me-C_{25} (Ginzel et al., 2003a). Moreover, male *N. a. acuminatus* recognize mates by the presence of 7Me-C_{27}, with two additional branched compounds, 9Me-C_{27} and 7Me-C_{25}, acting as synergists (Lacey et al., 2008). These multi-component blends often are composed of compounds that are homologues, as is the case here with 2Me-C_{26} and 2Me-C_{28}. However, to our knowledge, these compounds are the first 2-methylalkanes and even-chain length hydrocarbons to be identified as contact pheromones in the Cerambycidae. Nevertheless, both 2Me-C_{26} and 2Me-C_{28} are part of the wax layer of other cerambycid species, suggesting that some hydrocarbons may be common to the family. For example, 2Me-C_{26} was found in the cuticular hydrocarbons of male and female *N. a. acuminatus* (Lacey et al., 2008), both sexes of *M. robiniae* (Ginzel et al., 2003b), and in the wax layer of female *M. caryae* (Ginzel et al., 2006). Interestingly, 2Me-C_{28} is more abundant in the wax layer of male than female *M. robiniae* (Ginzel et al., 2003b), whereas it is more abundant in the cuticular hydrocarbons of female than male *M. caryae* (Ginzel et al., 2006).

The solvent extracts of female *M. dasystomus* contained 13 compounds that were essentially absent or greatly reduced in extracts of males. Similar sexual differences were noted in the cuticular lipid profiles of *M. robiniae* and *X. colonus*, in which the compounds constituting the contact pheromones were not completely absent from extracts of males, but were greatly reduced in abundance when compared to extracts of females (Ginzel et al., 2003a, 2006). Here, the differences in the relative amounts of the *M. dasystomus* contact pheromone components, 2Me-C_{26} and 2Me-C_{28}, were obvious, with these two compounds being co-dominant in the hydrocarbon profiles of females. Furthermore, 2Me-C_{26} was significantly more abundant in SPME wipe samples than in hexane extracts of female cuticular hydrocarbons, supporting our previous observations that contact pheromones are present in greater abundance in SPME samples when compared to whole-body extracts. SPME may more accurately represent the compounds that are accessible to the male antennae on the surface of the wax layer, and provide a clearer representation of those hydrocarbons that act as contact pheromones.

During this study, we also observed females displaying behaviors associated with long-range pheromone production. These "calling" behaviors included lowering the head, raising the abdomen, and extending the ovipositor, often in a rhythmic pumping motion. While "calling", one female everted a clear cylindrical, fluid-filled sac from her ovipositor. Female *Prionus californicus* release a powerful sex pheromone from a similar structure (Cervantes et al., 2006; Rodstein et al., 2009). In fact, the calling behaviors of female *M. dasystomus* are almost identical to those of *P. californicus* (Barbour et al., 2006), and similar to other species of insects in which females produce volatile pheromones (e.g., Burkholder et al., 1974; Hammack and Burkholder, 1981). In the subfamily Cerambycinae, the scanty available evidence suggests that mate location might involve three sequential behavioral steps: 1) both sexes are independently attracted to volatiles emanating from the larval host plant; 2) once on the host, males attract both sexes from some distance with aggregation pheromones; and 3) having recruited conspecifics, males recognize females by contact pheromones (Ginzel and Hanks, 2005). In contrast, beetles in the subfamily Prioninae may rely exclusively on sex pheromones to bring the sexes together (Barbour et al., 2006; Cervantes et al., 2006; Rodstein et al., 2009), and then, once in close proximity, the final steps of mate recognition may be mediated by contact pheromones. Although this is the first contact pheromone identified from a cerambycid beetle in the subfamily Prioninae, we anticipate that the use of contact pheromones will be widespread in this subfamily (e.g., Barbour et al., 2007), as it is in many other insect groups.

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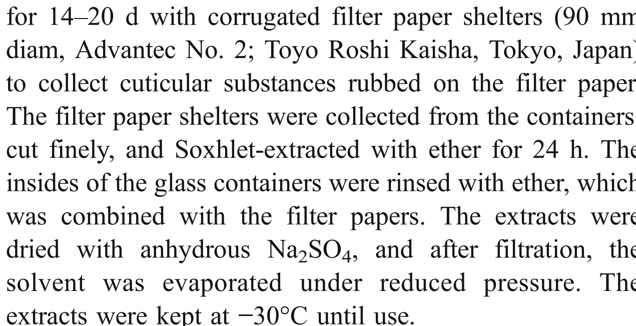
successful mating. Although the process of mate selection is conserved by the necessity to perceive and locate a suitable mate, pheromonal signals tend to be highly divergent across species (Johansson and Jones, 2007).

Callosobruchus species (Coleoptera: Chrysomelidae: Bruchinae) are serious pests of stored pulses, cowpeas, pigeonpeas, and other legumes (Rees, 1996, 2004), and are found worldwide, particularly in Eurasia. During mating, two types of sex pheromones are used sequentially. First, a sex attractant pheromone released by the female attracts the male from a distance. Second, a contact sex pheromone causes the male to extrude his genital organ for copulation at close range (Tanaka et al., 1981). Generally, sex attractant pheromones are volatile, whereas contact sex pheromones have low volatility and act as contact or gustatory stimuli (Birch, 1974). The contact sex pheromones have been characterized from *Callosobruchus chinensis* (L.) and *C. maculatus* (F.) as a mixture of monoterpene dicarboxylic acids and some hydrocarbons (Tanaka et al., 1981, 1982; Nojima et al., 2007). However, comparisons of cross-copulatory activity (i.e., interspecific copulatory activity) among four congeneric species revealed that both beetles lacked the ability to discriminate their conspecific and/or heterospecific partners (Shimomura et al., 2010). Meanwhile, we found that male *C. analis* (F.) exhibited copulatory behavior only in response to conspecific females among the four species, and we clarified the presence of a contact sex pheromone in this species (Shimomura et al., 2010). *Callosobruchus analis* is distributed widely in tropical Asia and Africa (Tuda et al., 2005).

Although the sex attractant pheromone of *C. analis* was determined to be a short chain, methyl branched fatty acid (Cork et al., 1991), the contact sex pheromone has not been investigated chemically. In this research, we characterized the contact sex pheromone of *C. analis*, and we discuss the specificity of mate recognition in this species among congeneric species based on the contact sex pheromone.

Methods and Materials

Insects A laboratory-maintained colony was used in this research. The beetles were reared on *Vigna angularis* in a dark incubator at 28°C with ambient humidity. Newly emerged adults from beans were separated immediately by sex. Female beetles that were to be presented to test males were freeze-killed and kept in a freezer (−30°C) until they were used, and males were kept separately in a glass vial (15 mm diam, 35 mm high) and conditioned for 1 d at 27°C with ambient humidity in a dark incubator.

Pheromone Collection Each set of 300–400 virgin females was kept in a glass container (90 mm diam, 100 mm high) 
for 14–20 d with corrugated filter paper shelters (90 mm diam, Advantec No. 2; Toyo Roshi Kaisha, Tokyo, Japan) to collect cuticular substances rubbed on the filter paper. The filter paper shelters were collected from the containers, cut finely, and Soxhlet-extracted with ether for 24 h. The insides of the glass containers were rinsed with ether, which was combined with the filter papers. The extracts were dried with anhydrous Na_2SO_4, and after filtration, the solvent was evaporated under reduced pressure. The extracts were kept at −30°C until use.

Purification of Pheromone Compounds

Acid-Base Partition The crude pheromone extract was fractionated into basic, acidic, and neutral fractions. The extract was dissolved in 30 ml ether and extracted twice with 20 ml of 0.5 M HCl. The acidic, aqueous layer was alkalized with 5 M NaOH and extracted twice with 20 ml of ether to recover the basic compounds, and this ether layer was washed with brine and dried with anhydrous Na_2SO_4. Acidic compounds were extracted from the original ether layer twice with 20 ml of 0.5 M NaOH. The alkaline aqueous layer was acidified with 5 M HCl and extracted twice with 20 ml of ether to recover the acidic compounds, and this ether layer was washed with brine and dried with anhydrous Na_2SO_4. The remaining ether layer, from which basic and acidic compounds had been extracted, and which contained neutral compounds, was washed with brine and dried by anhydrous Na_2SO_4. After filtration, each fraction was concentrated under reduced pressure.

Column Chromatography The acidic compounds were redissolved in ether and concentrated to dryness with 200 mg of silica gel (Wakogel C-200, Wako Pure Chemical Industries, Osaka, Japan) in a rotary evaporator. The silica gel was applied to the top of a prewetted 500 mg silica gel column. The acidic compounds were eluted sequentially with 7 ml each of hexane, 20% EtOAc in hexane, 50% EtOAc in hexane, EtOAc, 50% EtOAc in methanol, methanol and 0.1% TFA in methanol. The neutral compounds were redissolved in ether and concentrated to dryness with 700 mg of silica gel (Wakogel C-200) in a rotary evaporator. The silica gel was applied to the top of a prewetted 1.5 g silica gel column. The neutral compounds were eluted sequentially with 20 ml each of hexane, 5% ether in hexane, 10% ether in hexane, 20% ether in hexane, 50% ether in hexane, and ether. The basic compounds were not subjected to column chromatography because they had no behavioral activity in the copulation assay (see "Results").

High Performance Liquid Chromatography (HPLC) The active fraction was further purified by normal phase HPLC with LC-10A (Shimadzu, Kyoto, Japan), equipped with a

silica gel column (Inertsil SIL-100A, 3 μm, 4.6 mm× 250 mm, GL Science, Tokyo, Japan) and eluted with 5% 2-propanol in hexane (1 ml/min). The effluent was monitored with a photodiode array detector SPD-M10Avp (Shimadzu) and collected (10 ml/fraction). The active fraction was purified again on the same column; eluted with 2% 2-propanol in hexane (1 ml/min); and the effluent was collected (5 ml/fraction).

Methyl Esterification An aliquot of the active acidic fraction was added to a 50-μl V-shaped vial (Nichiden-Rika Glass, Hyogo, Japan). A volume of 10 μl of sample was mixed with 5 μl methanol and 5 μl of 10% trimethylsilyldiazomethane in hexane solution (Tokyo Kasei, Tokyo, Japan). The mixture was left at room temperature for 30 min, then subjected to analysis by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analysis GC-MS analysis was carried out with a Shimadzu GC 17A instrument equipped with a DB-5 or DB-23 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA), coupled to a Shimadzu QP-5000 quadrupole MS in EI mode (70 eV). Helium was used as the carrier gas at a head pressure of 100 kPa and a flow rate of 1.6 ml/min. Oven temperature was set initially at 60°C for 3 min and increased at a rate of 10°C/min to 280°C for the DB-5 column and 220°C for the DB-23, and held for 10 min. The injector and interface temperatures were set at 250°C and 280°C, respectively.

Determination of Stereoisomeric Compositions of Active Compounds The stereoisomeric compositions of the active compounds were determined by using the Ohrui-Akasaka method (Akasaka et al., 1997, 1998; Akasaka and Ohrui, 1999; Yajima et al., 2006, 2007a). An aliquot of the active fraction was dissolved in MeCN:toluene=1:1. (*R*)- or (*S*)-2-(2,3-anthracenedicarboximide)-1-propanol (2A1P-OH, excess amount); 2-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC, excess amount); and 4-*N*,*N*-dimethylaminopyridine (DMAP, excess amount) were added successively. The mixture was stirred at room temperature overnight. An aliquot then was loaded onto a silica gel TLC plate (10×10 cm, silicagel 60 F254, Art-5744, Merck, Lindenplatz, Haar, Germany) and developed with hexane:EtOAc=2:1. The target spot, detected by fluorescence, was collected and eluted with EtOAc. After evaporation of the solvent with an N_2 stream, the residue was dissolved in methanol and purified by 2D-HPLC. The HPLC conditions were as follows: 1) first column, Develosil ODS-HG-3 (4.6×150 mm, Nomura Chemical, Aichi, Japan); flow rate, 0.4 ml/min; mobile phase, methanol; column temperature, 0°C; and second column, Develosil ODS-A-3 (4.6×150 mm, Nomura Chemical); flow rate, 0.4 ml/min; mobile phase, methanol:MeCN:THF=2:2:1; and column temperature, −37°C. Fluorescence intensity was monitored at 462 nm (excitation at 298 nm) by using a FP-920 fluorescence detector (Jasco, Tokyo, Japan) to detect the desired compound.

Chemicals Four stereoisomers of 2,6-dimethyloctane-1,8-dioic acid (**1**) were synthesized as described in Nakai et al. (2005). (2*S*,6*R*)-**1** consisted of 83.8% d.e. and >99% e.e.; (2*S*,6*S*)-**1** consisted of 89.4% d.e. and >99% e.e.; (2*R*,6*R*)-**1** consisted of 90.4% d.e. and >99% e.e.; and (2*R*,6*S*)-**1** consisted of 95.8% d.e. and >87.1% e.e.. (*R*)-Callosobruchusic acid, (2*E*,7*R*)-3,7-dimethyl-2-octene-1,8-dioic acid, was synthesized as described in Yajima et al. (2007a) and consisted of >90% e.e.

Synthesis of (S)-Callosobruchusic Acid Column chromatography was performed with Wakogel C-200 (Wako). NMR spectra were recorded on a JNM-ECA400 (JEOL, Tokyo, Japan) (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts were expressed in ppm relative to the residual solvent peak. IR spectra were recorded on an IR-4100 spectrometer (Jasco) and are reported in terms of frequency of absorption (cm^{-1}). Optical rotation was measured on a P-2100 polarimeter (Jasco). Elemental compositions were analyzed on a Microcorder JM10 (J-Science, Kyoto, Japan).

(4S,2'E,6'E)-3-[6'-Methyl-8'-(tert-butyldimethylsilyloxy)-2,6-octadienoyl]-4-phenylmethyl-1,3-oxazolidin-2-one (**5**) A mixture of **3** (500 mg, 2.06 mmol) and (*S*)-**4** (1.20 g, 2.70 mmol) was dissolved in benzene (15 ml). The mixture was stirred for 12 h at 80°C and the solvent was evaporated. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=10:1) to afford **5** (855 mg, 93%) as a colorless oil. $[\alpha]_D^{23}$=+42.5 (*c*=0.92, $CHCl_3$). IR (film): ν=1783, 1684, 1635 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.06 (s, 6H), 0.90 (s, 9H), 1.65 (s, 3H), 2.20 (t, *J*=7.7 Hz, 2H), 2.43 (dt, *J*=7.9, 7.7 Hz, 2H), 2.79 (dd, *J*=9.5, 13.5 Hz, 1H), 3.33 (dd, *J*=3.1, 13.5 Hz, 1H), 4.19 (m, 4H), 4.73 (m, 1H), 5.35 (m, 1H), 7.15–7.37 (m, 6H).

(4S,6'E)-3-[6'-Methyl-8'-(tert-butyldimethylsilyloxy)-6-octenoyl]-4-phenylmethyl-1,3-oxazolidin-2-one (**6**) To a stirred and cooled (−78°C) solution of **5** (758 mg, 1.71 mmol) in dry THF (10 ml) was added a solution of lithium tri-*sec*-butylborohydride (1.0 M in THF, 3.42 ml, 3.42 mmol) under Ar. After stirring for 3 d, the mixture was poured into saturated NH_4Cl aq. The aqueous phase was extracted with ether. The combined extracts were washed with water and saturated NH_4Cl aq., and dried with Na_2SO_4. The organic layer was concentrated under reduced pressure. The residue was purified by SiO_2 column

chromatography (hexane/EtOAc=10:1) to afford **6** (590 mg, 77%) as a colorless oil. $[\alpha]_D^{22}$=+26.2 (*c*=1.38, $CHCl_3$). IR (film): ν=1784, 1697 cm^{-1} (C=O). ^1H NMR (400 MHz, $CDCl_3$): δ=0.06 (s, 6H), 0.90 (s, 9H), 1.46–1.70 (m, 4H), 1.62 (s, 3H), 2.05 (t, *J*=7.6 Hz, 2H), 2.77 (dd, *J*=9.5, 13.5 Hz, 1H), 2.94 (m, 2H), 3.33 (dd, *J*=3.1, 13.5 Hz, 1H), 4.18 (m, 4H), 4.67 (m, 1H), 5.32 (t, *J*=6.3 Hz, 1H), 7.15–7.37 (m, 5H).

(4S,2'S,6'E)-3-[2',6'-Dimethyl-8'-(tert-butyldimethylsilyloxy)-6-octenoyl]-4-phenylmethyl-1,3-oxazolidin-2-one (**7**) To a stirred and cooled (−78°C) solution of **6** (590 mg, 1.33 mmol) in dry THF (5 ml) was added a solution of sodium bistrimethylsilylamide (1.0 M in THF, 1.59 ml, 1.59 mmol) under Ar. After stirring for 30 min, methyl iodide (248 µl, 3.98 mmol) was added to the mixture. The mixture was stirred for 12 h at the same temperature and then the reaction mixture was poured into saturated NH_4Cl aq. The aqueous phase was extracted with ether. The combined extracts were washed with water and brine, and dried with Na_2SO_4. The organic layer was concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=10:1) to afford **7** (95 mg, 16%) as a colorless oil. $[\alpha]_D^{26}$=+53.7 (*c*=0.26, $CHCl_3$). IR (film): ν=1784, 1698 cm^{-1} (C=O). ^1H NMR (400 MHz, $CDCl_3$): δ=0.06 (s, 6H), 0.90 (s, 9H), 1.22 (d, *J*=6.7 Hz, 3H), 1.30–1.45 (m, 3H), 1.58 (s, 3H), 1.72 (m, 1H), 2.00 (t, *J*=7.4 Hz, 2H), 2.76 (dd, *J*=9.5, 13.5 Hz, 1H), 3.26 (dd, *J*=3.1, 13.5 Hz, 1H), 3.71 (sxt, 1H, *J*=6.7 Hz, 1H), 4.18 (m, 4H), 4.68 (m, 1H), 5.29 (dt, *J*=1.1, 6.3 Hz, 1H), 7.15–7.37 (m, 5H).

(4S,2'S,6'E)-3-[8'-Hydroxy-2',6'-dimethyl-6-octenoyl]-4-phenylmethyl-1,3-oxazolidin-2-one (**8**) To a stirred solution of **7** (90 mg, 0.20 mmol) in THF (0.8 ml) was added a mixture of solution of tetrabutylammonium fluoride (1.0 M in THF, 4.90 ml, 4.90 mmol) and acetic acid (290 mg, 4.90 mmol). After stirring for 4 h, the mixture was poured into water. The aqueous phase was extracted with EtOAc. The combined extracts were successively washed with saturated NH_4Cl aq., saturated $NaHCO_3$ aq. and brine, and dried with Na_2SO_4. The organic layer was concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=2:1) to afford **8** (56 mg, 82%) as a colorless oil. $[\alpha]_D^{26}$=+62.6 (*c*=0.33, $CHCl_3$). IR (film): ν=3428 cm^{-1} (O–H), 1770, 1697 cm^{-1} (C=O). ^1H NMR (400 MHz, $CDCl_3$): δ=1.23 (d, *J*=6.7 Hz, 3H), 1.30–1.50 (m, 3H), 1.66 (s, 3H), 1.74 (m, 1H), 2.00 (t, *J*=7.4 Hz, 2H), 2.76 (dd, *J*=9.5, 13.5 Hz, 1H), 3.26 (dd, *J*=3.1, 13.5 Hz, 1H), 3.71 (sxt, 1H, *J*=6.7 Hz, 1H), 4.14–4.23 (m, 4H), 4.68 (m, 1H), 5.41 (dt, *J*=1.1, 6.3 Hz, 1H), 7.15–7.37 (m, 5H).

(2E,7S)-Callosobruchusic acid (**2**) To a stirred solution of **8** (52 mg, 0.15 mmol) in CH_2Cl_2 (1 ml) was successively added $NaHCO_3$ (76 mg) and Dess-Martin periodinane (Dess and Martin, 1983) (180 mg, 0.45 mmol) at 0°C. After stirring for 30 min, the reaction was quenched by the addition of a saturated $NaHCO_3$ solution. The aqueous phase was extracted with CH_2Cl_2, and the organic extract was washed with brine and dried with Na_2SO_4. After concentrating under reduced pressure, the residue was dissolved in a mixture of *t*-BuOH (1 ml) and 2-methyl-2-butene (0.4 ml). The mixture then was cooled to 0°C, and a solution of 20% NaH_2PO_4 solution (0.5 ml) and $NaClO_2$ (140 mg, 1.50 mmol) was added. The solution was stirred for 1 h and then poured into water. The aqueous phase was extracted with EtOAc, and the organic extract was washed with brine, dried with Na_2SO_4. The organic layer was concentrated under reduced pressure. The residue was dissolved in THF (1 ml) and water (0.5 ml). To the solution was added solid LiOH (10 mg, 0.40 mmol). After stirring for 30 min, the mixture was poured into water. The aqueous phase was extracted with CH_2Cl_2. The aqueous phase was acidified with 6 N HCl and extracted with EtOAc. The organic extract was washed with brine and dried with Na_2SO_4. The organic layer was concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (hexane/EtOAc=1:5) to afford **2** (2 mg, 7% in three steps) as a colorless solid. $[\alpha]_D^{27}$=+10.1 (*c*=0.10, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ=1.22 (d, *J*=6.7 Hz, 3H), 1.45 (m, 1H), 1.54 (quint, *J*=7.5 Hz, 2H), 1.68 (m, 1H), 2.15 (s, 3H), 2.17 (m, 2H), 2.48 (sep, *J*=6.7 Hz, 1H), 5.69 (s, 1H). The physical properties were in good accord with previous report (Nanda and Scott, 2004).

*Determination of the Enantiomeric Purity of the Synthetic (S)-**2*** (*R*)- and (*S*)-2A1P-O bis-ester of (*S*)-**2** were prepared as described above and were analyzed by HPLC. The HPLC conditions were as follows. Column; Develosil ODS-A-3 (4.6 mm i.d.×150 mm, Nomura Chemical), mobile phase; MeOH:MeCN:THF=2:2:1, temp; −40°C, flow rate; 0.4 ml/min. Detection was carried out by monitoring fluorescence intensity at 460 nm (excitation at 362 nm) by using a FP-920 fluorescence detector (Jasco). The retention times of the (*R*)-2A1P-O bis-ester of (*S*)-**2** and (*S*)-2A1P-O bis-ester of (*S*)-**2** were 64.1 min and 77.0 min, respectively.

Behavioral Bioassay Males in vials were removed from the incubator and acclimated to conditions in an assay room for 1 h before the copulation assay. The assay was performed at 27°C in the light.

(1) Female dummy: Freeze-killed females were warmed to room temperature and washed sequentially with ether, methanol, and ether to remove the substances of the cuticular surface. After the solvent had evaporated,

the solvent-washed female was stabbed in the abdomen with insect pins and presented to the male in the vial to see if the male displayed his genital organ.

(2) Glass rod dummy: The tip of a glass rod (2 mm i.d., 100 mm length) was rounded by flame on a gas burner and abraded with sandpaper to imitate the female's body.

Five microliters of the extracts or synthetic compounds dissolved in ether were applied to each dummy with a micro syringe. After the solvent had evaporated, the dummy was presented to the male in the vial. Copulatory activity was evaluated by observing whether males tried to copulate and display their genital organ for at least 2 min in each assay. Each male and each female dummy was tested only once. The sample dose was adjusted in terms of female equivalents (feq; note that feq calculations were based on filter paper extracts, not on female whole-body extracts). The numbers of males that extruded their genital organs to the treated or solvent control were compared with a Fisher's exact probability test. Multiple comparisons for male copulatory activity were performed by Fisher's exact probability test followed by a series of sequential Bonferroni-corrections ($P<0.05$) (Rice, 1989).

Results

Purification and Identification of Acidic Pheromone Compounds Female contact sex pheromone was extracted from the filter papers taken from female containers. The yield of crude extract from approximately 2,100 females was 201.8 mg. The crude extract elicited strong copulatory behavior as previously reported (Shimomura et al., 2010); all male beetles tested attempted to copulate with female dummies treated with 0.5 feq crude extract. When the crude extract was fractionated into acidic, neutral, and basic fractions, only the acidic fraction elicited strong copulatory behavior that matched the behavioral response elicited by the crude extract (100%). The acidic fraction was purified by silica gel column chromatography, and seven fractions were obtained. When each fraction was assayed, only the 50% EtOAc in hexane fraction (Fr. 3) elicited strong copulatory behavior (80%; Fig. 1). This fraction was further purified by normal phase HPLC, and copulatory behavior was elicited by the 0–10 min fraction eluted with 5% isopropanol in hexane (95%; Fig. 1). The active fraction was purified further by normal phase HPLC, and copulatory behavior was elicited with the 5–10 min fraction eluted with 2% isopropanol in hexane (90%; Fig. 1). The yield of the fraction was 300 µg. The small portion of acidic compounds in this active fraction were derivatized to methyl esters and subjected to GC-MS analysis.

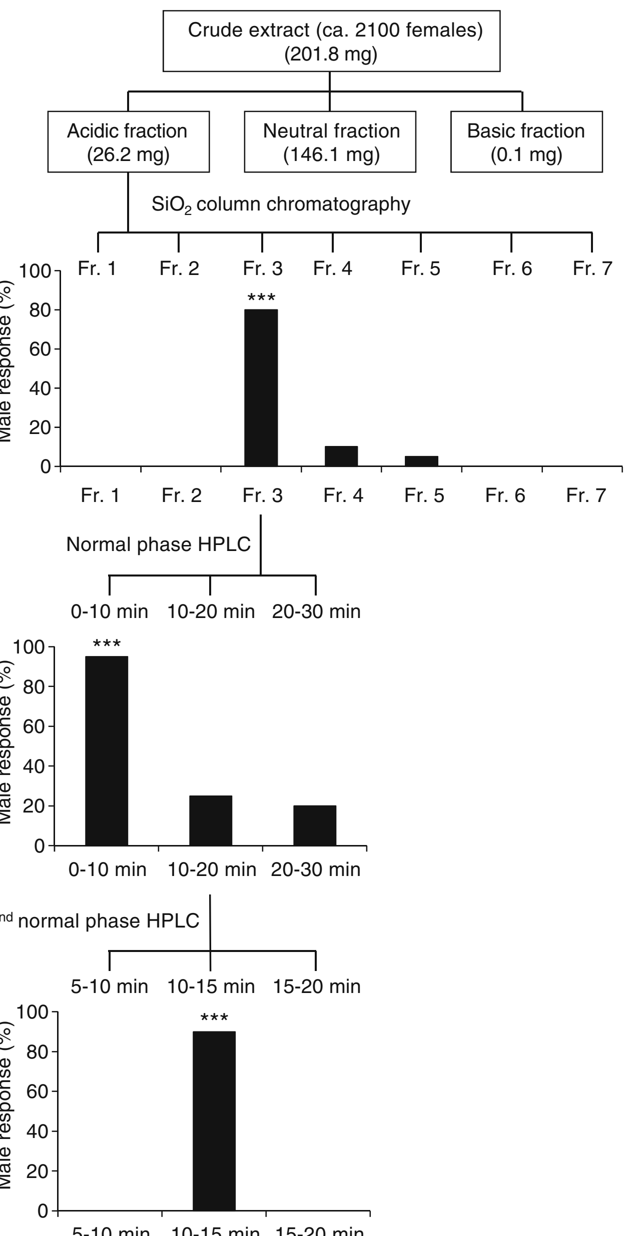


Fig. 1 Fractionation procedure for crude extract collected from female *Callosobruchus analis* by filter paper method and male copulation responses to female dummies applied with each fraction. Twenty males were tested for each fraction. All fractions were tested at 0.5 female equivalents. *Asterisks* above bars indicate significant differences between test fraction and negative control (solvent alone) (Fisher's exact probability test, ***; $P<0.001$)

GC-MS analysis detected two compounds in the active fraction, compound **1** and **2** (Fig. 2a). The mass spectrum of compound **1** was similar to that of a dimethyl ester of 2,6-dimethyloctane-1,8-dioic acid, the contact sex pheromone of *C. maculatus* (Nojima et al., 2007) (Fig. 2b). The mass spectrum of compound **2** was also similar to that of a dimethyl ester of (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid, the contact sex pheromone of *C. chinensis* (Tanaka et al.,

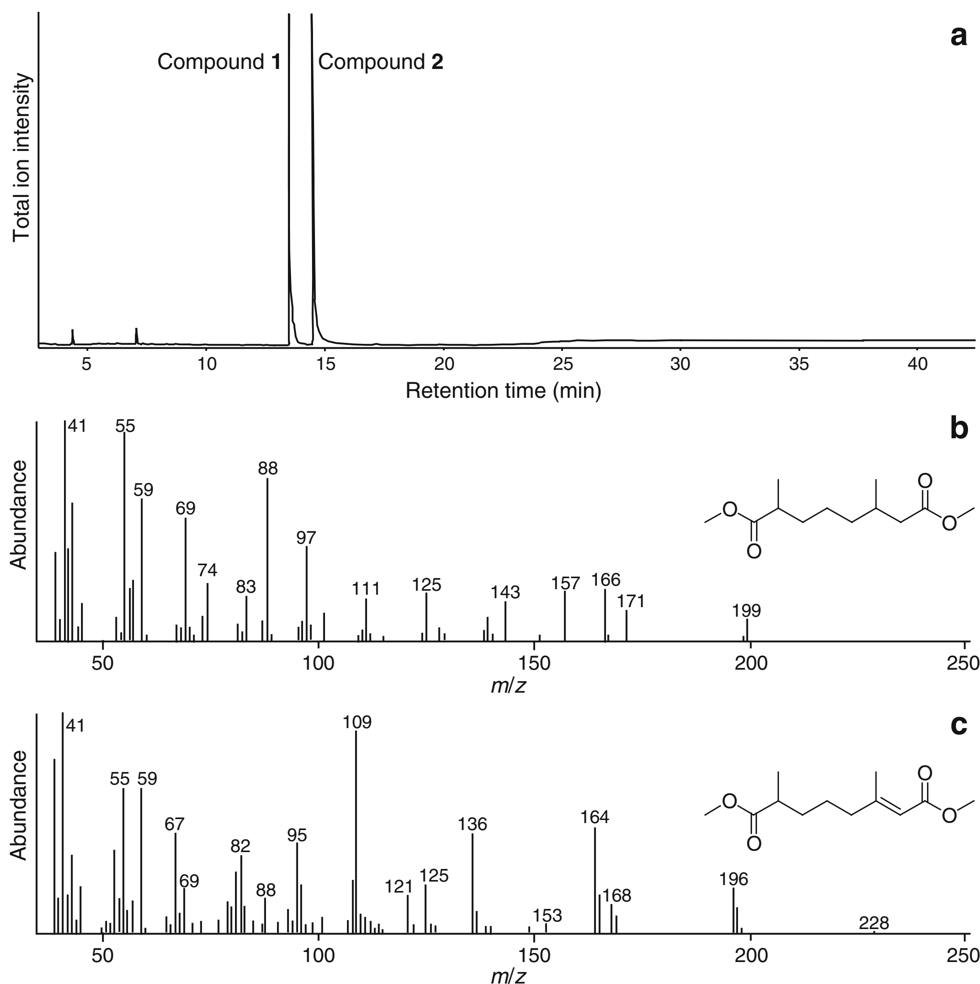


Fig. 2 GC-MS spectra of the active fraction from the original acidic fraction from female *Callosobruchus analis*. Total ion chromatogram of the active fraction (**a**). EI-mass spectra of methyl esterfied natural compound **1** identified as 2,6-dimethyloctane-1,8-dioic acid (**b**) and natural compound **2** identified as 3,7-dimethyl-2-octene-1,8-dioic acid (**c**)

1981) (Fig. 2c), in which the differences in the relative abundance of fragment ions are due to the different electron voltages used each study. The mass spectra and retention times of the two compounds on two different columns matched those of synthetic standards.

Determination of the Chemical Composition 2A1P-O derivatives of synthetic standards were analyzed by 2D-HPLC. During the first column analysis, all stereoisomers of 2A1P-O derivatives of synthetic compound **1** and **2** were eluted in fraction 14.7–19.5 min. This fraction was injected directly onto the second column by on-column injection with a six-way bulb system to avoid incorporation of impurities on the second column. All stereoisomers of (*S*)-2A1P-O derivatives of synthetic compound **1** and **2** were separated clearly by the second column (Fig. 3a). Likewise, the (*S*)- and (*R*)-2A1P-O derivatives of the active acid fraction were analyzed by 2D-HPLC. The (*S*)-2A1P-O derivatives of the active acid fraction were separated into a series of peaks (Fig. 3b). In this chromatogram, peaks 2 and 6 eluted with retention times indicative of (*S*)-2A1P-O derivatives of (*S*)-**2** and (2*S*,6*R*)-**1**, respectively (Fig. 3b). Subsequent analysis of (*R*)-2A1P-O derivatives of the active acid fraction revealed a series of peaks and that the retention times of each enantiomer were inverted with respect to each other (Fig. 3c). The retention times of peaks 1 and 3 were consistent with those of (*S*)-2A1P-O derivatives of (*S*)-**2** and (2*S*,6*R*)-**1**, respectively (Fig. 3c). The peak area ratios were almost identical; peak 2:peak 6= 63.3:36.7 for [(*S*)-2A1P-O derivatives] and peak 1:peak 3= 64.5:35.5 for [(*R*)- 2A1P-O derivatives]. The other peaks did not match the retention time for (*S*)- and (*R*)-2A1P-O derivatives. Thus, the chemical composition was determined as (2*S*,6*R*)-**1**:(*S*)-**2**=1.8:1. The amounts of compounds (2*S*,6*R*)-**1** and (*S*)-**2** in the active fraction from the filter paper extract were quantified as 92 and 51 ng/female, respectively.

Synthesis of Callosobruchusic Acid Although several asymmetric syntheses of callosobruchusic acid (**2**) have been reported (Mori et al., 1983; Gramatica et al., 1985; Giersch and Schulte-Elte, 1990; Abo and Mori, 1993; Yu et al., 2002; Nanda and Scott, 2004), we developed a new asymmetric synthesis of **2** to obtain an optically pure

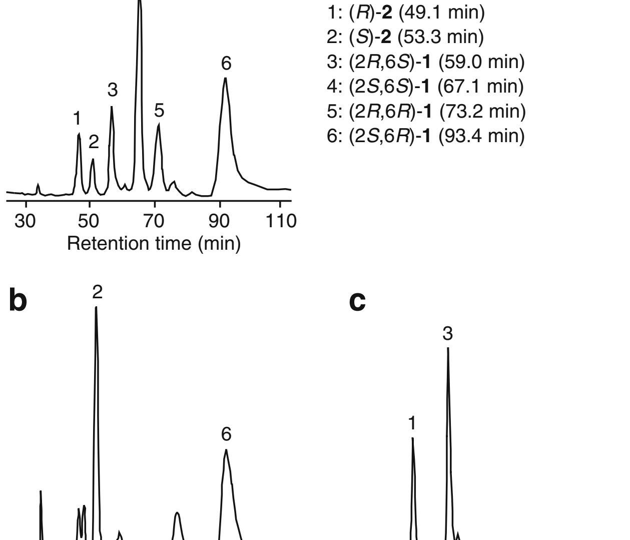


Fig. 3 2D-HPLC chromatograms of (*S*)-2-(2,3-anthracenedicarboximide)-1-propanyl (2A1P-O) derivatives of synthetic standards (**a**), (*S*)-2A1P-O derivatives of active fraction (**b**) and (*R*)-2A1P-O derivatives of active fraction (**c**)

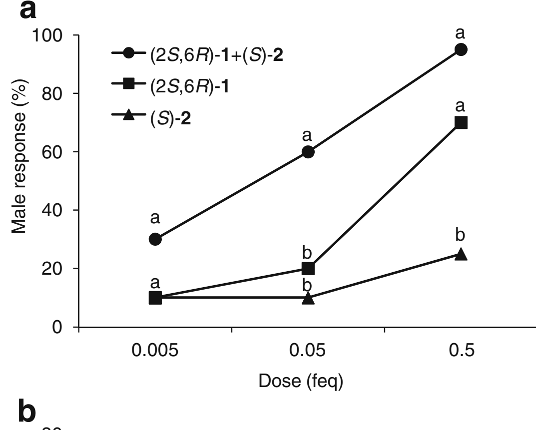
sample of (*S*)-**2** (Scheme 1). The synthesis is based on the strategy for the synthesis of 2,6-dimethyl-1,8-octane-dioic acid, the contact sex pheromone of *C. maculatus* (Nakai et al., 2005). The known aldehyde **3** (Imamura et al., 2004) was coupled with (*S*)-**4** (Evans and Weber, 1987; Nemoto et al., 1995) by the Wittig reaction. The resulting conjugate double bond of **5** was chemoselectively reduced by lithium tri-*sec*-butylborohydride (L-Selectride) to give **6**. The diastereoselective alkylation (Evans et al., 1982) of **6** with methyl iodide in the presence of sodium bistrimethylsilylamide (NaHMDS) afforded **7**. Although the chemical yield was very low, ^{1}H NMR analysis suggested that **7** was stereochemically pure. The *t*-butyldimethylsilyl (TBS) group of **7** was removed under mild conditions to prevent the epimerization of the chiral center (Yajima et al., 2008) to give **8**. Finally, the hydroxyl group of **8** was oxidized under mild conditions (Yajima et al., 2007b), and the chiral auxiliary group was carefully removed by LiOH to give the desired (*S*)-**2**. The enantiomeric purity of synthetic (*S*)-**2** was determined according to the reported procedure (Yajima et al., 2007a). HPLC analyses of (*R*)- and (*S*)-2A1P-O bis-esters of (*S*)-**2** produced a single peak, indicating that synthetic (*S*)-**2** is optically pure (>99% e.e.).

Behavioral Assay: 1. Female Dummy The pheromonal activity of both synthetic stereoisomers was confirmed in copulatory assays by using female dummies. The mixture of the natural ratio of (2*S*,6*R*)-**1** and (*S*)-**2** resulted in dose-dependent copulatory activity, and 95% of tested males exhibited copulation responses at 0.5 feq (Fig. 4a). In a single compound assay of (2*S*,6*R*)-**1**, 70% of males exhibited copulatory responses to this stimulus at 0.5 feq, but only 20% of males exhibited copulatory responses at 0.05 feq (Fig. 4a). In a single compound assay of (*S*)-**2**, only 25% of males exhibited copulation responses at 0.5 feq (Fig. 4a).

We next compared the stereochemistry-activity relationship for **1** at 0.5 feq. A small percentage of males exhibited copulatory behavior with (2*S*,6*S*)-**1** (25%), and a small, but significant percentage of males responded to (2*R*,6*R*)-**1** (40%). No copulatory behavior was observed with (2*R*,6*S*)-**1** (Fig. 4b). Only 15% of males exhibited copulation behavior with the mixture of (2*S*,6*R*)-**1** and (2*R*,6*S*)-**1** (Fig. 4b)

Behavioral Assay: 2. Glass Rod Dummy Male *C. analis* exhibited a significant copulatory response to a glass rod applied with 0.5 feq of crude extract or 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** plus 0.5 feq of the neutral fraction. No copulatory behavior was observed

Scheme 1 Synthesis of callosobruchusic acid. *a* (*S*)-**4**, benzene (93%); *b* L-Selectride, THF (77%); *c* NaHMDS, MeI, THF (16%); *d* TBAF, acetic acid, THF (82%); *e* Dess-Martin periodinane, $NaHCO_3$, CH_2Cl_2; $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, *t*-BuOH, H_2O; LiOH, THF, H_2O (7%)



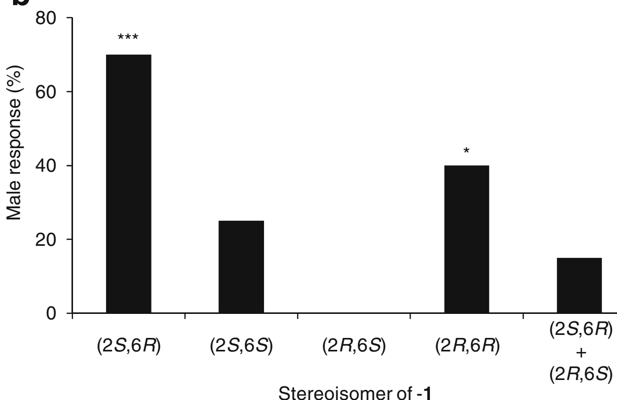
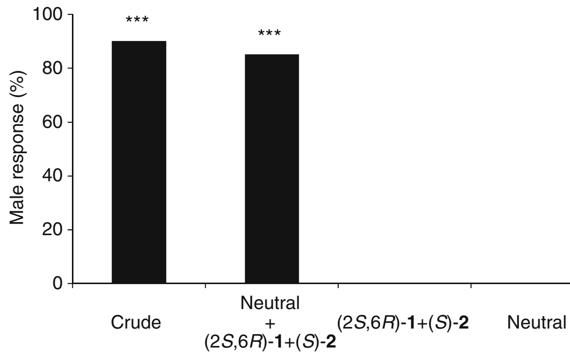


Fig. 4 Copulatory response of male *Callosobruchus analis* to female dummies applied with synthetic compounds. **a** Dose-behavioral assay of (2*S*,6*R*)-2,6-dimethyloctane-1,8-dioic acid (**1**) and (2*E*,7*S*)-3,7-dimethyl-2-octene-1,8-dioic acid (**2**). The ratio of mixture (2*S*,6*R*)-**1**+(*S*)-**2** was applied at the natural ratio of (2*S*,6*R*)-**1**:(*S*)-**2**=1.8:1 (see "Results" section). Twenty males were tested at each dose. *Different letters* within the same dose indicate significant differences (Fisher's exact probability test followed by a sequential Bonferroni test: $P<0.05$). **b** Stereoisomer-behavioral assay of **1**. All assays were tested at 0.5 female equivalents. Twenty males were tested for each isomer. *Asterisks* above bars indicate significant differences between test fraction and control (solvent applied) (Fisher's exact probability test, *; $P<0.05$, ***; $P<0.001$)

when the rod was applied with 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** or with 0.5 feq of the neutral fraction (Fig. 5). The neutral fraction was purified further into six fractions by column chromatography, and each fraction or combination of fractions was assayed with 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2**. The combined hydrocarbon fraction (hexane elute fraction: Fr. 1) with 5% ether elute fraction (Fr. 2) plus 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** exhibited comparable copulation activity to those of the original neutral fraction plus 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** (Fig. 5). However, marginal activity was elicited from hydrocarbon fraction (Fr. 1) plus 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** or 5% ether fraction (Fr. 2) plus 0.5 feq synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** (Fig. 5).

Discussion

Previously, *C. analis* has been shown to use a contact sex pheromone in its mating system, and, based on cross-copulatory analysis, this contact sex pheromone might be species specific (Shimomura et al., 2010). The extracts collected directly from whole bodies of female *C. maculatus* did not elicit copulatory activity from males, possibly due to the large quantities of chemicals and the sticky nature of the extracts (Nojima et al., 2007). Meanwhile, the extracts collected by the filter paper method from the containers that had housed female *C. maculatus* and *C. chinensis* elicited copulation activity from male *C. maculatus* and *C. chinensis*, respectively (Tanaka et al., 1981; Nojima et al., 2007). Therefore, we used the same male copulatory behavior in an assay to guide our fractionation procedure. Because male *C. analis* did not show copulatory behavior against females of *C. maculatus* and *C. chinensis* (Shimomura et al., 2010), we, at first, hypothesized that *C. analis* might use a different kind of compound as the contact sex pheromone from that of



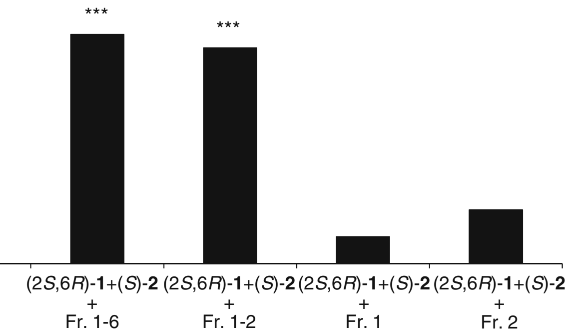


Fig. 5 Copulatory response of male *Callosobruchus analis* to glass rod dummies applied with test samples. Twenty males were tested for each sample. Each fraction and/or compound was combined so that the total mass reflected 0.5 female equivalents. *Asterisks* above bars indicate significant differences between test sample and control (solvent applied) (Fisher's exact probability test, ***; $P<0.001$)

C. maculatus and *C. chinensis*. However, GC-MS analysis of the purified active fraction revealed two compounds: 2,6-dimethyloctane-1,8-dioic acid (**1**), previously reported as a contact sex pheromone of *C. maculatus* (Nojima et al., 2007), and callosobruchusic acid, (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid (**2**), which was reported as a contact sex pheromone of *C. chinensis* (Tanaka et al., 1981, 1982).

In insect pheromonal communication, it is clear that bioactivity frequently may depend on chirality (reviewed in Mori, 2007). Therefore, we expected that the chirality of the two compounds was related to the species-specific copulation activity of male *C. analis*. Since the stereoisomeric compositions of the *C. maculatus* contact sex pheromone, 2,6-dimethyloctane-1,8-dioic acid (**1**), and the *C. chinensis* contact sex pheromone, (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid (**2**), were determined by the 2D-HPLC-Ohrui-Akasaka method as (2*R*,6*S*):(2*S*,6*R*):(2*S*,6*S*):(2*R*,6*R*)=43:38:18:trace for *C. maculatus* (Yajima et al., 2006), and (*R*):(*S*)=3.3–3.4:1 for *C. chinensis* (Yajima et al., 2007a), we applied the same methodology in this case. As a result, the chemical composition was determined as (2*S*,6*R*)-**1**:(*S*)-**2**=1.8:1, which meant that both compounds were stereochemically pure, unlike the case of *C. maculatus* and *C. chinensis*. The copulation assay with synthetic compounds clarified the role of both compounds. The synthetic mixture showed the same copulatory activity as the crude extract. However, (2*S*,6*R*)-**1** alone exhibited significantly less activity than the synthetic mixture at 0.05 feq, and (*S*)-**2** alone exhibited marginal activity, even at 0.5 feq. Therefore, we confirmed that (2*S*,6*R*)-**1** functions as a main compound, and (*S*)-**2** acts as an additive compound; both compounds were needed for full copulatory activity.

While female *C. maculatus* uses **1** as the contact sex pheromone, male *C. analis* did not exhibit copulation behavior with female *C. maculatus* in a cross-copulation experiment (Shimomura et al., 2010). To clarify this inconsistency, we compared the stereochemistry-activity relationship by using four stereoisomers of **1**. Intriguingly, the males exhibited weak copulation behavior to (2*R*,6*R*)-**1** and (2*S*,6*S*)-**1**, namely diastereomers of the natural isomer, but no males exhibited copulation behavior to the (2*R*,6*S*)-**1**, enantiomer of the natural isomer. In the contact sex pheromone of *C. maculatus*, (2*R*,6*S*)-**1** is the most abundant stereoisomer on the cuticular surface (Yajima et al., 2006). Therefore, we next combined (2*S*,6*R*)-**1** with (2*R*,6*S*)-**1**. The pheromonal activity of (2*S*,6*R*)-**1** was masked clearly by the (2*R*,6*S*)-**1** enantiomer. These results revealed partial evidence of mate recognition specificity of male *C. analis*.

There are many examples where contact sex pheromones are active even when applied on artificial objects (e.g., gelatin capsules, plastic vials, glass rods, or stainless steel spatulas) (see Tanaka et al., 1981; Fukaya and Honda, 1992; Zhang et al., 2003; Sugeno et al., 2006). Since males of *C. chinensis* and *C. maculatus* also exhibited copulation behavior to a glass rod treated with contact sex pheromone compounds (Tanaka et al., 1981; Nojima et al., 2007), we attempted to use the glass rod as a dummy for *C. analis*. As expected, male *C. analis* exhibited significant copulation behavior with the glass rod applied with 0.5 feq of crude extract. However, unlike in the case of the female dummy, no males exhibited copulation behavior to the glass rod applied with 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2**. As for the copulation activity of male *C. maculatus*, the glass rod applied only with the neutral fraction collected from the females by the filter collection method elicited copulation activity, albeit weakly (Nojima et al., 2007). In the case of *C. analis*, no copulation behavior was observed to the glass rod applied only with the female neutral fraction. Cuticular hydrocarbons acted as a synergist for the contact sex pheromone of *C. chinensis* and *C. maculatus* (Tanaka et al., 1981; Nojima et al., 2007). Therefore, we combined 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** with the neutral fraction, and copulation activity was similar to that of the crude extract. Although, in the case of *C. chinensis* and *C. maculatus*, the hexane eluted fraction (containing some hydrocarbons) of the neutral fraction was enough for the synergistic effect (Tanaka et al., 1981; Nojima et al., 2007), in the case of *C. analis*, marginal activity was exhibited when 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** was combined with the hexane eluted fraction of the neutral fraction. Meanwhile, the copulation activity elicited by 0.5 feq of the synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** combined with the hexane eluted fraction and 5% ether fraction of the neutral fraction was similar to that of the crude extract. Marginal activity was exhibited with 0.5 feq synthetic mixture of (2*S*,6*R*)-**1** and (*S*)-**2** combined with the 5% ether fraction of the neutral fraction. We, therefore, suggest that there may be another synergist for *C. analis*, and male *C. analis* may discriminate the female with a multi cue system, but further work is needed.

There have been few reports on stereoisomeric composition and/or the stereochemistry-activity relationship regarding contact sex pheromones. The German cockroach, *Blattella germanica*, for example, uses 3,11-dimethylnonacosan-2-one as the major component of contact sex pheromones (reviewed in Gemeno and Schal, 2004). The stereochemistry of this dimethylketone was confirmed as (3*S*,11*S*) (Mori et al., 1981; Nishida and Fukami, 1983), and the four stereoisomers were active individually (Nishida and Fukami, 1983; Eliyahu et al., 2004). In the comparative analysis of stereochemistry-activity relationship for *C. maculatus*, although the natural stereoisomeric ratio was the most active, each stereoisomer was active individually (Yajima et al., 2006). As for *C. chinensis*, each enantiomer also was active.

(*R*)-**2** was half as active as (*S*)-**2** (Mori et al., 1983). Meanwhile, in the case of *C. analis*, the enantiomer of the natural pheromone exhibited no copulation activity and masked the activity of the natural isomer. There are numerous examples of enantiomeric antagonistic effects in sex attractant pheromones. Indeed, in closely related and sympatric species, each species is suppressed by addition of the enantiomer produced by the other species, as a system of premating, reproductive isolation (Millar, 2000). The distributions, host plants, and habitats of *Callosobruchus* species overlap considerably, and the sympatric population of these three beetles also has been reported (Haines, 1989; Tuda et al., 2005). We, therefore, suggest that rigorous mate recognition of *C. analis*, including antagonistic effects, might have arisen during species differentiation.

The present study is the third example of a contact sex pheromone in *Callosobruchus* seed beetles. In all three cases, monoterpene dicarboxylic acids were used as the main compounds. Compounds **1** and **2** had been identified previously from the urine of some mammals treated with squalene synthase inhibitor (Bostedor et al., 1997). Furthermore, it was determined that these compounds were derived from farnesol, and the biosynthetic pathway was proposed (Bostedor et al., 1997). Similar biosynthesis could be occurring in the contact sex pheromones of *Callosobruchus* species, but this remains to be clarified.

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ovaries. In this study, we measured the 9-ODA content of normal and laying workers, and tested whether 9-ODA elicits attacks against laying workers.

Methods and Materials

Measurement of 9-ODA Content of Mandibular Pheromone

Bees were sampled randomly until 6 workers showing no ovarian activation on dissection were obtained from each colony. After this, all queens were removed from the colonies. Two weeks later, workers bees were sampled again until 6 workers were obtained with fully developed ovaries. The heads of the two types of bees were stored in 200 µl dichloromethane for 2 month at −15°C.

The extracts were evaporated to dryness under nitrogen, and the residues were re-dissolved in 20 µl of an internal standard, octanoic acid and tetradecane in dichloromethane, and 20 µl of bis-trimethylsilyltrifluoroacetamide. Extracts were injected splitlessly into a Hewlett Packard 6890 gas chromatograph, equipped with a 25×0.32 mm i.d., methyl silicone column, programmed from 60°C (held for 1 min) to 110°C at 50°C.min^{-1}, then to 220°C at 3°C.min^{-1}. Helium, at 1 ml.min^{-1} was the carrier gas. The compound 9-ODA was identified, based on comparison of retention time with that of an authentic standard, and quantified in relation to the internal standards.

Observation of Workers' Responses to 9-ODA

Experiments were conducted at Yunnan Agricultural University, Kunming, China. Twenty worker bees from each of three queenright and queenless colonies of *Apis cerana* were anesthetized with CO_2 and marked. They were divided into equal groups: one treated with 10 µg 1% w/w 9-ODA, similar to the quantity produced by a laying worker, another with 10 µg 200% w/w 9-ODA, similar to that produced by a queen (Tan et al., 2009), and the third (control) treated with 10 µg distilled water. When the anesthetized workers recovered, they were returned to their hives on a single frame and monitored for 3 min with digital video cameras, one on each side of the frame, to record any aggressive behaviour.

Statistical Analyses Independent *t*-tests compared the amounts of 9-ODA between queenright *A. cerana* workers and queenless laying workers. *Chi-square* tests were used to compare the aggression behavior directed to treated workers, between test and control groups, and between queenright and dequeened colonies. Count data were used in the *chi-square* calculations.

Results

Mean 9-ODA content in extracts of *A. cerana* workers was different (t_4=4.8, P<0.009) between queenright [0.014± 0.005 µg (SD)] and queenless [0.096 µg±0.029 (SD)] colonies. In queenright colonies, within 3 min after workers treated with 9-ODA (either 0.1 µg or 0.2 mg/bee) were released, greater percentages $(0.1\mu g : \chi^2_1 = 25.5,\ P < 0.001; 0.2mg : \chi^2_1 = 38.6, P < 0.001)$ of treated workers were attacked compared to the control groups (Table 1). In queenless colonies, greater percentages $(0.1\mu g : \chi^2_1 = 18.3, P < 0.001; 0.2mg : \chi^2_1 = 21.8, P < 0.001)$ of treated workers also were attacked after release, compared to the control groups (Table 1). However, overall, there were fewer attacks against treated workers in queenless colonies than in queenright colonies. This effect was significant for treatment with 0.2 mg of 9-ODA ($\chi^2_1 = 12.3, P = 0.001$), but not quite significant for treatment with 0.1 µg ($\chi^2_1 = 3.4, P = 0.067$). In both queenright and queenless colonies, workers were

Table 1 Number of treated *Apis cerana* workers attacked by nestmates (Mean±SD%), (N=10 workers per colony, 3 colonies)

Treatment 9-oxo-2-(*E*)-decenoic acid amount	Colony	Queenright		Queenless	
		Control	Test	Control	Test
0.1 µg	1	0	6	0	4
	2	1	6	0	4
	3	1	9	0	6
	Total	2 (6.7±5.8%)[c]	21 (70.0±17.3%)[a]	0 (0.0±0.0%)[c]	14 (46.6±11.5%)[a]
0.2 mg	1	1	9	0	5
	2	1	9	0	5
	3	2	10	0	6
	Total	4 (13.3±5.8%)[c]	28 (93.3±5.8%)[b]	0 (0.0±0.0%)[c]	16 (53.3±5.8%)[a]

[a] Mean percentages within one column or one row followed by a different letter are significantly different

more sensitive to the higher dose of 9-ODA. The difference was significant for the queenright colonies but not significant for queenless colonies $\left(\text{queenright}: \chi_1^2 = 5.5, P = 0.019;\ \text{queenless}: \chi_1^2 = 0.3, P = 0.606\right)$ (Table 1).

Discussion

Within a week of being dequeened, some 40% of workers in an *A. cerana* colony have activated ovaries (Tan et al., 2009). We found that pheromonal changes corresponded with this ovarian activation, with the mandibular gland extract of a laying worker containing approximately seven times as much 9-ODA as a worker without activated ovaries.

In our behavioral tests, we showed that other workers attacked workers with a queen-like 9-ODA bouquet, suggesting that detection of these pseudoqueens is mediated by the pheromonal cue (Hepburn, 1992). Although laying workers increased their 9-ODA content, it was still considerably less than that produced by queens. In *A. mellifera*, such a difference in mandibular gland content is sufficient for laying workers to be perceived as such by their nestmates and be attacked (Plettner et al., 1995).

Interestingly, more workers treated with 9-ODA were attacked in queenright colonies than in queenless ones. This effect may, in part, be due to greater numbers of attacking bees lacking ovarian activation in queenright colonies than in queenless ones (Tan et al., 2009). However, it also may be due to an increased tolerance of laying workers in queenless colonies over that in queenright colonies, perhaps mediated by prior 9-ODA levels in the respective colony types.

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Table 1 Chemicals used in the field tests with references to their purities and preparation

Compound	Abbreviation[a]	Chemical purity[b]	Stereochemical purity[b]	Ref.[c]
Main attractant pheromone component				
R=CH₃				
Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol				
Prepared by Japanese group	**(*SSS*)AcJ**	>99	>99	2
Prepared by Swedish group	**(*SSS*)AcS**	>99	>97	1, a,b
$R=CH_3CH_2$				
Propionate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol	**(*SSS*)Pr**	>99	>97	1, a, b
$R=CH_3(CH_2)_2$				
Butyrate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol	**(*SSS*)Bu**	>99	>97	1, a, b
$R=(CH_3)_2CH$				
iso-Butyrate of (2*S*,3*S*,7*S*)-3.7-dimethyl-2-pentadecanol	**(*SSS*)*i*Bu**	>99	>97	1, a, b
Antagonists, synergists				
Acetate of (2*S*,3*R*,7*S*)-3,7-dimethyl-2-pentadecanol	**(*SRS*)Ac**	>98	>97	1, a, b
Acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol	**(*SRR*)Ac**	>98	>97	1, a, b
Analogues				
(CH_3 is missing from **1**) Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol				
Prepared by Japanese group	**(*SS*–)AcJ**	>99	>98	3 and this work
Prepared by Swedish group	**(*SS*–)AcS**	>98	>99	4
(CH_3 is missing from **1**) Acetate of (2*S*,3*R*)-3-methyl-2-pentadecanol	**(*SR*–)Ac**	>99	>98	3 and this work
(CH_3 is missing from **1**) Acetate of (2*S*,7*S*)-7methyl-2-pentadecanol	**(*S*–*S*)Ac**	>99	>96	4
(CH_3 is missing from **1**) Acetate of (2*S*,6*S*)-2,6-dimethyl-2-tetradecanol	**(–*SS*)Ac**	>99	>97	4

[a] The hyphen in acronyms indicates a missing methyl group relative to compound **1**

[b] Chemical purity (%) : 100 × (Amount of the stereoisomers of the indicated compound/Amount of all compounds). Stereochemical purity (%) : 100 × (Amount of the indicated stereoisomer/Sum of amounts of all stereoisomers)

[c] 1) Högberg et al. (1990), Anderbrant et al. (1992b), and Hedenström et al. (2006), 2) Tai et al. (1990), 3) Tai et al. (1992), 4) Hedenström et al. (1992)

Table 2 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its analogs (100 µg per bait) (Test 1)

Bait	Compound	Mean catch ± SD[a]
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	32.4±24.4
B	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	1.6±1.1 a
C	Acetate of (2*S*,7*S*)-7-methyl-2-pentadecanol [**(*S*–*S*)Ac**]	0.2±0.4 a
D	Acetate of (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol [**(–*SS*)Ac**]	0.2±0.4 a
E	Unbaited	0.4±0.5 a

Test run in Uppland, Sweden, Aug. 6–Sept. 11, 1991

[a] Means followed by the same letter are not significantly different ($P<0.05$), according to ANOVA on log(catch + 1) transformed data followed by Tukey's test ($N=5$)

main attractant (Wassgren et al., 1992; Anderbrant et al., 1995; Bergström et al., 1995), suggesting that the length of the carbon chain is an essential characteristic of the pheromone for a given species. On the other hand, these two, and several other *Neodiprion* species, also are attracted to two or more compounds in which the acetate group of the main pheromone compound has been replaced with other acyl moieties (Anderbrant et al., 1992a, 1997, 2005; Anderbrant, 1999).

The most widespread diprionid species, *N. sertifer*, has a well characterized sex pheromone, with the acetate [**(*SSS*) Ac**] (Table 1) or propionate [**(*SSS*)Pr**] of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol (diprionol) as the main component. The (2*S*,3*R*,7*R*)-isomer of the acetate [**(*SRR*)Ac**] can act as a synergist to these major components in certain geographic areas, as an antagonist in other areas, and can have no effect when added in other areas (Tai et al., 1990; Anderbrant et al., 1992b, 2000). The (2*S*,3*R*,7*S*)-isomer of the acetate [**(*SRS*) Ac**] is able to replace the synergistic function of **(*SRR*)Ac** in Siberia and the antagonistic function in Europe, although, in the latter case, it has to be in much larger relative amounts (Anderbrant et al., 1992b, 2000).

To evaluate whether simpler molecules could mimic pheromonal activity, Tai et al. (1992) synthesized the diprionol acetate analogs, (2*S*,3*S*)-3-methyl-2-pentadecanol acetate [**(*SS*–)Ac**] and (2*S*,3*S*)-3-methyl-2-pentadecanol acetate [**(*SR*–)Ac**] (Table 1). These analogs lacked the methyl group in the 7-position (the hyphen in our compound acronyms indicates a missing methyl group relative to diprionol, see Table 1). Field tests in Japan showed that the analogs were active to *N. sertifer* males. To evoke the same response as the natural pheromone compound, however, the analogs had to be applied in an amount approximately 50 times greater.

In order to get a more complete picture of what molecular elements are important for pheromonal activity in this species, we investigated the effect of the two analogs tested by Tai et al. (1992), as well as of analogs lacking the methyl group at the 3-position, (2*S*,7*S*)-7-methyl-2-pentadecanol acetate [**(*S*–*S*)Ac**], or the first methyl group of the carbon chain, (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol acetate [**(–*SS*)Ac**, Table 1]. Field trials were performed both in Sweden and in Japan. In addition, we tested whether

Table 3 Catch of male *Neodiprion sertifer* in two trials of traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol alone or in combination with each of its analogs (100 µg per compound), (Test 2)

Bait	Compound	Mean catch ± SD[a]	
		Trial I	Trial II
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	11.4±10.6 ab	24.3±29.3 a
B	A + acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	13.6±13.9 a	43.3±28.0 a
C	A + acetate of (2*S*,7*S*)-7-methyl-2-pentadecanol [**(*S*–*S*)Ac**]	13.6±8.6 a	63.0±47.8 a
D	A + acetate of (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol [**(–*SS*)Ac**]	15.5±10.9 a	24.7±26.7 a
E	Unbaited	2.9±5.0 b	0.0±0.0 b

Test run in Uppland, Sweden, Aug. 8–Sept. 29 (Trial I) and Sept. 11–29 (Trial II), 1991

[a] Means within a trial followed by the same letter are not significantly different ($P<0.05$), according to ANOVA on log(catch + 1) transformed data followed by Tukey's test (Trial I $N=8$, Trial II $N=3$)

Table 4 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its analogs at three different concentrations (Test 3)

Bait	Compound	Amount (μg)	Mean catch ± SD[a]
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	10	83.6±100.1 b
B	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	10	2.5±8.0 a
C	**(*SS*–)AcS**	100	2.9±9.4 a
D	**(*SS*–)AcS**	1000	2.7±3.0 a
E	Acetate of (2*S*,7*S*)-7-methyl-2-pentadecanol [**(*S*–*S*)Ac**]	10	3.2±9.0 a
F	**(*S*–*S*)Ac**	100	1.7±5.5 a
G	**(*S*–*S*)Ac**	1000	2.8±8.3 a
H	Acetate of (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol [**(–*SS*)Ac**]	10	3.7±11.8 a
I	**(–*SS*)Ac**	100	4.5±11.3 a
J	**(–*SS*)Ac**	1000	3.7±4.1 a
K	Unbaited	–	2.1±6.6 a

Test run in Östergötland, Sweden, Aug. 12–Oct. 6, 1993

[a] Means followed by the same letter are not significantly different ($P<0.05$) according to ANOVA on log(catch + 1) transformed data followed by Tukey's test ($N=13$)

N. sertifer males were attracted to esters other than acetate and propionate, of the major pheromone component, as has been found for *D. pini* (Anderbrant et al., 2005).

Methods and Materials

Chemicals used were of high chemical and stereogenic purities, and were synthesized according to Högberg et al. (1990), Hedenström et al. (1992), Tai et al. (1990, 1992), and Hedenström et al. (2002). Purity details and abbreviations of compounds used below are given in Table 1. Two of the compounds were produced both in the Japanese and one of the Swedish laboratories (MSU), and the batches are labeled J or S, respectively, at the end of the chemical acronym. Chemicals were dissolved in *n*-hexane or *n*-heptane before being applied to dispensers.

Field experiments were conducted in young Scots pine, *Pinus sylvestris* L., plantations in the provinces of Uppland (in 1991), Östergötland (in 1993 and 1995) and Skåne (in 1997), situated in the east and south of Sweden. Additional experiments were carried out in young Japanese red pine, *Pinus densiflora* Siebold. & Zucc., stands in Hyogo and Osaka prefectures, southern Honshu, central Japan (in 1995 and 1997). Lund-I sticky traps (Anderbrant et al., 1989) were used with the chemicals released from dental cotton rolls (Celluron No. 2, Paul Hartmann S.A., France). This dispenser shows a relative decrease in release rate of diprionol acetate that is independent of load (Anderbrant et al., 1992a), thus releasing roughly constant ratios of

Table 5 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its analogs, alone or in combination with the (2*S*,3*R*)-isomer (Test 7)

Bait	Compound	Amount (μg)	Mean catch ± SD[a]
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcJ**]	5	92.2±104.9 b
B	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcJ**]	50	46.5±34.9 b
C	B + acetate of (2*S*,3*R*)-3-methyl-2-pentadecanol [**(*SR*–)Ac**]	50+0.5	61.5±34.9 b
D	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	50	9.2±10.7 ab
E	D + acetate of (2*S*,3*R*)-7-methyl-2-pentadecanol [**(*SR*–)Ac**]	50+0.5	6.5±5.5 ab
F	Acetate of (2*S*,7*S*)-7-methyl-2-pentadecanol [**(*S*–*S*)AcS**]	50	0 a
G	Acetate of (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol [**(–*SS*)AcS**]	50	3.5±4.0 a

Traps set up at four sites, with no trap rotation; Hyogo and Osaka, Oct. 6–Nov. 12, 1995.

[a] Means followed by the same letter are not significantly different ($P<0.05$) according to ANOVA on log(catch + 1) transformed data followed by Tukey's test ($N=4$).

Table 6 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its (2*S*,3*S*)-analog (Test 9)

Bait	Compound	Amount (µg)	Total catch[a]		
			Hyogo I	Hyogo II	Osaka
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcJ**]	5	61	68	3
B	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	5	51	–[b]	12
C	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcJ**]	100	43	25	4
D	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcJ**]	100	33	55	12
E	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	100	32	27	13

Traps set up at three sites, Hyogo, Oct. 22–Nov. 20, and Osaka, Oct. 12–Nov. 15, 1997.

[a] Trap positions rotated three times, but only total catches are available.

[b] Trap lost.

compounds over time if the compounds tested are similar. For instance, the release rate is about 10% of the original load after 2 d at 20°C and 1 m.s^{-1} wind speed, and 1% after 10 d, for dispensers loaded with 6, 60, or 600 µg diprionol acetate (Anderbrant et al., 1992a). Traps were placed at least 30 m apart, at about 2 m above ground, in pine trees. To reduce positional effects, traps were moved to a new random position after each check, in most tests.

In 1991 the activities of the **(*SS*–)Ac**, **(*S*–*S*)Ac** and **(–*SS*)Ac** analogs were compared with **(*SSS*)Ac** using one dose only. In test 1, each compound was tested alone, while in test 2 the analogs were combined with **(*SSS*)Ac** to evaluate possible synergistic or antagonistic effects. In 1993 (test 3), three different doses of each analog were used to make a comparison with the results in Japan of Tai et al. (1992) possible. In 1995, the effects of addition of **(*SR*–)Ac,** **(*SRR*)Ac**, or **(*SRS*)Ac** analogs to **(*SSS*)Ac** were tested (tests 4 and 5]. Tests 6–9 (in 1995 and 1997) investigated whether higher doses of the analogs, **(*SS*–)Ac** (of either Swedish or Japanese origin) and **(*SR*–)Ac**, could be used as substitutes for **(*SSS*)Ac** and **(*SRR*)Ac**. In 1997 (test 10), the attraction to four different esters of diprionol was compared. To compensate for the different release rates of the different esters, the amount of the propionate applied on the dispenser was doubled, while the amounts of the *iso*-butyrate and butyrate were quadrupled, compared to that of the acetate (c.f., Anderbrant et al., 2005).

Results

When tested in 1991 and 1993, none of the three analogs [**(*SS*–)Ac**, **(*S*–*S*)Ac**, and **(–*SS*)Ac**] alone caught significant numbers of *N. sertifer* males in Sweden, despite the presence of a relatively large population, as judged by the catch in the trap loaded with **(*SSS*)Ac** (test 1, Table 2). Further, addition of any of these analogs to **(*SSS*)Ac** did not yield increased catches of *N. sertifer*, relative to traps baited with **(*SSS*)Ac** alone (test 2, Table 3). The lack of activity of the analogs, in Sweden, was confirmed in test 3, in which doses up to 100 times that of **(*SSS*)Ac** were tested (Table 4). Only few individuals were caught in the analog-baited

Table 7 Catch of male *Neodiprion sertifer* in two trials of traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its (2*S*,3*S*)-analog (Test 6)

Bait	Compound	Amount (µg)	Mean catch ± SD[a]	
			Trial I	Trial II
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	4	1.5±1.0 a	1.0±1.2 ab
B	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	40	57.2±23.4 c	8.0±13.4 ab
C	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	400	0.8±1.0 a	2.5±1.9 ab
D	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecol [**(*SS*–)AcJ**]	400	14.0±4.2 b	12.0±9.6 b
E	Unbaited	–	0.5±0.6 a	0.2±0.5 a

Test run at two sites, Östergötland, Sweden, Sept. 2–Oct. 7, 1995.

[a] Means within a trial followed by the same letter are not significantly different ($P<0.05$). according to ANOVA on log(catch + 1) transformed data followed by Tukey's test (Trial I $N=4$, Trial II $N=4$).

Table 8 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol or its (2*S*,3*S*)-analog (Test 8)

Bait	Compound	Amount (μg)	Total catch[a]	
			Trial I	Trial II
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	5	13	1
B	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcJ**]	100	8	3
C	Acetate of (2*S*,3*S*)-3-methyl-2-pentadecanol [**(*SS*–)AcS**]	100	0	0
D	Unbaited	–	0	0

One trap set up at two sites, Östergötland, Aug. 28–Nov. 8, 1997.

[a] Trap positions rotated one and two times, respectively, but only total catches are reported.

traps, regardless of dose, not different from that of the unbaited trap; catches in traps baited with **(*SSS*)Ac** were much greater. However, in Japan, the **(*SS*–)Ac** analog, particularly of Japanese origin, attracted significant numbers of males (relative to the control) when tested in amounts 10 times higher than that used for **(*SSS*)Ac**-baited traps (tests 7 and 9, Tables 5 and 6). The **(*SS*–)AcJ** also was attractive to Swedish *N. sertifer* males (tests 6 and 8, Tables 7 and 8). In Trial II of test 6, the catch in traps baited with **(*SS*–)AcJ** was not different from the catch in traps baited with **(*SSS*)Ac** (albeit at one tenth the dose). However, in Trial I, the catch of *N. sertifer* in traps baited with **(*SS*–)AcJ** was less (but still higher than in control traps) than in traps baited with **(*SSS*)Ac**.

In Sweden, addition of the analogs **(*SRR*)Ac**, **(*SRS*)Ac**, or **(*SR*–)Ac** to **(*SSS*)Ac** resulted in a decrease in catch, relative to that in traps baited with **(*SSS*)Ac** alone (tests 4 and 5, Tables 9 and 10). At the same dose (1.0 μg) of analog, the antagonistic effect was significant for **(*SRR*)Ac** and **(*SR*–)Ac**, but not for **(*SRS*)Ac** (test 5, Table 10). In Japan, however, addition of **(*SR*–)Ac** to other attractive compounds [but not to **(*SSS*)Ac**, which was not tested] had no effect on trap capture (test 7, Table 5).

When the different esters of diprionol were tested in Sweden in 1997, **(*SSS*)Ac** and **(*SSS*)Pr** were attractive to *N. sertifer*, but **(*SSS*)*i*Bu** and **(*SSS*)Bu** were not (test 10, Table 11). At the higher dose (to compensate for lower volatility), **(*SSS*)Pr**-baited traps caught a greater number of *N. sertifer* than traps baited with **(*SSS*)Ac**; in contrast, catches in traps baited with the higher doses of **(*SSS*)*i*Bu** and **(*SSS*)Bu** were not different from the blank.

Discussion

Based on the results presented here, and earlier studies (Tai et al., 1990, 1992; Anderbrant et al., 1992b, 2000), Swedish and Japanese populations of *N. sertifer* differ from each other in several ways with respect to response of males to pheromone and related compounds. In both countries, the

Table 9 Catch of male *Neodiprion sertifer* in two trials of traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol alone or in combination with other isomers or the (2*S*,3*R*)-analog (Test 4)

Bait	Compound	Amount (μg)	Mean catch ± SD[a]	
			Trial I	Trial II
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	100	35.5±40.5 b	62.2±98.7 b
B	A + acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol [**(*SRR*)Ac**]	100+0.3	2.3±2.5 ab	3.0±2.8 a
C	A + acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol [**(*SRR*)Ac**]	100+3.0	3.2±4.5 ab	0 a
D	A + acetate of (2*S*,3*R*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SRS*)Ac**]	100+10	5.8±6.9 ab	4.2±3.4 a
E	A + acetate of (2*S*,3*R*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SRS*)Ac**]	100+100	0 a	0.6±0.9 a
F	A + acetate of (2*S*,3*R*)-3-methyl-2-pentadecanol [**(*SR*–)Ac**]	100+10	0 a	0.2±0.4 a
G	A + acetate of (2*S*,3*R*)-3-methyl-2-pentadecanol [**(*SR*–)Ac**]	100+100	0 a	0.2±0.4 a
H	Unbaited	–	1.5±1.3 ab	0 a

Test run in Östergötland, Sweden, Sept. 2–Oct. 7, 1995.

[a] Means within a trial followed by the same letter are not significantly different ($P<0.05$) according to ANOVA on log(catch + 1) transformed data followed by Tukey's test (Trial I $N=4$, Trial II $N=5$).

Table 10 Catch of male *Neodiprion sertifer* in traps baited with acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol alone or in combination with other isomers or the (2*S*,3*R*)-analog, (Test 5)

Bait	Compound	Amount (μg)	Mean catch ± SD [a]
A	Acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SSS*)AcS**]	100	134.0±95.1 b
B	A + acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol [**(*SRR*)Ac**]	100+0.1	43.7±24.7 b
C	A + acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol [**(*SRR*)Ac**]	100+1.0	0 a
D	A + acetate of (2*S*,3*R*,7*S*)-3,7-dimethyl-2-pentadecanol [**(*SRS*)Ac**]	100+1.0	108.3±100.0 b
E	A + acetate of (2*S*,3*R*)-3-methyl-2-pentadecanol [**(*SR*–)Ac**]	100+1.0	3.7±3.2 a
F	Unbaited	–	0 a

Test run in Östergötland, Sweden, Sept.14–Oct. 7, 1995.

[a] Means followed by the same letter are not significantly different ($P<0.05$), according to ANOVA on log(catch + 1) transformed data followed by Tukey's test ($N=3$).

(2*S*,3*S*,7*S*)-isomer of acetate or propionate esters of diprionol are the only compounds that, in relatively low doses, attract males by themselves. However, the isomer **(*SRR*)Ac** is strongly antagonistic [when added to **(*SSS*)Ac**] in Sweden, but is apparently without effect in Japan [this study; Anderbrant et al. (2000)]. Earlier field tests in Japan indicated a small synergistic effect of **(*SRR*)Ac** over a wide range of **(*SRR*)Ac**/**(*SSS*)Ac** ratios (Tai et al., 1990, 1992).

In Sweden none of the three analogs, **(–*SS*)Ac**, **(*S*–*S*)Ac**, or **(*SS*–)Ac**, elicited catches of males when used in traps by themselves, and, further did not did not affect the catches of males when added to **(*SSS*)Ac**. This suggests that the analogs did not bind effectively to the antennal receptors tuned to **(*SSS*)Ac** (Hansson et al., 1991). In Japan, however, **(*SS*-)Ac**, in amounts 10–20 times higher than that of **(*SSS*)Ac**, caught male *N. sertifer* in traps (tests 7 and 9, Tables 5 and 6). Unfortunately, no unbaited traps were used in these two trials, but the other two analogs, **(*S*–*S*)Ac** and **(–*SS*)Ac**, caught lower numbers. The weak, but distinct, activity of **(*SS*–)Ac** in Japan is in accordance with results obtained by Tai et al. (1992). They used 100 times more of this analog, compared with **(*SSS*)Ac**, to get similar catches. When the amount of compound used is so much greater, as it is in some of these tests, there is an increased risk of other contaminants or isomers influencing male responses. However, in this case it is unlikely, as the amount of contaminating **(*SSS*)Ac** acetate had to be very low and would not explain the attraction to **(*SS*–)Ac** in Japan (Table 1). We speculate that ***SSS***-tuned receptors differ between Japanese and European populations, with receptors of Japanese *N. sertifer* males binding relatively effectively to analogs lacking the methyl group in the 7-position, but receptors of Swedish males requiring this structural feature for effective binding. This is supported also by the lack of effect of addition of the three analogs to **(*SSS*)Ac** in test 2 (Table 3); i.e., the analogs do not seem to

Table 11 Catch of male *Neodiprion sertifer* in traps baited with different esters of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol [(***SSS***)ester] 1997 (Test 10)

Bait	Ester	Amount (μg)	Mean catch ± SD[a]	
			Trial I	Trial II
A	Acetate **(*SSS*)AcS**	50	7.6±8.0 b	6.2±5.1 b
B	Propionate **(*SSS*)Pr**	50	11.6±5.5 bc	12.8±12.7 b
C	*iso*-Butyrate **(*SSS*)*i*Bu**	50	0 a	0 a
D	Butyrate **(*SSS*)Bu**	50	0 a	0 a
E	Propionate **(*SSS*)Pr**	100	17.0±9.0 c	13.2±4.6 b
F	*iso*-Butyrate **(*SSS*)*i*Bu**	200	0 a	0 a
G	Butyrate **(*SSS*)Bu**	200	0.2±0.4 a	0 a
H	Unbaited	–	0 a	0 a

Test run in Skåne, Sweden, Sept. 2–30 (Trial I) and Sept. 2–24 (Trial II).

[a] Means within a trial followed by the same letter are not significantly different ($P<0.05$) according to ANOVA on log(catch + 1) transformed data followed by Tukey's test (Trial I and II $N=5$).

bind at all to the receptors of *N. sertifer* males of Swedish origin.

An earlier investigation in Japan showed that the attraction to **(*SS*–)Ac** was synergized by addition of **(*SR*–)Ac** over a wide range of ratios (Tai et al., 1992). However, catches were small, and no repositioning of traps was carried out in these trials. No such effect of **(*SR*–)Ac** was found in the present investigation (test 7, Table 5). In fact, in Sweden **(*SR*–)Ac** showed antagonistic activity. These results are consistent with the response to **(*SRR*)Ac** in the two countries, and maybe explained by **(*SR*–)Ac** binding to the same receptor as does **(*SRR*)Ac**. Further, test 5, and to some extent test 4, showed that **(*SR*–)Ac** is a stronger antagonist than **(*SRS*)Ac** (Tables 9 and 10), suggesting that the presence of the methyl group at carbon 7 in an *R*-configuration is problematic for a receptor tuned to a *S*-methyl configuration at this position.

While the presence of a methyl group at each of the three stereogenic carbons apparently is essential to evoke an adequate response from males, the functional group on carbon 2 appears less critical; **(*SSS*)Ac** and **(*SSS*)Pr** are similarly active, both electrophysiologically and behaviorally (this study; Hansson et al., 1991; Anderbrant et al., 1992a, 2000). *Diprion pini* shows an even broader tolerance to different esters, with the acetate, propionate, and *iso*-butyrate esters of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol being attractive (Bergström et al., 1995; Anderbrant et al., 2005). It is interesting to note that the *S*-configuration at carbon 2 is important, and apparently conserved, within the Diprionidae (only one investigated species, *Gilpinia pallida*, employs a compound with 2*R* configuration, see Hedenström et al., 2006).

However, the terminal end (i.e., farthest away from the functional group) of the molecule is essential for the attractive effects. For *N. sertifer*, homologs of **(*SSS*)Ac** with one or two carbons less, or one carbon more, in the chain are completely inactive in the field (Wassgren et al., 1992). These homologs actually were found in small quantities in female extracts and also induced antennal responses during gas chromatography—electroantennographic detection recordings (Wassgren et al., 1992). Also *D. pini*, which has a main pheromone component with (2*S*,3*R*,7*R*)-configuration, showed electrophysiological, but not behavioral, responses to esters of (2*S*,3*S*,7*S*)-diprionol and its homologs (Anderbrant et al., 1995). Whether these homologs stimulate the same receptor as the pheromone or other receptors is unknown.

Neither *N. sertifer* nor *D. pini* has been subjected to a thorough investigation of single sensillum recordings in response to the various analogs/homologs. One obstacle to such a study is that the main pheromone component in both species stimulates several neurons with different amplitudes (Hansson et al., 1991; Anderbrant et al., 1995), making reliable quantifications difficult. Detailed molecular structure-electrophysiological response studies have been undertaken for other systems. For instance, recordings of 42 analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, were subjected to quantitative structure-activity modeling (Norinder et al., 1997 and references therein).

In sawflies, the combination of a complex, chiral pheromone molecule and a need for high stereogenic purity has stimulated the search for active analogs that are easier to synthesize for practical use. The results presented in this study indicate that it might be difficult to find such an analog. Instead, a more practical solution may be a simpler synthetic approach that produces mixtures containing the actual pheromone along with inactive stereoisomers (Hedenström et al., 2002). Successful population monitoring with such a mixture has been performed for *Diprion jingyuanensis* Xiao et Zhang in China (Zhang et al., 2005).

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2007), and Omura et al. (2002a) found that *Niponia nodulosa* Verhoeff, a cryptodesmid polydesmidan, secretes 1-octen-3-ol and geosmin. Cyanogenesis was not detected in either case. Such findings led to the assumption that cyanogenesis is not universal among polydesmidan millipedes.

Duffey et al. (1977) and Omura et al. (2002b) found that the chemical content of the defensive secretion varied considerably among twelve xystodesmid millipedes, and that the variable chemical composition might be used chemotaxonomically to distinguish some members of this group.

In this study, we investigated three sympatric species: *Polydesmus complanatus* (L.) (distributed in central, eastern, northern, and southeastern Europe), *Brachydesmus (Stylobrachydesmus) avalae* Ćurčić & Makarov (endemic to Serbia), and *Brachydesmus* (*Stylobrachydesmus) dadayi* Verhoeff (distributed in Bulgaria, Hungary, Serbia, and Slovakia) (all Polydesmidae). The objectives were: 1) to determine and identify the chemical components of the defensive secretions from three European polydesmids; 2) to test for possible intergeneric and interspecific variation of the chemical constituents, especially in closely related forms; and 3) to confirm the consistency of cyanogenesis in the members of the Polydesmidae.

Methods and Materials

Collection and Handling of Millipedes Individuals of *P. complanatus*, *B. avalae*, and *B. dadayi* were collected in the autumn (September–November 2009) at Mt. Avala, near Belgrade. Millipedes were stored in plastic boxes with a layer of partly decomposed litter and kept in the laboratory for a few days at 10°C in the dark. Humidity in the boxes was kept high by spraying water on the litter every day.

Dissection and Anatomical Description of Glands Defense glands are not present on each pleurotergite in the three polydesmids: in *P. complanatus* they are present on somites 5, 7, 9, 10, 12, 13, and 15–19, and in both *Brachydesmus* species on somites 5, 7, 9, 10, 12, 13, and 15–18. In these pleurotergites, the defense glands are located on lateral sides on both paranotal expansions. Five specimens of each of these polydesmid species were cold anaesthetized, and the defensive glands were excised from the pleurotergites. Each gland was placed carefully on a slide and examined under the microscope (Axioskop 40, Carl Zeiss; magnification 400×).

Chemical Extraction For the collection of secretions, ten females and ten males of each of the three species were extracted in hexane (0.5 ml) for 10 min. To eliminate the effects of composition-altering oxidation and degradation of compounds, a portion of the extracts was subjected to gas chromatography-mass spectrometric (GC-MS) analysis immediately after preparation.

Chemical Analyses Analyses were performed on an Agilent 7890A GC system equipped with a 5975C inert XL EI/CI MSD and a FID detector connected by capillary flow technology through a 2-way splitter with make-up gas. An HP-5 MS capillary column (Agilent Technologies, Santa Clara, CA, USA, 25 mm i.d., 30 m length, 0.25 μm film thickness) was used. Samples were injected in splitless mode. The injection volume was 1 μl, and the injector temperature was 250°C. The carrier gas (He) flow rate was 1.1 ml/min, whereas the column temperature was programmed linearly in a range of 60–310°C at a rate of 10°C/min with initial 2-min and final 8-min holds. The transfer line temperature was 280°C. The FID detector temperature was 300°C. EI mass spectra (70 eV) were acquired in *m/z* range of 35–550, and the ion source temperature was 230°C.

A library search and mass spectral deconvolution and extraction were performed by using NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, ver. 2.64. We used retention index (RI) calibration data analysis parameters at a 'strong' level with a 10% penalty for compounds without an RI. The retention indices were determined experimentally by the method of van Den Dool and Kratz (1963). This method is based on the retention times of *n*-alkanes, which were injected after the sample under the same chromatographic conditions. The search was performed against our own library containing 4,951 spectra, and the commercially available NIST05 Willey07 library containing approximately 500,000 spectra (identifications supported by data in Table 1 and Supplemental Data Figs. 1 and 2).

Percentages (relative) of the identified compounds were computed from the corresponding GC-FID peak areas.

Detection of Hydrogen Cyanide Hydrogen cyanide secreted from live millipedes was examined qualitatively by using filter paper that had previously been impregnated with a saturated solution of picric acid and was air-dried. After the filter paper was sprayed with 5% sodium bicarbonate, and without drying, a live millipede was placed onto the paper and squeezed forcefully (five specimens of each species). If the millipede secretions contained hydrogen cyanide, the color of the portion of the paper stained by the secretions gradually turned orange (Noguchi et al., 1997).

Results

Eight compounds were identified in the *n*-hexane whole-body extracts of the three polydesmid species (Table 1,

Table 1 Chemical composition of the defensive secretions in three polydesmid millipede species analyzed by GC-FID and GC-MS

Peak	t_R (min)[a]	RI[a]	Compound	Relative abundance (%)[b]		
				Polydesmus complanatus	*Brachydesmus dadayi*	*B. avalae*
1	5.86	969	Benzaldehyde	41.6	15.0	12.9
2	6.93	1,038	Benzyl alcohol	–	5.9	1.2
3	7.93	1,103	Benzoylnitrile	48.4	66.7	64.1
4	8.46	1,138	Benzyl methyl ketone	0.9	–	–
5	9.12	1,180	Benzoic acid	–	–	18.3
6	9.89	1,235	Benzyl ethyl ketone	3.1	2.3	T[c]
7	11.18	1,330	Mandelonitrile	3.2	–	–
8	18.21	1,951	Mandelonitrile benzoate	2.8	10.1	3.5

[a] Calculated from GC-MS data

[b] Calculated from GC-FID peak areas

[c] Trace (less than 0.1%)

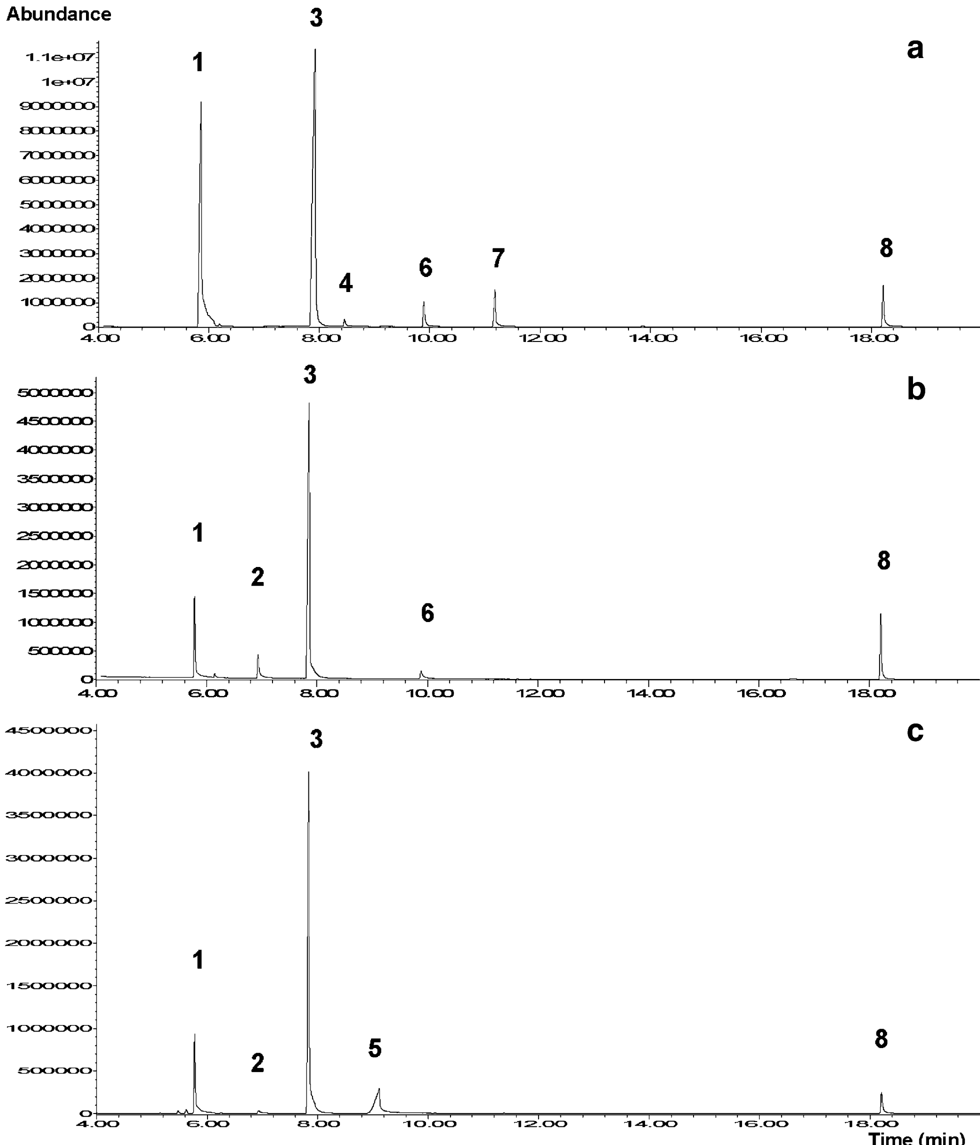

Fig. 1 Total ion chromatograms of the hexane whole-body extract from adult millipedes, *Polydesmus complanatus* (**a**), *Brachydesmus* (*St.) dadayi* (**b**), and *Brachydesmus (St.) avalae* (**c**). Peak 1: benzaldehyde; peak 2: benzyl alcohol; peak 3: benzoylnitrile; peak 4: benzyl methyl ketone; peak 5: benzoic acid; peak 6: benzyl ethyl ketone; peak 7: mandelonitrile; and peak 8: mandelonitrile benzoate

Supplemental Data Table 1 and Supplemental Data Figs. 1 and 2). The extracts showed different GC-MS patterns (Table 1 and Fig. 1). The extract of *P. complanatus* contained six compounds (Fig. 1a). The major components were benzoylnitrile and benzaldehyde; the minor components were mandelonitrile, benzyl ethyl ketone, mandelonitrile benzoate, and benzyl methyl ketone. The extract of *B. dadayi* contained only five compounds (Fig. 1b). The major component was benzoylnitrile; the minor components were benzaldehyde, mandelonitrile benzoate, benzyl alcohol, and benzyl ethyl ketone. The extract of *B. avalae* had six compounds (Fig. 1c). The major component was benzoylnitrile; the minor ones were benzoic acid, benzaldehyde, mandelonitrile benzoate, benzyl alcohol, and a trace amount of benzyl ethyl ketone. In all species, we confirmed the presence of hydrogen cyanide. No significant difference in the amounts of the components was observed between the sexes in the three analyzed species.

Discussion

In all three polydesmids that we analyzed, benzoylnitrile was a major component of the whole body extract. The second major component was benzaldehyde, which, along with hydrogen cyanide, is known as the most common defensive substance in various millipedes. A review of the literature shows that benzyl ethyl ketone, benzyl methyl ketone, and benzyl alcohol were detected for the first time from representatives of the order Polydesmida.

The dissection of glands in the three polydesmids showed that they have a typical structure, consisting of an inner elongated compartment or reservoir and a smaller compartment or vestibule. In *P. complanatus*, the reservoir stores mandelonitrile, whereas the vestibule contains an enzyme that catalyzes the breakdown of mandelonitrile into hydrogen cyanide and benzaldehyde (Hopkin and Read, 1992). Because we did not confirm the presence of mandelonitrile in both *Brachydesmus* species, we hypothesize that mandelonitrile benzoate is a cyanogenic precursor in these two species.

There is a controversy about the taxonomic position of the genera *Polydesmus* and *Brachydesmus*. Namely, these genera are differentiated mainly according to the number of pleurotergites (19 pleurotergites in *Brachydesmus* species vs. 20 in *Polydesmus* species), but in many forms of both genera, pleurotergites and gonopods show similar structure. Strasser (1971) speculated about the validity of the genus *Brachydesmus*, and even about some species with 19 pleurotergites from the genus *Polydesmus* [(e.g., *Polydesmus gradjensis* (Jawlowski) or *Polydesmus jugoslavicus* (Jawlowski)]. Hoffman (1979) treated *Brachydesmus* as a subgenus of the genus *Polydesmus*. However, Enghoff and Kime (2005) retained full generic status for both groups. The present study clarified the differences in chemical composition of the defensive secretions in these two polydesmid genera. Mandelonitrile was detected only in *P. complanatus*. On the other hand, benzyl alcohol was detected only in the *Brachydesmus* species. The presence of benzyl alcohol and absence of mandelonitrile may be a chemogeneric characteristic of *Brachydesmus* relative to *Polydesmus*. This difference supports the validity of the genus *Brachydesmus*.

Additionally, we demonstrated intrageneric variation in the chemical composition of whole-body extracts in the *Brachydesmus*, including the presence of benzoic acid only in *B. avalae* (Table 1). As another example of intrageneric variation, Casnati et al. (1963) and Eisner et al. (1975) showed that *P. collaris collaris* and *P. vicinus* produced HCN, benzaldehyde, mandelonitrile benzoate, and mandelonitrile. In contrast we have shown that whole-body extracts of *P. complanatus* contain benzoylnitrile, benzyl methyl ketone, and benzyl ethyl ketone, which were absent in *P. collaris collaris* and *P. vicinus* (Table 1). In most previous analyses of the Polydesmidae, only the major chemical components of the millipede exudates were identified (Casnati et al., 1963; Eisner et al., 1975; Duffey et al., 1977; Conner et al., 1977). Our study demonstrates that most differences among the polydesmid defensive secretions are found among the minor components, and that the chemical patterns in the defensive fluids may be useful in chemotaxonomy.

Shear et al. (2007) noted that the families of Xystodesmidae and Gomphodesmidae are cyanogenic and also produce benzoylnitrile (=benzoyl cyanide), which is not found in other families. However, Conner et al. (1977) found benzoylnitrile in the defense secretion of *Pseudopolydesmus serratus* (Polydesmidae). Thus, we confirmed the presence of benzoylnitrile in representatives of the family Polydesmidae.

The primary function of defensive secretion in the polydesmids is protection against potential predators (Duffey et al., 1977; Hopkin and Read, 1992; Omura et al., 2002a). Duffey et al. (1977) found that benzoylnitrile, the most abundant component, possesses anaesthetizing properties for potential predators, especially ants, which are abundant in the habitat from which we collected all of our specimens. A secondary function of the defensive secretion may be antimicrobial and antifungal. The three species that we analyzed are likely exposed to microbial attack. *Polydesmus complanatus* is a stratobiont, restricted mainly to litter and the uppermost soil layers. Both representatives of the genus *Brachydesmus* are minute geobionts, which inhabit deeper soil layers. Roncardi et al. (1985) found that the mixture of benzaldehyde, benzoic acid, benzoylnitrile, and mandelonitrile benzoate in some

xystodesmid millipedes was a more effective spore germination inhibitor than any individual compound. Furthermore, they noted that the antifungal function of the defensive secretion mixture could be a factor in preventing invasion of the cyanogenic gland and contamination of the millipede with microorganisms. Our results showed that the extracts of *P. complanatus* and *B. dadayi* contained benzaldehyde and mandelonitrile benzoate, whereas the extracts of *B. avalae* contained benzaldehyde, benzoic acid, and mandelonitrile benzoate. Mixed with other detected compounds, the defensive secretion in the species that we analyzed likely has a protective function for the cyanogenic glands, as well as the cuticle.

Our results confirm cyanogenesis in the family Polydesmidae. Benzyl ethyl ketone, benzyl methyl ketone, and benzyl alcohol were detected for the first time in the order Polydesmida. The specificity in the chemical composition of defensive secretions might serve as criteria for the chemotaxonomy in polydesmid species. Further studies are necessary in order to clarify differences in chemical composition in additional polydesmid representatives.

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Fig. 1 A proposed model of the chemical relationships between the privet tree and privet specialists

Oleuropein in privet leaves

Activation by foliar β-glucosidase and polyphenol oxidase

Quinone

Glutaraldehyde-like structure α,β-unsaturated aldehyde

Reaction with amino residue in the side chain of lysine in protein
Protein denaturation and lysine decrease

Competitive inhibition

Lysine in protein

Glycine and GABA in the digestive juice of privet-specialist insects

exceeded 50 mM (Konno et al. 1997). An injection experiment with ^{15}N-labeled glycine showed that glycine is actively transported from hemolymph to the midgut lumen against the concentration gradient in *B. wallichii* (Konno et al. 2001), suggesting that the glycine secreted in digestive juice has physiological functions. Glycine completely inhibited the lysine-decreasing activity of privet leaf extract and enzymatically activated oleuropein in *in vitro* experiments (Konno et al. 1997, 1998). Further, our recent experiments also showed that glycine functions as adaptation *in vivo*. When the non-specialist caterpillars, i.e., the Eri silkworm, *Samia ricini,* were fed privet leaves coated with free glycine, both the decrease in lysine concentration of the midgut contents and the retardation of growth observed in larvae fed privet leaves were completely prevented, and the larvae grew successfully (Konno et al. 2009). These results indicate that glycine secretion in the digestive juice of several privet specialists is an adaptive trait against the oleuropein-based chemical defense of privet trees. Further, as a chemical model that might explain this phenomenon, we hypothesized that amino residues in glycine inhibit the lysine-decreasing activity by competing with the amino residue in the side chain of lysine for oleuropein (Konno et al. 1999, 2009) (Fig. 1).

In the present study, we attempted to address several remaining questions. First, we wanted to examine the generality of glycine secretion among privet-feeding insects. Second, we were interested in knowing whether or not glycine is the only amino acid or chemical that can function as an adaptation against the privet defense and that has been adopted by privet specialists for that purpose. Our chemical model predicted that not only glycine, but also other amino acids containing amino residues could function in the adaptation to the privet defense. To address the first and second points, we extended the survey to investigate the presence of glycine or other amino acids in the digestive juice of additional lepidopteran species, both privet feeders and non-feeders, and also some hymenopteran species. We found that most privet specialists, whether Lepidoptera or Hymenoptera, have glycine or other amino acids such as

GABA in their digestive juice. The third question was what structure in amino acids is important for inhibiting the privet activity. Fourth, we asked whether the concentrations of amino acids found in the digestive juice were sufficient to inhibit the lysine-decreasing activity. These last two points were analyzed and clarified by *in vitro* experiments. We speculate on the evolutionary processes and characteristics of these convergent adaptive traits in privet specialists, and how the cost of adaptation may have affected the coevolutionary process between the privet tree and its specialists.

Methods and Materials

Insects The Eri silkworm, *Samia ricini*, and the true silkworm, *Bombyx mori* are maintained in our institute as experimental insects. Larvae of all other lepidopteran and hymenopteran species used in the present study were collected from wild populations in Tsukuba, Ibaraki, Japan (36°N, 136°E). These species are listed in Table 1.

Analysis of Amino Acids in the Digestive Juice and Midgut Lumen To examine whether the glycine secretion observed in the larvae of several privet-specialist lepidopteran species is a general trait found in all privet-feeding insect species, we analyzed the digestive juice of larvae of 42 herbivorous lepidopteran and hymenopteran species. The digestive juices (N=3–10) were collected from the last instar larvae fed their host plants at 3 d after the last ecdysis as described previously (Konno et al. 1996, 1997). In short, larvae were starved for 1 d prior to the collection in order to decrease contamination of leaf material. Then, an electric shock was administered to the larvae, and the vomited digestive juice was collected. The fact that the larvae of most species vomited a large amount of fluid compared to their body sizes (e.g., an Eri silkworm with 5 g weight vomited more than 1 ml of fluid) suggests that the vomited fluid came from the midgut. Further, the glycine concentration in vomited digestive juice and that in samples collected from the anterior parts of midgut lumen by dissection showed good agreement both in *Bombyx mori* (8.98 mM and 5.72 mM in vomited digestive juice and sample collected from midgut lumen, respectively) (Konno et al. 1996) and in *Brahmaea wallichii* (50.05 mM and 52.94 mM, respectively) (Konno et al. 1997, 2001). These facts indicate that the digestive juice collected using the electric shock method well represents the condition of digestive juice in the midgut lumen or what is called midgut fluid. The collected digestive juice was analyzed by an auto amino acid analyzer (Model L5000; Hitachi, Tokyo) based on HPLC and the ninhydrin reaction. The digestive juice of *Artopoetes pryeri*, a privet-specialist that did not have glycine nor 19 other protein amino acids in its digestive juice, but had an unidentified ninhydrin-positive compound, was further analyzed by ^{1}H and ^{13}C -NMR. For this analysis, digestive juice was collected from 50 larvae by dissection. The ninhydrin-positive peak in the digestive juice was purified by HPLC, and ^{1}H and ^{13}C-NMR spectra were measured.

Analysis of the Lysine-decreasing Activity of Privet Leaves and Inhibitory Activity of Amino Acids, Amines and Ammonium Ion The analyses were performed as described previously (Konno et al. 1997, 1998, 1999). In short, the lysine-decreasing activity of the privet leaves was reproduced *in vitro* by mixing together an oleuropein-containing fraction free of activating enzymes, and an activating enzyme-containing fraction free of oleuropein, both of which were prepared from privet leaves and lacked any lysine-decreasing activity alone. The oleuropein-containing solution was prepared by homogenizing steamed privet leaves (100°C, 4 min) with 5 volumes of water and collecting the supernatant by centrifugation. The solution contained approximately 0.6% oleuropein. The activating enzyme-containing solution was prepared from fresh privet leaves by collecting an organelle fraction by differential centrifugation. Analyses were performed in 750 µl of sodium phosphate buffer solution (pH 7.0) that contained 1% ovalbumin, 500 µl of oleuropein-containing solution prepared from 100 mg of steamed privet leaves, 100 µl of enzyme-containing fraction prepared from 80 mg of privet leaves, and the indicated amounts of amino acids, amines, or ammonium ion. These conditions were determined in order to simulate roughly the conditions in the midgut lumen of caterpillars feeding on privet leaves. This assumes that fresh leaf material contains 25% of dry matter, that the dry leaf matter contains 20% of protein, and that fresh leaf material is diluted by five volumes of digestive juice in the midgut lumen. Reactions were performed at 25°C for 2 h, and stopped by the addition of 6 M HCl. After the reactions, the ovalbumin was hydrolyzed in 6 M HCl at 110°C for 22 h. The lysine content in hydrolyzed ovalbumin was analyzed by the same auto amino acid analyzer described above.

Results

Occurrence of High Concentrations of Glycine in the Digestive Juice of Privet-Specialist Lepidopteran and Hymenopteran Larvae We found very high concentrations of glycine in many of the privet-feeding species (Table 1). The average concentration of glycine for 11 privet-feeding species was 40.396 mM, and was significantly higher than that for 32 non-privet-feeding species (2.198 mM) (Mann-

Table 1 Concentrations of glycine and other amino acids in the digestive juice of herbivorous Lepidoptera and Hymenoptera larvae

	Privet feeders	(mM)	Non-privet feeders	(mM)
Lepidoptera				
Sphingidae				
	Dolbina tancrei	44.139	*Cephonodes hylas*[a]	0.223
	Psilogramma increta[a]	10.994	*Daphnis nerii*[a]	3.997
			Theretra nessus[a]	8.288
			Theretra japonica[a]	12.052
			Theretra oldenlandiae[a]	3.430
			Callambulyx tatarinovii[a]	0.087
			Agrius convolvuli	14.322
			Phyllosphingia dissimilis	5.540
			Ambulyx scauffelbergeri	0.036
Brahmaeaidae				
	Brahmaea wallichii	52.934		
Bombycidae				
			Bombyx mori[a]	5.717
Saturniidae				
			Antheraea yamamai[a]	0.062
			Rhodinia fugax[a]	1.860
			Samia ricini[a]	0.031
			Caligula jonasii	0.960
			Caligula japonica	0.510
Lasiocampidae				
			Malacosoma neustria[a]	0.059
Noctuidae				
	Crainophora fasciata	0.921	*Spodoptera litura*	0.033
	(β-alanine	9.851)		
	Pangrapta trimantesalis†	19.778	*Eutelia geyeri*	1.203
	Amphipyra monolitha	0.111	*Amphipyra monolitha*	1.569
	(on privet) (β-alanine	34.757)	(on cherry) (β-alanine	5.471)
Arctiidae				
	Spilosoma imparilis	0.280	*Hyphantria cunea*[a]	0.201
Geometridae				
	Naxa seriaria	58.347	*Ectropis crepuscularia*	0.127
	Inurois fletcheri	88.390		
Lymantriidae				
			Lymantria dispar[a]	0.056
Notodontidae				
			Hupodonta corticalis[a]	0.484
Papilionoidae				
	Artopoetes pryeri	3.661	*Ussuriana stygiana*	1.817
	(GABA	60.812)	(GABA	19.417)
			Japonica saepestriata	0.967
			(aspartate	44.063)
			Favonius orientalis	1.340
			(GABA	0.767)
			Atrophaneura alcinous	0.127
			Papilio bianor[a]	0.189
			Pieris rapae	0.136
			Cyrestis thyodamas[a]	0.180

Table 1 (continued)

	Privet feeders	(mM)	Non-privet feeders	(mM)
			Papilio polytes	0.017
Hymenoptera				
Symphyta				
	Macrophya timida	164.803	*Macrophya carbonaria*	4.710
Average(glycine)		40.396*		2.198*
		* Mann-Whitmey U-test, P=0.002		
Average (the most abundant amino acids)		49.553**		4.217**
		** Mann-Whitmey U-test, P<0.001		

The values of concentrations not otherwise indicated are the concentrations of glycine

GABA and β-alanine were not detected in the species unless the concentrations of these amino acids were otherwise indicated in the parentheses

The values for each species are an avarage from 3–10 individuals

[a] The data for this species was taken from Konno et al. (1997)

Whitney's U-test, P=0.002). Six out of eleven privet-feeding species showed glycine concentrations higher than 15 mM, and 7 out of 11 showed glycine concentrations higher than 10 mM. In *Dolbina tancrei* (Sphingidae), *Brahmaea wallichii* (Brahmaeidae), and *Naxa seriaria* (Geometridae), the concentrations were around 50 mM (0.4%), and in *Inurois fletcheri* (Geometridae) the concentrations reached 88 mM (0.7%). Further, in the privet-specialist sawfly, *Macrophya timida* (Hymenoptera), the glycine concentration reached 164 mM (1.2 %) (Table 1). In *Brahmaea wallichii*, a glycine-specific secretory mechanism has been identified in the midgut (Konno et al. 2001). In contrast, none of the 32 non-privet-feeding species showed a glycine concentration higher than 15 mM, and more than half of the species eventually had no glycine in their digestive juices (Table 1).

GABA and β-Alanine in the Digestive Juice of Some Privet Specialists Among the 11 privet-feeding species examined, 4 species did not have glycine in their digestive juice; these 4 species included *Artopoetes pryeri* (Papilionoidae), *Crainophora fasciata* (Noctuidae), *Amphipyra monolitha* (Noctuidae), and *Spilosoma imparilis* (Arctiidae) (Table 1). We further analyzed the digestive juice of the larvae of a Lycaenidae butterfly, *Artopoetes pryeri* (Fig. 2a), for amino acids other than glycine. GABA (γ-amino butyric acid), an amino acid known as a neurotransmitter (Waagepetersen et al. 1999), was present in high concentration (60.812± 40.992 mM, N=10) (Fig. 2b, c, d, e) instead of free glycine, while the concentrations of other free amino acids were low. The unexpected presence of GABA was confirmed further by ^{1}H-NMR analyses (Fig. 2c, d). The spectra of the purified ninhydrin-positive compound were as follows. For ^{1}H-NMR(400 MHz, D_2O):δ 1.80 (2H, quint, J=7.4 Hz), 2.20 (2H, t, J=7.4), 2.91 (2H, t, J=7.6). For ^{13}C-NMR (100 MHz, D_2O): δ 23.77, 34.51, 39.40, 181.63. These spectra were identical to those of the pure GABA standard. Further, in the digestive juice of two other privet-feeding species, *Crainophora fasciata* (Noctuidae) and *Amphipyra monolitha* (Noctuidae), 9.851 mM and 34.757 mM of β-alanine was observed, respectively. In total, 10 of 11 privet species retained high concentrations of either glycine, β-alanine, or GABA, with the only exception being *Spilosoma imparilis* (Arctiinae). The average concentrations of the most abundant amino acids in the digestive juices were significantly higher in privet-feeding species (49.553 mM) than in non-feeding species (4.217 mM) (Mann-Whitney U-test, P<0.001) (Table 1). No detectable GABA, β-alanine, and only a trace amount (less than 2 mM) of glycine were observed in the water extract of privet leaves extracted with five volumes of water. This observation together with our previous observation on active secretion of glycine in the digestive juice in *Bramaea wallichii* (Konno et al. 2001) indicated that high concentrations of glycine, GABA, and β-alanine found in the digestive juice of prevet specialists is derived from insects rather than from privet leaves.

Comparison of the Abilities of Amino Acids, Amines, and Ammonium Ion to Inhibit Lysine-decreasing Activity To examine whether the glycine in the digestive juice has an adaptive role at the concentrations found in the digestive juice of privet specialists, we performed an *in vitro* experiment in which protein (ovalbumin) was mixed with oleuropein activated by enzymes in the presence of glycine, thus imitating the conditions in the midgut lumen of privet specialists. The lysine content of treated protein was analyzed (Fig. 3a). Untreated ovalbumin contained 2.962±0.075 (μmol / 10 mg ovalbumin) of lysine, and in the ovalbumin treated by enzymatically activated ovalbumin without the

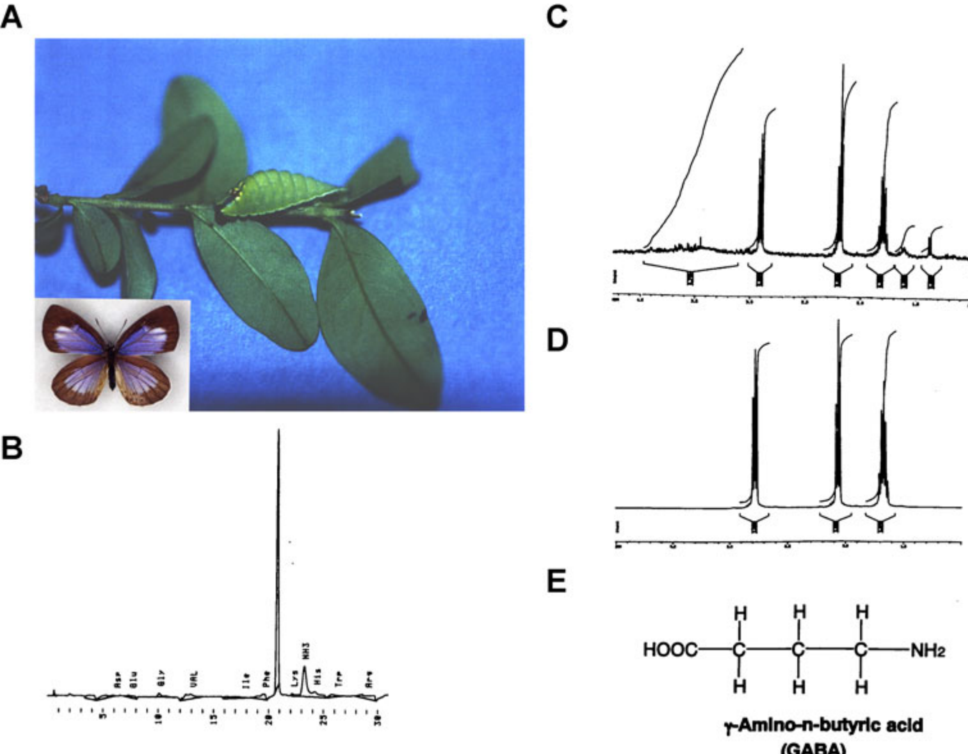

Fig. 2 A high concentration of GABA (γ-amino butyric acid) found in the digestive juice of larvae of a Lycaenidae butterfly, *Artopoetes pryeri*. **a** A larva and an adult (bottom left) of *A. pryeri*. **b** A ninhydrine-positive peak in the digestive juice of larvae of *A. pryeri* detected using an auto amino acid analyzer. **c** The ^{1}H-NMR spectrum of the chromatographically purified ninhydrine-positive peak in the digestive juice. **d** The ^{1}H-NMR spectrum of pure GABA. **e** The molecular structure of GABA

addition of glycine, the lysine content decreased to 0.619±0.102 (μmol / 10 mg ovalbumin). When ovalbumin was treated in the presence of 13.3 mM and 26.6 mM of glycine, the lysine contents in protein were 2.414±0.151 and 2.557±0.086 (μmol / 10 mg ovalbumin), respectively, and the lysine decrease was inhibited. The *in vitro* results that 13.3–26.6 mM glycine inhibited the lysine-decreasing activity suggest that the amount of glycine found in privet-feeding insects (15 mM–150 mM) would prevent the lysine-decreasing activity of the privet leaves and its adverse effects also *in vivo*. In fact, our recent *in vivo* results showed that the presence of 30 mM of glycine in the midgut lumen due to concomitant administration of glycine during the oral administration of privet leaves prevented the loss of lysine and significantly improved the growth performances of the non-specialist Eri silkworms, *Samia ricini* (Konno et al. 2009).

GABA also inhibited the lysine-decreasing activity as effectively as glycine (Fig. 3a). When ovalbumin was treated by enzymatically activated oleuropein in the presence of 13.3 mM and 26.6 mM of GABA, the resulting lysine contents were 2.286±0.019 and 2.576±0.109 (μmol / 10 mg ovalbumin), respectively, thus indicating that the GABA found in digestive juice has an adaptive role. β-Alanine also efficiently inhibited the lysine-decreasing activity of oleuropein (Fig. 3a).

Since glycine, GABA, and β-alanine are amino acids, and since glutaraldehyde and iridoid aglycones, which are similar to glutaraldehyde, have been reported to bind to amino residues of free amino acids and to the side chain of lysine in protein (Richards and Knowles 1968; Kawahara et al. 1992; Touyama et al. 1994; Bianco et al. 1999a, b), we hypothesized that the amino residues in glycine, GABA, and β-alanine inhibit the lysine-decreasing activity by competing with the amino residue in the side chain of lysine for oleuropein (Fig. 1). To examine this possibility, we analyzed the inhibitory activity of amino acids, amines, ammonium ion, and related compounds (Fig. 3a, b). Alanine, propyl amine, ammonium acetate, ammonium chloride, GABA, glycine, and β-alanine showed inhibitory activity, while sodium acetate and urea, which do not have amine properties (such as basicity), did not inhibit the lysine-decreasing activity. These results indicated the relevance of our hypothesis (Fig. 1). However, the inhibitory activity differed significantly among the amino acids and amine-related compounds tested. While glycine, GABA, and β-alanine inhibited lysine-decreasing activity almost completely at lower concentrations (13.3 mM–26.6 mM) and propylamine also showed strong inhibition, alanine and ammonium ion did not inhibit the activity completely even at concentrations 5 times higher (53.3 mM–133.3 mM) (Fig. 3a, b). These results can be attributed to the similarity in structure among glycine, β-alanine, GABA, and propylamine, all of which have amino residues at the ends of the molecules, vs. alanine (as well as all other protein forming amino acids except lysine) which does not. Its amino residue is half hidden by other parts (i.e., side chains) of the molecule. Thus, the amino acids that

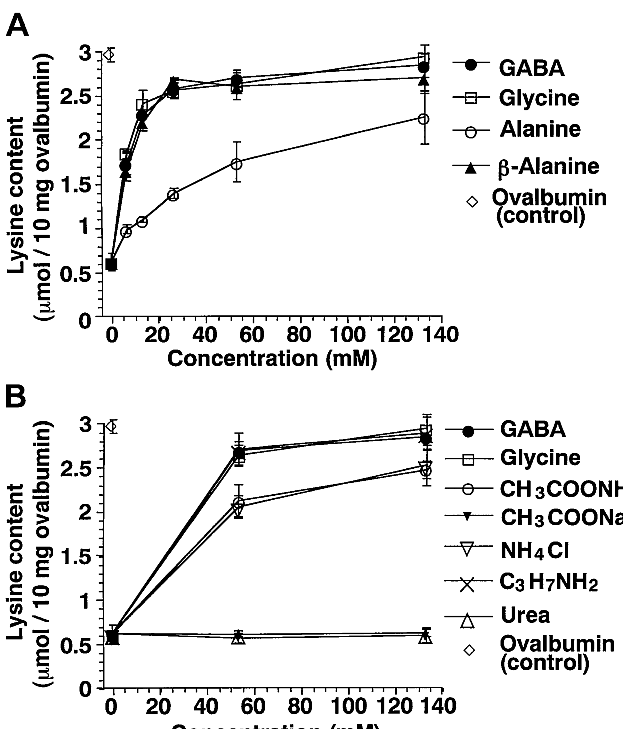

Fig. 3 Inhibitory effects of glycine, GABA, and other amino acids, amines, and ammonium ion on the lysine-decreasing activity of privet leaves. Untreated ovalbumin contains approximately 3 μmol lysine per 10 mg ovalbumin. When ovalbumin was exposed to the lysine-decreasing activity of the privet leaves, the lysine decreased to approximately 0.6 μmol per 10 mg ovalbumin. The indicated amounts of amino acids, amines, and ammonium ion were added in the treatment, and the amounts of lysine in treated ovalbumin were measured (average±SD, N=3)

have been adopted by privet specialists worked most effectively.

Discussion

In this study, we found that a number of phylogenetically distinct privet-feeding insects have convergently developed similar molecular strategies based on the same chemical principle, namely the secretion of amino acids such as glycine, GABA, and β-alanine as a neutralizer, in order to feed on the privet tree defended by an iridoid glycoside, oleuropein. To our knowledge, this is the first study to identify a biological role of animal-derived GABA in plant-herbivore interactions, and also the first to establish a biological role of animal-derived GABA that does not involve neurons, such as a role in neurotransmission or neurodevelopment (Waagepetersen et al. 1999).

In this study, we found that 7 of 11 privet specialists secrete glycine, that 10 of 11 privet specialists secrete glycine, GABA, or β-alanine, and that the concentrations of these amino acids are significantly higher in privet specialists than non-privet specialists. Further, our biochemical analyses showed that these amino acids inhibit the decrease in lysine caused by oleuropein *in vitro* (Fig. 3), and that glycine inhibits the decrease in lysine and improves the non-specialists' performance *in vivo* (Konno et al. 2009). All these findings indicate that amino acid secretion in the digestive juice is a general and efficient adaptive trait of privet-specialists against the privet defense based on oleuropein, the iridoid glycoside in its leaves.

The present experiments are the first to elucidate a physiological mechanism of insect adaptation to an iridoid glycoside-based defense, which is found widely in plants (Bowers 1991). It is an interesting question whether similar adaptive mechanisms exist in the specialist herbivores of iridoid-defended plants other than privet trees. Glutaraldehyde-like alkylators (di-aldehyde) are not only produced by the activation of iridoids by β-glucosidase (Konno et al. 1999), but also by the activation of caulerpenyne by esterase activity in an invasive sea green algae, *Caulerpa taxifolia* (Jung and Ponert 2001). In both cases, they exert lysine-decreasing activity *in vitro* (Konno et al. 1999; Weissflog et al. 2008), although the structures of the precursors, oleuropein and caulerpenyne, are very different. It would be interesting to examine whether caulerpenyne also functions in the defense of the sea algae and whether amino acid secretion also exists in the digestive juices of specialist feeders of the sea algae as an adaptation in the marine ecosystem.

It is clear that the iridoid-based defense of privet trees can be classified as an anti-nutritive plant defense in which essential amino acids of nutritive proteins are targeted and destroyed by the plant itself (Felton and Gatehouse 1996; Zhu-Salzman et al. 2008), similar to the defenses that involve phenolics and tannins (Felton et al. 1989; Felton and Gatehouse 1996), proteinase inhibitors (Felton and Gatehouse 1996; Lawrence and Koundal 2002), and arginase and threonine deaminase (Chen et al. 2005). Despite the importance of such anti-nutritive plant defenses, adaptive mechanisms against them have been established only in limited cases, such as the excretion of tannin-binding proline-rich protein in mammalian saliva that defend against tannin (Shimada 2006), the production of catalase against peroxidase-activated phenolics (Felton and Duffey 1991), or the induction of insensitive proteinase against proteinase inhibitor (Broadway 1995). Our study on this molecular adaptive mechanism of privet specialists against the iridoid-based anti-nutritive defense of privet trees contributes a unique example of insect adaptation to an anti-nutritive defense.

Figure 1 presents our latest chemical model of the privet-privet specialist relationship based on our present and previous studies (Konno et al. 1997, 1998, 1999, 2009). The model suggests that one amino residue in a molecule of

an amino acid is required to prevent the loss of one molecule of lysine in dietary protein. The model also predicts that not only glycine but also all other amino acids and molecules with amino residues could prevent lysine loss and protein denaturation. Our experiments (Fig. 3) showed that the prediction holds, and the results imply that any amino acid is a potential neutralizer of the protein-denaturing / lysine-decreasing activities exhibited by privet leaves.

However, the amino acid found in the digestive juices of the privet specialists we examined was glycine in many cases, β-alanine in two cases, and GABA in a single case; no other amino acids were found in the juices from privet specialists. This pattern could be explained only if we assume the costs incurred by a privet specialist in adapting to a chemical defense of the privet tree. It is clear that, in adapting to the privet tree chemical defense, herbivores incur adaptive costs by adopting an amino acid secretion strategy, since it is necessary to consume one amino residue of some molecule to save one molecule of lysine, an essential and valuable amino acid. The cost of adaptation must be less than the benefit it provides. For this reason, the amino acids used and consumed by herbivores must be non-essential amino acids, such as glycine, or derivatives thereof that insects can synthesize by themselves, such as GABA and β-alanine, which can be easily produced from glutamate and aspartate, respectively. For the same reason, the amino acids used by privet specialists must have sufficient neutralizing effects at low concentrations. We observed that glycine, GABA, and β-alanine are five times more effective than alanine (Fig. 3a). The digestive juices of many privet-specialist species contain 40–160 mM of GABA or glycine (0.3–1.2% for glycine). These concentrations are probably necessary and sufficient for these specialists. If they need to substitute glycine, β-alanine, or GABA with alanine, it is estimated that 200–800 mM (1.8–8.9%) of alanine would be necessary. However, the cost to secrete such high concentrations of alanine (the cost of alanine itself and the cost to transport a large amount of alanine across the midgut) would be quite high. Further, such a high concentration (8%) of alanine would be harmful because of high osmotic pressure, and also 8% alanine is close to saturation (the solubility of alanine to water at 25°C is 121 g/l, 12.1%), and this could make secretion difficult. Therefore, glycine, β-alanine, and GABA seem to be the only amino acids that are at once economical and efficient, and for which the benefits of production exceed the costs.

The evolution to amino acid secretion seems to have occurred at least four times convergently, once for Lepidoptera with GABA, once for Lepidoptera with β-alanine, and at least once each, but probably more, in Lepidoptera and Hymenoptera with glycine. The secretion of glycine has evolved more frequently and is distributed more widely among insects than the secretion of β-alanine or GABA (Table 1). This pattern also could be explained from the standpoint of the cost of adaptation, since β-alanine and GABA are likely to be costlier than glycine because of the cost of carbon. All our results suggest that in privet /privet-specialist systems, herbivores incur costs in adaptation and evolve into these adaptive traits only if benefits exceed costs. The trait with the cheapest cost of adaptation is selected for. Due to the lack of empirical observations, it has not been clear whether herbivores incur adaptive costs in adapting to plants with chemical defenses (Gould 1988; Futuyma and Keese 1992; Agrawal 2000). The present study provides empirical observations of the cost of adaptation.

Our results show a rare example of physiological adaptation to a quantitative defense (Lambers et al. 1998) (that is, a defense with compounds, such as phenolics and tannins, that reduce digestibility and exist in large quantities). In the evolution of a quantitative defense, it seems reasonable to suppose the existence of “quantitative” types of coevolutionary arms races. These may include corresponding escalations of the amounts of defense and adaptive substances and/or mechanisms in plants and herbivores, respectively, that may finally reach an equilibrium condition after the extinction and/or exclusion of less well adapted species, and decrease in the population of herbivores on the plant species. In the evolution of the privet-privet specialist relationship, it is possible that such “quantitative” types of coevolutionary arms races coexisted with the classical “qualitative” types of coevolutionary arms races, which include upgrading of and innovations in the defense chemicals and adaptive mechanisms, as has been reported in the coevolution of coumarin-containing plants such as Umbelliferae and herbivores feeding on them (Berenbaum 1978, 1991). Indeed, most qualitative defenses have a quantitative (dose-dependent) aspect. Oleuropein, which has a much stronger lysine-decreasing activity than other iridoids (Konno et al. 1999) would be a “second-generation” or “upgraded” iridoid that may have evolved from less active iridoids through a “qualitative” type of coevolutionary arms race. In contrast, the surprisingly high concentration of oleuropein in privet leaves (ca. 3% of leaf wet weight, 55 mM) (Konno et al. 1998) and of glycine (164.8 mM, 1.24%) and GABA (60.8 mM, 0.63%) in the digestive juice of specialist insects may have resulted from a “quantitative” type of coevolutionary arms race to destroy (strategy of plant) and/or save (strategy of herbivores) ca. 5 mM (Konno et al. 2009) of lysine in the gut lumen, since lysine is crucial for herbivores.

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hypothesis (Gauld et al., 1992, 1994), which posits that tropical parasitoids are not more diverse because sequestering hosts are more toxic and thus less available to parasitoids. Indirect support for this component of the nasty host hypothesis comes from studies that show a negative relationship between levels of sequestered host defensive compounds and parasitoid success (Campbell and Duffey, 1979; McDougall et al., 1988; Sime, 2002; Nieminen et al., 2003; Singer and Stireman, 2003; Lampert et al., 2008).

We used larvae of the catalpa sphinx, *Ceratomia catalpae* Boisduval (Lepidoptera: Sphingidae), and its parasitoid, *Cotesia congregata* Say (Hymenoptera: Braconidae), to test some assumptions of the nasty host hypothesis. The catalpa sphinx specializes on species of *Catalpa* (Bignoniaceae) (Baerg, 1935; Bowers, 2003), which contain the iridoid glycosides catalpol and catalposide (Nayar and Fraenkel, 1963; von Poser et al., 2000), terpenoids that are unpalatable to a range of generalist herbivores (Bowers, 1991). However, these compounds are used as feeding stimulants by catalpa sphinx larvae (Nayar and Fraenkel, 1963), which also sequester them for their own chemical defense (Bowers and Puttick, 1986; Bowers, 2003). Catalpa sphinx larvae hydrolyze catalposide to catalpol before sequestration, and caterpillar catalpol concentrations (5–20% total dry weight) can be several times higher than catalpa leaf iridoid concentrations (2–5% dry weight) (Bowers and Puttick, 1986; Bowers, 2003). Most of the iridoid glycosides are stored in the hemolymph, which contains approximately 50% dry weight catalpol (Bowers, 2003). Further, larvae regurgitate onto potential predators to repel them, and this regurgitant contains iridoid glycosides (Bowers, 2003).

The gregarious koinobiont parasitoid, *Cotesia congregata*, is the major parasitoid of catalpa sphinx larvae in the Eastern U.S. (Baerg, 1935; Ness, 2003a, b). *Cotesia congregata* generally are restricted to larvae of Sphingidae as hosts; however, Krombein et al. (1979) list only 15 sphingid species as hosts and also list *Trichoplusia ni* (Noctuidae) as a host. In laboratory experiments, *Hyles lineata* (Sphingidae) also was shown to be a permissive host, *Pachysphinx occidentalis* was a refractory host, showing complete encapsulation, and *Sphinx vashti* was considered semi-permissive, showing some encapsulation (Harwood et al., 1998). In another experiment, *T. ni* was a semi-permissive host (Beckage and Tan, 2002). *Cotesia congregata* attacks its hosts during the 2nd through the 4th instars by rapidly injecting eggs. Larvae develop in the hemocoel, bathed in and eating hemolymph, over approximately 2 weeks, then exit through the host cuticle, and pupate inside silken cocoons attached to the host. *Cotesia congregata* successfully parasitize catalpa sphinx larvae despite catalpol sequestration by this host.

The effects of sequestered iridoid glycosides on predators are well documented. Checkerspot butterflies that sequester these compounds induce vomiting in birds that eat them (Bowers, 1980, 1981), and invertebrate predators will reject or perform poorly when offered caterpillars sequestering iridoid glycosides (de la Fuente et al., 1994/1995; Dyer and Bowers, 1996; Camara, 1997; Strohmeyer et al., 1998; Theodoratus and Bowers, 1999; Rayor and Munson, 2002). There is mixed evidence that sequestered iridoids harm parasitoids. The specialist braconid, *Cotesia melitaearum,* grew faster when developing in caterpillar hosts (*Melitaea cinxia*, Nymphalidae) with higher levels of iridoid glycosides, and development of the generalist ichneumonid, *Hyposoter horticula,* was not affected by levels of iridoid glycosides in the host (Harvey et al., 2005). In contrast, a field survey found that *M. cinxia* feeding on *Plantago lanceolata* (Plantaginaceae) plants that were low in iridoids were more likely to be parasitized than larvae feeding on high iridoid glycoside containing plants (Nieminen et al., 2003).

In this study, we tested one assumption of the nasty host hypothesis by examining the effects of catalpol sequestration by catalpa sphinx larvae on the performance of its parasitoid *Cotesia congregata*. We examined the leaf chemistry of several populations of catalpa trees across the Eastern United States, and tested for correlations with sequestered iridoids in catalpa sphinx larvae from those populations. We then determined whether different levels of average sequestration at a site were related to differences in parasitism success and performance of *C. congregata*. In this system, one assumption based on the nasty host hypothesis is that increased catalpol sequestration is associated with decreased parasitoid success.

Methods and Materials

Collections Sixty-seven separate sites with *Catalpa bignonioides* were located and surveyed throughout the Eastern U.S., ranging from Southern New Jersey to Western North Carolina, from 14–19 August, 2007. Most stands were individuals or small groups of trees in residential or public lots. Six of the stands were attacked by catalpa sphinx larvae, and catalpa sphinx larvae parasitized by *Cotesia congregata* (as determined by the presence of emerged cocoons) were found at all locations except for Cape May Co., New Jersey (Table 1).

Each population was sampled according to the following protocol. All catalpa sphinx larvae within reach (~2.8 m) were removed from trees along with the leaves upon which they fed. Larvae were a combination of 3rd, 4th, and 5th instars (~20%, 64%, and 16%, respectively) during these collections. Leaves and larvae were placed in 1 l plastic Ziploc® boxes and stored in a cooler until they could be processed in the laboratory at the University of Colorado, Boulder. Additional catalpa sphinx larvae were shipped in late August from a

Table 1 Sites with *Catalpa bignonioides* from which catalpa sphinx (*Ceratomia catalpae*) larvae were collected

	Site	Latitude	Longitude	Trees	*C. congregata*
1	Cumberland Co., VA	37°42′44.38″	78°10′43.14″	~30	Present
2	Johnston Co., NC	35°41′18.24″	78°23′53.07″	10	Present
3	Patrick Co., VA	36°37′37.37″	80°07′40.92″	1	Present
4	Botetourt Co., VA	37°37′01.84″	79°58′09.69″	3	Present
5	Adams Co., PA	39°47′05.63″	77°16′04.47″	10	Present
6	Cape May Co., NJ	39°14′39.43″	74°50′58.30″	15	Absent

collection made at the Clemson University Experimental Forest (Pickens Co., South Carolina).

Plant and Insect Chemistry Upon arrival in Colorado, a subset of five to ten unparasitized larvae from each population were starved for 8 h, weighed, and frozen at −80°C for chemical analysis with gas chromatography [extraction protocol and instrument setup followed those described previously by Bowers (2003)]. The remaining larvae were removed from leaves, placed in a growth chamber set to 25°C with a 16:8 h, L:D photoperiod, and maintained on washed *C. bignonioides* leaves collected from the University of Colorado campus until either pupation or parasitoid emergence. The damaged leaves on which caterpillars had been collected were washed with distilled water to remove caterpillar frass, pooled by population, oven-dried at 50°C, then ground into a fine powder for chemical analysis. Leaves from the Pickens Co., South Carolina, population arrived almost entirely eaten and were not included in the chemical analysis. We extracted iridoids from a 50 mg subsample of the leaf powder from each population and quantified catalpol and catalposide. Iridoid extraction methods and instrument setup were the same for leaves and caterpillars.

Leaf iridoid glycoside amounts (mg) were divided by the weight of the extracted sample to obtain a percentage dry weight of catalpol and catalposide for each population. Because leaf samples were pooled, we could not statistically compare the iridoid glycoside concentrations of leaves among populations. To estimate caterpillar iridoid glycoside concentrations on a dry weight basis, we used a conversion factor (D.W./F.W.= 0.1193, r^2>0.98) obtained from a separate set of 30 starved 4th and 5th-instar *Manduca sexta* larvae that were killed, weighed, dried, and weighed again. We applied this conversion factor to the wet weight of the catalpa sphinx larvae to calculate larval dry weight to allow direct comparisons with leaf iridoid concentrations, We did not have sufficient numbers of catalpa sphinx larvae to obtain fresh-dry weight conversions from this species. Larval catalpol concentration was compared among six populations using analysis of variance (ANOVA) (SPSS version 9.0). Populations were treated as a random factor. We then used linear regression to determine whether sequestered iridoids were dependent on leaf chemistry.

Parasitism by Cotesia congregata *and Caterpillar Sequestration-Parasitoid Relationships*. When *C. congregata* cocoons appeared on the remaining larvae, larvae were isolated in individual plastic cups to allow parasitoid adults to develop. Any adult *C. congregata* were anesthetized and removed, while any hyperparasitoids of *C. congregata* were killed and preserved for voucher specimens. After all *C. congregata* and their hyperparasitoids had emerged, catalpa sphinx larvae were killed by freezing and then dissected to examine them for the presence of parasitoid larvae. Any cocoons that did not yield adults were dissected to identify the occupant (*C. congregata* or hyperparasitoid). We added the total number of dead *C. congregata* larvae inside catalpa sphinx hosts to the number of cocoons to calculate total parasitoid clutch size, and divided the number of *Cotesia* cocoons by total clutch size to determine the proportion that survived until pupation. Average total clutch size and arcsine-square root transformed mortality were compared among populations, treating population as a random factor, using 1-way ANOVA. Parasitism level was calculated as the number of parasitized catalpa sphinx divided by the total number of larvae collected at each site and compared among populations using a χ^2 test.

Results

Insect Collections and Parasitoid Success We collected over 500 individual catalpa sphinx larvae from seven populations. Trees typically were heavily attacked by dozens to hundreds of larvae, often to the point of defoliation. Approximately one-third of the larvae collected were parasitized by *C. congregata*, which were absent only at the Cape May Co., New Jersey site.

Levels of parasitism by *C. congregata* varied five-fold among populations, ranging from 15% to 80% (χ^2= 182.51, P<0.001) (Fig. 1). The average total parasitoid clutch size (adults + larvae dead inside catalpa sphinx host) among all sites was 24.52±1.69 individuals per

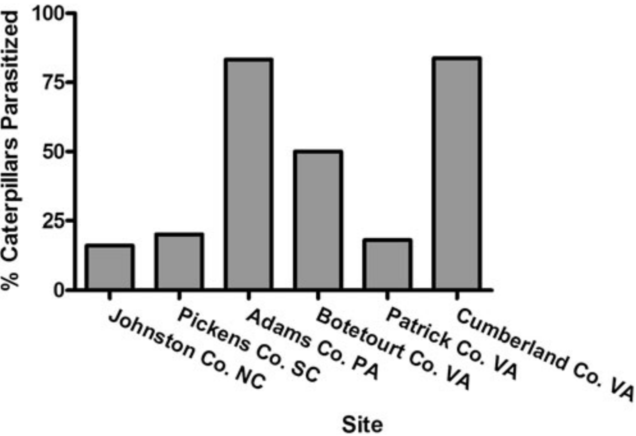


Fig. 1 *Cotesia congregata* parasitism levels for six populations of catalpa sphinx larvae (*Ceratomia catalpae*)

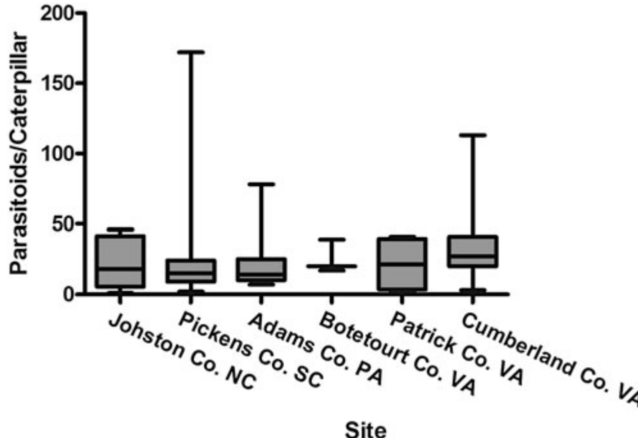


Fig. 2 Comparison of *Cotesia congregata* total clutch size among populations. *Lines* within boxes represent medians, *box* margins represent 25th and 75th percentiles, and *whiskers* represent the extreme low and high values

brood with a median of 21, which did not differ significantly among the surveyed populations ($F_{5,164}$= 1.79, P=0.12) (Fig. 2). Clutch size varied widely from 1 to 172. Only 11 broods contained over 100 individuals. Within-brood survivorship was over 90% for all populations, and it varied significantly among populations ($F_{5,164}$=3.33, P=0.007). This result was driven by the results from the Cumberland Co., Virginia, population (the only population to have catalpa sphinx larvae that contained over ten dead parasitoid larvae); if this population is excluded from analysis, parasitoid survivorship to pupation did not significantly vary among the other populations ($F_{4,82}$=1.35, P=0.26).

Hyperparasitoids were found only in *C. congregata* from parasitized catalpa sphinx larvae collected from the Adams Co., Pennsylvania, and Cumberland Co., Virginia sites. At least one hyperparasitoid individual emerged from 16% of *Cotesia* broods from the Adams Co. population and 6% of those from the Cumberland Co. population. Hyperparasitoid species included those that attacked larval stages [e.g., *Mesochorus* sp. (Ichneumonidae) (Baur and Yeargan, 1994)] and pupal stages (e.g., *Hypopteromalus* (Pteromalidae) (Gaines and Kok, 1999) of *Cotesia*.

Plant and Caterpillar Chemistry Iridoid glycoside concentrations in catalpa leaves varied over 3-fold, ranging from 3–10% dry weight depending on the population (Fig. 3). Leaves contained much higher concentrations of catalposide than its precursor catalpol (Fig. 3). Caterpillar iridoid glycoside concentrations also varied among populations ($F_{5,29}$=5.63, P=0.001), ranging from 6.5% to 22.5% dry weight catalpol (mean: 13.04%±0.87). Larvae from populations with higher concentrations of leaf iridoids tended to sequester higher concentrations of catalpol, although this was only marginally significant (r^2=0.20, P=0.09; caterpillar catalpol = 0.55*leaf iridoids + 8.63) (Fig. 4a).

Caterpillar Chemistry and Parasitoid Fitness Responses - There was a weak positive but not significant relationship between sequestration and total clutch size of *C. congregata* in each population (r^2=0.04, P=0.09) (Fig. 4b). Within-brood survivorship was over 90% for all broods and decreased as caterpillar sequestration increased (r^2= 0.29, P=0.03), when all broods were considered (Fig. 4c). Again, this relationship was driven by results from the Cumberland Co., Virginia population. If this population is excluded, there is no negative relationship between brood survivorship and sequestration (r^2=0.85, P= 0.07).

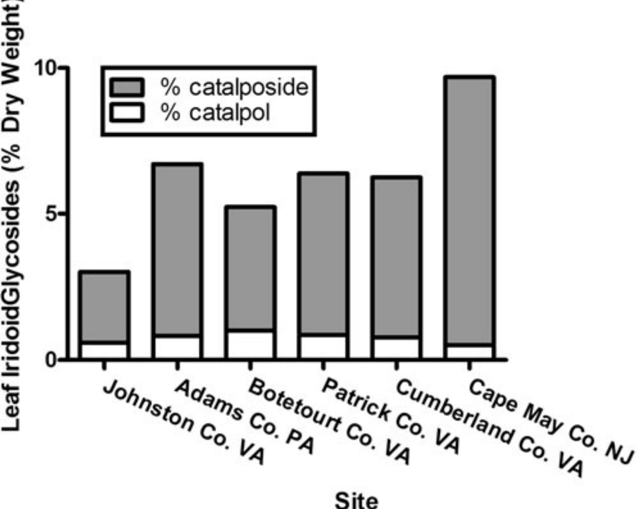


Fig. 3 Iridoid glycoside content of *Catalpa bignonioides* leaves from five sites in the Eastern U.S. *Bars* represent amounts detected in samples pooled from one to ten trees

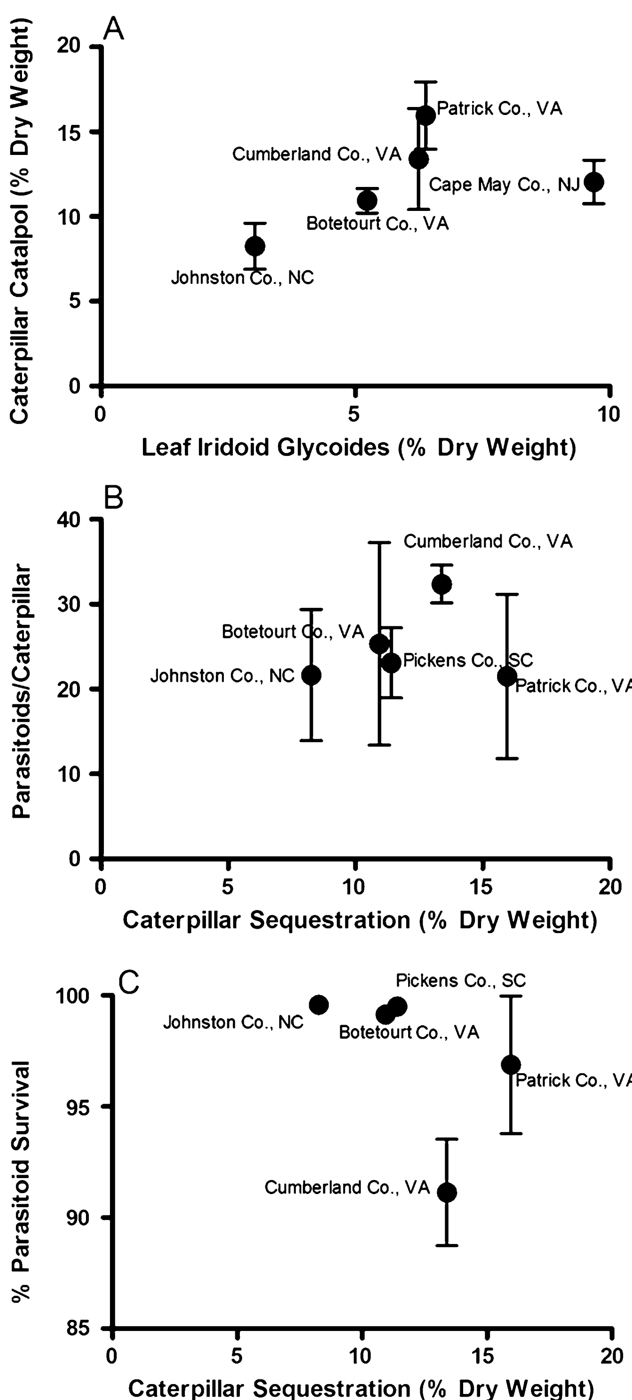
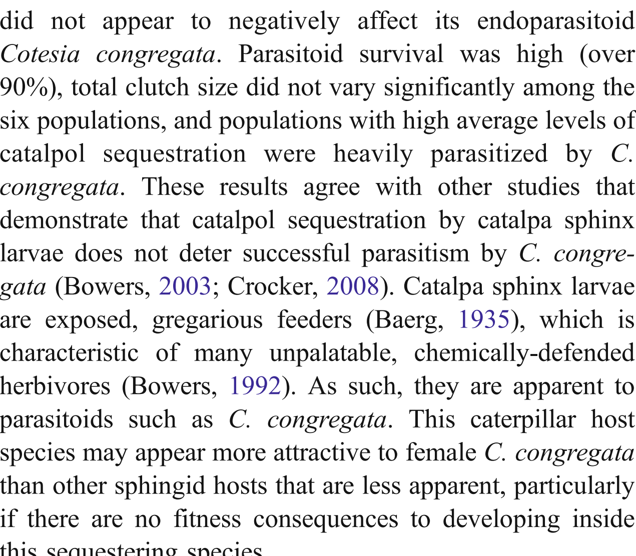


Fig. 4 Relationship between **a** *Catalpa* iridoid glycosides and catalpol sequestration by catalpa sphinx (*Ceratomia catalpae*). **b** *Catalpa* iridoid glycosides and *Cotesia congregata* total clutch size, and **c** *Catalpa* iridoid glycosides and *Cotesia congregata* percentage of larvae surviving to adulthood

Discussion

The nasty host hypothesis assumes a deleterious effect of host sequestered plant toxins on insect parasitoids (Gauld et al., 1992, 1994). Catalpa sphinx catalpol sequestration



did not appear to negatively affect its endoparasitoid *Cotesia congregata*. Parasitoid survival was high (over 90%), total clutch size did not vary significantly among the six populations, and populations with high average levels of catalpol sequestration were heavily parasitized by *C. congregata*. These results agree with other studies that demonstrate that catalpol sequestration by catalpa sphinx larvae does not deter successful parasitism by *C. congregata* (Bowers, 2003; Crocker, 2008). Catalpa sphinx larvae are exposed, gregarious feeders (Baerg, 1935), which is characteristic of many unpalatable, chemically-defended herbivores (Bowers, 1992). As such, they are apparent to parasitoids such as *C. congregata*. This caterpillar host species may appear more attractive to female *C. congregata* than other sphingid hosts that are less apparent, particularly if there are no fitness consequences to developing inside this sequestering species.

Our data do not support the component of the nasty host hypothesis that posits that chemically defended hosts are unsuitable to parasitoids (Gauld et al., 1992, 1994). Instead, parasitism levels and clutch size tended to increase in populations of caterpillars that were more chemically defended. There was an overall decrease in larval *C. congregata* survival as average caterpillar chemistry increased; however, the significance of this effect was heavily influenced by seven out of 83 parasitoid broods collected from a single population. Parasitoid survival was not negatively affected by chemistry if these broods, which were the only broods out of almost 200 with 10 or more dead larvae, were not considered.

A potential explanation for our results is that sequestering herbivores are “safe havens” for developing endoparasitoids (Dyer and Gentry, 1999; Gentry and Dyer, 2002; Smilanich et al., 2009). According to this hypothesis, parasitoids that develop inside chemically defended hosts receive fitness benefits in that they are protected from their own natural enemies, including hyperparasitoids and predators of the host. This hypothesis is supported by studies in which parasitoids perform better when reared from more toxic individuals of a single host species (Zvereva and Rank, 2003; Harvey et al., 2005). Large scale surveys of parasitoid incidence have shown that chemically defended insects typically have high levels of parasitism in the field (Cornell and Hawkins, 1995; Gentry and Dyer, 2002).

Sequestering catalpa sphinx larvae provide a safe haven for developing *C. congregata* parasitoids if these sequestering caterpillar hosts are protected from natural enemies such as caterpillar predators and hyperparasitoids of *C. congregata*. The importance of catalpol sequestration as a predator deterrent for both catalpa sphinx and emerged *C. congregata* pupae remains unknown. Catalpol is a deterrent to ants (Dyer, 1995; Dyer and Bowers, 1996), as well as to

a variety of other predators (de la Fuente et al., 1994/1995; Theodoratus and Bowers, 1999; Rayor and Munson, 2002). Ants are major antagonists of catalpa sphinx larvae in the Eastern US and can repel larvae from entire trees (Ness, 2003a, b). Ants may act as an important selective agent for catalpol sequestration, and high catalpol levels may allow catalpa sphinx larvae to avoid ant harassment.

The presence of hyperparasitoids would suggest catalpa sphinx larvae may not be completely safe havens for primary parasitoids. Hyperparasitoids successfully developed from *C. congregata* cocoons in two different populations. Catalpol is sequestered by *C. congregata* (Bowers, 2003). It is currently unknown whether hyperparasitoid development is adversely affected by catalpol sequestered by *C. congregata*, and how such fourth trophic level effects might very among different hyperparasitoid taxa. In another study, *Mesochorus* sp. (Ichneumonidae) (*Mesochorus* was the most common hyperparasitoid genus reared in this study) adults contained small amounts of catalpol when reared from parasitoids of sequestering checkerspot caterpillars, thus indicating that hyperparasitoids do consume catalpol (Reudler Talsma, 2007). If hyperparasitoids are unaffected by the relatively low levels of iridoid glycosides sequestered by *C. congregata,* then the warningly colored catalpa sphinx larvae could provide a strong visual cue for hyperparasitoid host location.

Although our results did not reveal any strong negative relationships between caterpillar chemical defense and parasitoid success, future study is needed to determine whether catalpol might be intrinsically toxic to developing *C. congregata* larvae or any other parasitoid species. Catalpol acts as a feeding deterrent to non-adapted insects (Bowers and Puttick, 1988), and *C. congregata* larvae may lack catalpol-sensitive gustatory receptors. However, there are also post-consumption toxic effects of catalpol on several herbivore species (Bowers and Puttick, 1988, 1989). Catalpol exposure and consumption may have more subtle effects on *C. congregata,* such as prolonged development time; measuring this was not possible with host larvae parasitized in the field.

Further study is necessary to determine the ecological importance of the safe haven hypothesis. In particular, field studies that closely examine the palatability of chemically defended host species to generalist predators are needed to evaluate whether their parasitoids are less prone to attack by predators. In addition, the potential of hyperparasitism deterrence by developing inside chemically defended hosts deserves consideration. Controlled lab experiments that manipulate dietary catalpol and catalpol sequestration of parasitized catalpa sphinx larvae would provide further evidence that *C. congregata* are not negatively affected by catalpol sequestration. Finally, the role of chemical defense in determining parasitoid host choice deserves future study. The safe haven hypothesis predicts that parasitoids may prefer unpalatable hosts, and this preference for chemically defended hosts would provide strong support for the adaptive use of hosts unpalatable to natural enemies.

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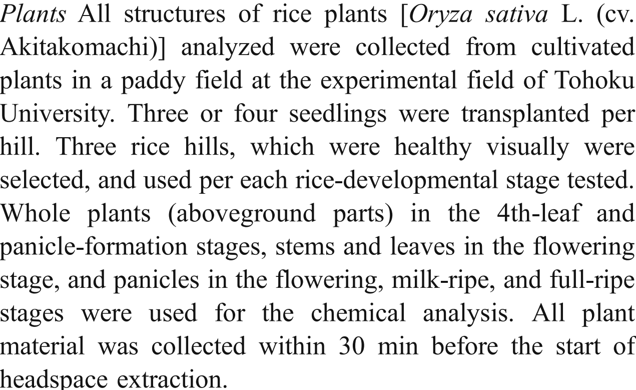
generation hatches and disperses to dominant gramineous weeds such as *P. annua* in the spring (Higuchi et al., 2001; Kikuchi and Kobayashi, 2003). Initially, they reproduce mainly on gramineous weeds such as *P. annua* and *L. multiflorum*. The invasion of adults into rice paddy fields gradually increases with the heading of the rice and rapidly increases with flowering (Okuyama, 1974; Hachiya, 1999; Ishimoto, 2004). Afterwards, the invasion ceases abruptly. In autumn, the bugs disperse to gramineous weeds such as *D. ciliaris*, where they lay diapausing eggs (Higuchi et al., 2001). However, the connection between the invasion of the bugs into the paddy fields and the flowering of the rice has been unclear for many years.

Many phytophagous insects, including mirid bugs, use host plant odors as olfactory cues to find hosts (Visser, 1986; Groot et al., 2003; Blackmer et al., 2004; Hori, 1999; Hori et al., 2006). In previous studies, we investigated the behavioral responses of rice leaf bugs to rice plant odors with a linear track olfactometer. The attractiveness of plants to bugs differed with the rice growth stage and with the part of the plant. Although *T. caelestialium* adults were attracted to the odors of whole rice plants in the panicle-formation stage and to panicles in the flowering stage, they were not attracted to other structures tested (Niiyama et al., 2007). In addition, the host odor preferences of the bugs changed between rice plants and gramineous weeds according to the developmental stage of the rice (Fujii et al., 2010). Bugs tended to prefer flowering rice panicles to gramineous weeds, whereas they preferred gramineous weeds to rice plants before the rice flowering stage. After the flowering stage, they showed no preference between rice plants and weeds. Therefore, we hypothesized that the volatile compounds released by rice change during development, and that flowering rice panicles emit compounds attractive to the bugs. In this study, we analyzed rice plant volatiles from various developmental stages, and we investigated the attractiveness of a characteristic compound emitted from flowering panicles.

Methods and Materials

Insects Rice leaf bugs were collected from a field of Italian ryegrass (*Lolium multiflorum*) and a grassland in experimental fields of the Agricultural Experiment Stations of Akita Prefecture and Tohoku University, Japan. The bugs were maintained on seedlings of wheat (*Triticum aestivum* L.) in an acrylic cage at 25±1°C, and were kept under a reversed photoperiod (8D:16L). The olfactory response of the rice leaf bug is not strongly influenced by mating experience or adult age (Niiyama et al., 2007). Therefore, 2- to 10-d-old adult mated males and females were used for all trials.


Plants All structures of rice plants [*Oryza sativa* L. (cv. Akitakomachi)] analyzed were collected from cultivated plants in a paddy field at the experimental field of Tohoku University. Three or four seedlings were transplanted per hill. Three rice hills, which were healthy visually were selected, and used per each rice-developmental stage tested. Whole plants (aboveground parts) in the 4th-leaf and panicle-formation stages, stems and leaves in the flowering stage, and panicles in the flowering, milk-ripe, and full-ripe stages were used for the chemical analysis. All plant material was collected within 30 min before the start of headspace extraction.

Standard Chemicals β-Caryophyllene (purity: >90.0%), (−)-limonene, α-humulene, and farnesene were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 2-Heptanone, 3-octanone, 6-methyl-5-heptene-2-one, (*Z*)-3-hexenyl acetate, methyl benzoate, linalool, methyl salicylate, *n*-dodecene, *n*-dodecane, *n*-decanal, *n*-tridecene, *n*-undecanal, *n*-undecanol, and geranyl acetone were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). (*Z*)-3-Hexenol, α-copaene, and (*Z*)-thujopsene were purchased from Sigma-Aldrich Co. (St. Louis, Mo., USA). (*Z*)-3-Hexenal was supplied by Zeon Co. (Tokyo, Japan). β-Elemene was supplied by Nippon Terpene Chemicals Inc. (Kobe, Hyogo, Japan).

Extraction of Headspace Components Headspace components of rice plants were extracted with solid-phase microextraction (SPME) and analyzed by gas chromatography-mass spectrometry (GC-MS). Headspace components were analyzed three times. Each replication was done with a new set of plant materials from different rice hills. Fresh plant material (5 g) from a rice hill was gently curled and/or folded, and placed into a sample vial (40 ml) with a Mininert® valve (As One Corp., Osaka, Japan). Headspace of the plant materials in the vials was extracted via SPME (100 μm polydimethylsiloxane; Supelco, Bellefonte, PA, USA) at 22±1°C for 30 min.

Chemical Analysis by GC-MS Headspace components extracted with SPME were subjected immediately to GC-MS. They were identified by comparing gas chromatograph (GC) retention times and mass spectra with those of the authentic standards. Compounds that were not commercially available were identified with a National Institute of Standards and Technology (NIST) mass spectral database and a mass spectral data book by Adams (2001). GC-MS was conducted with a Shimadzu GCMS-QP2010 equipped with a DB-5MS column (30 m×0.25 mm inner diam, 0.25 μm film thickness, J&W). Mass data were analyzed by a Shimadzu GCMS Solution with a NIST mass spectral database. Helium was used as carrier gas at a column head pressure of 100 kPa. The GC was set for

splitless injection (splitter opened after 1 min). The temperature program of the column oven was as follows: isotherm for 5 min at 35°C, 4°C/min gradient to 200°C, 30°C/min gradient to 300°C, and isotherm for 10 min at 300°C. The injector and detector temperatures were 220°C and 250°C, respectively.

Bioassay The attractiveness of β-caryophyllene was evaluated with a linear track olfactometer modified from an original design (Sakuma and Fukami, 1985; Hori, 1998). It was made of transparent acrylic tubing and steel rods. The rods formed a T-junction at the point where the airstreams carrying the treatment and control airflows met from two side arms in the olfactometer. At the T-junction, the bugs chose the direction in which to proceed. A rubber septum (Precision Seal®, white, for 7-mm outer diam glass tubing, Sigma-Aldrich Co., St. Louis, Mo., USA) impregnated with 100 μl of a hexane solution of β-caryophyllene [concentrations (*v/v*%): 0.0001, 0.001, 0.01, 0.1, or 10%] was placed on the treatment side, whereas a septum impregnated with only hexane was placed on the control side. An airflow filtered through activated charcoal was sucked out by pump at a rate of 1.0 l/min regulated by an airflow meter. Ten bugs were placed in a pot installed at the base of the central vertical tube of the olfactometer. It was difficult to observe the orientation behavior of bugs under the light conditions because bugs were strongly influenced by light. We had previously confirmed that bugs fed similarly in the dark and under lights. Therefore, all bioassays were conducted in a dark room kept at 25±1°C to remove the influence of light. The numbers of bugs in the traps of the treatment and control sides were counted 2 hr after the start of the assay. In preliminary tests, approximately equal numbers of bugs entered each side trap when both side chambers were empty. Each comparison was replicated 12 times, and the treatment and control sides were alternated after half of the replications had been completed. The bugs were used once only.

Quantification of β-Caryophyllene Emitted from Rice Plants Quantities of β-caryophyllene emitted from rice plants were calculated by GC-MS as described above. The peak area of the total ion chromatogram of β-caryophyllene was measured, and the concentration was calculated using a calibration curve. The calibration curve was obtained from the peak area of an authentic standard of β-caryophyllene emitted from a rubber septum treated with it. The rubber septum treated with 100 μl of a hexane solution of β-caryophyllene was placed into a sample vial (40 ml) with a Mininert® valve, and its headspace was extracted by SPME (100 μm polydimethylsiloxane) at 22±1°C for 30 min. From the calculation result, it was shown that the concentration of β-caryophyllene in the headspace of flowering rice panicles (5 g) was equivalent to that of a rubber septum treated with 100 μl of 0.0012% β-caryophyllene.

Statistical Analysis The numbers of bugs in the traps of the treatment and control groups were analyzed by a Wilcoxon matched-pairs signed-ranks test. The attractiveness of β-caryophyllene to the bug was estimated by an excess proportion index (*EPI*) according to the formula:

$$EPI = (nt - nc)/(nt + nc),$$

where *nt* and *nc* represent the total number of bugs in the treatment and control traps, respectively (Sakuma and Fukami, 1985). Differences in relative contents of each volatile compound among different rice developmental stages and plant parts were analyzed by a Games-Howell test.

Results

Volatile Components of Rice Plants The volatile composition of rice plants changed according to developmental stage (Table 1). In addition, the volatile compositions of plants in the flowering stage differed between the panicles and the stems and leaves. The relative content of geranyl acetone was high in all rice plant structures analyzed. However, the relative geranyl acetone contents of whole plants in the panicle-formation stage and flowering panicles were relatively lower than those of the others, showing significant differences from that of milk-ripe panicles. Relative content of green leaf volatiles (GLVs) was relatively high in whole plants in the fourth-leaf stage (relative level of (*Z*)-3-hexenol was high, although a significant difference was not obtained) and in panicles in the full-ripe stage (relative levels of (*Z*)-3-hexenal and (*Z*)-3-hexenol were high, although, again, significant differences were not obtained), whereas relative GLV levels in the other rice plant structures were low. In contrast, relative terpene amounts emitted from whole plants in the panicle-formation stage, and from panicles and stems and leaves in the flowering stage were higher than those of the other rice plant structures. However, terpene composition of stems and leaves in the flowering stage was different from those of whole plants in the panicle-formation stage and panicles in the flowering stage: the relative level of linalool was higher, although a significant difference was not obtained. In addition, the relative level of β-caryophyllene was lower. The composition of terpenes was similar between whole plants in the panicle-formation stage and panicles in the flowering stage (in both cases, the relative levels of β-caryophyllene and β-elemene were high). The relative β-caryophyllene content in flowering panicles was significantly higher than those of other rice structures except for whole plants

Table 1 Volatile components in the headspace of rice plants (CV.Akitakomachi) in different growth stages

Compounds[b]	Retention times	Mean values of relative contents (%)[a] (N=3)					
		Fourth-leaf stage Whole plant	Panicle-formation stage Whole plant	Flowering stage Stems & Leaves	Flowering stage Panicle	Milk-ripe stage Panicle	Full-ripe stage Panicle
(*Z*)-3-Hexenal[c]	5.36	1.92±1.24 a[d]	6.03±1.70 a	4.69±3.94 a	0.39±0.19 a	ND[e] a	24.15±6.40 a
(*Z*)-3-Hexenol[c]	7.90	18.21±14.80 a	1.47±1.47 a	0.07±0.07 a	ND a	ND a	14.45±3.82 a
2-Heptanone[c]	9.30	2.03±0.52 ab	0.33±0.17 a	1.97±1.00 ab	3.68±0.27 b	ND a	ND a
n-Nonane	9.70	0.46±0.03 a	0.07±0.07 a	0.26±0.16 a	0.61±0.13 a	1.34±0.43 a	0.12±0.12 a
3-Octanone[c]	13.66	ND a	ND a	ND a	2.01±0.18 b	ND a	ND a
6-Methyl-5-heptene-2-one[c]	13.69	8.47±0.89 b	0.93±0.29 a	0.51±0.26 a	ND a	0.84±0.84 a	4.90±0.76 ab
(*Z*)-3-Hexenyl acetate[c]	14.62	1.20±0.27 a	0.92±0.44 a	0.22±0.09 a	ND a	ND a	0.25±0.12 a
Limonene[c]	15.40	5.33±0.52 b	1.77±0.42 a	0.85±0.29 a	2.87±1.17 ab	0.35±0.35 a	ND a
2-Nonanone	18.21	0.46±0.31 a	0.40±0.24 a	1.03±1.03 a	ND a	ND a	ND a
Methyl benzoate[c]	18.28	ND a	0.10±0.10 a	ND a	2.37±2.06 a	ND a	ND a
Linalool[c]	18.58	ND a	ND a	7.81±7.81 a	1.88±1.68 a	ND a	ND a
Methyl salicylate[c]	22.17	1.86±0.73 ab	5.27±0.62 b	9.31±1.44 ab	8.42±5.14 ab	ND a	ND a
n-Dodecene[c]	22.21	ND a	ND a	ND a	ND a	4.17±1.24 a	1.50±0.47 a
n-Dodecane[c]	22.50	0.57±0.06 bc	0.46±0.20 ab	0.89±0.78 abc	2.35±0.25 c	ND a	ND a
n-Decanal[c]	22.78	10.69±2.53 ab	1.73±1.73 ab	2.92±2.35 ab	11.09±1.16 b	ND a	ND a
n-Tridecene[c]	25.92	ND a	ND a	0.18±0.09 a	5.10±0.70 a	1.84±0.98 a	0.84±0.22 a
n-Undecanal[c]	26.52	1.88±0.43 a	1.31±0.58 a	0.93±0.64 a	2.00±0.33 a	ND a	2.41±0.39 a
Unidentified	28.50	0.28±0.15 a	2.29±2.29 a	1.85±1.13 a	0.30±0.18 a	ND a	0.08±0.08 a
Unidentified	28.71	ND a	2.63±2.63 a	ND a	ND a	ND a	ND a
α-Copaene[c]	28.79	0.02±0.02 a	1.14±0.21 a	2.91±0.66 a	1.78±0.51 a	5.78±1.72 a	ND a
β-Bourbonene	29.06	0.05±0.05 a	1.41±1.00 a	ND a	ND a	ND a	ND a
β-Elemene[c]	29.28	1.48±0.57 a	10.13±0.66 b	3.27±1.64 ab	7.50±1.29 ab	ND a	ND a
n-Undecanol[c]	29.42	0.40±0.13 a	1.16±0.35 a	4.80±2.82 a	2.25±0.64 a	3.99±1.16 a	1.01±0.14 a
β-Caryophyllene[c]	30.27	4.21±1.52 a	28.91±3.77 ab	5.33±1.67 a	16.22±1.30 b	ND a	ND a
(*Z*)-Thujopsene[c]	30.79	ND a	ND a	3.31±1.68 ab	4.49±0.06 b	9.37±1.08 b	ND a
Geranyl acetone[c]	31.29	33.16±9.44 ab	10.38±4.07 a	25.29±10.77 ab	16.89±3.03 a	59.16±4.87 b	41.37±7.20 ab
α-Humulene[c]	31.47	0.35±0.13 a	2.85±0.49 a	0.60±0.36 a	0.52±0.11 a	ND a	ND a
α-Zingiberene	32.82	ND a	0.58±0.35 a	1.77±0.97 a	0.75±0.34 a	ND a	ND a
α-Farnesene[c]	33.15	ND a	2.36±0.26 b	0.40±0.24 a	ND a	ND a	ND a
δ-Amorphene	33.35	ND a	2.68±0.65 a	2.81±1.41 a	ND a	ND a	ND a
(*E*)-Cadina-1(2),4-diene	33.98	ND a	0.33±0.33 a	2.14±1.11 a	ND a	ND a	ND a

[a] Relative contents ± SE were calculated by the peak area ratio in a total ion chromatogram

[b] The compounds showing the relative contents above 1% for at least one of the growth stages analyzed are listed in this table

[c] Compounds identified by comparison to authentic standards

[d] Values followed by different letters in the same row are significantly different (Games-Howell test, $P<0.05$)

[e] ND = not detected

in the panicle-formation stage. The relative β-elemene content of whole plants in the panicle-formation stage was significantly higher than those of other rice structures except for both plant parts in the flowering stage. In milk-ripe panicles, relative terpene content was decreased, and in full-ripe panicles, terpenes were not detected at all. Statistical analysis showed that relative contents of 2-heptanone, 3-octanone, n-dodecane, n-decanal, and (*Z*)-thujopsene in flowering panicles also were high among rice structures tested. In flowering panicles, the relative tridecene content also was higher than in other rice structures, although, again, a significant difference was not

obtained. In whole plants in the panicle-formation stage, the relative α-farnesene content was significantly higher than those of the other rice structures.

Amounts of β-Caryophyllene Emitted from Rice Plants Amounts of β-caryophyllene from whole plants in the fourth-leaf stage, whole plants in the panicle-formation stage, flowering panicles, and stems and leaves in the flowering stage were equivalent to those from a rubber septum treated with 100 μl of hexane solutions of β-caryophyllene at concentrations of 0.0012, 0.0031, 0.0012, and 0.0015%, respectively. β-Caryophyllene was not detected from the headspaces of the panicles in the milk-ripe and full-ripe stages.

Behavioral Response of the Rice Leaf Bug to Authentic β-Caryophyllene Standard The behavioral response of the rice leaf bug to β-caryophyllene is shown in Fig. 1. Adult females were significantly attracted to β-caryophyllene at a concentration of 0.001% (T=7, Z=2.089, P=0.037). However, females were significantly repelled by β-caryophyllene at a high concentration, such as 10% (T=5, Z=2.0732, P=0.038). In contrast, adult males were neither attracted nor repelled by β-caryophyllene at any concentration tested.

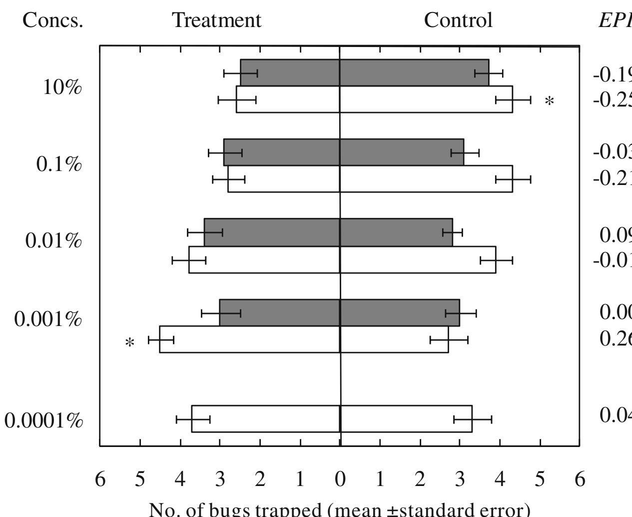


Fig. 1 Behavioral response of the adult rice leaf bug to β-caryophyllene. Gray bars: males; white bars: females. $EPI = (nt - nc)/(nt + nc)$, where nt and nc are the total number of bugs in the treatment and control traps, respectively. Significant difference: * P<0.05 in Wilcoxon matched-pairs signed-rank test (N=12)

Discussion

The invasion of rice leaf bugs into rice paddy fields gradually increases with the heading of rice, and rapidly increases with flowering (Hachiya, 1999; Ishimoto, 2004). In previous studies, we demonstrated that the attractiveness of rice plant odor to bugs differed with the rice developmental stage and the part of the plant (Niiyama et al., 2007). Bugs were attracted to whole plants in the panicle-formation stage and to panicles in the flowering stage. In addition, the preference of the bugs for either rice plant odors or gramineous weed odors changed according to the stage of development (Fujii et al., 2010). Bugs tended to prefer rice plants in the flowering stage to gramineous weeds but preferred gramineous weeds to rice plants in the vegetative stage. Bugs showed no preference between rice plants and gramineous weeds after the milk-ripe stage of the rice. In the present study, rice plant odors contained ubiquitously occurring plant volatiles; the quantitative composition of the volatile blends released differed among the developmental stages or plant parts; Therefore, it is thought that the amounts of active compounds and the ratio of volatile compounds are what is important for the plant's attractiveness to the bugs.

In whole plants in the panicle-formation stage and panicles in the flowering stage, the relative levels of sesquiterpenes—especially, β-caryophyllene—were higher than those in the other rice plant structures. β-Caryophyllene was not detected in the headspace of panicles in the milk-ripe nor full-ripe stages. In the olfactometer test, *T. caelestialium* females were attracted to β-caryophyllene at a concentration of 0.001%. These results suggest that β-caryophyllene is one of the attractants in the rice plant odor that acts as a key compound in the bugs' invasion into paddy fields. However, β-caryophyllene also was detectable in the odor of whole plants in the fourth-leaf stage and in stems and leaves in the flowering stage, which did not exhibit attractiveness to the bugs. Furthermore, the amounts of β-caryophyllene emitted from these rice plant structures were similar (i.e., amounts from whole plants in the fourth-leaf stage, whole plants in the panicle-formation stage, flowering panicles, and stems and leaves in the flowering stage were equivalent to those from a rubber septum treated with β-caryophyllene at concentrations of 0.0012, 0.0031, 0.0012, and 0.0015%, respectively). Thus, it appears that it is the relative content of β-caryophyllene and also the composition of the other compounds that are important for the attractiveness. Volatiles from whole plants in the fourth-leaf stage or from stems and leaves in the flowering stage, may contain compounds that mask the attractiveness of β-caryophyllene and/or compounds that repel the bugs. Rice leaf bugs were attracted only to 0.001% β-caryophyllene and not to the other concentrations tested. This result suggests that amount of β-caryophyllene—as well as the relative content of β-caryophyllene and composition of the other compounds—is important for attractiveness.

The attractiveness of β-caryophyllene to female bugs at a concentration of 0.001% was lower than that of flowering

rice panicle odor (*EPI* by β-caryophyllene: 0.26 [present study]; *EPI* by flowering panicles: 0.48 [previous study by Niiyama et al., 2007]). In some insect species, it has been reported that several host plant volatiles act synergistically as attractants (Nojima et al., 2003; Tasin et al., 2007). Other volatiles of flowering panicles likely act synergistically with β-caryophyllene.

Adult male bugs were not attracted to any tested concentration of β-caryophyllene. In our previous study, although males tended to be attracted to panicles in the flowering stage, this attraction was not significant (Niiyama et al., 2007). The difference in olfactory response to β-caryophyllene between females and males may be one of the causes of the difference in response to flowering rice panicles. However, males are attracted to the odor of whole plants in the panicle-formation stage, as are females (Niiyama et al., 2007). It is thought that rice odor must contain compounds other than β-caryophyllene that are related to male attraction.

Although β-caryophyllene is distributed widely among plant species (e.g., Weissbecker et al., 2000; Asaro et al., 2004; Bertoli et al., 2004; Deng et al., 2004), it has been demonstrated that this compound is used by several insect species as an olfactory cue for host finding. For example, the damson-hop aphid *Phorodon humuli* (Schrank) (Campbell et al., 1993), the western corn rootworm beetle *Diabrotica virgifera virgifera* LeConte (Hammack, 2001), the grapevine moth *Lobesia botrana* Den et Schiff. (Tasin et al., 2006), the boll weevil *Anthonornus grandis* Boheman (Minyard et al., 1969), and the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Khalilova et al., 1998) use this sesquiterpene for host plant location.

Similar to the rice leaf bug, many species of stink bugs cause pecky rice (e.g., Harper et al., 1993; Jahn et al., 2004; Takeuchi et al., 2004) and invade paddy fields after the heading of rice (Nakasuji, 1973; Rashid et al., 2006). Flowering rice panicle odor also may be attractive to other species of rice-ear bugs and may cause their invasions into paddy fields. Hori (2009) has demonstrated that the sorghum plant bug *Stenotus rubrovittatus* (Matsumura) also is attracted to flowering rice panicle odor. The rice bug *Leptocorisa chinensis* Dallas too is attracted to rice plants with flowering panicles (Kainoh et al., 1980). We suspect that β-caryophyllene may play the role of an olfactory cue in finding the suitable developmental stage of rice plants for other species of rice-ear bug as well.

In this study, we analyzed volatile components of cut plant parts that had shown attraction of bugs in our previous studies (Niiyama et al., 2007; Fujii et al., 2010). We extracted volatile constituents efficiently from plants of various developmental stages by SPME. In addition, we compared volatiles between panicles and stems and leaves by using cut plant parts. However, cut plant odors may differ from intact plant odors (e.g., Lou et al., 2005). Therefore, it will be necessary to investigate the volatiles released from intact plants.

Our findings suggest that β-caryophyllene is just one of the attractants in flowering rice panicle odor, which causes the invasion of rice leaf bugs into paddy fields. Because, the attractiveness of β-caryophyllene was lower than that of flowering rice panicle odor, and because there were several other compounds whose relative levels in whole plants in the panicle formation stage and/or panicles in the flowering stage also were higher than those in the other rice plant structures, it is necessary to investigate how these other compounds relate to the attractiveness of flowering rice panicle odor. Attractants released from flowering rice panicles may make it possible to establish new control methods for the bug, including traps.

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elicit specific VOCs that attract belowground natural enemies to the roots (Rasmann et al., 2005). Both aboveground and belowground emitted VOCs can act as indirect defenses, attracting natural enemies that eventually kill the herbivore. Aboveground and belowground induced responses, however, are not mutually independent. For example, signaling hormones induced in one compartment may be transported throughout the plant and trigger systemic responses in the undamaged plant parts as well (Bezemer and van Dam, 2005). Recent studies show that root herbivory alters VOC profiles induced by aboveground herbivores feeding on the same plant, which affects the preference of aboveground parasitic wasps searching for aboveground feeding hosts (Rasmann and Turlings, 2007; Soler et al., 2007). These examples illustrate the importance of belowground induced responses for aboveground multitrophic interactions associated with plants.

When VOC blends contain relatively few compounds or when belowground feeding causes a general repression of VOC emissions—as is the case for maize (Rasmann and Turlings, 2007)—the differences between aboveground and belowground induced plants are relatively easily identified. However, in plant species such as *Nicotiana attenuata* and several Brassicaceous species, which emit complex VOC profiles that contain several hundreds of different individual compounds belonging to various biosynthetic groups, pinpointing shifts in VOC blends is more challenging (van Dam and Poppy, 2008; Bruinsma et al., 2009; Gaquerel et al., 2009). The analytical challenge in detecting shifts in these VOC blends goes beyond detecting a single 'responsible' compound. VOCs, like all metabolites, are produced via intricate biosynthetic networks in which the production of various compounds is interrelated. Another complicating factor is that damage by belowground or aboveground herbivores may cause several VOCs in the profile to change in different directions (Soler et al., 2007; Bruinsma et al., 2009). As 'pure chemicals are rare in nature' and 'real odors are mixtures of volatiles' (Bargmann, 2006), it is seldom that single VOCs are associated with the complete behavioral response of an organism; it is more likely that multiple compounds in the plant-emitted VOC blends serve as cues. Moreover, different compounds in the blend may elicit similar responses, and a single compound may elicit only a behavioral response when offered in the proper background of other plant VOCs (Mumm and Hilker, 2005). Under such conditions, a comprehensive and system-wide approach is needed to identify the biosynthetic shifts that occur in these complex blends, especially when the aim is to correlate multiple changes in VOC blends to binary parasitoid preference tests. Traditional statistical approaches, such as series of ANOVAs on each individual compound, do not provide this comprehensive overview. Therefore, novel bioinformatic approaches based on multivariate data analysis are required to characterize these complex VOC data sets, and link the outcome to ecological data such as preference tests (van Dam and Poppy, 2008).

Multivariate approaches have been used widely in plant metabolomics studies. Only recently are they more commonly implemented for the (unsupervised) analysis of large VOC data sets (Leitner et al., 2008; van Dam and Poppy, 2008; Bruinsma et al., 2009; Gaquerel et al., 2009). Multivariate analyses are tailored to deal with complex data sets that contain variables that are correlated. Interrelated variables are also common to VOC data sets, because they contain groups of VOCs derived from communal biosynthetic pathways, and even from single enzymes producing a range of products (e.g., terpene biosynthetic enzymes; Schnee et al., 2006; Tholl, 2006). Hence, multivariate analyses are more appropriate to extract the biologically relevant information from VOC blends than multiple single ANOVAs, which ignore these internal correlations. Last but not least, multivariate analyses provide a better understanding of the system because they summarize the variation of potentially hundreds of compounds in a limited number of—typically two or three—factors. These consist of 'scores' that are indicative for the compositional difference of VOC blends for each subject (plant), while the relative importance of each VOC in a factor is quantified by model 'loadings' (Jansen et al., 2010). Scores and loadings can be plotted in two-dimensional figures that provide attractive visual support for whether and how different VOC profiles differ from each other.

Two types of multivariate models can be distinguished based on their objective: unsupervised models, of which Principal Component Analysis (PCA) is most widely used, describe all information in the data as well as possible. Different origins of the information (e.g., experimentally induced or stochastic variation) are not distinguished. Supervised methods, on the other hand, focus on *a priori* defined differences between plants, corresponding to treatments imposed by the experiment. Supervised models therefore are more appropriate to distinguish differences between VOC blends emitted by plants that are experimentally induced (Jansen et al., 2010). Partial Least Squares-Discriminant Analysis (PLSDA) is the method that is most widely used to this end in metabolomic analyses (Barker and Rayens, 2003). This model consists of a 'prediction' of whether each plant was treated or not, and quantifies the importance of each VOC in the separation between treatment groups. This latter quantification is referred to as a Discriminant Function. PLSDA is often applied to dichotomous problems, for example, to compare induced with control plants. However, choice tests in biological experiments often are more complex than that and require

multiple comparisons between treatment groups. This can be solved by defining ‘protected contrasts’ between more than two groups based on experimentally or biologically relevant combinations of the treatments, as is often employed in analysis of variance models (Sokal and Rohlf, 1995). We employed such protected contrasts in our PLSDA model to explicitly correlate consistent shifts in the VOC profiles due to root or shoot induction with the host plant choices of parasitic wasps.

Here, we present a case study to illustrate how the novel combination of PLSDA with protected contrasts can identify biological relevant shifts in complex VOC blends. The aim of the analysis is: 1) to identify shifts in VOC blends elicited by shoot feeding herbivores on feral cabbages (*Brassica oleracea*) that were previously induced at the roots or the shoots; and 2) to link these shifts to parasitoid preference data in a statistically sound and visually attractive fashion. For the initial induction of the cabbage plants, we used jasmonic acid (JA), an endogenous plant hormone that is part of the signaling cascade induced by chewing insects and biotrophic leaf pathogens (De Vos et al., 2005). External JA application triggers various herbivore-induced responses in *Brassica* spp. including VOC emissions that attract natural enemies to the plant (Loivamäki et al., 2004). Here, we induced the cabbage plants either via the roots or on the shoots. The use of JA as the initial inducer has the advantage that both roots and shoots can be induced quantitatively and in a similar fashion, which is not achievable when using real insect herbivores. For our case study, this experimental advantage was prioritized over the fact that external JA application does not exactly mimic herbivore induced VOC in cabbages (Bruinsma et al., 2009). Three days after JA treatment, we placed ten *Pieris brassicae* or ten *P. rapae* caterpillars on the leaves of each plant to analyze the effects on JA pretreatment on aboveground VOCs induced by natural herbivore damage. Both caterpillar species are known to induce the production of similar groups of VOCs in various cabbage species, but differ in the amount of damage they do (Geervliet et al., 1997; Smid et al., 2002). Plants with caterpillars were presented in pairs or triplets to female *Cotesia glomerata*, a gregarious parasitoid wasp that frequently parasitizes both *Pieris* species in the field (Brodeur and Vet, 1995; Geervliet et al., 2000). Similarly treated plants with caterpillars were used to collect the dynamic headspace of control, root JA-induced and shoot JA-induced plants on direct thermodesorption traps. After GC-MS analyses of the VOC profiles, we used the experimental design plus the hierarchical preferences of the parasitoids to develop a PLSDA model combined with contrast analyses to reveal which shifts in the VOC blends may explain the wasps' behavior.

Methods and Materials

Plant Growth and Induction Feral *Brassica oleracea* L. (Brassicacea) seeds from a batch that was originally collected from several individuals in a wild population near Heteren (see van Dam et al., 2004) and propagated for one generation in the communal garden at the NIOO (open pollination), were germinated on glass beads. After 1 week, the seedlings were transferred to 1.3 l pots filled with a sand-peat mixture. Plants were watered with tap water as needed. When plants had six true leaves, they were assigned to the following treatment groups: shoot jasmonic acid induction (SJA); root JA induction (RJA); or control (CON). Plants in the SJA group received 500 μg jasmonic acid (JA; Sigma, St Louis, IL, USA) in a 0.1% Triton X-100 solution on the oldest two leaves, and the RJA group received 500 μg JA in 10 ml 0.1% Triton solution injected in the soil near the roots. CON plants and untreated organs of the JA-induced plants were treated with similar volumes of acidic solution (pH=3.7 with HCl; 0.1% Triton) to control for acid induced responses (see van Dam et al., 2004 for a detailed description of the procedure). This amount of JA has been shown to increase leaf glucosinolates 3–7 day after application (Qiu et al., 2009). The control plants received a solution with a similar pH to control for induction effects due to the acidity of the JA solution.

Parasitoid Choice Tests Pieris brassicae L. (Lepidoptera; Pieridae; large white) and *P. rapae* L. (Lepidoptera; Pieridae; small white) caterpillars were obtained from a culture maintained on *B. oleracea* cv. *gemmifera* var. Cyrus at the Laboratory of Entomology, Wageningen University, The Netherlands. Three days after the JA treatments, 10 first instar larvae per plant were placed on two untreated leaves. The larvae were allowed to feed for at least 3 h before plants were used in the choice experiment. *Cotesia glomerata* (Hymenoptera; Braconidae) wasps were obtained from parasitized *P. brassicae* larvae reared as above. After emergence from the cocoons, adult wasps were kept in a communal cage with access to honey and water. After 3–6 day, female wasps were isolated from the culture and given a single oviposition experience with a first instar caterpillar presented on a paint brush (Smid et al., 2007). The experienced wasps then were introduced singly via a sliding observation window into a cage (100×70×70 cm) covered with fine mesh gauze on three sides and the roof. Two plants with caterpillars were placed 30–40 cm apart in the back of the cage. When there were three plants, they were placed in a triangular configuration (30 cm apart). Wasps were released individually on the bottom of the cage equidistant to all plants. Per set of plants, 10–15 female wasps were tested for their preference. After five tests, the

positions of the plants were swapped. Individual plants were used for one set only. The preference observations were performed over several days. On each day, at least one replicate of each possible treatment combination was included to control for day-effects. A positive choice was scored as the first landing of the wasp on a plant, after which the wasp was removed immediately. Individual wasps were used only once for a preference test. The preference data were analyzed with replicated G-tests (Sokal and Rohlf, 1995). Within each comparison, each set of plants and the 10–15 females that chose between them, were considered as one replicate in the G-test.

Volatile Trapping and GC-MS Analysis A separate group of plants was treated as above with JA. Three days later, CON, SJA, and RJA groups all received 10 caterpillars on two untreated leaves. The plants were transferred individually to 17 l glass bell jars (41 cm high, 24.5 cm diam) placed on a stainless steel bottom plate in a controlled climate cabinet (21°C, 70% r.h.). At the top, the jars were supplied constantly with pressurized air (200 ml/min; Hoekloos, Rotterdam, The Netherlands) cleaned over a Zero Air generator to remove hydrocarbons (Parker Hannifin Corp, Tewksbury, MA, USA). Volatiles were collected by pulling headspace air over a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) with a vacuum pump. Traps were mounted at shoot level to an outlet on the side of the jar. Flow rates over the traps were set to 100 ml/min using mass flow regulators (Sho rateTM, Brooks Instrument, Hatfield, PA, USA). After 45–60 min, traps were removed, capped, and stored at 4°C till analysis. Four jars with individual mass flow regulators were sampled in parallel. Six to seven full series of 4 plants and two background VOC profiles from an empty jar were sampled between 9:00 and 17:00 h. Thus, we sampled the following numbers of individual plants in each experiment: P. brassicae experiment 15 CON, 14 SJA, 15 RJA, and 6 untreated plants without caterpillars for background trapping; P. rapae experiment: 13 CON, 13 SJA, 13 RJA, and 12 untreated plants without caterpillars.

VOCs were desorbed from the traps using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) at 200°C for 12 min (He flow 30 ml/min) and focused on an internal Tenax cold trap (−10°C). After 1 min of dry purging, trapped VOCs were introduced into the GC-MS (model Trace, ThermoFinnigan, Austin, TX, USA) by heating the cold trap for 3 min to 270°C. Split ratio was set to 1:4, and the column used was a 30 m× 0.32 mm ID RTX-5 Silms, film thickness 0.33 μm (Restek, Bellefonte, PA, USA). Temperature program: from 40°C to 95°C at 3°C/min, then to 165°C at 2°C/min, and finally to 250°C at 15°C/min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 0.4 scan/sec). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2005 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral libraries. Additionally, mass spectra (minimum match factor=85/100) and linear retention indices (LRI, window set to +/−2) of chromatographic peaks were compared with values reported in the literature (Adams, 2007), based on which we compiled a database of over 13.000 literature LRI entries from studies using similar phases as the RTX-5 column (5% phenyl equivalent polysilphenylene-siloxane). Additionally, we obtained reference spectra from several authentic standards (farnesene, limonene, methyljasmonate, methylsalicylate, dimethyldisulfide, dimethyltrisulfide, octanal, nonanal, decanal, cis-3-hexen-1-ol, 2-phenylethylalcohol, indole, benzylcyanide, and phenylethylisothiocyanate; Sigma- Aldrich, St. Louis, Il, USA). The integrated signals generated by the AMDIS software from the MS-chromatograms were used for comparison among the treatments. Peak areas in each sample were divided by the total volume in ml that was sampled over the trap to correct for differences in sampling volume between experiments.

Multivariate Data Analysis of VOC Data Only those VOCs with a peak area larger than the background and found in more than 50% of the samples of either treatment group were selected for multivariate analysis. Thus, in the P. brassicae experiment 116 compounds, and in the P. rapae experiment 136 compounds were statistically analyzed. Prior to analysis, the P. rapae data were transformed as log (x+1) and for both caterpillar spp. the data were unit-variance scaled. We analyzed two contrasts that were of interest: the difference between all JA-treated and control plants (CON vs. (SJA+RJA)), and between RJA and SJA plants. These two ‘protected contrasts’ were modeled using two separate Partial Least Squares Discriminant Analysis (PLSDA) models per caterpillar species (Barker and Rayens, 2003). The first contrast describes the general effect of JA application, regardless of where the JA was applied, by contrasting the VOC profiles emitted by plants in the control group against those emitted by both JA-treated groups (CON vs. (RJA-SJA), lower horizontal axes in Fig. 2a, b). This axis thus indicates which VOCs explain the preference of parasitic wasps for JA-induced plants in general. The second contrast was that between the SJA and RJA plants, thus revealing which VOCs are responsible for the parasitoids’ preference for shoot induced plants. For validation purposes, 1000 models with randomly permuted class labels were fitted alongside every model. The number of latent variables required for both models was determined using cross-validation (Geladi and Kowalski, 1986). To rule

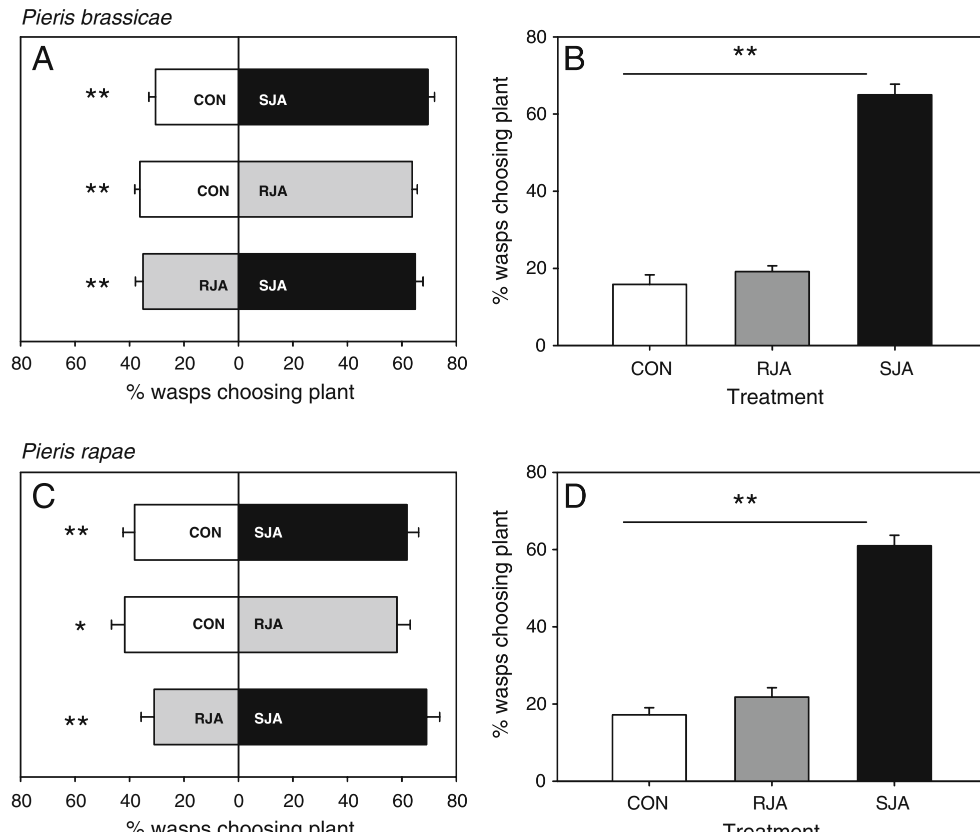


Fig. 1 Preference of *Cotesia glomerata* wasps expressed as the percentage of wasps preferring the plant (±s.e.m. calculated over 8–18 replicate test runs of 10–15 females per run). **a** and **c** Dual-choice tests between plants treated with jasmonic acid (JA) to the roots (RJA), to the shoots (SJA) or with acidic water (CON) 3 d before 10 Pieris brassicae (**a**) or 10 Pieris rapae (**c**) larvae were added. *Asterisks* denote a significant difference in preference between the two plants (* P Gp<0.01, ** P Gp<0.001, see Table 1. **b** and **d)** Triple choice tests between CON, RJA and SJA plants. *Asterisks* denote an overall significance in preference between the three plants (** P Gp<0.001)

out model over-fit, the predictive capacity of each model from the cross-validation was compared to that of permuted models of equal complexity. The permutation test also was used to determine the likelihood of each VOC being involved in the modeled contrasts: this is done by comparing the value of the discriminant function for each VOC to those of the same VOC in the discriminant functions of the randomly permuted data sets (which form a 'null distribution'). This comparison can be translated into a *P*-value for each VOC in each contrast.

Results

In dual choice tests, 5-day old *Cotesia glomerata* females always preferred JA-induced plants over control plants, regardless of where the JA was applied or the *Pieris* species that was used (Fig. 1a, c; Table 1). However, when given the choice, wasps consistently and significantly preferred SJA plants over RJA plants, irrespective of the caterpillar species that was feeding on the leaves (Fig. 1a,c). This strong and significant preference for SJA plants also was evident when plants from the three different treatment groups were presented simultaneously to the wasps (Fig. 1b, d).

In both VOC analyses, the first discriminant axis represents the contrast between the control and both the RJA and SJA plants (Fig. 2a, b, horizontal axes). The importance of each VOC in this contrast is represented by the horizontal position of the compound, and its significance for each contrast is indicated by both point labels. This showed that the increased emission of monoterpenes is shared by RJA and SJA plants (compounds 6–15 in Fig. 2a, b, and Table 2), which contributed highly to the separation of both JA-treated groups from the CON group. The induction of monoterpene emissions by JA-application was consistent for both *Pieris* species (Fig. 3). One VOC decreased after JA application: in the *P. brassicae* experiment, the emissions of dimethyldisulfide (DMDS) were lower in JA-treated plants (compound 5 in Fig. 2a). Significant decreases in DMDS emissions in JA-induced plants were not observed in the *P. rapae* experiment.

Interestingly, we found a clear contrast in VOC profiles between root and shoot JA-induced plants (RJA vs. SJA; vertical axes in Fig. 2). Shoot JA application strongly induced the emission of several sesquiterpenes (compounds number 16–18, 20–23) and a homoterpene (number 19), whereas root induction did not result in elevated emissions of these compounds (Table 2; Fig. 2a, B; Fig. 4a, b). Again, this pattern was similar for both herbivore experiments and was also observed for unidentified sesquiterpenes (Table 2,

Table 1 Goodness-of-fit tests (g-test) of *Cotesia glomerata* preference

Test combination	Replicates[a]	Total wasps[b]	P Gh[c]	P Gp[d]
Caterpillar : *Pieris brassicae*				
CON—SJA	13	191	0.91	**<0.001**
CON—RJA	13	196	0.99	**<0.001**
RJA—SJA	13	198	0.72	**0.001**
CON—RJA-SJA	13	197	0.84	**<0.001**
Caterpillar: *Pieris rapae*				
CON—SJA	16	180	0.09	**<0.001**
CON—RJA	15	171	0.07	**0.017**
RJA—SJA	18	140	**0.003**	**<0.001**
CON-RJA-SJA	8	120	0.98	**<0.001**

a. Number of replicates (plant pairs). b. numbers of wasps tested. c. P Gh indicates whether the replicates are heterogeneous; if P Gh>0.05 the choice of the different sets of wasps has been consistent over the replicates. d. P Gp indicates that, based on the pooled results the null-hypothesis stating that wasps have no preference for one of the treatments may be rejected. P-values<0.05 are in bold

CON control

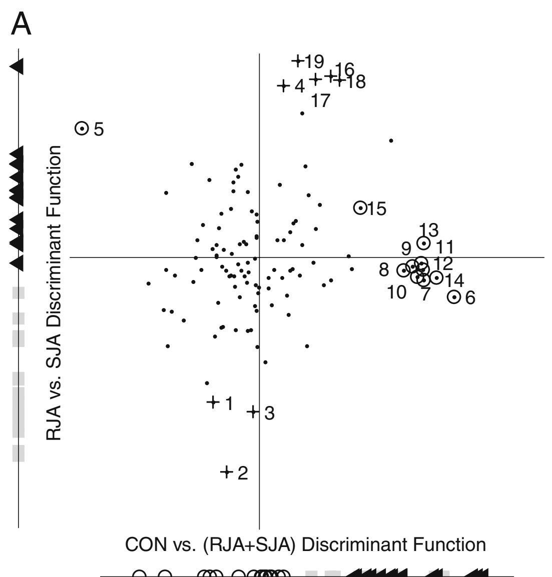
SJA shoot jasmonic acid induction

RJA root jasmonic acid induction

Fig. 4b). The levels of a few compounds were lower after shoot than after root JA treatment; in the *P. brassicae* experiment, several aldehydes (compounds 1–3), and in the *P. rapae* experiment, 2,4-pentadione (compound 24), were more pronounced in control and root induced than in shoot induced plants (Table 2, Fig. 2a, b). In contrast to the differences in terpene emissions, these effects were observed for only one of the *Pieris* species.

Discussion

Based on the results of our combined PLSDA–protected contrast analysis, we could easily identify which VOCs are associated with parasitoid preference. In all choice experiments, *C. glomerata* females strongly preferred aboveground JA-induced plants that had the highest emissions of sesqui- and homoterpenes, even when neighboring belowground induced plants showed equally increased monoterpene levels. This indicates that sesquiterpenes are more attractive cues for this parasitoid species than monoterpenes are. This does not preclude that monoterpenes themselves are attractive as well, but our results show that their attractiveness was context-dependent. The emission of various VOCs from other biosynthetic classes changed after JA application as well, but only the shifts in terpene emissions were consistent for both experiments. Several other studies have shown that plants with enhanced levels of monoterpenes or sesquiterpenes/homoterpenes are more attractive to *C. glomerata* females (van Poecke et al., 2001;



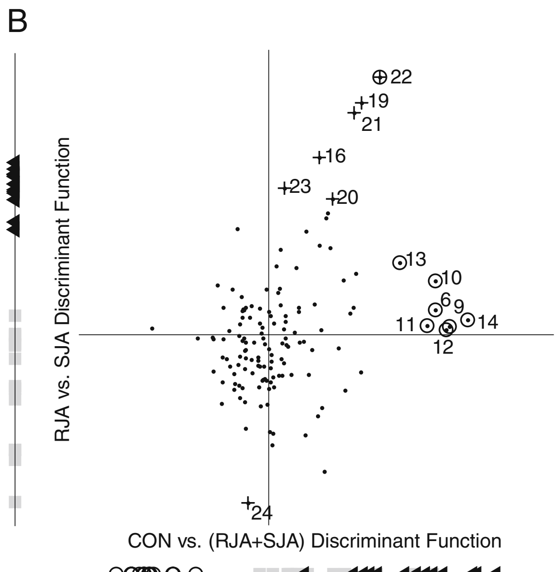


Fig. 2 Two-dimensional PLS-Discriminant fingerprints for VOCs emitted by *Brassica oleracea* plants with **a**. 10 *Pieris brassicae* larvae or **b** 10 *P. rapae* larvae. The separation of individual plants on each discriminant function are given on the *left vertical* (RJA vs. SJA) and *lower horizontal* (CON vs. (RJA+SJA)) *axes* in each figure. Open circles=control plants, grey squares=root-induced (RJA) plants; black riangles=shoot-induced (SJA) plants. The two-dimensional plots in the middle show the contribution of each VOC to the separation. The position of each point is determined by its importance for the contrasts. The numbered VOCs (see Table 1) contributed significantly to the separation of treatment groups (encircled: (CON vs. (RJA+SJA)), crosses: for (RJA vs. SJA). Statistical significance was determined by testing the discriminant function value of a VOC against a null distribution of 1000 models on randomly permuted treatment group assignments ($P<0.05$)

Table 2 Differentially induced volatile organic compounds in the headspace of jasmonic acid induced feral Brassica oleracea plants

No.	Compound name[a]	RI [b]	Reported in [c]	Compound class	*Pieris brassicae*		*Pieris rapae*	
					P(CON-JA)[d]	P(RJA-SJA)	P(CON-JA)	P(RJA-SJA)
1	Nonanal*	1103	A, B	aldehyde	0.2698	**0.0400**	–	–
2	Decanal*	1204	A, B	aldehyde	0.3606	**0.0308**	–	–
3	2-Undecanal	1362	A, B	aldehyde	0.4718	**0.0388**	–	–
4	Hexylacetate	1016	A, B	ester	0.3520	**0.0110**	–	–
5	Dimethyldisulfide*	738	A, B	sulfide	**0.0330**	0.0890	–	–
6	1,8-Cineole	1028	A, B	monoterpene	**0.0024**	0.3500	**0.0098**	0.2982
7	alpha-Pinene	927	A, B	monoterpene	**< 0.001**	0.3238	–	–
8	alpha-Terpinolene	1084	A, B	monoterpene	**0.0172**	0.4698	–	–
9	alpha-Thujene	921	A, B	monoterpene	**< 0.001**	0.4254	**0.0056**	0.4456
10	beta-Myrcene	990	A, B	monoterpene	**< 0.001**	0.3354	**0.0228**	0.2496
11	Limonene*	1027	A, B	monoterpene	**< 0.001**	0.4564	**0.0156**	0.4218
12	Sabinene	967	A, B	monoterpene	**< 0.001**	0.3988	**0.0088**	0.4612
13	(*E*)-Sabinene hydrate	1094	A, B	monoterpene	**0.0156**	0.4602	**0.0292**	0.1934
14	(*Z*)-Sabinene hydrate	1064		monoterpene	**0.0012**	0.3634	**0.0052**	0.3968
15	Monoterpene 1	1024		monoterpene	**0.0418**	0.2138	–	–
16	beta-Elemene	1389	A, B	sesquiterpene	0.1300	**<0.001**	0.2832	**0.0074**
17	Zingiberene	1491		sesquiterpene	0.2270	**0.0068**	–	–
18	Sesquiterpene 1	1500		sesquiterpene	0.1222	**0.0024**	–	–
19	(3*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT)	1117	B	homoterpene	0.2926	**0.004**	0.1232	**0.0024**
20	Sesquiterpene 2	1460		sesquiterpene	–	–	0.2076	**0.0216**
21	Sesquiterpene 3	1464		sesquiterpene	–	–	0.1120	**< 0.001**
22	Sesquiterpene 4	1467		sesquiterpene	–	–	**0.0458**	**< 0.001**
23	Sesquiterpene 5	1469		sesquiterpene	–	–	0.4204	**0.0118**
24	2,4-Pentadione	779		ketone			0.4082	**0.0374**

a. The table contains only those volatile organic compounds (VOCs) that were found to significantly contribute to the separation of the treatment groups. The numbers in the first column refer to the numbers in the contrast plot in Fig. 2a and b. Compounds indicated with a * were identified by comparison of pure standards, the other compounds were identified based on MS reference manuals (Adams, 2007), NIST 2005, Wiley 7th Ed. MS library, and our own MS and LRI literature library. All plants had 10 *Pieris brassicae* or 10 *P. rapae* caterpillars feeding on their shoots at the time of VOC collection. b. RI=retention index on the GC-MS; column RTX5-MS. One monoterpene and five sesquiterpenes could not be sufficiently identified based on their RI value and mass spectrum using our criteria, and were given sequential numbers. c. indicates whether this compound had been reported earlier in various *Brassica* species (B, based on references Bruinsma et al., 2009; Geervliet et al., 1997; Gols et al., 2008; Soler et al., 2007) or *Arabidopsis* (A, based on reference Rohloff and Bones, 2005). d.The P-values were determined by testing the discriminant function value of a VOC against a null distribution of 1000 models on randomly permuted treatment group assignments. Values in bold are $P<0.05$

Mumm et al., 2008). The strength of our approach, however, lies in the fact that we could readily contrast the relative importance of these two terpene classes within the VOC blend and could directly relate differences in VOCs between JA-treatments to parasitoid choice behavior. Our analysis, however, cannot reveal whether one, several, or all of the terpenes together are responsible for the observed preferences. This could be assessed experimentally by presenting isolated compounds—in combination with the appropriate background odors—to parasitoids in choice tests, or by using plants that are genetically modified with terpene synthase genes (Kessler and Baldwin, 2001; Ibrahim et al., 2005; Kappers et al., 2005; Mumm and Hilker, 2005). The latter approach has shown that single terpene synthases generally yield mixtures of similar terpenes within a class (Kappers et al., 2005; Schnee et al., 2006; Tholl, 2006). This is consistent with the induction patterns we saw in our experiments, in which several members of a biosynthetic class responded uniformly to the induction treatments. The absence of a strong selection on terpene synthases to produce a single product, underscores our assumption that plant VOC cues likely consist of 'bouquets' of different VOCs, and that it is the quality of the blend rather than the quantity of a single compound that determines the behavioral response (van Poecke et al., 2001).

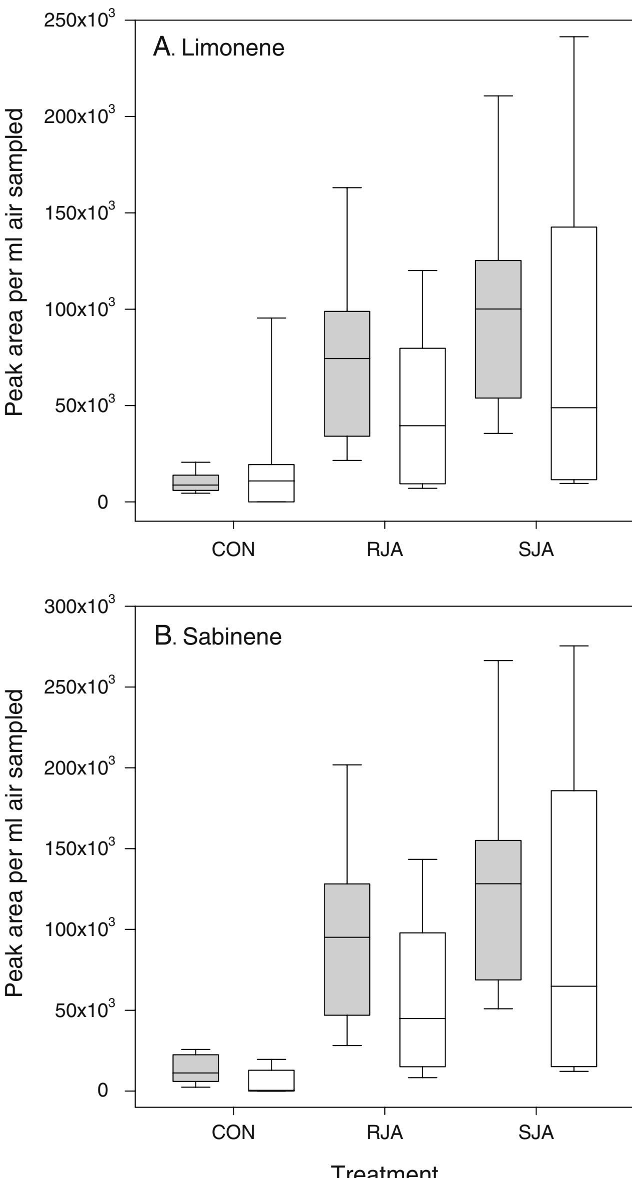


Fig. 3 Box plots showing the median value, the 25th percentile, and 75th percentile of the peak area per ml sampled air for **a** limonene, and **b** sabinene. *Grey bars*: values in Pieris brassicae experiment, *white bars*: values in P. rapae experiment. CON=control plants, RJA=root jasmonic acid (JA) induced plants, SJA=shoot JA induced plants. The *error bars above* and *below the box* indicate the 90th and 10th percentile

As expected, JA application only partly mimicked herbivore-induced responses. Similar to the induced responses caused by root herbivore damage, only shoot induction with JA significantly increased the emission of sesqui- and homoterpenes, whereas root herbivory and root JA application did not (Soler et al., 2007). JA application to the roots, however, did not increase the emissions of sulfides, such as DMDS, which have been found to be induced by cabbage root fly larvae. These sulfides also are of biological relevance, but they play a dual role. It has been suggested that *C. glomareta* wasps use these as cues to avoid root infested plants (Soler et al., 2005), whereas soil dwelling beetle predators of root fly larvae, and parasitoids of *Plutella xylostella*, another leaf feeding cabbage specialist, may use DMDS as a positive cue to locate their host or prey (Ferry et al., 2007; Kugimiya et al., 2010). As JA application did not increase the emission of sulfide compounds in root induced plants, we could not asses the ecological role of this compound in our set-up. For *C.*

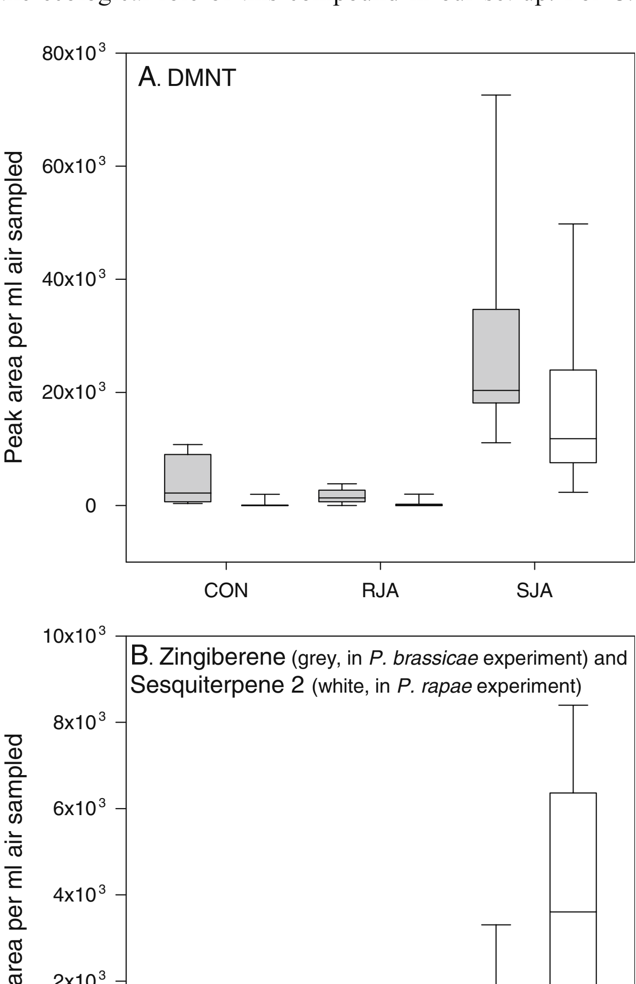


Fig. 4 Box plots showing the median value, the 25th percentile, and 75th percentile of the peak area per ml sampled air for homo-and sesquiterpenes. **a** DMNT.'<3E>-4,8-dimethyl-1,3,7-nonatriene; **b** Zingiberene plus sesquiterpene 2 (zingiberene alike); Legend (DMNT) *Grey bars*: values in Pieris brassicae experiment, *white bars*: values in P. rapae experiment. CON=control plants, RJA=root jasmonic acid (JA)-induced plants, SJA=shoot JA-induced plants. The *error bars above and below the box* indicate the 90th and 10th percentile

glomerata, it is of ecological and evolutionary relevance to assess the difference between root and shoot-infested plants, as root-infested plants yield hosts of inferior quality or her offspring. Wasps emerging from caterpillars on root-infested plants attain lower body masses, which eventually results in a lower fitness (Soler et al., 2005). In an earlier study, we found that parasitoid fitness parameters were reduced significantly on shoot JA-induced plants (Qiu et al., 2009). Yet, we found that shoot JA-induced plants were strongly preferred by female wasps. The discrepancy between parasitoid performance and preference may be explained partly by the fact that JA-induction does not provide the full complement of volatile cues that this parasitoid needs to locate the most suitable hosts.

There is ample evidence that female *C. glomerata* wasps have the ability to detect terpenes and various plant odors with their antennae and can learn to prefer different odors when rewarded (Vet et al., 1995; Smid et al., 2002, 2007). Much less is known about how parasitoids perceive and integrate compositional shifts in complex VOC mixtures, and how they learn to respond to these changes within complex odor plumes (but see Vet and Groenewold, 1990). Learning more about sources of variation in plant odor signals is important for our understanding of the evolution of plant-parasitoid interactions and the role of VOC therein (Vet, 1999; Allison and Hare, 2009; Schuman et al., 2009). The signal-to-noise ratio of the odor profiles determines the reliability of the information, and this may influence the way parasitoids respond to plant cues, innately or through learning. *Vice versa*, the way parasitoids perceive and learn to distinguish between plant VOCs may influence the evolution of plant VOC emissions with regards to the quantitative and qualitative composition of the odor blend in response to herbivore damage (Vet, 1999; Allison and Hare, 2009). Our study demonstrates that parasitoids are able to specifically detect enhanced emissions of sesqui- and homoterpenes against a background of equally increased monoterpene emissions in JA-induced plants. This suggests that the presence of especially sesqui- and homoterpenes in the VOC mixtures may serve as a reliable cue that signals the presence of shoot induction by actively feeding herbivores on a plant that is free from root herbivores.

Unexpectedly, our results also reveal that the terpene synthases of the cytosolic mevalonate (MVA) pathway, leading to the synthesis of sesqui- and homoterpenes (Degenhardt and Gershenzon, 2000; Hopke et al., 1994; Tholl, 2006), are induced by shoot JA application only. In contrast, the biosynthesis of monoterpenes via the methyl-erythritol phosphate (MEP) pathway, which is located in the plastid, is equally elicited by root and shoot JA application. It has been known longer that terpene and sesquiterpene synthases are induced by internal as well as external JA (Ozawa et al., 2000; Tholl, 2006). However, it was hitherto unknown that terpene synthases belonging to these two major biosynthetic pathways may respond differently to JA application, solely depending on where the JA was applied on the plant. It is unlikely that the differential induction of mono- and sesquiterpenes is merely a matter of signal dilution in root induced plants. First, monoterpene emissions were equally enhanced in root and shoot JA-induced plants. Moreover, similar regulatory differences have been found for the induction of glucosinolates, which serve as direct defense compounds in Brassicaceae (Hopkins et al., 2009). Shoot JA induction increased the levels of indole glucosinolates that are derived from tryptophan, whereas root induction triggered the production of the aliphatic glucosinolates (van Dam et al., 2004; Jansen et al., 2009). The latter are produced from methionine via a different biosynthetic and regulatory pathway. Taken together, these results imply that root induced responses are regulated fundamentally different from responses triggered by shoot herbivory. This may provide plants with the option to optimize their inducible defense responses in natural environments where both root and shoot herbivores may interact with the plant (Bezemer and van Dam, 2005). Eventually, the differential expression of both direct and indirect induced defenses after root and shoot herbivory may have profound effects on the performance of above-ground herbivores and parasitoids associated with plants (Masters et al., 2001; Soler et al., 2005; Qiu et al., 2009). In addition to population-based variation in the sensitivity to induction hormones (Schuman et al., 2009), root induction may be yet another level of variation that determines differences in induced VOC profiles between plants.

Here, we have shown how novel bioinformatic analyses successfully bridge the gap between ecologically relevant observations and JA-induced changes in VOC blends. Our supervised PLSDA coupled to protected contrast analysis provided strong statistical support as well as visually appealing information on shifts in chemically complex VOC profiles. It enabled us to assess the relative importance of shifts in different VOC blends and directly link these to the behavior of higher trophic levels associated with these plants. This could not have been achieved with a conventional discriminant analysis that focused only on how each treatment group differed from all other groups. We expect that this method can be applied easily to similar experiments with real herbivores, which may yield novel information regarding the relative importance of specific (groups) of VOCs that are differentially induced by aboveground and belowground induction. The results also show that multivariate analyses do not only provide answers to experimental questions, but also may provide additional insight and generate novel, testable hypotheses about the internal regulation and coordination of above-

ground and belowground induced responses in plants. Bioinformatic analyses, thus, are an invaluable tool to 'see the woods from the trees' that should be applied more frequently to analyze plant VOC blends and other complex chemical data sets.

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and soil fertility (Dixon and Paiva, 1995; Lillo et al., 2008). A number of ecological theories have been formulated to model the plant carbon allocation to phenolic secondary metabolites. The carbon-nutrient balance hypothesis postulates that in nitrogen-limited plants secondary metabolism is directed towards carbon-rich metabolites, such as phenylpropanoids (Coley et al., 1985). That hypothesis and other related theories consider modulation of the synthesis of phenolic secondary metabolites to be an indirect effect of nutrient deficiency, which limits the plants' ability for growth (Tuomi et al., 1990; Jones and Hartley, 1999). Many earlier studies either supported or contradicted this hypothesis, which now appears to be over-simplified. Recent studies have shown that carbon and nitrogen metabolisms are linked by a sophisticated regulatory network (Matt et al., 2002; Fritz et al., 2006). Factors that affect photosynthesis, such as low light intensity can, via a decrease in primary metabolites, provoke a decrease in phenylpropanoids under normal nutrient conditions (Matt et al., 2002). Alternatively, the concentrations of foliar phenolics, particularly flavonoids, may be regulated by a need for photoprotection that depends on the amount of light in excess of the capacity of the photosynthetic enzymes for utilizing light (Close and McArthur, 2002).

The environmental growing conditions in Finland differ considerably from south to north, ranging from southern boreal to northern boreal and subarctic climatic vegetation zones. Consequently, the quality and quantity of phenolics are expected to differ among bilberry leaves growing in different parts of the country. Previous studies have shown that the phenolic content in juniper (*Juniperus communis*) and birch (*Betula pubescens*) leaves increases with latitude (Stark et al., 2008; Martz et al., 2009). However, although the effect of altitude and/or latitude on AC content of bilberry fruit has been previously shown (Lätti et al., 2008; Rieger et al., 2008), no data are available about changes of phenolic composition in bilberry leaves along large geographical gradients. In addition to the latitudinal gradient, the synthesis of phenolics in bilberry leaves may change along the successional stages of the forest. Furthermore, a change in phenolics as leaves develop has been demonstrated previously (Jaakola et al., 2004).

The aim of the present study was to determine the variations in the soluble phenolics in bilberry leaves following leaf development and along environmental gradients. We investigated changes in the biosynthesis and composition of phenolics during development of bilberry leaves collected from plants naturally growing in three sites in northern boreal latitudes (forest, clear cut, and fell). In addition, we conducted a study at 116 sites along a large geographical gradient ranging from south to north boreal latitudes, analyzing the soluble phenolic composition and antioxidant capacity of mature bilberry leaves. Our hypotheses were that: (i) the biosynthesis of leaf-soluble phenolics vary along with leaf development in different growing conditions, and (ii) more environmentally stressful growing conditions such as those found towards higher latitudes promote higher concentrations of leaf-soluble phenolics.

Methods and Material

Study Sites and Collection of Plant Material

Experiment I To investigate changes in composition and biosynthesis of bilberry (*Vaccinium myrtillus* L.) leaf phenolics during leaf development in northern boreal environments, leaves were collected from naturally growing plants on 12 June, 27 June, 17 July, and 5 September 2006 in the Rovaniemi area in northern Finland. Three sites were selected: a clear cut area, (C), which was logged in 2000, an old successional Norway spruce (*Picea abies*) forest (F), only about 50 m from C (66°21′N 26°21′E, 70 m a.s.l.), and a higher altitude fell (T) (Vennivaara, 66°32′N 25°36′ E, 229 m a.s.l.). The fell contained characteristic high-altitude vegetation with sparsely growing old-growth Scots pine (*Pinus sylvestris*) trees. Five plants separated by a minimum of 3 m were selected each time, transported to the laboratory in an icebox, and the leaves were detached immediately, sorted, frozen in liquid nitrogen, and stored at −80°C until phenolic and RNA were extracted. Healthy individuals representing the average development stage at the sampling site were selected.

Experiment II To investigate variation in composition of the leaf phenolics along environmental gradients, samples were collected at 116 study sites that belong to the National Forest Inventory (NFI) network in Finland. The latitudes of these sites range from 59º49′ N to 70°04′ N (about 400 km north of the Arctic Circle). With an average of 143 m, altitudes in Finland generally are low. However, increases in latitude coincide with increases in altitude, and in the northern latitudes, even a small shift in altitude can have great significance for growing conditions. The geobotanical zones range from south boreal forests in the south of Finland to north boreal and subarctic conditions in the northernmost parts. We utilized sites selected for the BioSoil project (Finnish Forest Research Institute), which included

626 sites all belonging to the NFI site network. For this study, we first selected sites with minimum bilberry coverage of 10% in the ground vegetation, and then used a systematic selection that ensured even distribution of sites in different parts of the country. In the north, every second BioSoil site with bilberry coverage of at least 10% was selected, but in the south, every third site was selected. The NFI sites belong to several different forest development classes that correspond to successional stages of the forest. To ensure that the role of latitude or altitude is not influenced by differences in the age structure of sites, we chose only development classes from advanced thinning to mature stands (classes 3–6 in NFI classification), and excluded open clear cuts and forests with young seedlings (mean height of seedlings <1.3 m, classes 0–2 in NFI classification). Phenolic content and composition did not differ among the rest of the forest development classes (classes 3–6) ($P>0.05$, not shown), and thus we did not include the forest class in testing for the effects of latitude and altitude. Correlations between the concentrations of phenolics in bilberry leaves and other site characteristics in NFI site database or the soil nutrient concentrations analyzed in connection with the BioSoil project will later provide further opportunities to investigate correlations between leaf quality and growth conditions, and will be published elsewhere.

Latitudes and altitudes of the selected sites ranged from 60º00′ N to 69°60′ N and from 20 m to 370 m, respectively (Fig. 1a). The predominant tree species most commonly was Norway spruce (*Picea abies*), but in the north, Scots pine (*Pinus sylvestris*) and mountain birch (*Betula pubescens* ssp. *czerepanovii*) also were common. At each site, a composite sample of 20 young and healthy shoots cut from 20 individual phenotypes was collected randomly to exclude from our results the role of genetic variation among bilberry individuals. The sampling period extended from 3 July 2006 to 30 August 2006 (Fig. 1b). For practical travelling reasons, samples collected in Experiment II were not frozen in liquid nitrogen as in Experiment I, but rather were stored in paper bags after harvesting and sent to the laboratory where the branches were weighed and dried at 40°C for 48 h. Leaves then were detached, milled, and stored in sealed plastic bags in the dark at 4°C until extraction of soluble phenolics.

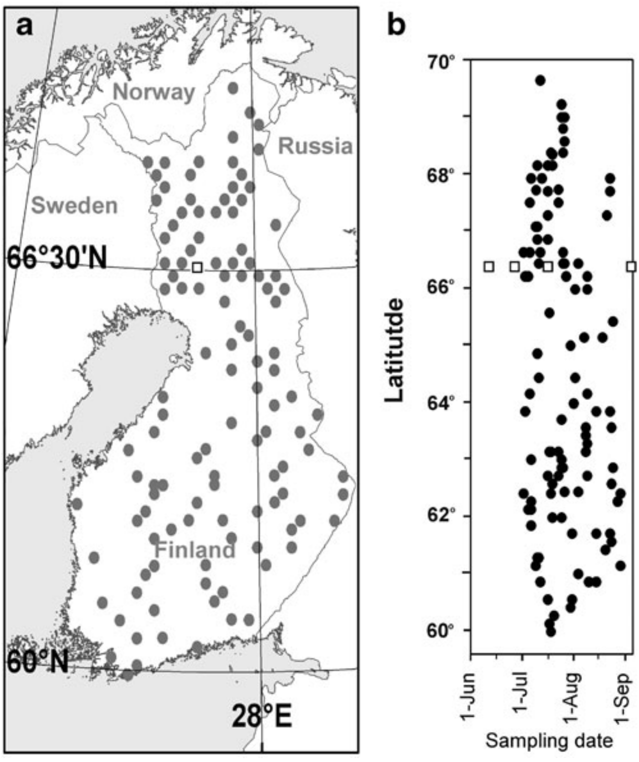


Fig. 1 Location of Experiment II sampling plots in Finland (**a**) and distribution of the sampling during the summer 2006 (**b**). Sampling plots for Experiment I are also indicated by *open squares*

Chemical Analysis of Leaves In this study, the term “phenolics” refers to soluble phenolics only, since cell-wall bound phenolics were not extracted. Leaf pigments and phenolics from dried leaves were extracted by incubating and shaking 1 g of milled leaves in 10 ml of acidic water (0.1% HCl) for 1 h in the dark at 4°C. After gross filtration over filter paper, samples were centrifuged for 10 min at 3,000*g,* and the supernatants were stored at −20°C until analysis. Control extractions showed a similar composition after longer extraction or extraction in boiling water for 5 min. Frozen leaf samples from Experiment I were ground in liquid nitrogen and extracted once with 10 volumes of acidified methanol /water (*v*/*v*) (0.1% HCl) by shaking for 1 h at 4°C in the dark. After centrifugation, the supernatant was transferred into glass tubes and stored at −20°C until analysis.

Due to the large number of samples in Experiment II, a fast and reliable analysis protocol was adopted. Acid hydrolysis of crude leaf extracts (incubation in 2 N HCl for 1 h at 80°C, see below) leads to the production of ACs from PAs, caffeic acid from CGAs, *p*-coumaric acid and other HCA derivatives, and

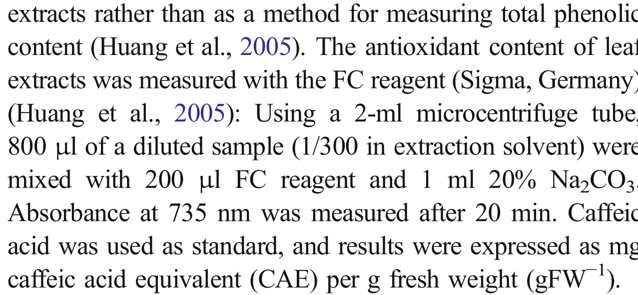
flavonols from flavonol glycosides. HPLC analysis of the samples from Experiment I (N=60) before and after acid hydrolysis allowed us to compare both methods for quantification of phenolics. Statistically significant linear correlations were found between quantification results obtained with or without acid hydrolysis, especially for the percentage of CGAs, HCA derivatives (*p*-coumaric and cinnamic acid derivatives other than caffeic and ferulic acids), flavonols and PAs (R^2=0.714, 0.961, 0.839, and 0.955, respectively). The total amount of phenolics (sum of quantified compounds) measured before and after acid hydrolysis was, however, about 50% lower after hydrolysis (not shown), but both values remained significantly proportional (R^2=0.369, F=36.34, P<0.001). The use of fresh and dry material, different extraction solvents and the inability to quantify catechins from hydrolyzed extracts due to technical reasons may partly explain this difference. Consequently, acid hydrolysis of crude extracts followed by HPLC analysis was adopted as a general protocol for comparison of the samples from Experiment II.

Phenolic composition of hydrolyzed and unhydrolyzed plant extracts was established by HPLC using a Spherisorb ODS II column (4.6×250 mm, particle size 5 µm) and precolumn (Waters, Milford, MA, USA). Samples were eluted from the column by a solvent gradient consisting of Solvent A [0.1% (*v*/*v*) H_3PO_4] and Solvent B [100% MeOH] as previously described (Keski-Saari and Julkunen-Tiitto, 2003). Peaks were identified according to their retention times, UV spectra, and by using HPLC/MS (Keski-Saari and Julkunen-Tiitto, 2003). Quantification was carried out with a UV/visible diode-array detector (Waters PDA 996) at 320 nm for all peaks except catechin, which was determined at 280 nm. The following phenolics were used as references for quantification: CGA (for non-hydrolyzed extracts), caffeic acid (for hydrolyzed extracts), *p*-coumaric acid for all other HCAs, (+)-catechin, hyperin (for non-hydrolyzed extracts), and quercetin (for hydrolyzed extracts) for all flavonol glycosides. Chlorogenic acid (CGA) (3-O-caffeoylquinic acid), caffeic acid, *p*-coumaric acid, (+)-catechin, and hyperin were obtained from Sigma (Germany). Quercetin was purchased from Aldrich (Germany).

Monomeric anthocyanin (AC) content was measured using the differential pH method with cyanidin-3-O-glucoside (BioChemika, Germany) as reference (Lee et al., 2005).

Proanthocyanidins (PAs) were analyzed after their conversion to ACs by incubation of the extracts in 2 N HCl for 1 h at 80°C under conditions optimized for bilberry extracts (Zhang et al., 2004). After cooling, the solution was neutralized by the addition of NaOH, centrifuged, and 20 µl were analyzed by HPLC as described above, with detection at 535 nm using cyanidin-3-O-glucoside as standard.

The Folin-Ciocalteu (FC) reaction can be interpreted as a method for estimating the antioxidant capacity of food or plant


extracts rather than as a method for measuring total phenolic content (Huang et al., 2005). The antioxidant content of leaf extracts was measured with the FC reagent (Sigma, Germany) (Huang et al., 2005): Using a 2-ml microcentrifuge tube, 800 µl of a diluted sample (1/300 in extraction solvent) were mixed with 200 µl FC reagent and 1 ml 20% Na_2CO_3. Absorbance at 735 nm was measured after 20 min. Caffeic acid was used as standard, and results were expressed as mg caffeic acid equivalent (CAE) per g fresh weight (gFW^{-1}).

Radical scavenging activity (RSA) of the samples was measured by using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, Germany). Five µl of a diluted sample extract (1/10 in 50% aqueous methanol), representing 50 µg of dried leaves, were mixed with 1 ml of 0.25 mM DPPH at 20°C. Absorbance at 517 nm was measured immediately (A_0) and after 15 min (A_{15}) and 30 min. Decrease in absorbance was still linear after 15 min of reaction time but a plateau usually was reached at 30 min, so that the value measured at 15 min (A_{15}) was used to calculate RSA. RSA was expressed as the percentage of quenched DPPH after 15 min of reaction time, and was calculated by using the equation: $RSA\,(\%) = ((A_0 - A_{15})/A_0)*100$. Under these experimental conditions, 1.38 µg ascorbic acid were necessary to obtain an activity of 20%.

RNA Extraction and Gene Expression Analysis To complement leaf phenolic composition data from Experiment I, we analyzed the steady state expression of selected genes of the flavonoid pathway. Total RNAs were isolated as described previously (Jaakola et al., 2001) and reverse transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quality of the isolated RNA was verified by measuring the absorbance spectrum with a NanoDrop N-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and by electrophoresis in a 1% (*w*/*v*) ethidium bromide-stained agarose gel. The transcript accumulation of bilberry flavonoid biosynthetic genes chalcone synthase (CHS; AY123765), dihydroflavonol 4-reductase (DFR; AY123767), anthocyanidin synthase (ANS; AY123768), and anthocyanidin reductase (ANR; FJ666338) was detected using a LightCycler® SYBR Green I Master qPCR kit (Roche Molecular Biochemicals, Germany). Quantitative real-time PCR analyses were performed with a LightCycler 480 instrument and software (Roche Molecular Biochemicals, Germany). PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec (ramp rate 4.4°C/sec), 60°C for 20 sec (ramp rate 2.2°C/sec) and 72°C for 10 sec (ramp rate 4.4°C/sec). The melting curve was measured at 95°C for 0.5 sec (ramp rate 4.4°C/sec), 57°C for 15 sec (ramp rate 2.2°C/sec), and 98°C for 0 sec (ramp rate 0.11°C/sec). The primers 5′-CCAAGGCCATCAAGGAATG-3′ and 5′-TGATACATCATGAGTCGCTTCAC-3′ were used for amplifying CHS,

5′-GAAGTGATCAAGCCGACGAT-3′ and ATCCAAG TCGCTCCAGTTGT-3′ for DFR, 5′- TCTTCTAC GAGGGCAAATGG-3′ and 5′-ACAGCCCATGAAATC CTGAC-3′ for ANS, and 5-GCTGGTGTTTCT CCCACAAT-3′ and 5′-CAGGCAACACCTTACCAACA-3′ for amplifying ANR. Quantification of PCR products was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AY123769, primers 5′-CAAACTGTCTTGCCC CACTT-3′ and 5′-CAGGCAACACCTTACCAACA-3′) as a control gene. Results were calculated with the LightCycler®480 (Roche) software (release 1.5.0 SP1), using the calibrator-normalized PCR efficiency-corrected method (technical Note No. LC 13/2001, Roche Applied Science). Specificities of PCR products were verified by melting curve analysis.

Soil Carbon and Nitrogen Analysis We analyzed soil nutrient status from sites of Experiment I in the Rovaniemi area. At each site, we sampled 6 soil cores extending through the whole humus layer (max. 5 cm depth). Soil was dried (60°C, 48 h), milled, and analyzed by CHN-analyzer (CHN-1000 Elemental Analyzer, LECO Corporation, MI) in an accredited laboratory (Finnish Forest Research Institute, Central Laboratory Services). Carbon (C) and nitrogen (N) contents (% of dry weight) were measured.

Statistical Analyses SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) for Windows was used for statistical analysis. Normality of variables was checked before performing the statistical analysis described below. To investigate differences among the three sites (no replication) and the sampling time effect (four time points) in Experiment I, one-way ANOVA followed by pair-wise comparisons using Tukey's HSD test were made. For Experiment II, regression analyses were done to measure the coefficient of determination R^2 and ANOVA F and P values between the variables and latitude, altitude, and sampling date. In order to determine which factor (latitude, altitude, or latitude and altitude) was most significant in predicting the variables, information-theoretical approach and Aikaike's Information Criterion (AIC) were used (Burnham and Anderson, 2002). This approach is used commonly in observational studies such as in the present experiment to determine which model best fits the data.

Results

Soluble Phenolic Composition of Mature Leaves Bilberry leaves were mature in mid-July, so only samples collected on 17 July 2006 in Experiment I are described in this section. At that time, fruits were purple at T and C sites but still green at F site. The level of phenolics in mature leaves varied considerably (from 30.16 to 80.03 mg gFW^{-1}) between sites, but the same compounds were detected throughout the summer, and only the relative proportions of the different compounds changed (data not shown). In every sample, the most abundant compounds were CGAs (Table 1), including caffeoylquinic acid comprising about 85% of the CGAs and a methyl-chlorogenic acid derivative accounting for less than 15%. The second most abundant class, flavonol glycosides (Table 1) included mainly quercetin derivatives such as quercetin-glucuronide, hyperin, quercetin-arabinoside, but also contained kaempferol-glucuronide and two myricetin derivatives. Other compounds present in lower proportions were catechins (3 peaks), HCAs comprised of over 90% *p*-coumaric acid derivatives, and PAs (including procyanidins and prodelphinidins). In mid-July, ACs (two cyanidin glycosides) were detected only in samples collected at C and T sites. High standard deviations calculated over all the samples collected in mid-July (N=15) can be explained by the different growing conditions, as described below.

Table 1 Soluble phenolic content and composition of mature bilberry leaves collected on 17 July 2006 in the Rovaniemi area

Compound	$mggFW^{-1}$ [a]	(%) [b]
Sol. phenolics	55.39±16.61	(100)
CGAs	29.85±7.29	(55.5)
Flavonol glycosides	17.52±8.74	(29.6)
Catechins	3.91±0.73	(7.4)
HCAs	3.40±0.87	(6.3)
PAs	0.69±0.42	(1.1)
ACs	0.016±0.022	(0.02)

[a] Values are mean values ± SD (N=15)

[b] % of the total soluble phenolics

The average RSA of the leaf extracts estimated by using the DPPH method was 21.43±7.46% (N=15) in mid-July. The amount of antioxidants estimated by the FC method was 28.19±7.91 mg CAE gFW^{-1} in the same period.

Effect of Environment and Foliar Development on Phenolic Composition Although in every site the total phenolic levels were similar in young leaves, at the end of the growing season compound levels were lower in the forest (F) than in the clear cut (C) or fell (T) sites (Fig. 2a). F samples had a significantly higher water content (F=10.52, P=0.002) than the T and C samples (57.60±2.41, 52.05±0.32, and 54.29±2.28% H_2O in F, C, and T samples, respectively) but the differences were not big enough to explain the differences measured in phenolic composition between the samples. The C and T samples had similar compositions (Fig. 2b–g) with the total phenolic content slightly increasing during leaf development. HCAs strongly decreased during early leaf development stages, CGAs remained relatively stable in all stages, while the flavonol glycoside content increased rapidly until the mature stage in mid-July. The flavonol content, however, did

Fig. 2 Concentrations ($mg g FW^{-1}$) of total phenolics (**a**), HCAs (hydroxycinnamic acids) (**b**), CGAs (chlorogenic acid derivatives) (**c**), flavonol glycosides (**d**), catechins (**e**), PAs (proanthocyanidins) (**f**), ACs (anthocyanins) (**g**), RSA (radical scavenging activity) (**h**), and antioxidants (Folin-Ciocalteu values) (**i**) in bilberry leaves collected in the Rovaniemi area in summer 2006 in three different sites (Experiment I). On the same sampling date, samples statistically different from the others at $P=0.05$ are indicated by a star. Values are means ± SD ($N=5$). *F* forest; *C* clear cut; *T* fell

not significantly change in mature leaves from mid-July to early September ($F=1.29$, $P=0.289$, and $F=2.170$, $P=0.179$ for C and T sites, respectively). The PA content showed a similar trend to flavonol glycosides. Phenolic content of F samples was not only lower but its composition was different from that of the C and T samples. F leaves were characterized by lower contents in CGAs, flavonols, and PAs compared to C and T leaves. Moreover, CGA content decreased, whereas flavonol content did not significantly increase during leaf development ($F=0.755$, $P=0.536$). The proportion of kaempferol glucuronide remained stable during all stages in all samples but interestingly, it was always significantly higher in F compared to C and T samples (21.96±6.66, 10.13±2.27, and 10.12±3.16% of all flavonol glycosides in F, C, and T samples, respectively, all sampling times included). The AC amount measured in F leaves also was lower than in C and T leaves, but was not statistically different due to high individual variation (93.79±89.31, 193.38±107.7, and 284.18±262.48 $\mu g\ gFW^{-1}$ in F, C, and T samples, respectively).

Expression of the key genes of the flavonoid pathway was studied in the C, F, and T leaves using quantitative RT-

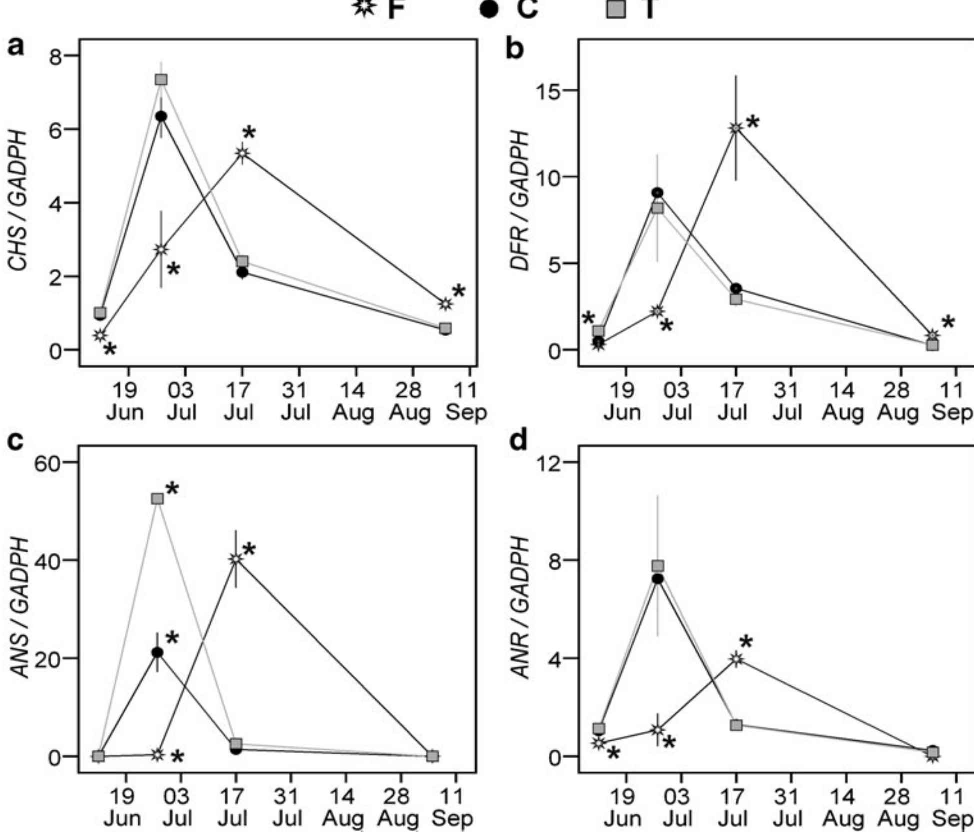


Fig. 3 Expression analysis of the *CHS* (**a**), *DFR* (**b**), *ANS* (**c**), and *ANR* (**d**) genes in bilberry leaves growing in the Rovaniemi area in different sites (Experiment I) in summer 2006 using qPCR. *GADPH* gene expression was used as reference. On the same sampling date, samples statistically different from the others at $P=0.05$ are indicated by a *star*. Values are means ± SD ($N=3$–5). *F* forest; *C* clear cut; *T* fell

PCR. Whatever the site, the expression level of the selected genes was similar in young (12 June) and old leaves (5 September) (Fig. 3). Differences, however, were observed in the developmental profile of transcript accumulation in C and T compared to F samples. At the end of June, the expression of flavonoid synthesis genes was several-fold higher in leaves growing in open areas (T and C samples) compared to forest leaves (F), which was consistent with the accumulation of the phenolics (Fig. 2).

The RSA activity measured with the DPPH test increased three-fold from young leaves to old leaves in F and C sites, but activity was always higher in the C compared to the F samples (Fig. 2h). A significantly higher activity was measured in T compared to C and F samples in young leaves in early June, but similar values were measured in early September in all samples. A similar developmental pattern was observed for the antioxidants quantified by using the FC method: they increased in all samples during foliar development, with similar values in T and C samples and significantly lower values in F samples from the end of June onwards (Fig. 2i). When analyzing correlations between biochemical parameters and RSA activity or FC values (antioxidant content), it appeared that the content of PA was the parameter most strongly correlated with RSA activity and FC values (R^2= 0.753 and 0.909, respectively). Strong linear relationships also were found with catechin content (R^2=0.747 (RSA) and 0.715 (FC)) and flavonols (R^2=0.473 (RSA) and 0.677 (FC)), but less with total phenolics (R^2=0.224 (RSA) and 0.426 (FC)). Thus, antioxidant capacity was best explained by PA and flavonols but not total phenolics.

To establish whether the nutrient status of the study sites was correlated with the concentration of leaf phenolics, soil samples collected in the C, F, and T sites were analyzed for carbon and nitrogen content. Results showed that among the three sites, the T site had the lowest nitrogen availability (high carbon/nitrogen ratio) (Table 2). Similar values were observed for the C and F sites which are separated by only 50 m, but the highest N availability was measured at the C site, which was logged in 2000.

Effect of Latitude and Altitude on Phenolics Experiment I showed that most of the changes in bilberry leaf composition occurred in early development stages before mid-July. We, therefore, tested the effect of maturation on leaf properties in Experiment II by linear regressions between biochemical

Table 2 Soil nutrient status in bilberry collecting sites in Rovaniemi area

Site	Carbon/Nitrogen[a]
C	31.44±3.02
F	38.76±3.88
T	44.09±4.51

[a] Values are mean values ± SD ($N=6$)

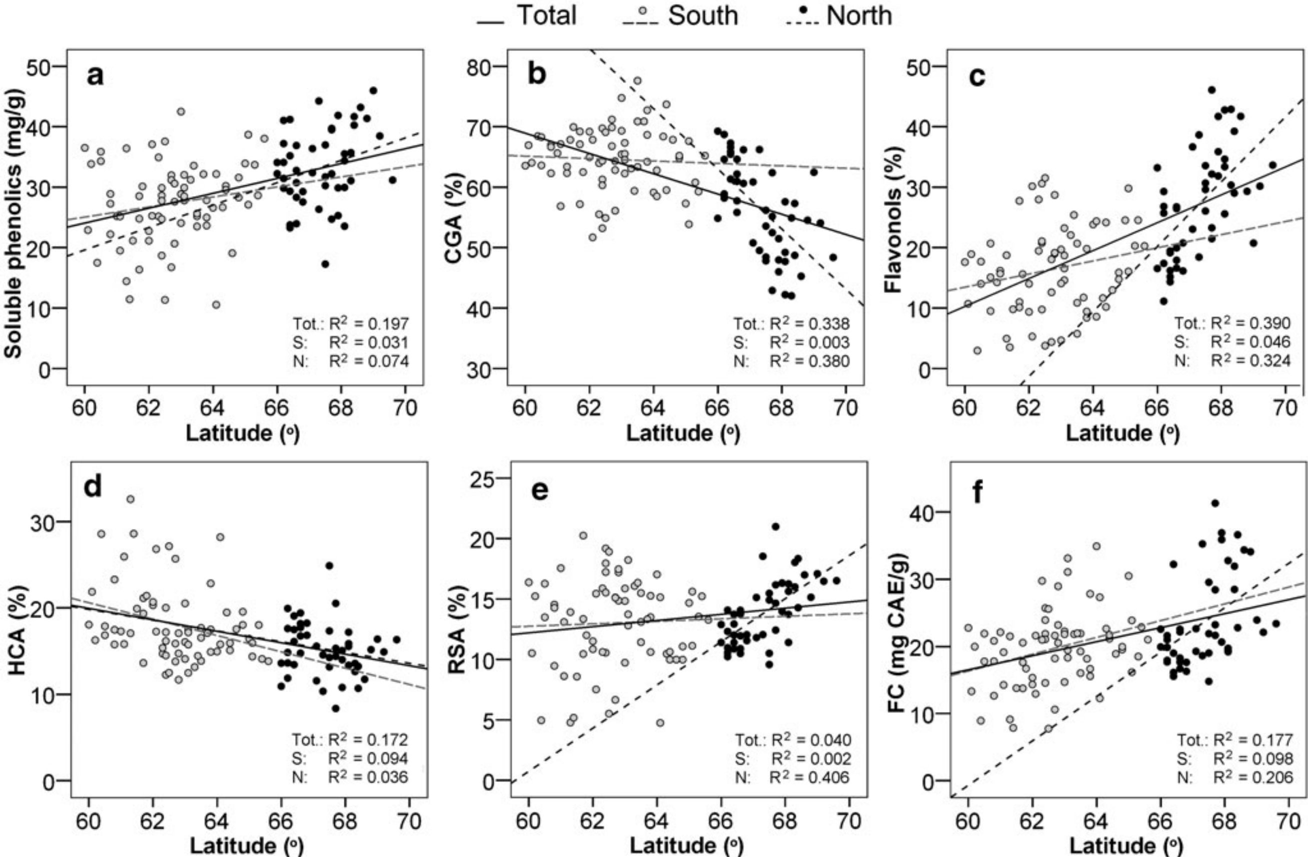
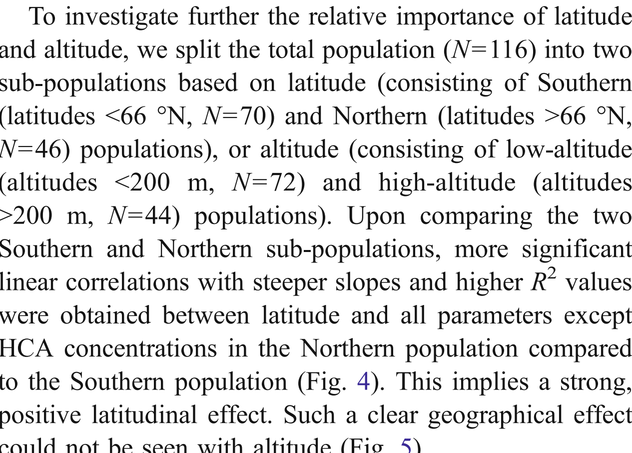


Fig. 4 Effect of latitude on the content of phenolics (mg per g dry weight (mg gDW^{-1})) (**a**), the proportion of CGAs (chlorogenic acid derivatives) (**b**), flavonols (**c**), HCAs (hydroxycinnamic acids) (**d**), the RSA (radical scavenging activity) (**e**), and the Folin-Ciocalteu values (**f**) in bilberry leaves. The total population (N=116) was divided into two sub-populations based on the latitude: the South (N=70, latitudes <66°N) and the North (N=46, latitudes >66°N) populations. Linear regression lines and coefficients of determination R^2 are indicated in each panel for each population. *Tot*. Total population, *S* Southern population, *N* Northern population

parameters and the date of sampling. There was a significant relationship between the date of sampling (P<0.001) with the proportion of ACs (R^2=0.212) but with only a minor effect and little consequences on leaf composition due to the low abundance of ACs in bilberry leaves (0.02±0.04% in hydrolyzed leaf extracts, N=116). There were no significant relationships between the date of sampling and the total phenolic content, the proportion of CGAs, flavonols, HCAs and PAs, the RSA activity, or the FC values (P=0.382, 0.076, 0.707, 0.005, 0.004, 0.008 and 0.031, respectively).

The northern parts of Finland are characterized by higher altitudes, and significant changes in the leaf composition were measured with increasing latitude and/or altitude (Figs. 4 and 5). Since latitude and altitude are closely correlated (R^2=0.465, F=106.68, P<0.001), we applied the Information Theoretic approach with Aikaike's Information Criterion (AIC) values to determine which model, latitude, altitude, or latitude and altitude, best fit the variation in leaf phenolics. The models that included both latitude and altitude were best in explaining the contents of all analyzed parameters (Table 3).


To investigate further the relative importance of latitude and altitude, we split the total population (N=116) into two sub-populations based on latitude (consisting of Southern (latitudes <66 °N, N=70) and Northern (latitudes >66 °N, N=46) populations), or altitude (consisting of low-altitude (altitudes <200 m, N=72) and high-altitude (altitudes >200 m, N=44) populations). Upon comparing the two Southern and Northern sub-populations, more significant linear correlations with steeper slopes and higher R^2 values were obtained between latitude and all parameters except HCA concentrations in the Northern population compared to the Southern population (Fig. 4). This implies a strong, positive latitudinal effect. Such a clear geographical effect could not be seen with altitude (Fig. 5).

Antioxidant capacities were higher at higher latitudes and altitudes (Figs. 4 and 5, Table 3). No analysis of linear correlations between RSA or FC values with the different leaf parameters were completed because phenolic content and composition were measured only after acid hydrolysis had occurred (such data could be used only for sample comparison, see Methods and Material).

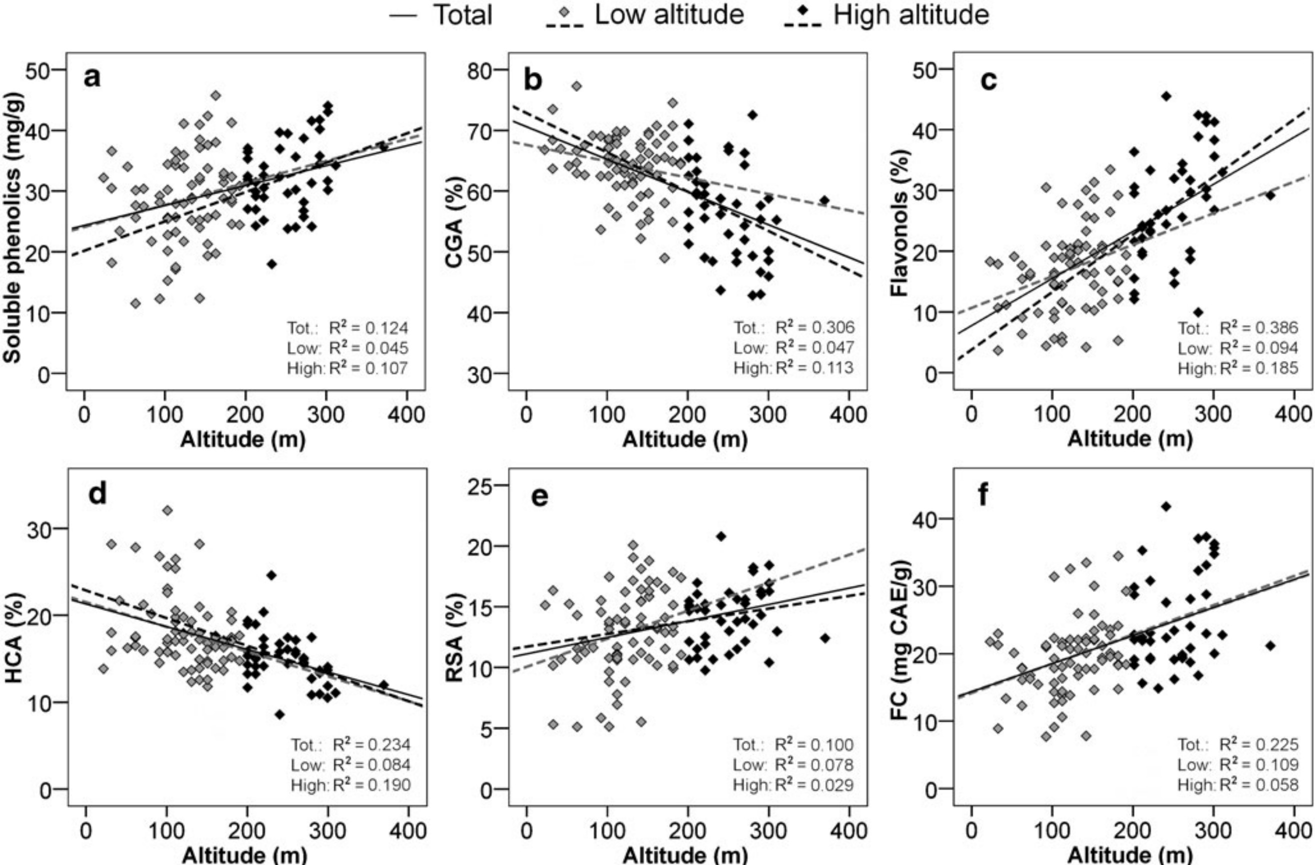


Fig. 5 Effect of altitude on the content of phenolics (mg gDW^{-1}) (**a**), the proportion of CGAs (chlorogenic acid derivatives) (**b**), flavonols (**c**), HCAs (hydroxycinnamic acids) (**d**), the RSA (radical scavenging activity) (**e**), and the Folin-Ciocalteu values (**f**) in bilberry leaves. The total population (N=116) was divided into two sub-populations based on the altitude: the low-altitude (N=72, altitudes <200 m) and the high-altitude (N=44, altitudes >200 m) populations. Linear regression lines and coefficients of determination R^2 are indicated in each panel for each population. *Tot*. Total population, *Low* low-altitude population, *High* high altitude population

Discussion

Bilberry leaf composition measured in this study is in agreement with other previously published reports (Witzell et al., 2003; Jaakola et al., 2004; Riihinen et al., 2008), with CGAs and flavonol glycosides as the most abundant compounds. Flavonol glycosides included quercetin and kaempferol as previously reported (Fraisse et al., 1996; Jaakola et al., 2004; Harris et al., 2007), and also myricetin derivatives. Catechins, HCAs (mainly *p*-coumaric derivatives), PAs, and ACs were minor components.

Although only three sites were studied in Experiment I without replication, clear differences in the leaf phenolic composition were measured among the sites. Similar

Table 3 Models Akaike's Information Criterion (AIC) and R^2 values of the different biochemical parameters of bilberry leaf and the geographical factors (latitude and altitude)

Compounds	Latitude		Altitude		Latitude + Altitude	
	AIC value	R^2	AIC value	R^2	AIC value	R^2
Sol. phenolics (mg g^{-1DW})	482.4	0.268	481.0	0.187	469.7	0.269
CGA (%)	477.7	0.403	475.0	0.310	456.8	0.413
Flavonol (%)	531.6	0.441	526.1	0.365	505.8	0.469
HCA (%)	341.8	0.192	330.6	0.200	328.2	0.228
PA (%)	−116.4	0.024	−117.2	0.050	−115.3	0.051
AC (%)	−868.9	0.069	−830.0	0.002	−840.1	0.094
RSA (%)	302.7	0.077	290.5	0.095	292.1	0.098
FC (mg g–^{1FW})	466.1	0.236	449.7	0.239	445.4	0.277

composition and developmental variations were measured in leaves collected in open areas under high light conditions (clear cut (C) and fell (T) leaves), with most importantly, a high increase in flavonols during early stages of leaf development. Leaves that developed in the low-light forest environment (F) displayed lower phenolic content and a different composition from C and T leaves, with particularly lower amounts of flavonols and PAs. Expression analysis for key genes of the flavonoid pathway suggested that the synthesis of flavonol and PAs was delayed in F leaves compared to C and T leaves. The proportion of kaempferol derivatives in flavonols also was higher in F compared to C and T leaves, in accordance with an earlier study in which an increased quercetin:kaempferol ratio was detected in bilberry leaves growing under direct sun exposure (Jaakola et al., 2004). In all types of leaves, composition did not significantly change in mature leaves after mid-July.

Light induces expression of flavonoid pathway genes (Hartmann et al., 2005; Matus et al., 2009), but nitrogen and temperature also can affect this pathway (Witzell and Shevtsova, 2004; Lillo et al., 2008; Usadel et al., 2008). Clear cutting sometimes can increase soil temperatures and enhance soil nitrogen availability through changes in nutrient release rates from litter and soil organic matter (Palviainen et al., 2005; Thiel and Perakis, 2009). Concentrations of leaf phenolics generally are higher in nutrient-poor than in nutrient-rich soils (Close and McArthur, 2002; Witzell and Shevtsova, 2004), but we observed the lowest soil carbon/nitrogen ratio (highest nitrogen availability) in the C site, which also showed the highest concentration of leaf phenolics. Thus, the variation in concentration of leaf phenolics among the three sites did not seem to correlate with soil nitrogen availability.

A previous study showed that production of phenolics was higher in upper bilberry leaves exposed to direct sunlight than in lower shaded ones (Jaakola et al., 2004). Our results are in agreement with this result, but we were able to show here that the genes involved in flavonoid synthesis are highly expressed in shade leaves as well but with a different timing. Similar maximum relative levels of gene expression were measured in leaves growing under different conditions, but surprisingly the accumulation of the corresponding phenolics remained lower in old F leaves. However, steady-state expression levels of key genes of the flavonoid pathway do not measure the actual enzymatic activity. The less favorable growing conditions in August compared to July at such latitudes might prevent full enzymatic activity and thus inhibit efficient synthesis of secondary compounds.

On the basis of our results, higher light intensity seemed to be a more important factor regulating the concentration of phenolics than the soil nutrient status, but this needs to be verified with investigations that use replicated study sites. Light has been proposed previously as having a stronger effect than soil nutrient availability on the production of flavonols in bilberry leaves (Witzell and Shevtsova, 2004).

The antioxidant capacity of bilberry leaves, measured with the DPPH or the FC method, increased during development, with higher capacities measured in C and T leaves compared to F leaves. PAs appeared to have an important role in the antioxidant capacity, which is in line with previous results (Määttä-Riihinen et al., 2005).

We also conducted an investigation on bilberry leaf phenolic composition along a large environmental gradient (60º00′ N to 69°60′ N with altitudes ranging from 20 to 370 m) (Experiment II). In line with our hypothesis, we found an increase in leaf phenolics with increasing latitude. By selecting similar forest successional stages that had similar light conditions, we excluded the role of forest development stage from the trends along the environmental gradients. Furthermore, in samples collected from July to the end of August, there was no effect of sampling date on the composition of the mature bilberry leaves. The data are consistent with the results from the analysis of mature leaves presented above (Experiment I). The northern parts of Finland are characterized by higher altitudes, with altitude closely correlated with latitude; we, therefore, also tested whether latitude, altitude, or latitude + altitude is the most powerful determinant of phenolic content and composition of bilberry leaves. Statistical analyses showed that all phenolic groups were best correlated with a model that included both latitude and altitude. These results are in line with previous work on bilberry fruits, in which ACs are a major component and where latitude and altitude were shown to affect their content (Lätti et al., 2008; Rieger et al., 2008).

The higher content of secondary metabolites found in bilberry leaves in the subarctic and north boreal sites, as compared to the south boreal sites, may reflect the adaptation of bilberry to northern growing conditions. The northernmost sampling sites in this study correspond to a subarctic environment, where plants are continuously subjected to several abiotic stresses, such as frequent frost spells, almost continuous light during the growing season, and nutrient-poor soils. Higher altitudes, especially in combination with northern latitudes, also contribute to severe growing conditions with low mean temperatures and high light intensity due to a discontinuous tree canopy. All of these factors, especially higher ultraviolet B radiation, affect secondary metabolic pathways (Dixon and Paiva, 1995; Witzell and Shevtsova, 2004; Lillo et al., 2008; Usadel et al., 2008). High need for photoprotection in the north can be explained by the fact that photo-oxidative stress induced by light intensity may be increased at low temperatures, because of the limiting effect

of temperature on photosynthesis (Close and Mcarthur , 2002). Flavonoids prevent photo-oxidative damage that could result from excess light in relation to the photosynthetic capacity (Close and Mcarthur, 2002). In boreal forests, the overstory trees form an effective shade for understory vegetation. The density of the tree canopy is higher in the southern than in the northern boreal areas for the same forest developmental class (e.g., Salemaa et al., 2008), so need for flavonoids and other photoprotective compounds would be greatest at high latitudes.

Our results demonstrate the importance of the geographical patterns of latitude and altitude on the chemical quality of bilberry leaves, which underlines the importance of light conditions on the concentration of flavonoids in plants. These findings support the idea that synthesis of polyphenolic secondary compounds are means of active adaptation to environmental stresses, rather than an indirect effect of nutrient deficiency that limits the plant growth.

In summary, our results show that foliar development and abiotic stresses characterizing the growing sites have significant effects on quantitative and qualitative composition of phenolics in bilberry leaves. The leaves of plants growing in high-light intensity sites, higher latitudes, and/or higher altitudes have over a two-fold higher concentration of total phenolics compared to plants growing in lower altitudes and low-light intensity sites. This may have implications for potential allelopathic effects of bilberry on tree seed germination and root growth (Jäderlund et al., 1996; Mallik and Pelissier, 2000), on food intake and digestibility of bilberry leaves (Duncan and Poppi, 2008) and to the harvesting of bilberry leaves by the health-promoting natural product industry (Mäkitalo et al., 2006).

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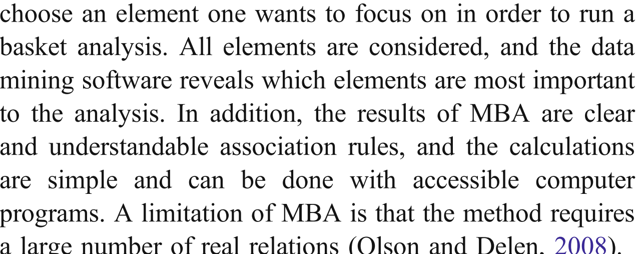
often have been used to monitor general atmospheric pollution levels on a regional scale, and substrate-plant relationships are important to differentiate between geogenic and anthropogenic element sources (Reimann and de Caritat, 2000).

Instruments currently used to investigate these problems usually are conventional statistical methods such as multivariate analysis of variance (MANOVA), factorial MANOVA, analysis of covariance (ANCOVA), regression analysis, and principal component and classification analysis (PCCA). There is, however, a need to find new statistical tools for environmental analysis that determine relations hidden in a set of data and present them as simple association rules. Innovative statistical methods are helpful to identify ecological trends and changes in response to environmental change (Samecka-Cymerman et al., 2007; Ferguson et al., 2008).

This research evaluated Market Basket Analysis (MBA), a novel data mining technique, for testing ecological and biogeochemical hypotheses. Until now, this technique has been used only to study the habits of shoppers in supermarkets and the contents of their shopping trolleys. MBA is a modelling technique based upon the theory that if a certain group of items is bought, this makes it more (or less) likely that another group of items also will be purchased. MBA is, thus, a technique that discovers relationships among pairs of products purchased together. This technique, however, also can be applied to other research areas to determine relations hidden in a set of data and present them as simple association rules (Agrawal et al., 1993; Agrawal and Srikant, 1994; Han et al., 2001; Witten and Frank, 2005). Association rule mining was first introduced by Agrawal et al. (1993), who were inspired by MBA and designed a framework for extracting rules from a set of transactions related to items bought by customers. They also proposed the *a priori* algorithm that discovers large (frequent) item sets. Since then, many advances have been introduced to handle various data types and structures (Messaoud et al., 2008).

MBA analysis is an intuitive application of association rules, striving to analyze customer buying patterns by finding associations between items that customers put into their baskets. Each customer's basket is represented as a Boolean vector, denoting which items are purchased. The vectors are analyzed to find which products are frequently bought together (by different customers), i.e., associated with each other (Cios et al., 2007). These co-occurrences are represented in the form of association rules: LHS⟹RHS where the left-hand side (LHS; Body) implies the right-hand side (RHS; Head).

MBA offers advantages over classical pattern recognition techniques such as PCCA or other types of data mining. MBA is undirected, and it is not necessary to



choose an element one wants to focus on in order to run a basket analysis. All elements are considered, and the data mining software reveals which elements are most important to the analysis. In addition, the results of MBA are clear and understandable association rules, and the calculations are simple and can be done with accessible computer programs. A limitation of MBA is that the method requires a large number of real relations (Olson and Delen, 2008).

We applied this statistical tool to determine relations hidden in a set of ecological data and to present them as simple association rules, assuming that the results obtained would be clear and simpler to interpret. The value of the method was tested by determining the chemical composition of the fern *Athyrium distentifolium*, sampled from sites with different types of bedrock in the Tatra mountains. This fern was selected because it grows abundantly in the Tatra mountains and accumulates elevated levels of metals when growing on serpentine and metalliferous soils (Cornara et al., 2007).

The Tatra mountains and the adjacent lowlands along the Polish–Slovak border form part of a Biosphere Reserve, created in 1993 in the context of the UNESCO MAB program. This area is a strictly protected region in the country (Bytnerowicz et al., 2003). It is practically free from local pollution and, thus, useful for investigations into relations between the bedrock and the chemical element contents of plants. *Athyrium distentifolium* grows in the Tatra mountains on lithosoils with a shallow layer of organic material, in sites characterized by different types of substrates: granites, gneisses, limestones, dolomites, and sandstones (Bac-Moszaszwili et al., 1979). The first two substrates in particular usually contain elevated levels of certain elements (Kabata-Pendias, 2001), and form special conditions for plant growth. The distribution of *A. distentifolium* offered an opportunity to compare the elemental composition of this species on different types of parent rock, in order to evaluate whether the concentrations of chemical elements in a plant correlate with parent rock geochemistry (Reimann and de Caritat, 2000; Neff et al., 2006). The aim of the present study was to use MBA as well as PCCA to investigate the influence of bedrock on concentrations of a number of elements in *A. distentifolium*, and to compare the results obtained with the two different techniques. We investigated the hypothesis that different types of rock would influence the concentrations of elements in this fern.

Methods and Materials

Fern Sampling A total of 118 sampling sites in the Tatra mountains were selected, on different substrates. Thirty sites were situated on limestones and dolomites (later

combined as limestones), 18 were selected on quartz sandstones, and 70 on granites and gneisses (later combined as granites). At each site, five *A. distentifolium* samples were collected within a 25×25 m area. Each sample consisted of a mixture of three subsamples of fronds. As required by the rules set by the Environmental Monitoring and Data Group (Markert et al., 1996) and the European Heavy Metal Survey (Harmens et al., 2004), the collected ferns had not been exposed directly to canopy throughfall, as they were collected in open areas.

Chemical Analyses Before analysis, plant material was washed for a few seconds in distilled water and dried at 50°C to constant weight. Fern samples were ground and homogenized in a laboratory mill. Samples (300 mg dry weight, in triplicate) were digested with nitric acid (ultra pure, 65%) and perchloric acid (ultra pure, 70%) in a microwave oven (MARS5 CEN Corporation). After dilution to 50 ml, the plant digests were analyzed for Fe, Mg, Mn, and Zn by using Flame Atomic Absorption Spectrophotometry and for Cd, Cr, Cu, Ni, and Pb by using Furnace Atomic Absorption Spectrophotometry (AVANTA PM GBC Scientific Equipment). Ca, Na, and K were determined with a flame photometer (PFP7 JENWAY Ltd.). All elements were determined against standards (Atomic Absorption Standard Solution, Sigma Chemical Co), and blanks were prepared in the same way as samples. Blanks and standards contained the same matrix as samples. All results for plants were calculated on a dry weight basis.

The recovery rates of elements (percentages with SD) were as follow: Ca (98±3), Cd (96±5), Cr (101±4), Cu (98±3), Fe (98±2), K (98±3), Mg (97±3), Mn (102±2), Na (100±3), Ni (95±5), Pb (94±5), and Zn (103±3). The standard reference material consisted of bush branches and leaves (DC73348, LGC).

Statistical Analyses Differences between sampling sites in terms of concentrations of elements in ferns were evaluated by ANOVA on log-transformed data to obtain a normal distribution of features according to Zar (1999). The normality of the analyzed features was checked by means of *Shapiro-Wilk's W* test, and the homogeneity of variances was checked by means of *Bartlett's test* (Sokal and Rohlf, 1994; Zar, 1999). Sampling sites were divided into three groups according to the type of substrate. Sampling sites in group one consisted of granites and gneisses, those in group two of limestones and dolomites, and those in group three of sandstones. We grouped granites and gneisses together as both are more acid types of primary rock, while limestones and dolomites were grouped together as basic types of rock (Kabata-Pendias, 2001).

A *post-hoc LSD test* (Zar, 1999) was used to compare the concentrations of elements in ferns in groups 1, 2, and 3.

Market Basket Analysis MBA was used to classify the ferns by means of association techniques in terms of the concentrations of elements in the plants, in order to identify groups that tend to occur together within the sampling sites and to identify relationships between them (Witten and Frank, 2005; Han and Kamber, 2006).

The *a priori* algorithm is a popular and efficient algorithm for deriving association rules from large data sets, based on some user-defined "threshold" values. An association is an expression of the form: Body –>Head (Support, Confidence). The confidence value denotes the conditional probability of the Head of the association rule, given the Body of the association rule. The support value is computed as the joint probability (relative frequency of co-occurrence) of the Body and the Head of each association rule. Rules originating from the same item set have identical support but can have different confidence values (Agrawal et al., 1993; Agrawal and Srikant, 1994; Han et al., 2001; Witten and Frank, 2005; Nisbet et al., 2009). The *Lift* (also called improvement factor) describes the predictive power of a rule. It shows how much better a rule is at predicting the result than just assuming the result in the first place. The Lift is the ratio of the density of the target after application of the left-hand of the Body and Head rule to the density of the target in the population. In other words, it is the ratio of the records that support the entire rule to the number that would be expected, assuming that there is no relationship between the items. A Lift greater than 1 (100%) means that the resulting rule is better at predicting the result than guessing whether the resultant item is present, based on individual item frequencies. The higher the Lift of a rule, the better its predictive power (Berry and Linoff, 2004; Banek et al., 2006). For the purpose of MBA, we divided the concentrations of each element in the plants into quartile groups. Each group was arbitrarily categorized as follows: very low (values between minimum and Quartile 1), low (values between Quartile 1 and Quartile 2), high (values between Quartile 2 and Quartile 3), and very high (values between Quartile 3 and maximum).

Principal Component and Classification Analysis Principal component analysis (PCA) often is used in ecology to reduce the data and stabilize subsequent statistical analyses (Vaughan and Ormerod, 2005). PCCA previously has been applied in environmental sciences (Deng et al., 2007; Otto et al., 2008). PCCA is based on PCA and offers a practical and clear classification of a set of data for a number of objects (Legendre and Legendre, 1998). We established

whether relations between ferns growing on particular types of substrate and their elemental concentrations derived from PCCA would also be found by MBA. Thus, we could evaluate whether MBA also can be applied in ecology and environmental protection research.

All calculations were performed with Statistica version 8.0 (StatSoft Inc. 2008).

Results and Discussion

The concentrations of the chemical elements in ferns from three substrate types in the Tatra mountains (Table 1) differed significantly (ANOVA, $P=0.05$). *Athyrium distentifolium* from limestones contained the highest concentrations of Ca and Mg (*post hoc* LSD test, $P<0.05$), while ferns from granites contained the highest concentrations of Cd and Pb, and those from sandstones contained the highest concentrations of Cr (*post hoc* LSD test, $P<0.05$). The results for granites and limestones are in agreement with data published by Kabata-Pendias (2001), while Reimann et al. (2007) also found increased levels of Cr in plants from sandstones.

MBA was used to classify the ferns in terms of concentrations of elements and associations with substrate type (Table 2). The higher the Lift of a rule, the greater its predictive power (Banek et al., 2006). Based on the Lift values, we can conclude that the relation “If Body then Head” was more likely to occur in limestones (Lift 367–432%) and sandstones (Lift 419%) than in granites (Lift 158–174%). This information (Table 2) leads to the conclusion that ferns from limestones contained very low concentrations of Cd, Fe, Mn, and Pb, and very high concentrations of Ca and Mg (confidence 85–100%). Ferns from granites contained high concentrations of Mn, and very high concentrations of Cd and Pb (confidence 82–91%). Ferns from sandstones contained a very high level of Cr (confidence 100%).

The PCCA ordination of the sampling sites (Fig. 1) shows that the first principal component discriminated between ferns growing on limestones (positive scores) and those growing on granites (negative scores). The ferns growing on sandstones had negative scores for the second principal component.

Projection of the variables onto the factor plane indicated that factor 1 was positively related to Ca and Mg and negatively related to Cd, Pb, and Mn, while factor 2 was negatively related to Cr. Ferns growing on limestones were differentiated by the value of factor 1, which related positively to Ca and Mg. Ferns growing on granites also were differentiated by the value of factor 1, which was negatively related to Cd, Pb, and Mn. Ferns growing on

Table 1 Minimum/maximum values (mg kg^{-1}), mean and standard deviation (S.D.) of concentrations of elements in *Athyrium distentifolium* from the Tatra mountains

Substrate	Element	Min	Max	Mean	S.D.	Element	Min	Max	Mean	S.D.
gr	Ca	1,100	3,000	2,100	500	Mg	960	2,700	1,600	520
li		3,000	5,300	3,700	460		1,300	3,500	2,600	540
s		1,900	3,200	2,700	400		1,100	2,600	2,000	480
gr	Cd	0.2	0.9	0.5	0.2	Mn	27	900	200	160
li		0.01	0.3	0.1	0.1		11	280	40	50
s		0.03	0.7	0.3	0.2		14	450	170	130
gr	Cr	0.03	0.7	0.3	0.1	Na	80	200	120	28
li		0.1	0.8	0.3	0.2		80	580	140	86
s		0.3	0.9	0.8	0.1		80	180	120	30
gr	Cu	2.2	10	4.0	2.0	Ni	0.1	6.0	1.2	1.3
li		2.3	7.0	5.0	1.0		0.01	10	1.5	2.3
s		2.1	5.0	4.0	1.0		0.3	6.0	1.5	1.5
gr	Fe	41	200	70	20	Pb	2.0	6.0	3.0	0.8
li		30	100	60	18		0.8	2.0	2.0	0.3
s		50	140	80	24		0.6	4.0	2.0	0.9
gr	K	7,000	17,200	11,300	2,200	Zn	7.0	60	20	8.0
li		4,100	16,000	10,200	2,900		6.0	30	14	5.0
s		6,100	15,600	10,400	2,500		11	80	26	4.3

gr granites, *li* limestones, *s sandstones*

Table 2 Summary of the market basket analysis for concentrations of elements in the fern *Athyrium distentifolium* from different substrates in the Tatra mountains

If Body	then	Head	Confidence [%]	Lift [%]
Mn-vl, Pb-vl, Ca-vh	==>	li	100	432
Cd-vl, Pb-vl, Ca-vh	==>	li	100	432
Cd-vl, Ca-vh	==>	li	96.2	416
Mn-vl, Ca-vh	==>	li	95.7	413
Ca-vh, Mg-vh	==>	li	95.5	413
Cd-vl, Mg-vh	==>	li	95	411
Cd-vl, Mn-vl, Ca-vh	==>	li	95	411
Cd-vl, Ca-vh, Mg-vh	==>	li	95	411
Mn-vl, Ca-vh, Mg-vh	==>	li	94.7	410
Cd-vl, Fe-vl, Ca-vh	==>	li	94.4	408
Fe-vl, Ca-vh	==>	li	94.4	408
Ca-vh	==>	li	87.9	380
Pb-vl, Ca-vh	==>	li	92	398
Mn-vl, Pb-vl	==>	li	90.5	391
Cd-vl, Mn-vl	==>	li	87	376
Cd-vl, Pb-vl	==>	li	87	376
Mn-vl, Mg-vh	==>	li	85.7	371
Cd-vl, Fe-vl	==>	li	85	367
s	==>	Cr-vh	100	419
Mn-h	==>	gr	82	158
Pb-vh	==>	gr	91	174
Cd- vh	==>	gr	86.2	369

Minimum support for the model 13%, minimum confidence 82%

li Limestones, *gr* granites, *s* sandstones, *vh* very high, *h* high, *l* low, *vl* very low

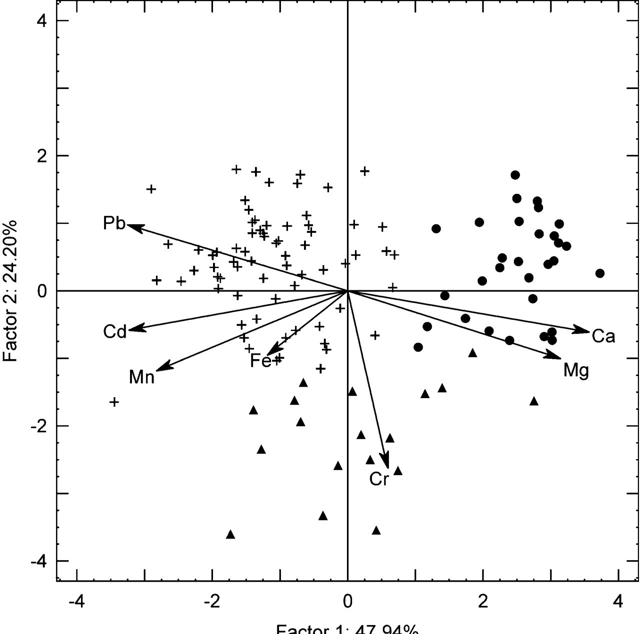


Fig. 1 Ordination of the 132 sampling sites by *PCCA* based on concentrations of elements in *Athyrium distentifolium* and projection of the concentrations of metals in ferns onto the factor plane; ▲ = granites, + = limestones, ● = sandstones

sandstones were differentiated by the value of factor 2, which was negatively related to Cr.

Ordination with PCCA resulted in a classification of the ferns from the Tatra mountains which was similar to that obtained by MBA (Table 3). Both analyses yielded the same elements that distinguished ferns growing on the same type of parent rock. MBA delivered more information on the relations between concentrations of elements in the ferns and the type of bedrock than PCCA, because MBA revealed not only the dominant elements (as PCCA did) but also elements with lower concentrations in ferns, in relation to a particular substrate. The highest concentrations of Ca and Mg were found in ferns on limestones, while the highest concentrations of Cd, Mn, and Pb were found in ferns on granites, and the highest concentration of Cr was found in ferns on sandstones. The lowest concentrations of Cd, Fe, Mn, and Pb were found in ferns on limestones (Table 3).

MBA is an undirected method, which does not require choosing specific items and finding their associations with other items, and also can reveal associations that may not have been expected by the investigator. In addition, data for this analysis are allowed to have certain characteristics, such as very large numbers of records, sparseness, and heterogeneity (Orson and Delen 2008). MBA as a data mining method was designed for the analysis of large data sets, thus making it useful for the data sets created by ecologists (McIntosh et al., 2007). We showed that MBA is a useful method for analyzing bioaccumulation of elements in plants and relations between plants and their environment. MBA possibly could discover associations and correlations among items in large data sets collected on a national or even larger scale. A disadvantage may be the necessity to group data before analysis, as was done in our investigation.

Table 3 Comparison of results of PCCA and MBA on elements distinguishing *Athyrium distentifolium* growing on different types of bedrock

Parent rock	PCCA	MBA
li	Ca, Mg	Ca-vh, Mg-vh; Cd-vl, Fe-vl, Mn-vl, Pb- vl
gr	Cd, Mn, Pb	Cd-vh, Mn-h, Pb-vh,
s	Cr	Cr-vh

li Limestones, *gr* granites, *s* sandstones; *vh* very high, *h* high, *l* low, *vl* very low

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Saliva consists of macromolecular compounds and small organic substances, including volatile organic compounds (VOCs), but the composition and functions of VOCs are not as well defined as some macromolecules. Saliva includes antibacterial, anti-viral, and antifungal proteins, such as lysozyme, lactoferrin, peroxidases, carbonic anhydrase, cystatins (cysteine proteinase inhibitors), and secretory immunoglobulin A. Antimicrobial peptides, histidine-rich histatins, and β-defensins, also are found in saliva from inflammation sites (Marcotte and Lavoie, 1998; Dodds et al., 2005). The von Ebner gland protein (VEGh), a member of the lipocalin family, represents one of the several saliva components that also are present ubiquitously in other body secretions (e.g., tears and semen). Lysozyme, also found in saliva, is present in other body fluids such as blood and sweat (Nieuw Amerongen and Veerman, 2002). Digestion-aiding salivary enzymes, such as α-amylase and a human-specific, hydrophobic lipase, participate in the first step of food digestion in the oral cavity (Marcotte and Lavoie, 1998; Isenman et al., 1999). Saliva also contains glycosylated (mucin-type) proteins that are responsible for the viscous gel-like properties of saliva (Wu et al., 1994). The small organic molecules previously described in saliva include hormones, amino acids, peptides (Marcotte and Lavoie, 1998), and nitric oxide (Benjamin et al., 1994; Palmerini et al., 2003).

More than 300 bacterial species have been found in the oral cavity, which also contribute to saliva chemical composition through secretion of their metabolic by-products (Scannapieco, 1994; Marcotte and Lavoie, 1998). Analysis of the sulfur-containing volatile compounds in breath has been reported in the investigation of sources for malodors in the oral cavity, and anaerobic bacterial activity contribute particularly to malodorous breath (Ochiai et al., 2001; Rodríguez et al., 2002; van den Velde et al., 2007). Small organic compounds of environmental origin may be transported into the saliva via the digestive tract, through the lungs (Kostelc et al., 1981; Amorim and Cardeal, 2007), or via transdermal absorption through the skin into the blood stream, followed by filtration into saliva (Jimbo, 1983; Jiang et al., 1996; Cross et al., 1997; Chatelain et al., 2003).

Several papers report comparative measurements in plasma and saliva for single organic compounds such as steroid hormones or their metabolites at relatively high concentrations (Hill et al., 2001; Jönsson et al., 2003; Contrerars et al., 2004; Kumar et al., 2005; Higarshi et al., 2007). Additionally, drug metabolite measurements in pharmacokinetic (Matin et al., 1974; Guo et al., 2007) and forensic studies (Lo Muzio et al., 2005; Gognard et al., 2006) have been reported in saliva. Samyn et al. (2007) recently reviewed various analytical procedures when using oral fluids in drug abuse investigations. However, there are relatively few reports on the endogenous volatile compounds in saliva. Lochner et al. (1986) report finding 39 compounds after solvent extraction and derivatization followed by gas chromatography-mass spectrometry (GC-MS). This approach is relatively tedious and, therefore, not suitable for a large number of analyses. Sastry et al. (1980) have reviewed different analytical methods for measuring VOCs in the salivary headspace by gas chromatography. However, these approaches are not applicable for a large number of analyses. Ligor (2009) recently reviewed preconcentration methods used in breath analysis, including solid-phase microextraction (SPME) methods.

In a previous study, we described the VOC profiles found on human skin (Soini et al., 2006; Penn et al., 2007), and in the present work we examined the VOCs in saliva samples from the same large group of subjects living in the Austrian Alps (Penn et al., 2007). As a prelude to systematic studies of a human population, it was necessary to develop a reliable analytical procedure for the large number of samples. We describe here an efficient and precise quantitative method for screening the native volatile compounds present at low levels in human saliva. We used a novel static stir bar sorptive extraction step followed by chemical analysis by GC-MS. The data from a large number of volatile compound profiles in human saliva and skin were evaluated by using a previously described chemometric pattern recognition technique (Dixon et al., 2006, 2007; Penn et al., 2007; Xu et al., 2007). The analytical method may have further potential in physiological, metabolic, pharmacokinetic, environmental, and forensic applications that require high analytical throughput and high sensitivity in analyzing various volatile compound levels in human saliva.

Methods and Materials

Sample Collection Saliva samples were collected in a non-stimulated fashion from 175 healthy volunteers within a geographically distinct area in a village in the Austrian Alps (Penn et al., 2007). Subjects did not have any dietary restrictions. Their food intake was documented in a questionnaire, and they were instructed to rinse their mouths with water before saliva collection, without brushing their teeth or using any mouthwashes. After 5 min of the water rinse, about 3 ml of saliva per person were accumulated in a plastic container by spitting. Samples were frozen at −20°C until analyses.

Reagents and Materials All analytical standard compounds were purchased from Sigma-Aldrich/ Fluka Chemical Company (Milwaukee, WI, USA) or TCI America (Portland, OR, USA). Stir bars (Twister™, 10 mm, 0.5 mm film

thickness, 24-μl polydimethylsiloxane (PDMS) volume) used for sample collection were provided by Gerstel GmbH (Mülheim an der Ruhr, Germany). The stir bars were conditioned prior to and between individual runs in the TC-2 tube conditioner (Gerstel GmbH) at 300°C under a purified stream of helium. Saliva samples (0.5 ml) were placed in 10-ml headspace vials (Gerstel) with screw caps equipped with high purity silicon/PFTE septa (Gerstel). Samples were diluted with 2.0 ml of high-purity water (OmniSolv®, EM Science, Gibbstown, NJ, USA). As an internal standard, 8 ng 7-tridecanone (Aldrich) were added in 5 μl of methanol (Baker Analyzed, Mallinckrodt Baker, Inc., Phillisburg, NJ, USA) to each vial, followed by the addition of a preconditioned stir bar into the liquid phase. The vials were closed and were subsequently incubated at 40°C for 3 h in an oven. The sampling bars were not stirred, but rather equilibrated in the vials in a static mode. After sampling, stir bars were rinsed with high-purity water (OmniSolv®), dried with paper tissue, and placed in thermal desorption autosampler device (TDSA) tubes for the analysis. After the analysis, TDSA tubes were washed with 50% OmniSolv® acetonitrile (EMD Chemicals, Darmstadt, Germany) in water containing 0.1% trifluoro-acetic acid (99%, Aldrich Chemical Company, Milwaukee, WI, USA), followed by a rinse with high-purity Omni-Solv® water and acetone (EMD Chemicals), and dried in the oven at 85°C for 60 min.

Analytical Instruments The GC equipment consisted of an Agilent 6890N gas chromatograph connected to the 5973i MSD mass selective detector (Agilent Technologies, Inc., Wilmington, DE, USA). Positive electron ionization (EI) mode at 70 eV was used with the scanning rate of 4.51 scans/sec over the mass range of 35–350 amu. The MSD transfer line temperature was set at 280°C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. Samples were thermally desorbed in a TDSA automated system (Gerstel), followed by injection into the column with a cooled injection assembly, CIS-4. The TDSA was operated in splitless mode. The TDSA temperature program for desorption was 20°C (hold for 0.5 min), then a 60°C/min ramp to 250°C (final hold of 3 min). Temperature of the transfer line was set at 280°C. The CIS-4 was cooled with liquid nitrogen to −80°C. After desorption and cryotrapping, CIS-4 was heated at 12°C/sec to 280°C, with the hold time of 10 min. The CIS-4 inlet was operated in the solvent vent mode, a vent pressure of 14 psi, a vent flow of 50 ml/min, and a purge flow of 50 ml/min. Compounds in the samples were separated on a DB-5MS [20 m × 0.18 mm, i.d., 0.18 μm film thickness] capillary column from Agilent (J&W Scientific, Folsom, CA, USA). The temperature program in the GC operation was 50°C for 1 min, then increased to 160°C at a rate of 5°C/min, followed by the second ramp at a rate of 3°C/min to 200°C (hold time: 10 min). The carrier gas head pressure was 14 psi (flow rate, 0.7 ml/min at constant flow mode and under retention time-locking conditions).

Data Analysis Saliva and skin volatile compound data were combined and evaluated with a newly developed chemometric pattern recognition method as described in Dixon et al. (2006); Penn et al. (2007); and Xu et al. (2007).

Method Development and Optimization All volunteers for the initial optimization study provided informed consent to participate in this study (05-9661), as approved by the Indiana University Bloomington Institutional Review Board. Saliva samples from healthy donors (by their own recognition) first were pooled and 0.5 ml aliquots were used for method development. The highly viscous sample matrix appeared to retain small organic volatile compounds, effectively competing with the PDMS material used in sorptive extraction. We learned this when stir bar extraction with stirring in the aqueous phase resulted in low recoveries and a high variation in the peak areas (40–50% relative standard deviation, RSD). Similar results were obtained by using a conventional headspace approach (Soini et al., 2005) with the stir bars. When the saliva samples and stir bars were set in the extraction vials at elevated temperatures for 1 hr without stirring (35 and 40°C), the recoveries increased, but peak area variability still exceeded 20% (RSD). At 40°C, extraction times of 1, 2, 3, and 4 hr revealed that peak areas reached the maximum plateau with a 3-h extraction time (data not shown), whereas the reproducibility of peak areas was less than 5% (RSD, N=4) for most of the compounds.

Results

Reproducibility Chromatographic analysis of seven selected salivary components in a pooled sample showed relatively good within-day reproducibility (Table 1). An internal standard was not added to the aliquots of pooled saliva. The long-term reproducibility of the method is reflected in the consistent variability (RSD=14.7%) in integrated peak area of the internal standard during an analysis of 175 samples (Fig. 1).

Characterization of Volatile Organic Compounds (VOCs) in Saliva In our previous report of the axillary skin VOCs for the same subject group, we found 373 repeatedly appearing compounds on the skin among about 5,000 VOCs for the 196 subjects (Penn et al., 2007). The chemometric data evaluation of the 175 salivary VOC profiles [consisting typically of

Table 1 Within-day reproducibilitiy of the peak areas (x 10^6) of selected salivary compounds in four aliquots of a pooled saliva sample

Compound	Retention time (min)	Average peak area counts (N=4)	SD[a]	RDS[a] (%)
4-methylbenzaldehyde[b]	9.2	22.97	0.43	1.87
borneol[a]	11.63	4.18	0.05	1.20
geraniol	13.76	1.54	0.03	1.95
1-dodecanol	19.66	35.26	0.84	2.38
1,12-dodecanediol[b]	26.32	2.99	0.10	3.34
caffeine	28.30	3.06	0.17	5.55
hexadecanoic acid	31.92	11.82	1.68	14.21

[a] *SD* standard deviation; *RSD* relative standard deviation

[b] Tentative identification

100–300 peaks (Dixon et al., 2006; Penn et al., 2007)], revealed that 166 of the 373 compounds from the constant axillary sweat compound group (i.e., 44.5%) also were found in saliva. We identified or tentatively identified 90 saliva VOCs (Table 2) among the 166 compounds that were found in common to saliva and skin (Penn et al., 2007). Thus, among the compounds in common, 76 remain unidentified. There was a substantial profile variability of total ion chromatograms (TIC) among the individual saliva samples (Fig. 2). All lactones, and amides (100%) and nearly all carboxylic acids (89%) and aldehydes (83%) found in the group of constant compounds on skin also were present in saliva. Smaller overlap was found for ketones (60%), ethers (50%), hydrocarbons (47%), esters (44%), alcohols and phenols (42%), and amines (12%). No androgen steroids were found in saliva, possibly due to the low detection limits.

Discussion

Individual variability observed among the salivary VOC profiles likely is due to several variables, including personal habits such as diet, environment, and genetics. Interestingly, almost half of the repeatedly appearing 373 VOCs on the skin found in the previous study (Penn et al., 2007) also were found in saliva. This suggests that these compounds could be endogenous. Because of the many possible entry routes for VOCs into the salivary flow, elucidation of their origin and route of entry is likely to be a difficult and complex task. For example, small organic compounds, such as pharmaceuticals, can move from blood to saliva via passive transcellular diffusion, ultrafiltration, or active transport mechanisms. Lipophilicity and molecular weight of the compounds of interest are among properties that influence the blood-saliva transfer rate (Höld et al., 1995). Targeted metabolic experiments containing a particular compound could verify some of the hypothesized excretion routes into saliva through the body and clarify which compounds are exogenous (externally transferred) or endogenous (internally transferred) in origin.

Salivary VOC composition could be an excellent indicator of environmental and occupational chemical exposure through transdermal absorption, inhalation through lungs, or via food intake. Environmental exposure to phthalates has been well documented in different body fluids. Multifunctional phthalate compounds can originate from soaps, shampoos, cosmetics, plastics, paints, and pesticide formulations. Their metabolites have been found

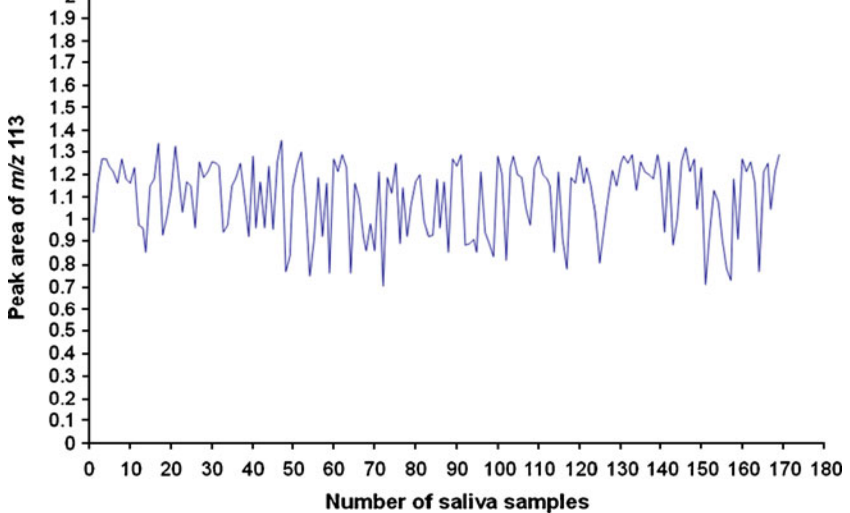

Fig. 1 Peak areas for the internal standard 7-tridecanone measured at m/z 113 (base peak) by sample number analyzed over a 17-d period (relative standard deviation = 14.7%, N=175)

Table 2 Compounds identified in saliva that have also been found on skin in a previous study of the same subject group (Penn et al., 2007)

Rt (min)	Compound
Alcohols and phenols	
Rt (min)	Compound
12.47	γ-terpineol[a]
12.90	2-phenoxyethanol[a]
13.82	geraniol
16.48	eugenol
27.75	a hexadecadienol[a]
Aldehydes	
Rt (min)	Compound
12.74	decanal
13.93	*p*-anisaldehyde[a]
14.38	geranial
15.46	undecanal
18.10	dodecanal
20.28	lilial[a]
20.61	tridecanal
23.00	tetradecanal
23.58	pentylcinnamaldehyde[a]
26.04	*E*-2-hexylcinnamaldehyde[a]
Ketones	
Rt (min)	Compound
8.98	acetophenone
14.99	2-undecanone
17.47	jasmone[a]
17.69	2-dodecanone
18.27	α-ionone[a]
19.66	β-ionone[a]
20.22	2-tridecanone
20.54	*Z*-α-irone[a]
22.52	2-tetradecanone
23.09	benzophenone[a]
24.92	2-pentadecanone
28.70	2-acetyl-3,5,5,6,8,8-hexa-methyl-5,6,7, 8-tetrahydronaphthalene [a]
Carboxylic acids	
Rt (min)	Compound
12.24	octanoic acid
14.76	nonanoic acid
21.90	dodecanoic acid
23.18	9-methyldodecanoic acid[a]
24.20	tridecanoic acid
25.74	10-methyltridecanoic acid[a]
26.80	myristic acid (tetradecanoic acid)
28.36	a methyltetradecanoic acid[a]
28.65	a methyltetradecanoic acid[a]
29.31	pentadecanoic acid
31.70	9-hexadecenoic acid[a]
31.96	palmitic acid (hexadecanoic acid)
34.37	9-heptadecenoic acid[a]
34.94	heptadecanoic acid

Table 2 (continued)

37.31	oleic acid
38.03	stearic acid (octadecanoic acid)
Esters	
Rt (min)	Compound
11.40	benzyl acetate[a]
16.37	α-terpineyl acetate[a]
17.16	geranyl acetate
21.24	α-trichloromethylbenzyl acetate[a]
21.95	pentyl salicylate[a]
22.21	isooctanediol dibutyrate[a]
23.30	isopropyl dodecanoate[a]
23.73	methyl-*cis*-dihydrojasmonate[a]
24.15	3*Z*-1-hexenyl salicylate[a]
24.46	1-hexyl salicylate[a]
27.53	2-ethylhexyl salicylate[a]
28.23	isopropyl hexadecanoate[a]
30.93	methyl hexadecanoate (methyl palmitate)[a]
32.40	hexyl dodecanoate[a]
32.99	ethyl hexadecanoate (ethyl palmitate)
33.70	isopropyl hexadecanoate[a]
37.35	2-ethyl-hexyl-4-methoxycinnamate[a]
43.35	1-octyl-4-methoxycinnamate[a]
Lactones	
Rt (min)	Compound
12.27	γ-heptanolactone[a]
16.70	γ-nonanolactone
18.60	coumarin
Amines	
Rt (min)	Compound
15.19	an aliphatic amine[a]
Amides	
Rt (min)	Compound
17.18	methyl N,N-diethylthiocarbamate[a]
18.04	a hydroxyl acetanilide[a]
21.98	*n*-propylbenzamide[a]
Hydrocarbons	
Rt (min)	Compound
12.26	1-dodecene
12.50	dodecane
15.15	tridecane
17.55	1-tetradecene
17.77	tetradecane
18.22	β-caryophyllene
19.72	*trans*-muurola-4(14),5-diene[a]
19.96	a methyl biphenyl[a]
20.36	α-farnesene
20.38	pentadecane
21.77	4-methylpentadecane[a]
22.56	hexadecane

Table 2 (continued)

23.18	6-phenylundecane[a]
25.04	heptadecane
27.48	octadecane
30.22	nonadecane
32.93	eicosane (C-20 linear hydrocarbon)[a]
35.79	heneicosane (C-21 linear hydrocarbon)[a]
38.79	docosane (C-22 linear hydrocarbon)[a]
Other (ethers)	
Rt (min)	Compound
17.65	diphenyl ether[a]
24.43	dioctylether[a]
29.26	bis(benzyloxy)methane[a]

[a] Tentative identification

in human urine (Silva et al., 2004a), breast milk (Calafat et al., 2004), amniotic fluid (Silva et al., 2004b), serum (Kato et al., 2003), and saliva (Silva et al., 2005). In general, salivary sampling is a less invasive alternative compared to serum collection for many biomedical applications (Cook, 2002; Gröschl and Rauh, 2006; Chiappin et al., 2007).

Certain limitations may pertain in sampling for sufficient volumes of saliva from infants, geriatric subjects, or subjects with certain physiological states that cause reduced saliva production (Granger et al., 2007). Saliva collection methods also may influence sample composition and induce interference to the analytical methods. Stimulated (e.g., by chewing gum) vs. non-stimulated saliva flow, or use of certain devices such as cotton balls or swabs placed in mouth can influence the final composition of the saliva (Gröschl and Rauh, 2006; Schipper et al., 2007). In this study, a non-stimulated saliva collection approach was chosen because stimulating agents and polymer-containing collection devices could introduce further contaminants in the analysis of the innate salivary VOC analysis.

In summary, the static stir bar extraction method for analyzing small organic compounds in saliva provided a sensitive and precise analytical approach for a large variety of compounds with different functional groups. The long-term reproducibility of the method allowed the analysis of a large number of samples in a high-throughput fashion. In this study, there was a large number of volatile compounds identified in saliva from a group of 175 subjects that were in common with components in previously analyzed sweat samples from the same subject group. This suggests that saliva presents a systemic body fluid that is potentially suitable for monitoring small organic compounds in physiological, metabolomic, pharmacokinetic, forensic, and toxicological studies.

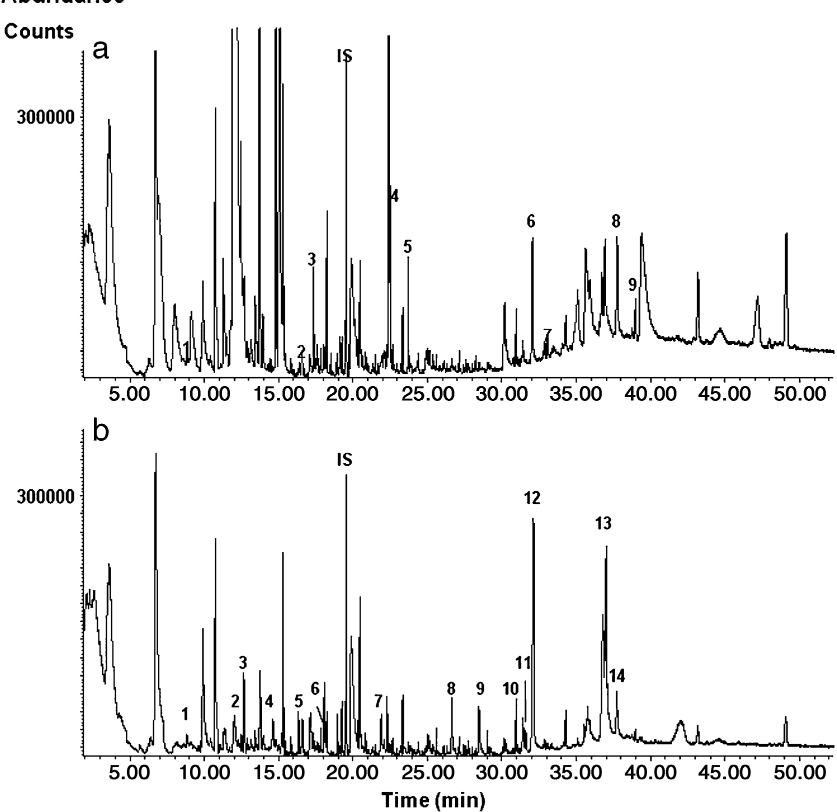

Fig. 2 Representative GC-MS total ion chromatograms of salivary VOC profiles from two subjects from different families: **a**: a female, and **b**: a male. Analytical conditions are described in the text. Numbers and names for selected compounds are **a**: 1=acetophenone, 2=eugenol, 3=β-caryophyllene, 4=hexadecane, 5=methyldihydro-*cis*-jasmonate, 6=hexadecanoic acid, 7=ethyl hexadecanoate, 8=oleic acid, 9=docosane and **b**: 1=acetophenone, 2=γ-heptanolactone, 3=decanal, 4=nonanoic acid, 5=nicotine, 6=tetradecane, 7=dodecanoic acid, 8=tetradecanoic acid, 9=caffeine, 10=methylhexadecanoate, 11=9-hexadecenoic acid, 12=hexadecanoic acid, 13=oleic acid, 14=octadecanoic acid. For **a** and **b**, IS=the internal standard (7-tridecanone)

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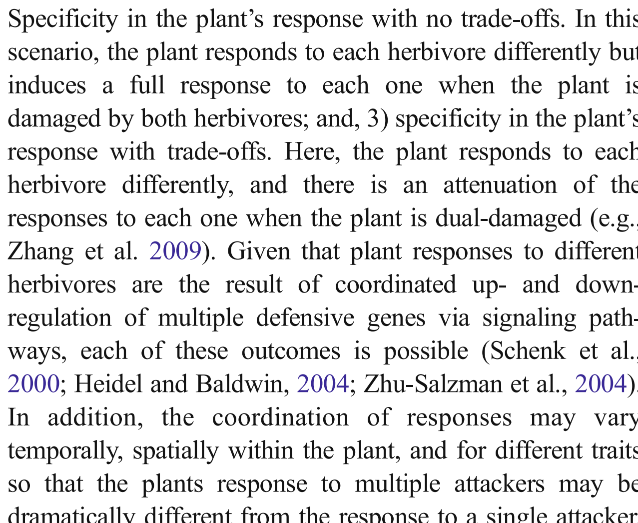
Introduction

Plants frequently are attacked by more than one herbivore species at the same time (e.g., Strauss, 1991; Vos et al., 2001; Hufbauer and Root, 2002). Specific plant responses can be related to feeding by insects from different feeding guilds (Walling, 2000), and can manifest themselves at different levels of biological integration, including transcriptional, biochemical, and organismal levels. Feeding by chewing caterpillars causes severe damage to plant tissues, most often accompanied by severe tissue loss. In addition, elicitors of plant defenses, including various fatty acid-amino acid conjugates, have been isolated from caterpillar regurgitant (Alborn et al., 1997; Pohnert et al., 1999; Halitschke et al., 2001). Mechanical wounding and elicitors in the caterpillar's oral secretions activate the jasmonate pathway, leading to the production of jasmonic acid (JA) and induction of defenses against many challengers (Farmer and Ryan, 1992; Karban and Baldwin, 1997; McCloud and Baldwin, 1997; Ohnmeiss et al., 1997; Moura and Ryan, 2001).

Compared to chewing caterpillars, phloem feeders cause limited tissue damage (Walling, 2000). Phloem-feeding insects, such as whiteflies and aphids, frequently activate the salicylic acid (SA) signaling pathway (Kempema et al., 2007; Zarate et al., 2007), and can manipulate resources within a plant by acting as sinks, causing increased translocation of nutrients to the attacked tissue (Walling, 2008). In addition, aphids secrete various, potentially signaling hydrolytic enzymes into the phloem during feeding including pectinases, glucosidases, peroxidases, and lipases (Miles, 1999; Mutti et al., 2008; De Vos and Jander, 2009). Aphids can not only alter localized expression of genes associated with the SA-dependent pathway, like pathogenesis-related (PR) proteins (Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003; Zhu-Salzman et al., 2004), but also JA-dependent genes, such as proteinase inhibitors (PI) (Martinez de Ilarduya et al., 2003; Voelckel et al., 2004). Aphid-dependent regulation of gene expression also can act systemically on JA- and SA-associated genes (Martinez de Ilarduya et al., 2003; Heidel and Baldwin, 2004; Voelckel et al., 2004), as well as on several housekeeping genes (Divol et al., 2005). However, some studies have not found significant systemic induction of PI (Heidel and Baldwin, 2004) or PR proteins (Divol et al., 2005) by aphids.

How plants cope with multiple co-occurring species of herbivores can be critical in insect-plant interactions (Shiojiri et al., 2001; Vos et al., 2001; Rodriguez-Saona et al., 2003, 2005; Dicke et al., 2009; Zhang et al., 2009). Plant responses to attack by multiple species of herbivores may result in three possible outcomes: 1) an additive response due to a lack of response specificity to different herbivores; 2)



Specificity in the plant's response with no trade-offs. In this scenario, the plant responds to each herbivore differently but induces a full response to each one when the plant is damaged by both herbivores; and, 3) specificity in the plant's response with trade-offs. Here, the plant responds to each herbivore differently, and there is an attenuation of the responses to each one when the plant is dual-damaged (e.g., Zhang et al. 2009). Given that plant responses to different herbivores are the result of coordinated up- and down-regulation of multiple defensive genes via signaling pathways, each of these outcomes is possible (Schenk et al., 2000; Heidel and Baldwin, 2004; Zhu-Salzman et al., 2004). In addition, the coordination of responses may vary temporally, spatially within the plant, and for different traits so that the plants response to multiple attackers may be dramatically different from the response to a single attacker.

Trade-offs in plant response can occur when different defense pathways are induced simultaneously, and there is increasing evidence that specifically the SA and JA signaling pathways can mutually affect each other. For instance, SA suppresses JA-dependent defense gene expression, either through inhibiting JA synthesis or its action (Doares et al., 1995). Similarly, induction of the JA pathway often inhibits the induction of the SA pathway (Stout et al., 1998; Preston et al., 1999; Felton et al., 1999; Paul et al., 2000; Thaler et al., 2002; Mur et al., 2006). Stout et al. (1998) showed that the negative effects of methyl jasmonate, a volatile derivative of JA, on beet armyworm caterpillar growth were eliminated by exposure of treated plants to SA. These trade-offs can occur as a result of simultaneous attack by herbivores and pathogens, and potentially when attacked by herbivores with different feeding habits (Stout et al., 2006). For example, growth rates of beet armyworm caterpillars increased when feeding on tomato plants previously fed upon by potato aphids compared to control plants (Stout et al., 1998).

In tomatoes (*Solanum lycopersicum* Mill.), caterpillars and aphids induce different plant responses. The beet armyworm, *Spodoptera exigua* (Hübner), induces the production of a variety of plant defenses including PIs (Broadway et al., 1986) via the jasmonate-signaling pathway (Thaler et al., 2002). In contrast, Fidantsef et al. (1999) found that feeding by the potato aphid *Macrosiphum euphorbiae* (Thomas) and the green peach aphid *Myzus persicae* Sulzer on tomato plants induces local expression of lipoxygenase and PR protein P4, but does not induce PI II. In a previous study, we investigated how single attack or attack by both beet armyworm caterpillars and potato aphids influence the adult preference and caterpillar performance of beet armyworm and its parasitoid *Cotesia marginiventris* (Cresson) in tomato plants (Rodriguez-Saona et al., 2005). We demonstrated that caterpillars and aphids induce different levels of plant resistance, but also

that dual-damaged plants were phenotypically distinct from plants damaged by either herbivore alone. For example, relative to undamaged plants, oviposition by moths was lower on caterpillar-damaged plants and higher on aphid-damaged plants compared to undamaged controls. Plants damaged by both herbivores, however, received equal oviposition as control plants. The widespread evidence across systems that aphids induce the salicylate pathway (e.g., Martinez de Ilarduya et al., 2003; Zhu-Salzman et al., 2004) suggests that aphid feeding may compromise resistance to caterpillars. Thus, we hypothesized that the plant's defensive response to caterpillars was weakened when aphids also fed on the plant.

In this study, we tested this hypothesis by employing an approach that integrates gene expression (microarray analysis and quantitative real-time PCR (qRT-PCR)) and biochemical measures (leaf carbon: nitrogen, protein quantification, and PI activity) with insect performance assays. Consistent with our hypotheses, we find a high level of attenuation and suppression of genes in dual-damaged plants compared to those that are damaged by a single herbivore.

Methods and Materials

Plants and Insect Colonies

Seeds of tomato plants (*S. lycopersicum* var. Castlemart) were planted in 500 mL pots filled with soil mix and 5–10 pellets of Nutricote (13-13-13 N-P-K). Plants were grown in a greenhouse between January and July under natural light supplemented with 400-W sodium halide lamps, watered daily, and fertilized weekly with a 15-20-15 N-P-K fertilizer. Plants, 25−27 d after planting, with two fully expanded leaves were used for experiments.

Two generalist insect herbivores were used to damage the tomato plants: the beet armyworm (*S. exigua*) and the potato aphid (*M. euphorbiae*). Beet armyworm caterpillars were obtained from the USDA Laboratory, Stoneville, MS, USA, and reared on an artificial diet (Southland Products, Lake Village, AR, USA) at room temperature (25°C). Potato aphids were collected in Southern Ontario from tomato plants and maintained on tomato plants in an environmental chamber (23°C, 14:10 L:D, and 60% relative humidity).

Experimental Treatments

Treatment procedures followed those described in Rodriguez-Saona et al. (2005). Plants (N=128) were randomly assigned to one of the following four treatments: (1) undamaged (control) plants; (2) plants damaged by 40 adult aphids on leaf 1; (3) plants damaged by one 3rd instar *S. exigua* caterpillar on leaf 2; and (4) simultaneous attack by both herbivores as in treatments 2 and 3. Leaves were numbered from the bottom of the plant, with the oldest leaf designated as "leaf 1." Please note: i) leaves 1 and 2 are strongly connected to leaf 3, but both leaves are only weakly connected to each other (Stout et al., 1996; Rodriguez-Saona et al., 2005); ii) the location of feeding, i.e., whether aphids or caterpillars fed on leaf 1 or 2, had no effect on the way leaf 3 responded to damage by these two herbivores (Rodriguez-Saona et al., 2005); and iii) there was no difference in the amount of leaf damage in the caterpillar only and dual damage treatments (Rodriguez-Saona et al., 2005). Both herbivores were confined to leaves 1 and 2 using spun polyester sleeves (35 cm wide×45 cm long) (Rockingham Opportunities Corporation, Reidsville, NC, USA). Control plants had sleeves but no herbivores.

Immediately after placing the insects on the plants, plants were transferred to a growth chamber (23°C, 14:10 h L:D, and 60% relative humidity, and a light intensity of 430 μmol/m^2/sec) for 5 d, a duration sufficient to induce systemic plant defenses (Rodriguez-Saona et al., 2005). After 5 d, plants had at least 4 fully-expanded leaves. Individual leaflets from leaf 3 of each plant (N=6, 8, 8, and 10 per treatment for trials 1, 2, 3, and 4, respectively) then were excised and used for either carbon and nitrogen analysis, total protein analysis, PI analysis, or gene expression analysis with cDNA tomato microarrays and quantitative RT-PCR. Leaflets from the same position of leaf 3 were used for each analysis. In addition, a leaflet from leaf 4 from each plant was used to conduct performance assays; similar to leaf 3, leaf 4 shares vascular connections with leaves 1 and 2 (Orians et al., 2000; C.R-S. unpublished data). The entire experiment was replicated 4 times (except for protein and PI which were replicated three times). Because aphids and caterpillars were placed on leaves 1 or 2 and the molecular, biochemical, and organismal measures were done on leaves 3 and 4, all the effects and interactions reported herein are systemic.

Gene Expression

Leaflets from leaf 3, taken from 5 different plants of the same treatment, were combined for each replicate (N=4 per treatment) and flash-frozen in liquid nitrogen for gene expression analyses. Tomato microarray chips (TOM 1 array) were purchased from the Boyce Thompson Institute (http://ted.bti.cornell.edu). The microarray target preparation and hybridization methods followed Dr. David Galbraith's (University of Arizona) protocols located at websites: http://www.maizearray.org/ and http://ag.arizona.edu/~dgalbrai/, and the instructions are paraphrased below.

Purification of tRNA and mRNA

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 5 g of powdered tomato leaf tissue for each treatment following the manufacturer's procedures. mRNA was purified from the total RNA samples using the Oligo (dT) Dynabead approach (Dynal Biotech, Inc., Lake Success, NY, USA) according to the manufacturer's protocol. We purified a minimum of 3 μg of mRNA in 4 rounds of purification of the total RNA through Oligo (dT) Dynabeads. Purified mRNA was stored at –70°C.

Direct Labeling of mRNA

Three μg of mRNA from each sample were reverse transcribed with Superscript II (Invitrogen) to produce one of two fluorescence-labeled targets, Cy3-dUTP or Cy5-dUTP (1 mM) (Amersham Biosciences, Piscataway, NJ, USA). Labeled cDNA was purified with Microcon YM30 columns (Millipore, Billerica, MA, USA). As an additional control, we included a dye-swap reversal for each treatment.

Hybridization of the Labeled cDNA to the Microarray Slides

The Cy3- and Cy5-labeled cDNA targets were denatured and subsequently hybridized onto preheated cDNA tomato microarray slides for 10 h at 60°C. The slides were washed according to the microarray hybridization protocol and dried prior to being scanned. A loop design was used for the hybridization scheme such that each sample was hybridized with every other as well as dye reversals for each of the samples. Eight true biological replicates of each treatment, including dye-swaps, were hybridized onto the microarrays. Ultimately, 6, 6, 7, and 3 samples of undamaged, aphid-damaged, caterpillar-damaged, and dual-damaged plants, respectively, were represented by the successful hybridizations.

Microarray Scanning and Analyses

The dry slides were scanned with a Gene Pix 4100 A (Axon Molecular Devices, Union City, CA, USA) at Western Illinois University. The laser intensity of the microarray scanner was set by the manufacturer. The photomultiplier detector was set such that the overall intensities of the scanned features for the two scanned images on a single microarray slide were close to equal. In addition, the detector was set at the highest level while maintaining the overall intensity below saturation. The scanned images were further normalized using the ratio of medians following the procedures outlined in the Acuity® 4.0 software (Axon Molecular Devices).

Each microarray slide was scaled to the slide with the highest signal intensity using Microsoft Excel. We ln-transformed the data and used the Acuity® 4.0 software to perform a one-way ANOVA for Multiple Groups, and then a 2-tailed *t-test* after testing the variance for each gene. The statistical cut-off used to assay significant genes was a *P*-value of ≤0.05 and at least a two-fold change in expression. The raw data are available in Table S1 (Supplemental Material). Genes that were statistically significant were organized further by hierarchal clustering using Acuity® 4.0 software. Genes were classified into biologically significant groups by using information about their known or expected function obtained though several search engines, gene databases, and protein databases. These protein databases included UniProt—Swiss-Prot Protein Knowledgebase (http://ca.expasy.org/sprot/) through The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). We also searched the Entrez Gene through the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov, which provided Gene References Into Function (GeneRIFs).

Quantitative Real-time PCR (qRT-PCR)

To validate the microarray results, we conducted qRT-PCR for the following herbivore defense-related genes: arginase, wound-induced PI II CEV 157, threonine deaminase, wound-induced PI I and II, lipoxygenase, and polyphenol oxidase D and F. Total RNA isolated during the RNA purification procedure described above was treated with TURBO DNase (Ambion, TX, USA) to remove any remaining DNA. DNase was inactivated and removed, and RNA was further purified by the RNeasy RNA cleanup MinElute columns (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA quantity was analyzed photospectrometrically, and RNA integrity was determined with RNA 6000 Nano Chips run on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Subsequently, 400 ng of DNA-free total RNA were converted into single-stranded cDNA by using a mix of random and Oligo-dT primers according to the ABgene protocol (ABgene, Epsom, UK). Gene-specific primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3) on the basis of sequences obtained for the genes of interest and 4 potential housekeeping genes (RPS18A, actin, beta-tubulin, eIF4A-2) to serve as the endogenous control ('normalizer'). qRT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene, La Jolla, CA, USA) using the Absolute™ QPCR SYBR® green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix. A dissociation curve analysis was performed

for all primer/probe pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. Furthermore, we tested the four housekeeping genes as invariant endogenous controls in the assay to correct for sample-to-sample variation in qRT-PCR efficiency and errors in sample quantitation, and found that both actin and eIF4A-2 performed best as endogenous controls ('normalizer'). The dynamic range of a given primer/probe system and its normalizer was examined by running triplicate reactions of five different RNA concentrations. Since the target and normalizer had similar dynamic ranges, the comparative quantitation method (ΔΔCt) was used, and data were transformed to absolute values with $2^{-\Delta\Delta Ct}$ for obtaining fold changes between treatments.

In addition, 5 stress-related genes (dehydrin, ethylene precursor 1-aminocyclopropane-1-carboxylate oxidase, allene oxide cyclase, peroxidase, and acidic endochitinase) and 3 pathogenesis-related genes (glutathione S-transferase, PR4, and PR-1A1) were validated by qRT-PCR. The same protocol described above was used, except that 400 ng of total RNA were converted into single-stranded cDNA by using a mix of random and Oligo-dT primers following the Verso™ SYBR® Green 2-Step qRT-PCR Low Rox Kit protocol (Thermo Fisher Scientific, Rockford, IL, USA). Then qRT-PCR was done in optical 96-well plates on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Quantification was performed on three biological replicates for each treatment.

Relative fold changes for each gene were compared to the aphid treatment, which was set to one. qRT-PCR fold change (relative to controls) was analyzed by using a one-way ANOVA with JMP (Sall and Lehman, 1996), followed by a Fisher's Least Significant Difference (LSD) test to assess separation of fold differences.

Carbon and Nitrogen

Leaflets for total leaf carbon and nitrogen analyses were oven-dried at 60°C for 48 h. Carbon and nitrogen concentrations were measured with 5 mg of dried ground leaf material by micro-combustion in an Elemental Combustion System 4010, CHNS-O analyzer (Costech Analytical Technologies, Valencia, CA, USA).

Total Protein

To determine total protein content, we followed methods modified from Jones et al. (1989). Buffer-soluble protein was extracted by homogenizing weighed leaf samples in 0.5 ml of 0.1 M NaOH (pH 11.8). The samples were vortexed for 3 sec and incubated at room temperature for 30 min. After incubation, samples were vortexed again and centrifuged at 11,000 rpm for 10 min at room temperature. Five µl of supernatant were mixed with 250 µl of Bradford reagent (Coomassie brilliant blue) in a cuvette, and the absorbance was measured at 595 nm. Total protein (mg of protein per gram of tissue sample) was calculated using a standard of bovine serum albumin in 0.1 M NaOH.

Proteinase Inhibitors

We measured the plant's ability to inhibit the hydrolysis of the artificial substrate azocasein by commercial trypsin in our four treatments. The procedure followed methods described in Rodriguez-Saona et al. (2005). Samples were ground in Tris HCl (pH 7.8) extraction buffer (3 µl/mg fresh weight), vortexed for 3 min, and centrifuged at 11,000 rpm for 10 min at 10°C. Sixty µl of the supernatant were added to 20 µl of Tris buffer, 50 µl of 2% azocasein in Tris buffer, and 20 µl of a 0.001 M HCl solution containing 200 ng of trypsin. A set of controls with no sample also was prepared for each sample using an identical procedure and adjusting the total volume with the Tris buffer. After incubation for 20 min at 28°C, 100 µl of trichloroacetate (100% w/v) were added to denature the substrate and stop the reaction. Samples then were centrifuged for 10 min at 8,000 rpm. One hundred µl of 1 M NaOH were added to 100 µl of the supernatant from each sample, and absorbance was measured at 450 nm. The activity of trypsin inhibitor is reported as one minus the percent ratio of sample to control absorbance (Orians et al., 2000).

Performance Assays

No-choice bioassays were conducted to test whether prior damage by aphids, caterpillars, or both herbivores differentially affected the performance of beet armyworm caterpillars compared to undamaged plants. Neonates were placed individually in 90-mm Petri dishes lined with moist filter paper and allowed to feed for 5 d on leaflets from one of the four treatments described above. All leaflets were excised from leaf 4. Petri dishes were kept at room temperature. Each trial had a total of 10 caterpillars per treatment ($N=40$) and was repeated 4 times. On day 5, all live caterpillars were weighed and mortality was recorded.

Statistical Analyses of Biochemical and Organismal Assays

The effects of aphids, caterpillars, and trial, and their interactions, on total leaf carbon:nitrogen ratio, total protein, PI activity, and caterpillar mass were analyzed using ANOVA (Systat 1998; SPSS Science, Chicago, IL, USA). *Post-hoc* pair-wise comparisons between treatments were performed using LSD tests. Percent data were arcsin-square root transformed and caterpillar mass were ln-

transformed prior to analysis to meet assumptions of normality and homogeneity of variance. Mortality data were analyzed using *G*-tests.

Results and Discussion

Gene Expression: Specificity of Induction

Specificity in the tomato responses to aphids and caterpillars at the transcriptomic level was detectable, and is represented in the microarray analysis where beet armyworm caterpillars induced the systemic expression of different genes than potato aphids (Fig. 1; Table 1). Herbivory systemically up- or down-regulated approx. 2% of the genes on the Cornell array (277 out of 13440) (Table S1). Eighty percent of the genes (222 out of 277) were up- or down-regulated by only one of the herbivores, confirming substantial specificity of the plant responses. Aphid feeding changed expression of 2.8 times more genes compared to caterpillar feeding (239 vs. 85, respectively) (Fig. 1). Ninety-three genes were up-regulated by aphid feeding, while 56 genes were up-regulated by caterpillar feeding compared to controls; twenty-five of these genes were up-regulated by both aphids and caterpillars (Fig. 1). One hundred and forty six genes were down-regulated by aphids, while 29 genes were down-regulated by caterpillars compared to controls; twenty-two of these genes were down-regulated by both herbivores (Fig. 1). Dual-damage induced changes in 11 genes; 8 of them were also induced by caterpillars alone, while the other 3 genes were induced by both caterpillars and aphids when feeding alone, and the expression of none of these genes was enhanced compared to when damaged by a single herbivore (Table 1).

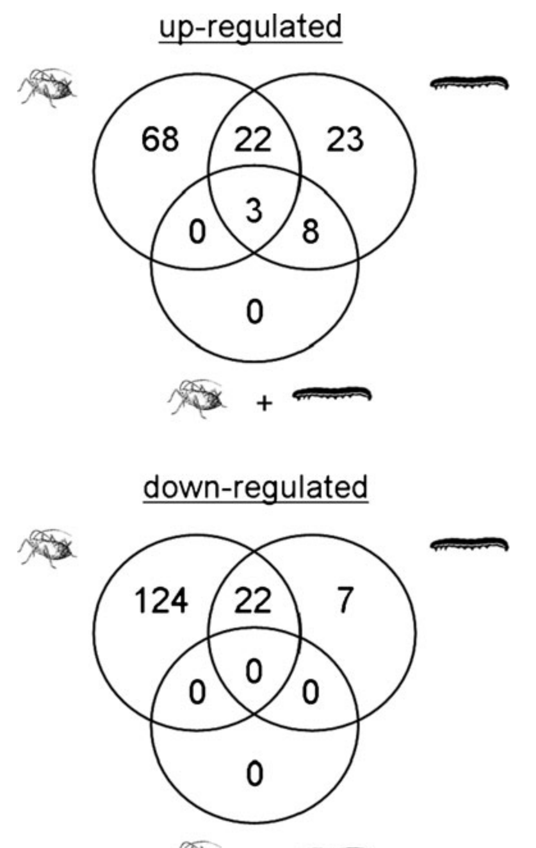


Fig. 1 Venn diagrams of the numbers of overlapping and non-overlapping transcriptional responses of tomato genes up-and down-regulated by aphids, caterpillars, or aphids and caterpillars. Numbers are up- or down-regulated genes that met our combined criteria of a minimum of 2-fold change in gene expression and a significant ANOVA (*P* values≤0.05)

Of all of the genes assayed, the most strongly systemically induced by herbivory were those related to herbivore defense, but gene expression was altered in many functional categories (Table 1). Seven herbivore defense-related genes were systemically induced by caterpillar feeding >10-fold greater than controls; including genes encoding for polyphenol oxidases, threonine deaminase, and an array of different protease inhibitors (Table 1A). These defense genes often are associated with the JA pathway (Fidantsef et al., 1999). In contrast, aphid feeding increased expression of fewer herbivore defense-related genes and to a much lesser extent than following caterpillar feeding (<6-fold induction).

Induction of pathogenesis-related proteins such as PR-1A1 and glutathione S-transferase was not specific to aphids (Table 1B). Similarly, salicylic acid methyltransferase that catalyzes the formation of methyl salicylate, the volatile derivative of SA, was induced by both herbivores (Table 1C). The lack of specificity in plant responses to aphid and caterpillar feeding in this category is surprising given previous results that aphids, but not caterpillars, are strong inducers of salicylate pathway-regulated genes (Walling, 2000). It is possible, however, for caterpillars to induce the SA pathway as shown for *S. exigua* caterpillars feeding on tobacco plants (Diezel et al., 2009). It is also possible for genes in the pathogen defense category to be regulated by JA (Li et al., 2004). An example is the translation-inhibitor protein that was strongly up-regulated by caterpillar feeding (Table 1B). Another possibility is that some of the SA pathway genes might have been missed because they may have been induced locally or soon after herbivore infestation.

Our findings are similar to a study by De Vos et al., (2005) who reported that the aphid *M. persicae* induces more *Arabidopsis* genes than the caterpillar *Pieris rapae* (L.), and a study in tobacco by Voelckel et al. (2004) that found that the magnitude of induction by *Myzus nicotianae* Blackman aphids was smaller than by the caterpillar *Manduca sexta* L.; although more genes were induced by

Table 1 Relative ratios of selected genes up- or down-regulated by aphid, caterpillar, and dual herbivory on tomato plants compared to non-wounded plants. For each gene, ratios with different letters indicate significant differences among treatments ($P \leq 0.05$)

Putative annotation+function	Gene name	Control	Aphid	Caterpillar	Caterpillar +Aphid
A. Herbivore defense related					
acid phosphatase	SGN-U145331	1.00(a)	1.80(a)	4.86(b)	1.80(a)
acid phosphatase	SGN-U145330	1.00(a)	2.08(a,b)	5.11(b)	1.85(a)
arginase	SGN-U145219	1.00(a)	1.06(a)	8.41(b)	4.79(a,b)
aspartic protease inhibitor 1 precursor	SGN-U143342	1.00(a)	3.31(a,b)	17.05(b)	10.79(b)
ethylene-responsive proteinase inhibitor I precursor	SGN-U144127	1.00(a)	2.05(a,c)	14.61(b,c)	9.03(b,c)
leucine-rich repeat resistance protein-like protein	SGN-U144588	1.00(a)	5.88(b)	1.58(a)	1.03(a)
polyphenol oxidase F (PPO) (Catechol oxidase)	SGN-U143365	1.00(a)	-1.63(a)	10.72(b)	2.04(a,b)
proteinase Inhibitor TYPE II TR8 Precursor	SGN-U143905	1.00(a)	4.60(b)	7.12(b)	8.35(b)
threonine deaminase	SGN-U143321	1.00(a)	2.18(a,b)	17.39(b)	10.23(a,b)
wound-induced proteinase inhibitor I precursor	SGN-U143552	1.00(a)	2.59(a,b)	18.49(b)	8.79(a,b)
wound-induced proteinase inhibitor I precursor	SGN-U143556	1.00(a)	3.80(b)	37.66(c)	14.33(b,c)
wound-induced proteinase inhibitor II precursor	SGN-U143329	1.00(a)	2.63(a,b)	20.35(c)	10.58(b,c)
wound-inducible carboxypeptidase	SGN-U148185	1.00(a)	4.21(b)	3.55(b,c)	1.16(a,c)
B. Pathogenesis related proteins					
catalase isozyme 1	SGN-U143191	1.00(a)	-2.67(a,b)	-2.29(b,c)	−1.17(a,c)
hypersensitive response assisting protein	SGN-U146564	1.00(a)	−1.14(a)	2.91(b,c)	1.94(a,c)
pathogenesis-related protein 1A1 precursor (PR-1A1)	SGN-U144656	1.00(a)	2.72(b)	2.03(a,b)	−1.08(a)
probable glutathione-S-transferase (Pathogenesis-related protein 1)	SGN-U143280	1.00(a)	3.79(b)	2.17(a,b)	−1.12(a)
remorin-like protein	SGN-U146116	1.00(a)	−2.53(b)	−2.05(a,b)	1.29(a,b)
translation-inhibitor protein	SGN-U143744	1.00(a)	1.80(a)	10.78(b)	7.62(b)
pto-responsive gene protein	SGN-U144888	1.00(a)	1.20(a)	4.12(b,c)	1.71(a,c)
C. Signaling related					
1-aminocyclopropane-1-carboxylate oxidase homolog	SGN-U143387	1.00(a)	11.41(c)	32.03(b,c)	2.03(a,b)
kinase interacting protein 1 (Petunia integrifolia)	SGN-U156478	1.00(a)	17.05(b)	6.65(a)	2.08(a)
lipase (class 3) family	SGN-U159040	1.00(a)	−4.57(b)	−2.84(a)	1.25(a)
MAP kinase	SGN-U146866	1.00(a)	−2.79(b)	−1.71(a)	−1.19(a)
S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	SGN-U146660	1.00(a)	2.04(b)	2.03(b)	1.00(a)
WIZZ [Nicotiana tabacum]	SGN-U143779	1.00(a)	−6.75(b)	−2.88(a)	1.72(a)
WRKY family transcription factor [Arabidopsis thaliana]	SGN-U145810	1.00(a)	3.33(b)	2.27(a,b)	1.39(a)
WRKY family transcription factor [Arabidopsis thaliana]	SGN-U144503	1.00(a)	−1.03(a)	2.11(b)	−1.23(a)
D. Photosynthesis related					
phytoene synthase	SGN-U143396	1.00(a)	−3.92(b)	−2.43(a,b)	1.43(a)
ribulose bisphospate carboxylase small chain 2A, chloroplast precursor	SGN-U143665	1.00(a)	−2.18(c)	−2.19(b,c)	−1.02(a,b)
triose phosphate chloroplast precursor	SGN-U143665	1.00(a)	−2.05(b)	−2.49(b)	−1.17(a)

caterpillars in the later study than by aphids. A higher number of genes were systemically down-regulated by potato aphid feeding than up-regulated in our study (146 vs. 93; Fig. 1). This is consistent with De Vos et al. (2005) who found a total of 1349 being down-regulated and 832 up-regulated by *M. persicae*. Although we did not find genes expressed only in the dual-damage treatment, we found a different pattern of gene expression in this treatment compared to aphid- and caterpillar-damage treatments (Fig. 2). In fact, our cluster analysis shows greater similarities in gene expression between dual-damaged and control plants than to those damaged by only aphids or caterpillars. Voelckel and Baldwin (2004) also found specificity in gene expression of tobacco plants singly or simultaneously attacked by a piercing-sucking mirid bug and the chewing caterpillar, *M. sexta*. In plants damaged by both herbivores simultaneously, they found a distinct transcriptional pattern from either herbivore alone. In

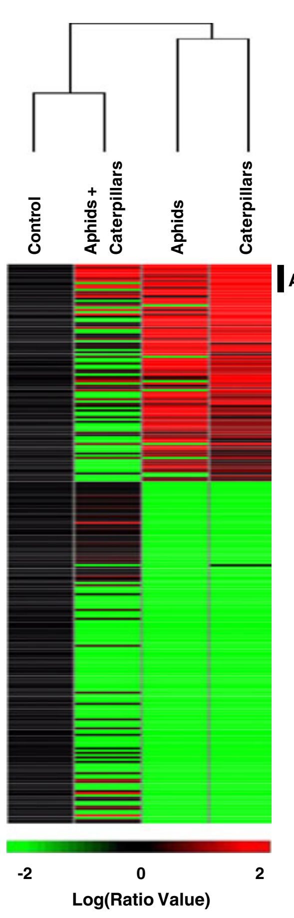


Fig. 2 Cluster analysis of the tomato genes up- and down-regulated in response to feeding by aphids, caterpillars, or aphids and caterpillars. The dendrogram on top of the cluster illustrates the relationship among the four treatments. Ratio values are calculated between herbivore-infested and non-infested control plants (set to 1). Genes up-regulated by treatment appear in red (positive log-ratio values), while those that were down-regulated appear in green (negative log-ratio values). Each row in the column corresponds to a single gene, and a color scale is presented below the figure. Letter A indicates a cluster of many of the plant defense genes associated with Table 1A

Fig. 3 Relative fold differences of arginase (**a**), wound induced proteinase inhibitor II CEV 157 (**b**), threonine deaminase (**c**), wound induced proteinase inhibitor I (**d**), wound induced proteinase inhibitor II (**e**), lipoxygenase (**f**), polyphenol oxidase D (**g**), polyphenol oxidase F (H), dehydrin (**i**), 1-aminocyclopropane-1-carboxylate oxidase (**j**), allene oxide cyclase (**k**), acidic endochitinase (**l**), peroxidase (**m**), glutathione S-transferase (**n**), pathogenesis-related PR4 (**o**), and pathogenesis-related PR-1A1 (**p**) determined with quantitative real time-PCR (qRT-PCR). Tomato plants were damaged by aphids, by caterpillars, or by both aphids and caterpillars. All of the qRT-PCR values are normalized to the aphid treatment (set to 1). Bars indicate means±SE. Different letters represent statistically significant differences between treatments ($P \leq 0.05$). $N=3$

contrast to our finding, however, Voelckel and Baldwin's study found only specificity after the first day of feeding, not in later days, whereas our study shows specificity five days after attack. These two studies thus show distinct genetic outcomes for the integration of specific plant responses when damaged by multiple herbivores.

Gene Expression: Bi-Directional Trade-offs

Suppression of systemic gene expression in the dual-damage treatment, where expression is changed to levels no longer different from controls, was found in 58% of the genes up- or down-regulated by one herbivore (135 out of 222), indicating a high degree of trade-offs in the expression of specific responses (Table 1). These trade-offs were reciprocal and occurred across functional categories. Caterpillar feeding suppressed up- or down-regulation of 66% (127 out of 192) aphid regulated genes, whereas aphid feeding only prevented up- or down- regulation of 8 out of 30 (27%) of the genes regulated by caterpillar feeding (Table 1). The expression of 12 caterpillar-induced genes was confirmed by qRT-PCR (Fig. 3a–l). Of these 12 genes, 5 showed attenuated expression in the dual damage treatment (i.e., expression in the dual-damage treatment was less than in the caterpillar-only treatment but more than in the aphid-only treatment), while 7 showed suppression (i.e., expression in the dual-damage treatment was different from the caterpillar-only treatment but not from the aphid-only treatment).

The pattern of attenuation of expression falls into two categories: 1) in some cases, aphids had no effect on the genes independently (e.g., threonine deaminase), but reduced the response of those genes to caterpillar damage (Tables 1 and 2) more remarkably, some genes (e.g., proteinase inhibitor gene I—SGN-U143556) that are strongly up-regulated by caterpillar feeding, and weakly induced by aphid feeding, the expression in dual-damaged plants was still reduced compared to the caterpillar-only treatment (Table 1). The same was true for some genes that showed aphid-dependent regulation: caterpillar alone had no effect (e.g., MAP kinase; Table 1), but attenuated the down-regulation of these genes to aphid damage in the dual attack and some genes that were strongly down-regulated by aphid feeding and weakly suppressed by caterpillar feeding (e.g., lipase; Table 1), still were attenuated in the dual-damage treatment compared to the aphid only treatment.

Thus, although aphids may minimize the magnitude of induction of plant responses by causing little cellular damage and avoiding detection, they also reduce the plant's ability to induce the responses to caterpillar feeding. This is consistent with the "decoy hypothesis," which states that by inducing the salicylate pathway aphids can suppress the jasmonate pathway defenses to their own benefit (Zhu-Salzman et al., 2004; Thompson and Goggin, 2006; Zarate et al., 2007). Our evidence shows that aphid feeding can suppress or attenuate induction of many jasmonate pathway-regulated genes, but we do not have evidence that this occurs due to induction of the salicylate pathway as the pathogen defense-related genes were induced similarly in caterpillar and aphid treatments (Table 1B; Fig. 3n–p). The genes in our "pathogen defense-related" category may or may not be regulated by the salicylate pathway. However, based on the microarray data even the two genes known to be salicylate-regulated PR proteins 1 and 1A1 were only weakly up-regulated by aphids (Table 1B), while qRT-PCR analyses detected no differences in PR protein induction

Quantitative RT-PCR

Arginase
a
Relative fold difference
12 9 6 3 0
a b c

Wound Induced proteinase inhibitor II CEV157
b
Relative fold difference
5.0 4.0 3.0 2.0 1.0 0.0
a b c

Threonine deaminase
c
Relative fold difference
16 12 8 4 0
a b c

Wound Induced Protease inhibitor I
d
Relative fold difference
100 80 60 40 20 0
a b c

Wound Induced Protease inhibitor II
e
Relative fold difference
50 40 30 20 10 0
a b c

Lipoxygenase
f
Relative fold difference
2.5 2.0 1.5 1.0 0.5 0.0
a b c

Polyphenol oxidase D
g
Relative fold difference
6.0 4.0 2.0 0.0
a b a

Polyphenol oxidase F
h
Relative fold difference
80 60 40 20 0
a b a

Quantitative RT-PCR

i Dehydrin

j 1-aminocyclopropane-1-carboxylate oxidase

k Allene oxide cyclase

l Acidic endochitinase

m Peroxidase

n Glutathione-S-transferase

o Pathogenesis-related PR4

p Pathogenesis-related PR-1A1

Relative fold difference

Fig. 3 (continued)

Table 2 Summary of 3-way ANOVAs for the effects of aphids, caterpillars, and trial on amounts of carbon, nitrogen, protein, and proteinase inhibitors in tomato plants, as well as the effects on *Spodoptera exigua* caterpillar mass. numbers indicate *F* values

Source	C/N	Protein	PI	Caterpillar mass
Aphids (A)	1.38 ns	0.13 ns	2.38 ns	0.06 ns
Caterpillar (C)	2.08 ns	15.06**	58.21**	38.93**
Trial (T)	7.68**	158.43**	3.34*	0.36 ns
A x C	5.11*	4.33*	0.09 ns	3.57[a]
A x T	0.44 ns	0.27 ns	1.78 ns	0.87 ns
C x T	2.15 ns	0.91 ns	6.26**	1.81 ns
A x C x T	2.03 ns	0.92 ns	0.57 ns	1.00 ns

[a] $0.07 \geq P > 0.05$; *=$0.05 \geq P > 0.01$; **=$P \leq 0.01$

between single and dual damaged treatments (Fig. 3o–p). In addition, we found attenuation of genes in many categories, not just those involved in defense (Table 1C–D). However it is achieved, attenuation of the jasmonate pathway expression may benefit the caterpillars and aphids, as both have lower performance on tomato plants treated with JA (Thaler et al., 2001; Cooper and Goggin, 2005).

Gene Expression: Lack of Specificity

There was an overlap of gene regulation in the case of 44 genes (out of the 277 genes influenced by herbivory), where both aphids and caterpillars, when feeding alone, up-regulated or down-regulated the systemic expression of the same genes (22 and 22 genes, respectively). Interestingly, however, for all 44 genes that lacked specificity the expression of these genes in the dual-damage treatment was not different from the controls. These included herbivore defense related genes (e.g., wound-inducible carboxypeptidase), photosynthesis related genes (e.g., triose phosphate chloroplast precursor), and signaling related genes (e.g., 1-aminocyclopropane-1- carboxylate oxidase homolog) (Table 1). For example, aphids and caterpillars down-regulated several genes associated with photosynthesis when feeding alone but not when feeding together on the same plant (Table 1C). A possible explanation for this scenario is that almost no gene is inducible only by a single transcription factor or signaling pathway. If two insects that induce different signaling pathways/transcription factors feed simultaneously on a plant, then these pathways/factors might interfere with each other. This could result in negative feedback or inhibition leading to a zero sum transcription of the respective subset of genes.

Ultimately the translation of these genetic changes to impacts on herbivores is what is critical, and such data from our system are discussed below.

Chemistry and Bioassays: Foliar Chemicals

Interactions between plant responses to caterpillar and aphid feeding impacted plant nutritional quality, as shown by the significant aphid×caterpillar effects on C/N and protein (Table 2). Aphid feeding decreased the C/N ratio in systemic leaves while caterpillar feeding had no effect (Fig. 4a). When both herbivores fed on the plant, however, the C/N ratio was equal to the control treatment, indicating that caterpillar feeding affected the plant's response to aphids. This effect is consistent with aphids manipulating the nutritional content of plants for their own benefit by increasing the proportion of nitrogen in leaves (e.g., Flynn et al., 2006), which is critical considering that leaf nitrogen is often a limiting resource for herbivorous insects (Mattson, 1980). This likely explains our previous finding that caterpillar consumption (in the first 7 days of development) increases on aphid-damaged plants compared to control plants, and also that the amount of caterpillar consumption on caterpillar-damaged plants was not affected by aphids (Rodriguez-Saona et al., 2005). Stout et al., (1998) also reported increased growth rates of beet armyworm caterpillars on tomato plants previously damaged by aphids compared to undamaged plants. Aphid and caterpillar feeding alone had no effect on the amount of protein in systemic leaves. However, both herbivores feeding on the plant reduced the amount of protein compared to aphid-damaged plants (Fig. 4b).

Our data show that caterpillars induced systemically high PI enzymatic activity, but this was not affected by aphids. Caterpillar-damaged plants had three times higher PI enzyme activity compared to control plants (Fig. 4c; Table 2), while aphids had no effect on PI activity, even though they did weakly induce PI gene expression (see results above). This is consistent with Stout et al. (1998) who found that local feeding by *Helicoverpa zea* (Boddie) increased PI activity, whereas feeding by potato aphids had no effect. However, the presence of aphids did not affect the PI activity induced in response to caterpillar damage even though our transcriptomic data indicated that aphid feeding strongly interfered with PI gene expression. Thus, although the results of the current study indicate that aphid feeding could both weakly induce expression of several PI genes and cause attenuation of PI genes induced by caterpillars, neither of these effects on gene expression led to a change in PI activity. Gene expression may not translate directly to protein activity due to a level of

Fig. 4 Effects of damage singly by aphids or caterpillars or simultaneous damage by aphids and caterpillars on amounts of carbon/nitrogen (C/N) ratio (**a**), protein (**b**), and proteinase inhibitors (**c**), as well as on *Spodoptera exigua* caterpillar mass (**d**) and mortality (**e**). Bars indicate means±SE. Different letters represent statistically significant differences between treatments ($P \leq 0.05$). N=3–4

transcript above which no more protein is made, post-transcriptional regulation, or interactions between gene products. Alternatively, the PI assay used simply may not be sensitive enough. In fact, in our previous study (Rodriguez-Saona et al., 2005) PI activity was about 20% lower in the dual-damage treatment than in the caterpillar only treatment, although this difference was not significant and not observed in the present study.

Chemistry and Bioassays: Caterpillar Performance

Prior caterpillar feeding decreased the mass of bioassay caterpillars by 54% (Fig. 4d) and increased caterpillar mortality by 28% (Fig. 4e). Bioassay caterpillars were 46% heavier and had 5% lower mortality on aphid-damaged plants compared to control plants, but these differences were not significant (Fig. 4d–e; G=0.27, P=0.6). Caterpillar performance on dual-damaged plants was similar to that on caterpillar-damaged plants (Table 2).

Therefore, caterpillar damage had a strong negative effect on subsequent caterpillar growth and survivorship, whereas aphid damage had only a weak and non-significant effect on caterpillar performance, and we found no attenuation of resistance in dual-damaged plants. These bioassays reveal the complicated relationship between gene expression, biochemical activity, and impacts on herbivores. In a previous paper (Rodriguez-Saona et al., 2005), we demonstrated that aphid feeding does influence compo-

nents of *S. exigua*'s ecology. For example, moth host acceptance was substantially lower on caterpillar-damaged plants, higher on aphid-damaged plants, and intermediate on dual-damaged plants. This finding is concordant with gene expression results from the current study and others that find conflict between some JA and SA regulated genes and synergisms between others (Schenk et al., 2000; Bodenhausen and Reymond, 2007). In our previous study, we further concluded that beet armyworm moth preference correlates with caterpillar performance only when plants were damaged by a single herbivore (aphids or caterpillars), but not in the dual-damaged treatment (Rodriguez-Saona et al., 2005). Insect preference and performance often are not correlated in part because different plant traits influence these aspects of herbivore biology (e.g., Prudic et al., 2005). Thus, some components of the plant's response to caterpillars that are influenced by aphids have impacts on other herbivores. Because interactions with multiple herbivores on the same plant are common under more natural conditions (Strauss, 1991; Hufbauer and Root, 2002) and because aphid densities are often reported to be very high in the field (Dixon, 1977), it is likely that under these conditions the interference by aphids in direct plant defenses against chewing herbivores can be even stronger than observed in our experiments.

In summary, we employed a combination of molecular, biochemical, and organismal approaches to investigate the interactions between tomato plants, the beet armyworm caterpillar, and the potato aphid. At the molecular level, both attacking insects influenced each other. At the biochemical level, the presence of aphids was too weak to change the PI activity (caterpillar-induced proteins), but the presence of caterpillars did alter the aphid influence on C/N ratios. Finally, at the organismal level, aphids did not overwrite the negative effects of caterpillars on the caterpillar's performance. However, our previous study (Rodriguez-Saona et al., 2005) shows that aphids do overwrite the repellency effects of caterpillars on the moth's oviposition preference.

When comparing across all three biological levels of organization, our data produced consistent results when considering each herbivore separately: caterpillars induced high expression of several JA-regulated defensive genes, resulting in high PI activity and reduced caterpillar performance, whereas aphids induced weak expression of only a few defensive genes associated with the JA pathway and did not induce PI activity or affect herbivore performance; but not when considering them together: when plants were attacked simultaneously by caterpillars and aphids, strong interactions exhibited at the molecular level translated into interactive effects on some chemicals, i.e., C/N ratio, but not PI activity, and some components of host suitability for herbivores, i.e., adult preference, but not caterpillar performance. Taken together, our study shows that caterpillar performance on plants could not be explained simply by the levels of defenses but by their balance with the nutritional content as a result of single or multiple herbivory. This study is a reminder that predictions by global gene expression studies need further verification across different levels of biological integration. Future studies will explore the mechanisms for attenuation in the dual treatment, characterize the transcriptional responses of JA and SA signaling genes in a time course experiment, and investigate aphid performance on single- and dual-damaged plants.

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opment (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Feussner and Wasternack, 2002), drought stress (Moons et al., 1997), accumulation of storage proteins (Beardmore et al., 2000) and leaf senescence (He et al., 2002) although the latter effect is controversial (see Seltmann et al., 2010). Jasmonates are especially well known for their roles played in defense against tissue damage, pathogen infection, and herbivory (van Kleunen et al., 2004; Arnold and Schultz, 2002; Howe, 2004; Babst et al., 2005; Truman et al., 2007), and serving as local and systemic signal molecules (Zhang and Baldwin, 1997; Stratmann, 2003; Ferrieri et al., 2005; Thorpe et al., 2007). They promote the transcriptional modification of numerous genes (Jung et al., 2007) that can impact whole-plant resource allocation of both nitrogen- and carbon-containing substrates (Meuriot et al., 2004; Babst et al., 2005; Gómez et al., 2010), as well as manifest in responses at cellular levels (Creelman and Mullet, 1997; Rickauer et al., 1997; Xie et al., 1998; Reymond and Farmer, 1998; Weber, 2002; Cheong et al., 2002; Farmer et al., 2003; Pauwels et al., 2008).

Such responses can manifest in the up-regulation of specialized biochemical pathways that have evolved as defenses against abiotic and biotic stressors (Osbourn, 1996; Farmer et al., 2003; Zhao et al., 2005). For example, the pathway of phenolic acid metabolism in plants requires the initial steps of general phenylpropanoid metabolism providing precursors for the synthesis of lignin, tannins, flavonoids, and other phenolics that serve to build physical barriers and/or reserves of alleochemicals in plant defense (Arnold and Schultz, 2002). It is well known that JAs will elevate the activity of key enzymes, including phenylalanine ammonia-lyase (Hudgins et al., 2004; Bower et al., 2005) and caffeoyl CoA *O*-methyltransferase (Lee et al., 1997), that promote the deamination of phenylalanine, the primary substrate of this pathway, as well as catalyze the *O*-methylation of key intermediates leading to increased lignification (Lee et al., 1997; Caño-Delgado et al., 2003; Hudgins et al., 2004).

Upstream from the phenylpropanoid pathway, the biosynthesis of aromatic compounds proceeds via the seven-step shikimate pathway to a branch point intermediate chorismate (Kloosterman et al., 2003). Chorismate subsequently is converted to three aromatic amino acids, tryptophan, tyrosine, and phenylalanine, as well as other aromatic compounds, via specific terminal pathways. This process is coupled tightly via metabolite feedback control mechanisms (see dashed lines in Fig. 1) impacting a subset of three key regulatory enzymes in the pathway: 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, chorismate mutase, and prephenate dehydratase (Bickel and Schultz, 1979; Herrmann and Weaver, 1999). Yet, little is known about the regulation of the shikimate pathway in concert with downstream phenolic metabolism.

Current "omics" research on plant defense responses has provided many new insights into induced plant responses and when coupled with physiological and biochemical studies at the whole plant level, can be illuminating. For example, Naoumkina et al. (2007), using cell cultures from *Medicago truncatula,* observed a 24 h delay in transcriptional modification of the early metabolic steps of the phenylpropanoid pathway after MeJA treatment, but a rapid (within hours) increase in medicarpin, an isoflavonoid produced from this pathway. This highlights that transcriptional up-regulation is not necessarily coupled with biochemical up-regulation.

Understanding the latent manifestations of highly integrated and interactive response networks can be difficult (Meuriot et al., 2004; Jung et al., 2007; Pauwels et al., 2008). In a recent prospectus, Schwachtje and Baldwin (2008) suggest that a key link in the defense network is reprogramming of primary metabolism, where such metabolites may themselves serve as signals that subsequently activate downstream pathways. Thus, primary metabolites may serve dual roles as signal and substrate for downstream secondary metabolism.

Here, we report on the use of the short-lived radioisotope, carbon-11 ($t_{½}$ 20.4 min) as $^{11}CO_2$ to measure in *Nicotiana tabacum* the partitioning of new carbon into metabolite pools of photosynthate, as well as into metabolites of the shikimate pathway. The use of carbon-11 for studying the partitioning of new carbon into metabolic pools enabled us to retest the same plant over short periods of time. These studies would be difficult to carry out if not impossible by using carbon-14 or carbon-13, as the metabolic pools would become enriched with tracer preventing a clear delineation between new and old carbon utilization. Furthermore, the use ^{11}C at true tracer concentrations placed us in a unique position to explore the effects MeJA had on [^{11}C]photosynthate pools unimpaired by the feedback control mechanisms described above. Most particularly, we were able to explore how such effects filtered down to regulation of the shikimate pathway and its coordination with up-regulation the phenylpropanoid pathway.

Here, we hypothesized that MeJA would cause rapid changes in [^{11}C]photosynthate pools, thus favoring increased partitioning of new carbon into amino acids. Furthermore, we hypothesized that MeJA would impart selective control over the shikimate pathway, thus giving rise to different partitioning of new carbon into its metabolites. We showed for the first time that defense induction, triggered by application of MeJA, can manifest in selective reprogramming of new carbon utilization in the shikimate pathway. As importantly, we show that metabolic reprogramming can be transient matching key transcript expression patterns.

Fig. 1 Shikimate pathway showing known metabolite feedback loops (dashed lines) that can affect metabolic regulation. This pathway is controlled by three key regulatory enzymes: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, chorismate mutase, and prephenate dehydratase. Metabolite feedback loops truncate at specific branch points within the pathway and are designated by+or—for promotion or inhibition (as adapted from Bickel and Schultz, 1979; Herrmann and Weaver, 1999)

Methods and Materials

General Approach In the studies described, we devised a protocol for measuring the effects of our treatment on the partitioning of recent carbon (as ^{11}C) and unlabeled endogenous carbon (as ^{12}C), into key metabolite pools. Specifically we dosed leaf-3 (counting down from the apex) of an intact plant with $^{11}CO_2$ tracer, and then 4 h or 15 h after treatment, we administered a second dose tracer to leaf-4 of the same plant. A 2 d study cycle (Fig. 2) shows the coordination in time between baseline and retest tracer administrations with MeJA treatment and the plant's light/dark cycle. The present study also required extraction of small amounts of tissue from the plant through leaf excision at its petiole. Prior to beginning our MeJA studies, we performed a proof-of-concept study to confirm that leaf excision would not induce metabolic reprogramming. At 4 and 15 h post leaf-3 excision, the [^{11}C]photosynthate composition was unchanged between leaf-3 and leaf-4 as was the ^{11}C amino acid profiles for tyrosine, phenylalanine, and tryptophan (data not shown). In summary, we found that cleanly excising a study leaf from the plant at its petiole does not induce changes in the metabolites of interest within the remaining tissues. With this knowledge, we designed an experimental protocol for testing same plants by using separate doses of ^{11}C before and after treatment.

Plant Material Tobacco plants (*Nicotiana tabacum* L. cv Samsun) were grown from seed in commercial potting mix

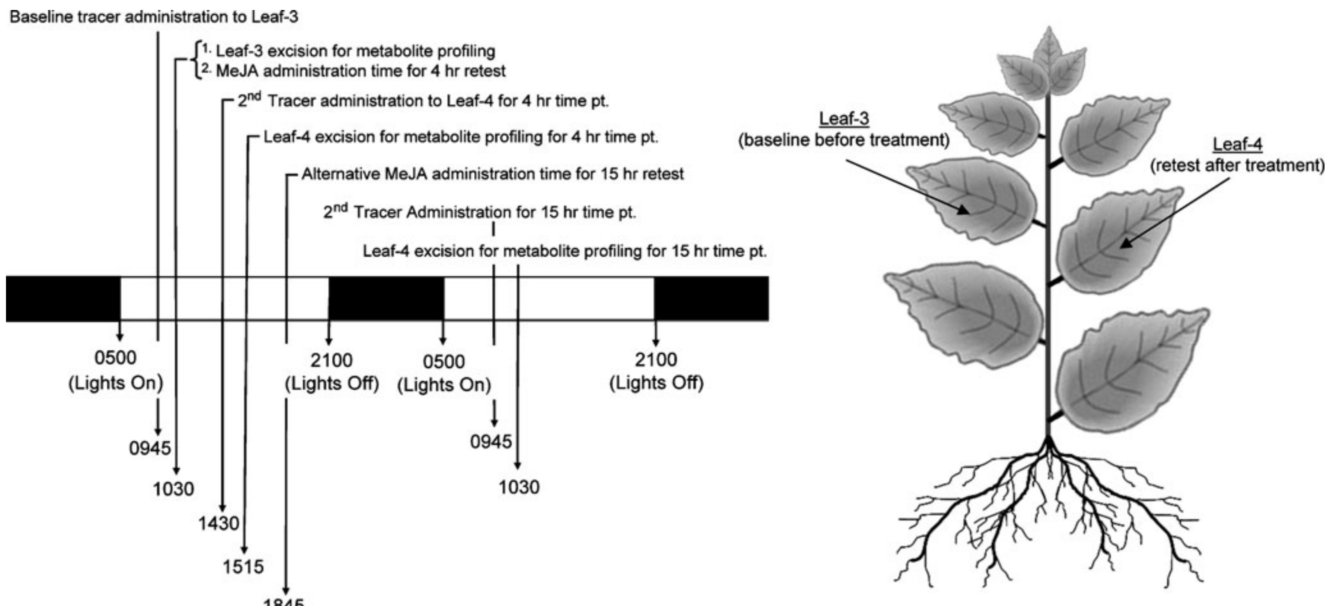


Fig. 2 Two-day study cycle of plants (*Nicotiana tabacum*) subjected to either a 4 h or 15 h metabolite profiling. Changes in metabolite profiles at either time point were always compared back to baseline responses from the same plant. Baseline ^{11}C tracer administration was always at 09.45 h Leaf excision for tissue extraction always occurred 45 min after tracer administration. Note that the 4 or 15 hr metabolite profiling time points dictated the time of day when control or MeJA treatments were applied to the study plant. Treatments were always applied during the light cycle

with a slow release fertilizer (Osmocote) under metal-halide lamps at 24°C with a 16/8 h-350 umol m^{-2}sec^{-1} photoperiod, and were used for experiments at their 7-leaf stage of development.

Radiotracer Production $^{11}CO_2$ was produced *via* the ^{14}N (p,α)^{11}C nuclear transformation (Ferrieri and Wolf, 1983) from 50 ml volume high-purity nitrogen gas target using 17 MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at Brookhaven National Laboratory, and captured on molecular sieve (4Å) (full details in Ferrieri et al., 2005).

Radiotracer Administration The $^{11}CO_2$ that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 400 ml min^{-1} as a discrete pulse for labeling a leaf in a 5×10 cm lighted cell (920 umol m^{-2}sec^{-1}) (Ferrieri et al., 2005). A PIN diode radiation detector (Bioscan Inc., Washington DC, USA) affixed to the bottom of the cell enabled continuous measurement of radioactivity levels within the cell. The difference between the amount of radioactivity registered in the pulse height, and residual radioactivity after the pulse had passed through the cell was a reflection of leaf tracer fixation. This value was used to normalize measurements to the same amount of carbon-11. In past studies (Ferrieri et al., 2005), we were able to correlate these measurements in radioactivity with direct changes in the CO_2 concentration entering and exiting the leaf cell by using infrared gas exchange, IRGA (Li-Cor model 6162).

We administered ^{11}C to leaf-3 and leaf-4 in series, before, and after treatment. Leaf designation was determined by counting down from the apex, with leaf-1 designated as the first leaf that was expanded to 50% of its full capacity.

Tissue Extraction and Derivatization Thirty minutes after the tracer was administered, the study leaf was cleanly excised at its petiole. The 5×10 cm area exposed to $^{11}CO_2$ was cut away from the remaining leaf tissue, weighed, and flash frozen in liquid nitrogen (LN_2). Using a mortar and pestle, the frozen tissue was ground to a fine particulate, and extracted in 3 ml of methanol (50% v/v in DI water) for 10 min at 90°C. The extract was separated by pipette and filtered through a 0.22 μM filter (Millipore Inc., Billerica, MA, USA). The total volume of the extract was measured and 150 μl aliquot delivered into a 2 ml brown-glass vial (Fisher Scientific, Inc., Pittsburgh, PA, USA). An equal volume of *o*-phthalaldehyde amino acid derivatizing reagent (OPA) containing 0.1% (v/v) mercaptoethanol and 0.1% (v/v) sodium hypochlorite (Sigma Life Science, St. Louis, MI, USA) was delivered into the same vial. The mixture was vortexed and then allowed to react at ambient temperature for 3 min. Primary amino acids are readily converted into UV absorbing iso-indole derivatives by OPA enabling their separation with reversed-phase high-performance liquid chromatography and quantification (Chow et al., 1987). While the OPA method has been

successfully used for measuring amino acids in purified plant extract, some have questioned how effective the method is for unpurified extracts (Martin et al., 1982). To confirm the validity of this method, we tested several amino acid standards in pure methanol/water (50% v/v), and in a methanol/water solution containing unpurified tissue extract, and subjected each sample to OPA reaction. Using the chromatography method described in the next section, we observed the same UV response from standards (within 5% standard error) in both types of formulations.

Analysis of Amino Acids A 50 μl volume of the derivatized mixture was injected onto a reversed-phased analytical HPLC column (Phenomenex, Torrance, CA, USA: Ultramex™ C_{18}, 10 μ particle size, 250×4.6 mm i.d.) using a pre-column gradient mixer (Isco, Lincoln, NE, USA) and a mobile phase comprised of A (DI water), B (0.01 M NaH_2PO_4-buffered at pH 6.8 using trifluoroacetic acid), and C (methanol). At injection, the mobile phase (1.5 ml min^{-1}) was sustained at 75% A: 25% B for 5 min and then programmed to attain 20% B: 80% C by 30 min. The mass levels of the amino acid derivatives were measured with a variable wavelength UV detector (Sonntek Inc., Upper Saddle River, NJ, USA) at 340 nm. The outlet of the UV mass detector was connected in series to a NaI gamma radiation detector (Ortec Inc, Oak Ridge TN, USA) that enabled direct measurement of the amount of radioactivity associated with each substrate eluting the column. Analog outputs from both the mass and radiation detectors were fed to a chromatography data acquisition station (SRI Instruments, Torrance, CA, USA) where integrated peak areas were measured from both inputs.

Compounds were identified by their elution times and correlated to those of authentic standards. Changes in endogenous substrate concentrations were correlated by correcting each chromatogram for the gram fresh weight of tissue that was used for a particular extraction. Samples also were corrected for analytical dilutions. Changes in radioactive substrate levels were normalized to a standard amount of ^{11}C tracer that was administered to a particular leaf. Radioactivity data was decay corrected back to a common time point owing to the extremely short half-life of ^{11}C (20.4 min). An additional 50 μl aliquot was taken in order to assay for total [^{11}C]photosynthate radioactivity in each tissue extract. This measurement was carried out using a separate static gamma counter. The efficiencies of both the flow and static gamma counters were calibrated against standards.

We note that although a full profile of [^{11}C]amino acids was measured, that data went beyond the scope of the present work and are not reported here. However, we pooled all observed radioactive amino acids and reported their total distribution relative to the soluble [^{11}C]photosynthate fraction.

Analysis of Radioactive Soluble Sugars A 50 μl volume of the methanol:water extract was injected onto a reversed-phased analytical HPLC column (Phenomenex, Torrance, CA, USA: Luna™ NH_2, 5 μm particle size, 250×4.6 mm i.d.). At injection, the mobile phase (1.5 mlmin^{-1}) was sustained at 80% acetonitrile: 20% water. Elution profiles of soluble sugars were matched against standards using a refractive index (RI) detector (Sonntek Inc., Upper Saddle River, NJ, USA). For radioactive extract analysis, the outlet of the RI detector was connected in series to a NaI gamma radiation detector (Ortec Inc, Oak Ridge TN, USA) that enabled direct measurement of the amount of radioactivity associated with each substrate eluting the column. Analog outputs from both the mass and radiation detectors were fed to a chromatography data acquisition station (SRI Instruments, Torrance, CA, USA) where integrated peak areas were measured from both inputs. The same corrections were made, as described above, for sample size and radioactive decay of the tracer. For the purpose of this work, all radioactive sugars were pooled and related back to their total distribution relative to the soluble [^{11}C]photosynthate fraction.

Measurement of Endogenous Cinnamic Acid Levels PAL catalyzes the formation of *trans*-cinnamic acid from phenylalanine. Therefore, measurement of endogenous cinnamic acid levels as a function of time after treatment should give us insight into the coordination between downstream phenolic metabolism and the shikimate pathway. In parallel studies, leaves were dried for 24 h in an oven at 70°C, weighed to obtain a dry mass, and extracted in tetrahydrofuran (Sigma Life Science, St. Louis, MI, USA) for 3 h at 25°C according to the procedure of Budi-Muljono et al. (1998). Gas chromatography analysis was performed on aliquots of tissue extract using a non-polar fused-silica column (30 m×0.53 mm i.d.) coated with methyl silicone gum (HP-1: Hewlett Packard Co. Rockville, MD, USA) at a film thickness of 2.65 μm. The gas chromatograph (Hewlett Packard 5890 Series II) was equipped with a flame ionization detector operated at a temperature of 300°C. The other instrument conditions were as follows: injector temperature, 230°C; carrier gas-helium; carrier gas inlet pressure, 100 kPa; oven temperature, 50–250°C at 10°Cmin^{-1}. Analyses were performed using the splitless injection mode. Data was acquired using a Vision IV chromatography station (Scientific Systems, Inc., State College, PA, USA). *Trans*-cinnamic acid was identified based on its elution time matched against that from an authentic sample. Peaks were integrated and correlated to a mass amount using a calibrated detector mass response curve.

Treatments Plants were sprayed uniformly with either DI water (as a control) or a 500 μM solution of MeJA in DI water immediately following excision of leaf-3 to acquire the baseline measurement of metabolic partitioning of tracer. Because MeJA was shown to distribute rapidly throughout the plant, we opted to treat the entire above-ground foliar tissue (Thorpe et al., 2007). After treatment, plants were housed individually in a positive air flow holding box illuminated using auxiliary halogen lights (300 $\mu mol m^{-2} sec^{-1}$). This ensured that interplant communication via plant volatile emissions would not compromise retest responses in study plants. Plants then were retested using leaf-4, and a second dose of ^{11}C tracer, at 4 or 15 h after treatment (Fig. 2).

Statistical Analysis One of the advantages of using short-lived radioisotopes is that they do not accumulate in the plant, and so retests can be performed over time and after treatment allowing the same plant to be used as its own control. All data derived from matched plant studies (baseline/post-treatment) and were presented as change in response relative to baseline values. As pointed out earlier, by using the same plant in a test-retest protocol, rather than averaging values across individual plants at baseline (untreated) and post-treatment (treated), we obtained a smaller standard error. Comparisons were made in the change of the ^{12}C and ^{11}C profiles from baseline levels to post-treated levels of control and MeJA treated plants using ANOVA single-variate analysis.

Results

The effects of MeJA on primary plant metabolism were evident from the changes seen in the [^{11}C]photosynthate composition (Fig. 3) 4 and 15 h after treatment. In unstressed plants (N=4), the typical [^{11}C]photosynthate comprised 90.2±2.0% soluble sugars, 3.0±0.6% amino acids and 6.8±2.1% of other labeled substrates that were not accountable in our sugar and amino acid analyses. Control treatments (N=5) had no effect on this distribution. However, MeJA (N=4) caused a significant decrease in the [^{11}C]sugar fraction to 83.7±2.3% (P=0.041) by 4 h post-treatment relative to controls with a commensurate increase in the [^{11}C]amino acid fraction to 6.3±0.9% (P=0.039) and an increase in the other [^{11}C]labeled substrate fraction to 10.0±2.5% (P=0.046). This trend was more significant by 15 h post-treatment using MeJA with the [^{11}C]sugar fraction decreasing to 59.4±6.6% (P<0.001), the [^{11}C] amino acid fraction increasing to 27.6±3.1% (P<0.001), and the [^{11}C]other fraction increasing to 13.0±7.5% (P=0.054). Alhough tissues were extracted just 30 min after tracer administration, leaf export of selected components comprising the [^{11}C]photosynthate mix might have altered its composition in the study leaf. However, we note that leaf export of [^{11}C]photosynthate by this time point was unchanged by MeJA treatment: 19.1±5.1% of fixed ^{11}C in untreated leaves and 19.6±2.8% 15 h after MeJA treatment (Gómez, unpublished).

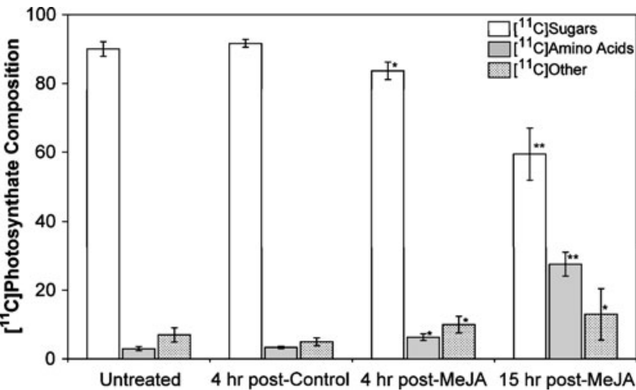


Fig. 3 Effect of treatments of *Nicotiana tabacum* at 4 h (control, N=5; MeJA, N=4) and 15 h (MeJA, N=4) on the partitioning of new carbon as ^{11}C into soluble sugar, amino acid and unknown substrate fractions of [^{11}C]photosynthate. Error bars denote standard deviations on means. Data bars denoted by * and ** indicate that change in ^{11}C-partitioning into metabolic pools from the untreated state to the control or MeJA treated states was significant at the 5% and 1% confidence levels, respectively, according to the ANOVA single variate analysis

Over the same timeline ^{11}C-partitioning into tyrosine (Fig. 4a) was only slightly elevated by MeJA at 4 h post-treatment though significantly (P=0.28) relative to controls. For phenylalanine (Fig. 4b) and tryptophan (Fig. 4c), ^{11}C-partitioning into these amino acids was much more responsive to MeJA with each showing a 12-fold elevation relative to baseline levels. These changes were significantly different from those of control treatments (P<0.001 and P=0.017, respectively). Interestingly, no change in the ^{12}C profiles of these compounds was noted during this time course. By 15 h, the elevated partitioning of ^{11}C into phenylalanine and tyrosine returned to baseline levels, and were not significantly different from those of controls. [^{11}C] Tryptophan levels, however, continued to grow in magnitude reaching a 30-fold elevation relative to baseline levels (P<0.001). Again, no change was noted in the ^{12}C profiles of these metabolites by 15 h post-treatment. Like others (e.g., Zangerl, 2003), we observed that MeJA reduced leaf level photosynthetic activity by 40% (by 15 h post-treatment) although this result was only marginally significant (P=0.075).

Finally, our studies assessed changes in endogenous cinnamic acid levels over the same timeline of treatment. PAL catalyzes the conversion of phenylalanine to *trans*-cinnamic acid which serves as a substrate for subsequent

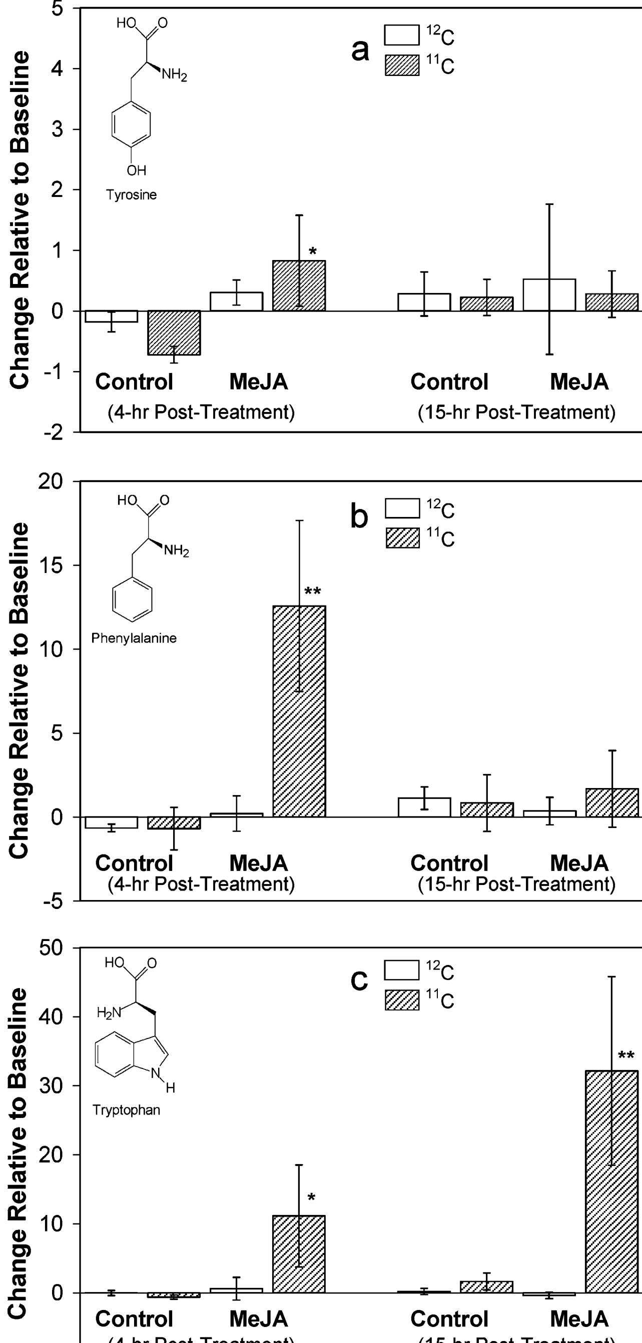
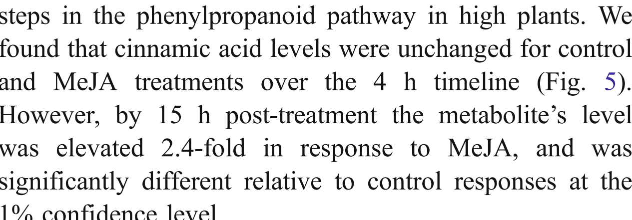


Fig. 4 Effect of treatments of *Nicotiana tabacum* at 4 h (Control, N= 4; MeJA, N=4) and 15 h (Control, N=4; MeJA, N=3) on the metabolic partitioning of ^{12}C and ^{11}C into the shikimate pathway involving biosynthesis of tyrosine (Fig. 4a), phenylalanine (Fig. 4b), and tryptophan (Fig. 4c) amino acids. Results presented as the change in ^{12}C and ^{11}C levels within individual compounds relative to their baseline values (untreated). Error bars denote standard deviations on means. Data bars denoted by * and ** indicate that the change in ^{11}C-partitioning into specific aromatic amino acids from the untreated state to the control or MeJA treated states was significant at the 5% and 1% confidence levels, respectively, according to the ANOVA single variate analysis


steps in the phenylpropanoid pathway in high plants. We found that cinnamic acid levels were unchanged for control and MeJA treatments over the 4 h timeline (Fig. 5). However, by 15 h post-treatment the metabolite's level was elevated 2.4-fold in response to MeJA, and was significantly different relative to control responses at the 1% confidence level.

Discussion

While compensatory photosynthesis is considered an important physiological response of plants (McNaughton, 1983; Crawley, 1983; Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999), it generally is restricted to re-growth tissue, and is not a universal response (Zangerl et al., 1997). A reduction in the available carbon resources through suppression of photosynthetic activity might reduce the endogenous pools of amino acids since there would simply be fewer carbon skeletons available. The fact that we see no change in the size of the endogenous aromatic amino pools, even though metabolic partitioning of new carbon (as ^{11}C) into these pools is seen to increase, may be a reflection of the balance between carbon input, metabolic reprogramming, and even resource re-allocation (i.e., physical transport from source tissues). Indeed, partitioning of new carbon (as ^{11}C) away from soluble sugars and into amino acids is evident with 27.6% of the [^{11}C]photosynthate comprising amino acids by 15 h post-treatment with MeJA. Given the short-lived nature of the isotope, we doubt that the increased [^{11}C]amino acid fraction after MeJA treatment is due to increased protein turnover. Furthermore, other studies by us have shown that MeJA will increase radioactive ^{13}N ($t_{½}$ 9.97 min) utilization as amino acids, as well as increase leaf export of these substrates (Gómez et al., 2010). Even so, there are many conditions (e.g., pathogen attack) that will reduce photo-

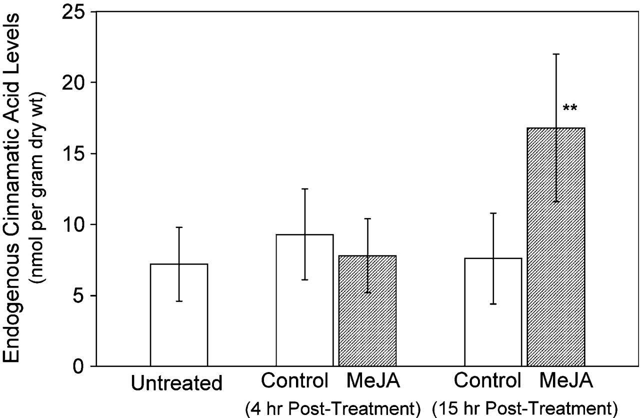


Fig. 5 Effect of control (N=4) and MeJA (N=4) treatments at 4 and 15 hr on endogenous levels of *trans*-cinnamic acid in tissue extract

synthesis, but will increase levels of soluble sugars and amino acids due to the higher energy demands of the plant during stress (Baena-González, 2010). Future studies will explore how the balance of carbon and nitrogen is maintained during early stress induction.

Most importantly, our results suggest that defense induction triggered by MeJA manifests in the reprogramming of only new carbon and not of existing carbon stores. This observation correlates with observations made by Arnold and Schultz (2002) that new carbon is incorporated into tannins during defense induction. It remains unclear why existing carbon stores were not utilized to up-regulate the phenylpropanoid pathway. This could reflect the fact that remobilization of existing carbon stores is a slower process and was not detected during the short timeline of this present study. We note that analyses of jasmonate-responsive genes in *Arabidopsis* (Jung et al., 2007) reveals a complex profile of both up-regulated and down-regulated transcripts differing in expression patterns over time for up to 24 h after MeJA treatment. In future studies, we need to explore whether longer time points give further insight into plant responsiveness to MeJA and its ability to utilize available carbon (both old and new) in defense.

Interestingly, 4 h after MeJA treatment, the level of [^{11}C] phenylalanine was transiently increased, but quickly returned to baseline levels by 15 h after treatment. As the first step in phenolic metabolism, this is a crucial biochemical reaction supporting both plant development and plant defense. Such behavior might be reasonable in light of findings by Jung et al. (2007) showing that MeJA will transiently induce transcript accumulation of numerous genes in *Arabidopsis* (e.g., At5g67300 associated with a MYB transcription factor in signal transduction). We note that many MYB transcription factors play essential roles in regulating phenolic metabolism during defense induction (Stracke et al., 2007; Malone et al., 2009). Furthermore, phenylalanine itself has been shown to promote PAL activity in *Cryptomeria* and *Perilla* cell suspension cultures (Ishikura et al., 1986), as did JA up-regulate transcripts associated with the phenylalanine ammonia lyase isoform 1 in *Populus nigra L*. (Babst et al., 2009). Indeed, we observed increased endogenous levels of cinnamic acid, but only 15 h after MeJA treatment, thus highlighting the need for studies that explore the coordination between the expression profiles of genes and relevant metabolism. Finally, we note that JA will up-regulate transcripts associated with DAHP synthase in *Populus* (Babst et al., 2009), as will wounding increase DAHP activity promoting increased carbon turnover within the shikimate pathway (Dyer et al., 1989). Our observation of increased partitioning of new carbon into [^{11}C]phenylalanine and other metabolites of the shikimate pathway may be a reflection of direct action of the phytohormone on DAHP and possibly other branch-point enzymes of that pathway. Future studies using transformed plants should provide additional insight here.

Like phenylalanine, a similar transient behavior was observed in the metabolic partitioning of ^{11}C into tyrosine, although not as large. Since phenylalanine and tyrosine derive from the same branch point (prephenate-to-arogenate) within the shikimate pathway, it seems reasonable that they exhibit similar transitory behavior.

Finally, MeJA treatment rapidly increases ^{11}C-partitioning into tryptophan, which continues to increase over a 15 h window after treatment. We note that tryptophan promotes chorismate mutase in the shikimate pathway, which may affect carbon partitioning from chorismate to tyrosine and phenylalanine (Bickel and Schultz, 1979). However, the endogenous concentrations of aromatic amino acids were unchanged over the timeline of our studies. Therefore, we rule out the possibility that feedback loops, that either promote or inhibit amino acid biosynthesis are likely responsible for the changes in ^{11}C-partitioning that we observed.

Tryptophan plays a major role in plant metabolism as the primary precursor in the biosynthesis of indole-3-acetic acid (IAA), a growth regulator that is part of the auxin family, key hormones that regulate growth and development (Doerner, 2008). Jung et al. (2007) reported a group of genes that were induced 1-2 h after MeJA treatment, reaching a maximum accumulation by 8 h and diminishing after 24 h. These middle-stage expression genes included those for transcription factors as well as tryptophan synthase subunits and the JR2 gene linked to IAA-Ala hydrolase. Therefore, it seems reasonable that ^{11}C-partitoning into tryptophan would remain elevated over the timeline of our study.

In summary, the use of ^{11}C as described in this paper offers unique opportunities to explore rapid temporal changes in new carbon metabolism. As we have demonstrated, studies of this nature can provide insight into how plants use their new carbon resources in response to stress.

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(2008) suggested that autumn colors could signal tree quality to myrmecophilous specialist aphids, which attract aphid-tending ants that defend the trees from other herbivores. Another indirect defense hypothesis (Holopainen, 2008) states that green foliage, e.g., in *Alnus* before leaf fall, can continue to produce herbivore-induced plant volatiles and maintain volatile-based indirect plant defenses against aphids until leaf abscission, but in yellowing foliage indirect defenses become weaker, thus creating an enemy-free space for specialist aphids. This hypothesis is based on the fact that herbivore-damaged plants attract the natural enemies of the herbivorous insect by releasing herbivore-induced plant volatiles (Turlings et al., 1990; Kessler and Baldwin, 2001; Kappers et al., 2005; Mäntylä et al., 2008; Dicke and Baldwin, 2010; Holopainen and Gershenzon, 2010). The induction of the terpene volatiles, however, in particular is strongly photosynthesis dependent (Arimura et al., 2008). During leaf maturation and the start of senescence, photosynthesis declines and expression of photosynthesis- and carbon fixation-related genes decrease (Kontunen-Soppela et al., 2010). Furthermore, phenolic compounds, which are responsible for the leaf autumn coloration (Wilkinson et al., 2002), are formed through the shikimate pathway and have phosphoenolpyruvate (PEP) as a fundamental precursor. Isoprene and monoterpenes, produced through the chloroplastic MEP pathway, share this precursor (Rosenstiel et al., 2003; Magel et al., 2006). Under limited substrate conditions in autumn, PEP might be preferentially allocated to form phenolic compounds instead of isoprenoids (Fares et al., 2010). Therefore, in deciduous trees, a reduced proportion of photosynthesis-related VOCs could be expected during the advance of senescence and leaf coloration. Moreover, this should lead to nearly total loss of MEP dependent compounds after abscission, as these compounds are not released from storage pools (Ghirardo et al., 2010) as in needle litter of terpene-storing conifers (Kainulainen and Holopainen, 2002; Isidorov et al., 2010).

Ougham et al. (2005) proposed that biochemical reactions related to autumn color development and leaf senescence may affect volatiles released from plant leaves. They expected that peak emission of volatiles would coincide with the phase when the leaf was yellow or red but still alive, and that insect herbivores may respond to these volatiles, rather than to bright colors. Indeed, some plant species emit the sesquiterpene (*E*)-β-farnesene, which is the major component of the aphid alarm pheromone. Transgenic *Arabidopsis* plants that over-express (*E*)-β-farnesene synthase and emit (*E*)-β-farnesene are repellent to aphids and arrest aphid parasitoids; the olfactory (*E*)-β-farnesene cues override the yellow visual cues that normally are strongly attractive to *Myzus persicae* aphids (Beale et al., 2006). Blande et al., (2010) found that aphid feeding upon green foliage of *Betula pendula* and *Alnus glutinosa* induces emissions of methyl salicylate (MeSA) in both tree species, and intensity of emission is dependent on the duration of feeding. MeSA is known to act as both an aphid repellent in spring (Glinwood and Pettersson, 2000a) and an attractant of autumn migrants (Pope et al., 2007), but also an attractor of foraging predators and parasitoids (Zhu and Park, 2005; Pareja et al., 2009).

Many volatile organic compounds (VOCs), particularly terpenes (Gershenzon, 1994; Loreto and Schnitzler, 2010) are costly (in glucose units) to produce, and as much as 10% of fixed carbon could be released to the atmosphere as volatiles (Penuelas and Llusia, 2003). Given the high cost, we tested a hypothesis that disintegration of chloroplasts and chlorophyll during autumn senescence may reduce the plant's ability to produce defensive VOCs.

Using a population of cloned *Betula pendula* that started autumn senescence in a field site, we addressed three specific questions. First, are the emissions of costly, photosynthesis-related terpenes and other herbivore-inducible VOCs lower in senescing (e.g., Bruggemann and Schnitzler, 2001) foliage than in green foliage? Second, are there emissions of specific aphid-repelling volatile compounds during the most active nutrient translocation period in yellowing leaves? Finally, does disintegration of chloroplasts and other cell organelles (Keskitalo et al., 2005) in the last stage of senescence lead to elevated emission of green leaf volatiles (GLVs), which could help aphids to avoid abscising leaves?

Methods and Materials

Plant Material Ten 1-yr-old micropropagated silver birch (*Betula pendula* Roth) saplings, originating from naturally regenerated birch forest (mixed *B. pendula* and *B. pubescens*) in Punkaharju (southeastern Finland; 61°48′ N, 29° 18′ E), were selected randomly. The potted saplings were grown at the Ruohoniemi experimental field at the Kuopio Campus Research Garden of the University of Eastern Finland (UEF) (62°13′ N, 27°13′ E, 80 m asl) in central Finland from May 2007 onwards (described e.g., in Blande et al., 2007 and Karnosky et al., 2007). Our surveys indicated that birch aphids did not appear on the saplings in this field site in summer and autumn 2007. Each sapling was healthy with green leaves, and no signs of senescence were visible prior to the start of the experiment (September 17) when they were brought from the field site to growth chambers. Some of the neighboring saplings already had distinctive foliage yellowing and were rejected during tree selection. However, we assumed that the autumn senescence process also had begun in our saplings, as the autumn senescence process, once initiated by environmental fac-

tors, seems to be a tightly controlled developmental program that is not significantly altered further by environmental factors (Keskitalo et al., 2005). The proportion of yellowing leaves in each sapling was monitored during the experiment. A leaf was considered yellow if one third or more of the leaf area was showing symptoms of yellowing. The proportion of green and yellow leaves was calculated on this basis.

All saplings were kept in two growth chambers under controlled conditions, which were based on the last 10 year mid-September weather and light conditions at the Ruohoniemi efield. The growth chambers were programmed with the following conditions; minimum temperature 8°C at 5:00, maximum 12°C from 15:00 to 17:00, relative humidity from 75% to 95%. Lights were on from 7:00 to 18:00, and the maximum light intensity (c.a. 350 $\mu mols^{-2}m^{-2}$) was maintained from 13:00 to 15:00 for 2 hr.

Collection of Volatiles from Shoots and Litter Emissions were collected four times from the main shoot of 9 saplings on September 18–19 (d 1), 21–22 (d 3), 24–25 (d 6), and 27–28 (d 9), 2007, until the fall of leaves began. After abscission, VOC emissions from the fresh litter were collected. Each shoot was enclosed in a pre-cleaned (oven-heated 120°C, 1 hr) multi-purpose polyethylene terephthalate (PET) cooking bag, (vol. 3 l, Look, Terinex Ltd., UK) (Ibrahim et al., 2010). The bags were fastened carefully to the shoot bark with gardening wire with care taken not to damage any foliage. One of the two outermost bag corners was cut and an air inlet tube inserted. Clean charcoal-filtered, and MnO_2 scrubbed air was pumped through Teflon tubing and into the bag at 400 ml/min to flush the system, and then reduced to 230 $ml min^{-1}$. The remaining bag corner was cut, and a stainless steel tube containing approximately 150 mg of Tenax TA-adsorbent (Supelco, mesh 60/80) was inserted and fastened into the opening. Air was pulled through the Tenax tube by battery-operated sampling pumps (Rietschle Thomas, Puchheim, Germany). The air flow through the Tenax tube was set to 200 ml min–1 with an M-5 bubble flowmeter (A.P. Buck, Orlando, FL, USA). Samples were collected between 9:00 and 15:00. After leaf abscission, emissions from leaf-free branches were sampled to detect possible bark emissions. Due to easy fall of leaves during the abscission phase, we sampled freshly abscised leaves to better evaluate emissions of the last stage. The newly generated litter was collected from these 5 saplings and stored separately in 5 pre-heated (1 h at 120°C to remove any adhered plant VOC from surfaces) and cooled 1.5 l glass containers in growth chambers. VOC emissions were sampled from these containers at +22°C by a similar method 6 day after the last sampling of foliar BVOCs. Each glass container held an average of 40 leaves. After VOC sampling, the leaves were dried in an oven at a temperature of 70°C until the dry weight was constant.

The VOC samples were analyzed with a gas chromatograph-mass spectrometer (Hewlett-Packard GC 6890, MSD 5973). Trapped compounds were desorbed with a thermal desorption unit (Perkin-Elmer ATD400 Automatic Thermal Desorption system) at 250°C for 10 min, cryofocused at −30°C, and injected onto an HP-5 capillary column (50 m×0.2 mm i.d.×0.5 μm film thickness, Hewlett-Packard) with helium as carrier gas. The temperature program was as follows: 40°C 1 min, 5°C min-1 to 210°C, 20°C min-1 to 250°C, 250°C 8 min. Compounds were identified and quantified by comparing the mass spectra to those of pure standards (one external standard mixture for terpenes and one for GLVs) and to spectra in the Wiley library (Vuorinen et al., 2004). Our terpene standard included pure compounds: α-pinene, sabinene, β-pinene, myrcene, (*E*)-β-ocimene, limonene, 1,8–cineole (eucalyptol), 1-chloro octan, γ-terpinene linalool, (*E)*-DMNT, alloocimene, α-copaene, longifolene, *trans*-β-farnesene, aromadendrene, α-humulene, δ-cadinene, and caryophyllene oxide. The GLV standard included: *cis*-3-hexen-1-ol, *trans*-2-hexenal, 1-hexanol, 1-octen-3-ol, *cis*-3-hexenyl acetate, 1-chloro octan, nonanal, *cis*-3-hexenyl butyrate, methyl salicylate, *cis*-3-hexenyl isovalerate, and *cis*-3-hexenyl tiglate. All compounds were purchased from Sigma-Aldrich (parent company of Sigma, Aldrich, Fluka and Supelco) except sabinene from CHEMOS GmbH, *trans*-β-farnesene and *cis*-3-hexenyl isovalerate from Bedoukian Research Inc. DMNT was synthesized in-house from commercial citral using methyltriphenylphosphonium bromide and butyl lithium (Ibrahim et al., 2008).

Data Analysis The quantities of emitted compounds in the headspace of plants and litter leaves were calculated in nanograms per g dry weight per h. Emission rates for each sample were compared with one-way ANOVA or non-parametric Kendall's W Test if data did not follow a normal distribution. The proportion of yellowing leaves was tested with Dunnet's test. A principal component analysis (PCA) (SIMCA-P 11.5.0.0, Umetrics AB, Umeå, Sweden) was used to divide the VOCs on the basis of the composition and ordination of the saplings for each sampling date (eigenvalues over 1 were extracted, and the original data compressed to two principal components PC1 and PC2).

Results

The proportion of green leaves in foliage was significantly affected ($F_{3,27}$=14.27, P<0.001) during the monitoring period with the proportion significantly lower on days 6

and 9 (Dunnet's test) than on the first day (Table 1). There were trends for total terpene emissions to decrease and total green leaf volatiles (GLVs) to increase (Fig. 1), but only total GLV emissions differed significantly among days ($F_{3,26}$=4,46, P=0.012) being higher on day 6 than on day 1. The emissions of the common photosynthesis-related monoterpenes (1,8-cineole, α-pinene, β-myrcene, β-pinene, limonene, and sabinene) were low during the yellowing process, and did not differ among the days (Table 1). Inducible terpenes, monoterpenes (*E*)-β-ocimene and linalool, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and the sesquiterpene α-copaene, were emitted only from saplings with green foliage. The inducible aromatic compound, methyl salicylate (MeSA), was emitted by green foliage and by one sapling already showing yellowing symptoms, but not from leaves in the later phases of senescence. MeSa emissions differed significantly (W=0.543, P=0.043) (nonparametric Kendall's W Test) among days. The GLVs *cis*-3-hexenol and *cis*-3-hexenyl acetate were emitted in minor amounts (17% of total emission) from green foliage. These compounds also were emitted from yellowing leaves and became more common volatiles just before leaf abscission. Principal component analysis (Fig. 2) showed that the emission of *cis*-3-hexen-1-ol was linked mostly to leaf senescence, and the emission was correlated positively with proportion of senescing leaves (r=0.682, P<0.01, N=30). The ratio of *cis*-3-hexen-1-ol to *cis*-3-hexenyl acetate concentrations in emissions changed from 0.8 in green and yellowing foliage to 4.0 just before abscission, and it increased to 100 after leaf abscission, as *cis*-3-hexenyl acetate was not detectable in fresh litter samples.

No volatile emissions were detected from the leafless shoots. Analysis of VOC emissions from abscised leaves in fresh leaf litter indicated that GLV compounds constituted more than 75% of the emission of birch leaves at the early decomposition stage (Fig. 3). Some emissions of monoterpenes and MeSa were detected, but the dominating photosynthesis related monoterpene α-pinene was not detected, while linalool, β-myrcene, and limonene were detected.

Discussion

VOC Emissions during Senescence There were sequential changes in the emission profiles during senescence of birch foliage, and principal component analysis was able to characterize and group the saplings separately on day 1 and 9 on the basis of the emission composition during the yellowing process. Inducible terpenes and photosynthesis-related monoterpenes were typical of green foliage emission profiles, and GLVs typical of senescing foliage. Our first hypothesis that a reduced proportion of costly photosynthesis-related VOCs will be emitted by senescing foliage is supported by our results, which show that VOCs known to be induced by herbivores (Vuorinen et al., 2007; Blande et al., 2010) or abiotic stress (Ibrahim et al., 2010) like DMNT, linalool, and (*E*)-β-ocimene are emitted by green leaves, but not by mostly yellow foliage. These

Table 1 Mean (±S.E.) proportion of green leaves in foliage of *Betula pendula* saplings and emissions of volatile compounds (ng g^{-1} DW h^{-1}±S.E.) for four sampling dates during the leaf yellowing process. (*N*=9)

Sampling date	Day 1	Day 3	Day 6	Day 9
Green leaves (%)	100	81.0±13.6	48.8±18.0[a]	21.6±11.8[a]
Terpenes				
1,8-cineole	1.12±1.12	0.61±0.61	0.12±0.12	0
(*E*)-β-ocimene	1.41±0.89	0	0	0
α-pinene	1.91±0.48	1.54±0.58	1.54±0,47	1.72±0.44
β-myrcene	0.11±0.11	0	0.27±0.12	0.10±0.10
β-pinene	0	0.15±0.15	0.17±0.17	0
Limonene	0.54±0.46	0.99±0.31	0.90±0.23	0.79±0.93
Sabinene	0	0.18±0.18	0.23±0.23	0
Linalool	0.21±0.21	0	0	0
DMNT	0.69±0.53	0	0	0
α-copaene	0.12±0.12	0	0	0
GLVs				
cis-3-hexen-1-ol	0.14±0.14	0.20±0.20	1.23±0.73	1.66±0.99
cis-3-hexenyl acetate	0.18±0.18	1.30±0.48	1.57±0.40	0.42±0.17
Aromatics				
MeSA	4.84±1.93	0.79±0.79	0	0
Other compounds				
Nonanal	2.4±0.1	1.9±0.5	0	0.8±0.2

[a] Significant difference from the control value (day 1) according to Dunnet's test

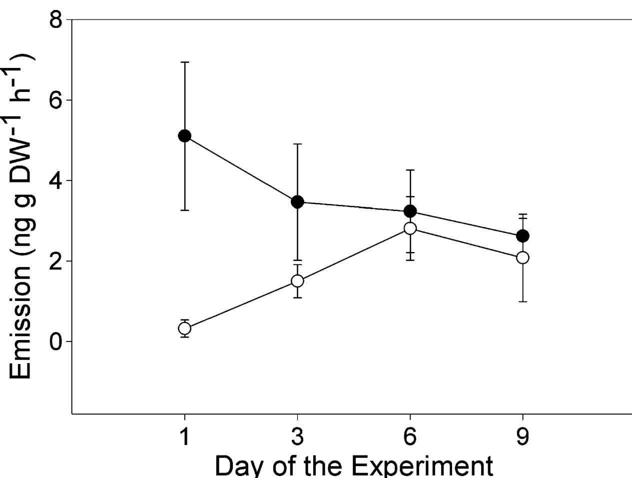

Fig. 1 Trend of total terpene (solid symbols, ●) and total GLV (open symbols, ○) emissions (mean±S.E.) from yellowing birch saplings *(N*= 9) over four sampling dates during the monitoring period (days 1—9) of leaf color change

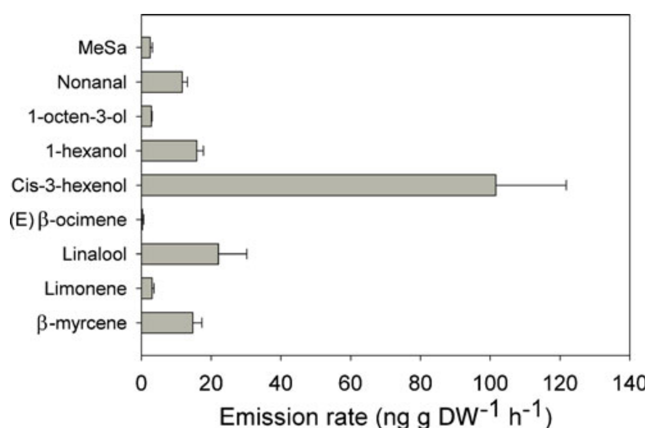

Fig. 3 VOC emissions (mean±S.E.) from fresh *Betula pendula* leaf litter. Sampling of volatiles at +22°C was conducted following 5 days incubation in growth chambers after leaf fall. *(N*=5)

inducible compounds can be found in small amounts (emission rates below 50 ngg^{-1} DW h^{-1}), in the emissions of actively growing intact *B. pendula* saplings (Vuorinen et al., 2005). Fungal pathogen infection, however, may increase (*E*)-β-ocimene emission by 30-fold, and chewing herbivore feeding increase the emission of these compounds by more than 10-fold (Vuorinen et al., 2007). We did not find the sesquiterpene (*E,E*)-α-farnesene in the emission profile, although during summer it is emitted abundantly by many *B. pendula* genotypes (Vuorinen et al., 2005, 2007; Ibrahim et al., 2010) and is a typical herbivore-induced VOC in several plant species (e.g., Blande et al., 2007; Pinto et al., 2007). However, α-pinene and β-pinene—light and photosynthesis-related monoterpenes (Hakola et al., 2001; Loreto et al., 2004)—were emitted from leaves in the last stage of senescence, which is against our hypothesis. Explanation for this could be that there were still 20% greenish leaves on the same shoot among senescent leaves, and these may be responsible for monoterpene emissions when analyzing emissions on a shoot or branch basis. Alternatively, α-pinene and β-pinene may have still unknown functions relating to degeneration of photosynthesis.

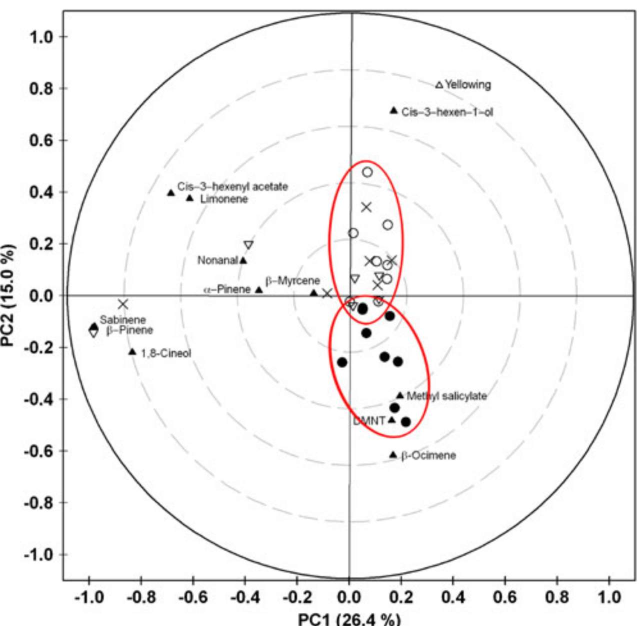

Fig. 2 A principal component bi-plot, showing the variation of volatile organic compound (VOC) composition and ordination of the *Betula pendula* saplings for each sampling date (day 1, 3, 6 and 9), on the basis of foliar VOC emission profile (1–4). Sapling symbols: ●=day 1, ▽= day 3, X=day 6, and o=day 9. Compounds are marked with the symbol ▲ and the symbol △ marks the proportion of yellow leaves

Against our second hypothesis, we did not find emission of the sesquiterpene (*E*)-β-farnesene—a known aphid alarm pheromone released by some plant species in nature (Beale et al., 2006)—or any other aphid repellent volatile that might have been expected according to the co-evolution hypothesis (Hamilton and Brown, 2001). MeSa is an aphid-induced compound in *B. pendula* (Blande et al., 2010); it is less reactive than most of the terpenes in the atmosphere, and emissions are induced even by abiotic factors such as ozone (Pinto et al., 2007). It is known to repel certain migrating aphids in spring (Glinwood and Petersson, 2000a), but mostly it is an attractant of autumn migrants (Pope et al., 2007). The cessation of MeSA emissions from leaves starting to change color together with the color signals could inform aphids of nutrient remobilization in leaves.

In accordance with our third expectation, GLVs dominated the VOC profile in the later stages of senescence, particularly in abscised leaves. GLVs commonly are emitted from mechanically damaged plant material (Fall et al., 1999; Vuorinen et al., 2005; D'Auria et al., 2007;),

including plant material damaged by chewing and piercing herbivores (Pinto et al., 2007). Some GLVs, for example *cis*-3-hexen-1-ol, can increase the predation rate of generalist predators (Kessler and Baldwin, 2001). Our saplings did not have any direct herbivore or mechanical damage, and the emissions of GLVs were rather low. Emissions of hexenol, hexanal, *cis*-3-hexen-1-ol, and *trans*-2-hexenal have been found to be associated with the senescence of grass crops (Karl et al., 2005). Physical stress leading to senescence strongly affects emission rates of GLVs (Fall et al., 1999). Our results suggest that these compounds might be related to the disintegration of cell organelles and dying cells (Keskitalo et al., 2005) during formation of necrotic leaf spots a few days before abscission. The dominance of *cis*-3-hexen-1-ol and concomitant decrease of *cis*-3-hexenyl acetate in leaf emissions in the last phase of senescence could be the olfactory cue, which female aphids might rely on to avoid falling from foliage with abscising leaves (Glinwood and Petersson, 2000b).

VOC Emissions after Leaf Fall The most common photosynthesis-related monoterpene (α-pinene) was not emitted from the fresh birch litter, which indicates that the emission of this highly volatile compound depends on photosynthesizing leaves in deciduous species that do not store monoterpenes. However, some other monoterpenes like β-myrcene, linalool, and (*E*)-β-ocimene were found in litter emissions. This could be due partly to emissions from aqueous and lipid phase stores (Noe et al., 2006) in abscised leaves due to limited volatility in cold autumn conditions. Furthermore, as the litter leaves were incubated for a few days in the same container, emissions per unit of leaf dry mass are not quantitatively comparable to foliage emissions due to possible adsorption and desorption of compounds onto the container surfaces (Schaub et al., 2010) and loss of leaf weight due to decomposition. The condensation and accumulation of terpenes on leaf and container surfaces could be an explanation for the appearance of linalool in litter samples, although it was detectable only in green foliage of saplings.

The dominance of GLV compounds in the VOC emission profile of fresh litter, and particularly the compound *cis*-3-hexen-1-ol, was most obvious and could be a result of the increasing proportion of necrotic leaf tissues during microbial decomposition. High concentrations of GLVs have been found in the forest atmosphere in autumn when senescing and litter leaves thaw after freezing spells of weather and emit GLVs (Karl et al., 2001). Fall et al. (1999) demonstrated with aspen, beech, and clover that after leaf detachment, emissions of GLVs were not dependent on light, and emissions were enhanced greatly as detached leaves dried out. Our results show that GLVs dominate in the emissions from senescing leaves and fresh leaf litter, although leaves did not undergo thawing or drying. Substantial *cis*-3-hexen-1-ol and *cis*-3-hexenyl acetate emissions from birch foliage and birch litter in autumn could have significant atmospheric and ecological impacts, as both compounds participate strongly in secondary organic aerosol formation in reactions with tropospheric ozone (Pinto et al., 2007; Hamilton et al., 2009) and hydroxyl radicals (Hamilton et al., 2009).

VOCs and the Coevolutionary Theory of Autumn Colors Handicap signals are strategic signals that indicate that an individual has possession of a great deal of some resources for costly investments (Zahavi, 1975). If the signal is not costly, then other individuals might mimic strong defense and thus diminish signal reliability (Hamilton and Brown, 2001). Green foliage is known to have high light use efficiency and photosynthetic capacity (Evain et al., 2004). There is a strong coupling of photosynthesis and phytogenic VOC emissions (Penuelas and Lusia, 2003), and among other functions these compounds are effective cues for predators and parasitoids toward herbivore-attacked plants (Holopainen, 2008; Dicke and Baldwin, 2010; Holopainen and Gershenzon, 2010). Our results indicate that particularly costly terpenes (Gershenzon, 1994; Loreto and Schnitzler, 2010) are diminished in volatile emission of yellowing birch leaves. This observation suggests that green foliage is capable of facilitating induced indirect defense based on volatile terpenes. It should be tested in behavioral experiments whether the color-changing senescing leaves are able to produce sufficiently volatile terpenes as odor signals for natural enemies of aphids and other herbivorous insects. Further research also should elucidate whether increasing GLV emissions from senescing foliage distinguishes herbivore-damaged leaves from undamaged leaves and whether carnivores can respond to this difference. Behavioral tests could give support for the hypothesis (Holopainen, 2008) that honest signals of herbivore damage for natural enemies of herbivorous insects are produced only by green foliage, and hence senescing foliage could provide an enemy-free space for aphids.

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2008; Himanen et al., 2009). Accumulated plant VOCs may volatilize into the atmosphere, depending on their concentration and physiochemical properties (Niinemets et al., 2004). Some VOCs, such as sesquiterpenes, may have allelopathic effects on neighboring plants (Fischer et al., 1989; Macías et al., 1996; Abdelgaleil and Hashinaga, 2007; Abdelgaleil et al., 2009; Wang et al., 2009).

Mikania micrantha H.B.K. (Compositae), a fast-growing vine species, is one of the world's most aggressive weeds. The plant originated from South and Central America (Maffei et al., 1999), and has led to serious ecological problems in Southeast Asia and the Pacific region (Zhang et al., 2004). In Southern China, *M. micrantha* has caused serious damage to crops and forests (Zhang et al., 2004; Wang et al., 2009). In natural ecosystems, rapid invasion of *M. micrantha* during recent years is correlated with extinction of native species (Lee and Klasing, 2004). Beside the efficient take-up of water and nutrients, *M. micrantha* releases phytotoxic compounds that inhibit germination and growth of neighboring plants (Cock et al., 2000; Ismail and Chong, 2002; Shao et al., 2005; Ni et al., 2006). Several studies indicate that allelopathy may be a phenomenon that increases fitness of invasive plants (Callaway and Aschehoug, 2000; Inderjit and Duke, 2003; Gómez-Aparicio and Canham, 2008). In general, allelopathy is more common under extreme environmental conditions, where water, light, or nutrients are limited (Anaya, 1999; Valladares et al., 2007). A similar stress situation might be true in the case of elevated CO_2 concentration.

β-Caryophyllene synthase catalyses the conversion of farnesyl diphosphate to β-caryophyllene, a common sesquiterpene of essential oils of many plants (Cai et al., 2002). β-Caryophyllene inhibits seedling growth of *Brassica campestris* and *Raphanus sativus* (Wang et al., 2009). Kil et al. (2000) reported that β-caryophyllene is a component of the essential oil of *Artemisia lavandulaefolia*, which suppresses seedling growth of *Achyranthes japonica* (Miq.). Overexpression of the sesquiterpene synthase gene namely Os*TPS3* [(*E*)-β-caryophyllene synthase)] in rice plants increases the production of the compound after methyl jasmonate (MeJA) treatment, and MeJA-treated transgenic rice plants attract more parasitoid wasps of *Anagrus nilaparvatae* than the wild-type (Cheng et al., 2007). These studies suggest that the β-caryophyllene synthase and β-caryophyllene may play a role in the indirect defense of plants.

We hypothesized that increasing atmospheric CO_2 concentrations may change allelochemical production and allelopathic potential of the invasive alien plant *M. micrantha*, which, in turn, may affect its interactions with native plants by affecting their germination and seedling growth.

Methods and Materials

Plant Materials Plants of *M. micrantha* H.B.K. (1.5 m high) were collected from a natural population in Qi Ao Island, Zhuhai (N 21°48′, E 113°3′) and maintained in a greenhouse in Guangzhou (N 23°8′, E 113°17′). They were cut into 10 cm pieces and transplanted into plastic pots (20 cm diam; 25 cm high) and allowed to climb on 1.5 m bamboo stakes. Uniform seedlings (about 1.2 m high) were selected and planted in 12 pots (one plant per pot). Transplanted pots were transferred into growth chambers at 350 or 750 ppm CO_2 concentration with 10 hr daylight 12 000 lx at 25°C; 14 hr night at 15°C and 80% relative humidity. Plants were watered with diluted Hoagland solution (25% *v*/*v*) and randomized twice a week to avoid internal chamber effects. Seeds of *R. sativus*, *B. campestris,* and *Lactuca sativa* were purchased from Guangzhou Seed Company (Guangzhou, China), and seeds of *M. micrantha* were collected from Qi Ao Island, Zhuhai. β-Caryophyllene (>98.5% purity) was purchased from Sigma-Aldrich Chemie GmbH (D-89555 Steinheim, Germany).

SSH Library Construction Suppression subtractive hybridization (SSH) was used to generate a cDNA library (Diatchenko et al., 1996). Briefly, 30-d-old *M. micrantha* plants grown at 350 ppm CO_2 were exposed to 750 ppm CO_2 for 6 d, or left at 350 ppm CO_2. Total RNA then was isolated from the 5th and 6th leaves (counted basipetally from the apex) using the standard guanidine thiocyanate method (Chomczynski and Sacchi, 2006). Double strand cDNA was synthesized using the SMART™ cDNA amplification Kit (Clontech). A subtracted cDNA library was constructed using the PCR-Select cDNA Subtraction Kit (Clontech). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and ligated into the pGEM-T easy vector (Promega). The constructs were transformed into *E. coli* Top10 (Invitrogen). Positive clones were verified by PCR using T7 and SP6 primers and sequenced with an ABI 3730 sequencer (Applied Biosystems, Inc.). All obtained sequences were compared with DNA databases using the BLASTX sequence comparison software (Altschul et al., 1997) at the NCBI website (http://www.ncbi.nlm.nih.-gov/BLAST). An identified expressed sequence tag (EST) named WGJ87 (658 bp) showed high level of sequence similarity (e^{-35}) to a characterized β-caryophyllene synthase gene from *Artemisia annua* (accession number AAL79181) (Cai et al., 2002), and this inspired us to do further analysis.

Real-time Quantitative PCR Real-time PCR was performed with RNA isolated from *M. micrantha* leaves to confirm activation of β-caryophyllene synthase expression in response to increased CO_2 concentration. Twelve pots of

M. micrantha (1.2 m high) were transferred into the growth chambers at 350 or 750 ppm CO_2 for up to 12 d. The 5th and 6th leaves of each plant from three pots were harvested at 3, 6, 9, and 12 d. Total RNA was isolated from the harvested leaves and quantified based on the absorbance at 260 nm. The integrity of RNA was checked with agarose gel electrophoresis. The RNA (1 μg) was treated first with DNase I (Invitrogen) to remove any genomic DNA contamination. The RNA then was reverse transcribed using oligo (dT) primer and ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's instructions.

Real-time PCR was performed on the ABI PRISM 7000 sequence detection system (Applied Biosystems) in a volume of 20 μl containing 0.2 μM of each primer, 10 μl of 2×SYBR Green I (Roche) and 1 μl of the cDNA temple. The PCR cycling conditions were: 95°C for 1 min, 40 cycles of 95°C for 15 sec, 55°C for 15 sec, 72°C for 45 sec, and 85°C for 20 sec for signal collection in each cycle. To assess the specificity of the PCR amplification, a melt-curve analysis was performed at the end of the reaction by increasing temperature from 55 to 99°C and held for 5 sec at every increment of 1°C, and a single peak was observed.

The PCR primers were WGJ87F1 (5′-TAAGAAGGAG CAAGAAAGAGTGC-3′) and WFG87R1 (5′-CTC TTTGATGTCTTCTTCCACTTC-3′) for β-caryophyllene synthase, and WGJ60 (5′-GATTCCACCAGACCAG CAAAGG-3′) WGJ61 (5′-CACCACGAAGACGAAGCA CAAG-3′) for ubiquitin. The primers were designed from the sequenced ESTs, and the ubiquitin gene was chosen as internal standard. Real-time PCR reactions were performed in triplicate. Analysis of relative gene expression data was performed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using *M. micrantha* plants exposed to 350 ppm CO_2 as a reference.

GC-MS and GC Analysis Twelve pots of *M. micrantha* grown at 350 ppm CO_2 for 30 d were exposed to 750 ppm for 3, 6, 9, and 12 d. Control plants were left at 350 ppm CO_2. The 5th and 6th leaves of each plant were harvested at 9:30 in the morning 3, 6, 9, and 12 d after exposure. For analysis of the volatiles sesquiterpenes, 500 mg fresh leaves were ground in liquid N_2. After addition of 5 g Na_2SO_4, the powder was enclosed in a 22-ml glass tube, and the 65-μm solid-phase microextraction (SPME) PDMS-DVB fiber (Supelco, Bellefonte, PA, USA) was inserted into the tube to collect volatiles at 80°C for 25 min. The SPME fiber was injected into a GC-MS system for analysis. For quantification, 11.3 ng of β-caryophyllene was added as an internal standard in one of the control samples.

GC-MS analysis of volatile sesquiterpenes was performed with a Finnigan Voyager using a BPX5 column (25 m×0.22 mm×0.25 μm), He (1 $ml\,min^{-1}$ gas flow rate), a splitless injection temperature 220°C, a quadrupole-type mass selective detector with a transfer line temperature 230°C, a source temperature 200°C, an ionization potential 70 eV, and a scan range 35 to 450 amu. The initial temperature was 60°C. After 3 min, the temperature was increased to 120°C (gradient of $10°C\,min^{-1}$); the temperature was then further increased to reach 180°C (gradient of $5°C\,min^{-1}$) and finally to 250°C (gradient of $25°C\,min^{-1}$). Individual sesquiterpenes were tentatively identified by a peak matching library search that used authentic standards and NIST (National Institute of Standard and Technology) and Wiley libraries.

Changes of the β-caryophyllene were analyzed by GC-FID using an HP-5MS column (30 m×0.25 mm×0.25 μm), N_2 (1 $ml\,min^{-1}$ gas flow rate) as carrier. Two ml headspace samples were analyzed using the parameters described above.

Bioassay of β-Caryophyllene Seeds of *R. sativus*, *B. campestris*, *L. sativa,* and *M. micrantha* were surface-sterilized with 0.5% $KMnO_4$ for 10 min and then washed × 3 with sterile distilled water. β-Caryophyllene was dissolved in ethyl acetate, and different volumes of this solution were added to filter paper in glass containers (6× 6×10 cm) to get 0.375, 3, and 24 $mg\,L^{-1}$ concentrations. After complete evaporation of ethyl acetate, 20 seeds of each target plant and 5 ml distilled water were placed in each glass container (Wang et al., 2009). Plants were grown in growth chambers at 350 or 750 ppm CO_2 (at 25±1°C with10 hr light, 14 hr dark and 80% relative humidity). Germination rate (3 d for *R. sativus* and *B. campestris*; 7 d for *L. sativa* and *M. micrantha*), root length, and shoot height of seedlings were recorded from each filter paper at the time of harvest (9 d for *R. sativus* and *B. campestris*; 16 d for *L. sativa* and *M. micrantha*). The magnitude of inhibition or stimulation in bioassay was used as the response index (*RI*) as per Williamson and Richardson (1988):

$$\mathrm{RI} = 1 - \mathrm{C}/\mathrm{T}(\mathrm{T} \geq \mathrm{C})$$
$$\text{or } \mathrm{RI} = \mathrm{T}/\mathrm{C} - 1(\mathrm{T} < \mathrm{C})$$

Where, C is control and T is treatment value. The absolute value of *RI* represents the phytotoxic effect. Value of $RI>0$ is considered stimulatory, while <0 is considered inhibitory.

Statistical Analysis Phytotoxicity of β-caryophyllene expressed as *RI* index was analyzed using one-way ANOVA followed by the Duncan's multiple range tests. Relative expression level of β-caryophyllene synthase gene and relative abundance of β-caryophyllene in *M. micrantha* leaves were analyzed using two-way ANOVA followed by the Duncan's *post hoc* tests using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA).

Results

Induction of β-Caryophyllene Synthase Real-time PCR analysis showed that elevated CO_2 significantly increased the transcript levels of β-caryophyllene synthase in the leaves of *M. micrantha* (Fig. 1). Transcript levels were increased 9.07, 18.95, 16.55, and 27.68 fold after the plants were exposed to 750 ppm CO_2 for 3, 6, 9, and 12 d, respectively. However, the gene did not significantly change its expression level when plants were grown in the chamber with CO_2 at 350 ppm.

Levels of β-Caryophyllene GC analysis indicated differences in the emission of β-caryophyllene between *M. micrantha* plants exposed to 750 ppm CO_2 and those kept at 350 ppm CO_2 (Fig. 2). Control plants grown at 350 ppm CO_2 did not significantly change during the experiment (12 d). However, plants at 750 ppm CO_2 varied in emission of β-caryophyllene during the experiment time. Maximum emission was found in plants exposed to 750 ppm CO_2 for 9 d. *M. micrantha* leaves in all plants exposed to 750 ppm CO_2 had significantly higher levels of β-caryophyllene than those exposed to 350 ppm CO_2 (Fig. 2).

Phytotoxicity of β-Caryophyllene β-Caryophyllene inhibited seed germination, root and shoot growth of *B. campestris*, *R. sativus*, *L. sativa,* and *M. micrantha*. The response indices (*RI*) of *B. campestris* grown under 350 ppm CO_2 were −0.08, −0.15, and −0.18 for the seed germination, root and shoot growth, respectively (Table 1). Root growth of *R. sativus*, *L. sativa* and *M. micrantha* was more sensitive than shoot growth. β-Caryophyllene at 24 mg L^{-1} significantly inhibited the seed germination and seedling growth of all four tested plant species (Table 1).

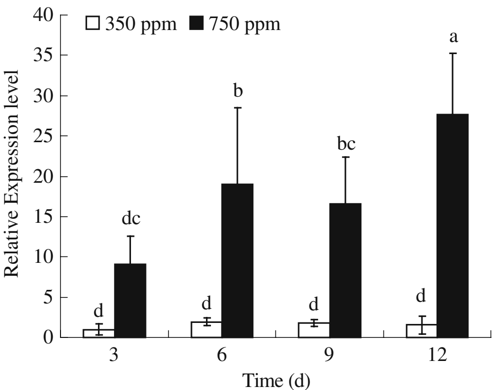

Fig. 1 Relative expression level of β-caryophyllene synthase gene in *Mikania micrantha* leaves as analyzed by real-time PCR. Plants were grown at 350 ppm CO_2 or 750 ppm CO_2 for the indicated period. Three replicates were used for treatment. Each bar represents means± SD. Different letters above the bars indicate significant differences ($P<0.05$) according to Duncan's multiple range tests. Two-way ANOVA: time effect, F=3.82, df=3, $P<0.05$ (P=0.031); the concentration of CO_2 effect, F=66.62, df=1, $P<0.001$; time×the concentration of CO_2 interaction, F=3.41, df=3, $P<0.05$ (P=0.043)

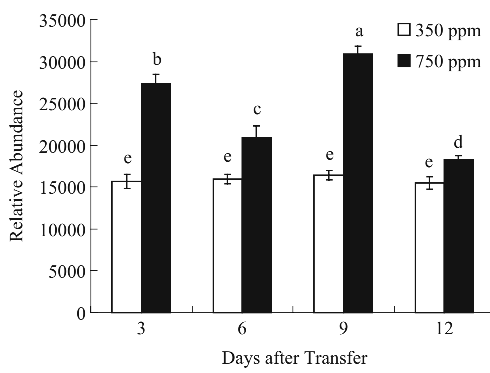

Fig. 2 Relative abundance of β-caryophyllene emission from *Mikania micrantha* leaves analyzed by GC. Plants were grown at 350 ppm CO_2 or 750 ppm CO_2 for the indicated period. Three replicates were used for treatment. Each bar represents means±SD. Different letters above the bars indicate significant differences ($P<0.05$) according to Duncan's multiple range tests Two-way ANOVA: time effect, F=69.88, df=3, $P<0.001$; the concentration of CO_2 effect, F=542.86, df=1, $P<0.001$; time×the concentration of CO_2 interaction, F=57.18, df=3, $P<0.001$

To determine the effects of elevated CO_2 on the phytotoxicity of β-caryophyllene, the seeds of the four plants were treated with β-caryophyllene in a similar way and then exposed to chambers with different CO_2 concentrations (350 and 750 ppm). β-Caryophyllene at a concentration of 24 mgL^{-1} inhibited root growth of *B. campestris*, *R. sativus*, *L. sativa,* and *M. micrantha* by 37, 38, 24, and 24% (corresponsive *RI*=−0.37, −0.38, −0.24, and −0.24), respectively, when CO_2 concentration was 350 ppm (Table 1). At elevated CO_2 concentration (750 ppm) β-caryophyllene at 24 mgL^{-1} inhibited root growth of *B. campestris*, *R. sativus*, *L. sativa,* and *M. micrantha* by 45, 58, 34, and 28% (corresponsive *RI*=−0.45, −0.58, −0.34, and −0.28), respectively (Table 1). Elevated CO_2 concentration enhanced the allelopathic potential of β-caryophyllene on all test species. Similar trends were observed with respect to seed germination and shoot growth of all the target plants.

Discussion

We identified a β-caryophyllene synthase gene sequence (GenBank FJ767894) in *M. micrantha.* Real-time PCR analysis showed that elevated CO_2 significantly increased the transcript levels of the β-caryophyllene synthase gene in the course of the experiment (Fig. 1). GC analysis confirmed that plants of *M. micrantha* did not significantly change their emission of β-caryophyllene when they were grown in the chamber with CO_2 at 350 ppm. However, plants exposed to 750 ppm CO_2 significantly increased the

Table 1 Effect of β-caryophyllene on seed germination and seedling growth expressed as Response Indices (*RI*) of four plants under 350 and 750 ppm CO_2. All data are presented as means±SD. Each value is the mean of three replicates. Different letters in the same row indicate significant differences ($P<0.05$) according to Duncan's multiple range test

Tested plant	Bioassay parameter	β-Caryophyllene concentration (mg L^{-1})					
		350 ppm CO_2			750 ppm CO_2		
		0.375	3	24	0.375	3	24
Brassica campestris	Germination	0.10±0.03 a	−0.08±0.06 b	−0.10±0.03 bc	0.03±0.05 a	−0.16±0.03 cd	−0.24±0.05 d
	Root length	0.17±0.15 a	−0.15±0.21 b	−0.37±0.14 c	0.06±0.15 a	−0.20±0.13 b	−0.45±0.08 d
	Shoot height	0.13±0.16 a	−0.18±0.24 c	−0.25±0.22 c	−0.08±0.16 b	−0.20±0.19 c	−0.32±0.16 d
Raphanus sativus	Germination	0.02±0.05 ab	−0.07±0.03 bc	−0.12±0.03 cd	0.07±0.03 a	−0.16±0.08 cd	−0.22±0.11 d
	Root length	0.05±0.15 a	−0.16±0.14 c	−0.38±0.13 e	−0.10±0.18 b	−0.25±0.14 d	−0.58±0.12 f
	Shoot height	0.09±0.14 a	−0.12±0.15 c	−0.33±0.11 e	0.02±0.13 b	−0.19±0.13 d	−0.52±0.16 f
Lactuca sativa	Germination	0.02±0.09 ab	−0.08±0.06 abc	−0.12±0.04 bc	0.06±0.03 a	−0.09±0.10 abc	−0.17±0.11 c
	Root length	0.01±0.27 a	−0.15±0.31 b	−0.24±0.18 c	0.05±0.12 a	−0.17±0.14 bc	−0.34±0.18 d
	Shoot height	0.04±0.17 a	−0.10±0.17 b	−0.17±0.16 bc	0.01±0.22 a	−0.19±0.31 c	−0.28±0.18 d
Mikania micrantha	Germination	0.02±0.04 a	−0.11±0.04 b	−0.15±0.07 b	0.06±0.09 a	−0.13±0.07 b	−0.19±0.04 b
	Root length	0.03±0.33 a	−0.17±0.43 bc	−0.24±0.19 bc	0.05±0.19 a	−0.16±0.19 b	−0.28±0.11 c
	Shoot height	0.04±0.21 a	−0.11±0.20 b	−0.17±0.21 bc	0.02±0.17 a	−0.15±0.18 bc	−0.20±0.19 c

emission of β-caryophyllene and reached the maximum level at 9 d after the exposure (Fig. 2). β-Caryophyllene emission capacity may depend on many factors, such as the rate of its synthesis, the rate of its conversion to other sesquiterpenes, and the rate of emission as volatile compounds from intact tissue. Our data showed clearly that elevated CO_2 concentration induced the expression of the β-caryophyllene synthase gene and increased the emission of β-caryophyllene in *M. micrantha*. These findings support our hypothesis that emission of VOCs from plants is particularly sensitive to changes of environmental factors such as CO_2. Leaves of *Quercus ilex* also increase volatile emission of monoterpenes at elevated CO_2 levels (Staudt et al., 2001). Elevated CO_2 typically has both physiological and biochemical effects on VOC emissions (Rosenstiel et al., 2003; Niinemets et al., 2004).

Allelochemicals play a role in mediating interspecific interactions (Dicke et al., 1990; Legrand et al., 2003; Duke, 2007). Terpenoids, a diverse group of secondary compounds, have a variety of ecological functions, including allelopathy (Gershenzon and Croteau, 1993; Sharkey and Singsaas, 1995). Allelopathy is a phenomenon whereby various plants enhance competitiveness and fitness (Singh et al., 2003; Kegge and Pierik 2010). The allelopathic potential of a given plant may vary in different habitats. Environmental stresses such as light, or nutrient or water deficiency may enhance allelopathic potential of plants (Anaya, 1999). β-Caryophyllene, a well-known volatile sesquiterpene with allelopathic potential, has been reported previously to inhibit development of seedlings of various plant species (Kil et al., 2000; Wang et al., 2009). A previously characterized β-caryophyllene synthase gene of *A. annua* also was induced previously by a fungal elicitor, suggesting a role for β-caryophyllene in plant defense (Cai et al., 2002). Emission rates of β-caryophyllene from orange trees (*Citrus sinensis* (L.) OSBECK, var. *Navel* and *Navel Late*) vary with light intensity and temperature (Hansen and Seufert, 2003). Here, at elevated CO_2 levels (750 ppm), phytotoxic effects of β-caryophyllene were enhanced on all tested plants, especially on *R. sativus*. Hence, CO_2 concentrations not only affected the emission of β-caryophyllene from *M. micrantha* leaves (Fig. 2), but also increased its phytotoxicity (Table 1). In other words, an increase in CO_2 concentrations may have multiple effects on plant-plant interactions.

Many studies suggest that climate changes may affect structure and function of ecological systems (Walther, 2003; Jump and Penuelas, 2005). Temperature changes in the subtropical north Indian plains seem to influence biosynthesis of essential oil constituents in *A. annua* (Bagchi et al., 2003). It has been suggested that rising atmospheric CO_2 concentrations increase abundance, biomass, and fitness of vines that may interfere with regeneration and diversity of forest ecosystems (Sasek and Strain, 1991; Granados and Körner, 2002; Zotz et al., 2006; Song et al., 2009). Increasing CO_2 concentration has been suggested previously as a factor that could facilitate exotic plant invasion (Song et al., 2009). Our experiment was conducted over 12 days. In nature, however, an increase in

CO_2 concentration is likely to occur over a long period of time for which the plant may even acquire adaptation. Recently, Klironomos et al. (2005) reported on the over estimation of plant responses in experiments that use abrupt increase of CO_2 concentration. Although 100 years (predicted time for doubling of CO_2 concentration) is a short time (in evolutionary terms) for acquiring such an adaptation, there is some evidence, however, that shows microevolution occurring in plants over short periods of time (Collins and Bell, 2004; Ward and Kelly, 2004). Based on such reports and our findings, we postulate that a future increase in atmospheric CO_2 levels may alter allelopathic potential of the invasive vine *M. micrantha*. Future studies are, of course, required to determine how the release of volatile allelochemicals and CO_2 concentrations will affect invasion of *M. micrantha* in Southern China.

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reliable indicators of their presence to parasitoids, but the detectability of these cues is low. On the other hand, stimuli emitted by the food plants of their herbivorous hosts are more detectable because they are emitted in larger amounts, but they do not necessarily indicate the presence of the herbivores (Vet and Dicke, 1992). In order to cope with this problem, parasitoids may combine stimuli from both their hosts and host food plants (Turlings et al., 1991a, 1993; Vet and Dicke, 1992; Dicke, 1999a, b; Fatouros et al., 2005; Tamò et al., 2006). Natural enemies, such as insect parasitoids, use infochemicals from host plants to locate the habitat of their hosts (Vinson, 1976; Dicke and Sabelis, 1988; Turlings et al., 1990; Vet and Dicke, 1992; Dicke, 1999b; Mumm and Hilker, 2006; Schnee et al., 2006; Heil, 2008). Natural enemies are able to discriminate between blends of volatiles produced by mechanically damaged and herbivore-damaged plants (Eller et al., 1988; Turlings et al., 1991b; Vet and Dicke, 1992; Dicke, 1999a). In addition to herbivore-induced plant volatiles, several other factors also may affect host searching behavior of a parasitoid, such as visual and vibrational stimuli from the host or host plant, the presence of competitors or natural enemies, previous oviposition experiences (learning), and physiological state of the parasitoid (Lawrence, 1981; Wardle, 1990; Wardle and Borden, 1990; Wäckers and Lewis, 1994; Casas et al., 1998; Dicke, 1999b; Dicke and Grostal, 2001).

The success of biological control agents in many projects has been attributed in particular to high host searching efficiency (Neuenschwander and Ajuonu, 1995; Ngi-Song and Overholt, 1997; de Moraes et al., 1999; Neuenschwander, 2001). For instance, the superiority of *Apoanagyrus (Epidinocarsis) lopezi* De Santis over *Apoanagyrus diversicornis* Howard (which is more fecund than *A. lopezi*) was attributed to its higher capacity to locate and parasitize young host instars at low densities (Neuenschwander, 2001). Similarly, *Cardiochiles nigriceps* Viereck displayed a higher host searching capacity, and consequently detected and parasitized more larvae of *Heliothis virescens* (Fabricius) compared to *Microplitis croceipes* Cresson (de Moraes et al., 1999).

Apanteles taragamae Viereck, the parasitoid that was used in the present study, is a solitary larval endoparasitoid of the legume pod borer *Maruca vitrata* Fabricius (Lepidoptera: Crambidae). It parasitized on average 63% of *M. vitrata* larvae on *Sesbania cannabina* (Retz) Pers. (Huang et al., 2003). The wasp also can transmit the multi-nucleopolyhedrovirus *Mavi*MNPV to larvae of *M. vitrata* (M. Tamò, personal communication).

Maruca vitrata is one of the key insect pests of cowpea, causing up to 80% of yield loss (Nampala et al., 2002). Damage by *M. vitrata* to grain legumes is made by its larvae (Taylor, 1978). Larvae feed on flower buds, flowers, and pods of cowpea (Taylor, 1978; Sharma, 1998). Infestation of flowers was found to be higher than that of flower buds and pods (Sharma, 1998). Larvae of this crambid also were reported to damage leaves of some wild leguminous plants, such as the peabush, *S. cannabina* (Huang et al., 2003).

An attempt to exert biological control of this insect pest is made through the importation of *A. taragamae* from Taiwan into Benin by the International Institute of Tropical Agriculture (IITA). The potential of the wasp as biological control agent is being evaluated.

In this study, we assessed the role of volatiles produced by *M. vitrata* larvae and two host plants, cowpea and peabush, in the host selection process by the parasitoid wasp *A. taragamae* by using a Y-tube olfactometer. Cowpea is the main cultivated host plant of *M. vitrata* in Benin whereas peabush is the host plant on which the parasitoid was collected in Taiwan.

Methods and Materials

Plant materials Seeds of the local cowpea variety Kpodji-guêguê and of peabush were sown in potted soil. Pots were placed in a greenhouse at 28±1°C, and 76±6% relative humidity (means ± SD). Plants were watered every 3 days during the experimental period. Experiments started at the onset of flowering. Flowers or stem portions were collected from cowpea (*Vigna unguiculata)* or peabush (*Sesbania cannabina)* plants to prepare the different odor sources used in the olfactometer tests.

Insect Materials

Mass rearing of Maruca vitrata Pupae of *M. vitrata* were obtained from a stock culture at the field station of the International Institute of Tropical Agriculture (IITA) in Benin. They were placed in open Petri dishes that were incubated in wooden cages (44×45×58 cm) with sleeves, having sides of fine screen and a glass top, and kept at 27.0±0.6°C and 60.9±4.6% relative humidity (means ± SD). Adults emerged inside the cages and were fed by using cotton fibers moistened with 10% glucose solution. Four-d-old female moths were transferred in groups of four or five individuals to transparent cylindrical plastic cups (3 cm diam.×3.5 cm high) and kept for 24 h to allow for oviposition, which occurred on the inner surface of the cups. Ovipositing females were fed with small pieces of filter paper moistened with 10% glucose solution, which were replaced every 24 h. Cups carrying eggs were kept under the same experimental conditions until the larvae hatched. Larvae were transferred to large cylindrical plastic containers (9 cm diam.×12 cm high) provided with artificial diet prepared according to Jackai and Raulston

(1988), and developed through five instars until pupation. The artificial diet contained 4 l water; 59.2 g Agar-agar; 400 g cowpea grain flour; 127.2 g wheat or maize germ flour; 60 g Wesson salt; 44.4 g sorbic acid; 6.3 g methyl p-hydroxy-benzoate; 25 g ascorbic acid; 50 ml acetic acid; 6 ml formaldehyde; 11 g aureomicin; 22 g potassium hydroxide; 29.6 ml choline chloride; and 30 ml vitamin B mixture (Jackai and Raulston, 1988). Pupae were collected and placed in cages. All larvae used in the experiments were obtained from the mass production.

Mass rearing of Apanteles taragamae Cocoons of *A. taragamae* were obtained from the stock culture at the IITA station in Benin that originated from parasitoids collected on *S. cannabina* infested by *M. vitrata* at the World Vegetable Center (AVRDC) in Taiwan. Emerged adults were kept in plastic cylindrical cups (4.5 cm diam.× 5 cm high). A hole (2 cm diam.) punched in the lid of the cups was covered with fine mesh. Adults of *A. taragamae* were fed with honey streaked on the fine mesh of the lid. To allow mated female wasps to parasitize hosts, they were offered, during 24 h, 2-d-old larvae of *M. vitrata* in a small cylindrical cup (3 cm diam.×3.5 cm high) containing a piece of artificial diet. The exposed larvae were reared until cocoon stage. Cocoons were collected and placed in cylindrical cups (4.5 cm diam.×5 cm high). Mass production of wasps took place in a climate chamber with a temperature of 25.3±0.5°C (mean ± SD) and a relative humidity of 78.9±5.6% (mean ± SD). Female wasps used for the different choice tests were obtained from this mass rearing.

Maruca vitrata Multi-Nucleopolyhedrovirus (MaviMNPV) *Maruca vitrata* multi-nucleopolyhedrovirus *(Mavi*MNPV) is a baculovirus isolated from infected larvae of *M. vitrata* on peabush in Taiwan (Lee et al., 2007; Chen et al., 2008). Infected larvae were sluggish, pinkish, and ceased feeding 3–4 day after virus exposure. When dead, larvae were found hanging from the top of the plant with the prolegs attached to the host plant. The virus attacks all larval stages with a high susceptibility of early instars (first and second stages). *Mavi*MNPV potentially could be used as a component in an Integrated Pest Management Programme against *M. vitrata* (Lee et al., 2007). It has been introduced to the IITA-Benin laboratory from AVRDC for experimental purposes.

Oviposition-experienced female wasps Emerged adult female parasitoids were kept together with males for 48 h in cylindrical plastic cups (4.5 cm diam. × 5 cm high) to allow mating. They were fed with honey. Mated females gained oviposition experience by parasitizing two 2-d-old *M. vitrata* larvae in cylindrical plastic cups (3 cm diam. × 3.5 cm high), 30 min prior to the olfactometer tests. The host larvae had been reared on artificial diet. These oviposition-experienced parasitoid females had not received contact with the odor sources used in the present study.

Odor-experienced female wasps Two-d-old mated female parasitoids were kept together with uninfested cowpea flowers for 24 h in cylindrical plastic containers (9 cm diam×12 cm high) where they were fed with honey streaked on the mesh cover of containers. Thirty min prior to the olfactometer test, they were allowed to parasitize 2-d-old larvae feeding on cowpea flowers. Odor-experienced females were used only to test for their response to the volatiles from uninfested cowpea flowers against clean air.

Dynamic olfactometer set-up The response of *A. taragamae* females to volatiles produced by cowpea, peabush flowers, and host larvae was investigated by using a glass Y-tube olfactometer similar to that used by Gnanvossou et al. (2003). Clean airflow was divided in two, and each subflow passed through one of the two odor sources connected to the arms of the glass Y-tube olfactometer. The windspeed in the olfactometer was controlled at 4 l/min.

Bioassay procedure Naïve mated females of *A. taragamae* (without oviposition experience) and oviposition-experienced wasps alternatingly were introduced individually at the entry of the Y-shaped glass tube. Their movement was observed for maximally 10 min. A test began when the wasp started to move. Female wasps remaining motionless for more than 5 min at the release point were discarded from the analysis. Parasitoid wasps that did not reach the end of the olfactometer arm were considered as non-responding wasps. After testing two naïve and two oviposition-experienced female parasitoids, the positions of the odor sources were exchanged to correct for any unforeseen asymmetry in the experimental set-up. Odor sources were renewed after testing eight naïve and eight experienced female wasps. A total of 16–20 naïve and oviposition-experienced female wasps were tested daily, and 60–70 naïve and oviposition-experienced females were tested in total for each choice situation. All female wasps used in this study were 3-d-old.

Bioassays on the response of naïve and oviposition-experienced female wasps to volatiles produced by cowpea flowers The influence of volatiles produced by cowpea flowers on the host selection behavior of both naïve and oviposition-experienced females of *A. taragamae* was investigated by testing the following odor combinations: a) 4 uninfested flowers vs. clean air; b) 4 caterpillar-infested flowers from which larvae were removed prior to the

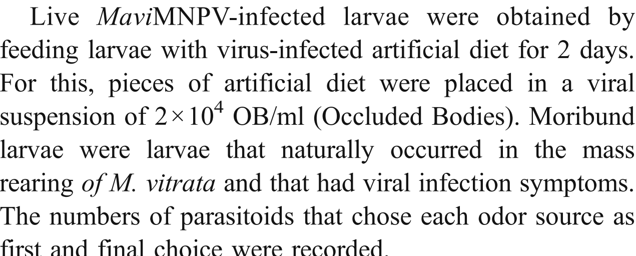
experiment vs. clean air; c) 4 caterpillar-infested flowers from which larvae and their feces were removed prior to the experiment vs. clean air; d) 4 mechanically damaged flowers vs. clean air; e) 4 uninfested vs. 4 caterpillar-infested flowers from which larvae were removed prior to the experiment; f) 4 uninfested vs. 4 mechanically damaged flowers; g) 4 caterpillar-infested flowers from which larvae and feces were removed prior to the experiment vs. 4 uninfested flowers; h) 4 caterpillar-infested flowers from which larvae were removed prior to the experiment vs. 4 mechanically damaged flowers.

Infested cowpea flowers consisted of racemes carrying 4 flowers, infested with 10 1-d-old larvae of *M. vitrata* for 24 h. Before the infestation, racemes were placed in water-filled cylindrical plastic vials (4.5 cm diam × 11.5 cm high) sealed with parafilm to keep racemes fresh and hydrated. Larvae were removed 15 min prior to using the flowers in an olfactometer experiment. Flowers were mechanically damaged 15 min prior to the experiment by three scratched lines onto flowers by using a clean needle.

Bioassays assessing the influence of previous contact with cowpea and peabush plants on the host searching behavior of A. taragamae The influence of an odor experience on the behavioral response to uninfested flowers was studied by using odor-experienced female wasps for odor combination (a) (clean air vs. uninfested cowpea flowers).

As the wasp strain in use was collected originally on peabush plants, the effect of leaves and flowers of this plant on the host searching behavior of the wasp was evaluated by using both naïve and oviposition-experienced females. The *M. vitrata* larvae cause damage to peabush mostly by destroying leaves (Huang et al., 2003). However, flowers of this leguminous shrub also may be damaged. Both naïve and oviposition-experienced female wasps were used to test the following odor combination: i) Uninfested stem portions carrying 4 leaves and 4 flowers of *S. cannabina* vs. clean air.

Bioassays on the effect of volatiles from host larvae on the host searching behavior of A. taragamae The effect of volatiles produced by *M. vitrata* larvae on the host selection behavior of *A. taragamae* was assessed by using healthy, moribund, and live *Mavi*MNPV-infected larvae. Virus-infection had occurred during the mass rearing of *A. taragamae*. Our objective was to assess whether the wasps, being capable of transmitting the baculovirus, avoid infected larvae. Both naïve and oviposition-experienced female wasps were used to test the following odor combinations: j) 10 healthy larvae vs. clean air; k) 10 moribund *Mavi*MNPV-infected larvae vs. clean air; l) 10 healthy larvae vs. 10 moribund *Mavi*MNPV-infected larvae; m) 10 *Mavi*MNPV-infected larvae vs. clean air; n) 10 *Mavi*MNPV-infected larvae vs. 10 healthy larvae.


Live *Mavi*MNPV-infected larvae were obtained by feeding larvae with virus-infected artificial diet for 2 days. For this, pieces of artificial diet were placed in a viral suspension of 2×10^4 OB/ml (Occluded Bodies). Moribund larvae were larvae that naturally occurred in the mass rearing *of M. vitrata* and that had viral infection symptoms. The numbers of parasitoids that chose each odor source as first and final choice were recorded.

Statistical analysis Analysis of data on the number of parasitoids per odor source was performed by using binomial tests with the null hypothesis that the distribution of the wasps over the two arms of the olfactometer was 50:50. Differences between naïve and experienced females wasps were tested with a 2×2 contingency table analysis based on *Chi-square*. Non-responding wasps were recorded but not included in the statistical analysis.

Results

Influence of volatiles from cowpea flowers on the host searching behavior of A. taragamae In all experiments, the responses of the naïve and the oviposition-experienced wasps were not significantly different (contingency table tests, $P > 0.05$). Therefore, we combined the data for naïve and oviposition-experienced wasps.

Females of *A. taragamae* were significantly attracted to volatiles from *M. vitrata*-infested cowpea flowers from which larvae had been removed prior to the experiment, when tested against clean air or uninfested flowers (Fig. 1). Similar results were obtained when the wasps were offered infested flowers from which larvae and feces had been removed prior to the experiment, against clean air or uninfested flowers. The parasitoids did not discriminate between volatiles from uninfested flowers and clean air, between volatiles from mechanically damaged flowers and clean air, or between volatiles from mechanically damaged and uninfested flowers (Fig. 1). Female wasps did discriminate between volatiles from *M. vitrata*-infested flowers and mechanically damaged flowers (Fig. 1).

Response to volatiles produced by uninfested peabush and cowpea flowers Volatiles from stem portions carrying uninfested leaves and flowers of the peabush *S. cannabina* attracted *A. taragamae* females when tested against clean air (Fig. 2). When wasps were given an odor experience, uninfested cowpea flowers were preferred over clean air (Fig. 3), while without the odor experience there was no effect of volatiles from uninfested flowers on parasitoid attraction (Fig. 1).

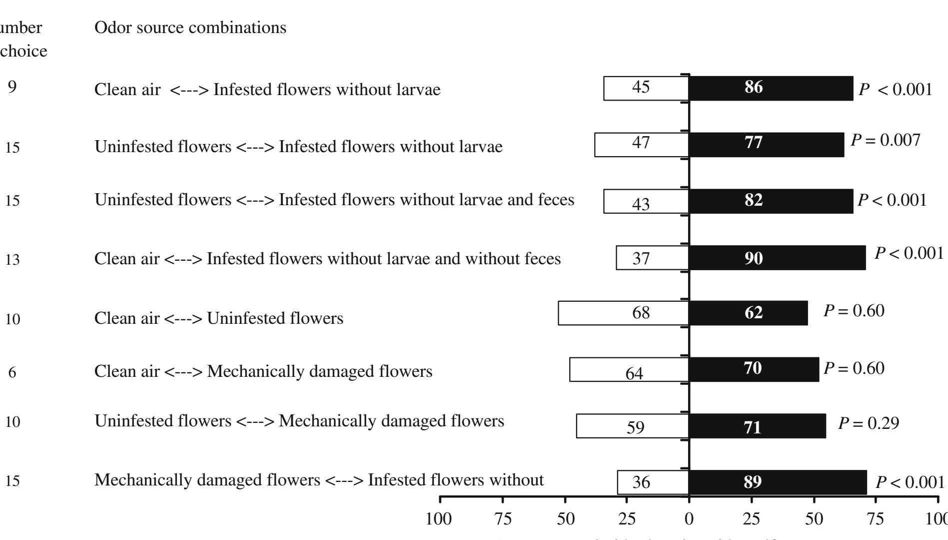


Fig. 1 Behavioral response of *Apanteles taragamae* females offered choices between volatiles from cowpea flowers, that were either uninfested, mechanically damaged, or infested with *Maruca vitrata*, and clean air in a Y-tube olfactometer. *Numbers in bars* represent the total number of parasitoids that chose the olfactometer arm. *P*-values given to the right of the bars are for the two-tailed binomial test

Response to volatiles from host larvae Females of *A. taragamae* displayed a preference for volatiles produced by healthy *M. vitrata* larvae when tested against clean air (Fig. 4). Wasps did not show any preference for volatiles from moribund *Mavi*MNPV-infected larvae over clean air, and they preferred volatiles emitted by healthy larvae over volatiles from moribund *Mavi*MNPV-infected larvae. The parasitoid females did discriminate volatiles from live *Mavi*MNPV-infected larvae against clean air (Fig. 4), but no preference was displayed when they were given a choice between volatiles from healthy larvae and live *Mavi*MNPV-infected larvae.

Discussion

Results from this study show the importance of *M. vitrata*-induced floral volatiles produced by cowpea in the host selection process of *A. taragamae*. Female wasps were attracted by volatiles emitted by *M. vitrata*-infested cowpea flowers from which the larvae had been removed. The parasitoids were not attracted to uninfested cowpea flowers, but this changed when they had received an odor experience. Indeed, long-range volatiles produced by undamaged or herbivore-damaged plants are known to attract natural enemies of herbivorous insects, thus increasing their efficiency in locating their hosts' habitat (Dicke and Sabelis, 1988; Turlings et al., 1990; Vet and Dicke, 1992; Ngi-Song et al., 1996; Du et al., 1998; Dicke, 1999b; Shimoda et al. 2005; Moayeri et al., 2007; Dicke and Baldwin, 2010). In most cases, studies have addressed the volatiles that are produced by leaves. In our study, however, volatiles were emitted by the previously infested flowers. Floral volatiles are known primarily as attractants for pollinators (Jervis et al., 1993; Pichersky and Gershenzon, 2002). However, herbivorous insects that feed or oviposit on flowers have been reported to rely on cues from flowers to locate their hosts (Ekesi et al., 1998; Jönsson et al., 2005; Andrews et al., 2007), and parasitoids may also use floral volatiles to locate a food source (nectar) (Wäckers, 2004). However, to our knowledge only one other study has shown that herbivore-damaged flowers emit volatiles that attract a parasitoid enemy of florivorous herbivores (Jönsson and Anderson, 2008). It will be interesting to investigate how volatiles from herbivore-infested flowers affect the behavior

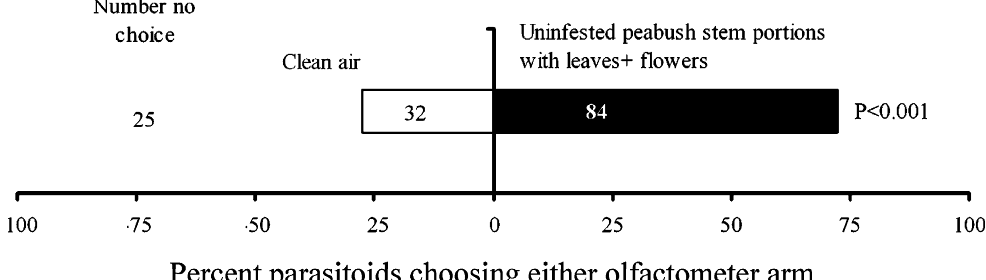


Fig. 2 Behavioral response of *Apanteles taragamae* females to uninfested leaves and flowers of peabush in a Y-tube olfactometer. *Numbers in bars* represent the total number of parasitoids that chose olfactometer arm. *P*-value given to the right of bars is for the two-tailed binomial test

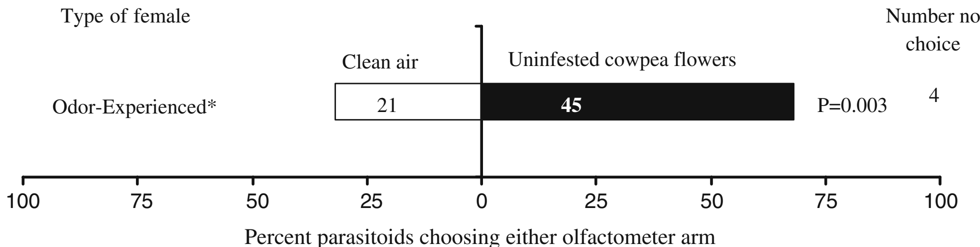


Fig. 3 Behavioral response of odor-experienced females of *Apanteles taragamae* to uninfested cowpea flowers in a Y-tube olfactometer. *Numbers in bars* represent the total number of parasitoids that chose olfactometer arm. *P*-value given to the right of bars is for the two-tailed binomial test. *: Odor-experienced female wasps are females that that had parasitized two 2-d-old larvae reared in the presence of host-infested cowpea flowers, 30 min prior to the olfactometer test

of pollinators. After all, herbivore-infested flowers are likely to be an inferior food source to pollinators.

The attraction of *A. taragamae* to uninfested peabush leaves and flowers may be a reflection of a genetic trait related to searching in peabush fields. Indeed, the current wasp species was imported from Taiwan, where it was collected from *M. vitrata* larvae feeding on peabush leaves. Volatiles from uninfested plants have been reported to be long-range attractants in some other parasitoid species (Elzen et al., 1983; Ding et al., 1989; Ngi-Song et al., 1996). Thus, *Cotesia flavipes* Cameron, originally collected in maize showed a preference for uninfested maize plants over sorghum in a dual choice olfactometer experiment (Ngi-Song et al., 1996). Likewise, the endogenous parasitoid *C. sesamiae* (Cameron) preferred volatiles produced by uninfested sorghum plants over those from maize. Indeed, both sorghum and *C. sesamiae* originate from Africa and share the same environment. The braconid *Macrocentrus grandii* Goidanish, a larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Hubner), was attracted to uninfested maize plants (Ding et al., 1989). However, herbivore-infested plants were found to be more attractive than uninfested plants (Turlings et al., 1991a; Du et al., 1998; Vet et al., 1998; Dicke, 1999b).

In the host habitat, short-range stimuli from the host itself are reliable indicators of its presence, but they usually are not well detectable (Vet and Dicke, 1992). Our study shows that the parasitoid wasp *A. taragamae* was significantly attracted to volatiles emitted by *M. vitrata* larvae. An oviposition experience through the parasitization of larvae fed with artificial diet did not affect the parasitoid's response to host larval volatiles. The use of kairomones by parasitoids for host location has been reported in many parasitoid species (Afsheen et al., 2008). The braconid parasitoid *Microplitis. croceipes* was attracted to odors of *Heliothis virescens* (Fabricius) larvae (Elzen et al., 1987; Röse et al., 1997). Similarly, the bruchid larval parasitoid *Eupelmus vuilleti* (Crw) was reported to respond to volatiles from *Bruchidius atrolineatus* (Pic) larvae (Cortesero and Monge, 1994). However, usually herbivore-induced plant volatiles are more attractive to parasitoids than herbivore-produced volatiles (Turlings et al., 1991a; Steinberg et al., 1992).

Herbivore-associated organisms such as microbes also may be a source of chemical information to parasitoids

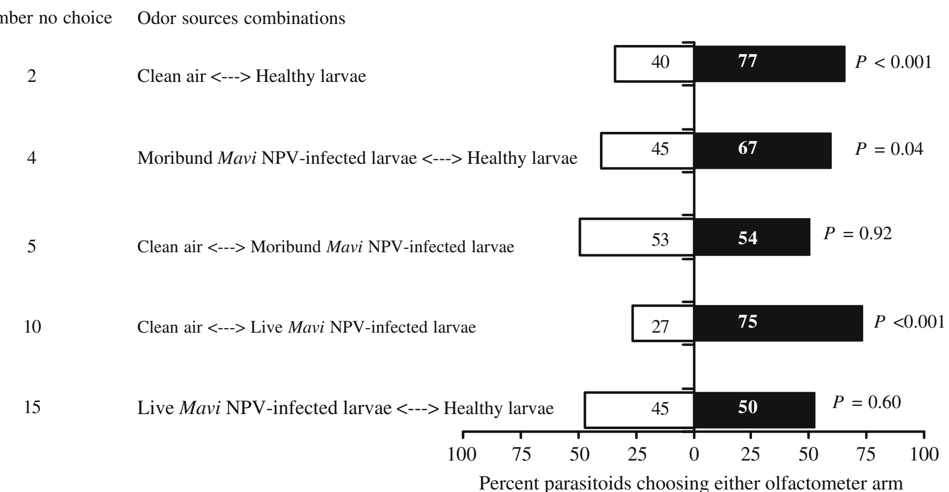


Fig. 4 Behavioral response of *Apanteles taragamae* females offered choices between volatiles from healthy *Maruca vitrata* larvae, moribund or live *Mavi*MNPV-infected larvae, and clean air in a Y-tube olfactometer. *Numbers in bars* represent the total number of parasitoids that chose olfactometer arm. *P*-values given to the right of bars are for the two-tailed binomial test

during host location (Vet and Dicke, 1992). *Apanteles taragamae* has been found to be a vector for the transmission of *Mavi*MNPV to larvae of *M. vitrata* and could acquire and transmit the virus over several generations (M. Tamò, personal communication). In this study, females of *A. taragamae* were attracted to *Mavi*MNPV-infected live larvae, but not to moribund larvae (Fig. 4). The wasps did not discriminate between volatiles from healthy and *Mavi*MNPV-infected live larvae, but they preferred the volatiles from healthy larvae over those from moribund larvae. Apparently, the viral infection only affected larvae attractiveness in a late stage of infection. The virus disease symptoms appear about 3–4 day after infection of the *M. vitrata* larvae (Lee et al., 2007). Our observations are similar to those reported for the parasitoid *Biosteres longicaudatus* Ashmead, which was unable to locate immobilized or dead hosts (Lawrence, 1981).

*Mavi*MNPV, like other baculoviruses, which are host-destroying viruses, is likely to affect negatively the development of *A. taragamae,* and it needs more attention for its management. Parasitoids and insect pathogens often are involved in scramble competition for host resources in dually infected and parasitized hosts. In such cases, some parasitoid species develop a strategy to enhance their developmental rate (Escribano et al., 2000). The temperature seemed to influence *Mavi*MNPV symptoms development in parasitized larvae. At 29°C, *A. taragamae* pupated on average 6 d after parasitization, and this limited the detrimental effect of the virus infection (Dannon et al. unpublished data). Interactions between the wasp and *Mavi*MNPV need to be investigated further to evaluate the effects of the virus on the parasitoid and to identify factors that avoid or limit the detrimental effect on the wasp reproduction.

In summary, this study shows that floral volatiles produced by *M. vitrata*-infested cowpeas flowers attracted *A. taragamae* females. Uninfested leaves and flowers of peabush also attracted the parasitoid. In contrast, the wasp was attracted to uninfested cowpea flower only after an odor experience. Olfactory cues from *M. vitrata* larvae also were used by the wasp in its host selection process. Further research should assess the influence of other key host plants of *M. vitrata,* such as *Pterocarpus santalinoides* L'herit ex DC.*,* and *Lonchocarpus sericeus* (Poir) H.B.K. on the host selection behavior of *A. taragamae.*

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the sex ratio of the progeny becomes important (Godfray, 1994). Both gregarious and solitary species can superparasitize hosts, but, in solitary parasitoids, the presence of supernumerary juveniles results in competition for survival and the death of all but one. This competition has been defined as a lethal larval combat (Ueno, 1997), physiological suppression (Vinson and Hegazi, 1998), or scramble competition (Mayhew and Hardy, 1998). Solitary parasitoids must, therefore, lay their eggs on unparasitized hosts to increase the chances of survival of their progeny. However, while the expected fitness gain per host is lower when females superparasitize, superparasitism by solitary species can be an adaptive behavior in certain circumstances (van Alphen and Nell, 1982), e.g., when the number of unparasitized hosts is small or when travel time between patches is long (van Alphen and Visser, 1990). Such behavior is adaptive only if the second egg laid on an already parasitized host can "win" the competition with the first immature (Lebreton et al., 2009).

Eupelmus vuilleti (Crw.) (Hymenoptera: Eupelmidae) is a solitary ectoparasitoid of the larvae of *Callosobruchus maculatus* Fabr. (Coleoptera: Bruchidae) in the tropical zones of West Africa. Females perceive various types of information from seeds that contain their hosts, thus enabling them to change their oviposition behavior. They can discriminate between unparasitized and parasitized hosts, preferring to lay eggs on unparasitized hosts, thus changing their offspring sex ratio (Darrouzet et al., 2007, 2008). They also can discriminate unparasitized hosts from hosts parasitized by a different wasp species, *Dinarmus basalis*: this discriminative capacity seems to be due to chemical cues that probably originate from the Dufour's gland, and that were deposited by previous ovipositing females (Jaloux et al., 2005). After oviposition by *E. vuilleti* females, hydrocarbons also are found on seeds (Darrouzet, personal observation) and can be perceived by other conspecific females, which could, in turn, alter their responses. However, in this species, the nature and origin of cues perceived by conspecifics are still unknown.

This study determined whether cuticular hydrocarbons deposited artificially on seeds are perceived by *E. vuilleti* females, and whether these females change their oviposition accordingly. Specifically, (1) we tested whether the CHC profiles from males and females are different, and (2) we presented seeds labeled with CHCs from males or females to ovipositing females.

Methods and Materials

Rearing Conditions Bruchid *Callosobruchus maculatus* (F.) (Coleoptera, Bruchidae) and wasp *Eupelmus vuilleti* samples were taken from populations that have been established in the laboratory since 1997, and which originated from Burkina Faso (West Africa). They were mass-reared in a climate-controlled chamber under conditions close to those at their place of origin: 12 hr light at 33°C, 12 hr dark at 23°C and 70% r.h.

Preparation of Reproductive Patches A circle of six equidistant gelatine capsules, each containing one *C. maculatus* L_4 larva, was fixed in an arena (diam=8 cm, height=2.5 cm; henceforth called a reproductive patch, Fig. 1). Each bruchid fourth instar (L_4) was placed inside the capsule after being removed from its seed by dissection and selected by weight (9.12±0.20 mg). The gelatine capsules mimic the bruchid pupal chamber in the seed and enable oviposition by *E. vuilleti* females (Darrouzet et al., 2003). Two different areas within the patch were prepared to provide aggregates of hosts with different properties (Darrouzet et al., 2007). Three adjacent capsules were labeled with cuticular hydrocarbons (CHCs) from males or females (L capsules), and the other three adjacent capsules remained unlabeled (unL capsules). CHCs from the abdomen of parasitoids were transferred onto the cap of the capsules by rubbing the individuals for 10 sec. Each capsule was labeled by using a single individual that was put to sleep at 4°C before rubbing. Control patches were prepared with six unlabeled capsules.

Sample Preparation and Chromatographic Procedures Cuticular hydrocarbons were extracted in conical vials with 1 ml dichloromethane (Sigma Aldrich) from males (3 replicates of 10 males), females (6 replicates of 10 females), CHC labeled capsules (50 upper part of capsules; i.e., the part in contact with the ovipositing parasitoid females), and from unlabeled capsules (50). After mixing for 1 min, the insects or capsules were removed, and the extracts were dried to 2 µl and analyzed by gas chromatography. Samples first were analyzed using a Perkin-Elmer Autosystem XL GC (Perkin-Elmer, Wellesley, MA, USA) with a flame ionization detector (FID)

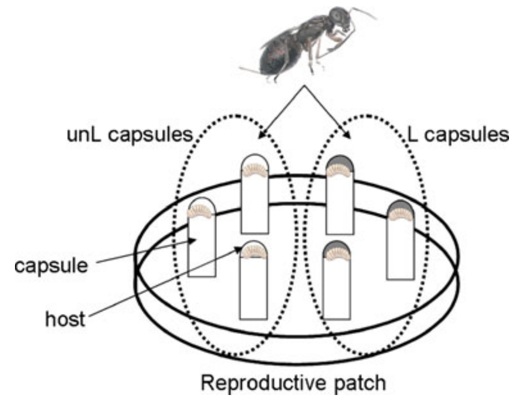


Fig. 1 Diagram of a reproductive patch

and Turbochrom software. Samples were injected into the GC-FID injector at 220°C in splitless mode and analyzed by using a BP1 capillary column (25 m, ID 0.32 mm), which was temperature programmed from 150°C (2 min) to 300°C (10 min) at 5°C/min.

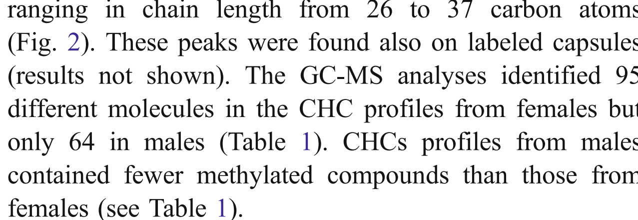
The components then were identified by GC-MS analysis by using a Hewlett-Packard 5890 GC system coupled to a 5989A MS, controlled by HP ChemStation software. Pools of males (30 individuals) and females (60 individuals) were injected into the GC-MS injector using the procedure described above. Hydrocarbons were identified by their mass spectra corroborated by their ECL indices (for review see Lockey, 1988; Blomquist, 2010), using the M^+ of diagnostic ions and the $M\text{-}15^+$ when visible to confirm their methylbranch numbers (Nelson, 1993).

Oviposition of Mated Females To obtain mated females, 2-h-old virgin females were mated with a 24-h-old virgin male. Two patches containing unlabeled (unL) and labeled (L) capsules were offered daily (2 exposure periods of 4 hr) to each mated female (24 individuals, 13 females with L capsules labeled with CHCs from males, and 11 females with L capsules labeled with CHCs from females). Oviposition was performed in the climate-controlled chamber. At the end of each period, the positions of each egg laid in each capsule were noted. After each period, old capsules were replaced by new ones. To determine the sex of eggs laid by mated females, at the end of each period, each egg laid on a capsule was transferred to a different host and deposited in a different cell on a Plexiglas sheet closed by a Plexiglas cover-slide until the emergence of the adult parasitoids (Darrouzet et al., 2003). On emergence, the sex ratio was measured as the proportion of females emerging from the cells on the Plexiglas sheets (i.e., the secondary sex ratio).

Results were compared with mated (19) females ovipositing in the same experimental conditions in reproductive patches containing a circle of six unlabeled capsules.

Statistical Analyses Data were tested for normal distribution and homogeneity of variance (*Levene test*) and then were analyzed by using a *Mann-Whitney's U-test* for unpaired samples, a *Wilcoxon test* for paired samples, and a *Student's t-test*. The sex ratios of eggs laid in parasitism and superparasitism were compared by using a standard χ^2*-test*. All statistical analyses were performed with Statistica 6.0 software (Statsoft, Inc.).

Results

Analyses of CHCs from Males and Females The GC-FID analyses distinguished 38 peaks in females and 24 in males, 
ranging in chain length from 26 to 37 carbon atoms (Fig. 2). These peaks were found also on labeled capsules (results not shown). The GC-MS analyses identified 95 different molecules in the CHC profiles from females but only 64 in males (Table 1). CHCs profiles from males contained fewer methylated compounds than those from females (see Table 1).

Oviposition of Females Within Control Patches (Unlabeled Capsules) Comparison of two random areas of three adjacent capsules showed that females had the same oviposition activity for the number of parasitized hosts (*Student t test*: t=0.44, df=18, P=0.67) (Fig. 3a), eggs laid (*Student t test*: t=0.30, df=18, P=0.77) (Fig. 3b), and self-superparasitized hosts (*Wilcoxon test*: Z=1.32, P=0.19) (Fig. 3c).

Oviposition of Females Within Patches when Capsules L Were Labeled with Female CHCs Ovipositing females parasitized more hosts and laid more eggs in unL capsules than in L capsules (*Wilcoxon test*: Z=2.28, P=0.023 and Z=2.03, P=0.043, respectively) (Figs. 3a and b). However, they self-superparasitized the same number of hosts in unL and L capsules (*Student t test*: t=1.63, df=22, P=0.12) (Fig. 3c). These females had the same oviposition activity in unL capsules as females ovipositing in three random adjacent capsules in control patches (number of eggs laid, *Student t test*: t=0.023, df=29, P=0.98; number of parasitized hosts: t=0.72, df=27, P=0.47; number of self-superparasitized hosts: t=0.54, df=29, P=0.59) (Figs. 3b, a, and c, respectively). However, they laid fewer eggs in L capsules than females ovipositing in three random adjacent capsules in control patches (*Mann-Whitney U test*: U=47.5, P=0.05) (Fig. 3b), and parasitized fewer hosts (*Mann-Whitney U test*: U=49.5, P=0.05) (Fig. 3a). But they self-superparasitized the same number of hosts (*Student t test*: t=0.21, df=29, P=0.83) (Fig. 3c).

Oviposition of Females Within Patches When Capsules L Were Labeled with Male CHCs Females had the same oviposition activity in unL and L capsules (parasitized hosts: *Student t test*: t=0.16, df=12, P=0.88; eggs laid: t=0.12, df=12, P=0.90; self-superparasitized hosts: *Wilcoxon test*: Z=0.25, P=0.80) (Figs. 3a, b, and c, respectively). Females parasitized the same number of hosts in unL or L capsules as females ovipositing in three random adjacent capsules in control patches (unL capsules, *Student t test*: t=−1.85, df=30, P=0.074; L capsules: t=−1.88, df=30, P=0.069), laid the same number of eggs (unL capsules: t=−1.12, df=30, P=0.268; L capsules: t=−1.38, df=30, P=0.176), and self-superparasitized the same number of hosts (unL capsules, *Mann-Whitney U test*: U=168.5, P=0.074; L capsules: U=121.5, P=0.95).

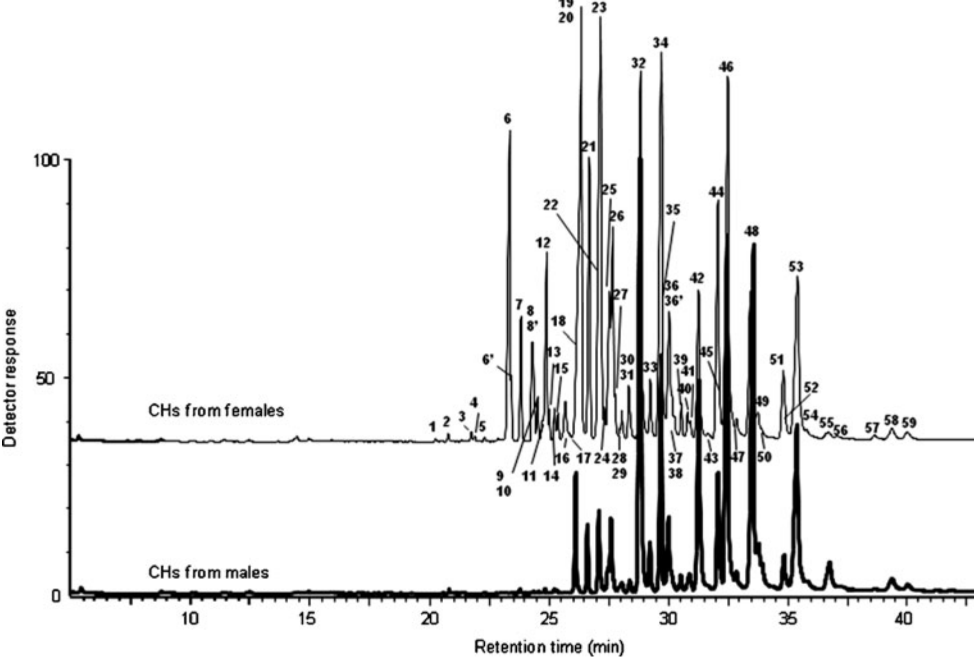


Fig. 2 GC-FID chromatogram of cuticular hydrocarbon profiles from *Eupelmus vuilleti* males and females

Oviposition of Females Between Separate Patches Females had the same oviposition behavior for patches containing L capsules labeled with male CHCs as females ovipositing in control patches (mean number ± SE of eggs laid: 33.23±1.42 vs. 29.84±1.58; *Student t test*: t=2.19, df=31, P=0.15; self-superparasitized hosts: 1.9±0.31 vs. 2.71±0.48; *Mann-Whitney U test*: U=160, P=0.157). When patches containing L capsules were labeled with female CHCs, females also had the same oviposition behavior in those patch as females in control patches (mean number ± SE of eggs laid: 25.83±1.17; *Student t test*: t=3.17, df=30, P=0.08; self-superparasitized hosts: 3.44±0.6; *Student t test*: t=0.86, df=25, P=0.36). However, females laid fewer eggs in the patch when L capsules were labeled with female rather than male CHCs (*Student t test*: t=15.90, df=24, P<0.001). Also, they self-superparasitized more hosts (*Student t test*: t=6.05, df=19, P=0.02).

Sex Ratio of Progeny When two capsule areas were compared within patches, we showed that females produced the same offspring sex ratio (proportion of females) in unL and L capsules, whatever the sexual origin of the CHCs deposited on L capsules (female CHCs: SR=0.68 in unL capsules vs. 0.62 in L capsules χ^2=1.07, df=1, P=0.30; male CHCs: SR=0.79 vs. 0.71, χ^2=2.38, df=1, P=0.12). Females within control patches also produced the same offspring sex ratio in two random areas (SR=0.65 vs. 0.62, χ^2=0.24, df=1, P=0.62).

Offspring sex ratios (L + unL capsules) were female biased, i.e., SR=0.66 for females ovipositing in patches containing L capsules labeled with female CHCs (χ^2=21.74, df=1, P< 0.001), 0.75 for females ovipositing in patches containing L capsules labeled with male CHCs (χ^2=68.00, df=1, P< 0.001), and 0.64 for females ovipositing in control patches (χ^2=28.31, df=1, P<0.001). However, females laid more daughters in patches containing L capsules labeled with male CHCs than in control patches or patches containing L capsules labeled with female CHCs (χ^2=9.52, df=1, P= 0.002 and χ^2=5.02, df=1, P=0.025, respectively) (Fig. 4).

Discussion

Eupelmus vuilleti females had different ovipositing activities depending on whether or not the seeds were labeled previously with CHCs. This adaptive oviposition shows that *E. vuilleti* females can detect these compounds present on seeds, and that their oviposition behavior is modified by the sexual origin of these CHCs. The presence of CHCs from other females modified egg distribution on hosts, whereas the male CHC label affected only the sex ratio of the progeny. These results demonstrate that parasitoid females can use (i) chemical residues present in the environment (such as CHCs deposited on seeds) and (ii) the sexual origin of such compounds to change their oviposition behavior.

In *E. vuilleti* species, males and females have different CHC profiles. CHCs from females contain certain mono- and dimethyl-branched compounds, which are absent from males. This shows a sexual dimorphism in the profiles. Although the functional significance of this sexual dimorphism in *E. vuilleti* remains to be tested, it may be significant, as is the case in some insects such as the cricket *Teleogryllus oceanicus* (Thomas and Simmons,

Table 1 Identification (using GC-MS) and relative amounts (% of total, using GC-FID) of the cuticular hydrocarbons from *Eupelmus vuilleti* males and females. A "/" separates carbon atom numbers when compounds have a methyl group that may be placed at either of these positions and cannot be discriminated using GC-MS

				Presence in			
				females		males	
Peak number	CHC identity	ECL	CN	mean	se	mean	se
1	4/2Me-C24	24.65	25	traces		–	
2	*n*-C25	25.00	25	traces		–	
3	4/2Me-C25	25.65	26	traces		–	
4	3Me-C25	25.70	26	traces		–	
5	*n*-C26	26.00	26	traces		–	
6 + 6′	4/2+3Me-C26 + C27:1	26.70	27	2.09	0.5	traces	
7	*n*-C27	27.00	27	1.83	0.23	0.37	0.14
8 + 8′ + 9	13+11+9+7Me-C27	27.35	28	0.57	0.07	–	
10	5Me-C27	27.49	28	0.31	0.07	traces	
11 + 12	4/2+3Me-C27	27.71	28	1.56	0.23	0.25	0.08
13	5,9diMe-C27	27.76	29	traces		–	
14	*n*-C28	28.00	28	0.52	0.03	0.22	0.05
15	3,7diMe-C27	28.06	29	0.21	0.07	0.15	0.07
16	14+13+12+11+10Me-C27	28.33	28	0.38	0.02	0.05	0.05
17	6Me-C28	28.41	29	0.04	0.03	–	
18	4/2Me-C28	28.69	29	3.53	0.4	3.1	0.12
19 + 20	C29:1 + 3Me-C28	28.75	29	1.84	0.23	traces	
21	*n*-C29	29.00	29	5.93	0.71	2.3	0.32
22	15+13+11Me-C29	29.33	30	6.92	0.69	2.47	0.59
23	9+7Me-C29	29.39	30	traces		–	
24	5Me-C29	29.46	30	0.32	0.04	0.18	0.04
25	4/2Me-C29 + 9,19+9,17+9,15+9,13diMe-C29	29.65	30 + 31	2.54	0.52	–	
26	3Me-C29	29.69	30	1.95	0.26	1.09	0.24
27	5,11+5,9diMe-C29	29.73	31	1.71	0.44	1.55	0.19
28 + 29	3,13+3,11+3,7diMe-C29	30.10	31	0.77	0.04	traces	
30 + 31	16+15+14+13+12Me-C30	30.32	31	0.98	0.08	traces	
32	4/2Me-C30	30.68	31	7.03	0.76	14.15	1.22
33	*n*-C31	31.00	31	1.43	0.09	1.38	0.22
34 + 35	15+13+11Me-C31	31.36	32	10.38	0.6	9.09	0.29
36 + 36′	11,21+11,19+11,17+11,15+9,13diMe-C31	31.53	33	3.1	0.37	2.07	0.44
37	7,13diMe-C31	31.59	33	traces		0.64	0.2
38	3Me-C31	31.65	32	0.38	0.04	–	
39	3,13diMe-C31	32.13	33	0.09	0.06	traces	
40	16+15+14+13+12+11Me-C32	32.32	33	0.58	0.02	traces	
41	C33:2	32.38	33	0.46	0.1	0.23	0.07
42	11,17+11,15diMe-C32+4Me-C32	32.72	34 + 33	4.19	0.31	5.96	0.85
43	*n*-C33	33.00	33	0.42	0.19	1.08	0.3
44 + 45	17+15+13+11MeC33	33.33	34	7.62	0.7	5.45	0.63
46	11,15diMe-C33	33.67	35	14.99	0.58	15.53	0.93
47	diMe-C33	34.16	35	traces		–	
48 + 49 + 50	C35:2+11,17diMe-C34+C35:1	34.4–34.6	35 + 36	4.18	0.69	22.05	2.68
51 + 52	17+15+13+11Me-C35	35.36	36	2.99	0.43	2.33	0.3
53	13,17+11,17diMe-C35	35.70	37	8.2	0.79	8.31	0.69
54	C37:2		37	Not	quantified		

Table 1 (continued)

Peak number	CHC identity	ECL	CN	Presence in females mean	females se	males mean	males se
55	C37:1		37				
56	11,17diMe-C36		38				
57	19+17+15+13+11Me-C37		38				
58	13,17+11,17diMe-C37		39				

ECL equivalent chain length, *CN* carbon number

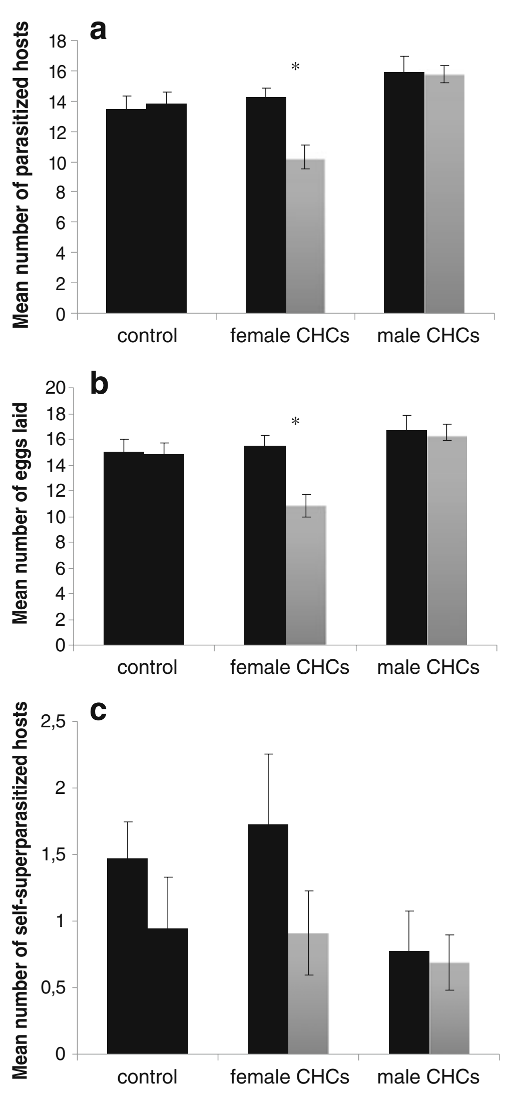


Fig. 3 Mean number and standard error of parasitized hosts (**a**), eggs laid (**b**) and self-superparasitized hosts (**c**) by *Eupelmus vuilleti* females ovipositing in unlabeled (unL) or labeled (L) capsules. *$P<0.05$

2008) or various species of Drosophila (Cobb and Jallon, 1990). If the chemical composition of CHCs is used as a contact pheromonal signal for mate choice, sexual selection may drive differentiation of these compounds. Various studies of several species of insects have demonstrated such differentiation (Howard and Blomquist, 2005; see appendix in Thomas and Simmons, 2008; Ferveur and Cobb, 2010). Particular compounds produced by only one sex can stimulate the sexual behavior of the other, or a specific sex ratio among hydrocarbons that are present in the two genders can influence mate choice (Ginzel et al., 2003). It also has been shown that some selected traits related to mate choice need maturation delay to be effective. This delay also may involve CHC profiles, as the differences among these compounds are more marked between sexes when insects are older (Chenoweth and Blows, 2005). The CHCs profiles of *E. vuilleti* were not analyzed according to age in this study. This trait may not be of great importance in this species, however, as adults are sexually mature when they emerge (Darrouzet, personal observation).

Many insects deposit chemical residues on different substrates, and these traces can provide intraspecific and/or

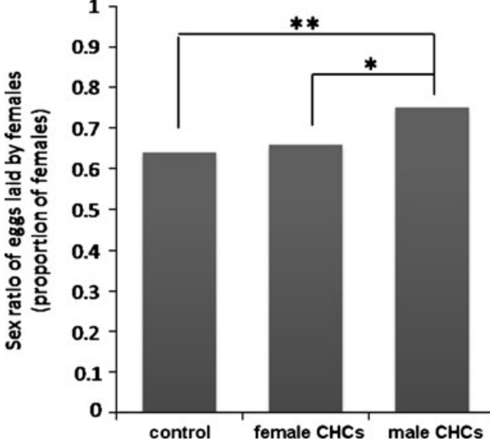


Fig. 4 Sex ratio of eggs laid by *Eupelmus vuilleti* females confronted with only unlabeled capsules (control), capsules labeled by female CHCs or male CHCs. *$P<0.05$; **$P<0.01$

interspecific cues that modify the behavior of receiving individuals. Marking pheromones are used by many parasitoid species, thus avoiding the laying of their eggs in hosts that have already been parasitized and that are less useful for the larval development of their offspring (Godfray, 1994; Nufio and Papaj, 2001). In a previous study, Darrouzet et al. (2007) showed that *E. vuilleti* females discriminated parasitized from unparasitized hosts. Under the same experimental conditions, the parasitized status of hosts was mimicked by labeling seeds containing unparasitized hosts with female CHCs. Females showed the same differences in oviposition strategy (in the location of eggs laid) when they were in the presence of either unlabeled or female CHC labeled seeds (in this study) as in the presence of both unparasitized and parasitized hosts (see Darrouzet et al., 2007). They laid more eggs on unparasitized hosts (unlabeled seeds in this study) than on parasitized hosts (labeled seeds). This mimicry was not obtained with seeds labeled with male CHCs. Hydrocarbons, thus, may be the chemical information used by ovipositing females to discriminate unparasitized from parasitized hosts. Jaloux et al. (2005) observed that *E. vuilleti* females used such compounds, deposited by *Dinarmus basalis* females after laying eggs, to superparasitize their hosts. In this interspecific competition, *E. vuilleti* females laid more eggs in superparasitism than in parasitism. The glandular secretion deposited by *D. basalis* females originates from the Dufours' gland.

Cuticular and Dufour's gland secretions may result in identical activity because of the similarity in hydrocarbon composition. However, there are differences in the relative concentrations between cuticular and Dufour's gland hydrocarbons, as is the case in *E. vuilleti* (Darrouzet, personal observation). This similarity seems to be a widespread phenomenon in insects as described, for example, in bumblebees (Oldham et al., 1994), polistes wasps (Dani et al., 1996), honeybees (*Apis mellifera*: Gozansky et al., 1997), and some parasitoid wasps (*Cephalonomia tarsalis*, *C. waterstoni*, *Anisopteromalus calandrae* and *Pteromalus cerealellae*: Howard and Baker, 2003; *Dinarmus basalis*: Jaloux et al., 2005).

Eupelmus vuilleti females drill the seeds with their ovipositor and oviposit on the bruchid larvae *C. maculatus,* which develop in seeds of a legume, the cowpea *Vigna unguiculata* (Walp.). The bruchid and its parasitoid emerge in granaries after the cowpea seeds have been harvested and reproduce in this storage environment. Several generations of *C. maculatus* and *E. vuilleti* develop in granaries throughout the dry season. *Eupelmus vuilleti*, therefore, lives in an environment where several types of seed are stored. Under these conditions, females exploring the surface of the seeds looking for bruchid hosts to oviposite, or males searching for females with whom to copulate, could touch seeds and thus deposit CHCs because of crowding. These chemical residues could then be perceived and used by conspecifics to identify the gender of individuals. The perception of female CHCs on a seed that contains a host could inform a conspecific female that the host may already be parasitized and the perception of male CHCs could indicate that the host is unparasitized. The lengths of the CHCs identified in this study range from 24 to 37 carbon atoms, which is similar to that found in other parasitoid species (Howard and Baker, 2003). Given their long chains, these compounds mainly are non-volatile and are, therefore, probably detectable for some time after being deposited. As female CHCs prevent other females from laying their eggs on the same hosts, they probably are perceptible for at least a period that will prevent the first individual laid from being affected adversely as a result of a second oviposition (Roitberg and Mangel, 1988). It is not known how long such a marker can last. Moreover, the way in which *E. vuilleti* deposits CHCs in a seed store still has to be determined. However, chemical residues may remain on a substrate as described in previous studies, and thus indirectly associated with the host (Borges et al., 2003; Conti et al., 2004). The footprint may consist of non-volatile lipids, secreted by specialized glands, identical to those found on the cuticle (Nakashima et al., 2004), or CHCs present in the wax layer of the cuticle (Colazza et al., 2007). The egg parasitoid species *Trissolcus basalis* uses the CHCs on the cuticle of their hosts (the stink bug *Nezara viridula*) as contact kairomones to discriminate gender (Colazza et al., 2007). Additionally, Müller and Riederer (2005) showed that compounds from a phytophagous insect could be adsorbed on a plant surface and be used as host-finding kairomones for parasitoids.

In Hymenoptera parasitoids, females have a haplodiploid genetic system for allocating the sex of their progeny, where males develop from unfertilized eggs and females from fertilized eggs (Godfray, 1994). This sex allocation can be modulated by various factors such as the number of conspecific females in the reproductive patch (Hamilton, 1967) or the presence of already parasitized hosts (Darrouzet et al., 2008). Although a single female is expected to lay the minimum number of sons able to mate with all their sisters, when the competition level increases (with more females or more already parasitized hosts), the same female would benefit by producing a higher proportion of sons in order to mate with both her own daughters and those of other females. This is predicted by Hamilton's local mate competition (LMC) theory (Hamilton, 1967). Moreover, additional information also could help the female to adjust her progeny allocation for already parasitized hosts, such as the sex of the juvenile in or on the host (Lebreton et al., 2010), its age (Lebreton et al., 2009), or its species (Gauthier et al., 1999). *Eupelmus vuilleti* females can alter their offspring sex ratio as predicted by the LMC theory. The

sex ratio changes in response to the presence of conspecific females and/or already parasitized hosts (Darrouzet et al., 2003, 2007, 2008).

Little attention has been paid to the role of males in influencing sex allocation, although males can influence female sex allocation in a number of ways in haplodiploid organisms (Henter, 2004). Males may change their fertilization ability by producing sperm unable to fertilize eggs: mated females thus would produce more unfertilized eggs, i.e., sons. A second possibility is that males could transfer sperm to females during mating, but that incompatibility between paternal and maternal genomes could lead to the death of daughters. Males also may actively search for mates and then influence female sex allocation because they only transfer their genes to the next generation when the females lay fertilized eggs, i.e., daughters.

Finally, according to the constrained model (Godfray, 1990), when the proportion of virgin females increases in the population, mated females should produce a higher proportion of daughters, thus compensating for the number of sons produced by these "constrained" females. However, mated females would benefit by detecting the proportion of virgin females present. This estimation could be performed on the basis of the encounter rate of courting males in the environment (Ode et al., 1997). Under our experimental conditions, there were no males. However, females produced more daughters when they perceived male odor on seeds. This suggests that males may influence female sex allocation by depositing their odor in the environment.

In summary, this study supports the idea that, in *E. vuilleti*, females adjust their oviposition behavior according to the sexual origin of cuticular traces left by conspecifics on seeds. When they detect traces of other females, they lay fewer eggs on these seeds but when they perceive traces of males, they produce more daughters. This result highlights the importance of the precise nature of the chemicals present in the environment on strategy adjustments.

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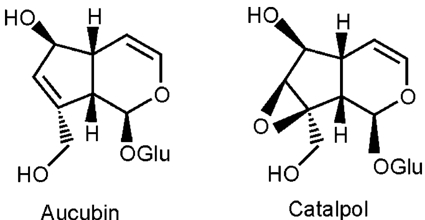

Fig. 1 Chemical structures of aucubin and catalpol

10–12% dry weight in leaves, although amounts can vary with leaf and plant age and among individuals (Bowers and Stamp, 1993); 2. *P. major*, which produces only aucubin in relatively low amounts of 0.5–2.0% in leaves (Barton and Bowers, 2006); or 3. both. *Junonia coenia* Hübner (Nymphalidae) is a specialist on iridoid glycoside producing plants, and larvae can sequester high amounts of iridoid glycosides, which render them unpalatable (Theodoratus and Bowers, 1999). *Estigmene acrea* (Drury) (Arctiidae) is a conspicuous grazing generalist (Bernays et al., 2004) that preliminary analyses suggested can sequester iridoids (Bowers, 2009), while *Spilosoma congrua* (Walker) (Arctiidae) is a cryptic, grazing generalist that can also sequester iridoids (Bowers and Stamp, 1997). All these species feed on both *Plantago* species.

Methods and Materials

Freshly hatched larvae were reared in groups of 10 until the third instar, when they were placed individually into 100 mm Petri dishes for the remainder of the trial. Larvae were reared upon one of four leaf diets: freshly excised leaves of *P. major*, *P. lanceolata*, *P. major* until the antepenultimate instar then changed to *P. lanceolata*, or *P. lanceolata* until the antepenultimate instar then changed to *P. major*. Leaves of a mixture of ages were collected daily from many individual plants in stands around Boulder, CO, USA. Leaves were of various ages, and no attempt was made to collect leaves from particular plants or of a certain age. Iridoid glycoside concentrations of leaves were not measured. Larvae were frozen at −80°C immediately upon reaching the final instar. Sample sizes ranged from 8–10 individuals/diet, except for *E. acrea* reared on *P. major* followed by *P. lanceolata*, where mortality was high and only 2 larvae were available. Larvae were prepared for analysis and iridoid glycosides [derivatized using Tri-sil Z (Pierce Chemical Company)] quantified by gas chromatography (methods in Bowers and Stamp, 1997). Fresh to dry weight conversions were obtained from separate sets of caterpillars, and all results are reported on a dry weight basis. Bonferroni-adjusted 1-way analyses of variance (ANOVA) were performed for each species, comparing the percent dry weight of total iridoid glycosides (analyzed as the arc-sine transformed proportion) and the proportion of catalpol (arc-sine transformed) sequestered among diets. Tukey's HSD post-hoc tests were applied where appropriate.

Results

Junonia coenia larvae sequestered the greatest concentration of iridoids, while *E. acrea* sequestered the lowest concentration, and *S. congrua* was intermediate (Fig. 2). All three species sequestered both aucubin and catalpol when both were available, although the relative proportion of the two compounds varied among both diets and species. Catalpol has not been reported in *P. major* (Barton and Bowers, 2006 and references therein), and thus, none was found in the larvae reared solely on this plant, so these larvae were not included in analyses of catalpol sequestration.

Estigmene acrea was a poor sequesterer and was particularly inefficient at sequestering catalpol, which could not be compared statistically (Fig. 2). Diet did not influence

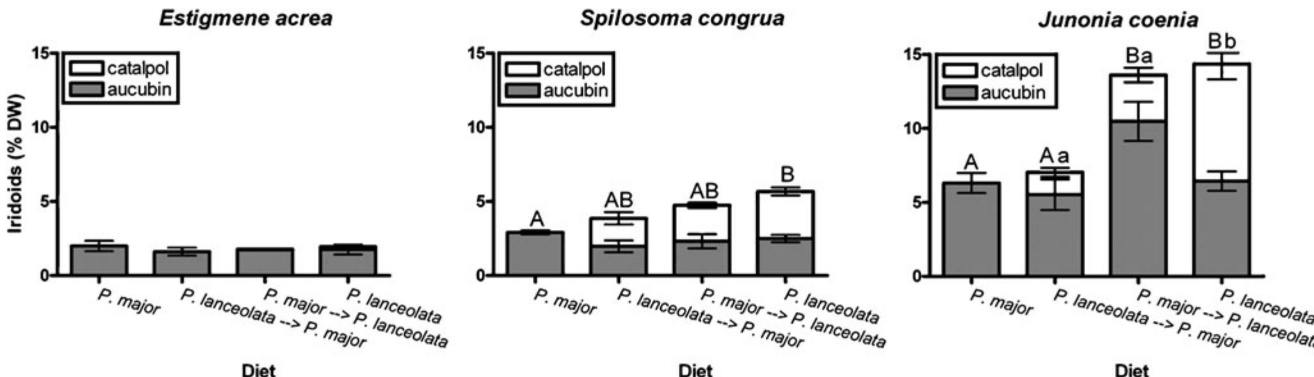

Fig. 2 Average (±1 SEM) iridoid glycoside sequestration of three caterpillar species varying in degree of specialization. *Uppercase letters* indicate significantly ($P<0.05$) different total iridoid glycoside means according to Tukey's Honestly Significant Difference post-hoc tests, while *lowercase letters* indicate significant differences in the proportion of catalpol

E. acrea sequestration ($F_{3,26}$=0.26, P=0.85). *Spilosoma congrua* was particularly effective at sequestering catalpol. Catalpol made up the majority of the sequestered iridoids and proportion of catalpol was not influenced by diet ($F_{2,21}$=0.71, P=0.50); however, diet did influence total iridoid sequestration ($F_{3,30}$=8.18, P<0.001) (Fig. 2). Diet influenced both the proportion of catalpol ($F_{2,23}$= 82.68, P<0.001) and total iridoids ($F_{3,31}$=5.80, P=0.003) sequestered by *J. coenia* (Fig. 1).

Discussion

The three species differed substantially in their sequestration ability. *Junonia coenia*, a specialist on iridoid glycoside containing plants, sequestered the highest amounts. The amount of total iridoid glycosides was almost three times higher when these larvae were fed solely *P. lanceolata* than when they were fed *P. major*. Feeding experiments with wolf spiders showed that such a difference renders *P. lanceolata*-reared *J. coenia* less palatable than those reared on *P. major* (Theodoratus and Bowers, 1999). In addition, however, catalpol appears to be a more toxic iridoid than aucubin (Puttick and Bowers, 1988), which may also contribute to this difference in palatability. *Spilosoma congrua* sequestered about one third the levels of iridoids sequestered by *J. coenia*. Of three *Spilosoma* species tested, *S. congrua* was the only species with the ability to sequester iridoid glycosides; its congeners, *S. virginica* and *S. latipennis* were unable to sequester iridoids (Bowers and Stamp, 1997). Interestingly, although *E. acrea* was able to sequester aucubin in about the same amounts as *S. congrua*, it was poor at sequestering catalpol. *Estigmene acrea* thus was particularly effective at breaking down or eliminating catalpol.

In both *S. congrua* and *J. coenia*, diet had a strong effect on the amount of iridoids sequestered. Since most food is eaten and most iridoids are ingested in the later instars, the host plant fed on during the later instars most affected the chemical content of the caterpillars. This was most apparent in *J. coenia*, in which feeding on *P. lanceolata* in the later instars doubled the amount of sequestered iridoids compared to larvae fed only *P. major* or *P. lanceolata* followed by *P. major*. Indeed, larvae fed *P. lanceolata* after *P. major* had levels of total iridoid glycosides almost as high as larvae reared exclusively on *P. lanceolata* (Fig. 2). However, feeding exclusively on *P. lanceolata* resulted in much higher levels of catalpol being sequestered compared to larvae fed on *P. major* followed by *P. lanceolata*. Although less dramatic, similar results were found for *S. congrua*. These results emphasize the importance of host plant choice in later instars, as that affects sequestration and consequent protection against natural enemies.

Both arctiid species in this study graze among several plant species, including species that vary widely in secondary chemistry (Bernays et al., 2004). Depending on the plant species consumed, these caterpillars can sequester quite different profiles of secondary compounds, and potentially vary dramatically in their palatability to natural enemies. Furthermore, *E. acrea* can sequester a quite different group of compounds, pyrrolizidine alkaloids, and retain these compounds through to the adult stage in amounts as high as approximately 4% dry weight (Hartmann et al., 2005). Thus, larvae of *E. acrea* are better at sequestering pyrrolizidine alkaloids than iridoids. However, given the polyphagous feeding behavior of this species, it could encounter both pyrrolizidine alkaloid and iridoid glycoside containing hostplants and could sequester both kinds of compounds.

In summary, this study provides the first quantification of sequestration of iridoid glycosides by *E. acrea*, and showed that only low levels are sequestered and that catalpol is inefficiently sequestered, if at all. Second, we showed that host plant species affects sequestration of iridoids in *S. congrua* and *J. coenia*, but not *E. acrea*. Third, the variation in sequestration among diets was greatest for *J. coenia*, the species sequestering the highest amounts and concentrations of iridoid glycosides. Overall, our results show that although plant defense compounds may be sequestered by both generalist and specialist insects, the amounts that different species contain and the efficiency with which those compounds are sequestered may vary considerably.

Acknowledgements We thank A. Smilanich and D. Wagner for collecting *Estigmene acrea* and *Spilosoma congrua*, E. Burke and A. Hill for rearing assistance and C. Quintero for comments. This study was funded by NSF grant DEB 0614883 to M.D. Bowers and L.A. Dyer.

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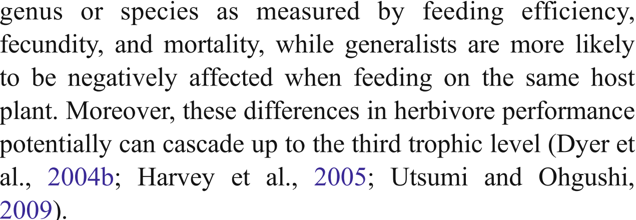
apparent lack of defensive properties for a variety of plant secondary compounds that have no antiherbivore function when tested separately from complex natural mixtures (Harborne, 1988; Ayres et al., 1997).

Quantification of the tritrophic effects of plant defenses has been the focus of many studies in chemical ecology. The bottom-up or "green desert" model suggests that plant defenses work directly to limit herbivore populations, which in turn limits natural enemy populations (White, 1978; Abe and Higashi, 1991). Plant defenses also have been found to mediate top-down forces by attracting natural enemies to herbivores (Vet and Dicke, 1992; De Moraes et al., 1998; Turlings and Wackers, 2004) or by decreasing herbivore resistance to parasitoids (Zvereva and Rank, 2003; Bukovinszky et al., 2009; Lampert and Bowers, 2010). In the latter example, sequestered toxins negatively affect a caterpillar's immune response by reducing its ability to encapsulate and kill parasitoid eggs, thus rendering them more vulnerable to successful attacks (Haviola et al., 2007; Smilanich et al., 2009). On the other hand, plant defenses can have detrimental effects on natural enemies. Herbivore sequestered plant defenses can provide resistance by being toxic or deterrent to natural enemies (Campbell and Duffey, 1981; Barbosa et al., 1986; Dyer, 1995; Ode, 2006; Singer et al., 2009). Parasitoid performance often is linked to the host herbivore performance, such that negative effects of plant chemistry on the herbivore are associated with negative effects on the parasitoid (Harvey et al., 2005; Soler et al., 2005; Gols et al., 2008). These deleterious effects of plant defenses on parasitoids, combined with higher toxicity and diversity of plant defenses in the tropics, are suggested to explain why there is no latitudinal diversity gradient for parasitic hymenoptera ("nasty" host hypothesis, Gauld et al., 1992).

While the effects of plant chemistry on the third trophic level vary among study systems, one strong bitrophic pattern is that there are marked differences in the response of specialist herbivores, which are adapted to specific host plant chemistry, and generalist herbivores, which are adapted to compounds from many different host plants but have a comparatively weaker response to specific defenses. Most studies that compare the effects of chemistry on specialists versus generalists have focused on single compounds (Bowers and Puttick, 1989; Van Dam et al., 1995; Wasano et al., 2009) or a broadly measured mixture of compounds (e.g., total phenolics, Bi et al., 1997, also see Coley et al., 2005) as opposed to examining synergy. However, there is growing evidence that synergistic defenses also affect specialist and generalist differently (Hagele and Rowell-Rahier, 2000; Dyer et al., 2003). Whether or not the defensive compounds are acting synergistically, specialists tend to be better adapted to the variety of secondary metabolites within a single host plant genus or species as measured by feeding efficiency, fecundity, and mortality, while generalists are more likely to be negatively affected when feeding on the same host plant. Moreover, these differences in herbivore performance potentially can cascade up to the third trophic level (Dyer et al., 2004b; Harvey et al., 2005; Utsumi and Ohgushi, 2009).



In this paper, we examine how synergistic imide and amide chemical defenses found in *Piper* spp. (Piperaceae), known collectively as *Piper* amides, affect specialist and generalist herbivores. We specifically test the hypotheses that: (1) *Piper* amides act synergistically to negatively affect herbivore growth and survivorship; (2) synergistic effects vary between specialist and generalist herbivores; and (3) there are negative synergistic effects on a specialist herbivore's resistance to parasitoids. To test these hypotheses we performed a series of experiments that examined the performance of specialist and generalist lepidopteran larvae on *Piper* amides found in two species of *Piper*—*P. cenocladum* and *P. imperiale.*

Methods and Materials

Piper The genus *Piper* is diverse with high intraspecific diversity of secondary metabolites, including the *Piper* amides (reviewed by Parmar et al., 1997; Kato and Furlan, 2007). These compounds contain a phenyl moiety with a variable length carbon side chain (typically with at least one unsaturation) ending in a carbonyl carbon. The nitrogen containing portion of the amide is derived from piperidine, pyrrole, or an isobutyl group, and may contain an unsaturation, an epoxide, or a carbonyl group. *Piper cenocladum* and *P. imperiale* are two species of particular interest due to their high densities in forest understories, high diversity of consumers, and previously recorded synergy among some of the *Piper* amides present in the leaves (Dyer et al., 2004b; Fincher et al., 2008).

Piper imperiale is a large shrub or small tree found in the lowland tropical wet forest of Costa Rica and is morphologically and ecologically similar to *P. cenocladum* (Burger, 1971). It contains three imides: 5′-desmethoxydihydropiplartine (an analog of piplartine found in *P. cenocladum*), piplaroxide (an epoxide derivative of 5′-desmethoxydihydropiplartine), and an alkene (Fincher et al., 2008); these compounds deter arthropods (Fincher et al., 2008). *Piper imperiale* also contains at least five different sesquiterpenes, and their role as a plant defense has not yet been determined (Fincher et al., 2008). Several facultative ant species have been found inhabiting *P. imperiale*. However, these plants do not produce food rewards for the ants, and it is unknown if the plants benefit from the presence of ants.

Piper cenocladum is a tall understory plant with large, long-lived leaves and is common in the lowland wet forests of Costa Rica (Burger, 1971). This shrub is defended by a combination of *Piper* amides and a mutualism with *Pheidole bicornis* Forel ants. Plants uninhabited by ants increase amide production by at least a factor of three (Dodson et al., 2000; Dyer et al., 2001). *Piper cenocladum* contains two imides and an amide at high concentrations (total amide content can be as high as 3.8% dry weight): 4′-desmethylpiplartine (0.18% dry weight with an ant colony and 0.45% without ants), piplartine (0.14% with ants and 0.58% without ants), and cenocladamide (0.09% with ants and 0.33% without ants, Dodson et al., 2000). Other experiments have demonstrated that these *Piper* amides deter arthropods and act synergistically against some herbivores (Dyer et al., 2003, 2004b).

Herbivores The species-rich genus, *Eois* (Geometridae), is specialized on the genus *Piper,* with *Eois* species specializing on an average of two *Piper* species (Connahs et al., 2009). *Eois nympha* feeds on both *P. cenocladum* and *P. imperiale* (Dyer and Gentry, 2002). In Costa Rica, *Eois* are parasitized at a rate of 23.4% by a combination of wasps (Braconidae and Ichneumonidae) and flies (Tachinidae) (Connahs et al., 2009). *Spodoptera frugiperda* (Noctuidae) is a *Piper* naïve generalist herbivore native to tropical regions of the western hemisphere and is a major crop pest in several regions of North America. Eggs were purchased from Agripest, North Carolina, USA and a lab colony was maintained at Tulane University.

Experiments The synergistic effects of *Piper* amides from *P. cenocladum* and *P. imperiale* were examined on specialist and generalist caterpillars. For experiments with *P. imperiale* imides, we used both a specialist and a generalist caterpillar, and for experiments with *P. cenocladum* amides, we used only the specialist. In each experiment, larvae were fed diets containing different concentrations of single amides or a combination of amides. The amides were synthesized in the laboratory at Mesa State College (Richards et al., 2001). Amide concentrations for experimental diets were chosen based on concentrations found in *Piper* leaves, and were calculated as the % dry weight of the leaf disk or artificial diet. These experiments were labor and material (field-collected larvae and synthetic amides) intensive. Thus, it was difficult to conduct them at the same time and with identical methods.

Piper imperiale Imides Before initiating experiments, we quantified the imide concentrations in fully expanded new leaves of 39 individual *P. imperiale* shrubs, using methods previously described by Dodson et al. (2000). Leaves were collected at 39 different sites, spread evenly across the forest at La Selva Biological Station, Heredia province, Costa Rica. The *P. imperiale* leaves contained 0.13% (±0.021 SE) dry weight total imides. Dry weight of individual imides was distributed as follows: 0.006% (±0.001 SE) alkene, 0.12% (±0.021 SE) piplaroxide, and 0.003% (±0.001 SE) 5′-desmethoxydihydropiplartine. We performed leaf disk bioassays on the specialist, *E. nympha*, at La Selva (Dec 2007–Feb 2008), using concentrations similar to these natural concentrations (but slightly lower). We collected early instar (1–2) larvae of *E. nympha* from the field, and randomly assigned them to petri dishes with 9 cm diam disks of fresh *P. imperiale* leaf cuttings. Before adding the caterpillars, we pipetted 1 ml of methanol solution containing amides onto the underside of each leaf disk, covering the entire surface, and allowed the methanol to evaporate. Control diets included methanol only. Imide concentrations added to the leaf disks were alkene (0.001, 0.003, 0.01, 0.05% dry weight), 5′-desmethoxydihydropiplartine (0.001, 0.003, 0.01, 0.05% dry weight), piplaroxide (0.001, 0.01, 0.1, 0.2%), and mixtures which were equal parts of each compound with total imide dry weights of 0.003, 0.06, 0.12, and 0.3% (0.001, 0.02, 0.04, 0.1% of each compound). These added imides supplemented existing leaf defensive chemistry. We placed six individuals on each diet concentration and recorded the survival; for caterpillars that reached pupation, we recorded pupal mass, which is a good measure of potential fecundity (Tammaru et al., 1996; Thiery and Moreau, 2005; Hazarika et al., 2007).

In a second synergy experiment with *P. imperiale* imides, we used newly hatched caterpillars from a *S. frugiperda* lab colony. The control diet consisted of 35 g powdered Fall Armyworm Diet (Southland Products, Inc. Lake Village, AR, USA) mixed with 201 ml distilled water and 1.5 ml linseed oil. For experimental diets, a single compound or a combination of compounds was added to the powdered control diet as follows, alkene: 0.001, 0.006, 0.01, 0.1%, 5′-desmethoxydihydropiplartine: 0.001, 0.003, 0.01, 0.1%, piplaroxide: 0.001, 0.12, 0.25, 0.5%, and mixtures that were equal parts of each compound with total imide dry weights of 0.003, 0.15, 0.3, and 0.9% (0.001, 0.05, 0.1, 0.3% of each compound). Each treatment started with 310 larvae and culled to 300 at Day 3 to have the same starting number across treatments. Survival was recorded daily from days 9–40 and again on days 43, 45 and 53. We used Day 53 as a cutoff since over 95% of the larvae had pupated by then (those that were still alive were assumed to make it to pupation). Due to high mortality on the mixed diets, we were unable to compare pupal mass for this treatment.

Piper cenocladum Amides We performed similar leaf disk bioassays of *E. nympha* with amides from *P. cenocladum.*

Diet treatments consisted of cenocladamide, piplartine, 4′-desmethylpiplartine and equal part mixtures. Added concentrations varied from 0.01 to 0.8% dry weight, with 7–9 different concentrations per diet. Fresh leaves were collected from plants with ants in the field that had a mean total amide dry weight of 0.41% (±0.02 SE), while concentrations without ants was 1.36% (±0.02 SE)(Dodson et al., 2000). Thus, the amide concentrations added were likely within the natural range of the plant. Caterpillars had been naturally exposed to parasitoids in the field prior to collection, and since they were randomly assigned treatments in the lab, we assumed equal parasitism frequency before placement on diets. Therefore, differences in mortality caused by the emergence of a parasitoid are due to post-treatment differences in the development of the immature parasitoid, which may be correlated with the immune response as well as nutritional indices (Smilanich et al., 2009). During the fourth instar, we measured weight gain, average weight, food consumed, and frass produced. Using dry weight conversions, we calculated relative growth rate (GR = larval dry weight gain / average larval dry weight during interval) and approximate digestibility (AD = (dry weight of food consumed−dry weight of frass) / dry weight of food consume) (Waldbauer, 1968). In summary, the response variables for this experiment were: survival, pupal mass, parasitoid related mortality, GR, and AD.

Analysis For both experiments, we used linear and logistic regressions to estimate the effects of diet concentration on pupal weights, survivorship, and parasitism rates. We further examined the relationships between *P. cenocladum* amide concentrations and *E. nympha* pupal mass and parasitism frequency with path analysis (Proc CALIS, SAS Institute Inc., NC, USA). Based on previous path models (Smilanich et al., 2009), we chose simple models *a priori* in which amide concentration had direct and indirect (via GR and AD) effects on pupal mass and parasitism frequency. Path analyses were conducted for all diets (three single amide diets and one mixed diet) and models yielding goodness of fit *chi-squares* with *P*-values greater than 0.05 were considered a good fit to the data.

An interaction index (α) is usually calculated in standard synergy studies (Tallarida, 2000). It is calculated as, $\alpha = Z/\sum(f_i A_i)$. Where f_i represents the fraction of a compound in the mixed diet and A_i is the concentration of that compound necessary to achieve a certain level of response when tested individually; Z is the concentration of the mixture of compounds necessary to achieve a certain level of response. Values of α significantly less than one demonstrate a synergy. However, our data were inappropriate for these calculations because in all experiments there were single amides that did not significantly affect the response variables, making it impossible to determine A_i. Thus, any increase in effect resulting from including these ineffective compounds was considered evidence of synergy.

Results

Piper imperiale Imides Piper imides had a synergistic effect on *E. nympha* pupal mass, but not on survival. The mixed imide diets reduced pupal mass (r^2=0.95, $F_{1,3}$= 38.77, P<0.05, Fig. 1). However, there was no significant relationship between change in pupal mass and piplaroxide (r^2=0.72, $F_{1,3}$=5.11, P=0.15) or 5′-desmethoxydihydropiplartine (r^2=0.70, $F_{1,3}$=4.66, P=0.16) concentrations. The alkene appeared to have a positive effect on *E. nympha*, since increasing the concentrations of alkene caused increases in pupal mass (r^2=0.99, P<0.01, $F_{1,3}$=219.93). There were no significant associations between *Piper* amide concentration and survival for any single or mixed diets (logistic regression, P>0.05). Mean survivorships were 100% for the control, 96% for the alkene diet, 92% for the 5′-desmethoxydihydropiplartine diet, 75% for the piplaroxide diet, and 75% for the mixture diet.

Piper imides had a synergistic effect on *S. frugiperda* survival. All concentrations of 5′-desmethoxydihydropiplartine produced results similar to the control (Fig. 2). The highest concentration piplaroxide diet (0.5% dry wt) and all concentrations of alkene caused reduced larvae survival. In contrast, larvae experienced 100% mortality on all mixed diets.

Piper cenocladum Amides Piper amides of *P. cenoncladum* had synergistic effects on the survival of *E. nympha.* While there was no relationship between individual *Piper* amide concentrations and *E. nympha* survival (logistic regression, piplartine failed goodness-of-fit test, 4′-desmethylpiplartine χ^2=4.44, *df*=1, P<0.05, β=−15.31±8.25, *Wald*=3.44, *df*=1, P>0.05, cenocladamide χ^2=0.59, *df*=1, P>0.05), a mixture of all three amides significantly affected survivorship (χ^2=25.50, *df*=1, P<0.001, β=−4.62±1.07, *Wald*=18.691, *df*=1, P<0.001). Mean survivorship for control, piplartine, cenocladamide, 4′-desmethylpiplartine, and mix diets were 83, 85, 89, 81 and 33%, respectively.

There was a significant treatment effect on parasitoid success (ANOVA, $F_{4,\ 138}$=6.78, P<0.01, Fig. 3). The mixture was the only treatment associated with an increased parasitism frequency (Logistic regression, χ^2=12.64, *df*=1, P<0.001, β=3.47±1.10, *Wald*=9.91, *df*=1, P<0.01). No larvae died of parasitism on piplartine or control diets, and there was no relationship between cenocladamide and parasitism, and 4′-desmethylpiplartine and parasitism (cenocladamide χ^2=0.01, *df*=1, P>0.05, 4′-desmethylpiplartine

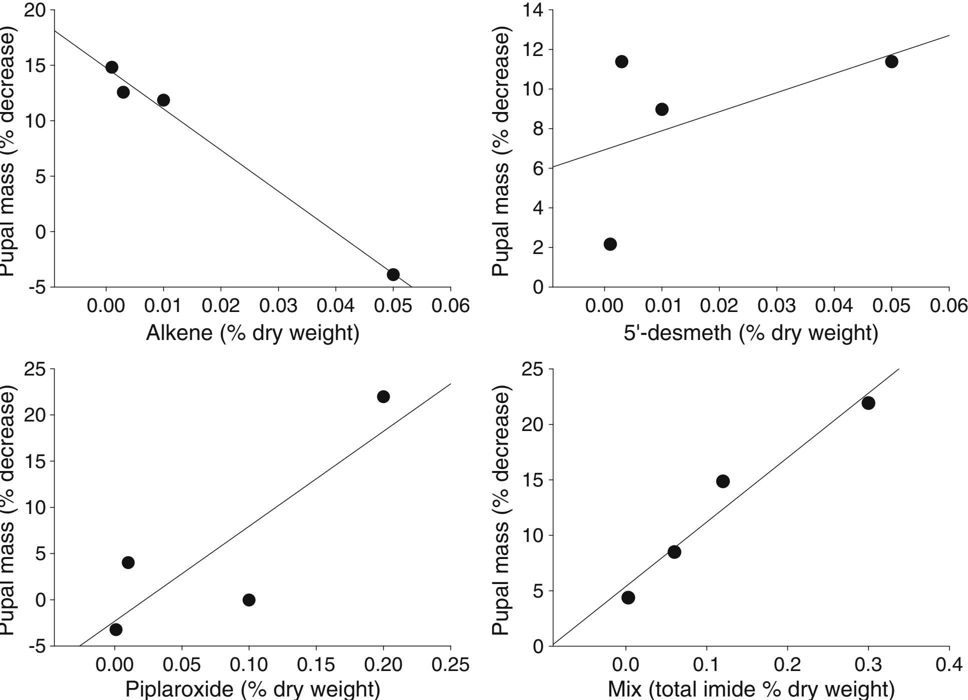


Fig. 1 Percent decrease in pupal mass of *Eois nympha* from control when reared on *Piper imperiale* leaf disks with alkene, 5′-desmethoxydihydropiplartine (5′-desmeth), piplaroxide and a mixture of all three imides added

χ^2=5.43, *df*=1, $P<0.05$, β=21.87±11.32, *Wald*=3.73, *df*=1, $P>0.05$). The parasitoid frequency path model demonstrated that mixed *Piper* amide concentration had a greater direct positive effect on parasitoid success than any single amide treatment (Fig. 4). Parasitoid success also was negatively affected via GR and AD, but there were no significant effect of amide treatments on GR and AD (Fig. 4).

There were negative effects of 4′-desmethylpiplartine and mixed amide concentrations on pupal mass of *E. nympha* (4′-desmethylpiplartine diet, r^2=0.36, $P<0.05$, $F_{1,11}$=6.18, mixed diet r^2=0.76, $P<0.001$, $F_{1,10}$=31.64), but pupal mass did not change with piplartine and cenocladamide concentrations (r^2=0.001, r^2=0.09, respectively, $P>0.05$). The pupal mass path model showed that

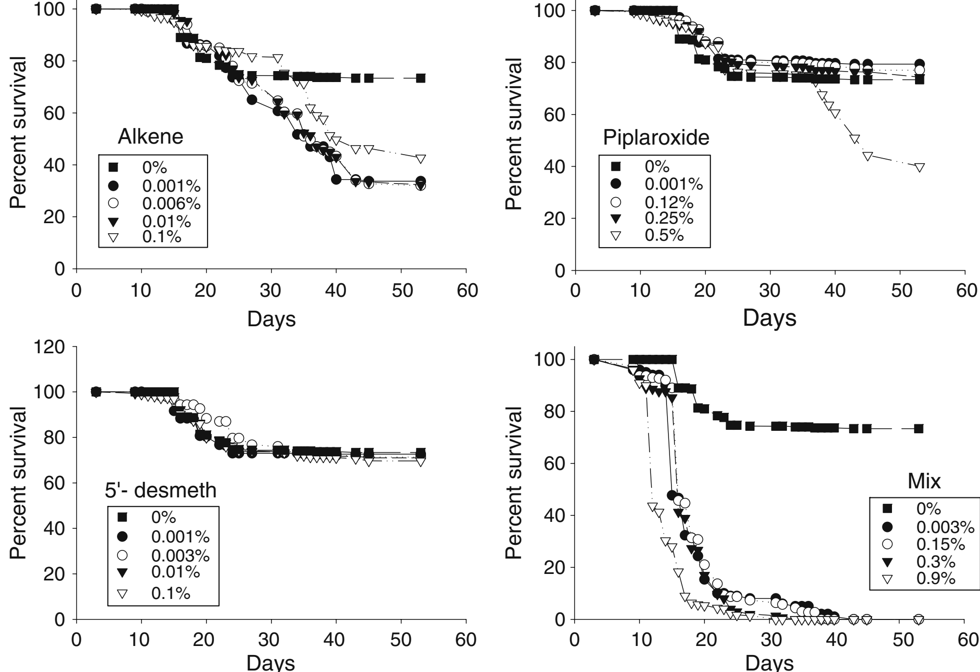


Fig. 2 Percent survival of *Spodoptera frugiperda* feeding on artificial diets with alkene, 5′-desmethoxydihydropiplartine (5′-desmeth), piplaroxide and a mixture of all three imides. Mix concentrations indicate the total imide concentrations

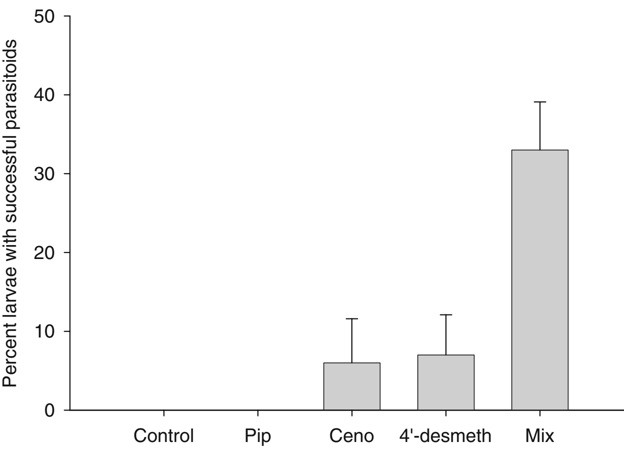


Fig. 3 Percentage of *Eois nympha* with successful parasitoids when reared on *Piper cenocladum* leaf disks containing methanol without amides (control, $N=12$) or with piplartine (Pip, $N=26$), cenocladamide (Ceno, $N=18$), 4′-desmethylpiplartine (4′-desmeth, $N=27$) and a mixture of the three amides ($N=60$) added. The mixture had significantly higher parasitoid success (*LSD*, $P<0.01$)

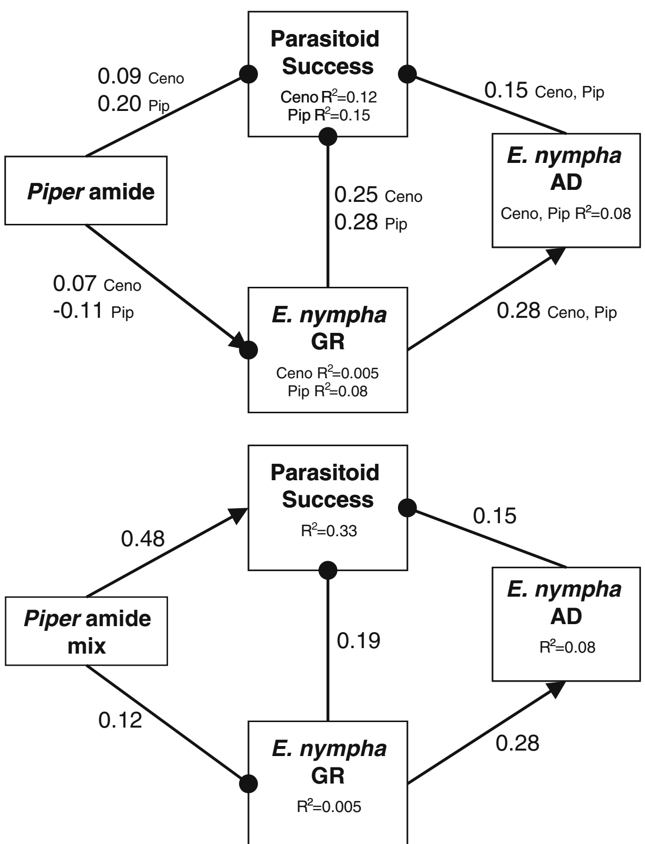


Fig. 4 Effects of *Piper* amides on parasitoid success in *Eois nympha* larvae reared on leaf disks with *Piper cenocladum* amides added (Parasitoid Frequency Model). Positive effects between variables are indicated by an arrow and negative effects are indicated by a bullet. Relationship strengths are indicated by the standardized path coefficient. R^2 values indicate the total variance explained in the model. The top diagrams the relationships for larvae on single amide treatments. 4′-desmethylpiplartine is not represented due to the lack of fit of the model ($\chi^2=41.84$, $df=1$, $P<0.001$). The model was a good fit for cenocladamide (Ceno, $\chi^2=0.22$, $df=1$, $P=0.64$) and piplartine (Pip, $\chi^2=0.17$, $df=1$, $P=0.81$). The bottom diagrams the relationships for larvae on the mixed amide treatment ($\chi^2=0.04$, $df=1$, $P=0.84$)

the mixed *Piper* amide treatment had the greatest direct negative effect on pupal mass compared to any of the single amide treatments (Fig. 5), and there was an indirect effect of amides on pupal mass via GR and AD. Again this relationship was strongest for the mixed treatment (Fig. 5).

Discussion

Piper amides synergistically affected both generalist and specialist herbivores via different mechanisms. Similar to a previous study with *P. cenocladum* amides (Dyer et al., 2003) and studies with other compounds (e.g., Roslin and Salminen, 2008), the mixture of amides in *P. imperiale* had dramatic toxic effects on survivorship of the naïve generalist *Spodoptera* caterpillars, while the negative effects on the specialist *Eois* caterpillars were more subtle. For *Eois*, increasing *Piper* amides caused increased parasitoid success and lower pupal mass, and this effect was more than doubled in response to increasing mixtures of amides as opposed to individual amides.

The synergistic effects of *Piper* amides on parasitism have interesting tritrophic implications. The traditional view of plant chemical defenses usually focuses on sequestration of toxins that confer a defensive benefit to the herbivore (Brower, 1984; Singer et al., 2009). However, our results are consistent with other recent studies that have shown an indirect positive effect of plant chemical defenses on parasitoids via disrupting the caterpillar immune response (Haviola et al., 2007; Smilanich et al., 2009). Since *Eois* species sequester *Piper* amides (Dyer et al., 2004a), the mechanisms for affecting parasitism include a direct negative effect on hemocytes responsible for the immune response or an indirect effect via decreasing overall herbivore vigor. Since the feeding efficiency measurements were not affected by ingestion of *Piper* amides, it is more likely that the increases in parasitism were due to direct toxicity of amides to immune cells. We did not directly measure the immune response, thus, there may be alternative explanations for these results, and further investigation is warranted. Interestingly, this *Piper*-*Eois*-parasitoid system is not consistent with the assumption of the “nasty” host hypothesis that herbivore sequestered plant defenses reduce host suitability for parasitoids (Gauld et al., 1992). We found the opposite; parasitoids were more successful in herbivores on more toxic mixed diets, which is consistent with previous studies that demonstrate that specialist parasitoids are less susceptible to changes in host

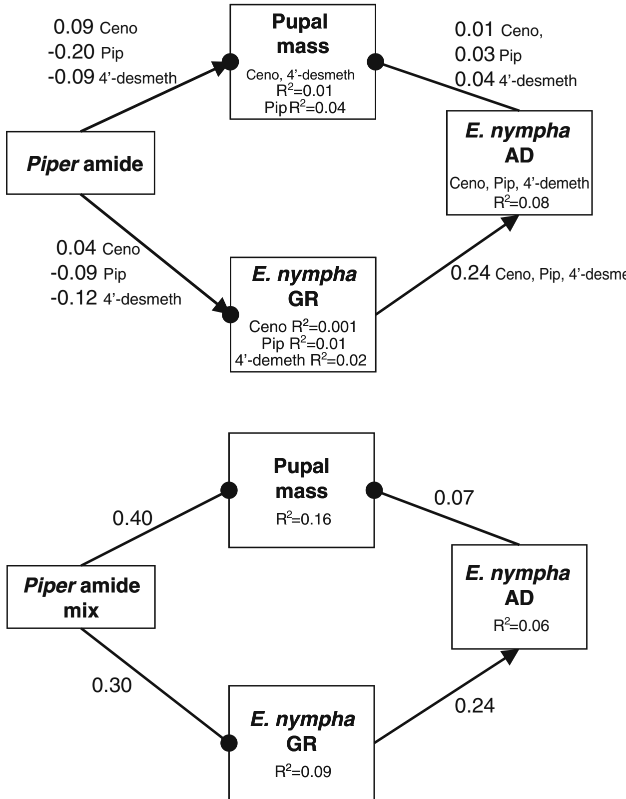


Fig. 5 Effects of *Piper* amides on pupal mass in *Eois nympha* larvae reared on leaf disks with *Piper cenocladum* amides added (Pupal Mass Model). For diagram description refer to Fig. 4. Model fit: Cenocladamide (Ceno) χ^2=2.23, *df*=2, *P*=0.33, Piplartine (Pip) χ^2=0.73,*df*=2, *P*=0.69, 4′-desmethylpiplartine (4′-desmeth) χ^2=3.52,*df*=2, *P*=0.17, Mix χ^2=0.15, *df*=2, *P*=0.93

chemistry than generalists (Gunasena et al., 1990; Barbosa et al., 1991; Harvey et al., 2003, 2005).

Results from the *P. cenocladum* experiments demonstrate that the effectiveness of different natural enemies is context dependent (Bronstein, 1994; Heil et al., 2002; McKey et al., 2005). The control leaves, which were collected from plants containing ants, had low amide concentration (Dodson et al., 2000; Dyer et al., 2001), high survival, and no parasitoid related mortality. Thus, when ants are present, they should be the main source of mortality and primary mechanism of *Eois* control. In comparison, the mixed amide leaves, which had concentrations similar to those found in plants without ants present (Dodson et al., 2000; Dyer et al., 2001), had high mortality from parasitoids. These two strategies of biotic and chemical plant defense may differ in their overall effectiveness and costs to the plants. For instance, producing high concentrations of amides may be costly to *P. cenocladum*, but the ecological cost of herbivory is lessened due to lower generalist herbivory and high mortality from parasitism on specialist herbivores. The same trade-off between physiological and ecological costs applies to *P. cenocladum* individuals that house ants, which produce low concentrations of amides while maintaining high levels of costly lipid and protein rich food bodies for the ants (Dyer et al., 2004b); in this case, it is the ants that maintain low levels of generalist and specialist herbivory (Letourneau et al., 2004). In contrast, *P. imperiale* does not have ant mutualists and suffers higher levels of herbivory by generalists and specialists (Fincher et al., 2008), even though relatively low levels of amides are necessary for synergistic defense against generalist caterpillars (a 0.9% mixture caused 100% mortality in 31 days) compared to *P. cenocladum* (a 1.34% mixture of *P. cenocladum* amides caused 100% mortality in 54 days, Dyer et al. 2003).

In summary, we found that *Piper* amides in two species of *Piper* have negative synergistic effects on specialist and generalist herbivores. *Piper* amides were highly toxic and acted as a strong bottom-up force on generalist herbivores, while specialists were indirectly affected by positive synergistic effects of amides on parasitoids, which can be an important top-down force in herbivore regulation. When considering these responses of generalist and specialist herbivores to synergistic plant defenses, some interesting questions arise: Did synergistic compounds evolve in response to specialist herbivores that circumvented the toxicity of individual defenses? What are the physiological mechanisms that result in effective defensive synergy of secondary metabolites? In our experiments, specialists were able to circumvent the toxicity and developmental effects of diets spiked with single amides via unknown physiological mechanisms, but were strongly affected by synergistic mixes via effects on parasitoid development. Because the fresh leaf disks already contained *Piper* amides, all treatments in the specialist experiments were to some degree mixed diets. This means that in response to selective pressures from herbivores, an increase in the concentration of a single amide would have little adaptive value. In contrast, a small increase in several *Piper* amides would result in significantly reduced pressure from *Eois* populations, through decreased pupal mass, fecundity, parasitoid resistance, and survival. Similar results in other plant-insect systems would have interesting implications for tritrophic theories of coevolution and specialization (e.g., Singer and Stireman, 2005).

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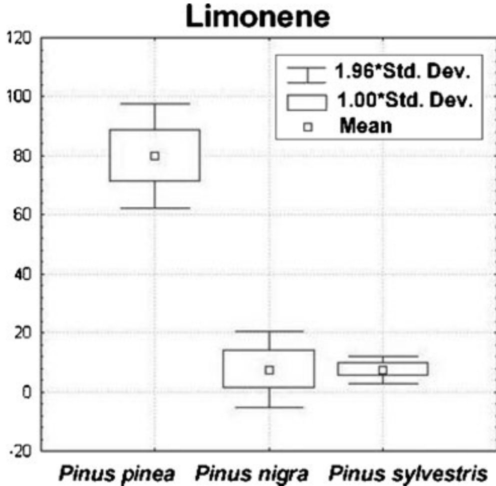

Fig. 1 Box-and-whisker plot showing mean percentage peak areas and relative standard deviations (SD and 1.95×SD) for limonene in three *Pinus* species

established extraction and identification technique widely used to analyze organic compounds in different matrices (Chai et al., 1993; Chai and Pawliszyn, 1995; Xiong et al., 2004; Ouyang and Pawliszyn, 2006; Bocchini et al., 2009). Recently Yassaa and Williams (2007) published a study dealing with the sampling of monoterpenoid compounds from *P. sylvestris* by SPME. An example of SPME/GC/MS analysis of volatile compounds *in vivo* is provided by Asaro et al. (2004) who studied the role of volatiles associated with preferred and non preferred hosts of *Rhyacionia frustrana* (Comstock) pest.

Here, we investigated the differential attractiveness of volatile compounds produced by three pine species on *N. sertifer* females. A SPME/GC/MS method for the qualitative and quantitative determination of volatiles from *P. sylvestris, P. nigra*, and *P. pinea* was set up. Our specific aim was to study the olfactory response by the sawflies to pine volatile compounds that were found to be characteristic for an attractive species (*P. sylvestris*) and a non-attractive species (*P. pinea*).

Methods and Materials

SPME/GC/MS Two different SPME fibres, 75 μm Carboxen™–PDMS and 70 μm Carbowax/Divinylbenzene (CW/DVB), specified by Supelco (Supelco, Milan, Italy) as suitable for sampling volatiles and semivolatile compounds, respectively, were tested. The SPME fiber, after sampling, was desorbed (10 min, 280°C) inside the injector of a Varian 3400 Gas Chromatograph (Varian, Walnut Creek, CA, USA) connected to a Varian Saturn 2000 Ion Trap Mass Spectrometer. The chromatographic capillary column was a DB-5MS J&W (30 m×0.25 mm I.D., 0.25 μm film thickness). The chromatographic elution was temperature programmed as follows: 50°C for 10 min, then from 50°C to 225°C at a rate of 5°C/min. The overall analysis time was 45 min. Mass spectra were acquired in the range *m/z* 40–450, at a rate of one scan per sec. Chromatographic peaks were identified by means of an automatic search of the NIST library of mass spectra and compared with mass spectra of terpenoid compounds reported in the literature (Adams, 2007). Standard compounds, namely α-pinene, β-pinene, myrcene, and limonene were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy); δ-3-carene was purchased from Carl Roth (Carl Roth GmbH, Karlsruhe, Germany). Standard compounds were used to confirm compound identification and to carry out the bioassays.

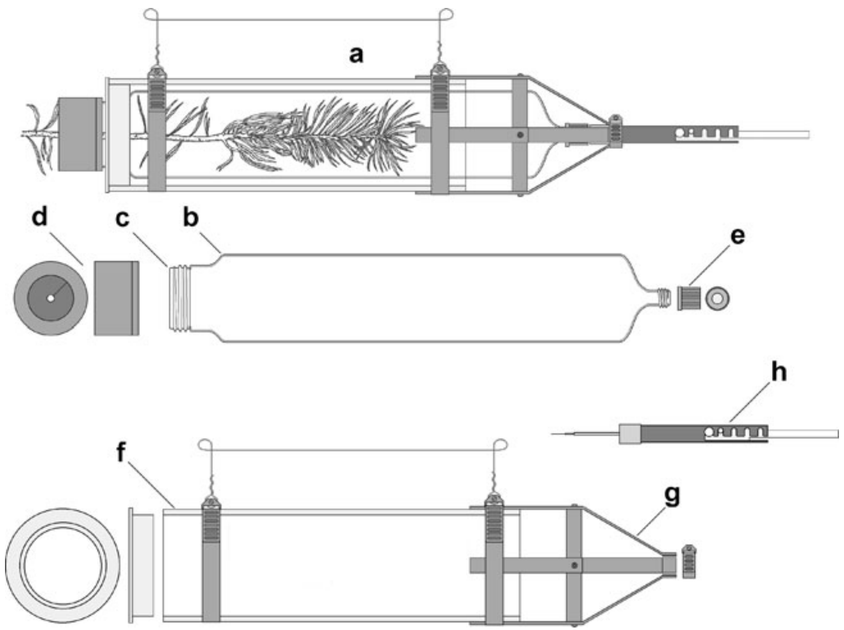

Fig. 2 Sampling structure used for the *in vivo* SPME sampling (**a**). The container was a glass cylinder with an internal volume of 1.2 l (**b**) and, at one end, an opening large enough to allow the branch to be placed inside it (**c**). The opening was fitted with a screw cap sealed with rubber/Teflon, (**d**) and can be tightly closed around the branch itself. At the other end of the glass cylinder, another opening was fitted with a hole screw cap (**e**) and was used for SPME sampling operations. The glass cylinder was housed inside a PMMA (polymetyl methacrylate) cylinder (285×90 mm) (**f**) provided with a metallic structure (**g**) in order to maintain the SPME holder (**h**) in a stable position during sampling operation

Method Set-Up A laboratory test was carried out in order to determine which of the two fibers (Carboxen–PDMS and CW/DVB) was more suitable for sampling volatiles emitted by pine needles. Known amounts of *P. nigra* needles were put inside a 40 ml vial sealed with screw caps with a PTFE septum. The two fibers were exposed to the headspace for 15, 30, and 60 min at ambient temperature and then desorbed. The sum of the areas of all the chromatographic peaks was calculated. For each sampling time, the calculated area was higher for the experiments carried out with the Carboxen–PDMS fiber. Due to this result, all successive experiments were performed using a Carboxen–PDMS fiber.

Sampling time is a critical step to be optimized in an SPME method. For this reason, sampling time vs. total area experiment was performed. The tested sampling periods were 5, 15, 30, and 60 min; a sampling time of 60 min was considered sufficient to allow a representative absorption of the volatiles.

Replicate analyses of volatiles (4 samples of needles for each pine species) from *P. pinea*, *P. nigra*, and *P. sylvestris* were performed. Relative standard deviations ranged from values as low as 10% for limonene in *P. pinea* samples to more than 100% for less abundant compounds.

Despite the high variability of results, a discriminant analysis allowed the identification of limonene as the compound that can successfully discriminate between *P. pinea* and the other pine species. In Fig. 1, a box-and-whisker plot for limonene (area percentage) as determined in the three pine species is shown.

In Vivo Laboratory Experiment An *in vivo* laboratory experiment was performed by using a Carboxen–PDMS fiber with a sampling time of 60 min. To better control absorption conditions, a container which can house a whole tree branch was custom-made (Fig. 2). The cylinder was suspended from the tree in such way as to maintain natural branch bearing.

Before starting the *in situ* sampling, an experiment was performed comparing the summed areas of the chromatographic peaks at 60 and 120 min for *P. sylvestris in vivo* sampling and 60 min long sampling of *P. sylvestris* needles under the same conditions described in the "method set-up" section. Data showed that the *in vivo* conditions required longer sampling times in order to reach a satisfactorily high amount of adsorbed volatiles. This was probably due to the higher volume of the glass branch container with respect to the amount of pine leaves present inside it. A sampling time of 120 min was considered sufficient to give a good representation of the volatile composition of living plants.

In Situ Sampling Experiments To test the analytical sampling system *in situ* and *in vivo*, a pinewood in Piedimonte (about 550 m above sea level, Province of Forlì-Cesena, Italy), 1.9 ha large and mainly formed by 20–30 yr-old *P. domestica*, *P. sylvestris*, and *P. nigra* trees, was used.

Sampling experiments were carried out on the last 10 d of November (mean temperature 15°C; mean illuminance 28.57 klx), in the afternoon (3–5 p.m.). This period of the year was chosen because it corresponds with the peak of flight period of *N. sertifer*, which in temperate regions, starts at the end of September and the beginning of October (Baronio et al., 1997). *In situ* sampling was performed on 3 different days by using 9 SPME fibers. Each day 3 samples for each pine species were collected. A total of 27 samples from different trees were collected. After sampling, SPME fibers were stored at −80°C and later analyzed by GC/MS.

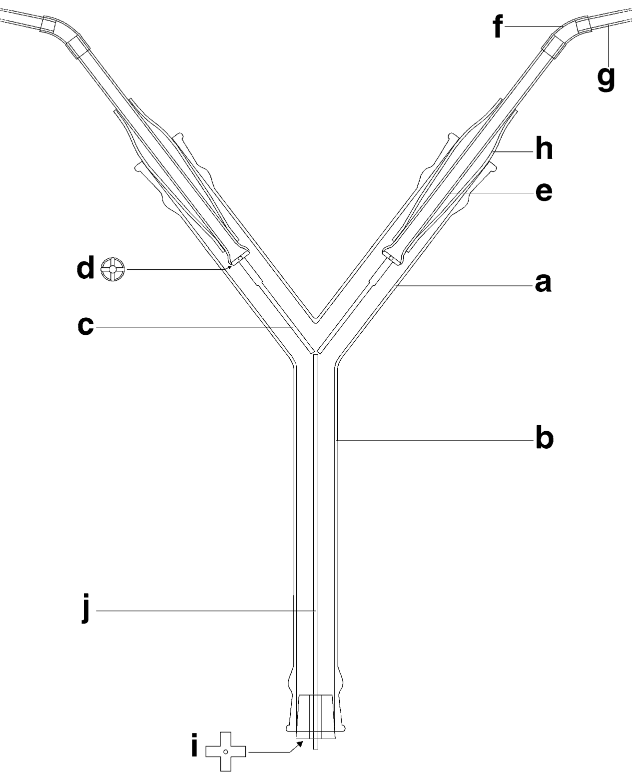

Fig. 3 The modified Y-tube olfactometer used in bioassays. The Y-tube had a diameter of 30 mm and a thickness of 2 mm; the two arms were 185 mm long, forming a 75° angle (**a**). The stem was 250 mm long (**b**). In each arm, a 40 mm long frosted glass stick with a thickness of 3 mm (**c**) was connected by a cross-shaped glass weld (**d**), to a glass tube 230 mm long and 10 mm large (**e**). These glass tubes were connected by 30 mm polyethylene tubes (**f**) with two PTFE (polytetrafluorethylene) tubes (**g**) and inserted into a spindle-like glass tube (**h**) which was fitted at the upper side of the two arms to maintain the frosted glass stick at the centre of the arms and parallel to them, leading to a contact of the glass sticks. A silicon rubber plug (**i**), placed at the base of the stem, was equipped of another frosted glass stick (**j**) with a thickness of 3 mm. The silicon rubber plug had a cross shape which allowed the air flow from the olfactometer

Bioassays—Insects We tested the olfactory response of *N. sertifer* females to pine volatiles in laboratory olfactometer bioassays. The sawflies were taken from a lab-rearing obtained from a population collected in two pine stands located in Piedimonte and Vessa (Forlì-Cesena province). Larvae were reared in plastic boxes (28×18×12 cm) kept at 20°C, 70% r. h., and 12 hr photophase, and were fed with *P. sylvestris* branches taken from the woodland. Female cocoons were sexed and stored inside Petri dishes until emergence. Female individuals chosen for the olfactometer test were 5–10 d-old virgin specimens. In this species, oviposition starts on average on the 5th day after cocoon emergence (Baldassari et al., 2003).

Bioassays—Olfactometer A modified Y-tube glass olfactometer was used. A glass "railroad" was inserted into the Y-tube, in a similar way to the wire "railroad" used in the Y-tube modified by Sabelis and Van de Baan (1983) (Fig. 3). The whole olfactometer was suspended from a wood board covered with white cardboard. The wood board was connected with a metallic hinge to another horizontal board; in this way the olfactometer could be angled from the vertical to the horizontal position.

A mechanical pump was used to force air flows inside the olfactometer's two upper arms. The air stream was humidified by passing through water, and filtered by using a carbon filter. Two flow-meters kept air flows to 2 l per min. Air was forced through two 100 ml Büchner flasks, one of which (odor chamber) was spiked with a known amount of the compounds to be tested. Each Büchner flask was connected via the two PTFE tubes to the two upper arms of the olfactometer.

By introducing CO_2 smoke inside the upper arms of the apparatus, we tested whether the two separate air flows mixed. No mixing was observed. We observed that *N. sertifer* female individuals could move easily and climb inside the olfactometer that was set in an upright position. Laboratory walls were painted white, and windows were closed during experiments. An halogen lamp was fixed to the ceiling, producing a light intensity of 1,000 lx at the crossing of the olfactometer arms.

Neodiprion sertifer females were tested singly by placing them inside the vertical shaft of the olfactometer on a platform made of brass mesh on the top of silicon plug. Each individual was tested once. If the individual did not reach the Y shape junction position within 5 min, the behavior was classified as "no choice". If the individual reached one of the two Y arms of the olfactometer within 5 min, this behavior was classified as "first choice". A "final choice" was assigned to individuals that reached the top of the chosen arm after more than 3 min. In the other cases, a "no choice" behavior was considered.

In order to add to the scent chamber an amount of volatile compound that mimics the plant release, the amounts of limonene and myrcene were quantified by the standard addition method. The apparatus used for *in vivo* laboratory experiments was utilized to quantify volatiles released from a branch of *P. sylvestris*. Briefly, known amounts of the compound to be quantified were added to the glass cylinder containing the branch (20 to 25 cm long), and the areas obtained after SPME/GC/MS analysis were plotted vs. the added amounts of standard. The calculated regression curve was used to determine the amount of compound initially present inside the glass cylinder. The

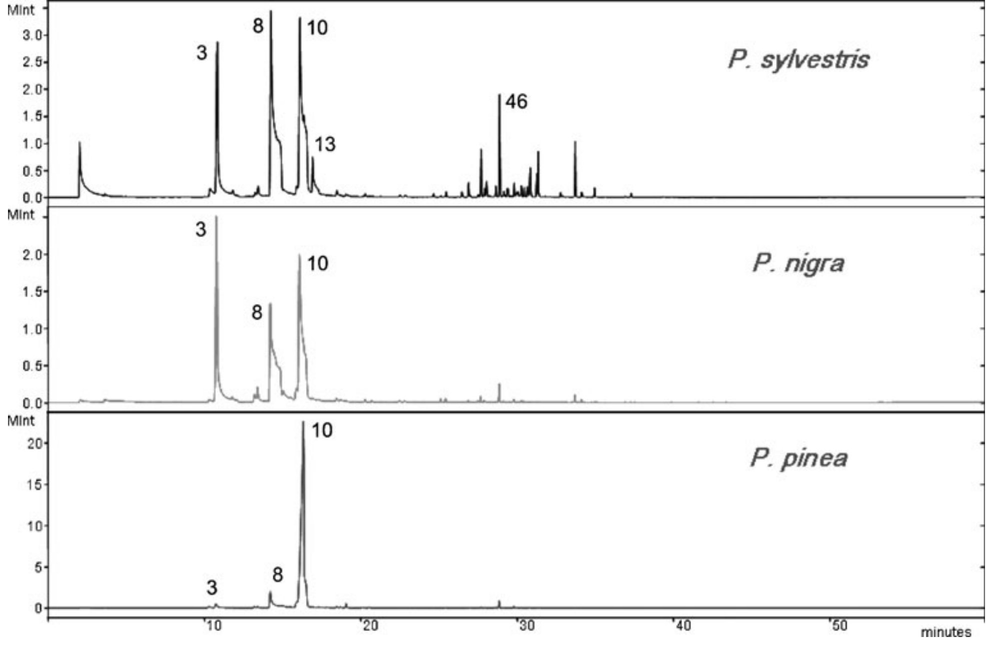

Fig. 4 SPME/GC/MS chromatograms of volatiles emitted from the three different *Pinus* species. Numbers as in Table 1 (3: α-pinene, 8: δ-3-carene + myrcene, 10: limonene, 13: terpinolene and 46: (Z)-caryophyllene)

obtained amounts of limonene and myrcene were in the range 1–2 μg/l. The volatile compounds were deposited in the scent chamber in excess with respect to the concentration measured in the glass cylinder in order to let a small fraction of it evaporate in the air flow, thus maintaining a constant concentration of the volatile compound during the experiment.

The scent chamber was regenerated after 30 min of testing, and observations were resumed after 5 min. After having tested the behavior of 10 individuals, the instrument was rotated 180° in order to avoid any position bias (Koschier et al., 2000). Olfactometer glass walls were washed with dichloromethane before each variation in concentration and before changing the tested chemical compound.

Statistical Analysis A discriminant analysis was used to ordinate the compositions of volatile blends of the three pine species. After GC/MS analysis, peak areas were measured in each chromatogram, and area percentage was calculated for the identified compounds. These values were subjected to a discriminant analysis using the software STATISTICA (RELEASE 5.1) (STATSOFT INC, 1995).

For statistical analysis of the olfactometer data, a *chi-square* test was used to statistically compare differences in choice between clean air and air spiked with the volatile

Table 1 Compounds identified in the chromatograms of volatiles sampled from *Pinus pinea*, *P. nigra* and *P. sylvestris* trees

N.	Retention time (scan)	Compound	N.	Retention time (scan)	Compound
1	622	Tricyclene	34	1,521	Phenyl ethyl acetate
2	644	α-Thujene	35	1,570	Bornyl acetate
3	692	α-Pinene	36	1,590	*n*-Tridecane
4	750	Camphene	37	1,644	δ-Elemene
5	789	Verbenone	38	1,671	α-Cubebene
6	823	Sabinene	39	1,680	α-Longipinene
7	852	β-Pinene	40	1,714	α-Ylangene
8	910	δ-3-Carene+Myrcene	41	1,724	α-Copaene
9	988	o-Cymene	42	1,738	β-Bourbonene
10	1,015	Limonene	43	1,746	β-Cubebene
11	1,050	(Z)-β-Ocimene	44	1,761	*n*-Tetradecane
12	1,117	*cis*-Linalool oxide	45	1,790	Longifolene
13	1,145	Terpinolene	46	1,799	(Z)-Caryophyllene
14	1,163	2,5-Dimethyl styrene	47	1,818	α-Gurjunene
15	1,186	Linalool	48	1,829	β-Gurjunene
16	1,188	*n*-Undecane	49	1,838	*cis*-Muurola-3,5-diene
17	1,255	*allo*-Ocimene	50	1,844	α-Guaiene
18	1,263	*trans*-Pinocarveol	51	1,859	α-Humulene
19	1,278	*neo-allo*-Ocimene	52	1,866	Germacrene D
20	1,287	*trans*-Verbenol	53	1,886	γ-Muurolene
21	1,314	Citronellal	54	1,896	Germacrene D (isomer)
22	1,322	Tetrahydro lavandulol	55	1,910	β-Selinene
23	1,321	*trans*-Pinocamphone	56	1,920	Valencene
24	1,330	Pinocarvone	57	1,946	γ-Cadinene
25	1,347	Borneol	58	1,952	δ-Cadinene
26	1,357	*cis*-Pinocamphone	59	1,959	*trans*-Calamenene
27	1,365	Terpinen-4-ol	60	1,978	α-Cadinene
28	1,380	*p*-Cymen-8-ol	61	1,990	α-Calacorene
29	1,399	α-Terpineol+Myrtenol	62	2,048	Caryophyllene oxide
30	1,430	*n*-Decanal+Verbenone	63	2,068	*n*-Hexadecane
31	1,462	Thymol methyl ether	64	2,603	Manoyl oxide
32	1,502	Sabinene hydrate acetate	65	2,629	*epi*-13-Manoyl oxide
33	1,516	Piperitone	66	2,712	Abietadiene

Table 2 Mean relative (%) total peak areas and standard deviations (SD) of the most abundant compounds in the headspace of three pine species (*in vivo* sampling, 9 samples for each *Pinus* species)

Compounds	*P. nigra*		*P. pinea*		*P. sylvestris*	
	Mean	SD	Mean	SD	Mean	SD
α-pinene	44.97	44	19.39	132	16.49	82
β-pinene	4.43	63	3.57	113	1.35	126
Myrcene	10.68	82	4.73	72	39.83	45
Limonene	18.30	64	66.12	48	23.40	54

Table 3 Discriminant analysis results showing the standardized coefficients of the variables, indicating the compounds which have more weight in separating the three groups

Compounds	Root 1	Root 2	
Myrcene	2.11	1.02	$P<0.001$
δ-Cadinene	1.63	0.54	$P<0.001$
Limonene	0.24	2.42	$P<0.001$
trans-Verbenol	−0.76	−0.80	$P<0.001$
β-Pinene	0.36	1.72	$P<0.001$
α-Pinene	0.65	1.01	$P=0.055$

compound to be tested. The null hypothesis was that the percentages of individuals choosing the spiked air and the pure air flows were 50%, respectively.

Results

In Situ Sampling Experiments—SPME/GC/MS Representative chromatograms obtained by analyzing the volatile fraction of the three pine species *P. pinea*, *P. sylvestris*, and *P. nigra* are shown in Fig. 4. A total of 66 chromatographic peaks were identified by GC/MS (Table 1). The most abundant compounds, in terms of absolute areas of the chromatographic peaks, were α- and β-pinene, myrcene, and limonene. Their relative amounts in the headspace of the three pine species are reported in Table 2 (areas of each identified compound are expressed as relative percentages).

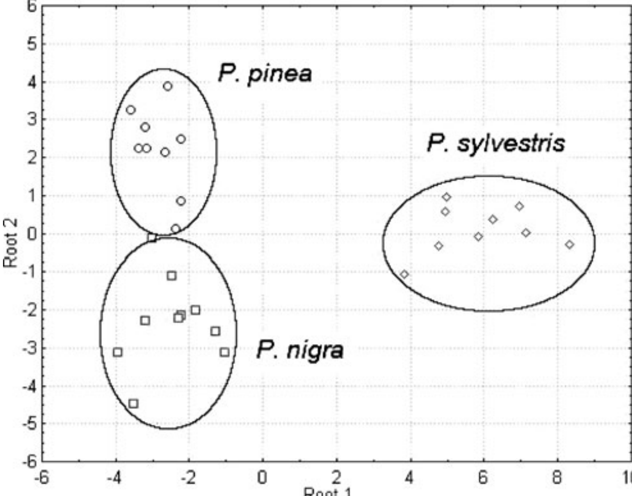

Fig. 5 Discriminant analysis applied to chromatographic data, showing significant differences in the composition of volatile blends emitted from three *Pinus* species. Each *symbol* represents an *in vivo* sampling experiment. The graph was generated applying root coefficients showed in Table 3

A discriminant analysis was applied to the quantitative GC/MS data, and the first two discriminant functions (i.e., root 1 and root 2) showed a clear ordination of the data into three groups (Fig. 5), each corresponding to a pine species. *Pinus pinea* can be differentiated from *P. sylvestris* and *P. nigra* mainly by limonene and myrcene, as evinced from the values of the standardized coefficients (Table 3). In particular, the first discriminant function shows the highest contribution for myrcene, while the second function is affected mainly by limonene. Limonene was the main component of the *P. pinea* volatile fraction. The mean percentage of the limonene peak area represents 66% of the *P. pinea* volatile blend composition, whereas the peak area of this compound accounts for 18% and 23% of all detected volatile components in *P. nigra* and *P. sylvestris*, respectively. Since the odor of *P. pinea* is characterized by its high relative amounts of limonene, we assumed that this terpenoid protects *P. pinea* trees from *N. sertifer* attacks. The highest mean percentage peak area of myrcene was found in *P. sylvestris*; the discriminant analysis revealed that *P. sylvestris* can be separated statistically from the two other species by this volatile compound.

Table 4 Olfactory responses by *Neodiprion sertifer* females (final choice) to limonene (1 µl and 2 µl added to the odor chamber) vs. air and to myrcene (1 µl) vs. air

Compound tested		Control		Statistics[a]	
N	%	*N*	%	χ^2	*P*
Limonene 1 µl		Air			
41	87.23	6	12.77	24.6	<0.001
Limonene 2 µl		Air			
14	31.11	31	68.89	3.67	=0.055
Myrcene 1 µl		Air			
26	57.78	19	42.22	0.20	=0.658

[a] Comparison was made by *Chi-square* test. Yates correction was used to calculate χ^2 values (*d.f.*=1)

Bioassays According to the results obtained by GC/MS, the appropriate volatile compounds to be used in the bioassay were limonene (1 µl and 2 µl added to the odor chamber) and myrcene (1 µl added to the odor chamber). Females' reactions were investigated by recording the reactions of 50 and 48 specimens, respectively.

Olfactometric reactions of female individuals to volatiles are shown in Table 4. Females showed a high percentage of choice. In particular, in the three experiments the percentages of choice were 94, 92, and 96% in the bioassay with 1 µl of limonene, 2 µl of limonene, and 1 µl of myrcene, respectively. *Neodiprion sertifer* females were attracted significantly by the lowest amount of limonene (1 µl in the odor chamber): a statistically significant percentage of individuals (87.23%) chose the arm of the olfactometer leading towards the limonene spiked odor chamber ($P < 0.01$, χ^2 test). This behavior changed when the amount of limonene added to the odor chamber was increased from 1 to 2 µl; in this second condition, most individuals (68.89%) chose the non-spiked odor chamber. Myrcene (1 µl) attracted *N. sertifer* females only in 57.78% of cases, showing no attractive pattern ($p > 0.05$, χ^2 test).

Discussion

Previous studies have shown that the sawfly *N. sertifer* avoids ovipositing on *P. pinea*, whereas *P. sylvestris* and *P. pinea* are accepted for oviposition. We suggest that the high amounts of limonene released by *P. pinea* may contribute to the avoidance of this tree since (a) high amounts of limonene were shown here to repel *N. sertifer* females, and (b) only *P. pinea* released high relative amounts of limonene, whereas no such high limonene emission was found in the two other species. While limonene was repellent at high doses, low amounts attracted the sawflies. This behavior has not been documented previously for *N. sertifer* although it has been detected in other insects living on coniferous trees. For example, α-pinene is repellent to many insects but it may be attractive to the same insects at lower concentration (Annila and Hiltunen, 1977).

It is remarkable that egg deposition of *N. sertifer* occurs in autumn when the limonene content in *P. pinea* needle is higher than earlier in the year (Tiberi et al., 1999). The pattern of *N. sertifer* females selecting *P. sylvestris* and *P. nigra* trees for laying eggs is similar to that of the pine processionary moth *Thaumetopoea pityocampa* which lays a limited number of eggs on *P. pinea*, but prefers *P. sylvestris* and *P. nigra* (Tiberi et al., 1999). Future studies need to investigate whether avoidance of *P. pinea* by this moth is mediated by the same volatiles as in *N. sertifer*.

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forest trees, where their abundance and diversity are greatest (Arnold et al., 2001).

The interaction between endophytes and their hosts varies from mutualistic to parasitic, depending on species, physiological state, chemistry of the host plant, environmental stress, and other abiotic conditions (Espinosa-García et al., 1993; Gilbert and Strong, 2007). These fungi may impact survival and fitness of plants in all terrestrial ecosystems, and may play a significant role in plant biogeography, evolution, and community structure (Rodríguez et al., 2009).

The secondary metabolites produced by endophytes or their infected hosts could be chemical defenses against herbivores, pathogens, or competitors (Schulz and Boyle, 2005; Strobel, 2006; Herre et al., 2007). *In vitro* competition experiments show that endophytic fungi inhibit the growth of other endophytic fungi (Espinosa-García et al., 1993) and phytopathogens (Yue et al., 2000; Arnold et al., 2003). Endophytes in the tissues of living plants are relatively unstudied as potential sources of novel natural products. These compounds offer an enormous potential for the discovery of new agrochemicals and drugs of natural origin (Tan and Zou, 2001; Schulz et al., 2002).

Muscodor has been isolated as an endophyte from tropical trees and vines in Central America, Australia, and Thailand. Four species of *Muscodor* are known: *M. albus* from small limbs of *Cinnamomum zeylanicum* in Honduras and *Myristica fragrans* in Thailand (Worapong et al., 2001; Sopalun et al., 2003); *M. roseus* from two monsoonal rainforest tree species, *Grevillea pteridifolia* and *Erythophelum chlorostachys* in Northern Australia (Worapong et al., 2002); *M. vitigenus* from *Paullinia paullinioides*, a liana growing in the understory of the rainforests of the Peruvian Amazon (Daisy et al., 2002a); and *M. crispans* from *Ananas ananassoides*, a wild pineapple in the Bolivian Amazon Basin (Mitchell et al., 2008). Based on morphological and DNA sequence analyses, González et al. (2009) found that *M. yucatanensis* is a new species. Colonies of this fungus are whitish and produce a strong musty odor when grown on potato dextrose agar (PDA). They grow slowly and do not produce reproductive structures. *Muscodor* species produce a mixture of toxic volatile organic compounds (VOCs) that are lethal to a wide variety of plant and human pathogens. The mixture of VOCs consisted primarily of alcohols, acids, esters, ketones, and lipids, including 3-methyl butyl acetate (isoamyl acetate), which was the most active compound (Ezra et al., 2004a,b; Strobel, 2006).

This paper describes part of a long-term project on the chemical ecology of endophytic fungi and their possible role in plant defense mechanisms (González et al., 2007; Macías-Rubalcava et al., 2008). We investigated the allelochemical potential of the endophytic fungus *Muscodor yucatanensis* and cataloged the compounds it produces *in vitro*.

We performed bioassays to test the toxicity of VOCs and organic extracts of the mycelium and culture medium. We assessed the effects of the extracts on the growth of other endophytic fungi from El Eden plant species, on some important phytopathogens, and on dicotyledonous and monocotyledonous plants. We also evaluated the chemical composition of the VOCs mixture and of the organic extracts from mycelium and culture medium.

Methods and Materials

Fungal Material *M. yucatanenis* was isolated from surface sterilized segments of healthy leaves of *Bursera simaruba* (Burseraceae) ('chacah' or 'palo mulato') collected at the Ecological Reserve El Eden, Quintana Roo, Mexico in September 2004. A specimen of the plant was deposited in the University of California, Riverside Herbarium (G.P. Schultz & R. Palestina # 1092/UCR 110695). The fungus was cryogenically preserved in liquid nitrogen vapor 10% glycerol, and was deposited in the fungal collection of Herbario Nacional de México (MEXU), UNAM, accession number MEXU 25511.

Effects of VOCs Bioassays were performed using six endophytic fungi isolated from *B. simaruba* and other surrounding plants in the El Eden Ecological Reserve: *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangifera* (isolated from the leaves of *Callicarpa acuminata*); the unknown isolate 120 and *M. yucatanensis* (isolated from *Bursera simaruba* leaves); and *Xylaria* sp. (isolated from *Pteridium aquilinum* leaves). As a complement to bioassays that used these endophytic fungi, we also tested VOCs on phytopathogenic microorganisms: fungi *Fusarium oxysporum, Alternaria solani,* and *Rhizoctonia* sp., and fungoids *Phytophthora parasitica* and *P. capsici.*

Bioassays were performed under sterile conditions using divided Petri dishes (9 cm) with two compartments containing PDA medium. The plastic walls dividing the plate prevented the diffusion of any soluble compounds produced by *M. yucatanensis* to the second compartment but did allow free exchange of VOCs. Due to the very slow growth of *M. yucatanensis* on PDA medium, an inoculum of it was placed in one of the compartments of the dish and grown at 25–27°C and a photoperiod of 12:12h fluorescent light (Octron 4100 K, Ecologic, 32 W) for 10 d before the bioassay. The inoculum from a test species was then placed in the other compartment of the dish, which was then wrapped with two layers of plastic wrap and incubated as above. Growth of the test organisms was assessed after different times of incubation, depending on individual growth rate. *Phytophthora capsici*, *P. parasitica*, *Colletotrichum* sp., *Phomopsis* sp., and *Rhizoctonia* sp. were examined after 3 d; *A. solani* and *F.*

oxysporum after 4 d, and *G. mangifera*, isolate 120, *Xylaria* sp. and *M. yucatanensis* after 10 d. The average of two perpendicular diameter measurements for each mycelium was recorded at the end of the bioassays and compared with the respective controls (Macías-Rubalcava et al., 2008). Experiments were performed following a complete randomized design with four replications per treatment.

Recovery of test fungi that were severely inhibited by *Muscodor* VOCs was evaluated by transferring an agar plug of the test microorganism to a new Petri dish with fresh PDA and incubating under the same conditions. Recovery was evaluated by measuring mycelial diameter as described above and comparing results with controls.

Phytotoxicity of the VOCs was tested on roots of two dicots, amaranth (*Amaranthus hypochondriacus*; Amaranthaceae) and tomato (*Lycopersicon esculentum* var. Pomodoro; Solanaceae), and one monocot, barnyard grass (*Echinochloa crus-galli*; Poaceae). We selected these test plants because we needed plants that have rapid, homogenous, high frequency germination, and because seeds of tropical plants including *B. simaruba* germinate poorly. Amaranth seeds were purchased at a local market at Tulyehualco, Mexico, D. F., tomato seeds in Semillas Berentsen, Celaya, Guanajuato, Mexico, and barnyard grass seeds were collected from plants growing in the greenhouse of Instituto de Ecología (UNAM).

The bioassays were performed with divided Petri dishes containing PDA and inoculated with *M. yucatanensis* in one compartment. The endophyte was grown at 28°C for different times (0, 3, 5, 10, 15, 20, 30, and 50 days) before introducing seeds into the other compartment that contained 1% water agar. At each time, 10 test seeds were sown and dishes were wrapped with two layers of plastic wrap and placed in a germination cabinet in the dark at 27°C. Root lengths of test plants were measured after 24 hr (amaranth), 48 hr (barnyard grass), or 72 hr (tomato) of exposure to *Muscodor* VOCs. Root lengths were compared to controls that did not have the *M. yucatanensis* inoculum. Experiments were performed following a complete randomized design with four replications per treatment.

We evaluated recovery of seeds and seedlings that were severely inhibited by *Muscodor* VOCs. Inhibited seeds and seedlings were removed from the divided Petri dishes and placed in new Petri dishes with fresh 1% water agar, and incubated as before. Root lengths were measured after 24 and 48 hr, and compared with their respective controls.

Organic Extracts of Culture Medium and Mycelium Fungi release some secondary metabolites into the environment while retaining others in the mycelium. *M. yucatanensis* was cultured in 18 Erlenmeyer flasks (1,000 ml), each containing 500 ml of potato dextrose broth (PDB). Each flask was inoculated with three agar plugs taken from a stock culture of *M. yucatanensis* on PDA, and was incubated with constant agitation on an orbital shaker at 150 rpm for 6 wk at 25–27°C and a photoperiod of 12:12 hr (fluorescent light).

At the end of the fermentation process, culture medium in each flask was separated from the mycelium by filtration. Culture medium (9 L) was extracted × 3 with 9 L of dichloromethane (CH_2Cl_2) and then × 3 with 9 L of ethyl acetate (EtOAc). The combined organic phases were filtered over anhydrous Na_2SO_4 and concentrated *in vacuo* to give 306.9 mg of a yellowish resinous product. The mycelium was extracted with CH_2Cl_2 and EtOAc (3×2 L). The combined mycelium extracts were evaporated to yield 2.73 g of a yellowish resinous product.

Organic extracts of the culture medium and mycelium were tested with the same eleven test microorganisms used in the bioassays described above. Both extracts were evaluated at 125, 250, 500, and 1,000 μg/ml in sterile PDA, added before the agar had solidified in 6-cm Petri dishes. The positive control was the commercial fungicide Prozycar® (carbendazim: methyl benzimidazol-2-yl-carbamate) added to agar at 200 μg/ml. This concentration of Prozycar completely inhibited test microorganisms in a preliminary bioassay. Unamended PDA was used as negative control. An inoculum (5 mm^2 agar plug) of each test microorganism was placed on the amended agar in a Petri dish and incubated in darkness at 27°C. The average of two perpendicular measurements of the mycelium was recorded after 3, 4, and 10 d of incubation depending on the growth rate of the test microorganism. The experiment used a complete randomized design with four replications per treatment.

A Petri dish bioassay was used to evaluate the effect of the organic extracts (culture medium and mycelium) on the root length of amaranth, tomato, and barnyard grass. Each extract was evaluated at 50, 125, 250, and 500 μg/ml by adding the extract to 1% water agar before the agar had solidified. Unamended water agar was used as negative control, and agar with 1,000 μg/ml of Rival® (Glyphosate: N-(phosphonomethyl) glycine) as positive control. This level of Glyphosate completely inhibited root growth in the test plants in a preliminary bioassay. Ten seeds of each test plant were sown directly on agar in 6 cm Petri dishes and incubated in the dark at 27°C. Root lengths were measured 24 hr after treatment for amaranth, 48 hr for barnyard grass, and 72 hr for tomato. The experiment used a complete randomized design with four replications per treatment.

Qualitative Analysis of VOCs M. yucatanensis mycelium was grown on PDA for 10 d in 15 ml SPME vials. A Solid Phase Micro Extraction (SPME) syringe was placed through a small hole drilled in the side of the vial through a Teflon septum and exposed to the vapor phase for 60 min. The SPME syringe (DVB–PDMS–Carboxen 50/30, Supelco Bornen, Belgium) was conditioned before use for 40 min at 250°C. After exposure to the VOCs, the syringe was inserted into the split (1:50 ratio) injection port of a gas

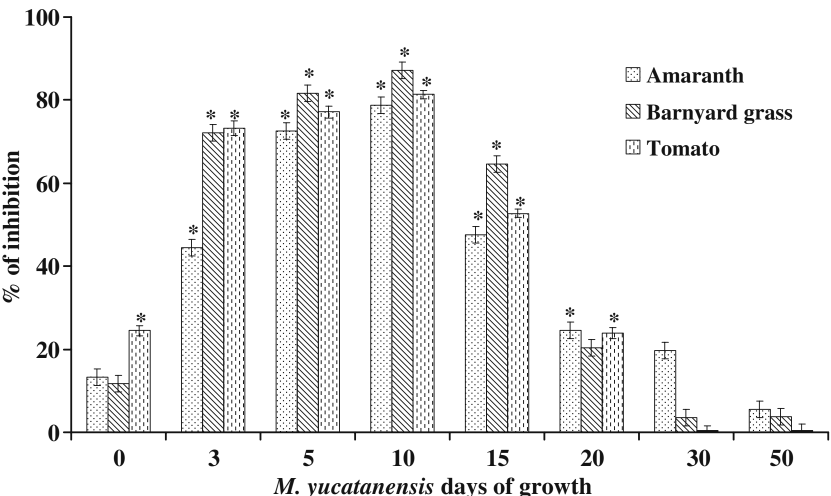


Fig. 1 Phytoinhibitory activity of VOCs produced by *Muscodor yucatanensis* after different periods of growth. The root lengths of amaranth, tomato, and barnyard grass seedlings were determined after 24 hr of exposure to VOCs (Amaranth): 48 hr of exposure (barnyard grass); or 72 hr of exposure (tomato). Vertical bars represent SD, $N=4$; $^{*}P<0.05$

chromatograph (GC) (Agilent 6890N) interfaced to a LECO time of flight mass spectrometer (MS-TOF) operating at unit resolution with electron ionization (EI) operating at 200°C . Separation on a 10 m × 0.18 mm I.D. DB5 J&W capillary column with a film thickness of 0.18 micron used the following temperature program: 40°C for 0.5 min, followed by 280°C for 2.5 min at 70°C/min. The carrier gas was ultra high purity helium at 1 ml/min. A 3 min injection time was used to introduce the sample into the GC. The MS was scanned at a rate of 10 scans/s over a mass range of 30–450 Da. Data acquisition and data processing were performed with the LECO ChromaTOF software system. We established that the SPME syringe effectively trapped fungal volatiles in preliminary tests. To evaluate background compounds, comparable analyses were conducted on vials containing only PDA, and the data were subtracted from the unknowns.

M. yucatanensis VOCs were identified by retention index (RI). Linear retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes with the parameters described above. Chemical identification was confirmed by comparison of mass spectra with the NIST database (National Institute of Standards and Technology).

Qualitative Analysis of the Organic Extracts Extracts were dissolved in CH_2Cl_2, and one μl of each extract was directly injected into the gas chromatograph equipped and operated as

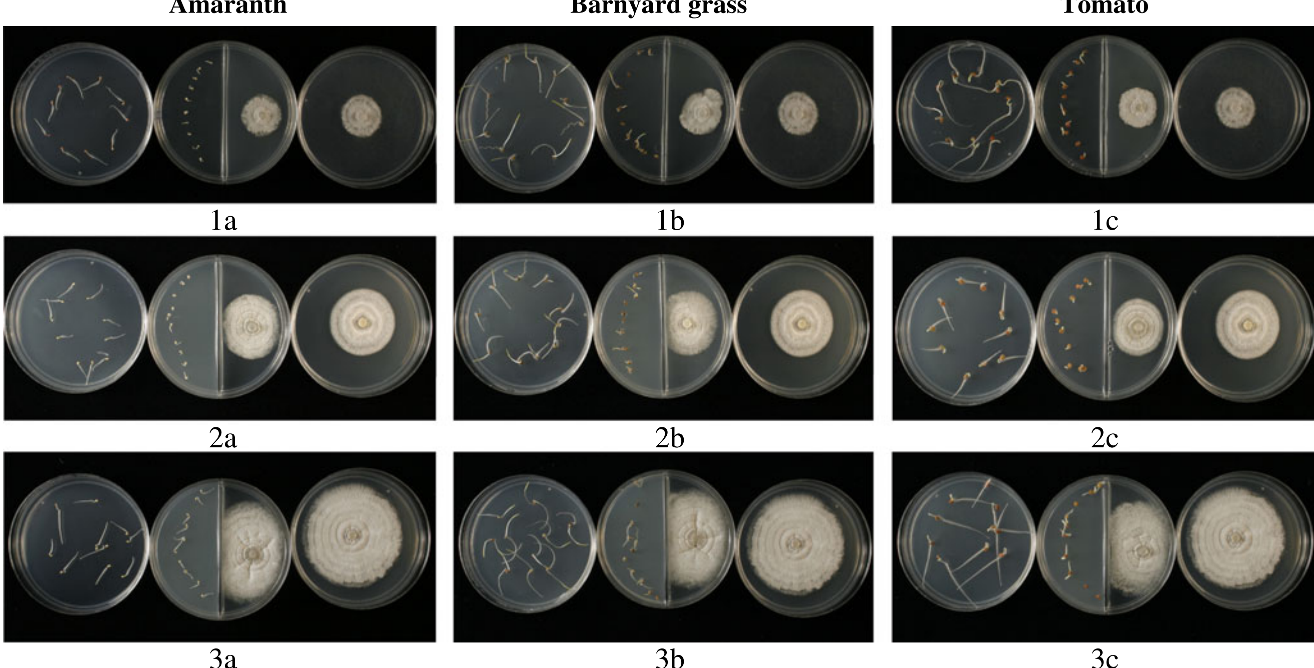


Fig. 2 Phytoinhibitory activity of VOCs from *Muscodor yucatanensis* grown for 5 (1), 10 (2), and 15 (3) days on the root length of amaranth (1a, 2a, and 3a), barnyard grass (1b, 2b, and 3b) and tomato (1c, 2c, and 3c)

Table 1 Percent recovery of the root length of amaranth, tomato and barnyard grass at 24 hr and 48 hr, after exposure to *Muscodor yucatanensis* VOCs

Muscodor yucatanensis days of growth	% of recovery[a]					
	Amaranth		Tomato		Barnyard grass	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
0	78.6	87.3	72.3	92.9	65.3	81.2
3	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	0	0
15	0	0	0	0	0	0
20	20.2	67.3	17.3	75.3	14.6	69.5
30	22.3	70.2	98.6	100	100	100
50	87.5	97.6	90.3	98.3	100	100

[a] Percent of root length recovery compared with the respective control

described above, but with the column temperature programmed as follows: 40°C for 1 min, followed by 280°C for 5 min at 20°C/min. The injector was set at 250°C with a 1:50 split ratio and solvent delay of 100 sec. The MS was scanned at a rate of 10 spectra/s over a mass range of 33–600 Da. Background was established with an extract of PDA.

Components were identified by comparison of their RI, determined relative to the retention times (RT) of a series of n-alkanes with linear interpolation on the HP-5 MS capillary column. They were also confirmed by comparison of their mass spectra using the NIST database. The relative amounts of individual components of the organic extracts were expressed as percentages of the peak area relative to the total peak area.

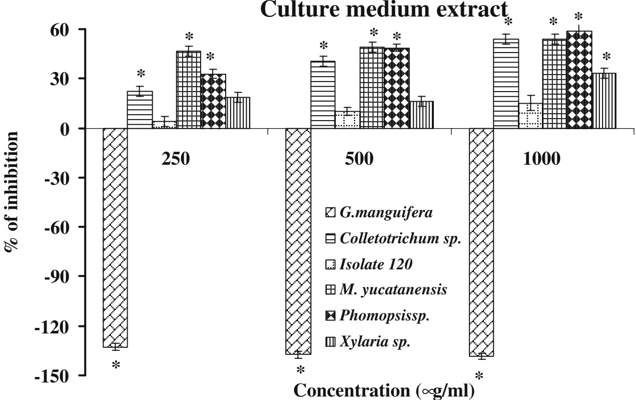
Statistical Analysis Bioassay data were analyzed by ANOVA and Tukey's tests (Mead et al., 2002) using Statistica 6.0. IC_{50} (50% inhibitory concentration) values for organic extracts were calculated by probit analysis (Zar, 2007) based on the average growth diameter of the mycelium and root length with SPSS 15.0 software. Data are represented as mean ± standard deviation (SD). A *P* value less than or equal to 0.05 was used to identify significant differences from controls.

Results

Effect of VOCs VOCs were lethal to the endophytes *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangiferae*, and to the phytopathogens *Phythophthora capsici*, *P. parasitica, Rhizoctonia* sp., and *Alternaria solani. Fusarium oxysporum* remained alive and recovered 86 % of its growth after exposure to VOCs. The three endophytic fungi were not significantly inhibited by these VOCs: isolate 120 was inhibited only slightly (3.3 %), *M. yucatanensis* was not inhibited (0 %), and *Xylaria* was non-significantly stimulated (18 %).

The phytoinhibitory activity of VOCs increased proportionally from the first day of *Muscodor* growth, and was most inhibitory after 10 d of endophyte growth (Fig. 1). However, phytotoxic activity decreased significantly from 15 to 50 d. Figure 2 is a qualitative example of phytotoxic activity after 5, 10, and 15 d of *Muscodor* growth. Roots of the most affected plants darkened before dying.

The recovery of germinating seeds after exposure to VOCs is shown in Table 1. VOCs produced by mycelium that was 3–15-d-old were very inhibitory to the three test plants. However, the roots of seedlings exposed to VOCs



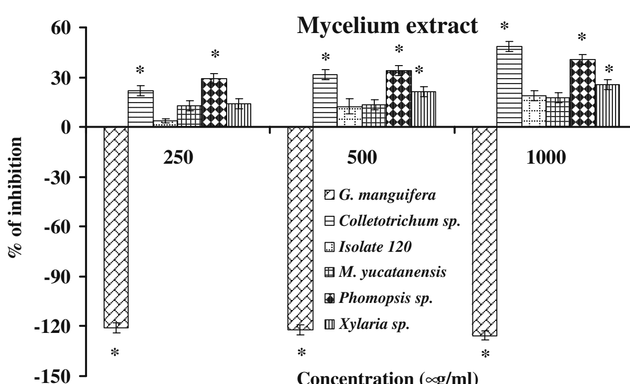


Fig. 3 Inhibitory activity of the culture medium and mycelium organic extracts from *Muscodor yucatanensis* against six endophytic fungi. Vertical bars represent SD, *N*=4; *$P<0.05$

from 1–3-d-old mycelia or 20–50-d-old mycelia recovered 1 to 2 d after exposure.

Effects of Organic Extracts Figure 3 shows significant dose-dependent fungal growth inhibition caused by both extracts of *M. yucatanensis* on almost all test endophytic fungi. *Colletotrichum* sp. and *Phomopsis* sp. were significantly inhibited by both extracts at all concentrations. The culture medium extract from *M. yucatanensis* caused a significant reduction in its own growth at all concentrations. In contrast, the mycelium extract did not significantly inhibit the growth of *M. yucatanensis*. *Xylaria* sp. was inhibited only by the highest concentration of either extract. IC_{50} values for the culture medium extract on the radial growth of *Colletotrichum* sp. was 794 μg/ml; for *M. yucatanensis,* 591 μg/ml ; and for *Phomopsis* sp., 631 μg/ml. IC_{50} for the mycelium extract for all endophytic fungi was >1,000 μg/ml.

G. mangifera was significantly stimulated by both extracts at all concentrations tested (Fig. 3). We estimated that the concentration required to stimulate a 50% increase in radial growth (SC_{50}) for *G. mangifera* was 94 μg/ml for the culture medium extract and 104 μg/ml for the mycelium extract.

The effects of the culture medium and the mycelium extracts on the radial growth of phytopathogenic microorganisms are shown in Fig. 4. In general, extracts were inhibitory only at 500 and 1,000 μg/ml, and the IC_{50} values for almost all phytopathogens were >1,000 μg/ml.

Both extracts inhibited root growth of the three test plants in a concentration-dependent manner (Fig. 5). The culture medium extract was more phytotoxic. The IC_{50} values of the culture medium extract were 112 μg/ml for tomato, 132 μg/ml for amaranth, and 435 μg/ml for barnyard grass. The crude culture medium extract was more active than Glyphosate which had IC_{50} values of 33 μg/ml for tomato, 234 μg/ml for amaranth, and 114 μg/ml for barnyard grass. All IC_{50} values for mycelium extract were >500 μg/ml.

Chemical Composition The main volatile secondary metabolites produced by a 10-d-old *M. yucatanensis* culture comprised 38 compounds that were identified by GC/MS (Table 2). The culture medium extract contained 32 compounds, while the mycelium extract consisted of 23 compounds (Tables 3, 4). In general, the compounds present in

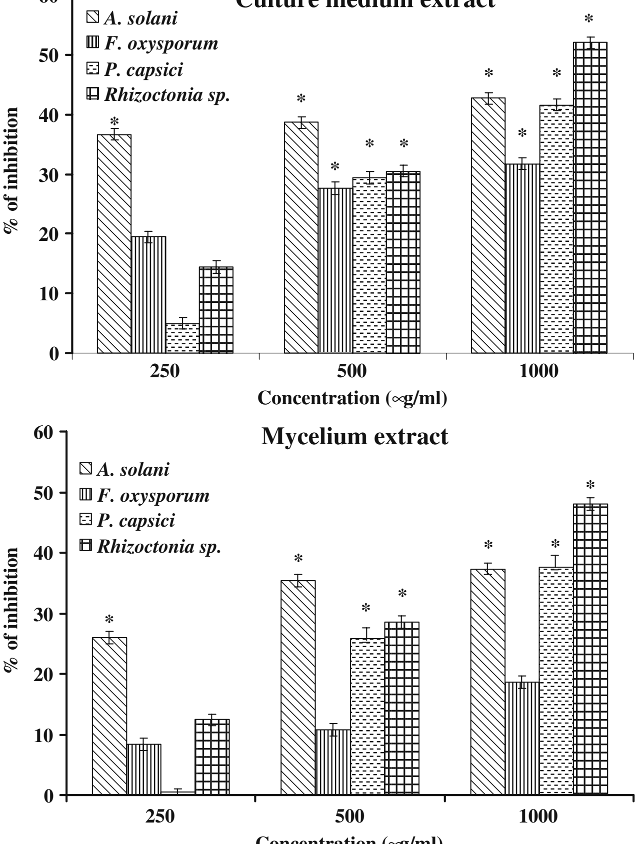


Fig. 4 Inhibitory activity of the culture medium and mycelium organic extracts from *Muscodor yucatanensis* against phytopathogenic microorganisms: *Alternaria solani, Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia* sp. Vertical bars represent SD, $N=4$; $*P<0.05$

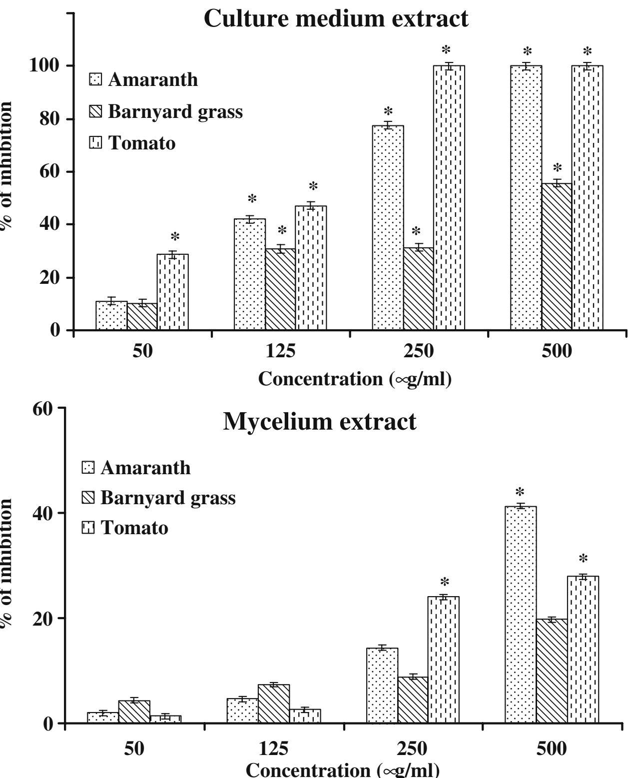


Fig. 5 Phytoinhibitory activity of the culture medium and mycelium organic extracts from endophytic fungus *Muscodor yucatanensis* on the root length of amaranth, tomato, and barnyard grass. Vertical bars represent SD, $N=4$; $*P<0.05$

Table 2 GC/MS analysis of the volatile compounds mixture (VOCs) produced by *Muscodor yucatanensis*

Compounds	Retention Index[a]	Total area (%)	MW	Molecular formula
2-methylbutan-1-ol	723.7	0.06	88	$C_5H_{12}O$
cyclohepta-1,3,5-triene	746.2	0.20	92	C_7H_8
heptane	655.0	0.42	100	C_7H_{16}
ethylbenzene	885.5	0.16	106	C_8H_{10}
octane	794.9	0.16	114	C_8H_{18}
propan-2-ylbenzene	988.3	0.04	120	C_9H_{12}
4-ethylcyclohexan-1-one	1004.3	0.08	126	$C_8H_{14}O$
methyl 2,3-dimethylbutanoate	843.1	0.17	130	$C_7H_{14}O_2$
2-methyl butyl acetate	874.9	0.49	130	$C_7H_{14}O_2$
1-methyl-4-(1-methylethyl)-benzene	1020.1	0.43	134	$C_{10}H_{14}$
1-methyl-3-(1-methylethyl)-benzene	1020.7	0.70	134	$C_{10}H_{14}$
α-Phellandrene	1026.2	5.02	136	$C_{10}H_{16}$
(1Z,5Z)-3,4-dimethylcycloocta-1,5-diene	1023.2	2.22	136	$C_{10}H_{16}$
2-pentyl furan	985.2	1.04	138	$C_9H_{14}O$
(E)-7-methylundec-4-ene	793.8	1.00	168	$C_{12}H_{24}$
3,4-dimethyldec-1-ene	1492.1	1.96	168	$C_{12}H_{24}$
4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene	1357.2	4.24	188	$C_{14}H_{20}$
1-oxacyclotetradeca-4,11-diyne	1578.9	0.37	190	$C_{13}H_{18}O$
2-iodo-pentane	916.7	1.11	198	$C_5H_{11}I$
1-iodo-3-methyl-butane	919.2	0.07	198	$C_5H_{11}I$
4,5-dehydroisolongifolene	1500.6	0.71	202	$C_{15}H_{22}$
2,5,9,9-tetramethyl-3,4,4a,5,8,9a-hexahydrobenzo[7]annulene	1398.8	5.32	204	$C_{15}H_{24}$
aristolene	1323.3	3.04	204	$C_{15}H_{24}$
caryophyllene	1376.8	15.84	204	$C_{15}H_{24}$
elemene	1340.6	0.34	204	$C_{15}H_{24}$
5,8a-dimethyl-3-prop-1-en-2-yl-2,3,4,4a,7,8-hexahydro-1H-naphthalene	1359.2	0.57	204	$C_{15}H_{24}$
1R,4S,7S,11R-2,2,4,8-Tetramethyltricyclo[5.3.1.0(4,11)]undec-8-ene	1379.5	23.44	204	$C_{15}H_{24}$
(Z)-4-(4,6,6-trimethyl-5-bicyclo[3.2.0]hept-3-enyl)but-3-en-2-one	1400	2.29	204	$C_{14}H_{20}O$
(3Z,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	1422.9	0.20	204	$C_{15}H_{24}$
(3Z)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-3-ene	1805.6	0.96	204	$C_{15}H_{24}$
2-methyl-2-(4-methyl-3-propan-2-ylpent-3-en-1-ynyl)cyclobutan-1-one	1321.8	2.94	204	$C_{14}H_{20}O$
aromadendrene	1375	12.19	204	$C_{15}H_{24}$
cyclopentyl 4-ethylbenzoate	960.52	4.85	218	$C_{14}H_{18}O_2$
5,6-dipropyldecane	1492.7	0.21	226	$C_{16}H_{34}$
cyclohexyl 4-ethylbenzoate	954.9	1.00	232	$C_{15}H_{20}O_2$
4-(butylsulfanylmethyl)-6-piperidin-1-yl-1,3,5-triazin-2-amine	971.29	0.05	281	$C_{13}H_{23}N_5S$
androstan-17-one, 3-ethyl-3-hydroxy-, (5a)-	1805	4.05	318	$C_{21}H_{34}O_2$
pentacosa-10,12-diynoic acid	1806	2.05	374	$C_{25}H_{42}O_2$

Compounds found in the control PDA plate are not included in this table

[a] Kovat´s indices calculated from retention time data on an HP-5 MS capillary column

both extracts are alcohols, esters, and ketones of saturated and unsaturated compounds, as well as benzene derivatives.

Discussion

Muscodor yucatanensis, like other *Muscodor* species (Ezra et al., 2004a,b; Strobel, 2006), produces a mixture of volatile compounds (VOCs) when cultured under *in vitro* conditions. These VOCs are selectively toxic to other endophytic and phytopathogenic fungi, and to plant roots. Strobel et al. (2001) found that VOCs of *M. albus* isolates had low inhibitory effects against fungi and bacteria. However, when these authors tested *M. albus* VOCs as a mixture they observed that they acted synergistically and killed a broad range of fungi and bacteria.

Table 3 GC/MS analysis of the compounds presents in the culture medium extract of *Muscodor yucatanensis*

Compounds	Retention Index[a]	Total area (%)	MW	Molecular formula
3-methyloxolan-2-one	950.7	0.03	100	$C_5H_8O_2$
ethylbenzene	857.0	0.66	106	C_8H_{10}
1,3-dimethylbenzene[b]	866.7	0.79	106	C_8H_{10}
5-propan-2-ylidenecyclopenta-1,3-diene[b]	889.9	0.75	106	C_8H_{10}
2-(hydroxymethyl)-2H-furan-5-one	1206.8	0.10	114	$C_5H_6O_3$
ethyl butanoate[b]	796.7	2.65	116	$C_6H_{12}O_2$
butyl acetate[b]	811.5	1.45	116	$C_6H_{12}O_2$
5H-cyclopenta[b]pyridine	1302.3	1.63	117	C_8H_7N
2-phenylethanol[b]	1119.3	5.62	122	$C_8H_{10}O$
3,3-dimethyl-2-hexanone	1235.7	0.76	128	$C_8H_{16}O$
ethyl 2-methylbutanoate	848.7	1.58	130	$C_7H_{14}O_2$
3-methylbutyl acetate	878.7	2.86	130	$C_7H_{14}O_2$
4-hydroxy-4-methyloxan-2-one[b]	1313.0	2.96	130	$C_6H_{10}O_3$
4-(2-hydroxyethyl)phenol[b]	1448.2	0.94	138	$C_8H_{10}O_2$
nonanal	1106.6	0.35	142	$C_9H_{18}O$
2,2,6-trimethylcyclohexane-1,4-dione	1172.0	0.40	154	$C_9H_{14}O_2$
1,7-dioxaspiro[5.5]undec-8-ene	1536.5	2.00	154	$C_9H_{14}O_2$
undecane[b]	1099.9	1.01	156	$C_{11}H_{24}$
8-hydroxy-3-methyl-3,4-dihydroisochromen-1-one	1542.6	5.60	178	$C_{10}H_{10}O_3$
2-(3,3-dimethylbut-1-ynyl)-3-methoxy-1,1-dimethylcyclopropane	1454.9	1.31	180	$C_{12}H_{20}O$
3,8-dihydroxy-3-methyl-4H-isochromen-1-one	1963.3	2.71	194	$C_{10}H_{10}O_4$
2,6-ditert-butyl-4-methylphenol [b]	1497.4	3.44	220	$C_{15}H_{24}O$
(E)-tetradec-9-enoic acid	2147.7	3.09	226	$C_{14}H_{26}O_2$
8,14-cedrandiol	2105.5	10.49	238	$C_{15}H_{26}O_2$
methyl octadecanoate	2141.3	31.00	298	$C_{19}H_{38}O_2$
methyl (13E,16E)-octadeca-13,16-dienoate	2146.3	6.90	294	$C_{19}H_{34}O_2$
hexadecanoic acid[b]	1982.9	0.40	256	$C_{16}H_{32}O_2$
(9E,12E)-octadeca-9,12-dienal[b]	2141.3	1.21	264	$C_{18}H_{32}O$
tridecan-3-yl 2-methoxyacetate	2662.7	0.09	272	$C_{16}H_{32}O_3$
octadecanoic acid[a]	2169.2	1.75	284	$C_{18}H_{36}O_2$
tert-butyl hexadecanoate	2184.6	1.09	312	$C_{20}H_{40}O_2$
(5-acetamido-3,4-diacetyloxy-6-carbamimidoylsulfanyloxan-2-yl)methyl acetate	2137.3	4.36	405	$C_{15}H_{23}N_3O_8S$

[a] Kovat´s indices calculated from retention time data on an HP-5 MS capillary column

[b] Compounds presents in the culture medium and mycelium organic extract of *Muscodor yucatanensis*. Compounds found in the control PDB broth are not included in this table

M. yucatanensis VOCs emitted by 3–15-d-old fungus significantly inhibited germination and root elongation of the three test plants. Many damaged seeds and seedlings could recover from inhibition if they had been exposed to VOCs from fungi that had been cultured 1–3 d, or more than 15 d. The loss of toxicity in older cultures may be due to depletion of nutrients from the culture medium, which may result in a change in VOCs production. Ezra et al. (2004b) investigated the VOCs emission profile of *M. albus* from 14 to 19 d growth and found that concentration of volatiles increased continuously, reflecting the increased growth of the fungus. After day 19, *M. albus* VOCs production declined, probably because of the depletion of carbohydrates in PDA.

Our bioassays with VOCs showed that, in general, they were lethal to endophytic and phytopathogenic fungi. However, the VOCs were not inhibitory to *M. yucatanensis* or to the endophytes *Xylaria* sp. or isolate 120, or to the phytopathogen *F. oxysporum*. Only five compounds that have been reported previously in other *Muscodor* species were found in the *M. yucatanensis* VOCs: octane; 2-methyl butyl acetate; 2-pentyl furan; caryophyllene, and aromadendrene (Atmosukarto et al., 2005; Strobel, 2006; Strobel et al., 2007). We did not find naphthalene in the VOC mixture of *M. yucatanensis*, but we found some derivatives of naphthalene. These compounds were reported in *M. albus*, *M. roseus,* and *M. vitigenus* (Strobel et al., 2001; Daisy et al., 2002b; Ezra et

Table 4 GC/MS analysis of the compounds presents in the mycelium organic extract of *Muscodor yucatanensis*

Compounds	Retention Index[a]	Total area (%)	MW	Molecular formula
ethylbenzene	860.9	2.80	106	C_8H_{10}
1,3-dimethylbenzene[b]	869.8	1.19	106	C_8H_{10}
5-propan-2-ylidenecyclopenta-1,3-diene[b]	891.2	2.96	106	C_8H_{10}
4-hydroxy-4-methylpentan-2-one	845.4	13.94	116	$C_6H_{12}O_2$
ethyl butanoate[b]	804.7	7.98	116	$C_6H_{12}O_2$
butyl acetate[b]	818.4	3.46	116	$C_6H_{12}O_2$
2-phenylethylethanol[b]	1121.1	0.66	122	$C_8H_{10}O$
4-hydroxybenzaldehyde	1408.9	0.88	122	$C_7H_6O_2$
4-hydroxy-4-methyloxan-2-one[b]	1345.6	14.70	130	$C_6H_{10}O_3$
4-(2-Hydroxyethyl)phenol[b]	1464.5	1.99	138	$C_8H_{10}O_2$
undecane[a]	1100.6	7.10	156	$C_{11}H_{24}$
methyl 1-methyl-2-oxocyclohex-3-ene-1-carboxylate	977.1	4.99	168	$C_9H_{12}O_3$
butyl 2-phenylacetate	1280.7	1.36	192	$C_{12}H_{16}O_2$
5,7,7-trichlorohept-6-en-2-one	1502.1	1.90	214	$C_7H_9C_{13}O$
2,6-ditert-butyl-4-methylphenol[b]	1500.5	10.04	220	$C_{15}H_{24}O$
3,5-di(phenyl)-1,2,4-trioxolane	963.0	4.10	228	$C_{14}H_{12}O_3$
(Z)-hexadec-11-enoic acid	2155.8	3.13	254	$C_{16}H_{30}O_2$
hexadecanoic acid[b]	1971.8	1.50	256	$C_{16}H_{32}O_2$
(9E,12E)-octadeca-9,12-dienal[b]	2134.0	2.86	264	$C_{18}H_{32}O$
octadec-9-ynoic acid	2153.6	12.46	280	$C_{18}H_{32}O_2$
octadecanoic acid[b]	2173.3	7.23	284	$C_{18}H_{36}O_2$
pregnane-3,20-diol, diacetate, (3a,5a,20R)-	1659.7	2.13	404	$C_{25}H_{40}O_4$
sitosterol	3162.8	1.51	414	$C_{29}H_{50}O$

Kovat´s indices calculated from retention time data on an HP-5 MS capillary column

[a] Compounds presents in the culture medium and mycelium organic extract of *Muscodor yucatanensis*

al., 2004a). The VOCs bioactivity suggested that *M. yucatanensis* plays an important mutualistic role by augmenting host defensive responses against pathogens and/or competitors and also provides defense against some of its own competitors.

The present study is the first to identify the chemical constituents of organic extracts from the *in vitro* culture medium and mycelium of a species of *Muscodor* and to report their *in vitro* bioactivity on the growth of other endophytic and phytopathogenic fungi and plants.

M. yucatanensis was not inhibited by the mycelial organic extract. However, its growth was inhibited 50% or more by the culture medium extract, apparently an autotoxic response.

The highly significant growth stimulation of *G. mangifera* by three concentrations of mycelial extract tested was unexpected. Rodrigues et al. (2004) estimated that *Guignardia* isolates were among the endophytes most frequently isolated from tropical woody plant species of Brazil. These authors thought that *Guignardia* species does not regularly co-evolve with hosts but instead 'jumps' to unrelated hosts. This species is polyphagous and occurs on many different and often unrelated plant species. We do not know if *Guignardia* grows more rapidly *in vivo* when it infects host plant tissues already infected with *Muscodor*. Further studies that target this potential interaction could provide insight into fungal community dynamics and competitive and facilitative strategies.

The culture medium extract of *M. yucatanensis* was toxic to roots of test plants at higher concentrations than the mycelial extract. The culture medium had 23 compounds. Both extracts share 12 allelochemicals including benzene derivatives, phenolic compounds, cyclopentadienes, esters, lactones, alkanes, aldehydes, and carboxylic acids. It is possible that the most abundant compounds present in the culture medium extract are responsible for its strong allelochemical potential. This may provide a defense mechanism for *M. yucatanensis* against other endophyte competitors, as well as against some competitors and phytopathogens of *B. simaruba*. Given that both extracts of *M. yucatanensis* stimulate growth of *Guignardia mangifera,* there may be some metabolites that enhance symbiotic relationships with other endophytic fungi.

We have limited understanding of the mechanisms that regulate the integrated functions responsible for the success of endophyte-plant relationships. The ecological functions of the thousands of endophytic fungi associated with tropical plants are currently understudied. Dry tropical forests in Yucatan

Peninsula, and natural protected areas such as the Ecological Reserve of El Eden, Quintana Roo in Mexico, are sources of biological resources that consist of diverse macro and micro-organisms. Further studies of endophytic relationships in plant communities will contribute to an increased knowledge of the biodiversity of microorganisms associated with plants, the allelochemicals they produce that gives them an aggregate value, and the tangled web of biotic and abiotic interactions they exhibit.

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antennal scape and foreleg femur than do females. After sexing, males and females were placed in separate plastic boxes (7.5×8.5 cm) and fed green maté branches. Insects were maintained at 25±2°C at 60±5% RH, under a 12:12 hrL/D photoperiod. The mating history of field-captured beetles was unknown.

Pheromone Collection and Analysis Groups of either 4 males or 4 females were placed in an all-glass aeration chamber, and the released volatiles were trapped on Super Q columns (200 mg; Alltech, Deerfield, IL, USA) as previously reported (Zarbin et al., 2003). Samples were collected continuously over 15 day by using a humidified and charcoal-filtered airstream (1 $l.min^{-1}$). Volatiles from each aeration were eluted from the Super Q columns with distilled hexane, with the adsorbent traps changed after 10 collections. Extracts were concentrated to 400 µl (one insect equivalent per 100 µl) using argon (Zarbin et al., 1999; Zarbin, 2001).

In order to investigate the effect of host plant on release of pheromone, beetle volatiles were collected as described above for 24 h over 3 day, either in the presence or absence of a small branch of host plant (N=3 per treatment). The same procedure was performed to determine the diel rhythm of volatile release from males during the photophase and scotophase and, subsequently, every 2 hr of the photophase (over 3 day; N=3 males per treatment). Plant volatiles were also collected from 67 g of green maté branches. Branches were placed in all-glass aeration chambers, and volatiles were collected over 24 hr. After elution from traps, the collections were concentrated (10 g of green maté branches/100 µl) under argon (Zarbin et al., 1999; Zarbin, 2001). Data were analyzed by ANOVA, followed by a Tukey *post-hoc* test using BioEstat 3.0 software (Ayres et al., 2003).

Extracts were analyzed by gas chromatography-mass spectrometry (GC–MS) using a Shimadzu gas chromatograph (model 17A) coupled to a Shimadzu QP5050A electron ionization mass detector. The GC was operated in the splitless mode, and was equipped with a DB-5 (30 m×0.25 m×0.25 µm) or a DB-Wax (30 m×0.25 m×0.25 µm) capillary column (both Agilent Technologies, Santa Clara, CA, USA). The column oven was maintained at 50°C for 3 min, and then increased to 250°C at 7°C.min^{-1} to 250°C. The concentration of the major chemical component from the sex-specific volatiles was determined by using an external standard curve (1, 10, 100, 500, 1,000, 1,500, and 2,000 ppm) based on tridecane.

Synthesis ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker ARX-200 spectrometer (200 and 50 MHz, respectively) in a $CDCl_3$ solution. Chemical shifts are expressed in ppm relative to $CDCl_3$ (7.27 and 77.23 ppm for ^{1}H and ^{13}C NMR, respectively). The infrared (IR) spectra were measured as films using a Bomem B100 spectrometer. Crude products were purified by flash or vacuum flash chromatography on silica gel (230–400 mesh). (*E*)-6,10-Dimethyl-5,9-undecadien-2-one (geranylacetone) **(A)** is commercially available (Fluka® 99.5% *E* isomer; Sigma-Aldrich, Milwaukee, WI, USA, 60/40 *E*/*Z* isomers). (*E*)-6,10-Dimethyl-5,9-undecadien-2-ol **(B)** and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate **(C)** were synthesized as described in Fig. 1.

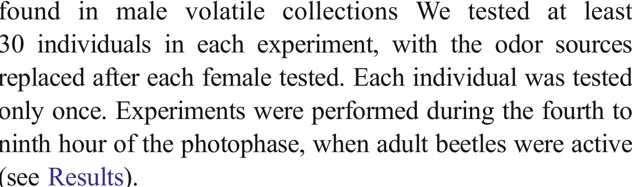
Synthesis of (E)-6,10-dimethyl-5,9-undecadien-2-ol (B) A dry THF solution (5 ml) of ketone **A** (Fluka®, 99.5%) (1 g, 5.1 mmol) was added slowly to a suspension of $LiAlH_4$ (0.290 g, 7.7 mmol) in THF (15 ml) at 0°C. The mixture was stirred for 5 hr at room temperature, then cooled to 0°C, hydrolyzed with $NaOH_{(aq)}$ (15%; 5 ml), and extracted several times with diethyl ether. The combined ether extracts were washed with water, dried over Na_2SO_4, and concentrated. The crude product was purified by flash chromatography (hexane/ethyl acetate, 8/2), yielding alcohol **B** (0.909 g, 90% yield). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.19 (d, 3H, J=6.16 Hz), 1.44–1.52 (m, 3H), 1.60 (s, 3H), 1.62 (s, 3H), 1.68 (s, 1H), 1.92–2.14 (m, 6H), 3.73–3.87 (m, 1H), 5.02–5.21 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.0, 17.7, 23.5, 24.4, 25.7, 26.6, 39.2, 39.7, 68.0, 123.9, 124.3, 131.4, 135.7. IR (ν Max, cm^{-1}): 824, 1082, 1127, 1377, 1447, 2853, 2915, 2966, 3349.

Synthesis of (E)-6,10-dimethyl-5,9-undecadien-2-yl acetate (C) An excess of acetic anhydride (0.5 ml, 5 mmol) and pyridine (0.5 ml) was added to alcohol **B** (0.800 g, 4.0 mmol) in dichloromethane (15 ml). The solution was stirred for 12 hr, diluted with dichloromethane (20 ml), and then washed with $HCl_{(aq)}$ (10%). The organic layer was

Fig. 1 Racemic synthesis of (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (**b**) and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (**c**) from (*E*)-6,10-dimethyl-5,9-undecadien-2-one (**a**)

washed with saturated $NaHCO_3$ solution before the dichloromethane solution was separated, dried over Na_2SO_4, and concentrated. After flash chromatography (hexane/ethyl acetate, 9/1), the acetate **C** was obtained at 85% yield (0.784 g). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.22 (d, 3H, J=6.26 Hz), 1.50–1.65 (m, 8H), 1.68 (s, 3H), 1.94–2.10 (m, 9H), 4.79–4.98 (m, 1H), 5.03–5.17 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.9, 17.7, 20.0, 21.4, 23.9, 25.7, 26.7, 36.0, 39.7, 70.7, 123.4, 124.3, 131.4, 135.7, 170.8. IR (ν Max, cm^{-1}): 1018, 1058, 1127, 1240, 1371, 1439, 1736, 2853, 2921, 2971.

Laboratory Bioassay of Synthetic Pheromone Female *H. betulinus* responses to synthetic pheromone racemic mixtures were tested in a binary choice Y-tube olfactometer, using humidified, charcoal-filtered air at 4 $l.min^{-1}$. The olfactometer consisted of a Y-shaped glass tube (4×40 cm) with two 20-cm arms. Hydrochloric acid and ammonium hydroxide were mixed to confirm the plume distribution of odor sources throughout the system (Baker and Linn, 1984). Odor sources were placed at the base of one arm of the olfactometer, and consisted of a 2×2 cm piece of filter paper loaded with synthetic pheromone, plant volatiles, or hexane (control). A single female beetle was placed at the base of the main olfactometer tube, and its behavior was observed for 20 min. A female that walked upwind and made contact, within 20 min., with a filter paper containing either the treatment or control odor source was recorded as a positive or negative response, respectively. Females that did not contact either the treatment or control odor source were excluded from the statistical analysis. The Y-tube was cleaned with alcohol and left to dry for 5 min after testing 4 females. The positions of the olfactometer arms were reversed upon changing the odor sources. A preliminary study showed that there was no difference in the responses of males or females in the olfactometer when both of the arms were blank, indicating a lack of positional effect of the experimental setup. Nine experiments testing female responses were performed: 1) to 10 μl of the plant volatiles extract; 2) to 5 μg (5 μl of a solution of 1 μg/μl hexane) of synthetic major pheromone component; 3) to 8 μg of synthetic major component; 4) to 5 μg of synthetic major component plus 10 μl of the plant volatiles collection; 5) to 8 μg of synthetic major component plus 10 μl of the plant volatiles collection; 6) to ternary mixture **I** (5 μg **C**: 0.43 μg **A**: 0.038 μg **B**); 7 to ternary mixture **II** (8 μg **C**: 0.69 μg **A**: 0.06 μg **B**); 8) to ternary mixture **I** plus 10 μl of the plant volatiles collection; and 9) to ternary mixture **II** plus 10 μl of the plant volatiles collection. The ternary mixture ratio [91.4:7.9:0.70 of racemic (E)-6,10-dimethyl-5,9-undecadien-2-yl acetate, (E)-6,10-dimethyl-5,9-undecadien-2-one and (E)-6,10-dimethyl-5,9-undecadien-2-ol, respectively] that was used in the experiment was similar to that



found in male volatile collections We tested at least 30 individuals in each experiment, with the odor sources replaced after each female tested. Each individual was tested only once. Experiments were performed during the fourth to ninth hour of the photophase, when adult beetles were active (see Results).

Data were analyzed by the Chi-squared test using BioEstat 3.0 software (Ayres et al., 2003).

Results

The chromatographic profile of volatiles from male and female *H. betulinus* showed three male-specific compounds, **A**, **B**, and **C**, with the following retention times (Rts) and Kovat's Indices (KIs) on the DB-5 column: **A**: Rt=16.65 min., KI=1,448; **B**: Rt=16.72 min, KI=1,454; **C**: Rt=18.84 min, KI=1,573. The ratio between these components was calculated as 7.9:0.7:91.4, respectively, based on the area of the GC peaks (Fig. 2). The mass spectrum of compound **A** was simple, with a base peak at *m/z* 43, a fragment at *m/z* 69, and a molecular ion of 194 Da (Fig. 3a). When this spectrum was compared to the NIST library, it was evident that the compound might be (E)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) or its 5*Z* isomer (nerylacetone). Compound (**A**) was identified as geranylacetone by co-injection of the crude extract with geranyl- and nerylacetone standards on the two GC columns. Identification was based on the similarity of retention times and fragmentation pattern.

Compound **B** had a similar retention time as component **A**. The mass spectrum (Fig. 3b) showed a molecular ion of *m/z* 196, a base peak at *m/z* 41, and other fragments at *m/z* 69 (90%) and 153 (34%), suggesting that **B** may be related to geranylacetone with hydrogenation at the carbon 5, or the corresponding alcohol, geranylacetol. Reduction of compound **A** with $LiAlH_4$ (Zarbin et al., 1998) yielded an alcohol with the same retention time and mass spectrum as compound **B**, confirming its chemical structure as (E)-6,10-dimethyl-5,9-undecadien-2-ol. In a previous study, this alcohol was identified as the primary component of the male-produced sex pheromone of the longhorn beetles, *Tetropium fuscum* and *Tetropium cinnamopterum*, members of the Spondylidinae subfamily, and was named fuscumol (Silk et al., 2007).

The mass spectrum of component **C** showed a base peak at *m/z* 109, fragments at *m/z* 43 (60%), *m/z* 69 (51%), and had a molecular weight of 238 Da (Fig. 3c). The molecular weight of component **C** was 42 Da greater than component **B**, and the retention time more than 2 min longer. These differences, in addition to the appearance of a fragment at *m/z* 43, suggested the presence of an acetyl group. This was

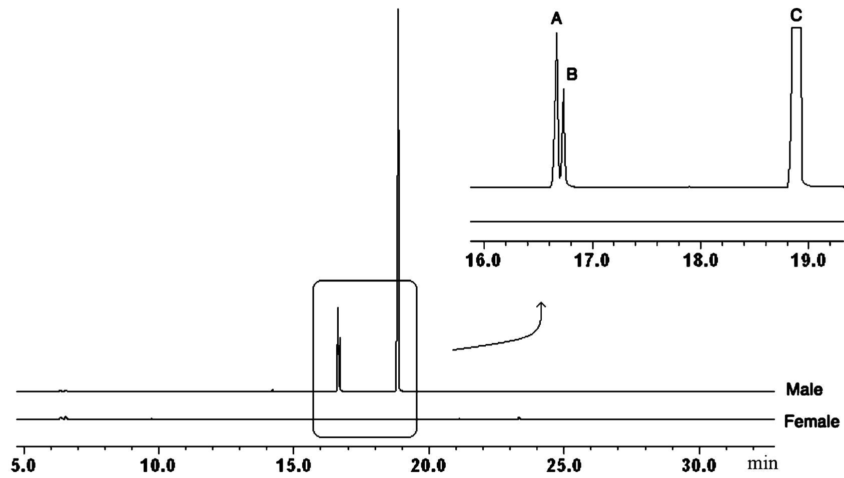


Fig. 2 Gas chromatographic analyses of volatiles obtained from male and female *Hedypathes betulinus*. The three male-specific compounds are (*E*)-6,10-dimethyl-5,9-undecadien-2-one (**a**), (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (**b**), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (**c**)

also supported by the appearance of a fragment at *m/z* 178, resulting from the loss of acetic acid (M^+ - 60). Acetylation of alcohol **B** by acetic anhydride and pyridine (Leal et al., 1999) resulted in an ester with a fragmentation pattern and retention time that perfectly matched that of compound **C**. Thus, the major compound was identified as (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate).

The production of (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate **C** was dependent on the presence of host plant. Beetles that fed on green maté released more total volatiles (344.2±86.5 μg/insect/day) than beetles that did not feed on green maté (0.53±0.35 μg/insect/day) ($F_{1,4}$=157.02, P< 0.05) (Fig. 4a). The release of pheromone components occurred during the photophase rather than the scotophase ($F_{1,4}$=14.93, P<0.05) (Fig. 4b), with maximum release occurring between 4 and 6 hr after the onset of the photophase (Fig. 4c). Six hours after the onset of the photophase, the amount of pheromone released decreased ($F_{5,12}$=94.08, P<0.001), from that of peak release, to an amount similar to that at the start of the photophase (Fig. 4c).

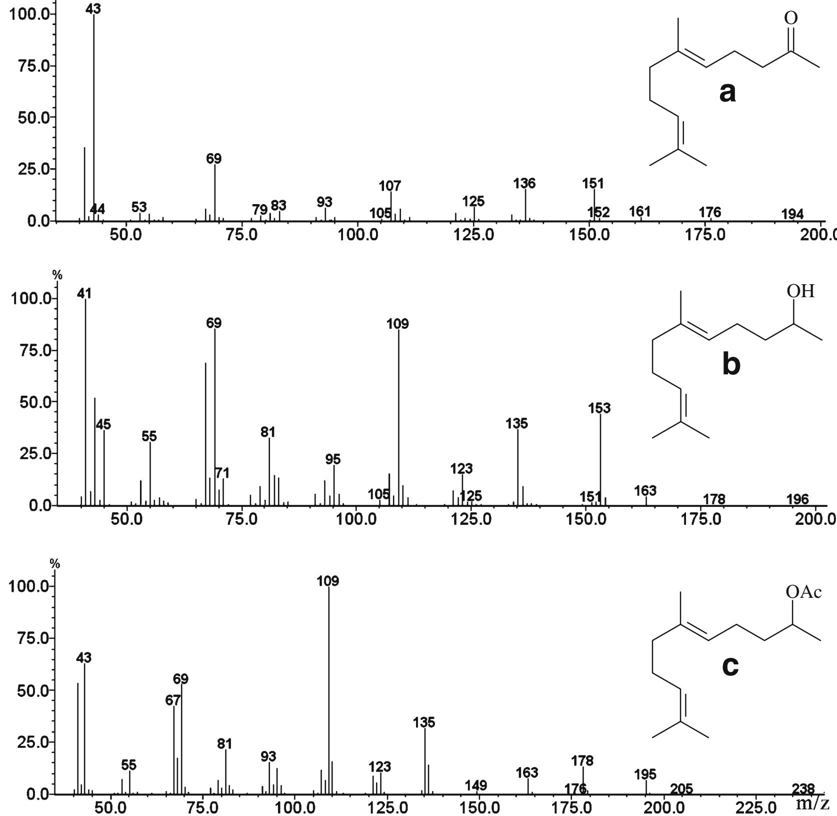


Fig. 3 Mass spectra of compounds **a** [(*E*)-6,10-dimethyl-5,9-undecadien-2-one], **b** [(*E*)-6,10-dimethyl-5,9-undecadien-2-ol], and **c** [(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate]

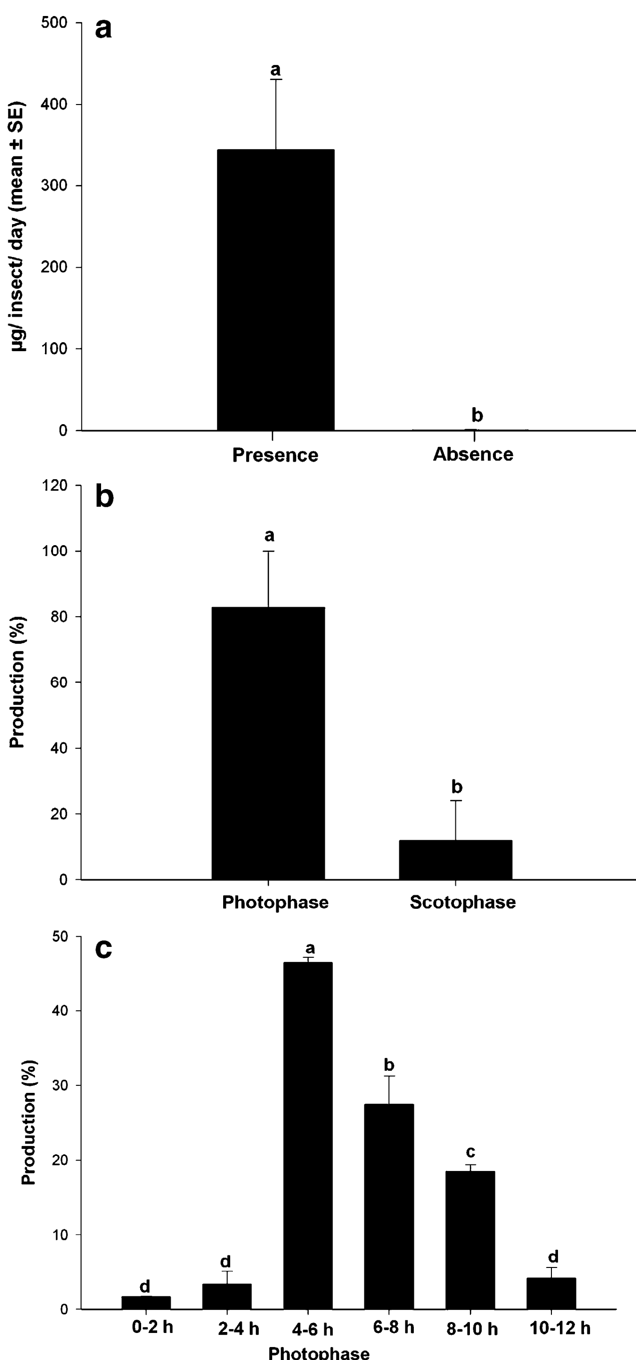
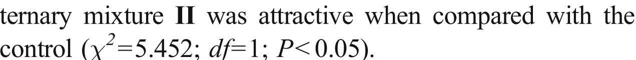


Fig. 4 Comparison of the amounts of the major component **C** [(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate] collected from aeration of *Hedypathes betulinus* males: **a** in the presence and absence of green maté branches; **b** between the photophase and scotophase; **c** among different times of the 12 hr of the photophase. Mean values followed by the same letter are not significantly different (ANOVA followed by a Tukey post-hoc test; *N*=3)

Female *H. betulinus* responses to the various treatments are shown in Tables 1 and 2. The major component alone at 5 µg (χ^2=0.034; *df*=1; *P*>0.05) and 8 µg (χ^2=0.143; *df*=1; *P*>0.05), and the ternary mixture **I** alone (χ^2=0.29; *df*=1; *P*>0.05) were not attractive to females (Table 1). However,


ternary mixture **II** was attractive when compared with the control (χ^2=5.452; *df*=1; *P*< 0.05).

Although plant volatiles alone were not attractive to females (X^2=0.133; *df*=1; *P*= 0.715), addition of these volatiles to the various pheromone component mixtures increased attraction. A greater number of females were attracted to the combination of host plant volatiles and component **C** at both doses tested, 5 µg (χ^2=7.53; *df*=1; *P*= 0.006) and 8 µg (χ^2=11.64; *df*=1; *P*< 0.001). Similarly, both doses of ternary mixture **I** (χ^2=5.828; *df*=1; *P*=0.015) and **II** (χ^2=9.783; *df*=1; *P*=0.001), as well as major component **C,** were attractive when combined with host-plant volatiles (Table 2).

Discussion

We identified the male-produced pheromone of *H. betulinus* as a mixture of three compounds: (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (major), (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone), and (*E*)-6,10-dimethyl-5,9-undecadien-2-ol. Within the sub-family Lamiinae, a male-produced volatile pheromone that attracts both sexes (i.e., an aggregation pheromone) has been identified previously for *Anoplophora glabripennis* (Zhang et al., 2002; Nehme et al., 2009). In field tests, addition of host-plant volatiles to this aggregation pheromone increased attraction of *A. glabripennis*, particularly for virgin females (Nehme et al., 2010). Evidence for a male-produced contact sex pheromone has been reported for *Steirastoma breve* (Liendo et al., 2005) and *Monochamus galloprovincialis* (Ibeas et al., 2008), while *A. glabripennis* females produce a contact recognition pheromone (Zhang et al., 2003). Our bioassay data indicated that the male-produced pheromone of *H. betulinus* is a sex pheromone, as conspecific males were not attracted to the pheromone. Thus, this is probably the first volatile sex pheromone identified from this subfamily. However, this needs to be confirmed in rigorous field tests by testing different ratios and concentrations of the compounds.

The male-produced compounds we identified in *H. betulinus* are different from male-produced sex or aggregation pheromones of other cerambycid species in the subfamily Cerambycinae (Allison et al., 2004; reviewed by Ray et al., 2006; Hanks et al., 2007; Lacey et al., 2008). The pheromones of most of these species typically are compounds with short chain (6–10 carbon) α-hydroxyl ketones or (α, β)-diols (Hanks et al., 2007; Lacey et al., 2008), although two exceptions are the male-produced aggregation pheromone of *Phymatodes lecontei* [(*R*)-2-methylbutan-1-ol] (Hanks et al., 2007), and a component of the sex pheromone of *Hylotrupes bajulus* (1-butanol) (Reddy et al., 2005). The pheromone components of *H. betulinus* also are different

Table 1 Responses of *Hedypathes betulinus* females to various synthetic racemate treatments in a Y-tube olfactometer

Odor sources		Responses (%) ♀
Main compound **C** (5 µg) vs. Control	Main compound **C**	41.7
	Control	38.9
	Not decided	19.4
	N=36	
Main compound **C** (8 µg) vs. Control	Main compound **C**	41.7
	Control	36.1
	Not decided	22.2
	N=36	
Ternary Mixture **I** vs. Control	Ternary mixture **I**	44.8
	Control	36.8
	Not decided	18.4
	N=38	
Ternary Mixture **II** vs. Control	Ternary mixture **II**	59.5*
	Control	24.3
	Not decided	16.2
	N=37	

*Statistically significant, Chi-square test, $P<0.05$

Hexane = solvent control; Ternary Mixture **I**=(5 µg **C**: 0.43 µg **A**: 0.038 µg **B**); Ternary Mixture **II**=(8 µg **C**: 0.69 µg **A**: 0.06 µg **B**); Ternary mixture ratio was calculated from male extract (91.4: 7.9: 0.70 of racemic (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate **C**, (*E*)-6,10-dimethyl-5,9-undecadien-2-one **A** and (*E*)-6,10-dimethyl-5,9-undecadien-2-ol **B**, respectively)

from the known female-produced pheromones of other cerambycids: N-(2′*S*)-methylbutanoyl-2-methylbutylamine is a pheromone component of *Migdolus fryanus* (Anoplodermatinae) (Leal et al., 1994), 3,5-dimethyldodecanoic acid is a pheromone component of *Prionus californicus* (Prioninae) (Rodstein et al., 2009), and (*S*)-10-oxoisopiperitenone has been identified as a pheromone of *Vesperus xatari* (Vesperinae) (Boyer et al., 1997).

Multi-component pheromones previously have been reported for some species of Cerambycidae. For example, Lacey et al. (2008) identified eight male-specific compounds from *Megacyllene caryae*, with initial field tests

Table 2 Responses of *Hedypathes betulinus* females to various synthetic racemate treatments and/or host plant volatiles (HPV) in a Y-tube olfactometer

Odor sources		Responses (%) ♀
HPV vs. Control	HPV	32.6
	Control	37.2
	Not decided	30.2
	N=43	
Main compound **C** (5 µg) plus HPV vs. Control	Main compound **C** plus HPV	56.8*
	Control	20.5
	Not decided	22.7
	N=44	
Main compound **C** (8 µg) plus HPV vs. Control	Main compound **C** plus HPV	69.4*
	Control	16.7
	Not decided	13.9
	N=36	
Ternary Mixture **I** plus HPV vs. Control	Ternary mixture **I** plus HPV	72.2*
	Control	13.9
	Not decided	13.9
	N=36	
Ternary Mixture **II** plus HPV vs. Control	Ternary mixture **II** plus HPV	66.7*
	Control	13.3
	Not decided	20
	N=30	

*Statistically significant, Chi-square test, $P<0.05$

Hexane = solvent control; Ternary Mixture **I**=(5 µg **C**: 0.43 µg **A**: 0.038 µg **B**); Ternary Mixture **II**=(8 µg **C**: 0.69 µg **A**: 0.06 µg **B**); Ternary mixture ratio was calculated from male extract (91.4: 7.9: 0.70 of racemic (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate **C**, (*E*)-6,10-dimethyl-5,9-undecadien-2-one **A** and (*E*)-6,10-dimethyl-5,9-undecadien-2-ol **B**, respectively)

determining that none of these compounds was attractive individually. Volatiles collected from male *Rosalia funebris* contained a major male-specific compound, (*Z*)-3-decenyl (*E*)-2-hexenoate, and several minor compounds (Ray et al., 2009). Despite the attraction, in the field, of both sexes of *R. funebris* to lures baited with synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate, the authors suggested that the minor components may form part of the natural pheromone, and that traps baited with a more complete blend could attract more beetles.

Our results demonstrated that addition of host-plant volatiles to the pheromone enhanced attraction to females (Table 2). Host plant volatiles often enhance an insect's response to sex pheromones, and this effect may result in true synergism; a response to the pheromone and plant volatile mixture that is greater than the sum of responses to the individual components (Reddy and Guerrero, 2004). Increased pheromone attraction due to the presence of host odors occurs in several insect groups, such as moths (Dickens et al., 1990, 1993; Reddy and Guerrero, 2000; Deng et al., 2004; Yang et al., 2004), beetles (Phillips et al., 1984; Byers et al., 1990; Nakamuta et al., 1997; Zhang and Schlyter, 2003), and flies (Landolt et al., 1992). In the Cerambycidae, host odor and pheromone synergism has been documented in *Anaglyptus subfasciatus* (Nakamuta et al., 1997), *Tetropium fuscum* and *T. cinnamopterum* (Silk et al., 2007), and *A. glabripennis* (Nehme et al., 2010). Only in *A. glabripennis* are adults attracted to plant volatiles alone, and in that case, this effect is more apparent for males than females (Nehme et al., 2010).

Studies of numerous cerambycid species suggest that conspecific location in these species is mediated first by attraction to host-plant volatiles and subsequently to pheromones (Hanks, 1999; Allison et al., 2004). Ginzel and Hanks (2005) hypothesized that cerambycine mate location and recognition results from three sequential behaviors: (1) both sexes are independently attracted to larval hosts by plant volatiles; (2) males attract females at short-range with pheromones; and (3) males recognize females by contact pheromones. In our study, female *H. betulinus* were not attracted to host-plant volatiles alone, indicating that mate location involves only stages 2 and 3, consistent with previous observations of the mating behavior of this species (Fonseca and Zarbin, 2009).

In summary, we have identified the sex pheromone of *H. betulinus* and described the temporal pattern of pheromone release. Future studies will determine the stereochemistry of components **B** and **C**, the biosynthesis and site of production of the components, as well as the activity of the pheromone and host-plant volatiles in the field.

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probably through the presence of a female-specific pheromone component. Upon contact or near contact, the male either retreats or mounts dorsally. He is especially likely to retreat if she has already mated (King et al., 2005); and retreats appear to be related to females that release an antiaphrodisiac when a male approaches, rather than from physical aggression from the female (King and Dickenson, 2008a). If a male mounts the female, he then continues to court her by vibrating his entire body on her. Fanning, mounting, and vibrating by a male do not require any active solicitation by the female, i.e., even dead females elicit such behaviors.

Pheromone components involved in mating behavior in other pteromalid species include pheromones used in attracting a mate and eliciting male courtship behaviors (Ruther et al., 2000). Among insects generally, female-produced pheromones are more common and better known (Keeling et al., 2004); however, in the parasitoid wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae), some male pheromones are known to affect a female's response to the male (e.g., Ruther et al., 2007). It is not uncommon for male and female parasitoids to have many of the same pheromones but in different quantities (Keeling et al., 2004; Ruther and Steiner, 2008), and this is true of *N. vitripennis* (Steiner et al., 2006). Prior to this study, no pheromone components of *S. endius* were known for either males or females. We report the identification of a pheromone component in *S. endius* that is very different from that reported for other pteromalids.

Methods and Materials

Spalangia endius *Colony* The *S. endius* were from a colony established from wasps collected in 1996 from Zephyr Hills, FL, USA and maintained by using pupae of a natural host, the house fly, *Musca domestica* L. (Diptera: Muscidae). Hosts were produced following techniques described in King (1988), but we used wood shavings in place of vermiculite as the rearing substrate. Parasitized hosts were isolated individually in glass test tubes prior to emergence of the wasp in order to obtain female virgin wasps. Virgin males were obtained from separate colonies parasitized by virgin females.

Semiochemical extraction and gas chromatography-mass spectrometry analyses Gas chromatography-mass spectrometry (GC-MS) was used to compare semiochemical extracts from males and females to determine if sex-specific compounds were detectable. The semiochemical extracts from males and females were obtained by two different methods, solvent extraction of whole insects and solid phase micro extraction (SPME). A third method (Super-Q trapping of volatiles) was used to prepare extracts from females alone. The purpose of solvent extractions was to remove all soluble compounds from the wasp's exoskeleton, whereas SPME was used specifically to collect emitted volatiles. For each solvent extraction, 10 wasps were killed by freezing at −17°C, transferred to a microtube, and extracted in dichloromethane (30 min, 60 µl). Extract volume was reduced to 20 µl under a gentle stream of nitrogen. For SPME, collections were made by exposing the fiber [65 µm polydimethylsiloxane/divinylbenzene blend (Supelco Inc., Bellefonte, PA, USA)] for 20 min to the static headspace of a 1.5 ml vial with a Teflon-lined septum (National Scientific, Rockwood, TX, USA) containing 10 insects.

Headspace volatiles were collected from females by using Super-Q porous polymer traps (Alltech Associates, Deerfield, IL, USA). This technique permitted a larger quantity of volatiles to be collected than the SPME method. For the trap collection of the volatiles, a Magnetek Universal Ser. 41ZF vacuum pump was used to pull the headspace of a 125 ml side-arm flask through 5-mm-diam. glass tubing that contained a 5 to 8-mm plug of Super-Q porous polymer. The Super-Q was held in place by a fine stainless steel screen (fused into trap glass wall) and glass wool. A variable number of 0-d-old females were placed in the flask. Females were replaced with new females every 2 d. Volatiles from a total of 200 females were collected over a 2-mo period. The flask opening was plugged with a cork with a hole, which was filled with glass wool to allow the free inflow of air. To recover the collected volatiles, the Super-Q traps were rinsed weekly with dichloromethane (2 ml). This was concentrated to 400 µl or 2 µl/ female equivalent. No food or water was fed to the insects during the collection period.

All three types of extracts were analyzed by a Hewlett Packard 5973 mass selective detector, interfaced with a Hewlett Packard 6890 GC (Agilent Technologies Inc., Santa Clara, CA, USA). A split/splitless inlet was used in splitless mode with either a 15-m DB-1 (0.25 mm i.d., 0.1 µm film thickness) or a 30-m DB-5MS (0.25 mm i.d., 1.0 µm film thickness) capillary column (J & W, Agilent Technologies Inc., Santa Clara, CA, USA). Carrier gas was He set at constant pressure (6 psi), and injector temperature was set at 280°C. The oven temperature started at 50°C for 1 min and increased to 280°C at a rate of 10°C/min.

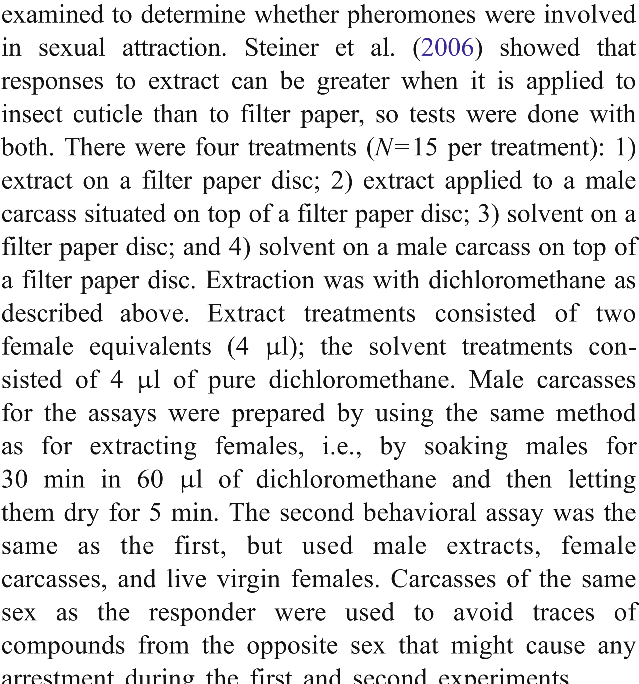
GC-Electroantennographic Detection (GC-EAD) analysis Male antennal responses to female-specific compounds from the SPME and Super-Q extracts were examined to determine how sensitively the female-specific compounds were detected. GC-EAD connections were made by inserting the base of an excised male antenna into a saline-filled Ag/AgCl glass pipet grounding electrode.

The suspended male antenna was maneuvered by a micromanipulator, into a stream of purified, humidified air (20 ml/sec) that emerged from a 20-cm-long L-shaped glass tube (7 mm i.d.) that ended 1 cm from the antenna. A second glass pipet Ag/AgCl-recording probe (Syntech, Hilversum, The Netherlands), fitted onto a second micromanipulator, was placed in contact with the distal cut end of the antenna. Stimuli were introduced into the air stream from the GC effluent (split between FID and EAD, GC-EAD interface temperature set at 280°C) through a hole in the glass tube positioned 10 cm from the antenna. The GC-EAD responses were amplified (500×) with an AC/ DC UN-6 amplifier (Syntech). Acquisition and analysis of the responses were performed by a computer equipped with an analog to digital conversion board (IDAC, Syntech) running GC-EAD software (Syntech). The GC was a Hewlett Packard 6890 GC (Agilent Technologies Inc.). A split/splitless inlet was used in splitless mode with 15-m DB-1 (0.25 mm i.d., 0.1 μm film thickness) capillary column (J & W, Agilent Technologies Inc.). Carrier gas was He set at a flow rate of 24 cm/sec, and injector temperature was set at 250°C. The oven temperature started at 50°C for 1 min and increased to 150°C at a rate of 10°C/min, followed by 30°C/min to 280°C.

Chemicals Methyl 4-methylsalicylate was purchased from Frinton (Vineland, NJ, USA). Methyl 3- and methyl 5-methylsalicylate were purchased from Aldrich (St. Louis, MO, USA). Methyl 6-methylsalicylate was synthesized from ethyl 6-methylsalicylate (APIN, Abingdon, Oxon) according to Castracani et al. (2003), except that the reaction ran for 5 hr. Reaction completion was checked by GC-MS.

Bioassays All behavioral assays were conducted in a small blue plastic dish (1.5 cm diam, 1 cm high) filled three quarters with dry sand and covered by a glass cover slip. A wasp was tapped from its glass tube into the dish. The dish contained a single treatment or control on filter paper (6 mm diam. Whatman No. 1), which was centered on top of the sand. Arrestment time of a test wasp was recorded for 5 min (adapted from Steiner et al., 2006). Arrestment time was the time that the wasp spent with a body part touching the filter paper (or carcass in the first experiment). No wasp was tested more than once, and all were between 0 and 1-d-old. Prior to data analyses, arrestment times were log transformed to improve normality and homoscedasticity. Log-transformed arrestment times were compared among treatments by using analyses of variance (ANOVA) or independent *t*-tests.

Response to extracts of the opposite sex In the first behavioral assay, male responses to female extract were



examined to determine whether pheromones were involved in sexual attraction. Steiner et al. (2006) showed that responses to extract can be greater when it is applied to insect cuticle than to filter paper, so tests were done with both. There were four treatments (N=15 per treatment): 1) extract on a filter paper disc; 2) extract applied to a male carcass situated on top of a filter paper disc; 3) solvent on a filter paper disc; and 4) solvent on a male carcass on top of a filter paper disc. Extraction was with dichloromethane as described above. Extract treatments consisted of two female equivalents (4 μl); the solvent treatments consisted of 4 μl of pure dichloromethane. Male carcasses for the assays were prepared by using the same method as for extracting females, i.e., by soaking males for 30 min in 60 μl of dichloromethane and then letting them dry for 5 min. The second behavioral assay was the same as the first, but used male extracts, female carcasses, and live virgin females. Carcasses of the same sex as the responder were used to avoid traces of compounds from the opposite sex that might cause any arrestment during the first and second experiments.

Responses to fractionated female-specific compounds The third bioassay experiment compared male behavioral responses to fractions containing one of the female-specific compounds or their combination. First, a female extract was obtained by soaking 150 females in 900 μl of dichloromethane for 30 min. Ninety female equivalents of the extract were blown dry under nitrogen and resuspended in 200 μl hexane prior to column chromatography on silica gel. The column was made with 5 cm of silica gel (70–230 mesh) in a Pasteur pipette with glass wool blocking the tapered part of the pipette. The female extract was loaded on the silica column, and three fractions were collected. Elution solvents were hexane, 1:1 diethyl ether: hexane, and diethyl ether (2 ml of each, in that order). The fractions were analyzed by GC-MS. One female-specific compound (previously identified as **A**) was present in the 1:1 diethyl ether/hexane fraction, and the second compound (previously identified as **B**) was found in the diethyl ether fraction. These two fractions were blown dry under nitrogen and resuspended in dichloromethane prior to behavioral analysis. The experiment compared the male behavioral responses to four treatments: 1:1 diethyl ether:hexane fraction (**A**), diethyl ether fraction (**B**), 1:1 blend of the two fractions (**A**+**B**), and solvent (dichloromethane) control (N=15 males each). Each treatment consisted of two female equivalents (4 μl) applied to a 6 mm piece of filter paper. The solvent control was applied in the same volume.

Response to methyl methylsalicylate isomers The fourth behavioral assay compared the male behavioral responses to authentic standards of four methyl methylsalicylate positional

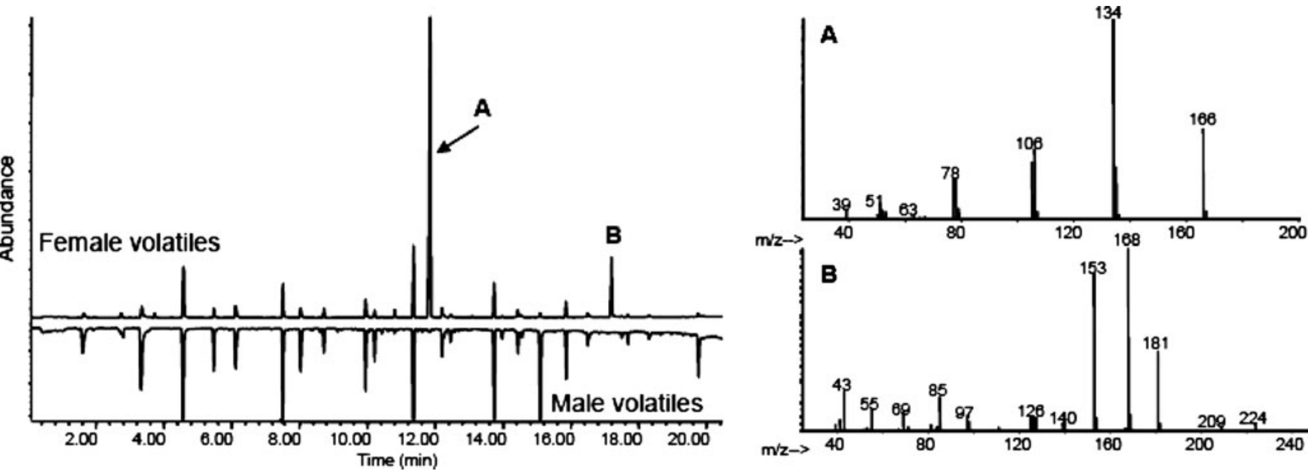


Fig. 1 Comparison of total ion chromatograms of SPME samples from male and female *Spalangia endius*. This illustrates the two female-specific compounds, **A** and **B**, and their mass spectra

isomers vs. vacuum-collected female volatiles. The two female-specific compounds were present in the vacuum collected volatiles. All methyl methylsalicylate isomers were applied at two female equivalents (about 300 ng). An arrestment time bioassay of virgin males was completed by using six different treatments, methyl 3-methylsalicylate, methyl 4-methylsalicylate, methyl 5-methylsalicylate, and methyl 6-methylsalicylate, along with vacuum-collected female volatiles (two equivalents), and a solvent control, all in 4 µl of dichloromethane. The temperature and relative humidity during these four behavioral experiments ranged from 21 to 24°C and 35% to 55%, respectively.

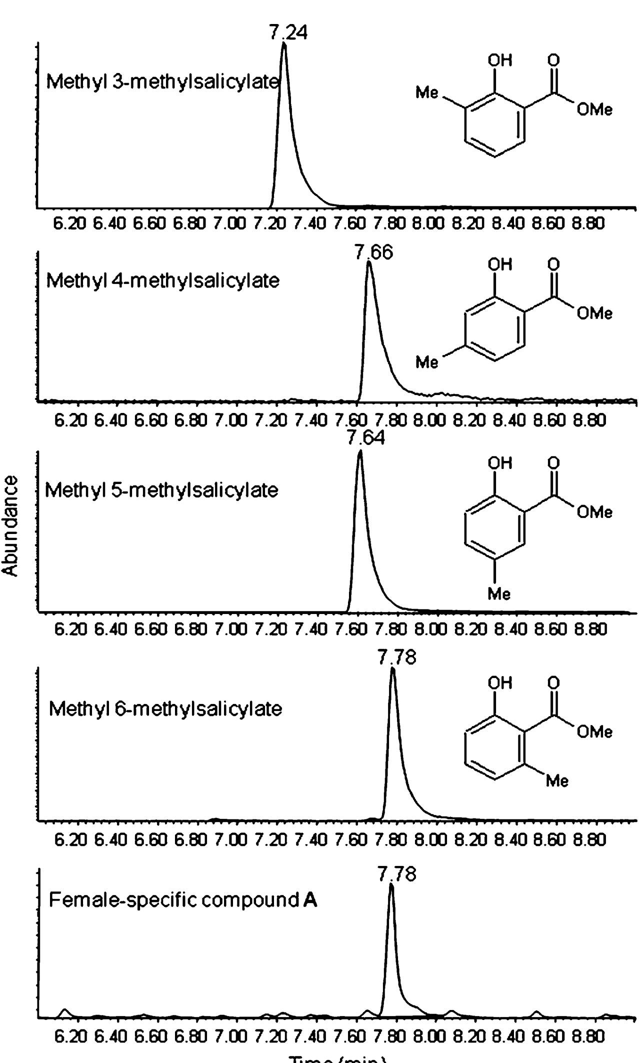


Fig. 2 Comparison of total ion chromatograms of authentic standards of four possible isomers of methyl methylsalicylate and female-specific *Spalangia endius* compound **A** (retention times from analysis on DB1 capillary column)

Results

Gas chromatography-mass spectrometry analyses Comparison of SPME collection (N=4) from adult *S. endius* males and females revealed two female-specific peaks (**A** and **B** in Fig. 1). The most abundant, **A,** was also present in all Super-Q collections (N=8); but only trace amounts of **B** could be detected in these collections. The two peaks were always detected in female body washes (N=5) in a 98:2 ratio, respectively. These two compounds were never detected in male volatiles collections or body extracts. The mass spectrum of compound **A** (Fig. 1) suggested an isomer of methyl methylsalicylate; no MS library (Wiley 1995) match was found for compound **B** (Fig. 1). Compounds **A** and **B** eluted from the silica column in 1:1 diethyl ether:hexane and diethyl ether, respectively, suggesting that both compounds are oxygenated.

The library mass spectra of the four possible isomers of methyl methylsalicylate were similar; thus authentic standards were acquired for all four for comparison to the natural product. The standard isomers all were separable by GC.

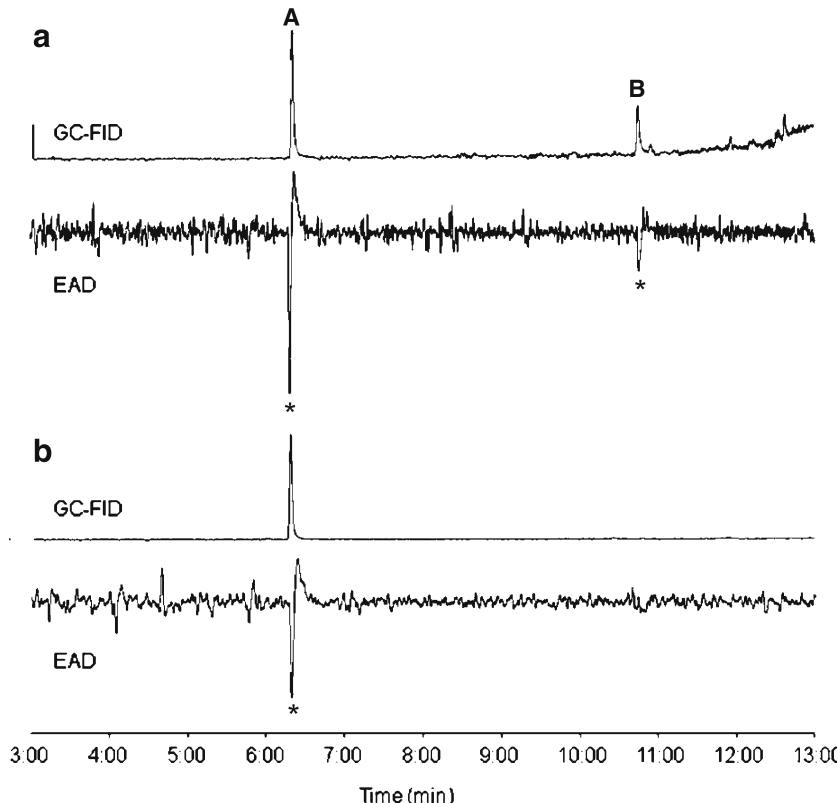


Fig. 3 Antennal response of male *Spalangia endius* to **a**) female-specific compounds **A** and **B** from the SPME sample and **b**) synthetic methyl 6-methylsalicylate; * indicates a positive response

Only methyl 6-methylsalicylate had an identical GC retention time and mass spectrum compared to compound **A** (Fig. 2).

Gas chromatography-Electroantennographic Detection (GC-EAD) analysis The GC/EAD analysis showed that male antennae (N=5) responded to the two female-specific compounds (Fig. 3 top), and that the male antennal response to natural **A** was identical in GC retention time compared to the response to synthetic methyl 6-methylsalicylate (Fig. 3 bottom).

Response to extracts of the opposite sex A 2-way ANOVA of arrestment time by extract (extract, solvent) and carcass (present, absent) was done for males and females separately. For males, there was no significant interaction between extract and carcass (F=0.09, df=1, 56, P=0.77), meaning the effect of each does not depend on the other. There were significant individual effects for both extract and carcass (F=45.79, df=1, 56, P=<0.001; F=21.62, df=1, 56, P=<0.001, respectively). Male arrestment was increased by the extract as can be seen by comparing solvent to extract and by comparing carcass with solvent to carcass with extract (Table 1). Male arrestment also was increased by the carcass being present, as can be seen by comparing extract alone to carcass with extract and solvent alone to carcass with solvent. For females, there were no significant effects of extract, carcass, or the interaction between them (F=0.31, df=1, 56, P=0.58; F=0.52, df=1, 56, P=0.47; F=1.66, df=1, 56, P=0.20). Thus, females did not appear to arrest to extract or carcass.

Responses to fractionated female-specific compounds A 2-way ANOVA of arrestment time by fraction-containing-**A** (present, absent) and fraction-containing-**B** (present, absent) was performed (Table 2). There was no significant interaction (F=0.013, df=1, 56, P=0.91) and no significant effect

Table 1 Arrestment times of *Spalangia endius* males and females to solvent and to extract of the opposite sex, with or without a carcass of same sex present[a]

	Solvent	Extract	Solvent with carcass	Extract with carcass
Male	0.29±0.09 (0–1.00)	0.97±0.14 (0–1.63)	0.75±0.10 (0–1.20)	1.49±0.06 (0–1.87)
Female	0.61±0.12 (0–1.26)	0.83±0.12 (0–1.53)	0.85±0.13 (0–1.59)	0.77±0.10 (0–1.18)

[a] Table entries are log-transformed mean ± s.e. (minimum–maximum), in log sec, N=15 per treatment per sex

Table 2 Arrestment times of *Spalangia endius* males and number of males that fanned to column fractions containing methyl 6-methylsalicylate and/or unknown compound and to solvent control[a]

Fraction content	Arrestment time	# Males that fanned
Solvent[b]	0.71±0.11 (0–1.49)	0
Methyl 6-methylsalicylate (A)	1.24±0.12 (0.30–2.19)	4
Mixture (A+B)	1.19±0.14 (0–2.39)	2
Unknown compound (B)	0.69±0.09 (0–1.40)	0

[a] Table entries are log-transformed mean±s.e. (minimum–maximum), in log sec, N=15 per treatment

[b] Dichloromethane

of the fraction containing **B** (F=0.071, df=1, 56, P=0.79), but there was a significant effect of the fraction containing **A** (F=20.09, df=1, 56, P<0.001). Thus, this experiment showed that relative to solvent control, males arrest to the fraction containing the **A** compound, but not to the fraction containing the **B** compound, and that the response to one fraction is not affected by the presence of the other fraction. Males periodically fanned during trials that included the **A** containing fraction.

Response to methyl methylsalicylate isomers To preserve statistical power, planned comparisons were used (Ruxton and Beauchamp, 2008). The first comparison of arrestment times in the bioassay (Table 3) was between the female-specific volatiles and methyl 6-methylsalicylate. Arrestment times did not differ (t=0.061, df=28, P=0.95), and so their times were combined into group 1. The next comparison was among the other three synthetic isomers (methyl 3-methylsalicylate, methyl 4-methylsalicylate, methyl 5-methylsalicylate), which did not differ either (F=0.57, df=2, 42, P=0.57); so the isomers' arrestment times were grouped and became group 2. Groups 1 and 2 then were compared, which showed that arrestment time was greater for female-specific volatiles and methyl 6-methylsalicylate than for other isomers (t=3.76, df=73, P<0.001; untransformed means: 20.27±8.01 s vs. 4.07±0.71 s, respectively). The last comparison was group 2 and the solvent control, which showed that males do not arrest significantly to the methyl 3, methyl 4, and methyl 5 isomers (t=0.87, df=58, P=0.39). Although males did not significantly arrest unless methyl 6-methylsalicylate was present, occasional courtship-fanning to methyl 6-methylsalicylate and other isomers was seen.

Table 3 Arrestment times of *Spalangia endius* males and number of males that fanned to isomers of methyl methylsalicylate, as well as to solvent and to volatiles of females[a]

Isomer or control	Arrestment time	# Males that fanned
Solvent[b]	0.61±0.10 (0–1.36)	0
3	0.44±0.11 (0–1.18)	1
4	0.47±0.12 (0–1.23)	3
5	0.60±0.11 (0–1.28)	2
6	0.95±0.12 (0–1.84)	5
Volatiles	0.93±0.17 (0–2.38)	1

[a] Table entries are log-transformed mean±s.e. (minimum–maximum), in log sec, N=15 per treatment

[b] Dichloromethane

Discussion

The study showed that female *S. endius* emitted two female-specific compounds, methyl 6-methylsalicylate and an unidentified second compound. The analyses of the extracts and the male behavioral results are consistent with the idea that methyl 6-methylsalicylate functions as a female-emitted pheromone component. Both female-specific compounds were biologically active when analyzed by GC-EAD. However, males arrested only to the silica fraction containing methyl 6-methylsalicylate and to the same degree as they arrested to the complete natural blend. In other words, the silica fraction with the unidentified material did not arrest males and did not increase arrestment to the silica fraction containing methyl 6-methylsalicylate.

The exact roles of methyl 6-methylsalicylate and the unidentified compound in intersexual interactions in *S. endius* remain to be determined. This study evaluated only male arrestment responses; it is possible that these two female-specific compounds, individually or as a blend, have long-range behavioral effects. Methyl 6-methylsalicylate might be described as a sex pheromone component because males fanned to the compound periodically, and fanning is seen only during male attraction to females (King et al., 2005; King, 2006). However, why only some males fanned is unclear. Given that males arrested to it, the compound might also be important in eliciting or maintaining the courtship

that males perform once mounted, as an attraction pheromone, or both.

Methyl 6-methylsalicylate has been identified before in some ants (e.g., Torres et al., 2001; Schönrogge et al., 2008) and some beetles (Moore and Brown, 1979; Gnanasunderam et al., 1984). It has never been identified before in the Pteromalidae or from any other wasps or bees. Although methyl 6-methylsalicylate has been identified from more than 20 species of ants, its function is known for only a fraction of them. It acts as a sex pheromone in *Polyergus breviceps* and *P. rufescens* (Greenberg et al., 2004; Castracani et al., 2005); a trail pheromone in *Tetramorium impurum* and *Mayriella overbecki* (Morgan and Ollett, 1987; Kohl et al., 2000); and as an alarm pheromone in *Gnamptogenys pleurodon* (Duffield and Blum, 1975). Sometimes methyl 6-methylsalicylate is mixed with other compounds to elicit a response, such as in *P. breviceps* and *P. rufescens* (Greenberg et al., 2004; Castracani et al., 2008); or it is the only compound needed to elicit a sexual response, such as in *P. rufescens* (Castracani et al., 2005). In *Camponotus* spp., *Bothroponera soror*, *P. breviceps, P. rufescens,* and *G. pleurodon*, methyl 6-methysalicylate comes from the mandibles (Brand et al., 1973; Longhurst et al., 1980; Blum et al., 1987; Greenberg et al., 2004; Castracani et al., 2005). In *T. impurum* and *M. overbecki*, methyl 6-methylsalicylate is found in the poison gland (Morgan and Ollett, 1987; Kohl et al., 2000). The location of methyl 6-methylsalicylate in *S. endius* currently is unknown, though preliminary experiments suggest it may be distributed over the entire cuticle.

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that approach dispensers. We recently have determined that the behavioral mechanism responsible for disruption of peachtree borer, *Synanthedon exitiosa* Say (Lepidoptera: Sesiidae) in orchards treated with polyethylene tube dispensers is competitive attraction (Teixeira et al., 2010). Here, our objective was to characterize the timing and duration of peachtree borer approaches to dispensers placed in orchards singly or at high density.

Another non-competitive behavioral mechanism proposed for mating disruption is the advancement of the male's diel rhythm of response, which causes the temporal decoupling of male and female flight (Cardé and Minks, 1995). According to this hypothesis, males perceive the pheromone permeated in a crop area, and begin flying much earlier than when females initiate calling, thereby decreasing male response when females release pheromone. There are no definitive tests that substantiate the importance of this mechanism, perhaps because in disrupted areas it is necessary to monitor a relatively large number of dispensers to obtain a sufficient number of data points for a robust analysis (Cardé et al., 1998). The use of field-deployed digital cameras and digital video recorders (DVR) to continuously monitor moths approaching dispensers can circumvent this difficulty, as multiple dispensers can be monitored simultaneously, and video can be examined later at a convenient timing. In this study, we used cameras and DVRs to record approaches by peachtree borer to a polyethylene tube dispenser placed singly in an orchard, and to 12 dispensers deployed in another orchard as part of a mating disruption trial.

The Sesiidae are unusual among moth families that use long-range sex pheromones in that they mimic Hymenoptera, both in appearance and flight behavior, and are primarily diurnal. Diel activity periods preferred by most species are the morning hours or late afternoon, usually not the hottest midday hours. (Eichlin and Duckworth, 1988). Peachtree borer originally infested several species of wild *Prunus* native in the eastern and central U.S., but became a pest of peaches, sweet cherries, and tart cherries when these were introduced into its range (Snow, 1990). Peachtree borer larvae feed on the phloem and vascular cambium of the lower trunk and roots. Along with stressing the tree and providing entryways for microbial diseases, borer feeding may girdle and kill young trees. Peachtree borer has only one generation per year, and the adult flight lasts most of the summer, peaking in August in Michigan. Volatiles emanating from the gum of infested trees, conspecific cocoons, and frass stimulate oviposition by female peachtree borer (Gentry and Wells, 1982; Derksen et al., 2007). The long seasonal period of activity and the sheltered feeding habit of borer larvae make these pests difficult to control with conventional insecticides.

Since the identification of (*Z,Z*)-3,13-octadecadienyl acetate as the pheromone of peachtree borer (Tumlinson et al., 1974), a number of studies have documented successful disruption of peachtree borer orientation to pheromone-baited traps, as well as reduction in pest density, through the use of synthetic sources of pheromone (McLaughlin et al., 1976; Snow, 1990). However, mating disruption to control peachtree borer has not been adopted widely, in part, because of pheromone cost and the labor involved in deploying dispensers. Characterization of moth interactions with synthetic pheromone dispensers may help maximize the efficacy of disruption and decrease costs. It also may help guide the development of other pheromone-based means of control, such as mass trapping and attract-and-kill.

Methods and Materials

Field Plots Isomale-Dual™ pheromone dispensers (Shin-Etsu Chemical Co. Ltd., Tokyo, Japan) were placed on 12 May 2009 in peach orchards (*Prunus persica* (L.) Batsch) located in Coloma, southwest Michigan, as part of a mating disruption trial. This dispenser is registered for mating disruption of peachtree borer and lesser peachtree borer *S. pictipes* (Grote & Robinson), and contains 33.3 mg of (*Z,Z*) and of (*E,Z*)-3,13-octadecadienyl acetates. The dispensers were deployed at 371 per hectare (150 per acre) in a 1.6 ha (4.0 ac) plot. On 11 July, one dispenser was removed from this plot and placed singly near the center of another 0.9 ha (2.3 ac) peach plot in the same farm located ~250 m west of the original plot. A standard red rubber septum lure (Trécé, Adair, OK, USA) containing a proprietary blend of the sex pheromone that consisted mostly of *(Z,Z)*-3,13-octadecadienyl acetate also was placed in the plot. Both orchards were planted with a mixture of peach cultivars approximately 10 years old, and known to be heavily infested with peachtree borer. Standard insecticide and fungicide sprays were conducted by the grower. No insecticide treatments were specifically directed toward peachtree borer.

Video Recordings Video recordings were conducted using a field-deployed DVR (model QH25DVR, QSee, Anaheim, CA, USA) powered by a 12 V Deka marine-type battery (DC31DT, Lyon Station, PA, USA). Power supply to the DVR was controlled by a 12 V DC timer. One DVR and two digital cameras (model QOCDC, QSee, Anaheim, CA, USA) were used in the orchard with the single dispenser and the lure. In the other orchard where dispensers had previously been deployed, 12 dispensers were monitored using three DVRs and four sets of cameras per DVR. The recording arena consisted of a 60×90 cm white corrugated plastic platform that provided a high-contrast background (Fig. 1). Dispensers and lures were placed hanging from a 15 cm wire placed on the center of the platform. The platform was located on the tree row, 120 cm from the ground. The single dispenser and the lure were placed 32 m

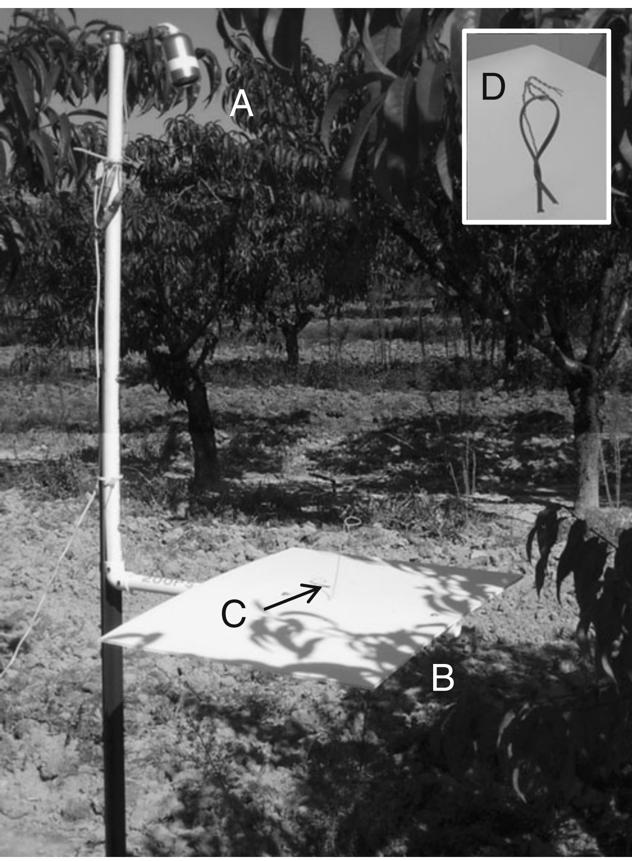

Fig. 1 Peachtree borer approach recording setup. **a** Camera mounted on a PVC pole attached to a metal pole. **b** Corrugated plastic platform. **c** Location of wire stand to hang the dispenser. **d** Detail of wire stand and dispenser

apart in the same row. In the other orchard, the platforms for each four camera set were 10 m apart. The dispenser in the platform was taken from the nearest tree so that the dispenser density in the area remained constant. Dispensers had been placed in 3 out of every four peach trees in the plot. The three sets of four cameras were placed in a transect across the plot, each set in the same row so that camera cables did not interfere with the passage of farm machinery between the rows. Recordings started on 11 July and ended on 18 August, and consisted of four 5 d to 6 d consecutive periods separated by 2 d to 3 d when the batteries were recharged. Recordings were made from 10:00 to 16:00 h. The same dispensers and lure were used throughout the study.

Video Analysis Video recordings were transferred from the DVR to a computer for visual evaluation. Preliminary observation of a sample of video records indicated that most approaches to the dispensers occurred in August, coinciding with the peak flight of peachtree borer in the area. The recording period from 13 to 18 August yielded the high number of approaches necessary for a robust statistical analysis. Therefore, we decided to use this period for detailed analysis of moth approaches to the dispensers. Focusing on a single week of data also allowed us to avoid variability due to changing environmental conditions over extended periods of time. Detailed scoring consisted of recording the time when a dispenser or lure was approached by a moth. An approach was defined as a period of time when a moth was observed casting while moving towards the dispenser, or remaining in sustained flight facing the dispenser. Events where moths were seen flying by but did not interact with the dispenser were not considered approaches. In the rare occasions when more than one moth was present, individual moths were tracked separately. Lesser peachtree borer and several other sesiid moths that approached the dispensers were excluded. The same observer scored all the approaches. The timing of approaches was determined based on the time stamp of the video recordings. When moths approached dispensers for less than 1 sec, the approach time was considered to be 1 sec. The duration of longer approaches was rounded up to the nearest second. The average approach timing (time of end plus time of beginning)/2 was calculated for each approach.

Statistical Analyses The average and standard deviation of approach timings was calculated for each observation date, and with data pooled by single dispenser, high-density dispensers, or lure treatment. The residuals of analysis of variance conducted on approach timing data were not normally distributed. Therefore, the non-parametric Kruskal-Wallis test as implemented in PROC NPAR1WAY of SAS (SAS Institute 2001) was used to determine differences in approach timing among treatments. Differences among treatments were determined by date, with daily approach timings, and with approach data over the entire period pooled by treatment. Approach timing data also were compared pooled by date to determine differences among dates. A frequency histogram was constructed using mean approach timing data pooled by treatment with bins consisting of 15 min time intervals during the diel flight period.

A frequency histogram of approach durations for each treatment, with bins consisting of 1 sec duration intervals, showed differences in the shape of the underlying frequency distributions. These differences strongly biased statistical tests towards the occurrence of significant differences among treatments. To avoid this bias, we characterized differences among treatments using the frequency histogram. In addition, we used PROC UNIVARIATE of SAS to calculate the median and interquartile range (IQR) for daily approach durations by treatment, and approach durations pooled by treatment over the entire period.

Trends in approach duration along the diel flight period were investigated using regression analysis by date and treatment, and with data pooled by date and by treatment, using PROC REG of SAS.

Results

Recordings during the period from 13 to 18 August were scored in detail, with the exception of 17 August when rainfall during the flight period almost completely stopped moths from approaching pheromone sources. The total number of moth approaches recorded during the 5-day period were 226, 175, and 292 to single dispenser, high-density dispensers, and lure, respectively. One of the cameras trained on a dispenser placed at high density malfunctioned during the whole period, and so the dataset corresponds to 11 dispensers only.

Approach Timing The mean approach timing (h:min:sec±SD) of peachtree borer to single dispenser, high-density dispensers, and lure was 11:33:12 ± 00:46:43, 11:43:52 ±00:45:58, and 11:41:21 ± 00:45:54 AM (Table 1). There were significant differences among approach timings to single dispenser, high-density dispenser, and lure when data were pooled by treatment (χ^2=8.7; *df*=2, *P*=0.01) and by date (χ^2=150.1; *df*=4, *P*<0.001). There were significant differences among treatments with daily data on 16 and 18 August (χ^2=6.1; *df*=2, *P*=0.05 and χ^2=7.9; *df*=2, *P*=0.01, respectively), but not on 13, 14, and 15 August (χ^2=0.8; *df*=2, *P*=0.64, χ^2=0.4; *df*=2, *P*=0.82, and χ^2=0.69; *df*=2, *P*=0.71, respectively). On 16 August, the earliest approach timing was recorded with the single dispenser, while on 18 August the earliest approaches were with the lure. The frequency histogram of approach timings recorded during the study period (Fig. 2) shows that approaches started between 10:15 and 10:30 AM and ended between 14:30 and 14:45. With all treatments, the highest percentage of approaches was recorded between 11:30 and 11:45 AM.

Approach Duration The frequency histogram of approach durations over the 5 days of this study showed distinct distributions among treatments (Fig. 3). Approaches to dispensers placed at high density were shorter than with the single dispenser or the lure, with over 50% of the approaches lasting less than 1 sec. In addition, few long approaches were recorded with dispensers placed at high density. The distribution of approaches to the single dispenser and lure were similar but a higher frequency of longer approaches was recorded with the lure. The relative rank of median approach durations was constant when analyzed per day (Table 2) with shorter approaches to dispensers placed at high density, then intermediate approach durations to the single dispenser, and the longest approaches recorded with the lure. With approach data pooled by treatment, the median was 1 (less than 1 sec) and IQR was 1 to 2 with dispensers placed at high density, indicating that 75% of approaches lasted less than 2 sec. With the single dispenser, the median was 3 sec and IQR was 2–4 sec, while with the lure the median was 4 sec and IQR was 2–5 sec.

There were no significant trends in approach duration vs. time of day with data pooled by treatment or date, or when data were analyzed separately by treatment and date (linear regression, *P*>0.05).

Discussion

Peachtree borer males in an orchard treated with a high density of pheromone dispensers for mating disruption did not approach the dispensers earlier than males in an orchard with a single dispenser. Therefore, this study does not support the hypothesis that one of the mechanisms of mating disruption is the advancement of male flight and the resulting loss of synchrony of male and female diel flight period caused by ambient permeation with artificial pheromone. Although there were statistical differences among the periods of approach to the single dispenser, high-density dispensers, or the lure when the 5 day data were pooled, treatments were not significantly different in three of the 5 days. On the dates when there were statistical differences among treatments, the relative timing of the approach period among treatments was not consistent. Considering that there was day-to-day variation in the relative timing of approach, and that differences among pooled treatment averages (~10 min) were much smaller than the standard deviation of treatment means (~45 min),

Table 1 Time of male peachtree borer approaches to single dispenser, high-density dispensers, and single lure placed in peach orchards

	Single dispenser		High-density dispensers		Lure	
	N	Mean±SD	*N*	Mean±SD	*N*	Mean±SD
13 Aug	31	12:16:42 ± 00:59:57	38	12:02:42 ± 00:38:41	49	12:09:29 ± 00:35:17
14 Aug	45	11:38:45 ± 00:30:41	40	11:40:48 ± 00:49:40	55	11:41:31 ± 00:43:14
15 Aug	50	11:30:30 ± 00:29:40	44	11:34:16 ± 00:29:33	89	11:40:11 ± 00:48:04
16 Aug[1]	75	11:00:14 ± 00:29:05	27	11:11:20 ± 00:37:53	62	11:32:06 ± 00:49:12
18 Aug[2]	25	12:13:37 ± 00:47:13	26	12:11:08 ± 00:54:54	37	11:41:31 ± 00:32:22
All dates[3]	226	11:33:12 ± 00:46:43	175	11:43:52 ± 00:45:58	292	11:41:21 ± 00:45:54

[1] Kruskal-Wallis χ^2 =6.1; *df*=2, *P*=0.05

[2] Kruskal-Wallis χ^2 =7.9; *df*=2, *P*=0.01

[3] Kruskal-Wallis χ^2 =8.7; *df*=2, *P*=0.01

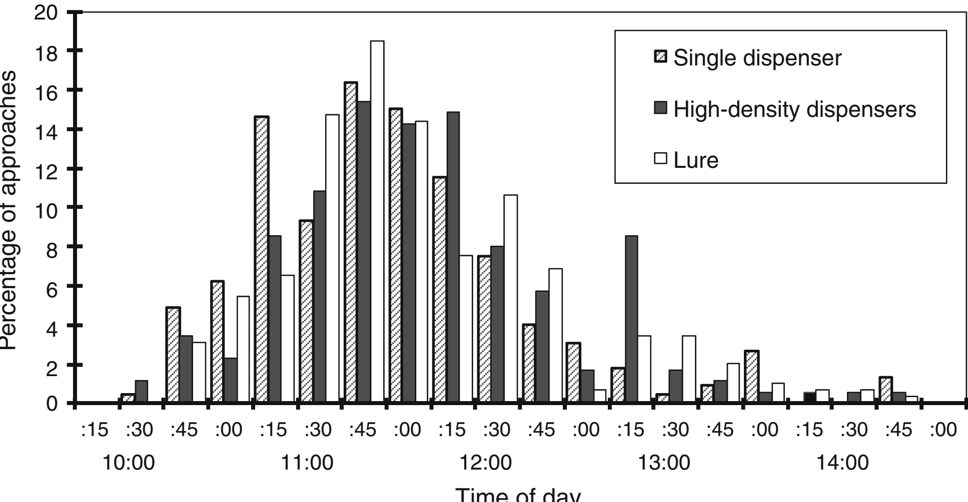

Fig. 2 Frequency histogram of the timing of male peachtree borer approaches to single dispenser, high-density dispensers, and single lure, at 15 min intervals during the diel flight period in peach orchards

we do not attribute biological significance to the differences among treatments. Video recordings suggest that day-to-day variation in the approach period was related to environmental conditions like cloudiness and wind speed. Peachtree borer approached artificial sources of pheromone mostly between 11:00 and 12:30 AM EST in Michigan, in mid August, which is similar to 11:00–12:00 AM CST determined by Greenfield and Karandinos (1979) in southwest Wisconsin, in late July.

In contrast to the timing of the approaches, placement of the dispensers singly or at high density had a consistent effect on the duration of the approaches. Most approaches to dispensers placed at high density lasted less than 1 sec, while approaches to single dispenser were longer, and approaches to the lure were the longest. This relative rank of approach duration was consistent on all days of the study. Therefore, we conclude that dispenser density affects the duration of male peachtree borer approaches to the dispensers. The release rate and/or pheromone blend composition also may affect the duration of the approach, as suggested by the difference between the single dispenser and lure. Peachtree borer males initiate mating while hovering near the female, and do not alight until coupling with the female by using their genital claspers (Slingerland, 1899; Barry and Nielsen, 1984). Possibly as a consequence of this specific behavior, most approaches to the dispensers or lure were relatively short. Nevertheless, several males spent a relatively long period of time hovering near the dispenser or lure, and some attempted mating by striking them with the tips of their abdomens. The frequency distribution of approaches to dispensers by three tortricid species observed by Stelinski et al. (2004) in non-disrupted plots was similar to that of peachtree borer, although approaches tended to be longer: most obliquebanded leafroller *Choristoneura rosaceana* (Walker) remained near the dispenser for less than 10 sec, and the majority of Oriental fruit moth *Grapholita molesta* (Busck) and redbanded leafroller *Argyrotaenia velutinana* (Walker) remained for less than 20 sec.

We hypothesize that differences in the duration of approaches to dispensers singly or at high density are related

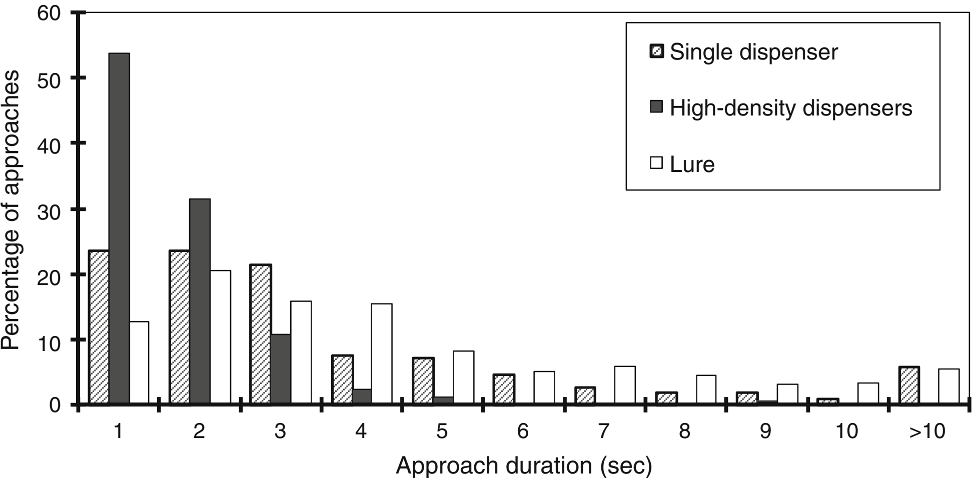

Fig. 3 Frequency histogram of the duration of male peachtree borer approaches to single dispenser, high-density dispensers, and single lure, at 1 sec approach duration intervals

Table 2 Duration of male peachtree borer approaches to single dispenser, high-density dispensers, and single lure placed in peach orchards

	Single dispenser		High-density dispensers		Lure	
	N	Median (IQR) sec	*N*	Median (IQR) sec	*N*	Median (IQR) sec
13 Aug	31	3 (2–5)	38	1 (1–2)	49	4 (2–6)
14 Aug	45	3 (2–6)	40	1 (1–2)	55	3 (2–6)
15 Aug	50	2 (1–4)	44	1 (1–2)	89	4 (2–7)
16 Aug	75	2 (1–3)	27	1 (1–2)	62	3 (2–4)
18 Aug	25	3 (2–6)	26	2 (1–2)	37	4 (3–7)
All dates	226	3 (2–4)	175	1 (1–2)	292	4 (2–6)

to competition among dispensers, or to changes in the behavior of moths exposed to dispensers at high density. Regarding the first hypothesis, we previously have determined that disruption of peachtree borer is mediated by competitive attraction (Teixeira et al., 2010). In the competitive-attraction equation (Miller et al., 2010), which relates male catch in a monitoring trap to the presence of competing pheromone sources like dispensers and females, the *findability* terms express the attraction potential of the different pheromone sources, measured as the proportion of moths that find an attractant source. The *retentiveness* terms measure the proportion of time that moths spend approaching a pheromone source and recovering the ability to follow another plume afterwards, in relation to the moth active sexual life time. The *retentiveness* term is deemed to be constant across dispenser densities, but our direct measurements show that, at least near the dispenser, the approach duration is not constant. It may be that competition among dispensers is expressed not only by the proportion of moths that find one attractant, but also by how much time moths spend interacting with each dispenser at a given density. Observations of moths suggest that competition among dispensers may be expressed behaviorally, as when moths find one dispenser but immediately shift to following a pheromone plume emanating from a nearby dispenser, which is more likely to happen as dispenser density increases.

Regarding the hypothesis that moth behavior changes as a result of exposure to dispensers at high density, shorter approach duration may be a symptom of the behavioral mechanism of disruption that changes from competitive-attraction to a non-competitive mechanism as dispenser density increases. When disruption is caused by non-competitive mechanisms, such as camouflage, sensory inhibition away from the dispenser, or sensory imbalance, moths do not approach the dispensers. It is possible that there is a continuum from competitive attraction to a non-competitive behavioral mechanism with increasing dispenser density. For example, male codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) responded to wax droplets in a manner consistent with competitive attraction at low density, but appeared to have become desensitized when wax droplet density was increased greatly (Miller et al., 2006b). The high density that we used, 371 dispensers per hectare, is much lower than that used with codling moth, but peachtree borer is extremely responsive to its pheromone (Tumlinson et al., 1974).

It is also possible that after multiple approaches to a dispenser, moths associate short-range visual or other cues from the dispenser with reduced probability of mating, and consequently reduce their effort near a dispenser. In a plot treated with high dispenser density, it is likely that a moth interacts multiple times with dispensers in the same day. Behavioral habituation, as opposed to physiological consequences of exposure to pheromone, may explain why approaches to dispensers at high density are shorter but moths still approach the dispensers.

With respect to the consequences for mating disruption of peachtree borer, shorter approach time with high dispenser density indicates decreased dispenser retentiveness. We measured only the duration of approaches near the dispenser, not the duration of the whole period when a moth is following the plume, or the recovery period following the approach. In addition, the overall impact of changes in retention time depends on the number of approaches to the dispensers that each moth makes, which is currently unknown. However, if moth inactivation from exposure to a high dose of pheromone near the dispenser is dependent on the duration of the exposure, then a small reduction in the duration of the approach may have a disproportionate impact on retention time because moths will recover faster. Our results suggest that there is a decrease in the disruptive activity of each dispenser with increasing dispenser density which, so long as the underlying moth behavioral mechanism remains competitive attraction, will lead to a higher number of moths caught in monitoring traps than expected as a function of dispenser density. Another and potentially more important consequence for mating disruption is that approaches to lures in monitoring traps placed in orchards with high dispenser density may also be shorter than when moths are placed in non-disrupted orchards. As a consequence, trap catch could be lower in disrupted blocks because of decreased trap efficiency and not because moths cannot find the trap. It

remains to be determined whether pheromone permeation affects the behavior of male moths that find females.

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Fig. 1 Approximate distribution of *Ostrinia nubilalis.* The European corn borer has been observed in most Eurasia as well as North Africa and North America. Because of a certain degree of confusion concerning the taxonomic status of *O. scapulalis* and *O. nubilalis* (Frolov et al., 2007) the Eastern limit of the distribution may be underestimated

clasper response (Klun, 1968). This observation provided a reliable bioassay for the presence of pheromone in extracts of female corn borers. The sex pheromone produced by female ECB was extracted for the first time in 1968 from 10,000 females (Klun, 1968). The active sex pheromone was isolated as a single component that could elicit male response. The male sex stimulant was identified as (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) (Klun and Brindley, 1970), a compound that since has been found to be part of the pheromone blend of more than 300 moth species (El-Sayed, 2010). Synthetic Z11-14:OAc was shown to possess biological properties identical to the natural pheromone in a close-range bioassay (Klun and Brindley, 1970). In the first field trials, the presence of the geometrical isomer (*E*)-11-tetradecenyl acetate (E11-14:OAc) was found to have a negative impact on trap catches (Klun and Robinson, 1971). However, contradictory results were reported from other field sites where males were specifically attracted to E11-14:OAc, Z11-14:OAc attracting comparatively few males in these locations (Roelofs et al., 1972). The hypothesis advanced then was that the distinct catches would correspond to different strains of corn borer that employed a different pheromone system (Roelofs et al., 1972). The pheromone polymorphism of the ECB had just been discovered.

When geometrically pure isomers could be synthesized and made available for field assays, previous conclusions regarding ECB pheromone communication could be revisited and modified. Male ECB were, indeed, weakly attracted to pure Z11-14:OAc (Klun et al., 1973). The addition of a small fraction of pure E11-14:OAc, however, dramatically increased trap captures, and the requirement of the E isomer for optimum attraction became obvious from field tests (Klun et al., 1973). Ultimately, the composition of female abdominal tips was scrutinized to identify pheromone components of the two behavioral strains, and females were found to produce either 97:3 Z/E11-14:OAc or 1:99 Z/E11-14:OAc (Kochansky et al., 1975). Tetradecyl acetate (14:OAc) also was found in gland extracts but had no effect on attraction (Kochansky et al., 1975). Similarly, trace amounts of (*Z*)-11-hexadecenyl acetate (Z11-16:OAc) were detected in extracts from E-strain females, but this compound was without noticeable behavioral effects (Peña et al., 1988).

Reproductive Isolation Between the Strains

It was shown through analyses of the sex pheromone of female moths that two strains of corn borer exist. The occurrence in nature of Z and E pheromonal phenotypes of the ECB was established through an extensive survey conducted in the mid-1970’s. In this survey, males at different geographic sites were found to respond to opposite isomeric blends of 11-14:OAc (Klun and Cooperators, 1975). Studies also revealed that the most prevalent ECB pheromonal phenotype in nature is the 97:3 Z/E11-14:OAc; the E strain having a more restricted distribution in Europe and North America probably because it may lack certain adaptive features possessed by Z strain individuals (Klun and Cooperators, 1975; Anglade and Stockel, 1984). This would explain the limited part of North America that was effectively colonized by the E strain, essentially close to the sites of introduction. Apart from the pheromone, there seem

to be no fixed differences between types, nor are there any morphological characters that permit differentiation.

In regions where sympatric populations of the Z and E strains were detected, males responding to a 1:1 blend occasionally were captured, and it was suggested that these males could be hybrid individuals produced from crosses between the two pheromonal types (Klun and Cooperators, 1975; Cardé et al., 1978). An alternative explanation may lie in a broader window of response in certain populations caused by genetic variability within these populations; these males would be a mixture of individuals that respond predominantly to Z or E blend (Klun and Cooperators, 1975; Cardé et al., 1978). Nevertheless, viable hybrids can be obtained under laboratory conditions (Liebherr and Roelofs, 1975), and interbreeding of opposite pheromonal types is not unlikely because aggregation of males and females of both strains occurs in similar grassy habitats (Showers et al., 1976; Dalecky et al., 2006). The inter-strain crosses, when successfully completed, give progeny showing heterosis, with the portion of individuals surviving each life stage being significantly higher (Liebherr and Roelofs, 1975). Similarly, F2 sibling crosses show 100% viable matings, and females do not exhibit a reduced fecundity (Liebherr and Roelofs, 1975). However, the hybrid crosses that occur under confined conditions are much less frequent than intra-strain crosses, and laboratory choice tests suggest that a degree of premating reproductive isolation exists between the two strains (Liebherr and Roelofs, 1975). A plausible explanation would be that a large proportion of the males are inhibited from attempting mating by the detection of the incorrect sex pheromone isomer ratio (Liebherr and Roelofs, 1975). Since an increase in the amount of the respective minor component eliminates male attraction, it was hypothesized that this presumably would cause reproductive isolation in the field and, therefore, support separate species status for the two strains (Kochansky et al., 1975).

Because of the relative rarity of inter-strain matings under laboratory conditions, it might be expected that such matings would be even rarer in nature. In addition, the few hybrids produced may not be able to mate at all (Liebherr and Roelofs, 1975). Analysis of the Δ11-tetradecenyl acetate isomeric composition of wild female moths indicated that sympatric pheromonal types mate assortatively with only a low level of hybridization detected (Klun and Maini, 1979; Roelofs et al., 1985; Klun and Huettel, 1988). Evidence that the Z and E pheromone strains of *O. nubilalis* are not freely interbreeding in areas of coexistence first came from allozyme data (Cardé et al., 1978). Although small, the genetic divergence detected between the Z and E strains is suggestive of ongoing speciation, and justifies that the strains could be considered as semispecies (populations that have acquired some attributes of species rank for which the speciation process is partially complete) or sibling species (Cardé et al., 1978). This was confirmed by a subsequent study that involved 30 loci corresponding to regulatory and non-regulatory enzymes in which the values of genetic distance obtained were indicative of taxa at the beginning of divergence (Cianchi et al., 1980). Areas in North America are secondary contact zones of recent origin where introgression could occur, though at a low level (Cardé et al., 1978).

The behavioral response of hybrid males was characterized to define precisely the response profile of hybrid males, which is crucial to understanding the interaction between sympatric Z and E individuals. Glover et al. (1991) reported that many hybrid males from reciprocal crosses failed to complete the behavioral sequence to locate a female, regardless of the blend they were exposed to. In addition, the few males that responded did not exhibit a preference for any particular blend but, rather, were attracted to a broad range of blends (Glover et al., 1991). Given that most hybrid males do not respond to any blend, and that the remaining ones are not tuned to any particular blend, F1 hybridization should be seen as a transient stage with a limited possibility of creating recurrent gene flow between the strains. However, the actual fate of hybrid individuals in the field remains obscure.

Pheromone Biosynthesis and Its Regulation

The pheromone components, E11-14:OAc and Z11-14:OAc, are produced through the fatty acid cycle. Palmitic acid is shortened to a 14-carbon intermediate through one cycle of β-oxidation. Desaturation then is accomplished by a Δ11-desaturase to give the (*E*)- and (*Z*)-11-tetradecenoyl precursors, which are subsequently reduced and acetylated to form the pheromone compounds (Roelofs et al., 1987) (Fig. 2). In both strains, the Δ11-desaturase produces the same ratio of the (*E*)- and (*Z*)-11-tetradecenoyl intermediates (Roelofs et al., 1987; Wolf and Roelofs, 1987). The enzyme has been characterized and expressed in a heterologous system, thus confirming its capacity to produce both geometric isomers of Δ11-tetradecenoyl from myristic acid (Roelofs et al., 2002). *In vivo* experiments that involve the use of labeled precursors have helped to determine that substrate selectivity occurs during the reduction step (formation of fatty alcohol from the acyl precursors); no selectivity of the acetylation step was noticed (Jurenka and Roelofs, 1989; Zhu et al., 1996b).

As in other moth species, the presence of lipid droplets in the pheromone gland cells has been reported in the ECB (Ma and Roelofs, 2002). These lipid droplets consist of triacylglycerols, the main pool of non-membrane lipids (Ma and Roelofs, 2002). Fatty acyl pheromone analogues, (*Z*)-

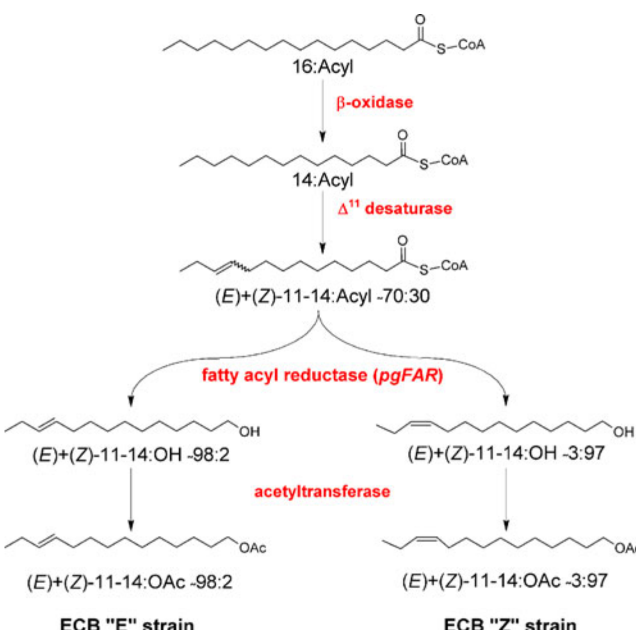
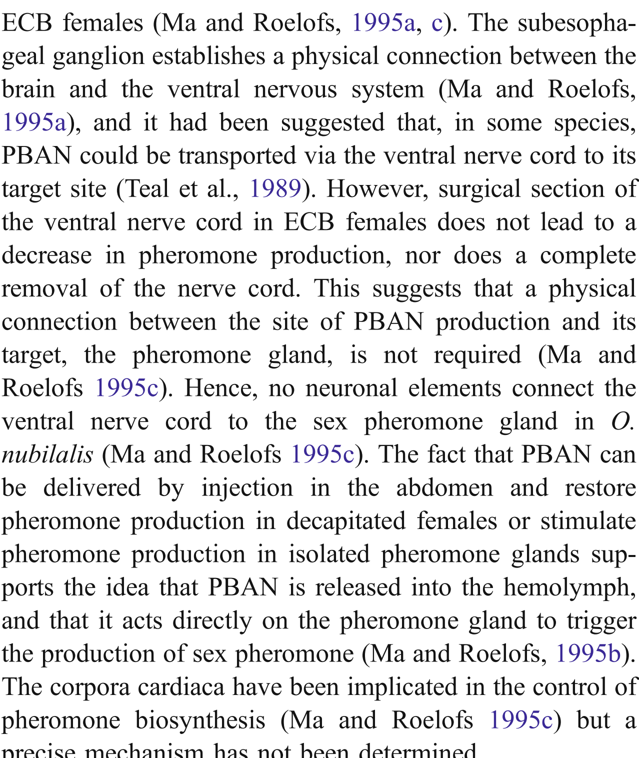


Fig. 2 Biosynthetic pathway towards the female *Ostrinia nubilalis* pheromone. *De novo* biosynthesis of (*Z*)- and (*E*)-11-tetradecenyl acetates starts from palmitoyl-CoA. One cycle of β-oxidation generates myristoyl-CoA, which is a substrate for a fatty acyl Δ11 desaturase. The (*E*)- and (*Z*)-11-tetradecenoyl moieties produced are converted into the corresponding alcohols by a fatty acyl reductase *pgFAR*. Finally, an acetyltransferase catalyzes the conversion of the fatty alcohol precursors into acetates

and (*E*)-11-tetradecenoate, are found predominantly in the triacylglycerols (Foster, 2004). The physiological role of these lipid deposits in the sex pheromone biosynthesis is two-fold. First, they seem to function as a sink for excess fatty acyl precursors, especially the direct precursor of the minor component (Foster, 2004). Second, they may be a source for the biosynthesis of pheromone, especially of tetradecanoate (14:Acyl), precluding the necessity for *de novo* biosynthesis of this acid during the period of pheromone production (Foster, 2004).

The sex pheromone production in female corn borers exhibits a cyclic pattern throughout the photoperiod, with a peak during the scotophase, and a valley with no pheromone during the photophase (Foster, 2004). The pattern changes slightly as females age, with the peak observed towards the end of the scotophase in young females, an apparent constant titer during the scotophase in 2 to 3-day-old females, and a peak in the early scotophase in older females (Foster, 2004; Kárpáti et al., 2007). This pattern apparently correlates with the mating activity as observed under laboratory conditions (Kárpáti et al., 2007). Pheromone biosynthesis is regulated by the neuropeptide pheromone biosynthesis activating neuropeptide (PBAN) (Raina and Klun, 1984; Raina et al., 1989; Ma and Roelofs, 1995b). PBAN is produced in three discrete sets of neurosecretory cells in the subesophageal ganglion of adult


ECB females (Ma and Roelofs, 1995a, c). The subesophageal ganglion establishes a physical connection between the brain and the ventral nervous system (Ma and Roelofs, 1995a), and it had been suggested that, in some species, PBAN could be transported via the ventral nerve cord to its target site (Teal et al., 1989). However, surgical section of the ventral nerve cord in ECB females does not lead to a decrease in pheromone production, nor does a complete removal of the nerve cord. This suggests that a physical connection between the site of PBAN production and its target, the pheromone gland, is not required (Ma and Roelofs 1995c). Hence, no neuronal elements connect the ventral nerve cord to the sex pheromone gland in *O. nubilalis* (Ma and Roelofs 1995c). The fact that PBAN can be delivered by injection in the abdomen and restore pheromone production in decapitated females or stimulate pheromone production in isolated pheromone glands supports the idea that PBAN is released into the hemolymph, and that it acts directly on the pheromone gland to trigger the production of sex pheromone (Ma and Roelofs, 1995b). The corpora cardiaca have been implicated in the control of pheromone biosynthesis (Ma and Roelofs 1995c) but a precise mechanism has not been determined.

PBAN interacts with a G protein-coupled receptor (Choi et al., 2003) and promotes the opening of ion channels leading to an influx of extracellular calcium ion into the ECB pheromone gland cells (Ma and Roelofs, 1995b). The increase of cytosolic calcium leads to the activation of secondary messengers such as cAMP that in turn activate kinases and/or phosphatases, which stimulate the pheromone biosynthesis pathway (Rafaeli, 2009). From investigations conducted in different moth species, it has been shown that various enzymatic steps are under the influence of PBAN, from the control of fatty acid synthesis to the metabolism of fatty acid precursors (Tillman et al., 1999). In *O. nubilalis*, PBAN affects pheromone biosynthesis during the reduction step, that is the conversion of (*Z*)- and (*E*)-11-tetadecenoyl precursors into the corresponding alcohols (Eltahlawy et al., 2007).

Genetic Basis of Pheromone Polymorphism

The finding that viable hybrids could be obtained when crossing the two pheromone strains has made possible a full series of investigations aimed at unraveling the genetic basis of polymorphism in the corn borer communication system, notably the genetics that regulates the geometric composition of the sex pheromone.

Hybrid females from reciprocal crosses have an isomeric complement that approximates 65:35 E/Z, which is intermediate from that of either of the parent strains (Klun and Maini, 1979). The difference in female pheromone production is

controlled primarily by one autosomal locus with two alleles under Mendelian inheritance (Klun and Maini, 1979; Roelofs et al., 1987). Recently, a genetic map based on crosses between the two strains confirmed that the pheromone production trait is encoded by a single autosomal locus (Dopman et al., 2004).

The E/Z ratio produced by females from two successive backcrosses is variable to a higher degree than predicted (Löfstedt et al., 1989). This additional variation in the blend of pheromone components could not be explained directly under the basic "1-locus-2-alleles" model and it was, therefore, considered that independently segregating modifier genes that affect the exact ratio produced by heterozygous females could be present in some populations (Löfstedt et al., 1989; Zhu et al., 1996a). It has been proposed that the basic model can be extended based on the idea that there are two variant alleles in the Z strain for the major pheromone production locus, while the E strain carries one allele at this locus (Zhu et al., 1996a). The genetic variation noticed in the Z strain could result from another locus than the major production locus itself, although the data available so far suggest that, if it exists, this locus would be in linkage disequilibrium with the major production locus (Zhu et al., 1996a).

The autosomal gene in question has been proposed to encode for a factor that affects the specificity of the final reduction step, because a specific change in this critical step would produce various pheromone blends of acetate components (Roelofs et al., 1987; Zhu et al., 1996a, b). Indeed, the last reduction step is certainly decisive for the determination of the final pheromone ratio since both parental strains, and F1 females possess a similar ratio of geometric isomers of the unsaturated 14-carbon intermediates (Roelofs et al., 1987). *In vivo* labeling experiments were used to demonstrate that the reductase system differs between the two strains (Zhu et al., 1996b). Recent molecular investigations have confirmed the existence of two fatty acyl reductase alleles with strain-specific substrate specificities (Lassance et al., 2010). A genetic mapping approach was used to confirm the link between the genotype at the fatty acyl reductase *pgFAR* locus and the phenotype for pheromone production (Lassance et al., 2010). The variation unraveled by Zhu et al. (1996a) can be interpreted as the consequence of polymorphism existing within each strain at the *pgFAR* locus. Future experiments should aim at determining the level of variation existing for the *pgFAR* gene in natural populations of ECB.

Location of Production Site

In the adult female *O. nubilalis*, nine abdominal segments can be distinguished; seven of them are covered with scales, whereas the remaining terminal segments form the ovipositor that normally remains telescoped within the preceding segments (Ma and Roelofs, 2002). When describing the isolation of the sex pheromone, Klun (1968) reported that it could be isolated from the fused 9th and 10th abdominal segments. Furthermore, the sex pheromone was localized on the surface of the ovipositor, which is characteristic of many moth species (Klun and Maini, 1979). Details on the ECB sex pheromone gland ultrastructure revealed that the gland is formed by a single layer of hypertrophied epidermal cells that are located in the dorsal fraction of the intersegmental membrane between the 8th and 9th/10th segments, thus forming a half-ring gland (Ma and Roelofs, 2002).

The cells that form the gland have some characteristic features such as the presence of numerous mitochondria, the presence of lipid droplets both in the cells and the overlying cuticle, apical plasma membrane foldings, and smooth endoplasmic reticulum (Ma and Roelofs, 2002). Gas chromatographic analysis has confirmed the presence of both the pheromone components and their immediate fatty acyl precursors in the dorsal intersegmental membrane (Ma and Roelofs, 2002). *In situ* hybridizations using the Δ11-desaturase as probe were performed in the sister species *O. scapulalis*, and revealed that the enzyme producing the fatty acyl precursors is expressed only in the dorsal part of the terminal abdominal segments. The molecular observations thus corroborate the biochemical results (Fukuzawa et al., 2006). Similar results are to be expected for *O. nubilalis.*

Male Behavioral Response to the Pheromone

In nature, the females release sex pheromone that attracts potential mates from a distance. The detection of pheromone by males results in a well-defined stereotypic behavioral sequence composed of successive steps: orientation towards the pheromone source, upwind flight, and location of the odor source (Glover et al., 1987). In the close vicinity of the pheromone source, males of *O. nubilalis* respond by taking part in a characteristic precopulatory courtship display (Klun, 1968).

As mentioned earlier, the identification of the sex pheromone was tightly linked to the understanding of the associated male behavioral response. Precopulatory behavior was used in a close-range bioassay to demonstrate the activity of Z11-14:OAc in the Z strain (Klun, 1968). This behavioral response could be elicited in the complete absence of the minor component of the pheromone (Webster and Cardé, 1984), suggesting that this behavior does not require a complete pheromone blend to be elicited or that the close-range bioassay used did not allow for a

determination of the behavioral function of the minor component. However, the results of both field-trapping and flight tunnel studies have shown the importance of the natural pheromone blend compared to the use of the single major component for orientation, upwind flight, and source location (Klun et al., 1973; Klun and Cooperators, 1975; Glover et al., 1987). Indeed, when males were flown to a series of pheromone sources that varied in the percentage of the minor isomer, very few insects completed the sequence of behaviors when none of the minor component was applied (Glover et al., 1987; Linn et al., 1997). The flight tunnel assay is very discriminating because males must fly upwind to the chemical source for a reasonable distance (1 m to 2 m in general), and show close-range behaviors that include landing close to the source and displaying. This type of bioassay established that the males show limited to no attraction to the blend of the opposite strain (Glover et al., 1987, 1990) (Fig. 3).

In a close-range bioassay, Z, E, and F1 hybrid males responded more intensively to the isomeric blend that corresponds to sibling females (Klun and Maini, 1979). However, in each male type, some males also responded to other isomeric combinations, with E males being the least specific in their response preferences (Klun and Maini, 1979). Among field-captured individuals, some males showed a broad response window, and exhibited a response to isomer ratios well outside that produced by their respective females (Klun and Huettel, 1988). The behavioral response of males was evaluated in wind tunnel assays, and revealed the ability of males to discriminate blend quality during flight. Typically, ECB males display increased specificity with successive steps in the flight sequence (Linn et al., 1997). Males of the two strains, as well as their hybrids, exhibited different levels of specificity for ratios: Z males were quite discriminating with fewer insects performing a full behavioral sequence when exposed to blends other than the Z blend (Roelofs et al., 1987; Glover et al., 1990; Linn et al., 1997). On the other hand, E males had a broader window of response, with some rare males even being attracted to the blend produced by Z females, which may indicate that E males are less canalized in their behavioral response compared to Z males (Roelofs et al., 1987; Glover et al., 1990; Linn et al., 1997) (Fig. 3). The variability reported for some males may help explain how individuals belonging to different pheromonal types can closely interact, thus leading to interbreeding in the field. As a rule of thumb, the peaks of male response (corresponding to source contact in the flight tunnel) are centered on the natural female-produced ratio (Linn et al., 1997). Hybrid males exhibit high levels of upwind flight and source contact to a wide range of E:Z ratios (Fig. 4) indicating that, unlike their parents, they are not tuned to a specific ratio; they are, however, characterized by a relative lack of response to any dose of the 99:1 ratio typically attractive to E males (Roelofs et al., 1987; Linn et al., 1997). Interestingly, an appreciable number of hybrid males (10%) can exhibit source contact with sources mimicking a 98:2 ratio (Linn et al., 1997).

The survey conducted by Klun and his collaborators in the 1970's indicates that the sex-attraction response specificities of populations of males vary somewhat from generation to generation and from one geographic location to another (Klun and Cooperators, 1975). In addition to the established pheromone polymorphism, populations may differ in their voltinism, the number of generation per year, with univoltine and bivoltine populations having been reported (Roelofs et al., 1985). In the U. S., Z strain populations can be either uni- or bivoltine, whereas the E strain is bivoltine (Roelofs et al., 1985). The two Z populations have different sensitivities for responding to the ratio of the minor component, with bivoltine Z borers requiring less E isomer to complete the behavioral sequence (Glover et al., 1987). The temporal and spatial heterogeneity of male responses can be seen as an expression of intraspecific genetic variability within the populations and strains. However, because of the somewhat broad window of response exhibited by males, pheromone traps cannot be used to accurately estimate hybridization level or assign the

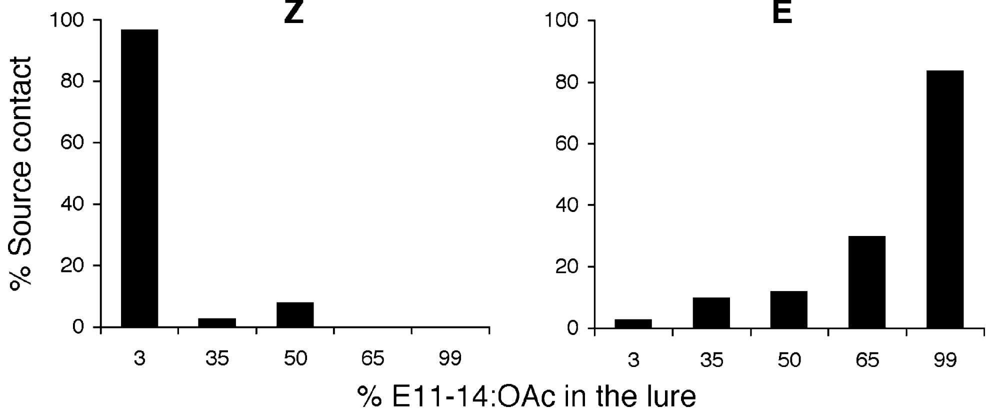


Fig. 3 Flight tunnel responses for pure *Ostrina nubilalis* pheromone races exposed to different blends of Δ11-14:OAc. Profiles represent the percentage of males that flew upwind and touched the pheromone source (adapted from Linn et al., 1997)

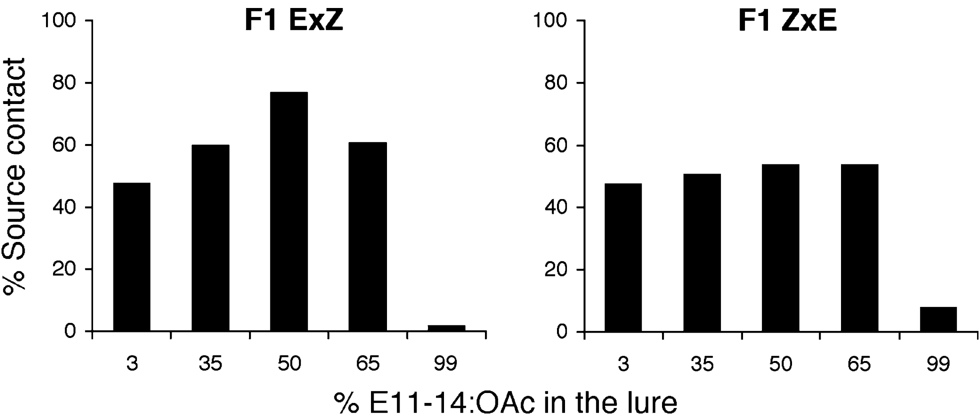


Fig. 4 Flight tunnel responses for male hybrids of *Ostrina nubilalis* pheromone races from reciprocal crosses exposed to different blends of Δ11-14:OAc. Profiles represent the percentage of males that flew upwind and touched the pheromone source (adapted from Linn et al., 1997)

pheromone-response genotype of captured males (Glover et al., 1990), and future surveys should include the use of appropriate molecular markers.

Behavioral Antagonists

Some compounds, when added to the sex pheromone blend can, dramatically affect its attractiveness. Such a molecule is said to be an antagonist. Here, I will not consider responses to chemical analogues of the sex pheromones, although studies devoted to explore this issue have been conducted; only naturally occurring compounds produced by species more or less related to the ECB are considered.

The isomer of the ECB pheromone components (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) is the most studied ECB behavioral antagonist. This compound is part of the pheromone used by two other extant species from the genus, namely *O. zealis* and *O. zaguliaevi* (Huang et al., 1998; Ishikawa et al., 1999a, b). Indeed, the ECB most likely evolved from an ancestral species that used a blend of Z9-14:OAc and Z/E11-14:OAc as pheromone components (Ishikawa et al., 1999b). Early data showed clearly that the addition of Z9-14:OAc reduced the attraction of ECB males to their sex pheromone blend (Klun and Robinson, 1972; Struble et al., 1987; Glover et al., 1989). Electrophysiological recordings suggested that males possess receptors that respond to Z9-14:OAc (Struble et al., 1987). It was first hypothesized that behavioral antagonists such as Z9-14:OAc would interact with the same chemoreception sites as the pheromone components (Nagai et al., 1977). However, single sensillum recordings demonstrated the existence of a specific detection channel in both E and Z strains for Z9-14:OAc (Hansson et al., 1987). The recent identification of an olfactory receptor from *O. scapulalis* that is primarily tuned to E11-14:OAc but inhibited by Z9-14:OAc opens the possibility for a direct action of the antagonist in distorting the firing rates of olfactory receptor neurons (ORN) expressing olfactory receptors (OR) that respond to the pheromone components (Miura et al., 2010). The antagonists may interfere through two distinct mechanisms: via the antagonist pathway itself, or via interferences with the agonist pathway. It is noteworthy that the behavioral antagonism-related ORN sometimes can respond to an agonist component, a phenomenon reported for the Asian corn borer, *O. furnacalis* (Takanashi et al., 2006; Domingue et al., 2007a). Investigations of the genetics of male response to pheromone components have revealed that the spike amplitude of cells that respond specifically to Z9-14:OAc do not appear linked to the factor that determines the development of Z and E11-14:OAc-responding ORNs (Cossé et al., 1995).

Other compounds have been reported for their antagonism of ECB pheromone, with the addition of as little as 1% in the pheromone blend leading to antagonism: (*E*)-9-tetradecenyl acetate (E9-14:OAc), (*E*)-and (*Z*)-9-dodecenyl acetate (E9-12:OAc and Z9-12:OAc, respectively) (Klun and Robinson, 1972; Klun et al., 1979; Struble et al., 1987; Glover et al., 1989), as well as (*Z*)-11-hexadecenal (Z11-16:Ald) (Gemeno et al., 2006; Linn et al., 2007a). These compounds have not been found as part of the pheromone blend of any *Ostrinia* species (Ishikawa et al., 1999b). As they have been found in other moth species, one could hypothesize that they have evolved under the private channel hypothesis, thus lowering the probability of cross attraction and, therefore, preventing male mistakes (Löfstedt, 1993; Cardé and Haynes, 2004). On the other hand, the antagonistic activity of some of these compounds may have no adaptive origin (no selective pressure for the emergence of the antagonism), but may be purely coincidental; these molecules may be able to interact with the

olfactory receptors implicated in the antagonist pathway because of their structural similarity to Z9-14:OAc (Linn et al., 2007a).

Smelling the Pheromone: The Male Olfactory System

The demonstration that the antennae of the ECB male are involved in the sex pheromone response came from a study by Klun in the late 1960's (Klun, 1968). This was consistent with observations reported earlier that established the antennae as the primary interface of olfactory reception in moths, and insects in general (Schneider, 1957, 1962). The antennae in both sexes are filiform, with about sixty flagellomeres, but the basal diameter of the antenna in the male is larger than in the female (Hallberg et al., 1994). The response resulting from the detection of a recognized odorant can be measured as an action potential, and electroantennograms (EAG) represent recordings of the summed electrical potentials being sent to the brain by ORNs for which odor stimulants elicit a response (Schneider, 1962, 1969; Mayer et al., 1984). The action potentials are transmitted so as to increase the response in the proximal direction only (Nagai, 1981). Typically, EAG potentials are visualized as negative change in the background potential.

The morphology of the antenna has been described, highlighting particular features of the antenna of male corn borers (Hallberg et al., 1994). Along the filiform antenna of corn borer males, the olfactory sensilla occupy the ventral surface, the dorsal surface being covered by scales (Cornford et al., 1973; Hallberg et al., 1994). Different sensillum types have been characterized, with the sensilla trichodea representing the most abundant type of sensillum found on the antenna (Hallberg et al., 1994). It was noticed that the highest EAG response was obtained when the ventral side of the antenna faced the air stream conveying the pheromonal stimulus (Nagai et al., 1977). This suggested that the sensilla housing olfactory receptor neurons sensitive to Z and E11-14:OAc are localized mainly on the scale-free ventral part of the male antenna and could, therefore, be the sensilla trichodea, given that these are more numerous on male antenna as opposed to female antenna (Cornford et al., 1973). Electrophysiological recordings from single sensilla trichodea indicated that the olfactory receptor neurons that respond to pheromone are housed within this type of sensilla (Hansson et al., 1987). In total, three subtypes of sensilla trichodea have been described on the ECB antenna: one longer type that is innervated by three sensory cells (trichodea A), and two shorter types innervated by two or one single sensory cell (trichodea B and C, respectively) (Hallberg et al., 1994). Note that in the female, only trichoid sensilla with two or three cells have been observed, and the trichoid sensilla appear shorter in females than in males (Hallberg et al., 1994). It was shown that the EAG response to the pheromone components is proportional to the length of the antenna that is stimulated, indicating that olfactory receptor neurons tuned to pheromone components occur regularly throughout the entire antenna (Nagai, 1981). Similarly, the fact that the ratio between the response to E and Z11-14:OAc remained constant, regardless of the length stimulated, suggested that the olfactory receptor neurons that respond to the major and minor pheromone components are distributed evenly along the antenna (Nagai, 1981). Therefore, the number of sensilla trichodea was predicted to be uniform per unit of surface area; this was confirmed by direct measurements (Nagai, 1981). Interestingly, the distribution of the various subtypes of sensilla trichodea along the antenna is uneven. The type A (three cells) is the most common type in the basal part, whereas in the distal part the majority of the sensilla are of the type B (two cells). In contrast, the type C sensilla (one cell) has an even distribution, and constitutes a large proportion of the sensilla present towards the tip of the antenna (Hallberg et al., 1994). The adaptive significance of this particular arrangement remains to be clarified.

Nagai et al. (1977) attempted to establish a link between antennal response recorded by EAG and the behavioral response of male exposed to the female sex pheromone by comparing the electrophysiological responses to Z and E11-14:OAc of Z and E strain males as well as to hybrid individuals. For individuals of the Z strain, Z11-14:OAc always gave a significantly larger EAG response than E11-14:OAc. Although the amplitude of the antennal response decreased throughout the lifespan of the preparation, the response E/Z ratio varied little over time; the E isomer eliciting a response about 70% of that elicited by identical amount of the Z isomer. On the other hand, no difference in the EAG response to the geometrical isomers could be detected when testing individuals of the E strain. Hybrids from reciprocal crosses were intermediate in their responses, although they appeared closer to the E strain males. However, other authors have reported that the E strain individuals have a stronger EAG response to E11-14:OAc than to Z11-14:OAc, making possible a distinction between the strains via EAG recordings (Linn et al., 1999). The amount used to stimulate the antenna during these tests appears critical, and this is certainly a factor that needs to be considered with particular attention.

Single sensillum recordings have revealed important differences between the subtypes of sensilla trichodea, and demonstrated that the strains are highly distinguishable in their electrophysiological responses (Hansson et al., 1987; Hallberg et al., 1994). In type A, one cell responds to the major pheromone component, the second cell to the minor

component, and the third to the behavioral antagonist. Type B sensilla houses cells that respond to the pheromone components only, whereas the single cell present in type C sensilla responds to either the major pheromone component or the behavioral antagonist (Hallberg et al., 1994). Typically, extracellular recordings of male antenna exhibit high-amplitude spike responses from an olfactory cell responding to the major pheromone component, and a low-amplitude spike from an olfactory cell responding to the minor component (Hansson et al., 1987; Hallberg et al., 1994). Furthermore, there is a positive correlation between the spike amplitude and the spike frequency of olfactory receptor neurons (Cossé et al., 1995). The existence of a correlation between spike size produced by stimulated pheromone-responding olfactory receptor neurons and the diameter of the neurons' dendrites has been demonstrated by morphometric measurements (Hansson et al., 1994). Thus, spikes with large amplitude are produced by receptor neurons that are larger than the receptor neurons that elicit spikes of small amplitude. One possibility is that a larger dendrite diameter may be facilitating the presence of more receptor sites specific for the pheromone component to which the olfactory receptor neuron is tuned; the consequence being a higher sensitivity to that component and an action potential of higher amplitude (Hansson et al., 1994; Cossé et al., 1995). Similarly, the outer dendritic segments taper distally in the sensillum (Hallberg et al., 1994). This gradual narrowing of the dendrite diameter towards the tip theoretically optimizes the charge transfer to the dendritic root while minimizing the dendrite volume (Cuntz et al., 2007).

The response profiles obtained from electrophysiological recordings of male corn borer antenna, as well as those for other species, suggested that the main pheromone component always elicits response from OR neurons displaying large spike amplitude, and that the behavioral response may be influenced by the spike amplitude and frequency evoked when these cells are stimulated. However, the results reported by Cossé et al. (1995) indicate that the behavioral response is not necessarily elicited by the component that evokes responses from the large spike amplitude neuron in olfactory sensilla because F2 hybrid males possessing Z-like antennae from an electrophysiological viewpoint responded behaviorally to the E blend. To test whether or not the antennal phenotype is a controlling factor in pheromone blend discrimination, Linn et al. (1999) used an antennal transplant technique to produce individuals with mixed phenotypes for antennal and behavioral responses. Their results were in agreement with the study of Cossé et al. (1995), and suggested that the sex pheromone preference of males is independent of the make-up of the peripheral sensilla and is, therefore, determined at a higher level. However, because the actual projection pattern of the ORNs is unknown, no firm conclusion can be drawn.

The investigations conducted to identify the female sex pheromone of the ECB have revealed also that the olfactory system of the male is organized such that it can detect small variations in the geometric composition of the attractant, and that each strain responds optimally to different geometrical proportions of Δ11-tetradecenyl acetate (Klun et al., 1973). Although the major sex pheromone component is Z or E11-14:OAc, the insect is obviously exquisitely sensitive to small amounts of the opposite isomer (Klun et al., 1973). No increase in EAG response was found when antennal preparations were stimulated with mixtures at the ratios found to be best in field trials (Nagai et al., 1977). Single sensillum responses from olfactory sensilla on the male antenna clearly indicated that the two pheromone-components are detected by different specialized receptors (Hansson et al., 1987; Löfstedt et al., 1989). Also, electrophysiological recordings from single sensilla indicated that, irrespective of the strains, all males have sensory cells sensitive to both pheromone compounds (Hansson et al., 1987). Taken together, these results can be interpreted as further indication that the pheromonal signal is integrated at a higher echelon than the peripheral level. The central nervous system input from the cells appears to be differentially interpreted to give the various phenotypic behavioral responses observed (Roelofs et al., 1987).

Understanding the central nervous processing is necessary when trying to link the processes that occur at the periphery with behavioral responses. Sex pheromone receptor neurons project into the antennal lobe, the first-order olfactory brain area, through the antennal nerve (Hansson, 1995). The antennal lobe comprises a number of glomeruli in which synaptic contacts are made between receptor neurons, projection neurons, and interneurons (Hansson, 1995). Each receptor neuron arborizes in a single glomerulus within the antennal lobe (Anton et al., 1997). Based on three-dimensional reconstructions that allow high resolution of the antennal lobe neuroanatomy, the number of glomeruli in the antennal lobe of Z strain male and female individuals was estimated to be approximately 66 and 64, respectively (Karpati et al., 2008). Located at the entrance of the antennal nerve of male moths, a few enlarged glomeruli dedicated to the reception of pheromonal information constitute the macroglomerular complex (MGC). The number of glomeruli that form the MGC compartments is variable between species and can be an indicator of the number of sex pheromone components, as well as pheromone antagonists detected by highly specialized olfactory receptor neurons. In *O. nubilalis*, the MGC of both E and Z strain males consists of three enlarged glomeruli (Anton et al., 1997; Karpati et al., 2008). Two

large highly convoluted and interdigitated glomeruli of variable shape and dimension receive information from E11 or Z11-14:OAc-responding ORNs, and the third glomerulus, located posteriorly to the two others, is dedicated to Z9-14:OAc, a behavioral antagonist (Anton et al., 1997; Karpati et al., 2008).

The two strains are indistinguishable on the basis of the neuroanatomy of their MGC: in both strains, ORNs that respond to the major pheromone component arborize in the largest medial glomerulus of the MGC, whereas the minor pheromone component-specific ORNs arborize in a smaller lateral glomerulus (Karpati et al., 2008). Olfactory output is made by projection neurons that have dendritic branches in the MGC and axons projecting through the inner antennocerebral tract to the calyces of the mushroom bodies and the lateral protocerebrum (Hansson, 1995; Anton et al., 1997). In the ECB, neurons are classified according to the component eliciting response at the lowest level of abundance, namely E or Z. E+Z-blend neurons respond equally to the same levels of Z and E isomers and mixtures of the two isomers (Anton et al., 1997). Recordings and staining of projection neurons responding to E or Z11-14:OAc show that they arborize only in the E or Z11-14:OAc-specific compartment of the MGC, respectively (Karpati et al., 2008). Similarly, projection neurons that exhibit a larger sensitivity to a blend rather than to either of the pheromone component alone arborize in both pheromone-sensitive MGC glomeruli (Karpati et al., 2008). Finally, neurons described in Anton et al. (1997) as generalist are most likely local interneurons arborizing in most if not all glomeruli (Karpati et al., 2008). Interestingly, the type of blend neurons that discriminate strain-specific blends is apparently consistent with the behavioral profile of each type of male (Anton et al., 1997). This might explain, in part, the ability of insects to discriminate behaviorally between the pheromone ratios of the different strains (Anton et al., 1997). However, it is still unclear whether these neurons are essential in the behavioral response that follows detection of different ratios of pheromone components (Karpati et al., 2008). Irrespective of their type, the antennal lobe neurons differ in their absolute sensitivity (Anton et al., 1997). It is worth mentioning that both receptor and antennal lobe neurons respond to both pheromone isomers when stimulated at high concentrations, while specific responses of the neurons are observed only at lower concentrations (Anton et al., 1997). Therefore, it is important to bear in mind that the pheromone concentrations and ratios used in electrophysiological experiments should be within the natural range that males may encounter.

The males of both pheromone strains and reciprocal hybrids exhibit the same neuron types, but the abundance of these types differs between the types of males, which indicates the existence of strain-specific characteristics at the antennal lobe level (Anton et al., 1997). The two strains have an identical MGC morphology accompanied by a reversed functional topology, with both olfactory receptor neurons and projection neurons displaying opposite innervation patterns (Karpati et al., 2008). Karpati et al. (2008) proposed that the occurrence of an interchange of olfactory receptors between pheromone receptor neurons within the same sensillum would explain the finding of a reversed functional topology, while olfactory receptor neuron and projection neuron arborization patterns in the MGC remained unchanged. The pheromone receptor neurons would always arborize to the same location in the MGC but the ORs expressed within the membrane of their dendrites would be tuned to a different pheromone component, thus leading to an opposite behavioral response. One may argue that such a receptor swap occurs when antennae are transplanted from one strain to the other, such as reported by Linn et al. (1999), who observed maintenance of the behavioral response of the implanted strain, which is not according to the prediction. However, because of a lack of information concerning the neuroanatomy of individuals that undergo antennal transplantation and, in particular, how the axonal targeting of olfactory receptor neurons is influenced, no definitive conclusion can be drawn from this type of experiment. Further studies are required to elucidate what factors determine the type of receptor expressed in a particular olfactory receptor neuron, and the arborization path followed by the sensory axon of that olfactory receptor neuron. Recently, a series of ORs responding to pheromone components has been identified in the ECB (Wanner et al., 2010). ORs appear to be narrowly and broadly tuned, with some responding nonspecifically to (*E*) and (*Z*)-11- or -12-tetradecenyl acetate, as found in *O. scapulalis* (Miura et al., 2010; Wanner et al., 2010); the latter E and Z12-14:OAc constitute the pheromone components of a close relative of the ECB, the Asian corn borer *O. furnacalis* (Ishikawa et al., 1999b). Previous electrophysiological recordings of ORNs indicated that ECB males possess ORNs responsive to E and Z12-14:OAc, and that these ORNs are, indeed, the same large- and small-spiking ORNs responsive to the ECB pheromone components (Domingue et al., 2006, 2007b). Some rare ECB males may be attracted to the Asian corn borer blend (Linn et al., 2003, 2007b); this alteration in the normal behavior might be caused by a firing ratio of ORNs responsive to E and Z12-14:OAc close to the ratio observed in response to the attractive ECB blend (Domingue et al., 2007b).

Many advances have been made during the last 30 years, and we have a much better understanding of

the ECB male olfactory system. Nevertheless, the precise mechanism leading to blend discrimination remains unknown. Most studies have used neurophysiological approaches, and the molecular bases of pheromone detection in ECB are yet to be unraveled. For example, the actual diversity of ORs in ECB, and what factors determine on which sensory dendrites they are expressed, remain essentially unexplored.

Genetics of Male Response

As the genetics underlying ECB sex pheromone differences was investigated, the heredity of male sexual response preferences toward specific mixtures of Δ11-tetradecenyl acetate was also scrutinized. Two types of response are distinguishable: the physiological response at the level of male antennae, and the behavioral response of males.

Electrophysiological recordings from single olfactory sensilla on male antenna showed that the reaction patterns of males from the two strains are distinguishable (Hansson et al., 1987; Roelofs et al., 1987). Males from the first filial generation were characterized as intermediate by displaying two types of olfactory cells that gave similar spike amplitudes to the E and Z isomers (Hansson et al., 1987). The inheritance of the response profiles is determined by a single autosomal gene with two alleles (Hansson et al., 1987; Roelofs et al., 1987). Because the locus controlling female pheromone production also was found to be autosomal, the possible linkage of the two loci or the existence of a single locus that determines both phenotypes came to be questioned. Evidence showed that even if the inheritance of pheromone production and the electrophysiological response of pheromone receptor cells are most often coupled in a complementary fashion, so that production and response are coordinated, their coordination can be uncoupled in matings among individuals of opposite types (Klun and Huettel, 1988). The experiments conducted by Löfstedt et al. (1989) demonstrated unambiguously the absence of a close linkage between the autosomal genes that determine the sex pheromone production and the organization of olfactory receptors in *O. nubilalis*. That was the first time where the degree of genetic linkage between characteristics of the sender and responder in a pheromone communication system had been investigated.

In terms of behavioral response profile, the F1-generation hybrid males from reciprocal crosses exhibited similar response profiles with about 50% of the males being attracted to different ratios of geometrical isomers (97:3, 65:35, 50:50, 35:65 Z/E), with the noticeable exception of the 1:99 Z/E blend that seldom attracted males (Roelofs et al., 1987; Glover et al., 1990; Linn et al., 1997). The remaining 50% of the F1 progeny do not respond to any blend (Roelofs et al., 1987; Glover et al., 1990). In Lepidoptera, males are the homogametic sex, usually denoted ZZ, and the females are the heterogametic sex, denoted ZW. The observation that paternal backcrosses to Z or E strains gave individuals with response profiles similar to that of the pure Z or E parent males indicated that the inheritance of the behavioral response is determined by a major sex-linked factor (Roelofs et al., 1987). It is not known if this major factor consists of a single gene or whether it consists of a set of closely linked genes. The demonstration that male behavioral response is determined by a locus present on the sex chromosome was made by using triose phosphate isomerase (TPI) an allozyme marker for the Z chromosome (Glover et al., 1990). A perfect match between the phenotype at TPI and the response profile of males indicated a complete linkage of the TPI locus and the locus controlling response to sex pheromone, thus confirming the sex linkage of male behavior (Glover et al., 1990). There was evidence for little recombination between the allozyme marker locus and the male behavior locus (Glover et al., 1990). It is worth mentioning that the phenotype at TPI may not always differ between populations of the E and Z strains, as reported by Cianchi et al. (1980). Genetic mapping of male response showed that TPI and the locus responsible for the difference in male behavioral response are not tightly linked, and a factor other than response to pheromone may maintain the linkage disequilibrium between TPI and response (Dopman et al., 2004, 2005).

Since the locus controlling male behavioral response is sex-linked, and the locus determining male electrophysiological response is autosomal, it appears evident that these loci are not linked and may segregate independently. For this reason, certain crosses between the E and Z strains should produce unusual males that respond behaviorally to one blend despite possessing antennae that respond electrophysiologically to the opposite blend. A study was undertaken to examine whether or not these unusual males possessing the wrong antennae can perform a complete behavioral sequence in a wind tunnel experiment. Males were produced through F2 crosses between the two races and the analysis of the spike amplitudes obtained with recordings of E-behavioral responders revealed that some males with Z-like antennae can fly to the E pheromone source (Cossé et al., 1995). This provided further support for the idea that the sex-linked factor associated with behavioral response affects the way an incoming signal from the antenna is processed in the central nervous system of male moths. The sex-linked factor that controls the behavioral response profile of males may, therefore, influence the architecture of the antennal lobe. The pattern

of neuron types found in the antennal lobes of hybrid or pure strain males coincide to some extent with the behavioral profile (Anton et al., 1997). Unfortunately, the limited number of recordings made did not allow the authors to draw any firm conclusion, despite striking coincidence. A study that focuses on males obtained from paternal backcrosses (EZxZ and ZExE), which should possess to a large extent only Z- or E-blend neurons, should give a clue regarding the link between discrimination of different ratios of pheromone components at the level of the antennal lobe and the behavioral response profiles of males, thus providing a physiological basis for the differences in behavior. The finding that the functional topology of the MGC in Z and E strain males is reversed (Karpati et al., 2008) indicates that further research is still required.

Given that the loci for sex pheromone production, male electrophysiological response, and male behavioral response are on different pairs of chromosomes, the response and the signal are not expected to be physically linked in this pheromone system.

Evidence for A Male-Produced Pheromone

Laboratory tests conducted under confined conditions have indicated that the two ECB strains are not freely interbreeding, which may result from the action of a chemical governing sexual behavior at close range (Liebherr and Roelofs, 1975; Pélozuelo et al., 2007). The detection of the incorrect substance may inhibit a large proportion of the females from accepting the mating overture (Liebherr and Roelofs, 1975). Males possess on both sides of the claspers and at the intersection between the 7th and 8th sternites two tufts of differentiated scales called hairpencils. The first clear evidence for the existence of a male pheromone in the ECB came from the observation that males deprived of their hairpencils have a decreased mating success (Royer and McNeil, 1993).Recently, the pheromone from hairpencils was demonstrated to play an crucial role in female choice, and was identified in the Z strain as a mixture of 16-carbon acetates, namely (*Z*)-9-hexadecenyl acetate, (*Z*)-11-hexadecenyl acetate, (*Z*)-14-hexadecenyl acetate, and hexadecanyl acetate (Lassance and Löfstedt, 2009). The bouquet produced by males of the E strain was found to be of similar composition but lacked (*Z*)-11-hexadecenyl acetate in most individuals (Lassance and Löfstedt, 2009). The behavioral significance of this difference in composition remains to be firmly demonstrated.

Males produce compounds that are structurally similar to those used by females, and both sexes appear to rely on the same genes to produce their pheromones. This finding raises interesting questions concerning the coevolution of the two traits, and especially for the coevolution of the two detection systems.

Concluding Comments

One of the purposes of investigating the European corn borer chemical ecology was to provide new tools to monitor and control the pest. Trapping systems that rely on synthetic sex pheromone have been used to catch the attracted males and monitor adult activity (Pélozuelo and Frérot, 2007). However, the information gathered from such pheromone-baited traps may be taken with circumspection as it is still unclear how well moth phenology correlates with trapping data and, while only males are captured, their mating status is unknown (McNeil, 1992; Pélozuelo and Frérot, 2007). The obvious potential of semiochemicals should not be questioned, but increasing our knowledge of the factors that influence their efficacy and how to interpret what we observe is necessary.

One line of ECB research that has not been sufficiently pursued is host plant selection by females. Whereas the ECB owes its name to the use of maize as a host, the insect can thrive on more than 200 plants species (Caffrey and Worthley, 1927). Little is known about what criteria determine the suitability of a host, in particular, what are the cues that make a host attractive to gravid females. The two pheromone strains may differ in their host preference, as exemplified by studies conducted in Canada, the U. S., and France (McLeod, 1981; Straub et al., 1986; Eckenrode and Webb, 1989; Bontemps et al., 2003; Thomas et al., 2003; Pelozuelo et al., 2004). The use of transgenic maize that express *Bacillus thuringiensis* toxin, and the associated strategies that prevent the emergence of resistance, require a better knowledge of the host range actually used by ECB in the field. Is *O. nubilalis* a truly opportunistic polyphagous species, or is it a mosaic of host-plant races hidden under the same name?

The two strains of *O. nubilalis* are sufficiently isolated to be considered as sibling species but still compatible enough from a genetic point of view to produce fertile offspring. As such, the ECB has become a model to study the evolution of sex pheromone communication (Smadja and Butlin, 2009). A series of studies have elucidated the genetic bases of the polymorphism observed in this sex pheromone communication system. While the gene responsible for the differences in female-produced sex pheromone has been characterized, those involved in the differences in male behavior and antennal responses are still unidentified. The two pheromone strains of *O. nubilalis* constitute a unique system to investigate how speciation affects different parts of the genome.

The apparent simplicity of the ECB communication system, as it turns out, has ample hiden complexity, and, as some who spent some years investigating the ECB chemical ecology say, "the more answers we obtain with the ECB, the more questions we get in return". The recent identification of the male-produced pheromone, a trait acting as a reproductive barrier between the strains, opens a new dimension of ECB research. Fascinating discoveries may be just around the corner, and corn borers probably have many more secrets to reveal.

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mass spectrometric analysis of ink supernatant indicated high levels of epinephrine in ink stripped from the intact ink sacs of dead specimens (Fig. 1). Since there were no previous reports of significant levels of epinephrine in cephalopod ink, we concluded that the finding was either a significant novel discovery or an artifact resulting from contamination by autolysis or the sampling method.

Catecholamines, such as dopamine, nor-epinephrine, and 5-hydroxytryptamine were documented from the nervous tissue of cephalopods as far back as 1971 (Juorio, 1971). Subsequently, epinephrine has been found in the haemolymph of the curled octopus *Eledone cirrhosa* (Lacoste et al., 2001) and also reported from other tissues such as nerves in trace amounts (Springer et al., 2005).

Consequently, the observation of an epinephrine spike in the ink of *S. australis* was of interest. The possibility was raised that epinephrine in its ink may have evolved as an allelochemical against vertebrate predators with respiratory surfaces exposed to sea water.

Of the other non-melanin components of cephalopod ink, the presence of the enzyme tyrosinase commonly is reported (e.g., Prota et al., 1981; Derby, 2007) and was suggested as the component responsible for anti-tumour activity in mammalian cells (Takaya et al., 1994; Naraoka et al., 2000). However, the physiological or behavioral significance of ejecting tyrosinase into sea water in response to a predator or as a signal of alarm to conspecifics is difficult to explain.

Along with DOPA, dopamine, and taurine, the presence of proteins in cephalopod ink is widely accepted. For example, Linh Tran et al. (2006) emphasised the claim that melanins *in vivo* are strongly bound to a protein host and that sample preparation frequently removes the protein, leaving subsequent studies to focus on the chromophore alone. Similarly, Meredith et al. (2006) suggested that the term melanin should 'encompass both the chromophore and the associated protein'. The presence of proteins within the eumelanin structure also has been suggested by Meng and Kaxiras (2008).

Two major obstacles have stood in the way of describing fully the chemical structure of the melanin complex. It has a monotonous absorbance pattern in the visible and near UV part of the spectrum. It is insoluble in all useful solvents, and thus, it defies structural analysis (Linh Tran et al., 2006).

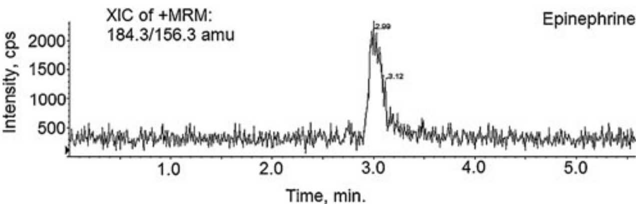

Fig. 1 Chromatogram of epinephrine in diluted ink from the ink sac of *Sepioteuthis australis*

Our initial discovery of a spike of epinephrine, along with a question about the biological justification for ejecting tyrosinase with ink led to this investigation. The aim was, therefore, to measure the levels of epinephrine and protein in the ink of *S. australis* specimens. Because of a suspicion that the epinephrine in our initial sample may have been an artifact, we undertook to compare the ink recovered by methods that minimized the chance of contamination from ink sac tissues (Syringed ink from freshly caught specimens), with that recovered by more traditional procedures (Stripping Method).

Histological studies of the ink sac were performed, along with comparative analyses of ink sampled from *S. australis* by each procedure.

Methods and Materials

Histological Structure of the Ink Sac The ink sacs of five *S. australis* were dissected from freshly-caught specimens and fixed in buffered formalin. They were infiltrated subsequently with paraffin wax, and a series of transverse and longitudinal histological sections was cut and stained with haematoxylin and eosin. Sections were viewed under the microscope, and representative views were photographed for analysis.

Ink Sampling Ink samples from specimens of *S. australis* were collected for comparison by two different methods:

> *Syringed Ink Method*. Squid were caught along the South Australian coast, placed 'live' on a bed of ice, and anaesthetized with a $MgCl_2$ spray (Messenger et al., 1985) before being killed by decapitation across the edge of the mantle (i.e., posterior to the pleuro-visceral ganglion) to avoid stimulation of ink release. The mantle cavity was opened immediately along its ventral surface, and the proximal end of the ink sac was freed from the surrounding tissue. The duct end of the ink sac was cut off with sharp scissors and held open with forceps before the ink was carefully drawn from the distal lumen with a 1 ml syringe fitted with a yellow (200 μl) automatic pipette tip. The sample then was transferred to an Eppendorf tube, taking care not to touch the outside surface of the syringe onto the lip of the tube. Samples were held on ice until they were stored at −80°C.
>
> Attempts to run this difficult collection protocol rendered only 6 ink samples with a sufficient volume of uncontaminated ink from approximately 30 squid caught. Many squid had emptied their ink sacs prior to

sampling attempts, or it proved impossible to introduce the tip of the syringe into the ink sac without contamination from external fluids. Problems also arose from the tendency of the pipette tip to adhere to the inside of the sac unless the fluid was drawn up very slowly. However, sufficient material was removed from stored material near the duct of the ink sac for analysis, representing uncontaminated ink. Of course, the nature of the ink could change once it was ejected into sea water, but that was not the focus of this study.

Milked Ink Method. Fresh squid were purchased within a day of being caught and held on ice (not frozen) by arrangement with Valente Seafoods, Adelaide, Australia. Dead animals were taken to the laboratory on ice, and the ink was extracted as soon as possible by the following method. The mantle cavity was opened along its ventral surface and the entire ink sac dissected out. The duct end was cut off and held over an Eppendorf collection tube while the ink was 'milked' by running forceps down its length, taking care not to introduce external fluids. Samples then were held on ice until stored at −80°C.

These samples represent ink contaminated with exudates from cells after autolysis, contents of the ink gland chambers, and possibly materials from the external surface of the ink sac.

Determination of Epinephrine, DOPA, Dopamine, and Taurine HPLC grade acetonitrile was obtained from Biolab (Australia). DL-DOPA, DL-dopamine, taurine, epinephrine/adrenaline, and ammonium acetate were purchased from Sigma-Aldrich (Australia). Water was purified for HPLC analysis by using a Milli-RQ ultrapure Water System (Millipore, USA).

Ink sampled by either method (about 50 mg) was vortex mixed with water, centrifuged, and the supernatants diluted 10 times with water. These unwashed samples were analyzed directly. Additional samples were washed repeatedly with water to remove soluble material, and the recovered melanin was suspended in 10 volumes of water and stirred for 2 hr at ambient temperature. The extract was centrifuged, and aliquots of the supernatants were transferred to autosampler vials for analysis.

The analytical equipment consisted of a solvent delivery system comprising two Shimadzu LC-10ADVP pumps used isocratically, a DGU-14A degasser, and SIL-HTC auto sampler (Shimadzu, Japan) held at 10°C. The HPLC system interfaced with a triple stage quadruple mass spectrometer (API 3000, Applied Biosystems, Canada) via an electrospray source. Instrument control, data acquisition, and data analysis were performed with Analyst software V1.4.1 (Applied Biosystems). For each analyte, MRM transitions were optimized in the positive mode by infusing the relevant reference compound; DOPA 298.3→152.3, dopamine 154.3→137.3, epinephrine 184.3→166.3, norepinephrine 170.3→152.3, and taurine 126.0→108.0. A Hilic C18 column (3×50 mm, Phenomenex) equipped with a matched guard was used with a mobile phase consisting of 30% acetonitrile and 10 mM aqueous ammonium acetate, delivered at a flow rate of 0.2 ml/min. The injection volume was 10 µl. Reference solutions were prepared fresh on the day.

Determining Protein in Ink Supernatants Mushroom tyrosinase, L-3, 4-Dihydroxyphenylalanine (L-DOPA), bovine pancreatic trypsin, subtilisin A, and pepsin were obtained from Sigma (St. Louis, MO, USA). SDS-PAGE was performed by using ready-made (NuPAGE) 4–12% (1.5 mm thick) SDS-acrylamide gels, MOPS-SDS running buffer, and SDS sample buffer. These were all obtained from Invitrogen Pty Ltd. Agarose was obtained from Amersham Biosciences Pty Ltd. All other chemicals used were of analytical grade.

To 300 µl of the ink sample, 100 µl of 50 mM Tris–HCl pH 8.0 was added. The mixture was vortexed at maximum speed for 30 sec and allowed to stand overnight at 4° C. The mixture was vortexed again for 30 sec and centrifuged at 13,000 rcf for 10 min. Fifty µl of the ink supernatant was mixed with SDS-PAGE sample buffer and incubated at 70° C for 10 min (all samples were non-reduced so that the tertiary structure of the proteins was preserved). Ten µl of the ink-SDS buffer incubate was loaded onto an SDS-acrylamide gel plate. The gel was run in a 1:20 dilution of MOPS running buffer made from a concentrated stock buffer solution. The gel was stained with a 0.1% (w/v) coomassie brilliant blue R-250 solution and de-stained in an acetic acid-water–methanol mixture (1:5:5).

Determining Electrophoretic Mobility of Proteins Present The method of Madaras et al. (2005) was used. Proteins in this method separate out on the basis of net protein electric charge, and the separation is not dependent on protein size. Electrophoresis was done by using a sodium barbitone buffer pH 8.6 where all the proteins that migrate towards the positive electrode possess a net negative charge. One µl of the Tris–HCl ink extract was loaded into an agarose gel plate sample well. Electrophoresis was performed by using 50 mM sodium barbitone buffer (pH 8.6, at ~2 Vcm^2). The gel was stained as described for SDS acrylamide gels. The stained protein bands were evaluated by the Gel-Pro Analyzer (Media Cybernetics USA) computer imaging software.

Enzyme Digestion of the Melanin Fraction Although no proteins could be detected in the Syringed Ink supernatant,

the possibility remained that some proteins were covalently linked to the melanin surface and were not detected in the supernatant or a melanin emulsion. To investigate this possibility, a series of experiments was performed using proteolytic enzymes to see whether proteins could be digested from the melanin. For this experiment, six ink samples from each group (Syringed or Milked Ink) were pooled (~600 µl) and washed repeatedly using un-buffered 0.15 M NaCl. The ink was suspended in the solution, vortexed at maximum speed for 30 sec, centrifuged at 13,000 rcf for 5 min, and the supernatant discarded. This process was repeated x 5. The washing process was designed to remove any freely soluble proteins present in the melanin ink leaving only the melanin particles.

The washed ink pellet was divided into two aliquots, and two different enzyme digests were prepared: one using 200 µl pepsin (0.05 mg/ml final concentration) at pH 1.0 in 0.1 M HCl, and the other using 200 µl trypsin (0.03 mg/ml) and subtilisin A (0.02 mg/ml final concentration) at pH 7.5 in tris-buffered saline pH 8.0. The washed melanin precipitate and enzyme mixtures were incubated at 37ºC for 4 h with occasional mixing. The digests then were centrifuged at 13,000 rcf for 10 min and examined for the presence of proteins by SDS-PAGE as described above.

As a positive control to demonstrate enzyme digestion, human albumin was used at 0.5 mg/ml concentration. The proteolytic enzyme concentrations and the conditions for the digestion of albumin were the same as those used for the melanin.

*Tyrosinase Assay T*yrosinase activity was assayed by the dopachrome method (Fling et al., 1963) as follows. The standard reaction mixture contained the substrate (5 mML-DOPA) in 0.1 M sodium phosphate buffer (pH 6.8), and 10 µl of the ink supernatant (as described above) were added to 990 µl of the L-DOPA substrate. The reaction took place in a micro cuvette with a path length of 1 cm, and the absorbance at 475 nm was monitored continuously for 10 min with a spectrophotometer (Shimadzu UV-1601) at 25º C. One unit of tyrosinase was defined as the amount of enzyme required to oxidize 1 µmol of L-DOPA per min under the above conditions, and calculated by using the molar extinction coefficient of dopachrome (3600 M−1 cm−1). For a positive control tyrosinase enzyme (mushroom> 1000 unit/mg) was used.

Results

Investigating the Histological Structure of the Ink Sac Histological sections revealed a mass of glandular tissue at the base of the ink sac, which sometimes extended over half way along the length of the lumen (Fig. 2). This mass seemed to comprise a series of compartments and ducts that increased in size as they matured and moved further into the lumen of the sac. The large spaces further away from the basal layer were filled with ink.

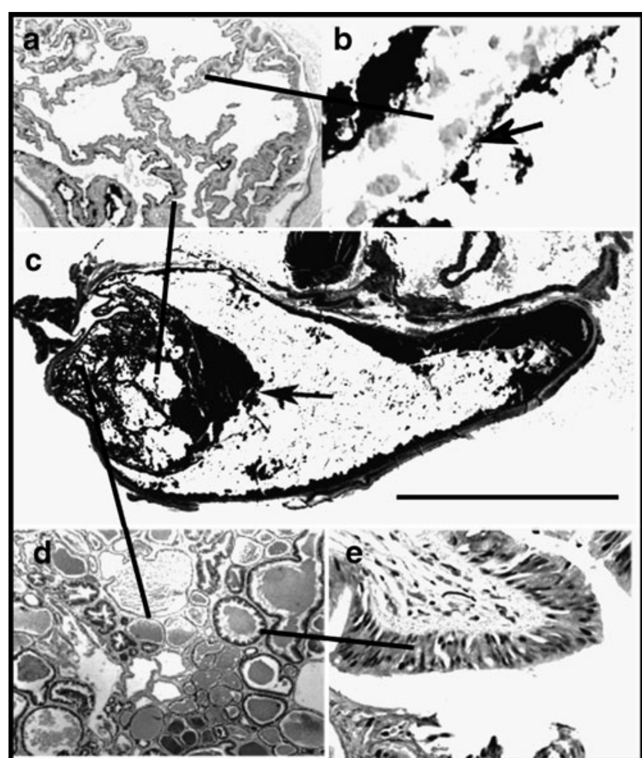

Fig. 2 Histological sections of the ink sac of *Sepioteuthis australis*. Legend: **c** Longitudinal section of whole ink sac (*arrow* indicates basal glandular area); Scale bar 10mm; **a** Melanin-producing reticulated tissue; **b** Melanin Granules in cells; **d** Mixed tissue (follicles and mucous ducts?) at the base of the gland; **e** Mucus-producing epithelium

Three distinct histological elements were found within the glandular tissue: apparently enclosed follicles that contained light-staining material (Fig. 2d); ducts lined with mucus-secreting cells (Fig. 2e); and reticulated epithelial layers with melanin granules visible in the cytoplasm of cells (Figs. 2a and b). It was not possible, without detailed serial-sectioning, to positively determine whether the glandular tissue was either comprised of closed follicles or tubular glandular epithelium, or a combination of both (Fig. 2).

Determination of the Presence of Epinephrine, DOPA, Dopamine, and Taurine After centrifugation of the diluted Milked Ink, substantial amounts of DOPA and taurine were recorded consistently (mid to high ppb range). Since the initial viscosity of the collected ink samples ranged from liquid to paste for different specimens, and analysis was performed directly after dilution, no attempts were made to quantitate the catecholamines. Dopamine was present to a lesser extent, but varied depending on the individual

samples. In some ink isolates, significant amounts (low ppb range) of epinephrine and nor-epinephrine were found (Fig. 1). These levels fluctuated among ink samples, and often no trace of either analyte was found in ink samples from different animals.

The same observations were made when melanin was separated from the supernatant. Suspensions of melanin, purified to different extents, were allowed to stir for 2 hr. After centrifugation and analysis of the supernatants, the presence of all the previously mentioned compounds was evident. Interestingly, dopamine was released into solution at a much slower rate than DOPA.

By contrast, supernatant from Syringed Ink showed no measurable traces of epinephrine or nor-epinephrine. This result matched that found for the protein content of Syringed Ink. However, DOPA, dopamine, and taurine were detected consistently in all ink supernatants, or were found to desorb from separated melanin, regardless of the method used to collect the original ink (Fig. 3).

Determining the Presence of Protein in Ink Supernatants Figure 4 shows the non-reduced SDS-PAGE of Milked and Syringed ink samples. No proteins were found in the Syringed Ink extracted from 'live' animals, while the Milked Ink samples yielded strong indications of protein. The purpose of the SDS surfactant was to mobilize all proteins according to size by negating charge. From the result, it was concluded that the protein present fell into a narrow mass range, or was mainly a single component.

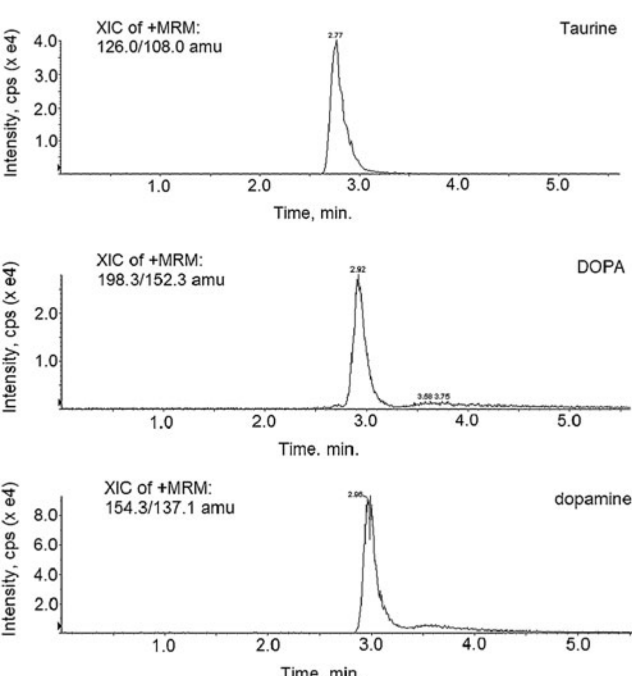

Fig. 3 Chromatograms of taurine, dopa and dopamine in ink collected by the Syringed method. The individual molecular ion→fragment mass transitions are shown separately and the scale varied according to each specific sample

Determining the Electrophoretic Mobility of the Proteins Present in Ink Sample Supernatants Figure 5 shows the agarose gel electrophoresis and protein scan on the pooled ink supernatants for which protein bands previously had been detected by SDS-PAGE (Fig. 4). Proteins in this method separated out on the basis of the net protein electric charge, and the separation was not dependent on the protein size. There was only one major protein band visible. This result, in conjunction with the SDS-PAGE result, once again indicated that the major protein in the ink found in these samples was composed largely of one high molecular weight protein that carried a net negative charge at pH 8.6.

Enzyme Digestion of the Melanin Fraction of Ink to Release Bound Proteins Figure 6 shows the results of the protein digestion experiments on the washed ink melanin. No protein could be detected when the melanin was obtained from Syringed Ink. However, protein was detected in both the pepsin and trypsin-subtilisin digested melanin collected by the Milked Ink Method. This most likely is due to precipitated and denatured protein that could not be removed by the washing process. Once exposed to the enzymes, soluble protein-peptides were released. The positive control experiment showed that both pepsin and trypsin-subtilisin completely digested albumin during the 4 hr incubation, and that the enzymes themselves are not detectable on the SDS-PAGE gel at the concentration used in the digestion experiment (Fig. 7).

Tyrosinase Assay on the Ink Samples Table 1 shows the residual tyrosinase activity in the Syringed Ink samples compared with the Milked Ink samples. While none of the Syringed Ink samples showed tyrosinase activity, the Milked Ink showed values comparable to the positive control that used commercial tyrosinase.

Discussion

The study of cephalopod ink involves several approaches. First, there is the biological approach, which seeks to understand the significance of inking behavior for the biology of a species. This may include an interest in the production of: 'smoke screens' and pseudomorphs (Bush and Robison, 2007), allelochemicals (Lucerno et al., 1994), or alarm signals to conspecifics (Wood et al., 2008). Recent elegant work has shown it to be a combination of these factors (Wood et al., 2010). The approach taken in the current study is, therefore, interested primarily in the nature of the ink as it would be ejected from the ink sac *in vivo*.

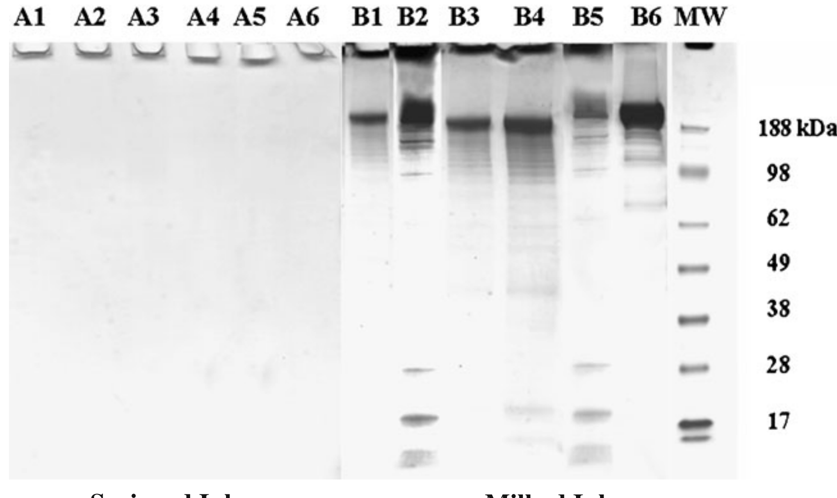

Fig. 4 Shows the non-reduced SDS-PAGE on ink samples; A1 to A6 were independent Syringed Ink samples from 6 different animals and B1 to B6 were independent Milked Ink samples from 6 different animals. MW is the molecular weight standards used

A second approach focuses on the cellular biochemical mechanisms of melanin synthesis where the intra- and extra-cellular location of enzymes and substrates is of interest (such as in melanosomes, etc.) (e.g., Palumbo et al., 1997; Palumbo, 2003). A third approach has no particular interest in cephalopod biology or melanin synthesis. Rather, its focus is on the utility of the ink for culinary or therapeutic purposes (Russo et al., 2003; Lei et al., 2007). Finally, the ink of cephalopods often is used in studies that attempt to determine the structure of melanin itself (Linh Tran et al., 2006; Meredith et al., 2006: Meng and Kaxiras, 2008).

However, despite the variety of perspectives taken in the study of cephalopod ink, no real attention has been given to the definition of its actual provenance. Because of the paucity of information about cephalopod ink sac structure and histology, no distinction seems to have been made between material located within the glandular chambers of the ink sac and that stored in the ink sac lumen and ready for ejection.

The results of experiments conducted on the ink of *S. australis* indicated that there was no epinephrine or protein naturally present in the supernatant of ink carefully syringed from the duct end of the ink sac in freshly-killed animals (Syringed Ink). By contrast, there were proteins (and possibly epinephrine) in samples milked from the ink sacs of dead specimens.

The SDS-PAGE experiment showed that all the protein present fell within a narrow mass range, while agarose gel

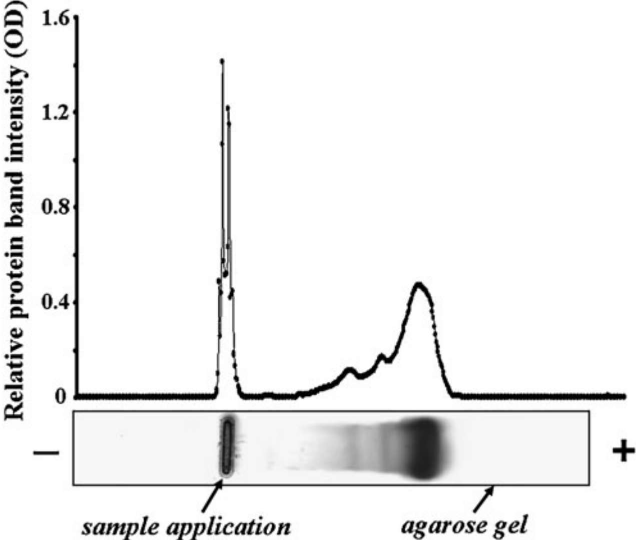

Fig. 5 The agarose gel electrophoresis and protein scan on the ink supernatant (samples B1 to B6 pooled) where protein bands were detected by the SDS-PAGE

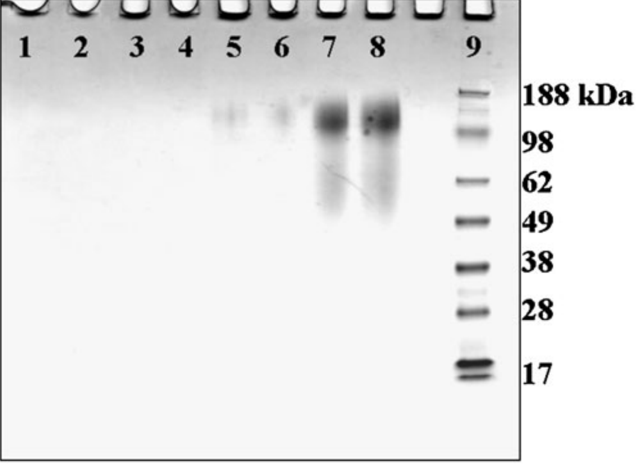

Fig. 6 Results of the protein digestion experiments on the washed ink melanin. Melanin samples from A1–A6 pooled and subsampled as 1–4 below. Melanin samples from B1–B6 pooled and subsampled as 5–8 below. Legend: Lanes *1* and *2*–Syringed Ink melanin after pepsin digest (pH 1.0); *3* and *4* Syringed Ink melanin after trypsin and substilisin digest (pH 7.5); *5* and *6* Milked Ink melanin after pepsin digest (pH 1.0); *7* and *8* Milked Ink melanin after trypsin and substilisin digest (pH 7.5); 9 Molecular Weight Standards

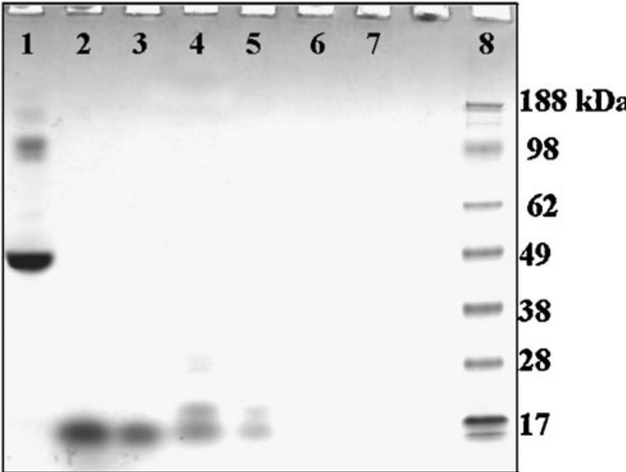

Fig. 7 Positive control for ink melanin protein digestion experiment. Legend: *1* - albumin (0.5 mg/ml); *2* and *3* albumin-pepsin digest (pH 1.0); *4* and *5* albumin-trypsin+substilisin digest (pH 7.5); *6* pepsin only (at the concentration used in the digestion experiment); *7* trypsin + substilisin only (at the concentration used in the digestion experiment). The enzymes alone are not detectable (at the concentration used in the digestion experiment), "8" molecular weight standards

electrophoresis, which separates proteins based on net electric charge, also indicated a single protein having an overall negative charge. Further analysis indicated that a single large molecular weight protein predominated in the non-melanin fraction of Milked Ink. Ink samples that contained this protein had strong tyrosinase enzyme activity.

In pursuit of proteins which may have been bound to the melanin fraction of the ink, and hence not evident in electrophoresis experiments, we performed digestion experiments to liberate such tightly bound species. No indication was found of the presence of bound proteins in the Syringed Ink samples. Hence, there was no detectable protein, either bound or free, in the ink carefully syringed from the duct area of the ink sac of freshly-killed *S. australis* specimens. In the case of Milked Ink, the presence of a protein that exhibited tyrosinase activity was most likely due to contamination from the rupture of glandular tissue at the base of the ink sac.

For comparison with other cephalopod studies, we analyzed ink supernatants for the presence of DOPA, dopamine, taurine, epinephrine, and nor-epinephrine. While traces of epinephrine and nor-epinephrine were recorded from Milked Ink samples, none was found in Syringed Ink collected from 'live' animals. On the other hand, in agreement with published reports (e.g., Lucerno et al., 1994), DOPA, dopamine, and taurine were consistently present in all ink samples analyzed, although in varying amounts. Exact quantification was not pursued, as the moisture content of the original ink samples varied from liquid to a paste consistency.

When seeking an explanation for our results, we searched for information on the gross histological structure of other cephalopod ink sacs and were surprised to find just a single published paper (Wang et al., 2008). This indicated the presence of a glandular mass at the base of the ink sac of the cuttlefish, *Sepiella maindroni*. Wang et al. (2008) described the basal secretory tissue as being highly branched, extending into the basal lumen of the ink sac and containing blood vessels, most of which were capillaries.

By comparing the histological structure of the ink sac of *S. australis* with that of *S. maindroni* (Wang et al., 2008), it was possible to note an important structural difference. The glandular tissue in *S. australis*, rather than being entirely dendritic and exposed to the ink sac lumen, as described for *S. maindroni,* seemed also to consist of a number of compartments, separate from the lumen (Fig. 2).

Using the structure of the *S. australis* ink sac as a guide, we propose that the ink along with its cargo of allelochemicals is synthesized in the basal mixed-glandular compartments and then released into the lumen of the sac for storage, perhaps by a break in the thin inactive compartmental walls. Indications are that the mucus component of ink may be synthesized in separate tubular structures.

We suggest it is this essentially protein-free material that is expelled by the animal for defensive and signalling purposes. Further, from this work, it is possible to suggest that tyrosinase activity in the ink of *S. australis* is present only when the basal glandular tissue is damaged, either during the sampling procedure or by autolysis after death.

A complicating factor may arise from the similarity between tyrosinase and haemocyanin, which is reputed to display tyrosinase-like activity (Schoot Uiterkamp and Mason, 1973; Jaenicke and Decker, 2004). Reported tyrosinase activity of cephalopod ink may be due to the

Table 1 Residual tyrosinase activity in the ink samples a1 to a6 (syringed) and b1 to b6 (milked), compared with mushroom tyrosinase (10 μg)

Samples	Tyrosinase activity (units)	Samples	Tyrosinase activity (units)
A1	0	B1	16
A2	0	B2	30
A3	0	B3	23
A4	0	B4	27
A5	0	B5	15
A6	0	B6	55
		tyrosinase enzyme	36

One unit of tyrosinase was defined as the amount of enzyme required to oxidize 1 μmol of L-DOPA per min under the assay conditions (see "Methods"), which was calculated using the molar extinction coefficient of dopachrome (3600 M^{-1} cm^{-1})

presence of hemocyanin, which has been included in samples by the rupturing of vessels in the ink gland.

While other studies have consistently documented the presence of proteins in the ink of cephalopod species (Prota et al., 1981; Takaya et al., 1994; Naraoka et al., 2000; Derby, 2007), we have been unable to detect free proteins or those associated with the melanin itself in Syringed Ink from *S.australis*.

There are at least three possible explanations. First, there may be significant differences among cephalopod taxa with regard to ink secretion and composition. Second, sampling procedures to date may have ruptured glandular cells, thus releasing proteins with tyrosinase activity. Third, there may be well-hidden (buried) proteins deep within the melanin superstructure that are not accessible to proteolysis. The latter alternative seems unlikely in view of recent findings by Meredith et al. (2006) showing that melanin consists of layers of pi-stacked 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole oligomer sheets.

Regarding the presence of DOPA, dopamine, and taurine in the ink supernatant, there is clear consistency with the findings of others who have studied different cephalopod species (Lucerno et al., 1994; Fiore et al., 2004). Our recordings of epinephrine and nor-epinephrine in some samples of ink preserved from dead animals can almost certainly be ascribed to contamination either from autolysis of cells after death or from damage while stripping of well-vascularised glandular tissue.

In order to understand the role of chemicals associated with the ink of *S. australis* there is a need for further work such as that of Gilly and colleagues (Gilly and Lucero, 1992; Lucerno et al., 1994) as well as that of Wood et al. (2010) on the effects of cephalopod ink allelochemicals on conspecifics and other marine organisms. A good example of such investigations on another mollusc taxon is that conducted by Derby and associates using the ink of *Aplysia* (e.g., Derby, 2007; Derby et al., 2007).

It is the various approaches and extraction methods for studying the ink of cephalopods that leads to confusion. The term 'ink' then is ambiguous. In some cases, it is regarded as the product of an ink sac being homogenized in a Waring blender (e.g., Naraoka et al., 2000). In others, it is the contents of an ink sac being removed after careful dissection (Liu and Simon, 2003; Fiore et al., 2004). Finally, it may be the dark material with its accompanying allelochemicals that may be ejected by many cephalopods when under threat or stress, as for *S. australis* in this study.

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Gross et al., 2010). Since its establishment in the 1980s, it has become the dominant ladybird in much of the USA and Canada (Koch and Galvan, 2008). One decade later, it was introduced into Europe and has now colonized 13 European countries (Brown et al., 2008).

One important characteristic of the harlequin ladybird is its high resistance to pathogens and predators. It was shown recently that adult *H. axyridis* were less susceptible to the entomopathogenic fungus, *Beauveria bassiana*, than the native European species, *Adalia bipunctata* and *Coccinella septempunctata* (Roy et al., 2008). *Harmonia axyridis* also was not a suitable host for the reproduction of various entomopathogenic nematodes (Shapiro-Ilan and Cottrell, 2005). Further, lower levels of parasitism by the wasp *Dinocampus coccinellae* were reported for *H. axyridis* compared to *C. septempunctata* (Koyama and Majerus, 2008).

In the immune system of insects, antimicrobial peptides are synthesized *de novo* following an infection (Boman and Hultmark, 1987). In recent years, due to the growing resistance of microbes to antibiotics, a number of studies have focused on the presence and effectiveness of antimicrobial peptides in insect hemolymph. Several of these peptides have been isolated and sequenced (Bulet and Stöcklin, 2005). Most studies involved model insects like *Drosophila melanogaster* and *Galleria mellonella* (Schmid-Hempel, 2005; Brown et al., 2009), but little is known about the disease resistance of Coleoptera, especially coccinellid beetles (Bulet et al., 1991; Gross et al., 2008).

In order to understand the rapid spread of *H. axyridis*, we evaluated several potential mechanisms that underlie the observed disease resistance of this species. As the survival of juvenile stages is one key factor for the successful establishment of invasive populations (Marco et al., 2002), we compared the antimicrobial efficacy of larvae and adults in *C. septempunctata* and *H. axyridis*. Antimicrobial defense of leaf beetles can be exerted either by the innate immune system or by volatile fumigants (Gross et al., 2008; Gross and Schmidtberg, 2009). Thus, we tested the hemolymph as well as several volatile components emitted by *H. axyridis* for antimicrobial potential. Furthermore, we investigated the mode of action of antimicrobial activities by determination of the minimal inhibitory activities (MIC) and activity measurement of key-enzymes of hemolymph obtained from live and frozen beetles.

Methods and Materials

Insects For establishing a laboratory rearing culture of *Harmonia axyridis* (Coleoptera: Coccinellidae), adults were collected in October 2008 in the surroundings of Dossenheim, Germany and reared permanently under controlled conditions in a climate chamber (24°C day, 19°C night, 60% humidity, 16:8 hr/L:D) at the Julius Kühn Institute, Dossenheim. Insects were kept in rearing cages (40×30×30 cm) and fed a diet of pea aphids (*Acyrthosiphon pisum*) reared on beans (*Vicia faba*), pollen (Ullmann, Erlensee, Germany), and water *ad libitum*. Eggs from *Coccinella septempunctata* (Coleoptera: Coccinellidae) were purchased (Katz Biotech, Baruth, Germany), and the beetles reared permanently under the same conditions. For all experiments, we used laboratory reared beetles (larvae and adults).

Microorganisms Bacillus subtilis, B. thuringiensis subsp. *tenebrionis* (strain 10 BI 256–82, isolated from *Tenebrio molitor* (Coleoptera: Tenebrionidae), Krieg et al., 1984), and *Micrococcus luteus*, were chosen as Gram-positive bacteria, and *Escherichia coli* (K12/D31) was used as Gram-negative organism. The yeast *Saccharomyces cerevisiae* (DSM 70499) was tested as an example for fungi (Gross et al., 1998, 2002). All organisms were obtained from the strain collection at the Julius Kühn Institute, Dossenheim. Bacteria were cultivated on Standard 1 agar plates (Roth, Germany) at 28°C (*Bacillus* and *Micrococcus*) or 37°C (*E. coli*), and the yeast on Sabouraud agar (Roth, Germany) at 28°C.

Agar Diffusion Assay

Insect Treatments Fourth instars and adults of both ladybird species were injected with 0.5 µl of sterile water or a dense (~10^{10} cells/ml) heat-inactivated (65°C, 60 min) suspension of *E. coli* with a microapplicator (Burkard, Germany) laterally into the abdomen 24 hr prior to hemolymph collection. A third group of larvae and adults served as untreated control.

Hemolymph Collection Hemolymph was collected from fourth instars or adults. Twenty-four hours after treatment, insects were pressed gently between two glass slides until they released their hemolymph by reflex bleeding from the joint between tibia and femur and elytral edges (adults), or setae (larvae). When this treatment was insufficient, a middle leg was cut with micro-scissors. The emanating hemolymph was absorbed directly from one individual insect onto small disks of sterile filter paper (3 mm diam). No distinction between males and females was made for either adults or larvae. Samples on paper disks were applied directly onto agar plates that contained the microorganisms and their respective growth media. Numbers of replications for treatments of adults were $N>9$, for treatments of larvae $N=12$. Untreated sterile filter paper disks were used as controls.

Bacteria For detection of hemolymph activity or synthetic compounds against bacteria, test plates were prepared as described by Gross et al. (1998, 2002).

Yeast Antifungal activity was determined with an inhibition zone assay against live *S. cerevisiae* according to Gross et al. (1998). The yeast was grown for 24 hr at 37°C in 100 ml of Sabouraud broth (Roth). Inhibition zone assays were performed on Sabouraud agar (Roth). After sterilization, the medium was mixed with 2 ml of yeast suspension from the submerged culture. Each Petri dish was filled with 5 ml of the inoculated medium.

Headspace The antimicrobial activity of eleven synthetic volatile organic compounds (VOCs) known from the headspace of adult *H. axyridis* (Cai et al., 2007) were tested against three model microorganisms (*E. coli, M. luteus, S. cerevisiae*) as described above: benzaldehyde (Aldrich), phenol (Roth), (+)-α-pinene (Fluka), (−)-α-pinene (Fluka), n-heptane (Merck), n-octane (Merck), n-nonane (Merck), toluol (Roth), R-(+)-limonene (Aldrich), 2-butanone (Fluka), 2-pentanone (Fluka). Two μl of undiluted VOCs were pipetted into a hole (3 mm diam) punched previously into the agar. Each test was replicated ten times.

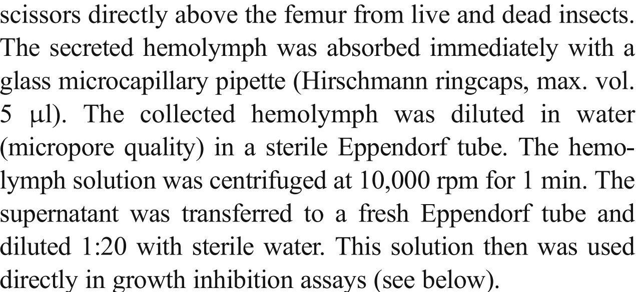
Evaluation of Antibacterial and Antifungal Activity After application of the hemolymph samples, Petri dishes were kept at 37°C for 24 hr. Diameters of inhibition zones caused by the samples through radial diffusion into the agar were measured after 24 hr. Series of similar inhibition zone tests were conducted using different concentrations of the antibiotic gentamicin (Serva) for all bacteria or nystatin (Serva) for yeast. The straight calibration lines obtained were used to calculate the antimicrobial activity in μg/ml of the respective antibiotic.

Activity Determination for Key Enzymes

Measuring of Enzyme Activity Muramidase (lysozyme-like) activity against cell walls of Gram-positive bacteria was measured by using the lytic zone assay with freeze-dried *M. luteus* (Sigma, Deisenhofen, Germany) as described previously (Gross et al., 2002). Some of the hemolymph samples caused clear zones in the turbid agar by dissolving the bacterial cell walls. Diameters of these lytic zones were measured after 24 hr of incubation at 37°C. Units/ml lysozyme were calculated using dilutions of chicken lysozyme (Sigma).

Determination of the Minimal Inhibitory Concentration (MIC)

Hemolymph Collection Hemolymph was collected from unchallenged adult beetles. The number of replications for each test group was >5. One hind leg was cut with micro-



scissors directly above the femur from live and dead insects. The secreted hemolymph was absorbed immediately with a glass microcapillary pipette (Hirschmann ringcaps, max. vol. 5 μl). The collected hemolymph was diluted in water (micropore quality) in a sterile Eppendorf tube. The hemolymph solution was centrifuged at 10,000 rpm for 1 min. The supernatant was transferred to a fresh Eppendorf tube and diluted 1:20 with sterile water. This solution then was used directly in growth inhibition assays (see below).

Assays to Determine the Minimal Inhibitory Concentration (MIC) of the Hemolymph The antimicrobial activity of hemolymph from live and dead beetles was evaluated with broth microdilution assays. Bacteria and yeast were grown overnight in liquid media with shaking at 28°C. Prior to experiments an inoculum of bacteria was transferred to liquid Mueller Hinton Broth (MHB) (Fluka), while the yeast strain was cultivated in Sabouraud Dextrose Broth (Roth). Microbial growth was monitored as absorbance of the overnight cultures at 600 nm. Aliquots were removed and plated on the respective nutrient agar in order to determine the numbers of CFU at 28°C. The optical density (OD) of each culture was determined, and cultures were diluted to a final concentration of 10^6 CFU. Ninety-six well flat bottom microtiter plates (Sarstedt) were used in the inhibition assays. The cultures were serially diluted. Forty μl hemolymph solution at a concentration of 5 μl/ml medium were pipetted into the first well and then 1:2 diluted with each of the following pipetting steps until the 12th well in each row. In each assay, the inhibitory effect of hemolymph from live and dead beetles was compared for the organism tested. Gentamicin at a concentration of 50 μg/ml was used as positive reference. In the first well, 20 μg/ml gentamicin were applied and 1:2 diluted until the 12th well. For MIC tests with *S. cerevisiae*, 50 μg nystatin per ml dimethylsulfoxid (DMSO) were used as reference. Aliquots of 100 μl of the bacterial and fungal cultures were added to each well. All microorganisms were tested separately. All tests were repeated five times with three rows from the same hemolymph pool each time. Microtiter plates were incubated in a shaker for 22 hr at 28°C. Antimicrobial activity was assessed by visually detecting turbidity in the wells. Growth inhibition was verified with a spectrophotometer at 600 nm (Microplate reader Floustar Omega, BMG Labtech). Data were collected with the software Omega 1.10 (BMG Labtech) and analyzed (Mars 1.11, BMG Labtech). The MIC was defined as the lowest concentration of the hemolymph or the antibiotic that inhibited all visually detectable microbial growth.

Statistical Analysis Differences in the antimicrobial activity of hemolymph from adults and larvae of the two ladybird species *C. septempunctata* and *H. axyridis*, as well as their

lysozyme-like activity were analyzed by Mann-Whitney *U* tests (Sachs, 1992). All statistical tests were performed with Statistica 5.5 software (StatSoft, 1999).

Results

Antimicrobial Activity The hemolymph of both adults and larvae of *C. septempunctata* showed a weak activity against Gram-positive bacteria (*M. luteus*), nearly no activity against Gram-negative bacteria (*E. coli*), and no activity against yeast (*S. cerevisiae*) (Table 1). While injection of sterile water increased the antibacterial activity in adults, only the injection of a dense bacterial suspension induced an increase in activity against Gram-negative *E. coli* in larvae. The activity against Gram-positive *M. luteus* increased slightly in larval hemolymph after water injection, but increased significantly when dead bacteria were injected ($P<0.05$, Mann-Whitney *U* test). In contrast, the hemolymph of *H. axyridis* strongly inhibited the growth of both bacteria and yeast (Table 1). Inhibition was strongest against *E. coli*, followed by *M. luteus*, and lowest against yeast. Compared to *C. septempunctata*, the antibacterial activity in *H. axyridis* hemolymph was between 640 (Gram-positive bacteria) and up to 16,000 times (Gram-negative bacteria) higher. These results were obtained irrespective of whether the ladybirds had been untreated or immune challenged ($P>0.05$, Mann-Whitney *U* test). The activity against yeast decreased after injection of water or an inactivated bacteria suspension into larvae, and after injection of bacteria in adults compared to untreated control ($P>0.05$, Mann-Whitney *U* test).

Minimal Inhibitory Concentration (MIC) The hemolymph of live *H. axyridis* inhibited the growth of yeast and all three bacteria tested. Inhibition was strongest for *E. coli* and *S. cerevisiae*, followed by *B. subtilis*, and lowest for *B. thuringiensis* subsp. *tenebrionis*. This species also was least susceptible to gentamicin (Table 2). After freezing the beetles at −20°C for a period of 13 to 66 days prior to hemolymph collection, the inhibitory activity decreased significantly, but the same susceptibility pattern was observed (Table 2). The concentration of gentamicin necessary to fully inhibit growth of *E. coli* was 0.2 µg/ml. The amount of hemolymph from live beetles (0.16 µl/ml) necessary for similar growth inhibition of *E. coli* was about four times lower than the hemolymph amount necessary to fully inhibit *B. subtilis* (0.63 µl/ml), and 2.5 µl/ml were necessary to inhibit the growth of *B. t. tenebrionis* (Table 2). The inhibitory effect of hemolymph from frozen beetles was eight times lower for *E. coli*, eight times lower for *B. subtilis*, and 2 times lower for *B. t. tenebrionis* compared to hemolymph from live beetles. The concentration of antifungal nystatin necessary to obtain complete growth inhibition of yeast was 3.13 µg/ml. A similar growth inhibition was observed by adding 0.16 µl hemolymph from live beetles to the medium. Its antifungal activity was four times higher compared to hemolymph from frozen beetles (Table 2).

Enzyme Activity Enzyme activity in the hemolymph of *H. axyridis* was higher than in *C. septempunctata* (Fig. 1a, b). In larvae of both species, hemolymph activity was higher than in adults. We observed an increase in the lysozyme-like activity in treated larvae (water, bacteria) compared to untreated larvae within both species (Mann-Whitney *U* test, $N=12$, $P<0.001$). There was no statistically significant difference between untreated and treated adults within either species ($P>0.05$, Mann-Whitney *U* test). Differences between treatments were not statistically significant ($P>0.05$, Mann-Whitney *U* test).

Table 1 Mean values and standard deviations of antimicrobial activity of hemolymph of two ladybird species *Coccinella septempunctata* and *Harmonia axyridis* in µg/ml antibiotic equivalent (gentamycin: *Escherichia coli* and *Micrococcus luteus*; nystatin: *Saccharomyces cerevisiae*) analyzed by agar diffusion assay

Stage	Microbe	*C. septempunctata*						*H. axyridis*					
		Untreated		Water		Bacteria		Untreated		Water		Bacteria	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Larva	*E. coli*	0^{a}	0	0^{a}	0	9^{b}	9	9064^{a}	4654	16460^{a}	9192	15249^{a}	14348
	M. luteus	0^{a}	0	41^{b}	94	116^{c}	63	6376^{a}	2585	8618^{a}	4365	8723^{a}	4592
	S. cerevisiae	0^{a}	0	0^{a}	0	0^{a}	0	223^{a}	86	131^{b}	60	110^{b}	41
Adult	*E. coli*	0^{a}	0	6^{b}	7	4^{b}	3	8818^{ab}	4021	6004^{a}	4070	11925^{b}	6807
	M. luteus	0^{a}	0	24^{b}	33	28^{ab}	41	23803^{a}	8941	21401^{a}	7786	18548^{a}	10291
	S. cerevisiae	0^{a}	0	0^{a}	0	0^{a}	0	1963^{a}	721	1681^{ab}	750	1090^{b}	571

Numbers of replications for treatments of adults are >9, for treatments of larvae are 12. Different letters indicate significant differences within treatments (*Mann-Whitney U test*, $P<0.05$)

Table 2 Minimal inhibitory concentrations (MIC) of hemolymph of live and frozen adult *Harmonia axyridis* (µl/ml) and antibacterial (gentamycin) or antifungal (nystatin) standards (µg/ml)

Agent	*E. coli*	*B. subtilis*	*B. thuringiensis*	*S. cerevisiae*
live	0.16	0.63	2.50	0.16
frozen	1.25	5.00	>5.00	0.63
Gentamycin	0.20	0.20	3.13	–
Nystatin	–	–	–	3.13

The number of replications for each group is >5

Headspace Three out of eleven tested VOCs known from the headspace of *H. axyridis* (phenol, (+)-α-pinene, benzaldehyde) inhibited the growth of bacteria, *E. coli* and *M. luteus*, as well as the yeast (Fig. 2). The other VOCs tested showed no inhibitory effects.

Discussion

The hemolymph from unchallenged *H. axyridis* strongly inhibited the growth of all tested microorganisms. By contrast, the hemolymph from unchallenged *C. septempunctata* showed no inhibitory effects against the same microorganisms. After injection of water or inactivated bacteria, the antibacterial activity in the hemolymph of *C. septempunctata* adults and larvae increased significantly. It is striking that neither treatment challenged the immune system of *H. axyridis*, but at the same time decreased the antifungal activity in its hemolymph. To date no other study has reported similar findings. This leads us to suggest a possible interference in the induction of different immune pathways. It was reported earlier (Lemaitre et al., 1997) that insects might respond to different challenges and, thus, adapt their disease resistance accordingly while

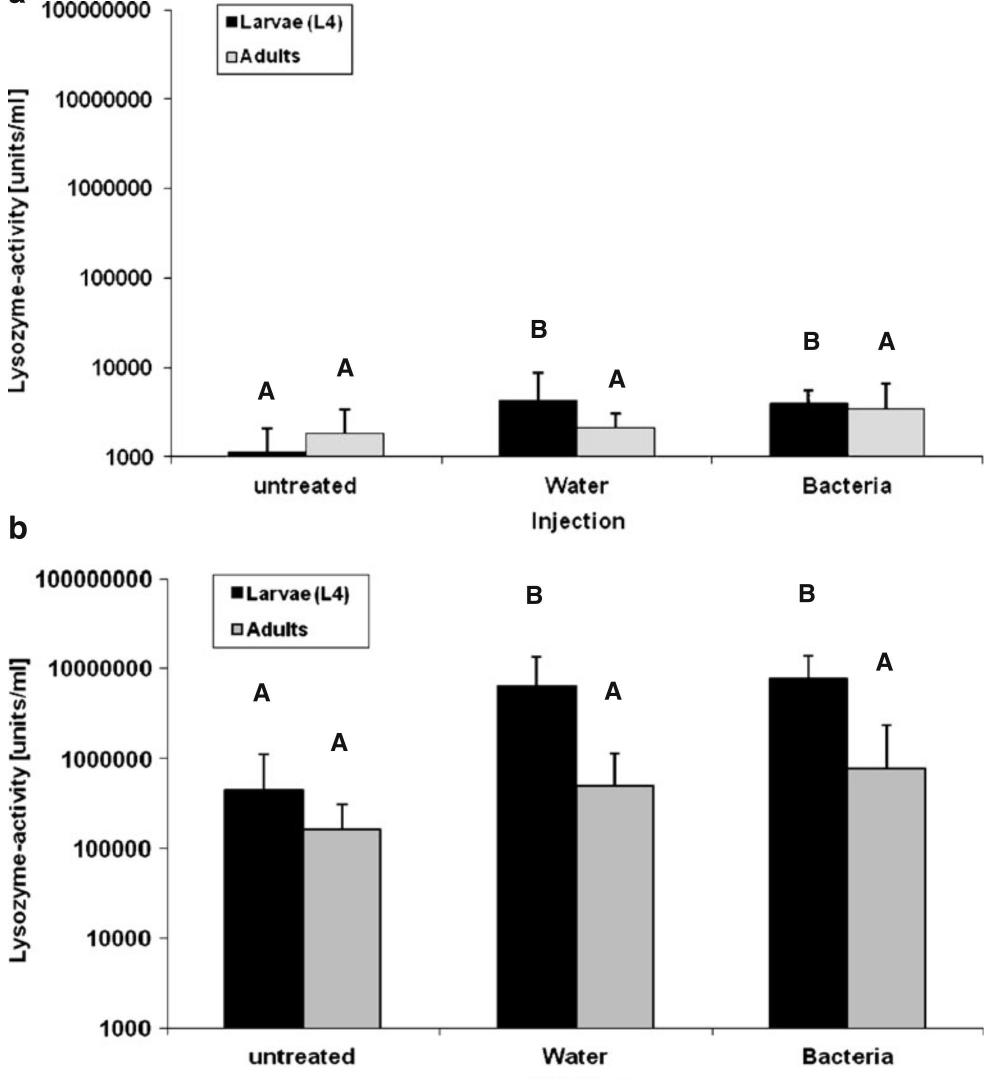


Fig. 1 Lysozyme activity [units/ml] of the hemolymph of ladybird beetles. **a**: *Coccinella septempunctata*. **b**: *Harmonia axyridis*. While there is no statistically significant difference between the three treatments of adults of both species (*grey bars*), there is an increase in the lysozyme-like activity in treated larvae (water, bacteria) compared to untreated larvae within both species (*black bars*; Mann-Whitney *U* test, N=12, P< 0.001)

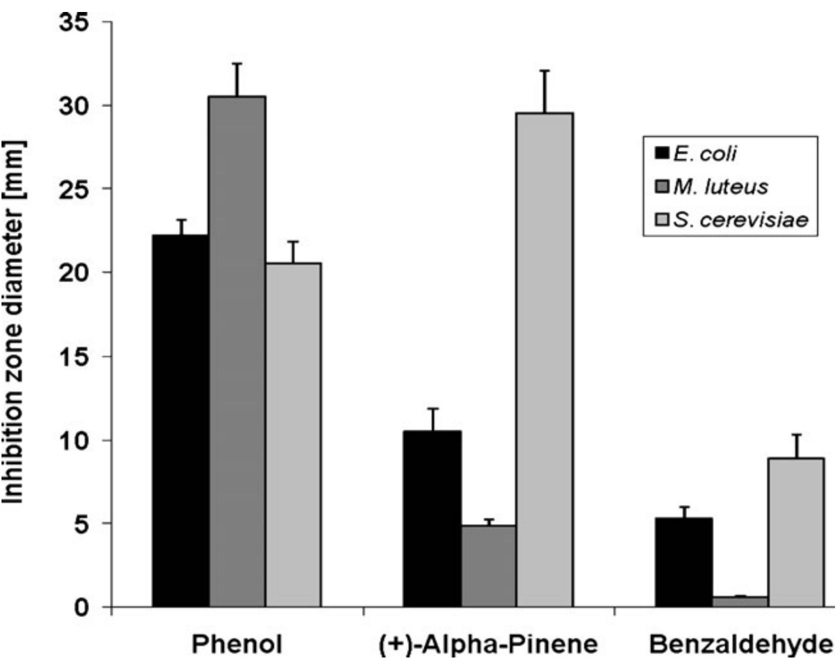


Fig. 2 Growth inhibition of bacteria (*Escherichia coli, Micrococcus luteus*) and yeast (*Saccharomyces cerevisiae*) by synthetic VOCs (phenol, (+)-α-pinene, benzaldehyde) present in the headspace of *Harmonia axyridis*. *Bars* represent mean and standard deviation of inhibition zone diameters (mm, N=10)

minimizing the resources necessary for this defensive response.

Lysozyme activity against *M. luteus* was more than hundredfold higher in the harlequin ladybird when compared to *C. septempunctata*. Induction increased the lytic activity in both larvae but not in adults of either species (Fig. 1). The higher activity of this enzyme might assure increased survival rates of larvae in the populations of the invasive *H. axyridis* compared to *C. septempunctata*. Furthermore, adults of *H. axyridis* additionally produce 38 volatile organic compounds (VOCs) (Cai et al., 2007). Three of them, phenol, (+)-α-pinene, and benzaldehyde, inhibited the growth of both bacteria and yeast in our assays. Additionally, a synergistic amplification of antimicrobial active VOCs is possible. These compounds may engulf the insects with an antimicrobial cloud. This phenomenon was recently reported for leaf beetles (Gross et al., 2008; Gross and Schmidtberg, 2009). Such a mechanism could be important, especially during overwintering, as *H. axyridis* forms aggregations consisting of hundreds of individuals in cavities under rocks or plants. Further studies will be necessary to test this hypothesis.

The MIC assays showed, that hemolymph of untreated adult *H. axyridis* inhibited the growth of Gram-negative *E. coli* and *S. cerevisiae* more strongly than the growth of Gram-positive bacteria (*B. subtilis* and *B. t. tenebrionis*). Moreover, the hemolymph from live beetles was significantly more efficient than hemolymph from frozen beetles against all organisms tested. We observed a difference in the susceptibility of *S. cerevisiae* between these two assays. The yeast was more susceptible to hemolymph in the liquid medium MIC assays compared to agar diffusion assays. This could be due to a better contact of the active compounds in the fluid than on the agar.

Antimicrobial peptides (AMP) effective against Gram-negative organisms known from invertebrates generally are produced after recognition of foreign compounds. They are, thus, considered induced defenses. We observed the contrary in *H. axyridis*; the hemolymph of unchallenged beetles caused in agar diffusion assays and MIC tests strong inhibition of microbial growth. This finding suggests high concentrations of constitutive antimicrobial compounds present in hemolymph. Inducible AMPs have been isolated from several insect orders, but few studies have been conducted with Coleoptera (Bulet et al., 1991). The chemical nature of the antimicrobial compounds in the hemolymph of *H. axyridis* is not yet known. The costs of maintaining a highly efficient immune system have been related to an inferior tolerance of starvation and desiccation in parasitoid resistant *Drosophila* (Hoang, 2001). Few data are available on stress tolerance of *H. axyridis* (Agarwala et al., 2008) and nothing is known how stress affects its immune system. We currently are conducting studies to evaluate various stress factors and their effects on the antimicrobial defense of *H. axyridis*.

Interestingly, in microtiter plate assays, we observed that the hemolymph of *H. axyridis* partially lost its activity after freezing live beetles at −20°C prior to hemolymph extraction. This effect was strongest against Gram-positive bacteria (*Bacillus subtilis, B. thuringiensis*) and weaker against Gram-negative *E. coli* and yeast (Table 2). A destruction of the antimicrobial compound due to the death

of the beetles is unlikely because the freezing of live beetles should stop immediately any destructive physiological processes. In an earlier study, it was suggested that low temperatures may change the qualitative and quantitative composition of antimicrobial peptides in insect hemolymph (Stephen and Johnson, 1962). The observed changes were, however, variable between species and age groups of the tested cockroaches. Duration of freezing had no effect on protein composition. Stephen and Johnson (1962) found qualitative changes of hemolymph proteins more pronounced for weak clot producing cockroach species. The hemolymph of multicolored lady beetles produces such clots only very slowly upon secretion (pers. observ.). Corresponding to Stephen's observations, protein stability is not assured after freezing and thawing of hemolymph samples. The reduction of the antimicrobial efficacy observed in our studies might be due to thawing of the frozen beetles prior to hemolymph collection. To our knowledge, no other studies have reported a similar loss of the inhibitory effect, since neither hemolymph from live nor from dead insects has been evaluated. Further assays on the kinetic of microorganism growth are necessary to verify the bactericidal/fungicidal or merely growth inhibitory effect of the hemolymph observed in our assays.

Gram-negative *E. coli* and the yeast *S. cerevisiae* were more susceptible to the antimicrobial substances in the hemolymph of adult *H. axyridis* than the two Gram-positive *Bacillus* species. In contrast, the third Gram-positive species tested, *M. luteus*, also was strongly inhibited. It has been reported that *M. luteus* is highly sensitive to chicken lysozyme (Altincicek et al., 2008). The high lysozyme activity in the hemolymph of *H. axyridis* compared to *C. septempunctata* may explain the strong inhibition of bacteria by both adult and larval hemolymph. It is not surprising that higher concentrations of antimicrobial compounds were necessary to inhibit the growth of insect pathogenic *B. t. tenebrionis*. This strain has been isolated from a beetle (*Tenebrio molitor*) and is mainly infective for beetles (Krieg et al., 1984). Recently, it was reported that the antimicrobial peptide rhinocerosin isolated from the coconut rhinoceros beetle is more active against Gram-positive bacteria with a MIC value of 1.0 µg/ml (Yang et al., 1998). A study on two different synthetic antimicrobial peptides found a wide variation in MIC values of 128 µg/ml, 16 µg/ml, and 4 µg/ml against *E. coli* (Kamysz and Turecka, 2005). For *H. axyridis*, it is not yet known whether antimicrobial proteins or other factors (review in Boman and Hultmark, 1987) are responsible for the observed microbial growth inhibition.

The differences in the antifungal activity between the two ladybird species tested are notable. Whereas the hemolymph from untreated adult *C. septempunctata* had no antifungal activity, the inhibitory activity of hemolymph from adult *H. axyridis* beetles corresponded to 100–1,000 µg/ml of the antimycotic nystatin. Little is known about the antifungal activity of insect hemolymph, probably because of a low search effort for antifungal proteins (Bulet and Stöcklin, 2005). Few studies, however, have reported strong growth inhibition of fungi by inducible insect hemolymph compounds (Lamberty et al., 1999), and defensins and cecropins are known for their antibacterial but also antifungal activities (Vilcinskas and Gross, 2005). Hence, our study is the first to report antifungal activity in an unchallenged beetle species. The presence and rapid response of the constitutive defenses in *H. axyridis* might be another factor that could help explain the competitive superiority of this exotic species when compared to native ladybird beetles.

In the present study, we did not differentiate between sexes or morphs with different elytral colors. Recent preliminary data suggest no differences in disease resistance between female and male *H. axyridis* (Gross, unpubl. data). Further studies of variations in hemolymph activity in different life stages, sexes, and morphs are necessary to understand its importance for the competitive success of *H. axyridis* (Fedorka et al., 2004).

Most studies have focused on behavioral differences between *H. axyridis* and native ladybirds in Europe (Soares and Serpa, 2007; Brown et al., 2008), Canada (Labrie et al., 2006), the USA (Yasuda et al., 2004) and Japan (Ware and Majerus, 2008). Larger size and physically better defended larval stages contribute to higher survival rates (Snyder et al., 2004) and to superiority in aggressive encounters with other ladybird species (Pell et al., 2008). Cannibalism and intraguild predation have been observed even when aphid prey is abundant (Pell et al., 2008). In Canada, *H. axyridis* has a shorter development time for younger larval stages than indigenous ladybird species (Labrie et al., 2006). Furthermore, *H. axyridis* adults are rarely preyed upon (Pell et al., 2008), and under laboratory conditions, adult beetles are rejected by the predatory hemipteran, *Podisus maculiventris* (Houg-Goldstein et al., 1996; DeClercq et al., 2003). Cottrell and Shapiro-Illan (2003, 2008) found that *H. axyridis* is more resistant to various *B. bassiana* strains when compared to highly susceptible native coccinellids. Roy et al. (2008) showed for three species of ladybirds that much higher concentrations of *Beauveria bassiana* conidia were required to artificially infect *H. axyridis*.

In summary, the observed disease resistance against antagonistic microorganisms is one more fitness parameter that contributes to the enormous capacity of *H. axyridis* to exploit new habitats and to out-compete other coccinellid species. The importance of disease resistance for life history traits and population dynamics of *H. axyridis* under different climatic and geographic conditions awaits further studies.

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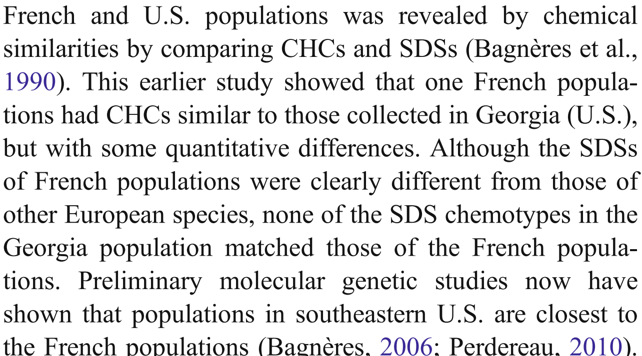
shown that environmental factors such as food, temperature, and social environment can affect the composition of CHC profiles (Florane et al., 2004; Dronnet et al., 2006). Although the processes underlying the production of chemical signatures are not well understood, analysis of these signatures has proved to be effective for termite classification. Qualitative differences in hydrocarbons can discriminate between species, whereas quantitative differences can discriminate between populations and colonies (Haverty et al., 1997; Page et al., 2002; Bagnères and Wicker-Thomas, 2010). Analysis of CHCs among Rhinotermitidae has helped to clarify the taxonomy of the Asian *Reticulitermes* (Takematsu and Yamaoka, 1999); the Australian *Heterotermes* and *Coptotermes* (Watson et al., 1989; Brown et al., 1990); the American *Coptotermes* and *Reticulitermes* (Bagnères et al., 1990; Haverty et al., 1991, 1996, 1997, 2000); and the European *Reticulitermes* (Clément et al., 2001).

In the Rhinotermitidae and Termitidae, soldiers have a frontal gland that secretes defensive compounds. These soldier defensive secretions (SDSs) are believed to play a role in defending colonies against predators and competitors (Zalkow et al., 1981), and they also may play a role in the production of primer pheromones in *Reticulitermes* (Henderson, 1998; Tarver et al., 2009). SDSs can be composed of alkanes, aldehydes, ketones, and terpenes, as well as more complex compounds (Quintana et al., 2003; Piskorski et al., 2007). They have often been studied in combination with CHC profiles as their composition differs geographically and among taxa (Bagnères et al., 1990), and they can be useful for identifying species (Bagnères et al., 1990; Haverty et al., 1996; Nelson et al., 2001, 2008; Clément et al., 2001; Piskorski et al., 2009).

The use of chemical compounds for taxonomy (chemotaxonomy) has been widely used and proved valuable in numerous termite species, particularly *Reticulitermes*. However, less attention has been paid to chemical variation within species, in particular with respect to invasive species (Haverty et al., 1990). This study analyzed both CHCs and SDSs within native and introduced populations of *Reticulitermes flavipes* (Kollar). In the United States, this termite is known as the eastern subterranean termite, but it has been introduced and established in other countries such as Canada, Chile, and Uruguay, as well as France and Germany (Clément et al., 2001; Austin et al., 2002, 2005; Su et al., 2006). For a long time, the introduced populations of France were considered as a European species, *Reticulitermes santonensis* (Feytaud, 1924). They are now considered to be introduced populations of *R. flavipes* on the basis of the homology of several mitochondrial and nuclear DNA sequences (Clément et al., 2001; Jenkins et al., 2001; Austin et al., 2002, 2005; Ye et al., 2004; Su et al., 2006), although the first correspondence between



French and U.S. populations was revealed by chemical similarities by comparing CHCs and SDSs (Bagnères et al., 1990). This earlier study showed that one French populations had CHCs similar to those collected in Georgia (U.S.), but with some quantitative differences. Although the SDSs of French populations were clearly different from those of other European species, none of the SDS chemotypes in the Georgia population matched those of the French populations. Preliminary molecular genetic studies now have shown that populations in southeastern U.S. are closest to the French populations (Bagnères, 2006; Perdereau, 2010).

This study determined and analyzed both CHCs and SDSs from three introduced populations in France and six native populations in the southern U.S. The main aims were (1) to determine whether an analysis of the chemical variation permits the discrimination of geographical populations and localities, and (2) to evaluate the degree of chemical similarity and variability among native and introduced populations.

Methods and Materials

Field Collection and Sampling In the U.S., samples were collected in Florida and Louisiana as it has been suggested that populations in France may have originated from these areas (Bagnères, 2006; Perdereau, 2010). In Florida, 3 populations were collected from 12 collection points, 3 in the Blackwater River State Forest, 3 in the Wakulla State Forest, and 6 in the Osceola National Forest (Fig. 1). In Louisiana, 3 populations were collected from 16 collection points, 6 in New Orleans, 6 in Jean Lafitte National Historical Park and Preserve, and 4 in Baton Rouge (Fig. 1). In France, 3 introduced populations were taken from 43 collection points: 20 in the Forêt de Saint Trojan in the south of the Ile d'Oléron (Charente Maritime), 20 in the Forêt d'Olonnes (Vendée), and 3 in Tours (Indre et Loire) (Fig. 1). Termites from these 9 populations were collected from 2006 to 2009. To draw comparisons on a similar scale, the distance between each population did not exceed 200 km, and transects within each population were less than 2 km, except for the populations in Oléron and Olonnes, which were on a larger scale (4 km). Samples from the 9 populations studied were collected from wood fragments or tree stumps at the 9 localities. At least 20 workers were taken from each collection point. For these 71 samples, the species was determined by morphological and chemical identification for the French populations and DNA analysis for the U.S. populations as described previously (Clément et al., 2001; Austin et al., 2002).

Analyses of Cuticular Hydrocarbons Twenty workers per collection point (71 in total with 12 from Florida, 16 from

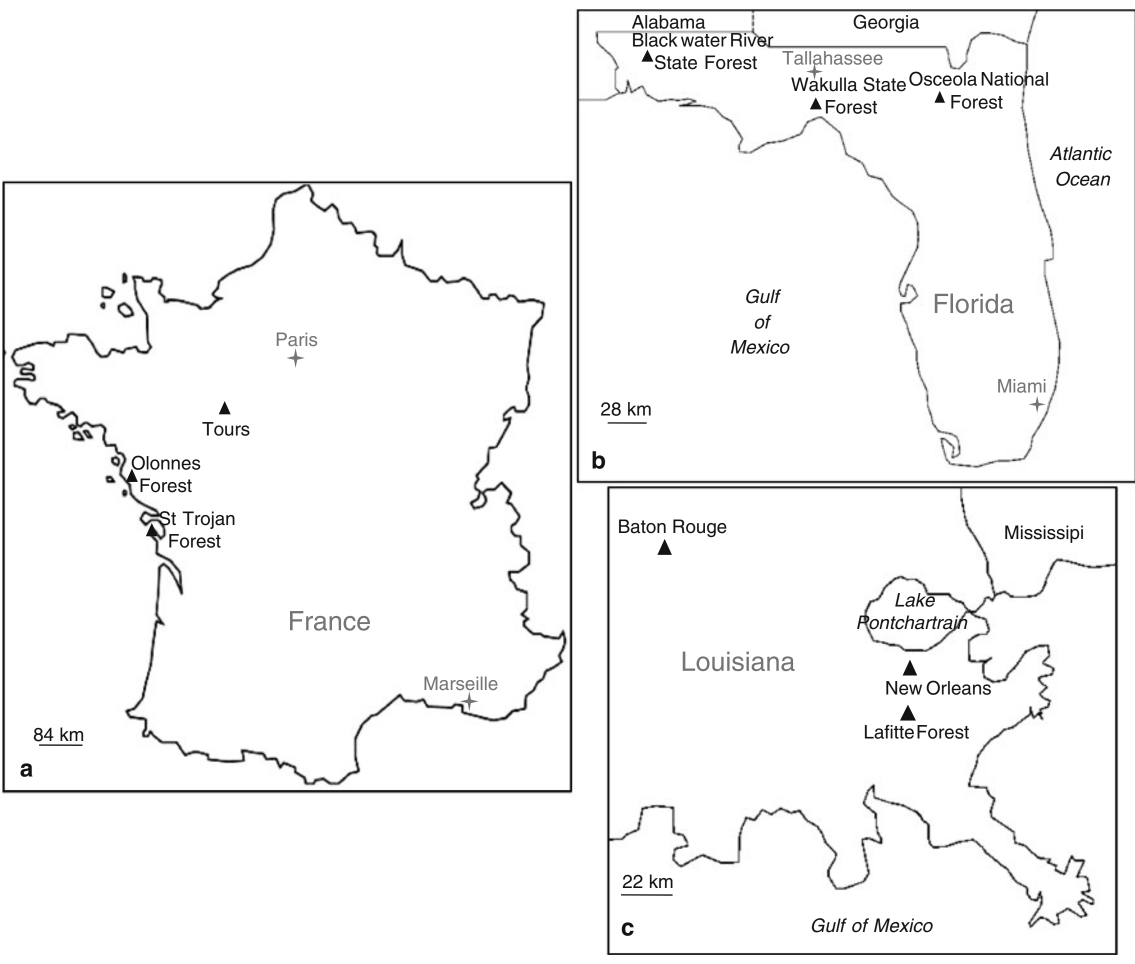


Fig. 1 Locations of *Reticulitermes flavipes* populations in (**a**) France, (**b**) Florida, and (**c**) Louisiana

Louisiana and 43 from France) were pooled for chemical extraction. The CHCs in each pool were extracted by rinsing individuals in a non-polar solvent (hexane in the U. S. and pentane in France) for 5 min, and then samples were dried for transport and stored at −20°C until analysis (1 month at the latest). Before injection, extracts were re-dissolved in 200 µl of pentane with 10 µl of 10^{-7} g/ml of *n*-eicosane (*n*-C20) as an internal standard. Samples (2 µl) were analyzed by GC with a Delsi Nermag DN 200, flame ionization detection (FID) (Alpha MOS, Toulouse, France), and a fused silica capillary column CP Sil 5 (WCOT) (Varian Inc., Palo Alto, CA, USA) (i.d. 0.25 mm×25 m×0.12 µm). The injection mode was splitless (15 sec), and the carrier gas was helium at a linear flow rate of 1.5 cm/sec. The temperature was programmed from 70°C to 150°C at 30°C/min and held at 150°C for 5 min, and then raised to 320°C at 5°C/min. Compound identification was based on previously reported analyses of CHCs with GC-MS (Howard et al., 1978; Bagnères et al., 1990), and checked by us by injecting 2 µl of pooled sample of each population in a GC-MS (Hewlett Packard 5890 GC (Agilent Technologies, Santa Clara, CA, USA) coupled to a Hewlett Packard quadrupole 5889A MS (Agilent Technologies) in electron impact mode (70 eV)) with the same column and temperature program as noted above for GC-FID, with an interface temperature of 250°C. Eighteen main CHCs present in all individuals were selected for analysis: 9-tricosene (*e1*), x-tricosene (*e2*), *n*-tricosane (*a3*), 11-methyltricosane (*m4*), 4/2-methyltricosane (*m7*), 9-tetracosene (*e8*), 3-methyltricosane (*m9*), *n*-tetracosane (*a11*), 11-methyltetracosane (*m12*), 5-methyltetracosane (*m14*), 4/2-methyltetracosane (*m16*), 9-pentacosene (*e17*), pentacosadiene (*n18*), *n*-pentacosane (*a19*), 11+13-methyl pentacosane (*m21*), 7,9-pentacosadiene (*n25*), 4/2-methylpentacosane (*m26*), and 3-methylpentacosane (*m29*). The areas under these 18 peaks were integrated by using Galaxie v.1.8.508.1 (Varian), and the relative proportions of each peak were calculated as described by Bagnères et al. (1990). Discriminant analyses were performed by using Rgui v.2.10.1. to determine whether pre-defined groups (i.e., the 3 localities and the 9 populations) could be discriminated on

the basis of their chemical profiles. This confirmed that the groups corresponded to the classification of the collection points. The same software was used to carry out principal component analysis (PCA) to determine the chemical relationship among the collection points.

The dissimilarity of hydrocarbon profiles among workers from different collection points was quantified by modifying Nei's standard genetic distance (Nei, 1987) as previously described (Queller, 1993; Dronnet et al., 2006) and by using Euclidean distances. The Nei and Euclidean distances were based on the relative amounts of chemical compounds. For each distance, dissimilarity matrices were constructed for all possible pairs of collection points from the mean relative areas of the CHC peaks at different levels: within each population, among populations within each locality (France, Florida, and Louisiana) and among each pair of localities (France/Florida, France/Louisiana, and Florida/Louisiana). Nei and Euclidean distances vary between 0 and 1; 0 indicates that the chemical profiles are identical, whereas 1 indicates that there are no shared compounds. Non-parametric Kruskal-Wallis tests were used for multiple independent comparisons of populations, localities, and groups of localities. Dunn's multiple comparison tests were carried out to define the specific difference between cuticular compound variations at each level of comparison by using XLSTAT v.2009.3.1.

Analyses of Soldier Defensive Secretions Defensive compounds were extracted from 50 soldiers (13 from France, 17 from Florida, and 20 from Louisiana). Because SDSs are volatile compounds, a special method was used for extraction and transport. Each extraction was performed by plunging one soldier into 20 µl of solvent (pentane or hexane) in a conical glass insert for 2 min. Each extract then was transferred to a Transferpettor (BRAND GMBH+ CO KG, Wertheim, Germany) cap (4×0.2 cm), one end of which was previously sealed. The other end of the glass cap was rapidly flame-sealed to avoid the evaporation of SDSs during transport to the laboratory. Two internal standards were used to check the extraction: one was added to the empty cap before extraction (*n*-octadecane) and the second was added just before injection (humulene). Two µl of each extract were analyzed by GC-MS with the instrumentation described above. The temperature program ran from 40°C to 200°C at 5°C/min and then increased at a rate of 8°C/min to 320°C with helium as carrier gas at a linear flow rate of 1.5 cm/sec. The chemotype of each sample then was determined according to the presence or absence of peaks on the GC traces. When present, determination of compounds was confirmed by comparing their retention times and mass spectra with data from previous studies on U.S. and French *R. flavipes* soldiers (Zalkow et al., 1981; Bagnères et al., 1990; Nelson et al., 2001).

Results

Proportions of Cuticular Hydrocarbons in French and American Populations The relative proportion of each cuticular hydrocarbon was determined for the 71 collection points. No qualitative difference was apparent in the CHC components of workers. All profiles had the same 18 hydrocarbons that had previously been found in *R. flavipes/ R. santonensis* (Bagnères et al., 1990) (Fig. 2). However, there were quantitative variations among the collection points.

Discriminant analysis revealed that the relative proportions of a large number of cuticular hydrocarbons discriminated both populations and localities. No single component could be used to separate the nine populations and the three localities, as nearly the whole of the chemical signature (72–83% of the peaks) was needed. The first discriminant analysis performed on relative amounts of the 18 peaks of each collection point discriminated the nine populations significantly. Of these, the relative proportions of 15 compounds (i.e., *e2*, *a3*, *m4*, *m7*, *e8*, *m9*, *a11*, *m12*, *m14*, *n18*, *a19*, *m21*, *n25*, *m26,* and *m29*) varied significantly among populations (*Wilks'* $\lambda<0.05$, $F=12.25$, $df=120, 354$, $P<0.001$). The two first principal axes accounted for 76.89% of the overall variance between groups (the first axis accounted for 46.14% and the second for 30.74%). A large percentage (97.18%) of the collection points were classified correctly in the original groups, with only two collection points of the Oléron population assigned to the Olonnes population. The second discriminant analysis performed on the 3 localities distinguished France, Florida, and Louisiana significantly (Fig. 3). Discriminant analysis selected 13 peaks (i.e.,. *e1*, *e2*, *a3*, *e8*, *m9*, *a11*, *m12*, *m14*, *e17*, *n18*, *a19*, *n25,* and *m29*), grouping all collection points within the assigned localities (*Wilks'* $\lambda<0.05$, $F=37.77$, $df=24, 114$, $P<0.001$). The two first axes accounted for 100% of the chemical variation between groups, with 70.27% of the variation explained by the first axis and 29.73% by the second axis. A large percentage (98.59%) of the collection points was correctly assigned to the original locality group, with only one collection point in Louisiana being grouped with the French colonies.

Principal component analysis based on cuticular hydrocarbon profiles of worker samples revealed that the first two principal components accounted for 41% of the total chemical variation (Fig. 4). The first axis accounted for most of the variation (25%) among Florida and Louisiana populations. The second axis accounted for 16% of the variation, distinguishing the Florida populations from the others. Principal component analysis showed that proportions of CHCs in French populations appeared closer to the three Louisiana populations than the three Florida populations. Chemical variations within and between populations

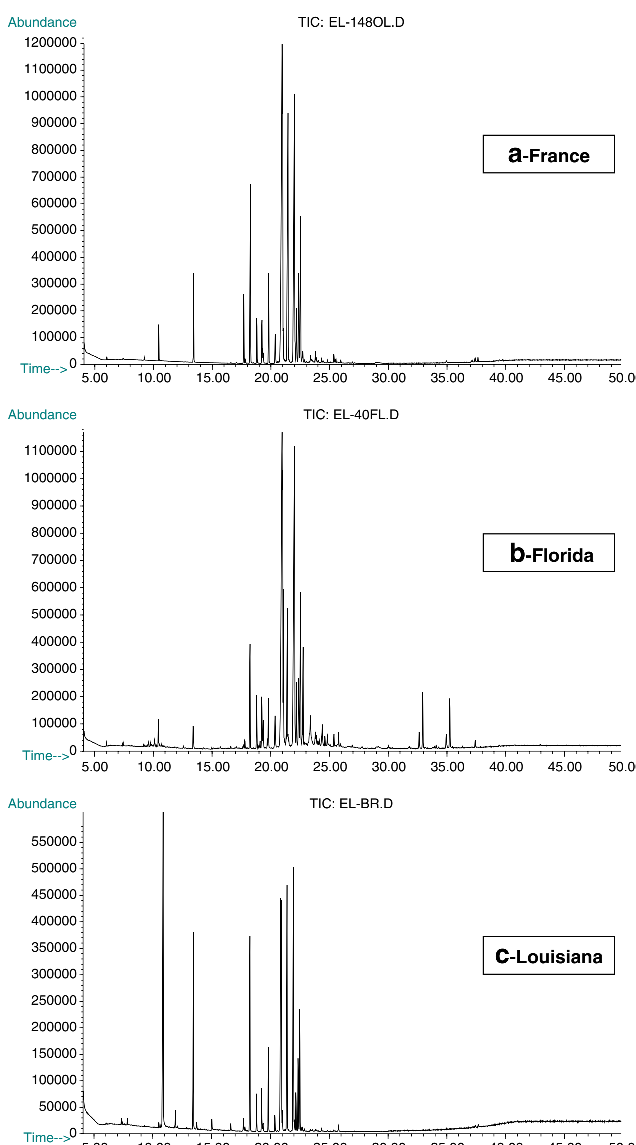


Fig. 2 Total ion chromatograms of GC-MS analysis of cuticular hydrocarbon extracts from populations of *Reticulitermes flavipes* from (**a**) France, (**b**) Florida, and (**c**) Louisiana. (the 2 first peaks between 10 to 14 min are the 2 internal standards)

in France appeared lower than in Florida and Louisiana. Although the French populations in St Trojan and Olonnes covered a greater geographical area than the American populations, their chemical profiles were less variable.

Chemical Dissimilarity Distances The chemical dissimilarity distances (Euclidean and Nei) within populations, between populations within localities, and between pairs of localities were calculated (Table 1). Non-parametric tests used to compare the chemical dissimilarity distances showed similar results for the Euclidean and Nei distances. The chemical distance within each population (Table 1) was similar for all the populations except for the Olonnes population which had a low variability in the CHC composition (Kruskal-Wallis test, $P<0.001$; Dunn's procedures for Olonnes-Oléron, $P<0.001$; Olonnes-Osceola, $P<0.001$; Olonnes-Black River, $P<0.05$; Olonnes-Wakulla, $P<0.05$; Olonnes-New Orleans, $P<0.001$; Olonnes-Lafitte, $P<0.001$), although it was similar to the chemical distances within the Tours and Baton Rouge populations. The chemical dissimilarity distances, both Euclidean and Nei distances, among populations within localities were significant (Table 1). The chemical distances between French populations were significantly less than those observed between populations in Florida and Louisiana (Kruskal-Wallis test, $P<0.001$; Dunn's procedures for France-Florida, $P<0.001$, France-Louisiana, $P<0.001$). Similarly, the chemical distances among the Louisiana populations were smaller than those observed among the Florida populations (Kruskal-Wallis test, $P<0.001$; Dunn's procedure, $P<0.001$). The chemical distance between localities (Table 1) showed that the distance between French and Louisiana was not significantly different from that between the French and Florida localities (Kruskal-Wallis test, $P<0.001$, Dunn's procedure, $P>0.05$). The chemical distance between the Florida and Louisiana localities was significantly greater than those between the French and Florida localities and the French and Louisiana localities (Kruskal-Wallis test, $P<0.001$, Dunn's procedures for Florida/Louisiana vs. France/Florida, $P<0.001$; Florida/Louisiana vs. France/Louisiana, $P<0.001$).

Soldier Defensive Secretions Six isoprenoid compounds were identified from the SDS extracts: α-pinene, β-pinene, limonene, γ-cadinene, cadinene aldehyde, and geranyl linalool. These compounds were the same as those found previously by Zalkow et al. (1981), Bagnères et al. (1990) and Nelson et al. (2001). Qualitative analyses of the SDSs on the basis of the presence/absence of peaks revealed 6 chemotypes, three of which were different from those found by Bagnères et al. (1990) (Table 2, chemotypes a, e, and f). γ-Cadinene, cadinene aldehyde, and geranyl linalool were present or absent in the various chemotypes, but the monoterpenes α-pinene, β-pinene, and limonene always were present. Two of the chemotypes were found in the French populations, one of which had already been reported (Bagnères et al., 1990; Quintana et al., 2003) with the monoterpenes and the geranyl linalool (Table 2 chemotype b), and the second, the most common in our samples, not previously reported, was composed only of monoterpenes (Table 2 chemotype a). Two chemotypes were observed in Florida and four in Louisiana. One chemotype (chemotype a) was common to France and Louisiana, but none was common to France and Florida.

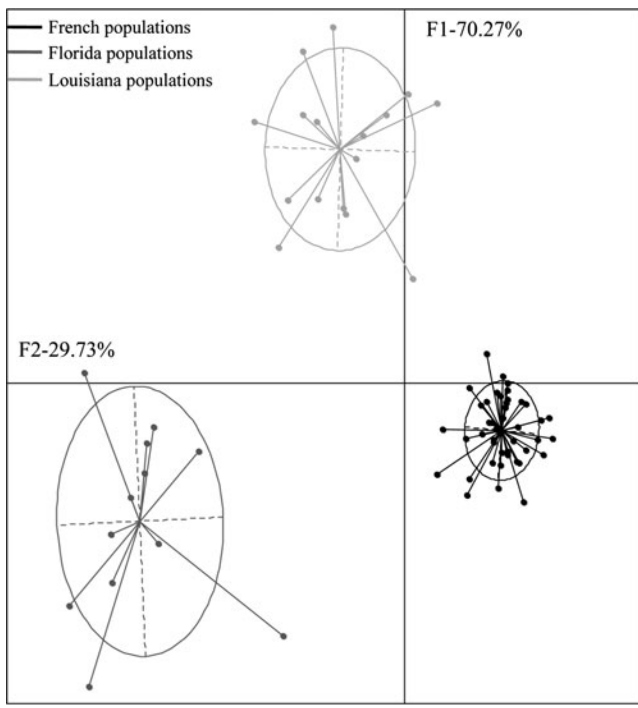


Fig. 3 Chemical differentiation of relative proportions of cuticular hydrocarbons of *Reticulitermes flavipes* among three localities (France, Florida, and Louisiana) on the two first axes of the discriminant analysis. Axes I and II account for 100% (70.27% and 29.73% respectively) of the total variation among localities

Discussion

This study showed that CHCs provide a useful marker for discriminating termite populations, since the results made it possible to discriminate localities and populations within *R. flavipes*. CHC proportions appear to be useful markers for distinguishing the various populations by determining the similarity within each geographical scale.

One major finding was the unexpected chemical homogeneity observed within introduced populations relative to the chemical variation within native populations. Even though chemical distances within populations were not significantly lower in France than in the U.S., the PCA revealed less divergence among the profiles for each collection point for the introduced populations than for the populations in Florida and Louisiana. This result is all the more significant as the comparison was drawn by using a similar geographical scale within populations, except for two French populations, which had a wider area. The use of two different solvents (i.e., hexane and pentane) cannot explain this variation, primarily because the polarities of the two solvents are similar if not identical. Furthermore, the dried samples all were dissolved a second time in pentane before GC analysis. Finally, if these solvents would have caused significant quantitative differences, then these should have been detected by the PCA. The U.S. populations would have been differentiated from the French populations, and the differences between Florida and Louisiana would have been less apparent.

The remarkable hydrocarbon homogeneity observed within the introduced populations of *R. flavipes* compared to the native populations has previously been detected in other introduced social insects. Recent research had revealed a similar change in CHC profiles within introduced ants, *L. humile* (Brandt et al., 2009) and *W. auropunctata* (Errard et al., 2005). These two studies showed that the CHC profiles of ants from native

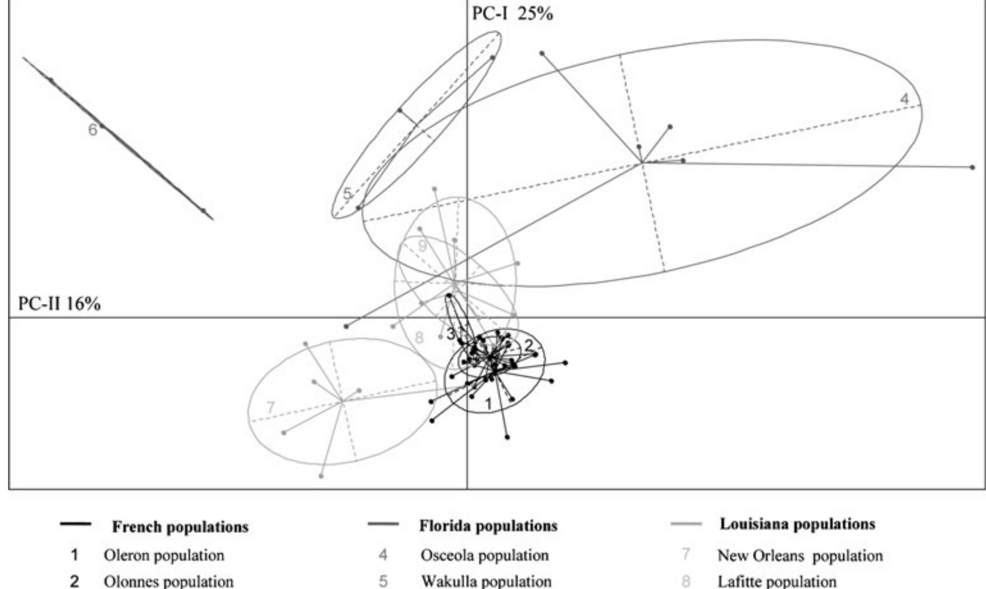


Fig. 4 Plot of the two first axes of the principal component analysis of the proportions of cuticular hydrocarbons from introduced French populations and native U.S. populations of *Reticulitermes flavipes*. Ellipses represent each population from France, Florida, and Louisiana

Table 1 Chemical distances (mean±SE) from a cuticular hydrocarbon analysis of populations of worker *Reticulitermes flavipes* from Florida, Louisiana, and France

		Chemical distance within populations [a]	
		Euclidean	Nei
Populations	Oléron	0.074±0.038 (b)	0.025±0.025 (b)
	Olonnes	0.039±0.017 (a)	0.007±0.006 (a)
	Tours	0.036±0.015 (a,b)	0.005±0.004 (a,b)
	Osceola	0.193±0.136 (b)	0.125±0.013 (b)
	Black river	0.131±0.031 (b)	0.051±0.016 (b)
	Wakulla	0.120±0.016 (b)	0.05±0.015 (b)
	New Orleans	0.098±0.033 (b)	0.041±0.027 (b)
	Lafitte	0.104±0.065 (b)	0.060±0.066 (b)
	Baton Rouge	0.062±0.011 (a,b)	0.015±0.005 (a,b)
Localities	France	0.065±0.033 (a)	0.018±0.019 (a)
	Florida	0.200±0.116 (b)	0.141±0.132 (b)
	Louisiana	0.105±0.052 (c)	0.054±0.055 (c)
Pairs of localities	France/Florida	0.093±0.067 (a)	0.049±0.071 (a)
	France/Louisiana	0.087±0.047 (a)	0.039±0.047 (a)
	Florida/Louisiana	0.158±0.093 (b)	0.102±0.100 (b)

[a] Different letters inside the parentheses indicate significant differences among groups (Kruskal-Wallis and Dunn *Post-Hoc* Tests).

populations were diverse, whereas the profiles of ants from various localities in introduced populations were uniform. This suggests that the introduction event into a new environment may be the cause of the reduced chemical variability of introduced populations. It is possible that, similarly to the introduced ant hypotheses, the reduced variability of recognition cues observed in introduced populations of *R. flavipes* is due to a reduction of genetic diversity through a genetic bottleneck (Tsutsui et al., 2000) or a selective process for the less common alleles of recognition (Giraud et al., 2002).

In all cases, the chemical homogeneity occurring in introduced populations of *R. flavipes* could explain two particular characteristics of the social organization in its French range. The first characteristic is the absence of aggression between colonies, which has been observed among all French populations of *R. flavipes* (Clément and Bagnères, 1998). Cuticular hydrocarbons generally are considered to have an important role in conspecific and colony member recognition (Clément and Bagnères, 1998; Blomquist and Bagnères, 2010). Thus, a low variability of chemical signature within introduced populations of *R. flavipes* could induce the recognition of non-nestmates as nestmates, thereby reducing intraspecific aggression. The second characteristic is the significant level of merging between separate colonies that has been found within one of the introduced French populations of *R. flavipes* studied (the Oléron population) (Perdereau et al., 2010). One of

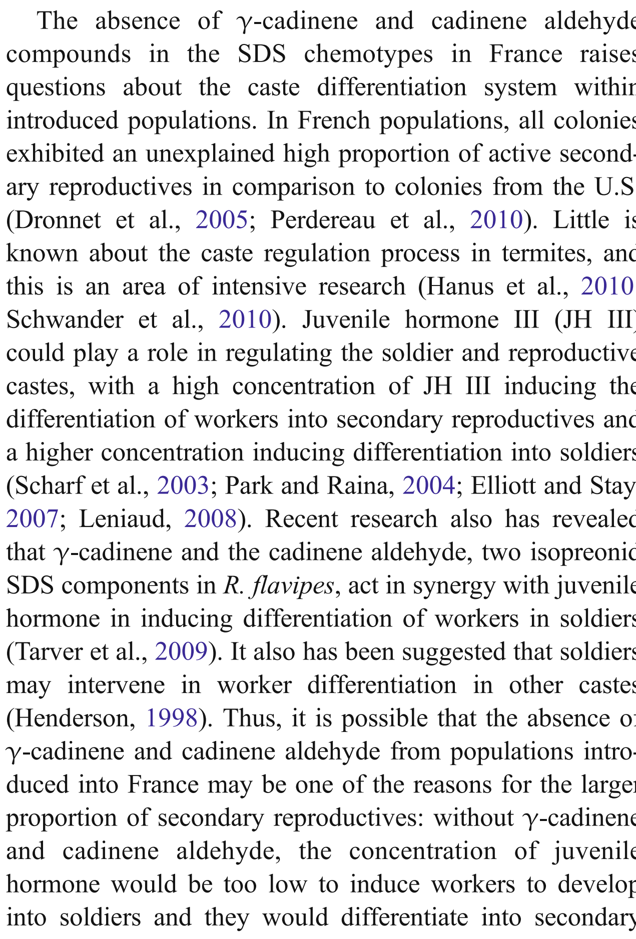
Table 2 Comparison of *Reticulitermes flavipes* soldier defensive secretion phenotypes by locality

Localities	Number of soldiers	Defensive secretion components					
		α-pinene	β-pinene	limonene	γ-cadinene	cadinene aldehyde	geranyl linalool
France							
Chemotype a	10	X	X	X	_	_	_
Chemotype b	3	X	X	X	_	_	X
Florida							
Chemotype c	15	X	X	X	X	X	_
Chemotype d	2	X	X	X	X	X	X
Louisiana							
Chemotype a	11	X	X	X	_	_	_
Chemotype c	6	X	X	X	X	X	_
Chemotype e	2	X	X	X	X	_	_
Chemotype f	1	X	X	X	_	X	_

the characteristics that appear essential for colony fusion is the absence of intraspecific aggression among individuals of the two parental colonies. The CHC homogeneity in introduced populations of *R. flavipes* could explain the lack of intraspecific aggression and, indirectly, the high rate of colony fusion within these introduced populations, as has been recently proposed for the Argentine ant, *Linepithema humile* (Vasquez et al., 2009).

This study also revealed that CHC profiles differed significantly among U.S. populations, whereas the three French populations exhibited similar CHC profiles. One possible explanation for this general pattern is that the three French populations all derived from a single original source population. This hypothesis is supported by several phylogeographic studies performed on *Reticulitermes* species, which showed that discrimination based on CHC profiles often is consistent with discrimination based on DNA markers (Jenkins et al., 2000; Clément et al., 2001; Copren et al., 2005; Austin et al., 2007). Another explanation that cannot be excluded is that the new habitat in France may have more consistent ecological factors, which may be the reason for the homogenous CHC profiles in all French populations of *R. flavipes*. Although studies have revealed that food, temperature, and social environment can affect the composition of CHC profiles (Florane et al., 2004; Dronnet et al., 2006), this second hypothesis is unlikely to explain the results obtained over this large geographic scale. Phylogeographic studies are needed to determine whether the French populations were founded from one or a few North American source population(s).

Concerning the source of the three introduced populations, results based on both CHC profiles and SDS chemotypes suggest that the French populations analyzed may have originated from Louisiana rather than from Florida. Principal component analysis showed that the CHC profiles of the three populations of Louisiana were closer to the chemical profiles of the three French populations than those in Florida. The analyses of SDSs showed, for the first time, a similar chemotype in the native and introduced ranges. This chemotype, not previously reported, is composed only of monoterpenes and is found in both France and Louisiana. The hypothesis that French populations came from Louisiana is also plausible from a historical point of view. During the 17th and 18th centuries, Louisiana was part of “New France” and New Orleans was the main trading port. Populations of *R. flavipes* may have been arrived in France on boats carrying agricultural and forestry products. In support of this, the first invasion of termites into France was reported in two major ports (Rochefort and La Rochelle) that were involved in international trade (Bobe-Moreau, 1843; Quatrefages, 1853).



The absence of γ-cadinene and cadinene aldehyde compounds in the SDS chemotypes in France raises questions about the caste differentiation system within introduced populations. In French populations, all colonies exhibited an unexplained high proportion of active secondary reproductives in comparison to colonies from the U.S. (Dronnet et al., 2005; Perdereau et al., 2010). Little is known about the caste regulation process in termites, and this is an area of intensive research (Hanus et al., 2010; Schwander et al., 2010). Juvenile hormone III (JH III) could play a role in regulating the soldier and reproductive castes, with a high concentration of JH III inducing the differentiation of workers into secondary reproductives and a higher concentration inducing differentiation into soldiers (Scharf et al., 2003; Park and Raina, 2004; Elliott and Stay, 2007; Leniaud, 2008). Recent research also has revealed that γ-cadinene and the cadinene aldehyde, two isopreonid SDS components in *R. flavipes*, act in synergy with juvenile hormone in inducing differentiation of workers in soldiers (Tarver et al., 2009). It also has been suggested that soldiers may intervene in worker differentiation in other castes (Henderson, 1998). Thus, it is possible that the absence of γ-cadinene and cadinene aldehyde from populations introduced into France may be one of the reasons for the larger proportion of secondary reproductives: without γ-cadinene and cadinene aldehyde, the concentration of juvenile hormone would be too low to induce workers to develop into soldiers and they would differentiate into secondary reproductives instead.

This study illustrates that hydrocarbon analysis is effective at discriminating populations of a *Reticulitermes* species. The CHC profiles reveal the possible origin of *R. flavipes* populations introduced into France and the history and routes of invasion. The homogeneity of the cuticular hydrocarbon profiles observed within introduced populations seems to be related to particular biological characteristics of introduced populations. Further studies should be carried out into the relationship between CHC variations and intraspecific aggression.

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Drywood termites are cryptic, seldom leaving obvious or visible external signs of their presence in wood. A commonly used sign for determining the presence of drywood termites is the occurrence of fecal pellets, ejected through a “kick-out hole” in the external surface of wood from the internal galleries (Ebeling, 1975). These pellets often are found as conical piles or are scattered on horizontal surfaces below infested wood. These hexagonally sided pellets are diagnostic for drywood termites, and can be used to distinguish damage from that by other wood-destroying insects (Ebeling, 1975; Moore, 1992).

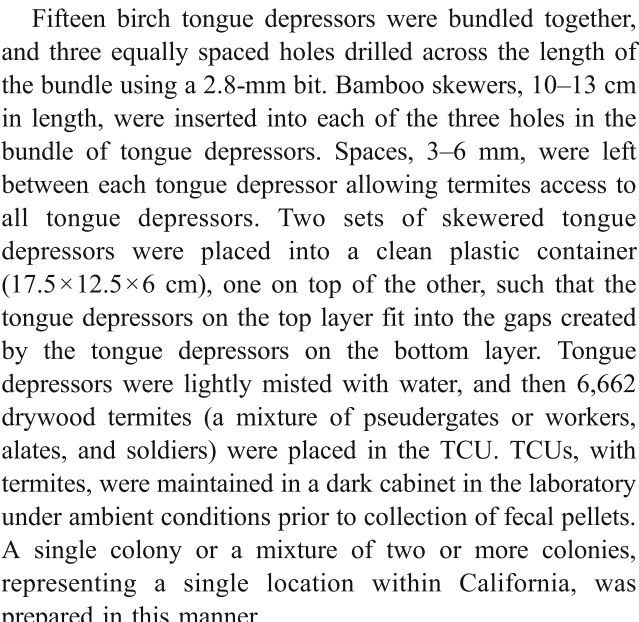
Grace and Yamamoto (2009) demonstrated the relationship between the cellulose and lignin content of the food utilized by small groups of *C. brevis* and *Incisitermes immigrans* (Light), and the quantity of fecal pellets produced over time. They also discussed the use of the size and number of fecal pellets for estimating both size and age of drywood termite colonies.

Drywood termites excrete feces in the form of hard, even-shaped fecal pellets. These fecal pellets contain the same mixture of hydrocarbons as the insects that produced them, albeit in slightly different proportions (Haverty et al., 2005). Because cuticular hydrocarbons are species specific in termites (Page et al., 2002), pellets can be used to identify the termite species that produced them. Rather than simply signaling the general presence of termites or providing a diagnosis of the species of termite inhabiting the wood, we postulated that these pellets could be chemically characterized so as to determine the status of a colony as active (alive) or inactive (dead).

Here, we report quantification of the hydrocarbons in pellets of *I. minor* aged for up to 1 year after they were produced. We document the changes in proportions of selected hydrocarbons as an indication of the length of time since the pellets were excreted.

Methods and Materials

Collection of Termites and Preparation of Termite Containment Unit Termites, *I. minor*, were removed from one naturally infested board (98.9×13.3×271.8 cm) collected on 19 July 2006 from Lakeview, California, and stored at the University of California Richmond Field Station. The board was cut across the grain into pieces, 5–8 cm thick, and stored at room temperature. A wood chisel was used to separate pieces of wood into smaller pieces, 0.5–1.0 cm thick. Termites were removed from all visible galleries with forceps. All remaining live termites, including soldiers and primary reproductives (alates), were placed in a termite containment unit (TCU). Termites thus collected were likely from a large mixed colony (Booth et al., 2010) or from multiple colonies. We were not able to assign the various galleries in the wood to any particular colony.



Fifteen birch tongue depressors were bundled together, and three equally spaced holes drilled across the length of the bundle using a 2.8-mm bit. Bamboo skewers, 10–13 cm in length, were inserted into each of the three holes in the bundle of tongue depressors. Spaces, 3–6 mm, were left between each tongue depressor allowing termites access to all tongue depressors. Two sets of skewered tongue depressors were placed into a clean plastic container (17.5×12.5×6 cm), one on top of the other, such that the tongue depressors on the top layer fit into the gaps created by the tongue depressors on the bottom layer. Tongue depressors were lightly misted with water, and then 6,662 drywood termites (a mixture of pseudergates or workers, alates, and soldiers) were placed in the TCU. TCUs, with termites, were maintained in a dark cabinet in the laboratory under ambient conditions prior to collection of fecal pellets. A single colony or a mixture of two or more colonies, representing a single location within California, was prepared in this manner.

Pellet Collection and Aging Process After all the TCUs had acclimated to laboratory conditions over several weeks, new holding chambers were prepared. A total of 3,402 live workers were transferred to a new TCU. All wood debris, pellets, dead termites, and damaged tongue depressors were discarded. The termites in the new holding chambers were maintained in the laboratory under ambient conditions for 2 weeks. At the end of this period, all pellets were removed, and sub-samples collected and readied for the aging study. Concurrently, three samples of 20 workers, three samples of 20 alates, and one sample of 20 soldiers were also collected for hydrocarbon analysis. Live termites were placed in a 20-ml scintillation vial and frozen until extraction.

Fecal pellets were separated from debris by sequentially sifting them through successively smaller sieves (Haverty et al., 2005). It was important to avoid contamination of samples with any extrinsic hydrocarbons. Thus, all substances such as hand lotion, lip balm, Parafilm, and waxed paper were kept away from the work area. Gloves were worn when handling pellets, and implements that came in contact with pellets were wiped with a paper towel dampened with absolute ethanol to remove any oils or waxes. Pellets were separated from fine debris by removing individual pellets with forceps or with an aspirator, until a sample weighing approximately 200 mg was obtained.

In developing this protocol we made one estimate and one critical assumption. The estimate was that at least 1,000 pellets were needed for each hydrocarbon analysis (based on preliminary analyses of pellets collected from drywood termites maintained in our laboratory). The critical assumption was based on work conducted by Scheffrahn et al. (1997) using *C. brevis* and *I. snyderi* (Light) from southern Florida. In that study, 40 *I. snyderi*

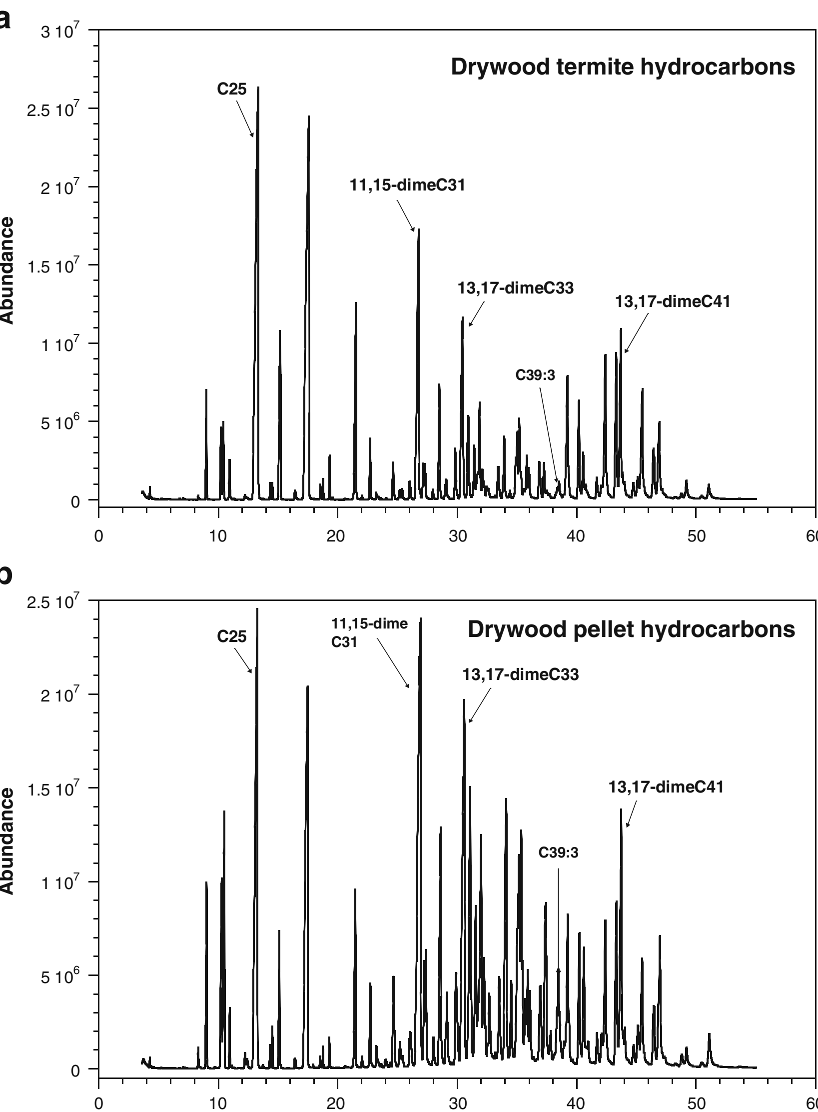


Fig. 1 Total ion chromatogram of cuticular hydrocarbons from **a** workers of *Incisitermes minor* (Hagen) and **b** from fecal pellets from the same collection. Acronyms for hydrocarbons are derived as follows: *X-me* location of methyl groups, the total number of carbons *CXX* in the hydrocarbon component excluding the methyl branch(es), and *CXX:Y* the number of double bonds following a colon

(pseudergates or workers) produced 573 pellets over 4 weeks, slightly less than 150 pellets/wk or about 3.5 pellets/individual/wk. In a study with small groups of *C. brevis* and *I. immigrans*, Grace and Yamamoto (2009) found that these species produced 4.9 to 7.0 pellets/individual/wk. For our study, we assumed that *I. minor* would produce pellets at roughly the same rate and, therefore, we needed about 3,000 to 5,000 individuals in a TCU for this study. We prepared one TCU to collect the requisite quantity of fecal pellets.

Four sub-sampling intervals, each replicated three times, were used: 0, 30, 90, and 365 days from the initial collection date. Sub-samples were stored in clean, 20-ml scintillation vials, sealed with 1.5×1.5 mm mesh screen. Vials were stored in a dark cabinet at the University of California Richmond Field Station at ambient temperature.

Voucher samples of *I. minor* pseudergates or workers and soldiers from this study (fresh, not dried) were preserved in 85% ethanol and deposited in the Essig Museum, University of California at Berkeley (Haverty et al., 2005).

Extraction Procedure and Characterization of Hydrocarbons Hydrocarbons from workers, alates, soldiers, and fecal pellets of *I. minor* were extracted, characterized, and quantified as previously reported (Haverty et al., 2005). Frozen termite samples were thawed and dried at 70°C for approximately 1 h before extraction. Each sub-sample of fecal pellets or termites was placed in a 20-ml scintillation vial, and immersed in 10 ml of *n*-hexane for 10 min. After extraction, hydrocarbons were separated from other compounds through 4 cm of activated Sigma silica gel (70–230 mesh) in

Table 1 Relative abundance[a] of hydrocarbons from pseudergates, alates, soldiers, and fecal pellets of *Incisitermes minor* (Hagen)

Hydrocarbons[b]	Pseudergates *N*=3	Alates *N*=3	Soldiers *N*=1	Fecal pellets *N*=12
2-meC22[c]	0.06 (0.02)	0.08 (0.02)	0.09	0.11 (0.02)
n-C23	1.30 (0.06)	1.34 (0.05)	1.48	1.35 (0.18)
2-meC23	1.45 (0.40)	1.72 (0.35)	2.11	2.13 (0.28)
3-meC23	1.31 (0.38)	1.38 (0.30)	1.71	2.06 (0.29)
n-C24	0.46 (0.01)	0.43 (0.02)	0.48	0.32 (0.05)
2-meC24[c]	0.13 (0.03)	0.19 (0.03)	0.19	0.15 (0.02)
3-meC24[c]	0.08 (0.02)	0.10 (0.02)	0.12	0.11 (0.02)
n-C25	16.35 (0.16)	11.75 (0.40)	12.64	8.65 (1.05)
2-meC25	0.18 (0.01)	0.27 (0.01)	0.24	0.14 (0.02)
3-meC25	0.23 (0.04)	0.36 (0.04)	0.34	0.26 (0.03)
n-C26	2.25 (0.15)	1.17 (0.07)	1.51	0.87 (0.12)
2-meC26[c]	0.14 (0.01)	0.13 (0.01)	0.14	0.07 (0.01)
n-C27	14.04 (0.55)	6.81 (0.31)	10.05	5.91 (0.65)
2-meC27[c]	0.13 (0.01)	0.09 (0.01)	0.13	0.06 (0.01)
3-meC27	0.20 (0.01)	0.19 (0.01)	0.25	0.12 (0.01)
n-C28[c]	0.43 (0.05)	0.17 (0.01)	0.35	0.15 (0.03)
n-C29	3.38 (0.33)	1.27 (0.07)	2.97	1.38 (0.18)
11-meC29[c]	0.06 (0.01)	0.15 (0.01)	0.10	0.09 (0.01)
9,13-dimeC29	0.70 (0.02)	0.82 (0.01)	0.91	0.63 (0.06)
9,13,17-trimeC29	0.11 (0.02)	0.27 (0.00)	0.16	0.22 (0.03)
n-C30[c]	0.04 (0.00)	0.07 (0.00)	0.10	0.00
13-meC30[c]	0.01 (0.02)	0.08 (0.00)	0.04	0.00
10,14-dimeC30	0.64 (0.06)	1.45 (0.02)	0.88	0.90 (0.03)
10,14,18-trimeC30	0.18 (0.02)	0.43 (0.01)	0.24	0.28 (0.02)
n-C31[c]	0.14 (0.01)	0.10 (0.01)	0.20	0.09 (0.01)
15-; 13-; 11-; 9-meC31[d]	0.36 (0.03)	0.82 (0.01)	0.42	0.49 (0.02)
13,17-; 11,15-dimeC31[d]	7.45 (0.68)	13.74 (0.52)	8.55	10.67 (1.80)
9,13,17-trimeC31	0.57 (0.05)	1.19 (0.02)	0.72	0.89 (0.06)
7,11,15-trimeC31	0.54 (0.04)	0.99 (0.01)	0.72	0.81 (0.09)
14-; 12-meC32[d]	0.17 (0.01)	0.33 (0.02)	0.19	0.27 (0.03)
12,16-dimeC32	2.01 (0.14)	3.57 (0.05)	2.22	2.97 (0.28)
10,14,18-; 8,12,16-trimeC32[d]	0.46 (0.03)	0.80 (0.02)	0.61	0.82 (0.06)
15-; 13-meC33[d]	0.93 (0.05)	1.57 (0.03)	0.92	1.30 (0.08)
13,17-; 11,15-; 9,13-dimeC33[d]	4.63 (0.31)	6.87 (0.11)	5.11	7.41 (1.03)
9,13,17-trimeC33	1.30 (0.08)	2.02 (0.02)	1.86	3.47 (0.33)
7,13,17-trimeC33	0.21 (0.02)	0.32 (0.01)	0.32	0.47 (0.07)
C35:3	1.01 (0.03)	2.93 (0.17)	1.64	1.76 (0.17)
C35:2[d]	2.31 (0.06)	4.46 (0.14)	2.71	3.55 (0.15)
12,16-dimeC34; C35:1[c,d]	0.68 (0.02)	1.16 (0.03)	0.85	1.40 (0.10)
10,14,18-; 8,12,16-trimeC34[c,d]	0.28 (0.00)	0.50 (0.04)	0.45	0.89 (0.14)
15-; 13-; 11-meC35[d]	0.56 (0.02)	0.85 (0.05)	0.67	1.11 (0.09)
13,17-dimeC35	1.27 (0.09)	1.96 (0.02)	1.83	3.95 (0.46)
9,13,17-trimeC35	0.14 (0.01)	0.28 (0.03)	0.27	0.93 (0.14)
C37:3[d]	1.74 (0.06)	3.33 (0.16)	2.72	3.28 (0.34)
C37:2[d]	1.67 (0.06)	2.55 (0.08)	2.18	2.90 (0.10)
12,16-dimeC36	0.17 (0.01)	0.34 (0.02)	0.28	0.71 (0.08)
C37:1	0.78 (0.02)	0.76 (0.03)	0.81	0.83 (0.06)
10,14,18-trimeC36; C37:1[c,d]	0.44 (0.03)	0.35 (0.05)	0.43	0.58 (0.10)
13; 11-meC37[d]	0.71 (0.01)	0.60 (0.04)	0.77	0.89 (0.14)

Table 1 (continued)

Hydrocarbons[b]	Pseudergates $N=3$	Alates $N=3$	Soldiers $N=1$	Fecal pellets $N=12$
13,17-; 11,15-dimeC37[d]	0.87 (0.05)	1.27 (0.01)	1.25	2.13 (0.22)
9,13,17-trimeC37	0.09 (0.01)	0.13 (0.01)	0.13	0.30 (0.06)
C39:3[d]	0.09 (0.02)	0.18 (0.02)	0.29	0.49 (0.16)
C39:3; 12-meC38[d]	0.49 (0.04)	0.56 (0.03)	0.76	1.03 (0.18)
14,18; 12,16-dimeC38[d]	0.02 (0.03)	0.14 (0.01)	0.14	0.24 (0.04)
C39:1; 10,14,18-trimeC38[d]	2.73 (0.19)	1.73 (0.04)	2.53	1.74 (0.23)
13-; 11-meC39[d]	1.75 (0.09)	1.03 (0.03)	1.70	1.26 (0.20)
13,17-; 11,15-dimeC39[d]	0.90 (0.01)	1.04 (0.14)	1.36	1.44 (0.08)
9,13,17-trimeC39[c]	0.41 (0.03)	0.17 (0.09)	0.11	0.20 (0.03)
12-; 10-meC40[d]	0.38 (0.05)	0.20 (0.01)	0.35	0.26 (0.07)
12,16-dimeC40	0.27 (0.00)	0.27 (0.02)	0.34	0.32 (0.05)
C41:1	3.31 (0.27)	1.70 (0.04)	2.79	1.58 (0.25)
13-; 11-meC41[d]	2.78 (0.20)	1.45 (0.06)	2.59	1.74 (0.31)
13,17-; 11,15-dimeC41[d]	3.34 (0.13)	2.90 (0.15)	4.00	3.33 (0.19)
9,13,17-trimeC41	0.79 (0.02)	0.30 (0.02)	0.33	0.42 (0.19)
12-; 10-meC42[d]	0.30 (0.03)	0.15 (0.01)	0.25	0.26 (0.27)
12,16-dimeC42	0.48 (0.04)	0.37 (0.02)	0.52	0.47 (0.12)
C43:1	2.72 (0.28)	1.25 (0.06)	2.27	1.31 (0.26)
15-; 13-meC43[d]	1.00 (0.14)	0.46 (0.05)	0.87	0.61 (0.16)
13,17-dimeC43	1.99 (0.16)	1.18 (0.16)	1.93	1.68 (0.28)
14,18-dimeC44[c]	0.12 (0.02)	0.07 (0.01)	0.12	0.13 (0.06)
C45:1[c]	0.42 (0.09)	0.16 (0.02)	0.33	0.21 (0.07)
13-meC45[c]	0.00	0.00	0.00	0.04 (0.03)
13,17-dimeC45[c]	0.37 (0.08)	0.18 (0.02)	0.34	0.43 (0.13)

[a] Mean percent (SD) of total hydrocarbon composition. Compounds are listed in elution order

[b] Acronyms for hydrocarbons are derived as follows: location of methyl groups (X-me), the total number of carbons (CXX) in the hydrocarbon component excluding the methyl branch(es), and the number of double bonds following a colon (CXX:Y)

[c] These hydrocarbons were not reported for *I. minor* pseudergates or fecal pellets in Haverty et al. (2005)

[d] An isomeric mixture or two or more compounds co-eluted in this peak

Pasteur pipette mini-columns. The resulting hydrocarbon fractions were evaporated to dryness under a stream of nitrogen and redissolved in 60 •l of *n*-hexane for gas chromatography-mass spectrometry (GC-MS) analysis. A 3-µl aliquot was injected into the GC-MS.

GC-MS analyses were performed on an Agilent 6890 gas chromatograph interfaced with an Agilent 5973 Mass Selective Detector, using Agilent Chemstation data analysis software (G1701CA version C.00.00). The GC-MS was equipped with an HP-1MS, fused silica capillary column (30 m×0.25 mm i.d, 0.25 µm film thickness), and was operated in split mode (split ratio of 30:1), using helium as carrier gas. The column oven was programmed from 200–320°C at 3°C min^{-1}, with a final hold of 11 min. Electron impact (EI) mass spectra were obtained at 70 eV.

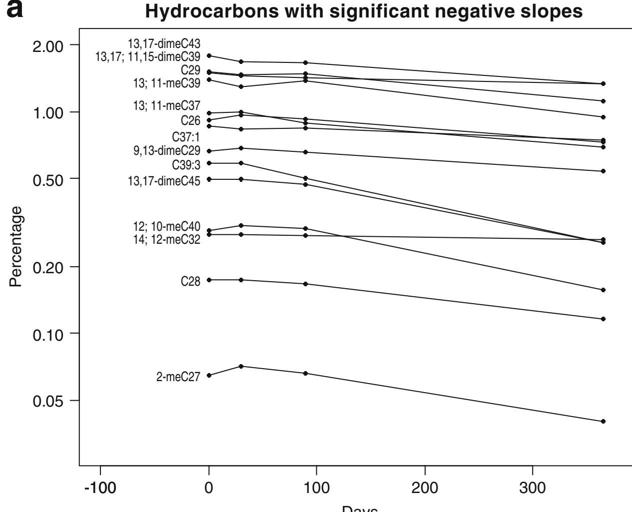
All chemicals of interest were identified by their retention times and mass spectra. Mass spectra of methyl-branched alkanes were interpreted as described by Blomquist et

Table 2 Relative abundance[a] of hydrocarbon classes from pseudergates, alates, soldiers, and fecal pellets of *Incisitermes minor* (Hagen)

Hydrocarbon class	Pseudergates	Alates	Soldiers	Fecal pellets
Normal Alkanes	38.39	23.12	29.77	18.71
Olefins[b]	18.38	21.11	20.31	20.66
Terminally branched methylalkanes	3.90	4.49	5.32	5.19
Internally branched methylalkanes	9.01	7.70	8.88	8.33
Dimethylalkanes	25.24	36.18	29.79	37.40
Trimethylalkanes	5.09	7.40	5.92	9.71

[a] Percent of total hydrocarbon composition

[b] All peaks with co-eluting olefins and saturated compounds are summarized as olefins



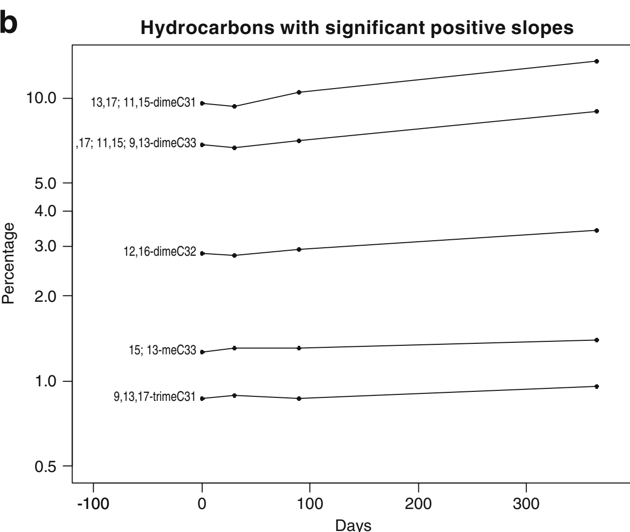
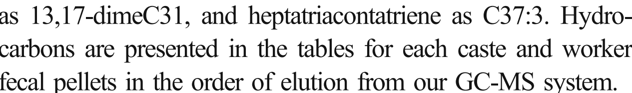


Fig. 2 Changes in percent content of individual hydrocarbons, over time, from fecal pellets of *Incisitermes minor* workers. **a** hydrocarbons with significant (α=0.05) negative slopes, and **b** hydrocarbons with significant positive slopes. Acronyms for hydrocarbons are derived as follows: *X-me* location of methyl groups, the total number of carbons, *CXX* in the hydrocarbon component excluding the methyl branch(es), and *CXX:Y* the number of double bonds following a colon

al. (1987) so as to identify methyl branch locations. Mass spectra of di- and trimethylalkanes were interpreted as described by Page et al. (1990) and Pomonis et al. (1978). Olefins were identified by their mass spectra, although double bond positions were not determined (Haverty et al., 2005).

In the text, tables, and figures, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the location of methyl groups (X-me), the total number of carbons (CXX) in the hydrocarbon component excluding the methyl branch(es), and the number of double bonds following a colon (CXX:Y). Thus, pentacosane is represented as *n*-C25, 11-methylnonacosane as 11-meC29, 13,17-dimethylhentriacontane


as 13,17-dimeC31, and heptatriacontatriene as C37:3. Hydrocarbons are presented in the tables for each caste and worker fecal pellets in the order of elution from our GC-MS system.

Statistical Analysis GC-MS peak (some peaks contained more than one compound or a mixture of positional isomers) areas were converted to percentages of total hydrocarbon fraction, enabling mean (±SD) relative amounts of each hydrocarbon peak for workers and alates, and overall mean (±SD) of each hydrocarbon peak for the fecal pellets, to be calculated.

The percentage of each hydrocarbon peak for pellets of each aging period was transformed to the log of the percentage (dependent variable Y) and regressed against the days of aging (independent variable X). Hydrocarbons with slopes different from 0 (α=0.05) were separated into two groups: those with a significant positive slope and those with a significant negative slope. For each aging period (0, 30, 90, and 365 day), an index of age (I_{age}) was created by subtracting the sum of the percentages of the hydrocarbons with a negative slope over time from the sum of the percentages of the hydrocarbons with a positive slope over time [$I_{age}=\sum(PEAK_{positive})$ minus $\sum(PEAK_{negative})$, for each aging period]. The I_{age} for each aging period then was regressed against the aging period (R Development Core Team, 2004).

Results and Discussion

The cuticular hydrocarbons for *I. minor* pseudergates and fecal pellets were characterized previously (Haverty et al., 2000, 2005). The composition of the hydrocarbon mixture

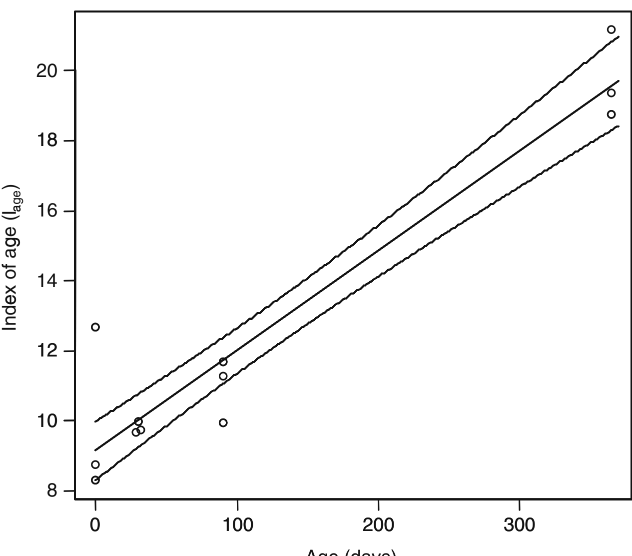


Fig. 3 Fitted line and 95% confidence intervals for a plot of the mean of index of age (I_{age}) against age. Values for 30 day are jittered a small amount so that all three data points can be seen

for this species and the hydrocarbons from their fecal pellets in the present study are displayed in Fig. 1 and summarized in Table 1.

Hydrocarbons from Termites *n*-Alkanes and dimethylalkanes were the predominant classes of hydrocarbons in all castes and in fecal pellets (Table 2), as previously reported by Haverty et al. (2000, 2005). *n*-Alkanes comprised from 23.12% to 38.39%, unsaturated components from 15.21% to 19.03% , terminally branched monomethylalkanes from 3.90% to 5.32%, internally branched monomethylalkanes from 7.70% to 9.01%, and dimethylalkanes from 25.25% to 36.18%, of the total hydrocarbon content. There was also a homologous series of trimethylalkanes, representing 8.26% to 9.48% of the total hydrocarbon content.

The hydrocarbons of *I. minor* characterized in this study included all of the compounds reported by Haverty et al. (2005), as well as 18 additional hydrocarbons (Table 1). These additional hydrocarbons were not abundant, never representing more than 0.85% of the total hydrocarbon (as for the mixture of 12,16-dimeC34 and C35:1 in soldiers) content, and usually much less. The detection of these additional hydrocarbons in this study are likely due to the greater concentration of the extracted hydrocarbons used or, possibly, colony to colony variation.

Hydrocarbons from Termite Fecal Pellets In general, the hydrocarbons from whole-body extracts of *I. minor* were represented in extracts of fecal pellets. As with whole-body extracts of *I. minor*, dimethylalkanes were the predominant class of hydrocarbon, followed by olefins and normal alkanes (Table 2). Only two hydrocarbons (*n*-C30 and 13-meC30; Table 1) were found in termites and not in fecal pellets, but these were present in small quantities, less than 0.1% of the total hydrocarbon content. The lack of these two hydrocarbons in fecal pellets may be a function simply of the concentration of the extracts.

Changes in Hydrocarbons of Fecal Pellets over Time We identified 73 hydrocarbon peaks in fecal pellets of *I. minor* (Table 1). Of these peaks (data from non-significant slopes not reported), 19 of 73 (26%) had a significant change, either positive (five) or negative (14), over time (Fig. 2). This is a much higher number of statistically significant slopes (different from 0), than would be expected by chance alone (5% of 73 peaks is <4 peaks).

The index, I_{age}, for each of the three replications (A, B, and C at 0, 30, 90, and 365 day) was plotted against the associated number of days (Fig. 3). The fitted line, with a 95% confidence interval for the means to show the variability of the replicates, had a high adjusted R^2 (= 0.888) value, suggesting that it might be possible to predict fecal age by the index.

We do not know the basis by which peaks increase or decrease over the 1-year period. In general, those that increased in relative abundance tended to have higher initial relative abundances; i.e., mono-, di-, or tri-methylalkanes with carbon chains of 31, 32, or 33. Those that decreased were represented in five of the six classes of hydrocarbons, ranging from C26 to C45. Only the terminally branched hydrocarbons were not represented in peaks with negative slopes.

The composition of the hydrocarbon mixture of insects is genetically controlled (Toolson and Kuper-Simbrón, 1989; Kaib et al., 1991; Page et al., 1991; Coyne et al., 1994). This composition can be slightly affected by diet and environmental conditions (Hadley, 1977; Espelie et al., 1994; Chapman et al., 1995; Howard, 1998; Woodrow et al., 2000). Haverty et al. (1996) demonstrated significant seasonal variation in quantities of some hydrocarbons of *Coptotermes formosanus* Shiraki from Hawaii; these differences were small and associated with the production of alates. However, the qualitative mix of hydrocarbons has been shown to be stable over decades among species of cone beetles in the genus *Conophthorus* (Page et al., 1990).

The motivation for our study was to devise a method for the evaluation of the success or failure of drywood termite treatments, including fumigation and local application methods. With the recent classification of sulfuryl fluoride (the only fumigant active ingredient registered in California) as a greenhouse gas (Mühle et al., 2009) and the anticipated increase in local treatments, we felt that there would be considerable interest by the industry, regulatory agencies, and consumers for a simple and accurate means to determine whether or not a targeted colony/infestation is still in a structure. Our method, based on changes in hydrocarbon chemistry over time, shows promise to this end. Future research includes: (1) validating these findings for additional species of drywood termites and geographic populations of the same species, (2) validating these findings at different times of the year, and (3) exploring seasonality in pellet production and hydrocarbon quality in pellets.

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Table 1 Attractiveness of combined visual and olfactory cues in comparison to decoupled visual (*Test 1*) and olfactory (*Test 2*) cues for experienced female and male *Episyrphus balteatus.* *The sex of one fly was not determined

	VISUAL		vs.	VISUAL+OLFACTORY	
Test 1	Landing	Approaching		Landing	Approaching
	0	0		18 (11♀; 7♂)	6 (5♀; 1♂)
	OLFACTORY		vs.	VISUAL+OLFACTORY	
Test 2	Landing	Approaching		Landing	Approaching
	7 (3♀; 4♂)	4 (4♀)		10 (8♀; 1♂)*	4 (3♀)*

EAD) in order to identify which compounds emitted by *C. arvense* inflorescences are perceived by the fly.

Methods and Materials

Plant Material and Volatile Collection The *Cirsium arvense* plants were from the Ecological Botanical Garden of the University of Bayreuth. *C. arvense* is a dioecious plant. Staminate and pistillate flower heads emit the same scent compounds, although the total amount of scent is higher in staminate flower heads (Theis et al. 2007; Primante and Dötterl unpublished data). The petals of both sexes reflect light mainly in the blue (max. 450 nm) and red range (650–700 nm) (Primante and Dötterl unpublished data). We, therefore, did not discriminate between flower sexes. Flowering branches were cut in the field and placed in water in the laboratory for immediate scent collection. Four to seven flower heads of *C. arvense* were enclosed in a polyester oven bag (Toppits, Germany), and over an 8 h period the emitted volatiles were trapped in an adsorbent tube filled with 20 mg of a 1:1 mixture of Tenax-TA (mesh 60–80, Supelco) and Carbotrap (mesh 20–40, Supelco) (Dötterl et al. 2005). Volatiles were eluted with 60 µl of acetone (SupraSolv, Merck KgaA, Germany) to obtain 6 odor samples for the electrophysiological experiments.

Electrophysiological Experiments and Chemical Analyses Electrophysiological analyses of the floral scent samples were performed with the GC-EAD system as described by Dötterl et al. (2005). Antennae from 23 females and 11 males of *E. balteatus* (one antenna per individual, one run per antenna) were tested between July and August 2008 on our 6 odor samples (3–5 female and 1–5 male antennae per sample). To identify the EAD-active compounds, 1 µl of the scent samples was analyzed on a Varian 3800 gas chromatograph fitted with a 1079 injector and a ZB-5 column (5% phenyl polysiloxane; length, 60 m; inner diam, 0.25 mm; film thickness, 0.25 µm; Phenomenex) and a Varian Saturn 2000 mass spectrometer (Dötterl et al. 2005). Component identification was carried out using the NIST 08 mass spectral database or MassFinder 3, and was confirmed by comparison of mass spectra and retention times with those of authentic standards.

Behavioral Assay To determine the relative importance of olfactory and visual cues for finding flowering *C. arvense* by flies experienced in foraging, we conducted two-choice bioassays in a flight cage at the end of summer 2009.

Pupae of *E. balteatus* were provided by Katz Biotech Ag (Baruth, Germany) and kept in a small gauze tent (60×60×60 cm) at 23°C until hatching. Immediately after the adults hatched, they all were transferred to a flight cage (7.20×3.60×2.20 m) that was set up in a greenhouse. The population of 55 female and 30 male adult flies were fed on fresh flowers of *C. arvense,* but all floral material was removed from the cage at least 12 h before conducting a bioassay.

We performed two two-choice bioassays to assess fly attraction to floral cues: 1) Visual only vs. visual and olfactory cues combined, and 2) Olfactory only vs. visual and olfactory cues combined. In the assays, flies were offered flowering branches (with 30–40 flower heads

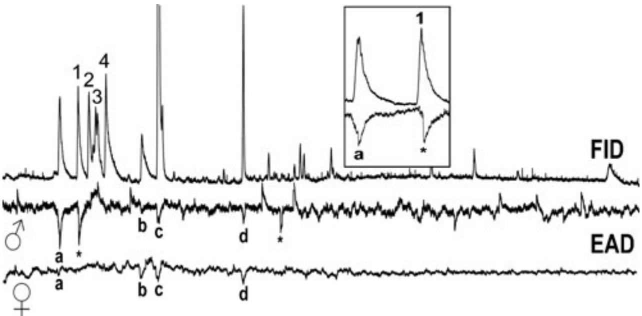

Fig. 1 Examples of coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *Cirsium arvense* inflorescence scent sample using antennae of female and male *Episyrphus balteatus*. a: phenylacetaldehyde; b: (*E*+*Z*)-pyranoid linalool oxide; c: methyl salicylate; d: dimethyl salicylate. The abundant compounds acetophenone (1), (*E*)-furanoid linalool oxide (2), coeluting linalool and methyl benzoate (3) and 2-phenylethanol (4) did not elicit responses. The responses marked with an asterisk are artifacts as shown in the box where we present an enlarged section of the FID and corresponding antennal response of the male. The antennal response did not occur simultaneously with FID peak 1

each) in quartz glass cylinders constructed to present either visual or olfactory cues, or both. The basic cylinder for testing attraction to olfactory and visual cues in combination consisted of a transparent quartz glass cap and body and a sleeve of Macrolon®, which connected and sealed the cap and body. The Macrolon® sleeve had 60 holes (diam 0.2 cm), arranged in three horizontal lines to allow diffusion of floral scents. The cylinders were mounted on a black polyvinyl chloride (PVC) disc (diam 11 cm) that was attached to a square wooden table. A tube coupled the cylinder to a membrane pump (flow 1 $1 min^{-1}$; G12/01EB, Rietschle Thomas, Puchheim, Germany). A modified transparent cylinder without holes and without connection to a pump was used for testing visual attraction only. A cylinder with holes and the pump, but painted black with semi-matte varnish, was used for testing olfactory attraction only.

The two test cylinders were set up 2 m apart for each of the two bioassays. Each test was conducted for 40 min, and 20 min after beginning the test, the position of the cylinders was exchanged. The behavior of the flies was classified as "approaching" when flies hovered in front of the cylinder but did not land, and "landing" when flies contacted the glass cylinder. To assure that an individual fly was counted only once in a specific test, responding flies were captured.

Results and Discussion

In the bioassays, *E. balteatus* preferred a combination of both cue modalities over visual cues but not over olfactory cues. When testing a combination of visual and olfactory cues against visual cues, 24 flies were attracted by the combination of both cues and no flies were attracted by the visual cues (*chi square* observed vs. expected test: χ^2= 24.0; df=1; P<0. 001) (Table 1). When testing a combination of visual and olfactory cues against olfactory cues, both types of cylinders had the same attractiveness (*chi square* observed vs. expected test: χ^2=0.36, df=1; P=0.55) (Table 1). These experiments demonstrate that experienced flies primarily use olfactory cues for seeking *C. arvense* flower heads, whereas visual cues do not play a significant role. We did not determine whether flies would respond to visual cues in the absence of *C. arvense* odors. *C. arvense* flowers reflect light in the blue range of light, and blue was somewhat attractive to *E. balteatus* (Sutherland et al. 1999).

The GC-EAD measurements revealed the candidate molecules responsible for attraction of flies. Four compounds occurring in the scent of *C. arvense* flower heads (Theis et al. 2007), consistently elicited antennal responses, in more than 50% of tested antennae from both female and male flies: phenylacetaldehyde, methyl salicylate, dimethyl salicylate, and pyranoid linalool oxide (Fig. 1). Less consistent responses were found for the two coeluting compounds methyl benzoate, which was not described by Theis et al. (2007), and linalool (47% response rate); and for 2-phenylethanol (41% response rate). Although we did not test the isolated EAD-active compounds for attractiveness, our results, and those of other researchers, led us to hypothesize that at least methyl salicylate and 2-phenylethanol are attractants for *E. balteatus*. These compounds were emitted in abundant amounts from the flower heads used in this study (Fig. 1), and have been described previously as syrphid attractants (Zhu and Park 2005). In a field experiment, they increased the attractiveness of yellow sticky cards (Zhu and Park 2005).

Recently, Majetic et al. (2009) found that augmentation of inflorescences of *Hesperis matronalis* with scent (collected at night from *H. matronalis*) increased visitation by syrphids, whereas the color of this color-polymorphic plant (white vs. purple) did not influence the visitation rate of flies. Their study also showed that syrphid flies respond to floral scent, but they performed the experiments with manipulated inflorescences. Our study demonstrates for the first time that a syrphid fly uses olfactory and not visual cues to find an unmanipulated pollen/nectar host-plant.

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are instead attracted to the mixture as a whole. Here, we seek to disentangle these alternatives.

In the present study, we investigated the behavioral response of the predatory mite *Phytoseiulus persimilis* to individual volatile compounds and to binary mixtures thereof. This predatory mite feeds on the highly polyphagous two-spotted spider-mite *Tetranychus urticae* (Bolland et al., 1998). The predatory mite relies entirely on olfactory cues to locate distant spider-mite-infested plants (Sabelis and Van der Baan, 1983; Sabelis et al., 1984; Dicke et al., 1990; Dicke and Dijkman, 1992). Different plants produce qualitatively and quantitatively different odor mixtures in response to spider-mite feeding (van den Boom et al., 2004). Consistent with the chemical diversity of the information associated with its prey this predatory mite responds to a wide variety of spider-mite induced plant odor mixtures (van den Boom et al., 2002) and a wide range of structurally different volatile compounds (Dicke et al., 1990; de Boer and Dicke, 2004a, b; Kappers et al., 2005; van Wijk et al., 2008). Discriminating the odor of a spider-mite-infested plant from an uninfested conspecific is a challenging task because many components of both odor mixtures will be present in both. Spider-mite-infested tomato, for example, emits substantial amounts of 19 compounds, 5 of which change significantly in concentration after spider-mite infestation.

Discrimination of the target odor from similar odor sources essentially can be achieved in two ways. Odor perception could be elemental, i.e., the mites perceive and respond to individual components of the mixture. As the components in a mixture are recognized, a component that is an attractant when presented individually is also in a mixture recognized as an attractant. Hence, if odor perception is elemental, the additive effects of all individually recognized attractants and repellents in the mixture will form a likely estimate of the attraction to the mixture. However, in most olfactory systems, the different chemical components of odor mixtures are not detected as independent elements because different odorants of the same mixture may compete for the same receptor site (Oka et al., 2004; Rospars et al., 2008), or because they may affect each other's representation through cross-glomerular circuit interactions in the olfactory system (Kay and Stopfer, 2006; Riffell et al., 2009). Consequently, odor perception becomes synthetic, the individual components of a blend may no longer be recognizable in the mixture, and the mixture is perceived as a distinct odor different from its individual components. This may facilitate the ability to discriminate between mixtures that share many components such as the odor of herbivore-infested plants and uninfested conspecifics. This is because synthetic perception releases the brain from the constraint of representing an odor in the same way, whether it is part of a mixture or not. Hence, correlated olfactory input can be decorrelated in such a way, that the ensemble of neurons encoding one odor, shares little or no overlap with the ensemble of neurons encoding the other. Consequently, when olfaction is strictly synthetic, the response elicited by a specific volatile compound may be entirely different from its contribution to the response elicited by a mixture that it is part of.

The central nervous system of *Phytoseiulus persimili*s consists of just 10.000 cells (van Wijk et al., 2006a). Its glomerular olfactory system is similar to the system found in insects and vertebrates (van Wijk et al., 2006b). The relative simplicity of this mite's olfactory system combined with its reliance on olfaction to locate distant prey makes it an ideal model organism to study how predators utilize the complex and variable source of olfactory information that herbivore- infested plants provide.

The olfactory response elicited by the individual components of an odor mixture serve as the input for three conceptual models that are used to predict the response elicited by the mixture. These model predictions were compared to the observed response elicited by the binary mixture in the predatory mite *P. persimilis*.

(1) One model is additive and therefore tests the assumption that the response elicited by each of the mixture's components contribute equally to the response elicited by the mixture.
(2) In a second model, the response elicited by an odor mixture is equal to the response elicited by the mixture's component that elicits the strongest response. Hence, this model is consistent with the frequently observed phenomenon of overshadowing, i.e., an olfactory system that receives competitive inputs by the different chemical components in the odor mixture. The odor that elicits the strongest response inhibits the perception of or the response to the other components in the mixture.
(3) The third model is intermediate between the other two models. Here, we assume that the response to a mixture is equal to the mean response elicited by its components. Hence, it is assumed that all of the mixture's components contribute equally to the response elicited by the mixture, while the perception of or the response to each component is reduced by the fraction of the total number of components in the mixture.

Because odors occur at different concentrations, we do not only investigate how the different components of odor mixtures affect the response that these mixtures elicit, but also, whether the same mechanism applies at different concentrations of the same odor mixture. This was examined in a separate series of experiments where we asked if a function that describes the response to a binary mixture as a function of the response to its components at

one concentration also describes the response to the same mixture at other concentrations.

Methods and Materials

Plants and Mites Lima been plants (*Phaseolus lunatus*) were grown from seeds in a climate room (22°C, 60% RH, 16:8 LD). After 2 week, these plants were infested with two spotted spider-mites (*Tetranychus urticae* Koch) to establish a culture of this mite. Predatory mites (*Phytoseiulus persimilis* Athias-Henriot) were reared in a climate room (25°C, 60% RH, 16:8 hL:D) on detached, spider-mite-infested Lima bean leaves. The predatory mites received fresh, spider-mite-infested Lima bean leaves every day, and the culture was harvested every day except for the weekend. This frequent harvesting of adult female mites ensured that most mites used in the experiments were one to a few days old after their molt to the adult phase. Predatory mites were obtained originally in 2001 from various locations near the coast of Sicily, Italy. Before choice tests, female predatory mites were kept in Eppendorf tubes and were deprived of water and food for 16–22 hr.

Odor Odors were selected from a panel of pure volatile compounds used to assess the olfactory response of *P. persimilis* (van Wijk et al., 2008). The odors selected for this study ensure that all possible combinations of repellents, attractants, and odors that elicited no response could be made. Most odors were obtained from Fluka with the exception of 2,3-dimethyl pyrazine that was obtained from Aldrich. Octan-1-ol and butan-1-ol were obtained from Sigma, dodecyl acetate from Aldrich, methyl salicylate (MeSA) from Sigma-Aldrich, and β-ocimene (70% *E*- and 30% *Z*- isomers) from R. C. Treat & co. The terpenoids (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were provided by Dr. W. Boland of the Max Planck Institute for Chemical Ecology, Jena, Germany.

Olfactory Response Tests Choice tests were conducted as described by van Wijk et al. (2008). In short, the response to the odors was assessed by using an experimental arena constructed from a Petri dish (diam 9 cm) positioned upside down. A radial airflow was established by the connection of a vacuum pump (flow 0.42 l/min) to an opening at the center of the bottom (lid) of the Petri dish. Prior to the experiment, groups of about 25 predatory mites were placed in cartridges that could be fitted between the vacuum pump and the experimental arena. For each replicate, the arena was provided with fresh odor sources and a new cartridge with a new group of predatory mites. Each odor was tested in a different set-up. To restrain predatory mites after their choice on each side of the arena, an insect glue barrier divided the arena into two halves except for an opening of 3 cm at the center of the bottom of the arena. This opening allowed mites to move from the cartridge to the side of their choice, and it allowed mites to move from one side to the other. One side contained filter paper (diam. 1 cm) with 0.5 μl of the odor (dissolved in hexane), and the other side contained a control filter paper with 0.5 μl of the solvent (hexane). Odor sources were prepared in a fume hut, and the solvent was allowed to evaporate for 1 min before the odor source was placed in the set-up. Mites were released from the cartridge, and after 3 min the mites at both halves of the choice dish were counted.

Control Experiments To assess whether there is a difference between the measured olfactory preference of *P. persimilis* released in groups and *P. persimilis* released individually, a control experiment was conducted. In this experiment, the olfactory preferences of mites released individually (40 mites) and mites released in groups (six groups each with ca. 20 individuals) were compared. Four different combinations were tested: 1) no odor at both sides of the Petri-dish; 2) a Lima bean leaf discs (1,5 cm) vs. no odor at the control side; 3) a leaf disc at both sides; 4) a spider-mite infested leaf disc (web, eggs and mites removed) vs. an uninfested leaf disc.

Experimental Design and Statistics The preference of nine groups, each consisting of ca. 25 mites was tested for each of the two odors and their binary mixture. The experimental design followed a 3×3 Latin square; the odors (A, B, and the mixture AB) formed one side of the square while the other consisted of the experimental sequence. Hence, each odor was perceived by three groups first, by three groups second, and so on, thereby ensuring that three clusters of three groups of ca. 25 mites received the three odors in three different sequences. All experiments in one Latin square were completed in ca. 75 min. The mites were held ca. 20 min. in Eppendorf tubes between subsequent choice tests.

Assuming individual mites make independent choices, the response to each of the three odors compared to the control was analyzed using the replicated *G*-test for goodness of fit (Sokal and Rohlf, 1995). Differences among choice experiments were determined by analysis of variance (*ANOVA*) of the arcsine square root transformed frequencies of the olfactory preference, followed by Bonferroni *post-hoc* testing. This not only allowed us to test if there was a difference between the responses to the odors, but also if there was an effect of the position in the sequence and whether there was a difference among the experimental sequences as a whole.

The experimental results were compared to three model predictions. These models predict the response that a binary mixture elicits as a function of the response to each of the mixture's components. The first model had the following additive structure:

$$E_{(AB)} = \left(0.5 + \left(O_{(A)} - 0.5\right) + \left(O_{(B)} - 0.5\right)\right)*100\%$$

Here, $E_{(AB)}$ is the expected attraction to the mixture AB in%, and $O_{(A)}$ and $O_{(B)}$ are the observed fractions of mites attracted to odor A and B, respectively. If $E_{(AB)}$ became greater than 100%, it was assumed to be 100%. Under the second model, it was assumed that the response that a mixture elicits was equal to the response elicited by the mixture's component that elicited the strongest response. Hence, there is always a complete overshadowing. Under the third model, the expected response elicited by the binary mixture was predicted to be equal to the mean of the response elicited by its components $\left[\left(E_{(AB)} = \left(\left(O_{(A)+}O_{(B)}\right)/2*100\%\right)\right]$. The observed attraction to the binary mixture was compared to the expected attraction under each of the three models using a X^2 for goodness of fit test.

Binary Mixtures with the Same Component Ratio at Different Concentrations Dose response curves (pure odor, 10, 100, and 1000 times diluted odor in hexane) were generated in a way similar to the experiments described by van Wijk et al. (2008). The response to each concentration of each odor was tested in 6 replicate experiments, each based on ca. 20 predatory mites. Dose response curves for the binary mixtures described here were measured simultaneously with the dose response curves for their components published earlier (Van Wijk et al., 2008).

Results

Control Experiments To examine if *P. persimilis* mites released in groups show the same preference for odors in the experimental set-up as *P. persimilis* released individually, 4 control experiments were conducted. The olfactory responses by *P. persimilis* assayed in groups did not differ from those tested individually (Table 1).

To examine whether the repetitive testing affected the responsiveness of the mites, a meta-analysis was performed on all experiments listed in Table 2. We regressed the responsiveness defined as absolute fraction of mites attracted to each odor minus 0.5, on the position in the experimental sequence. We found that the responsiveness did not vary with the position in the experimental sequence (equation of the regression line: $y = -0.0017x + 0.1244$, r^2=0.0002 with y being the responsiveness and x being the position in the experimental sequence). Additionally, we checked for each experiment whether there were differences among the different experimental sequences and among the positions of odors in the experimental sequence using ANOVA on the arcsine square root transformed frequencies of the olfactory preference followed by Bonferroni post-hoc analysis (Supplemental data Table 1). We did not detect any effect of the sequential testing on the olfactory preference except for experiment 1 (α-humulene and linalool). In this experiment, a significant effect of the position in the experimental sequence was found. This was due to the significant heterogeneity in the response to α-humulene (Table 2). In 9 choice tests (three times as the first, the second, and the third odor in the sequence), significant repellence was measured only once, when the odor was presented as the first odor in the sequence. Significant attraction was measured twice, when the odor was presented as the third odor in the sequence. The other six replicates revealed neither attraction nor repellence.

The Contribution of Each Component to the Mixture's Attractiveness We investigated the response of *P. persimilis* to 25 binary mixtures and to their individual components. Table 2 reports the fraction of mites (9 replicate experiments of ca. 25 mites per mixture) that were attracted to each binary mixture and its components. Table 2 also reports the predicted response under each of the proposed models, i.e., the additive response, the averaged response, and the overshadowing response model.

Based on the response to the mixtures' components, the experimental results can be divided into three groups: (1) experiments in which both components did not elicit a significant choice (G_p^{ns}) (experiment 1 to 7, Table 2); (2) experiments in which one of the components elicited a response (G_p*) whereas the other did not (experiment 8 to 17, Table 2); and (3) experiments in which both components elicited a response (G_p*) (experiment 18 to 25, Table 2). Figure 1 depicts the difference between the observed attraction to each binary mixture and the predicted attraction under each of the models.

In experiment 1 to 7 both components of the mixture elicited no response. Hence, in these experiments, the averaged response model and the overshadowing response model predict no significant attraction to or repellence of the mixture. Likewise, the additive response model predicts no response except when the response that each odor elicits borders significance and has the same direction. This latter case explains the significant difference between the observed absence of a response (G_p^{ns}) for the mixture and the expected response under the additive response model in experiments 2 (octan-1-ol and TMTT) and 5 (farnesol and

Table 1 Olfactory preference measured with mites released in groups and mites released individually

Group/ individual	No odor (%)	No Odor (%)	Replicated *G*-test for goodness of fit							*G*-test		Difference between mites released in groups and mites released individually ($\chi 2$ test)
			N	G_p	$P(G_p)$ *d.f.*=1	G_h	$P(G_h)$ *d.f.*=5	G_t	$P(G_t)$ *d.f.*=6	*G*	*P* (*G*) *d.f.*=1	$P(\chi 2)$ *d.f.*=1
6 groups	47.64	52.45	118	0.08	0.78	2.81	0.73	2.89	0.82			
Individuals	42.50	57.50	40							0.90	0.34	0.28
	Leaf disc (%)	Leaf disc (%)										
6 groups	48.74	51.26	119	0.08	0.78	2.81	0.73	2.89	0.82			
Individuals	52.50	47.50	40							0.10	0.75	0.41
	Leaf disc (%)	No odor (%)										
6 groups	52.54	47.46	118	0.31	0.58	5.21	0.39	5.52	0.48			
Individuals	45.00	55.00	40							0.40	0.53	0.10
	Infested leaf disc (%)	Leaf disc (%)										
6 groups	79.00	21.00	119	42.62	0.00	6.82	0.23	49.44	0.00			
Individuals	80.00	20.00	40							15.42	0.00	0.78

dodecyl acetate) (Fig. 1). The significant response to the mixtures (G_p*) in experiment 3 (dodecyl acetate, and 2,3-dimethyl pyrazine) and 6 (hexyl acetate and *cis*-3-hexen-1-ol) was significantly different from the predicted response under all three models. Given the variances among replicates, this does not necessarily imply that the difference among the components and the mixture differ significantly. As the experimental design followed a 3×3 Latin square, differences among odors were determined by analysis of variance (*ANOVA*). In this *ANOVA,* the arcsine square root transformed frequencies of the 27 individual choice experiments were analyzed for significant differences among the response to odors, position in the experimental sequence, and the three groups of different experimental sequences in the experiment. This analysis revealed that there were significant differences between the odors in experiment 6 (F_2=4.33, P<0.05) and in experiment 3 (F_2=18,34, P<0.05). *Post hoc* analysis revealed that the response to the mixture was significantly different from the response to its individual components. Hence, we conclude that in these experiments a mixture of two compounds that individually elicited no response, elicited a response when blended together in a binary mixture.

In experiment 8 to 16, one component of the binary mixture elicited no response whereas the other did. The overshadowing response model predicts that the response to the mixture is equal to the response elicited by the mixture's component that elicited a response. The additive response model predicts a response similar to that. Only the response to the mixture in experiment 8 (α-pinene and *cis*-3-hexen-1-ol) was significantly different from that predicted by the overshadowing response model. Experiments 8, 10 (dodecyl acetate and octan-1-ol) and 16 (linalool and MeSA) revealed a significant difference between the observed and the predicted response under the additive response model. In experiment 16, this appears to result from the fact that the repellence by the mixture was greater than the additive repellence by its components. *ANOVA* and the subsequent *post hoc* analysis revealed that this difference tended to be significant at P=0.07, *d.f.*=6. The reverse is seen in experiments 8 and 10. Here, the mixture elicited no response (G_p^{ns}) whereas one of its components did (G_p*). The *ANOVA* revealed that in both experiments the response to the mixture was not significantly different from the response to its components. Hence, although the responses to the mixture and its components were not significantly different, the replicated *G*-test indicates that the mites made no choice when provided with the mixture, whereas they did when offered one of its components. Moreover, the observed response in experiment 8 was significantly different from both the overshadowed response and the additive response model. In conclusion, in experiments in which only one of the mixtures' components elicited a response, the two models that predict overshadowing for the response to the mixture are most consistent with the observed response. There are, however, several cases where the component that elicited no response contributed to the response elicited by the binary mixture (experiment 8, 10, and 16).

In the last group of experiments, components in each mixture elicited a response. As in the previous groups of experiments, none of the models accurately predicts the response to all binary mixtures in this group. On one side of the spectrum, there are cases of overshadowing by the

Table 2 Observed and expected response to binary mixtures and their components

Experiment	Odor	Dilution factor odor	Fraction mites attracted	Replicated *G* -test for goodness of fit						Difference between observed response and model prediction $P(\chi 2)$, *d.f.*=1
				Gp	*P (Gp)* *d.f.=1*	*Gh*	*P (Gh)* *d.f.=8*	*Gt*	*P (Gt)* *d.f.=9*	
Where both components elicit no response										
1	α-Humulene	100	0.489	0.160	0.690	23.555	0.003	23.715	0.005	
	Linalool	100	0.541	1.731	0.188	9.508	0.301	11.240	0.260	
	α-Humulene+Linalool	100	0.502	0.004	0.951	10.118	0.257	10.121	0.341	
	Averaged response model		0.51							0.697
	Overshadowing response model		0.54							0.237
	Additive response model		0.53							0.402
2	Octan-1-ol	100	0.455	2.425	0.119	12.770	0.120	15.196	0.086	
	TMTT	100	0.465	1.629	0.202	16.098	0.041	17.727	0.038	
	Octan-1-ol + TMTT	100	0.520	0.474	0.491	4.579	0.801	5.053	0.830	
	Averaged response model		0.46							0.072
	Overshadowing response model		0.46							0.052
	Additive response model		0.42							0.002
3	Dodecyl acetate	10	0.518	0.387	0.534	10.578	0.227	10.965	0.278	
	2,3-Dimethyl pyrazine	10	0.492	0.081	0.776	5.852	0.664	5.933	0.747	
	Dodecyl acetate + 2,3-Dimethyl pyrazine	10	0.295	50.806	0.000	15.442	0.051	66.248	0.000	
	Averaged response response model		0.50							0.000
	Overshadowing response model		0.52							0.000
	Additive response model		0.51							0.000
4	DMNT	100	0.537	1.626	0.202	14.432	0.071	16.058	0.066	
	TMTT	100	0.540	2.025	0.155	8.494	0.387	10.519	0.310	
	DMNT + TMTT	100	0.548	2.961	0.085	8.707	0.368	11.668	0.233	
	Averaged response model		0.54							0.786
	Overshadowing response model		0.54							0.828
	Additive response model		0.58							0.368
5	Dodecyl acetate	100	0.549	3.166	0.075	33.353	0.000	36.519	0.000	
	Farnesol	100	0.526	0.826	0.363	12.615	0.126	13.441	0.144	
	Farnesol+Dodecyl acetate	100	0.495	0.029	0.864	8.831	0.357	8.860	0.450	
	Averaged response model		0.54							0.201
	Overshadowing response model		0.54							0.102
	Additive response model		0.58							0.015
6	Cis-3-hexen-1-ol	100	0.487	0.226	0.635	8.331	0.402	8.556	0.479	
	Hexyl acetate	100	0.520	0.610	0.435	10.105	0.258	10.715	0.296	

	Cis-3-hexen-1-ol+Hexyl acetate	100	0.395	17.079	0.000	11.244	0.188	28.323	0.001	
	Averaged response model		0.50							0.001
	Overshadowing response model		0.52							0.000
	Additive response model		0.51							0.001
7	Trans-2-hexen-1-ol	100	0.502	0.003	0.956	20.905	0.007	20.908	0.013	
	α-Terpinene	100	0.494	0.044	0.833	3.097	0.928	3.141	0.958	
	Trans-2-hexen-1-ol+α-Terpinene	100	0.442	4.746	0.029	33.538	0.000	38.284	0.000	
	Averaged response model		0.50							0.094
	Overshadowing response model		0.49							0.117
	Additive response model		0.50							0.107
Where one of the components elicits a response										
8	α-Pinene	10	0.458	1.864	0.172	4.942	0.764	6.805	0.657	
	Cis-3-hexen-1-ol	100	0.404	9.676	0.002	2.835	0.944	12.511	0.186	
	Cis-3-hexen-1-ol+α-Pinene	100+10	0.478	0.478	0.489	9.940	0.269	10.419	0.318	
	Averaged response model		0.43							0.150
	Overshadowing response model		0.40							0.023
	Additive response model		0.36							0.000
9	TMTT	10	0.468	1.091	0.296	7.262	0.509	8.353	0.499	
	Linalool	10	0.589	8.047	0.005	7.049	0.531	15.096	0.088	
	TMTT+Linalool	10	0.583	7.368	0.007	4.542	0.805	11.909	0.218	
	Averaged response model		0.53							0.099
	Overshadowing response model		0.59							0.864
	Additive response model		0.56							0.424
10	Dodecyl acetate	10	0.541	1.871	0.171	10.398	0.238	12.269	0.199	
	Octan-1-ol	10	0.579	7.662	0.006	22.416	0.004	30.078	0.000	
	Octan-1-ol + Dodecyl acetate	10	0.541	2.065	0.151	9.150	0.330	11.215	0.261	
	Averaged response model		0.56							0.570
	Overshadowing response model		0.58							0.246
	Additive response model		0.62							0.015
11	Octan-1-ol	10	0.569	5.640	0.018	8.234	0.411	13.874	0.127	
	α-Pinene	10	0.519	0.468	0.494	17.314	0.027	17.782	0.038	
	Octan-1-ol+α-Pinene	10	0.573	5.860	0.015	17.094	0.029	22.954	0.006	
	Averaged response model		0.54							0.383
	Overshadowing response model		0.57							0.893
	Additive response model		0.59							0.646
12	Cis-3-hexen-1ol	100	0.521	0.514	0.473	9.319	0.316	9.833	0.364	
	MeSA	100	0.369	20.475	0.000	11.625	0.169	32.100	0.000	
	Cis-3-hexen-ol+MeSA	100	0.365	20.563	0.000	10.156	0.254	30.719	0.000	
	Averaged response model		0.45							0.015

Table 2 (continued)

Experiment	Odor	Dilution factor odor	Fraction mites attracted	Replicated *G* -test for goodness of fit						Difference between observed response and model prediction P ($\chi 2$), $d.f.=1$
				Gp	P (Gp) $d.f.=1$	Gh	P (Gh) $d.f.=8$	Gt	P (Gt) $d.f.=9$	
	Overshadowing response model		0.37							0.902
	Additive response model		0.39							0.435
13	s-Limonene	100	0.483	0.379	0.538	23.261	0.003	23.640	0.005	
	MeSA	100	0.405	11.261	0.001	22.664	0.004	33.925	0.000	
	s-Limonene+MeSA	100	0.404	12.013	0.001	18.355	0.019	30.368	0.000	
	Averaged response model		0.44							0.225
	Overshadowing response model		0.41							0.965
	Additive response model		0.39							0.626
14	Linalool	10	0.496	0.025	0.876	13.094	0.109	13.119	0.157	
	MeSA	10	0.251	91.282	0.000	6.587	0.582	97.869	0.000	
	Linalool+MeSA	10	0.216	129.532	0.000	21.959	0.005	151.491	0.000	
	Averaged response model		0.37							0.000
	Overshadowing response model		0.25							0.234
	Additive response model		0.25							0.292
15	DMNT	100	0.506	0.033	0.855	18.408	0.018	18.441	0.030	
	MeSA	100	0.406	9.985	0.002	21.611	0.006	31.596	0.000	
	DMNT+MeSA	100	0.395	11.587	0.001	5.894	0.659	17.482	0.042	
	Averaged response model		0.46							0.068
	Overshadowing response model		0.41							0.739
	Additive response model		0.41							0.616
16	Linalool	100	0.493	2.350	0.125	4.985	0.759	7.335	0.602	
	MeSA	100	0.297	48.432	0.000	8.749	0.364	57.181	0.000	
	Linalool + MeSA	100	0.171	72.499	0.000	18.999	0.015	91.498	0.000	
	Averaged response model		0.42							0.000
	Overshadowing response model		0.30							0.000
	Additive response model		0.34							0.002
Where both components elicit a response										
17	Octan-1-ol	10	0.580	7.572	0.006	18.565	0.017	26.137	0.002	
	3-Octanone	10	0.402	11.439	0.001	8.871	0.353	20.310	0.016	
	Octan-1-ol+3-Octanone	10	0.404	10.204	0.001	15.082	0.058	25.286	0.003	
	Averaged response model		0.49							0.009
	Overshadowing response model		0.40							0.944
	Additive response model		0.48							0.019

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18	MeSA	10	0.317	36.344	0.000	17.687	0.024	54.032	0.000	
	3-octanone	10	0.307	42.757	0.000	6.463	0.595	49.220	0.000	
	MeSA + 3-Octanone	10	0.222	85.316	0.000	12.805	0.119	98.121	0.000	
	Averaged response model		0.31							0.004
	Overshadowing response model		0.31							0.006
	Additive response model		0.12							0.000
19	Dodecyl acetate	10	0.603	12.819	0.000	11.106	0.196	23.925	0.004	
	MeSA	10	0.268	62.738	0.000	16.640	0.034	79.378	0.000	
	Dodecyl acetate+MeSA	10	0.388	15.538	0.000	6.687	0.571	22.225	0.008	
	Averaged response model		0.44							0.156
	Overshadowing response model		0.27							0.000
	Additive response model		0.37							0.579
20	MeSA	10	0.264	67.894	0.000	14.436	0.071	82.330	0.000	
	β-Ocimene	10	0.385	15.369	0.000	8.478	0.388	23.847	0.005	
	MeSA + β-Ocimene	10	0.288	53.349	0.000	17.414	0.026	70.764	0.000	
	Averaged response model		0.32							0.249
	Overshadowing response model		0.26							0.404
	Additive response model		0.15							0.000
21	β-Ocimene	10	0.567	5.884	0.015	6.869	0.551	12.753	0.174	
	Octan-1-ol	10	0.574	7.380	0.007	11.133	0.194	18.512	0.030	
	β-Ocimene +Octan-1-ol	10	0.605	15.874	0.000	10.027	0.263	25.901	0.002	
	Averaged response model		0.57							0.290
	Overshadowing response model		0.57							0.339
	Additive response model		0.64							0.272
22	Benzyl benzoate	100	0.560	5.495	0.019	8.369	0.398	13.864	0.127	
	Farnesol	100	0.395	17.370	0.000	18.078	0.021	35.448	0.000	
	Benzyl benoate+Farnesol	100	0.471	1.320	0.251	12.704	0.122	14.023	0.121	
	Averaged response model		0.48							0.859
	Overshadowing response model		0.39							0.019
	Additive response model		0.45							0.611
23	Farnesol	10	0.447	4.286	0.038	14.368	0.073	18.655	0.028	
	2,3-Dimethyl pyrazine	10	0.424	8.412	0.004	21.891	0.005	30.303	0.000	
	Farnesol+2,3-Dimethyl pyrazine	10	0.179	168.757	0.000	11.333	0.184	180.090	0.000	
	Averaged response model		0.44							0.000
	Overshadowing response model		0.42							0.000
	Additive response model		0.37							0.000
24	Farnesol	100	0.442	4.800	0.028	18.151	0.020	22.951	0.006	
	3-Octanone	100	0.447	4.152	0.042	10.424	0.237	14.576	0.103	
	Farnesol + 3-Octanone	100	0.454	3.163	0.075	13.067	0.110	16.230	0.062	

Table 2 (continued)

Experiment	Odor	Dilution factor odor	Fraction mites attracted	Replicated *G* -test for goodness of fit						Difference between observed response and model prediction
				Gp	*P (Gp)* *d.f.=1*	*Gh*	*P (Gh)* *d.f.=8*	*Gt*	*P (Gt)* *d.f.=9*	*P* (χ2), *d.f.*=1
	Averaged response model		0.44							0.778
	Overshadowing response model		0.44							0.718
	Additive response model		0.39							0.045
25	Dodecyl acetate	100	0.568	6.386	0.012	18.570	0.017	24.956	0.003	
	Linalool	100	0.573	7.437	0.006	16.838	0.032	24.275	0.004	
	Dodecyl acetate+Linalool	100	0.547	3.234	0.072	19.978	0.010	23.212	0.006	
	Averaged response model		0.57							0.492
	Overshadowing response model		0.57							0.445
	Additive response model		0.64							0.004

component that elicited the strongest response, which was most obvious in experiment 17 (octan-1-ol and 3-octanone) and 20 (MeSA and β-ocimene) but this also applies to experiment 21 (β-ocimene and octan-1-ol), 24 (farnesol + 3-octanone), and 25 (dodecyl acetate and linalool). The additive response model accurately predicts the results obtained in experiment 19 (dodecyl acetate and MeSA), 20, 21, 24, and 25 (dodecyl acetate and linalool). Experiment 18 (MeSA + 3-octanone) revealed a weak additivity, which was significantly different from all three proposed models. There also are cases that are consistent with the additive response model experiment 19, 21, and 22 (benzyl benzoate and farnesol). On the other side of the spectrum, there was clear synergism in experiment 23 (farnesol and 2,3-dimethyl pyrazine), which was significantly different from the predictions under all three proposed models.

Binary Mixtures with the Same Component Ratio at Different Concentrations Here, we examined the hypothesis that a function that describes the attraction to an odor mixture as a function of the attraction to its components at one concentration also applies to other concentrations of the mixture. For example, if a mixture of two compounds is as attractive as the sum of the attractiveness of its components, we asked if this relation holds over a range of concentrations where the two components occur in the same ratio.

Dose response curves for the response to binary mixtures and the response to their components were constructed (Fig. 2). Apart from propionic acid, none of the odors elicited a significant response at the lowest concentration (1000× diluted). Since the individual components hardly elicited a response by the *P. persimilis* at this low concentration (van Wijk et al., 2008), and since the binary mixtures also hardly elicited a response (except for octan-1-ol + decan-1-ol), we only considered the three higher concentrations to test the null hypothesis. The null hypothesis was rejected in five (Fig. 2e to i) out of nine examined dose response curves of binary mixtures and their components.

In two experiments in which the null hypothesis was not rejected, the response elicited by one component overshadowed the response elicited by the other at all concentrations (Fig. 2a and b). Hence, the possibility that α-pinene was not perceived cannot be excluded (Fig. 2b). A mixture of two equally attractive components was not more attractive than either of its components (at all concentrations tested). Figure 2d shows a mixture of two compounds that were neither attractive nor repellent at any concentration. At the two highest concentrations, i.e. pure and 10× dilution, the response to the mixture was not significantly different from the response to its components. At the 100×

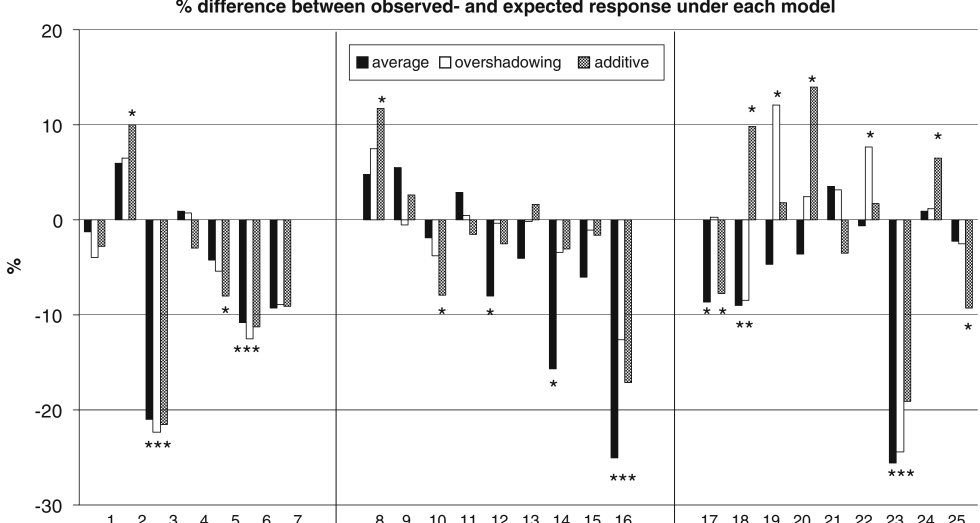

Fig. 1 Behavioral responses by *Phytoseiulus persimilis* to various odors. Below each group of three bars is the reference number of the experiment (also present in Table 2). Depicted is, for each experiment, the difference between the observed attraction (N=225 mites divided over 9 replicate choice tests) and predicted attraction under the averaging, the overshadowing and the additive response model respectively. A * indicates a significant difference ($X^2_{d.f.=1}$ P<0.05) between the observed response and the response under the model. The results are divided in three blocks representing experiments with both components of the mixture not eliciting a response, one component eliciting a response and both components eliciting a response, respectively

dilution, this difference was significant (F_2=4.72, P=0.026), however, the mixture was significantly attractive (G_p*) only at this dilution level while it tended to become significant at the 10× dilution (P (G_p)=0.055). Hence, this is an example where a mixture of two components that elicited no response when presented individually, triggered significant attraction when offered in combination. Although statistical significance for the difference between the mixture and its components was reached only at the 100× dilution, this result does not violate the null hypothesis tested here, as the qualitative contribution of each of the mixture's components to the response of the mites was the same at all concentrations.

The graphs depicted in Fig. 2e to i, all contain at least two points in the dose–response curve that require a different function to describe the attraction to the mixture as a function of the attraction to its components at different odor concentrations. Hence, we conclude that in five out of nine examined dose–response curves of binary mixtures of a constant component ratio, the relative contribution to the behavior elicited by each of the mixture's components varies with the odor concentration. Consistent with the results reported in Table 2 both components of the mixtures used in the dose–response experiments (Fig. 2) also do not appear to contribute equally to the response elicited by the mixtures. Sometimes the response to the mixture is very different from the response to both components. Figure 2e (100× dilution, 2,3-dimethyl pyrazine [(G_p^{ns}, G_h^{ns}), α-terpinene (G_p*, G_h*)]) shows an example of a mixture consisting of a neutral and an attractive compound that together are repellent (G_p*). Figure 2f (10× dilution: propionic acid (G_p*) and octan-1-ol, (G_p*)) shows an example of two attractive compounds that together are repellent (G*).

Discussion

Models based on neurophysiological principles have provided interesting insights in olfaction (Cleland and Linster 2005) and ideally are used to predict the response to odor mixtures. However, the neurophysiology of olfaction in organisms like *P. persimilis* is too poorly understood to construct models of olfactory information processing that incorporate morphological traits and physiological processes. This is why we used phenomenological models to test underlying general principles of information processing as they become apparent from behavioral responses.

The additive response model was based on the assumption that odors presented as individual compounds or as part of mixtures always elicit the same response. This requires that these odors are perceived strictly as elemental objects, and that each recognized object always triggers the same response. The responses to each compound are assumed to sum up to a response towards a mixture of these compounds. In the majority of mixtures studied (13 out of 25), the model prediction was significantly different from the observed results. This is consistent with results of

Fig. 2 The dose response relation of binary mixtures and their components. The experiments were conducted to assess if a function which describes the response of *Phytoseiulus persimilis* to a binary odor mixture as a function of the response to its components at one concentration also describes the response to the mixture at a different concentration. The y-axis represents the attraction of *P. persimilis* to the odor (N=120 mites divided over 6 replicates = 100%), the x-axis represents the four odor concentrations in decreasing sequence (pure, 10×, 100× and 1000× –diluted in hexane). The lowest concentration was omitted from the analysis as the mites hardly responded to the mixture. Above each odor concentration is an abbreviation which qualitatively describes the response to the odor mixture as a function of the response to its components. Abbreviations: ovA/ovB: overshadowing by compound A or B, eq: equal to the mixture's components, me: mean, sy: synergism, op: opposite of its components, at: a mixture of neutral compounds which is attractive, TMTT: (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

studies in spiny lobsters (Lynn et al., 1994; Derby et al., 1996), beetles (Thiery and Visser, 1986), and humans (Schiet and Cain, 1990; Laing et al., 1994) where the response to odor mixtures also was found to be different from the additive response to their components. We conclude that in the majority of the assessed mixtures, *P. persimilis* does not respond to components in odor mixtures as if each of these components is experienced in isolation.

The overshadowing response model is based on the assumption that a component that elicits the strongest response suppresses the detection of or the response to all other components in the odor mixture. This model was best applicable to the specific situation where only one of the mixture's components elicited a response. If both components elicited a response, it fared much more poorly, however. We conclude that the response to an odor mixture usually does not result from absolute overshadowing by the mixture's component that elicits the strongest response.

In its assumption that all components equally contribute to the response elicited by the mixture, the averaged response model retains a key feature of the elemental perception of odors. On the other hand, the contribution of each of the mixture's components to the elicited response is reduced by a factor equal to the number of other components in the mixture. This latter assumption states clearly that each component of an odor mixture affects the perception of or the response to all other components that are part of the mixture. The predicted response under the overshadowing and the averaged response model were significantly different from the observed response in respectively 8 and 9 out of 25 mixtures examined. We conclude that these two models, which assume that components of odor mixtures affect each other's perception, explain the observed response better than the additive model, which assumes an elemental perception of odor mixtures.

Our results suggest that there is no simple rule that will predict accurately the response of *P. persimilis* to binary mixtures as a function of the response to their components. For the simple binary mixtures tested here, the model that predicts the responses to mixtures best still fails to explain 32% of the observed responses. Although the three selected models together span a large part of the possible response spectrum we found that the observed response was significantly different from all three models in 20% of the mixtures assayed.

If we consider the results of both the experiments that tested model predictions and the dose response experiments, we find almost all possible interactions between the components in odor mixtures. There are examples of overshadowing, averaging, synergies, and even a case of attractive components that became repellent when offered in a mixture. There were components of mixtures that in isolation elicited no response, whereas they clearly affected the response elicited by mixtures they were part of. Furthermore, mixtures of the same two components at a constant concentration ratio gave—in the majority of the dose response curves assessed—rise to different interactions at different concentrations (Fig. 2). We conclude that the effect that different components in mixtures exert on the mite's response varies both with component identity and odor concentration. Consequently, knowledge about the repellence or the attractiveness of a specific odor has a limited predictive or explanatory value if this information is used in the context of the response that odor mixtures elicit in *P. persimilis*.

The response to some mixtures was so different from the response elicited by their components that they were probably perceived as something quite different from their components. Some species appear to perceive binary odor mixtures as elemental objects whereas, depending on the combination of odors in the mixture, binary mixtures appear to possess more synthetic properties in other species. Humans, for example, perceive the components of binary mixtures, while they fail to do so in mixtures with a complexity of three to four components (Laing and Francis, 1989; Livermore and Laing, 1996; Marshall et al., 2006). Binary mixtures appear to possess a varying degree of synthetic properties in other species such as rabbits (Coureaud et al., 2009), rats (Staubli et al., 1987; Linster and Smith, 1999; Wiltrout et al., 2003), and slugs (Hopfield and Gelperin, 1989; Sekiguchi et al., 1999).

Phytoseiulus persimilis is well known to acquire a preference for the odor of spider-mite-infested plants over the odor of uninfested plants. However, more remarkably, this predatory mite also learns to prefer odor from uninfested plants if this is associated with food (Drukker et al., 2000; van Wijk et al., 2008). If, in contrast to the results reported here, olfaction were strictly elemental, the mites could in theory associate each of the detectable components in an odor mixture with the presence of their prey. *Phytoseiulus persimilis* has been reported to acquire a preference for MeSA after it has been given the opportunity to associate MeSA containing plant odors with prey (de Boer and Dicke, 2004a), while other components of food-associated odors do not appear to be associated with prey (van Wijk et al., 2008). Parasitoids and bees also behaviorally generalize their conditioned response from a mixture to a small number of its components, while they do not generalize this response to other detectable components in the mixture (Meiners et al., 2003; Reinhard et al., 2010). Other arthropods may not generalize their response to any of a mixture's components. The black bean aphid is repelled by nine host compounds, while a mixture of these repellents is an attractant (Webster et al., 2010). Similarly, the moth *Manduca sexta* responds only to a full floral odor blend and not to its components (Riffell et al., 2009).

There is evidence that representations of odors become more distinct from their components with increasing mixture complexity and with the degree of similarity of the mixture's components. Measurements of the odor induced activity in second order neurons in the olfactory pathway of zebrafish have revealed that binary mixtures of pure odorants are often dominated by the responses induced by one of the components, while complex mixtures of food extracts that have many components in common are more distinct from their components (Tabor et al., 2004). Rats perceive mixtures of similar compounds as dissimilar from their components, while mixtures of dissimilar compounds are perceived as similar to their components (Wiltrout et al., 2003). In our experiments using simple binary mixtures, the mites responded to some mixtures as if they were odors distinct from their components. With increasing mixture complexity, there are more ways for components of odor mixtures to affect each others perception, thereby facilitating the mixtures distinctness from its components. This may well explain why the predatory mites do not generalize their response from the highly attractive odor of a spider-mite infested plant to most spider-mite induced components (van Wijk et al., 2008).

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tomatillo). Both species are difficult to control, and pheromones have been used for monitoring *H. virescens* moth flight and abundance (Chapin et al., 1997; Parajulee et al., 2004).

The chemical composition of *Heliothis* female sex pheromones have been studied in detail (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1982; Heath et al., 1991). The pheromone glands of female *H. virescens* produce at least seven compounds, not all of which have been shown to affect male behavior (Groot et al., 2009). The pheromone compound with the highest titer is (*Z*)-11-hexadecenal (*Z*11-16:Ald), which together with (*Z*)-9-tetradecenal (*Z*9-14:Ald) must be present to elicit oriented male flight behavior (Vetter and Baker, 1983). (*Z*)-11-hexadecenol (*Z*11-16:OH) also is produced by the pheromone gland of *H. virescens.* This compound inhibits the response of males when they are exposed to relatively high concentrations in a pheromone plume (Vetter and Baker, 1983), but when males are exposed to lower amounts of this alcohol in combination with *Z*11-16:Ald and *Z*9-14:Ald, they are attracted (Ramaswamy et al., 1985; Groot et al., unpublished data). *Heliothis subflexa* females produce all of the above compounds but in different ratios from those produced by *H. virescens* (Sheck et al., 2006). The males of *H. subflexa* must be exposed to Z11-16:Ald, Z11-16:OH, and (*Z*)-9-hexadecenal (Z9-16:Ald) to become attracted. Additionally, *H. subflexa* females produce (*Z*)-11-hexadecenyl acetate (*Z*11-16:OAc) which enhances *H. subflexa* male attraction (Groot et al., 2009) but inhibits responses of male *H. virescens* (Vetter and Baker, 1983).

Both *H. virescens* and *H. subflexa* have a similar set of odorant receptor proteins (HvORs and HsORs) that are preferentially produced in the male antennae (Krieger et al., 2004; Vásquez et al., in press). One of these receptors, HvOR13, has been shown to be the receptor of the major pheromone component *Z*11-16:Ald (Gohl and Krieger, 2006; Grosse-Wilde et al., 2007; Kurtovic et al., 2007; Wang et al., in press), and it is expected that the *H. subflexa* ortholog, *HsOR13*, has the same function. *HvOR15* and *HsOR15* have male antenna-biased expression (Krieger et al., 2004; Vásquez et al., in press), and were considered candidate receptors for the critical component *Z*9-14:Ald in *H. virescens* and *Z*9-16:Ald in *H. subflexa,* respectively (Baker, 2009; Krieger et al., 2009; Gould et al., 2010), but recent work with *H. virescens* indicates that this is unlikely (Wang et al., in press), although it is possible that *HvOR15* could be the receptor of (*Z*)-9-tetradecenoic acid (*Z*9-14:COOH), the oxidation product of *Z*9-14:Ald.

We recently have examined the expression levels of five candidate pheromone receptors in *H. virescens* and *H. subflexa*, including *HvOr13, HsOR13*, *HvOR15*, and *HsOR15*. We found that *HvOR13* and *HsOR1*3 transcript abundance was comparable between *H. virescens* and *H. subflexa* male antennae, while *HvOR15* was expressed at higher levels than *HsOR15* (Vásquez et al., in press). This study supported earlier findings (Krieger et al., 2004) showing that transcript levels of these receptors in *H. virescens* are lower in females than in males. Because all of the antennal material used for these experiments was obtained from 2 h post eclosion adults, we do not know if age and/or physiological state could affect their transcript levels. As moths age, male response to female pheromones in some moth species increases (Gadenne and Anton, 2000; Domingue et al., 2006; Evenden and Gries, 2008), and in others it remains constant (Delorme and Payne, 1984). These behavioral data suggest that pheromone receptor expression may vary with age in some species. In *H. virescens,* older males are more responsive to the female sex pheromone and also mate more frequently than younger males (Shorey et al., 1968), but we lacked data on whether this behavior correlated with increased production of pheromone receptor proteins and corresponding higher transcript levels. We also did not know whether production of pheromone receptor proteins decreases after males mate as might be expected based on the finding that *H. virescens* virgin males are more likely to mate than males that had mated (Klepetka and Gould, 1996).

The goal of this study was to examine the effect of age and mating status on *HvOR13* and *HvOR15* gene expression in *H. virescens*, and *HsOR13* and *HsOR15* gene expression in *H. subflexa*. We compared gene expression levels of each of these *OR* genes between virgin male moths collected at different ages (2 h, 1 d, 2 d, 4 d, and 8-d); and between 4-d-old virgin male moths and 4-d-old male moths that had mated multiple times. We hypothesized that receptor transcript levels would increase as the male moths age and become more likely to mate. We further hypothesized that males that had mated would have decreased receptor protein production via reduced *OR* transcript abundance.

Methods and Materials

Insect Rearing and Sample Collection Heliothis virescens and *H. subflexa* individuals used in this experiment were collected from laboratory colonies maintained at North Carolina State University. The *H. virescens* colony was established in 1988 from eggs collected from tobacco in Yadkin County, North Carolina (Gould et al., 1995). The *H. subflexa* colony was established in 1997 from larvae collected from *Physalis angulata* in Florence and Barnwell Counties, South Carolina (Sheck et al., 2006). Field and large cage experiments indicate that these insect strains have maintained normal mate calling and response behaviors (Groot et al., 2006; Gould et al., unpublished).

For our experiments, two cohorts of each insect species were reared at different time periods so that we would have independent biological samples. For *H. subflexa*, the first and second cohorts were reared in June 2008 and July 2008, respectively. For *H. virescens*, the first and second cohorts were reared in September 2008 and October 2008, respectively. For each cohort, a total of 378 neonate larvae of each species were placed individually into 30 ml plastic cups containing corn/soy meal artificial diet (Burton, 1970). Larvae were reared in these cups until the pupation stage, at which time they were separated by sex. Approximately 120 male pupae from each species were placed in small plastic containers (9 cm diam, 7.5 cm high) covered with cheesecloth with a maximum of 40 pupae per container. Pupae and the eclosed adults were kept in a reversed light cycle (14 L:10 D) at 27°C and 50% RH so that experimental procedures could be conducted during the day.

Male adult eclosion was checked at the start of the scotophase, and then every 2 h for the first 8 h of each scotophase. Each emerging male was placed in a 200 ml cup with a cheesecloth top and was provided with a 10% sugar water solution. This procedure was followed for 3–5 d for each species. Fifty adult males that were less than 2 h old were collected and were assigned in groups of ten to the following age treatments: 8 d, 4 d, 2 d, 1 d, and 2 h. For the 2 h treatment, moths were between 0 and 2-h-old. All age treatments correspond to the time at which antennae dissection was performed after eclosion. In addition, we singly paired ten 2-h-old male moths with virgin females in 200 ml cups and maintained them in this condition until the males were dissected. The antennae of these mated male moths were dissected 4 d after eclosion for comparison to 4-d-old virgin males. Mating was confirmed by successful egg hatch. For each cohort, two pools of 5 pairs of antennae were collected per treatment, and each pool represented a sample (experimental replicate). For each sample, the antennae were dissected and immediately placed in a 1.5 ml RNase free tube on dry ice. The antennae were stored at -80°C until RNA extraction. This rearing and dissection regime gave us two biological samples per treatment, per cohort, per species.

Expression Levels of HvOR13, HsOR13, HvOR15 and HsOR15 in Male Moth Antennae using qRT-PCR RNA was isolated from each pool of 5 pairs of antennae (sample unit) using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples ranged from 0.9 to 3.6 μg total RNA. cDNA was synthesized from 150 ng total RNA using Array Script reverse transcriptase (Ambion, Foster City, CA, USA) in a reaction mix that included random hexamers (Applied Biosystems, Foster City, CA, USA), 10X Array Script buffer (Ambion), RNAseOUT (Invitrogen, Carlsbad, CA, USA), and 10 mM dNTPs (Invitrogen). Primers were designed using PRIMER EXPRESS 2.0 software (Applied Biosystems) to target exons of the *OR* genes (*HvOR13*, *HsOR13, HvOR15 and HsOR15)* and two housekeeping genes (*RL31* and *RPS3*) (Vásquez et al., in press). The housekeeping genes *RL31* and *RPS3* were both used as control genes. The same primers were used for *H. virescens* and *H. subflexa* samples since they amplify highly similar homologous cDNA sequences as verified by qRT-PCR product sequencing and alignment to cDNA sequences (Vásquez et al., in press). The sequences for the primers (5′ to 3′) used are as follows:

qHOR13-F: CTGCAAACGCCACATACGAT
qHOR13-R: CCCTTGAATTTGGTAGTGTAGTCAAA
qHOR15-F: TTCTGGTCAAATGATGCTTGGT
qHOR15-R: TCCCGGCTGAGTAATTGTTATACA
qRPS3-F: GGGACCAGCAAGGCAAGA
qRPS3-R: GGGCTCTGTTACCAGGAT
qRL31- F: CTTACACAAACGGCTTCATGGA
qRL31-R: CTGCGAATTTACGGATTTCCTT

Expression levels of *HvOR13*, *HsOR13*, *HvOR15, HSOR15, RL31,* and *RPS3* were determined using quantitative real-time PCR (qRT-PCR) with an ABI Prism 7900 sequence detector and a 384-well optical reaction plate (Applied Biosystems). Each reaction well had a total volume of 10 μl and contained 2 μl cDNA sample, 5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 1 μl of a 10 μM stock of each primer, and 1 μl of nuclease free water (Invitrogen). The qRT-PCR program was run as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min, followed by 95°C for 15 sec and 60°C for 15 sec. A dissociation curve was used to determine primer specificity. A negative control (cDNA reaction without reverse transcriptase enzyme) was used to check for genomic DNA contamination. Three technical replicates (individual reaction wells) were run for each sample per primer pair. Each sample was run twice on separate plates so that we had two measurements (runs) for the same biological material per cohort and per species. A standard curve was created for each primer set using five serial dilutions of genomic DNA to calculate the relative quantities of RNA levels for each sample. The standard curve method was used for data analysis, thus, for each sample, triplicate wells were averaged and the ratio of the gene of interest to the housekeeping gene was calculated (ABI User Bulletin 2). Note that the *H. sublexa* and *H. virescens* samples were collected and analyzed by qRT-PCR at different times, and thus, this experiment was not intended to provide a direct comparison of expression levels between the two species. For a between species comparison see Vásquez et al. (in press).

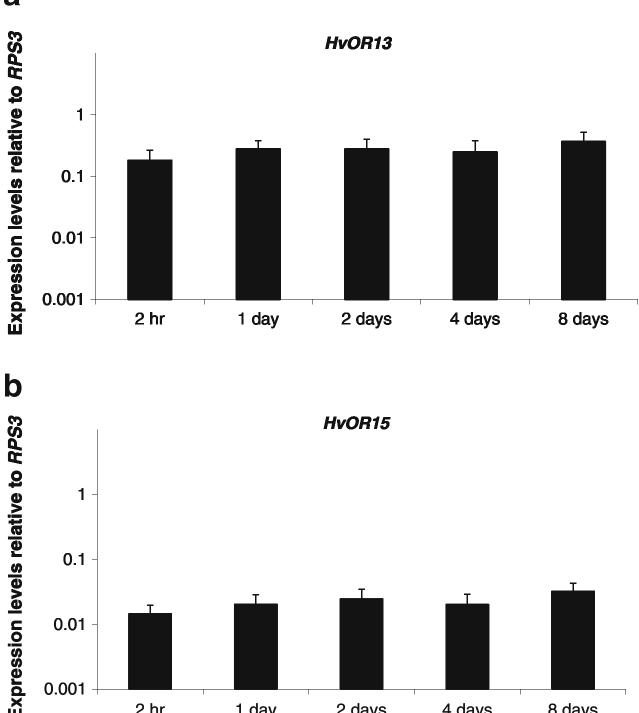
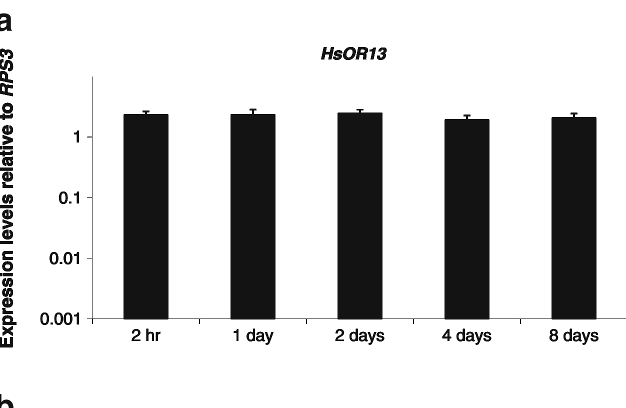


Fig. 1 Odorant receptor gene expression levels in *Heliothis virescens* male antennae across five ages determined by qRT-PCR. Expression levels of **a** *HvOR13* and **b** *HvOR15* were calculated relative to the control gene *RPS3* using the standard curve method. Bars on each column represent standard errors for the mean expression value (*N*= 4). Significant differences in levels of gene expression among treatments are indicated with different alphabetic letters



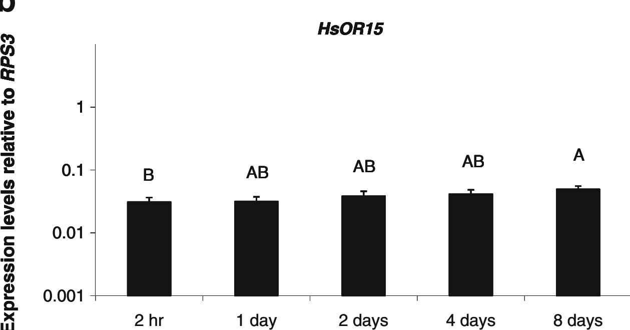


Fig. 2 Odorant receptor gene expression levels in *Heliothis subflexa* male antennae across five ages determined by qRT-PCR. Expression levels of **a** *HsOR13* and **b** *HsOR15* were calculated relative to the control gene *RPS3* using the standard curve method. Bars on each column represent standard errors for the mean expression value (*N*= 4). Significant differences in levels of gene expression among treatments are indicated with different alphabetic letters

Statistical Analyses A mixed model ANOVA was used to analyze gene expression data (log transformed) with gene expression as the response; cohort, age and the interaction treated as fixed effects; and run, run by cohort, run by age and experimental replicate nested within cohort by age treated as random effects. PROC MIXED (SAS, 2004) was used to perform the ANOVA as well as mean comparisons using LSMEANS. Gene expression in antennae of 4-d-old virgin and mated males was compared using *t*-tests.

Results

Housekeeping Genes Two housekeeping genes, *RPS3* and *RL31,* were used in this experiment. *RPS3* expression levels did not differ across *H. virescens* treatments (F=1.2, df=5, 12, P=0.364); or *H. subflexa* treatments (F=2.21, df=5, 4.84, P=0.206). Similarly, *RL31* expression levels were comparable across *H. virescens* treatments (F=2.73, df=5, 12, P=0.071); and *H. subflexa* treatments (F=4.36, df=5, 4.7, P=0.072). Therefore, we present only *HvOR* and *HsOR* expression results standardized to *RPS3* (for *RL31* results, see supplementary material).

Effects of Age Relative RNA quantities of pheromone receptor genes *HvOR13*, *HsOR13*, *HvOR15*, and *HsOR15* in the antennae of *H. virescens* and *H. subflexa* virgin males of five different ages (2 h, 1 d, 2 d, 4 d, 8 d) and 4-d-old mated males were calculated based on a standard curve and normalized to the housekeeping control genes. C_T values estimated for the *OR* and housekeeping genes are reported in Table S1 (Supplementary material). Transcript levels for *HvOR13* in *H. virescens* male antenna were similar (F= 0.72, df=4, 9.64, P=0.599) for all five ages examined (Fig. 1a). *HvOR15* was expressed at comparable levels (F= 0.73, df=4, 10.4, P=0.592) across all age treatments as well (Fig. 1b). In *H. subflexa* male antennae, no significant difference in expression levels of *HsOR13* (F=0.76, df=4, 10.1, P=0.575) was found across all five ages (Fig. 2a). In contrast, *HsOR15* expression levels were 1.6 times higher (F=3.69, df=4, 10.3, P=0.041) in 8-d-old males than those of 2-h-old males (Fig. 2b).

The mean *HvOR13*/*HvOR15* and *HsOR13*/*HsOR15* ratios for the 2 h treatment also were examined. *HvOR13* expression (normalized to *RPS3*) was 12.7 times higher than that of *HvOR15* (t=2.06, df=14, P=0.029), while *HsOR13* expression was 75.2 times higher than that of

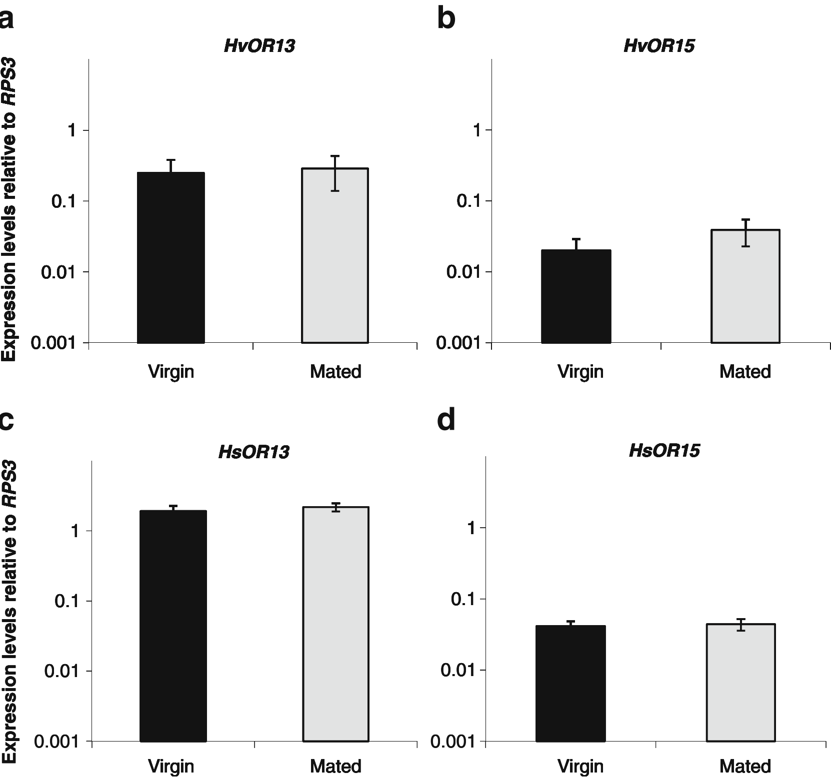


Fig. 3 Odorant receptor gene expression levels in antennae of 4-day-old virgin and mated *Heliothis virescens* and *Heliothis subflexa* males determined by qRT-PCR. Expression levels of **a** *HvOR13*, **b** *HvOR15*, **c** *HsOR13*, and **d** *HsOR15* were calculated relative to the control gene *RPS3* using the standard curve method. Bars on each column represent standard errors for the mean expression value (N=4). Significant differences in levels of gene expression among treatments are indicated with different alphabetic letters

HsOR15 (t=7.01, df=14, P<0.001). These gene expression ratios were approximately three times lower than those found by Vásquez et al. (in press) (*HvOR13*/*HvOR15* = 33.9, *HsOR13*/*HsOR15* = 268.4). These differences may be related to minor technical differences between studies that could affect RNA quantity estimates. However, the *HvOR13*/*HvOR15* ratio is consistently lower than the *HsOR13*/*HsOR15* ratio in both studies, thus, despite the differences in gene expression levels, both studies indicate that *OR13* expression is considerably higher than *OR15* in *H. subflexa* than *H. virescens*.

Effects of Mating Transcript levels of *HvOR13* and *HvOR15* were not significantly different between virgin and mated 4-d-old *H. virescens* male antennae (t=0.20, df=14, P=0.424 and t=1.09, df=14, P=0.147, respectively) (Fig. 3a, b). Likewise, *HsOR13* and *HsOR15* expression levels did not differ (t=0.56, df=14, P=0.292 and t=0.23, df=14, P=0.409, respectively) between virgin and mated 4-d-old *H. subflexa* male antennae (Fig. 3c, d).

Discussion

In biological systems, it is expected that gene expression levels will correlate with physiological and biological needs. For example, Fox et al. (2001) demonstrated that *Anopheles gambiae* females down-regulate one of their odorant receptor genes after taking a blood meal, presumably because these females are switching from searching for blood meals to searching for oviposition sites. Other studies in *A. gambiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* also have shown a correlation between changes in expression of a specific set of genes and behavioral and physiological responses (e.g., Peckol et al., 2001; Dierick and Greenspan, 2006; Abrantes et al., 2008; Zhou et al., 2009).

Male moths also could be expected to alter expression levels of odorant receptor genes based on their physiological and life history status. However, studies that investigate expression levels of odorant receptor protein genes or odorant binding protein genes in male insects, generally examine males of one post-eclosion age category and mating status, or different pre-eclosion age categories (e.g., Michalak et al., 2007; Xiu and Dong, 2007; Patch et al., 2009). In *H. virescens*, only one study has examined odorant receptor expression in male antennae at different times during pupal development (Krieger et al., 2009). A study that examined expression of one odorant receptor and one pheromone binding protein at nine time points during a 24 h period post eclosion in *Spodoptera littoralis* male antennae (Merlin et al., 2007) found no drastic changes in transcript abundance throughout this period of time. Another study that measured the effects of both age and

mating status in the diamondback moth, *Plutella xylostella* (Zhang et al., 2009), found that both factors affected expression levels of the pheromone binding protein gene, *PxylPBP1*. Transcript levels of this gene in male antennae decreased with time after emergence, and interestingly, 16-h-old mated males had considerably higher gene expression levels than unmated males of the same age. We could not find studies that examined effects of mating status and age over a period of time longer than 24 h on *OR* transcript levels.

Our results show that expression levels of *HvOR13*, *HsOR13,* and *HvOR15* do not change as the male moth ages over a period of eight days. It appears that quantities of *HsOR15* RNA increase slightly as the male *H. subflexa* moth ages, however, further replication is needed to confirm this trend. Mating status had no effect on expression levels of either gene in the two moth species. Our results indirectly suggest that males could be equally attuned to female pheromones regardless of age and previous mating experience. However, investment in pheromone receptor levels could be only a small component in the overall male responsiveness to females as other molecules (e.g., pheromone binding proteins, sensory neuron membrane proteins, pheromone degrading enzymes) may be involved in neuronal activation, or, furthermore, male response could be under central brain control. Another factor to consider is that RNA transcript levels do not always correspond with protein levels (Abreu et al., 2009; Bahrndorff et al., 2009), and this could be the case with pheromone receptor proteins.

Because there are no other studies that investigate changes in expression levels of pheromone receptor genes in male moths of ages beyond 24 h after eclosion or changes due to mating status, we compared our results to findings regarding effects of age and mating status on the actual behavioral response of male moths to pheromones. Some studies of male moths suggest that there is an increase in behavioral response to live females or synthetic female pheromones as virgin males age (Traynier, 1970; Baker and Carde, 1979; Gemeno and Haynes, 2000). Experiments with other species suggest there is an increase then decrease in virgin male response to live females or female pheromones (Kanno and Sato, 1978; Delisle, 1995; da Silva et al., 2006). In *Heliothis virescens*, the response of virgin males to female pheromone blends increases with age (Shorey et al., 1968). These behavioral results would not have been predicted from our findings as *OR* transcript levels in adult male antennae remained constant across time. The behavioral change in these insects could of course be due to changes in other aspects of the peripheral or central nervous system. For example, in a study with male *Agrotis ipsilon*, the corpora allata, which produces juvenile hormone (JH), was removed (Gadenne et al., 1993) and male sexual activity was dramatically lowered. Antennal sensitivity to the main pheromone component was measured in allatectomized and control males, and no statistical difference in the amplitude of the EAG response was found between these two groups. Morevover, JH controls behavioral responsiveness to pheromone by acting on central olfactory neurons (Anton and Gadenne, 1999).

In addition, there are a number of studies that have examined the effect of male moth age on electrophysiological response to female pheromones. The results of these indicate that male electrophysiological response varies across species. For *Ostrinia nubilalis*, based on a cut-sensillum technique, there was an increase in male electrophysiological response to female pheromone as the male moths aged (Domingue et al., 2006). Likewise, *Agrotis ipsilon* intracellular recordings indicated an increase in male electrophysiological response to female pheromone as the male moths aged (Gadenne and Anton, 2000). In contrast, in *Helicoverpa zea*, EAG response to the major pheromone component in the female blend remained constant in 1–3 day old male moths (Delorme and Payne, 1984). In cases where a change in electrophysiological response was found, a change in pheromone receptor gene expression could be involved, but this remains to be determined in the *Heliothis* species we examined. With regard to mating status, a study of *Vitacea polistiformis* showed that EAG responses of males to synthetic female pheromone were four times higher in virgin males than mated male moths (Pearson and Schal, 1999). In *H. virescens,* older males are less likely to mate than younger male moths (Klepetka and Gould, 1996). Thus, both studies suggest that production of pheromone receptor proteins could be higher in virgin male moths, and that mating could lead to a lower *OR* gene expression level. Nevertheless, our results do not support a correlation between mating and down-regulation of the *OR* genes examined.

There clearly is a high degree of variation among species in the effects of male age and mating status on behavioral and electrophysiological response to pheromones, but these results at the behavioral and electrophysiological level have not been linked to studies on expression level of genes involved in male response to pheromones. Our current work and that of Merlin et al. (2007) and Zhang et al. (2009) are a first step in this direction, but before any overall assessment of this relationship can be arrived at there is a need for similar gene expression studies in other moth species over the majority of their life spans.

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of (*Z*)-11-hexadecenal and (*Z*)-13-octadecenal, in a 1:1 ratio (Bjostad et al., 1981). This sex pheromone blend is used to monitor populations of this pest in Israel (Anshelevich et al., 1993), and attempts to control the insect by sex pheromone mating disruption also are underway in Israel (Ally R. Harari, personal communication). However, a possible drawback for the successful development of mating disruption of this insect is the instability of the natural pheromone. Aldehydes are unstable under field conditions, suffering from relatively rapid oxidation and polymerization. More stable and behaviorally active structural analogs of aldehyde pheromone components hence are potentially useful in pest management (Ujvary et al., 1993).

In this study, we report the electrophysiological and behavioral responses of *C. gnidiella* males to formate analogs of (*Z*)-11-hexadecenal and (*Z*)-13-octadecenal, namely (*Z*)-9-tetradecenyl formate and (*Z*)-11-hexadecenyl formate, respectively.

Methods and Materials

Insects Cryptoblabes gnidiella larvae were collected from an infested vineyard in Southern Uruguay (Juanicó, Canelones, 34°34′ S, 56°15′ W). The larvae were maintained on post-harvest grape remains, under a 14:10 hr (L:D) photoperiod at 22±1°C and 70–80% relative humidity. Pupae were sexed and maintained separately in cylindrical plastic boxes (12 cm diam., 11.5 cm high) until adult emergence. Adults were fed on a liquid diet, based on honey and sugar, until used in experiments.

Gas Chromatography-electroantennogram Detection (GC-EAD) Studies Analyses were carried out using an HP 5890 Series II Gas Chromatograph with simultaneous flame ionization and electroantennogram detection. The column effluent was split by a fused silica outlet splitter (Alltech Associates, State College, PA, USA), with N_2 makeup (30 ml.min^{-1}) added before the splitter. The outlet column to the EAD was heated to 220°C and passed through a water-cooled condenser tube (1 cm diam.), into a stream of charcoal-filtered and humidified air (300 ml.min^{-1}), to the antennal preparation. The antenna was located 1 cm downstream from the column exit and contacted antenna holders (Syntech, Hilversum, The Netherlands) via electrically conductive gel. The holders were connected to a pre-amplifying probe (10X, Syntech), and the EAD response was further amplified with a high-impedance amplifier (Syntech, Kirchzarten, Germany). The EAD signal finally was fed back to the GC motherboard for digitization and processing. The GC was equipped with an injector in the split mode and an Elite-5 column (Perkin Elmer, Fremont, CA, USA) (30 m × 0.25 mm, 0.25 μm), and operated with a constant carrier gas (H_2) flow of 2 ml.min^{-1}. The temperature program used was 70°C (held for 1 min) to 240°C at 10°C.min^{-1}.

To study the responses of antennae to pheromone components after exposure to formates, 1–3-d-old males (N=10) were placed individually in Petri dishes (9 cm diam.) with 150 holes (each, ca 1 mm diam.) evenly distributed over the base. Another Petri dish base, containing a piece of filter paper (2×2 cm) loaded with 5 μg of the formates (1:1 mixture), was connected to the bottom of the first, allowing the vapor of the formates to enter the upper Petri dish through the holes. The formates were dissolved in *n*-hexane, and the solvent allowed to evaporate before the tests. Males were exposed for 1 hr in the dark, transferred to a clean container, cooled for 5–10 min. before their antennae were excised for GC-EAD studies. The GC-EAD conditions were as described above, except a temperature program of 150°C (held for 1 min) to 240°C at 10°C.min^{-1} was used. The responses of antennae to the pheromone (1:1 mixture, 0.5 mg.ml^{-1}, 0.5 μl, 30:1 injector split ratio) were recorded as the height of the EAD signal, and compared (Mann-Whitney test) to the responses of antennae from control males (N=10) that had been treated under the same conditions without exposure to formates.

Wind Tunnel Experiments Experiments were conducted in a cylindrical acrylic tube (150 cm long × 40 cm diam.) equipped with a controlled suction pump, activated charcoal filters, and a metal screen to smooth the airflow (0.2–0.3 m.s^{-1}). Experiments were carried out at 21–25°C and 70–80% relative humidity. Two hours before the tests, 3–5-d-old virgin males were placed in individual glass tubes (8 cm long × 4.5 cm diam.) with mesh covering both ends. The tests were conducted during the dark period, and the behavioral responses of males were observed and recorded on a digital camera with night-vision technology (Sony DCR-SR45).

Four treatments were evaluated in the wind tunnel tests: **a)** a natural rubber septum containing 50 μg of pheromone (1:1 ratio of components); **b)** a septum loaded with 50 μg of pheromone (1:1 ratio) plus 50 μg of formates (1:1 ratio); **c)** calling virgin females (3–7-d-old); and **d)** calling virgin females along with a septum containing 50 μg of formates (1:1 ratio) placed outside, on top of, the tube enclosing the females. Treatments were hung at the tunnel entrance, inside a glass tube (8 cm long × 4.5 cm diam.; gauze-enclosed for the calling females). For the treatments with virgin females, 1–2 females were placed in the glass tube 2 hr before the experiments; in all replicates, at least one of the females was observed calling throughout the test period. Fifteen individual males were tested to each of the treatments, except (c), in which 20 males were tested.

Tests were run 1–5 hr before the photophase. Each tube containing a male was hung at the end of the tunnel, left

closed for 1 min to allow the odor plume to reach the male, the gauze removed, and the behavior of the male recorded for the next 10 min. The behaviors recorded were, oriented flight (directed toward the source) and source contact. Each male was tested only once. The behavioral data from the various treatments were compared using *Chi-squared* tests.

Field Tests Field trials were performed in an infested commercial vineyard (see insect collection for location), from mid January through early March 2010. Six different ratios of pheromone and formates were tested: 1:0, 0:1, 5:1, 1:1, 1:5, and 1:10. All septa (except 0:1 treatment) were loaded with 1 mg of pheromone (10 µl of a 100 $mg.ml^{-1}$ hexane solution, 1:1 ratio of components), and varying amounts of formates (100 $mg.ml^{-1}$ hexane solution; 1:1 ratio of components). Septa were hung in delta traps (white corrugated plastic; 24×15 cm base × 13.5 cm high), which were placed in vineyard rows (Gewürztraminer variety). Traps were spaced 20 m apart, with treatments randomly distributed in each of four independent blocks, separated by at least 40 m. Once a week, for 6 wk, male captures were checked, treatments within each block rotated, and septa in traps changed. In addition, sticky bases of traps were changed as needed, according to the captures of males. The number of males caught was transformed [$\sqrt{(x + 0.5)}$] for normality, and the data were analyzed by a factorial ANOVA, with blocks and time as independent factors (Statgraphics Plus 5.1). Differences among treatment means were assessed by a Tukey's test ($P \leq 0.05$).

Analytical Procedures Coupled gas chromatography-mass spectrometry (GC-MS) analyses were carried out using a QP-2010 Shimadzu machine, equipped with an OPTIMA-5-MS column (Macherey-Nagel) (30 m × 0.25 mm, 0.25 µm), operated at a constant carrier gas (He) flow of 1 $ml.min^{-1}$. The column oven temperature program was 70°C (held for 1 min), to 300°C at 7°$C.min^{-1}$, the injector temperature was 250°C, and the interface temperature 310°C. Injection (1 µl) was in the split mode. Electron ionization (EI; 70 eV) was used. NMR spectra (^{1}H, ^{13}C, DEPT) were recorded on a Bruker Avance DPX 400 (400 MHz) spectrometer, using $CDCl_3$ as solvent and TMS as internal standard, and are reported in δ values.

Synthesis of Compounds The sex pheromone components, (*Z*)-11-hexadecenal and (*Z*)-13-octadecenal, were obtained by oxidation of the corresponding alcohols with pyridinium chlorochromate (PCC) (1:2.2 molar ratio) in dichloromethane (Corey and Suggs, 1975). The crude product, in dichloromethane, was filtered through silica gel, the solvent evaporated, and the residue purified by flash chromatography (silica gel, hexane-ethyl acetate 9:1) to obtain the pheromone components (97 and 96% pure by GC-MS, respectively). (*Z*)-11-hexadecenal: ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 9.79 (s; 1H, CHO), 5.37 (t, *J*=5.4 Hz, 2H, CH = CH), 2.44 (m, 2H, $H_2C–CH_2O$), 2.05–2.04 (m, 4H), 1.29–1.65 (m, 12H, CH_2), 0.88–0.91 (m, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 203.2; 129.9; 44.3; 32.4; 32.0; 30.1; 29.8; 29.6; 27.6; 27.3; 23.1: 23.0; 22.7; 14.5. MS (EI) *m/z* (%): 238 (0.4); 237 (0.6); 209 (0.7); 152 (5); 149 (6); 138 (6); 137 (5); 136 (6); 135 (13); 125 (9); 124 (10); 123 (10); 122 (8); 121 (22); 112 (13); 111 (22); 110 (16); 109 (20); 108 (8); 107 (8); 99 (6); 98 (42); 97 (37); 96 (38); 95 (41); 94 (11); 93 (12); 85 (11); 84 (24); 83 (46); 82 (44); 81 (50); 80 (12); 79 (13); 71 (14); 70 (29); 69 (60); 68 (24); 67 (46); 57 (27); 56 (26); 55 (100); 54 (17); 53 (5); 43 (25); 42 (8); 41 (43). (*Z*)-13-octadecenal: ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 9.77 (s, 1H, CHO), 5.36 (t, J=4.6 Hz, 2H, CH = CH), 2.41–2.45 (m, 2H, $H_2C–CH_2O$), 1.98–2.07 (m, 4H, $H_2C–CH = CH–CH_2$), 1.60–1.67 (m, 2H, CH_2), 1.27–1.33 (m, 16H, CH_2), 0.89–0.93 (m, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 203.0; 129.8; 42.5; 30.9; 29.5; 27.2; 22.2; 14.6. MS (EI) *m/z* (%): 266 (2); 248 (5); 152 (5); 149 (6); 138 (6); 137 (5); 136 (6); 135 (13); 125 (9); 124 (10); 123 (10); 122 (8); 121 (22); 112 (13); 111 (22); 110 (16); 109 (20); 108 (8); 107 (8); 99 (6); 98 (42); 97 (37); 96 (38); 95 (41); 94 (11); 93 (12); 85 (11); 84 (24); 83 (46); 82 (44); 81 (50); 80 (12); 79 (13); 71 (14); 70 (29); 69 (60); 68 (24); 67 (46); 57 (27); 56 (26); 55 (100); 54 (17); 53 (5); 43 (25); 42 (8); 41 (43).

Both formates were synthesized from the corresponding alcohols, (*Z*)-9-tetradecenol and (*Z*)-11-hexadecenol (Pherobank, The Netherlands). Dicyclohexylcarbodiimide (DCC, 0.38 g, 1.84 mmol) was added to a solution of formic acid (70 µl, 1.84 mmol) in dried dichloromethane (6 ml), before (*Z*)-9-tetradecenol (0.3 g, 1.41 mmol) was added, along with 4-dimethylaminopyridine (DMAP) (catalytic amount). The same procedure was used with (*Z*)-11-hexadecenol (0.3 g, 1.25 mmol), adjusting the amount of reagents to maintain the same molar ratios (Neises and Steglich, 1978). The mixture was stirred for 4 hr under a nitrogen atmosphere at ambient temperature, before the crude product was diluted with dichloromethane (15 ml), filtered, and washed with a saturated solution of NaCl. After evaporation of the solvent, the residue was purified by flash chromatography (silica gel, hexane-ethyl acetate 9:1) to obtain 304 mg of (*Z*)-9-tetradecenyl formate and 302 mg of (*Z*)-11-hexadecenyl formate (90% yield and 99% purity, by GC-MS, for both formates). (*Z*)-9-tetradecenyl formate: ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 8.06 (s, 1H, OCOH); 5.30–5.39 (m, 2H, CH = CH); 4.16 (t, J=6.8 Hz, 2H, CH_2O); 1.99–2.02 (m, 4H, $H_2C–CH = CH–CH_2$); 1.62–1.69 (m, 2H, $H_2C–CH_2O$); 1.26–1.37 (m, 14H, CH_2); 0.89 (t, J=7.0 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 161.4; 130.1; 129.9; 64.3; 32.1; 29.8; 29.5; 29.3; 29.3; 28.6; 27.3; 27.1; 25.9;

22.5; 14.2. MS (EI) *m/z* (%): 194 (7); 138 (9); 137 (6); 124 (13); 123 (11); 111 (4); 110 (23); 109 (23); 97 (13); 96 (63); 95 (51); 83 (25); 82 (89); 81 (84); 80 (5); 79 (8); 70 (10); 69 (44); 68 (46); 67 (76); 66 (7); 57 (9); 56 (18); 55 (100); 54 (38); 43 (18); 42 (10); 41 (54). (*Z*)-11-hexadecenyl formate: ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 8.06 (s, 1H, OCOH); 5.30–5.39 (m, 2H, CH = CH); 4.16 (t, J= 6.8 Hz, 2H, CH_2O); 1.97–2.04 (m, 4H, H_2C–CH = CH–CH_2); 1.62–1.69 (m, 2H, H_2C–CH_2O); 1.27–1.37 (m, 18H, CH_2); 0.89 (t, J=7.0 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 161.4; 130.0; 130.0; 64.3; 32.1; 29.9; 29.7; 29.6; 29.6; 29.4; 29.3; 28.6; 27.3; 27.1; 26.0; 22.5; 14.2. MS (EI) *m/z* (%): 222 (7); 138 (8); 137 (6); 124 (14); 123 (12); 111 (6); 110 (23); 109 (21); 97 (17); 96 (60); 95 (41); 84 (5); 83 (29); 82 (79); 81 (65); 79 (6); 70 (10); 69 (46); 68 (40); 67 (58); 66 (5); 57 (12); 56 (18); 55 (100); 54 (33); 53 (6); 43 (22); 42 (11); 41 (60).

Results

GC-EAD Studies The sex pheromone analogs, (*Z*)-9-tetradecenyl formate and (*Z*)-11-hexadecenyl formate, elicited EAD responses from *C. gnidiella* male antennae (Fig. 1). Although these responses were not quantified, they were similar in magnitude to those of the pheromone components (Fig. 1). That a single antenna responded successively to an analog and pheromone component, respectively, suggests that the perception of the analog did not severely impair the subsequent responses of the antennal receptors. This is consistent with the results from the experiment in which males were pre-exposed either to formate vapors or control air before EAD responses to the pheromone components were recorded: the magnitude of the EAD responses to either pheromone component was similar regardless of pre-exposure (Fig. 2).

Wind Tunnel Experiments No behavioral responses were observed to the formates alone or the control septum in the wind tunnel (data not shown). Over 85% (13/15) of males showed oriented flight, and 60% (9/15) contacted the odor source when tested to synthetic sex pheromone (Fig. 3a). However, an antagonist effect was observed when males were tested to the mixture of pheromone and formates, with only 6.6% (1/15) of males exhibiting oriented flight and 0% contacting the odor source (Fig. 3a). This effect also was apparent when using calling virgin females. Using calling females alone, 45% (9/20) of males exhibited oriented flight and all of these contacted the source, whereas, when calling females were tested with the formates, no males exhibited oriented flight or contacted the source (Fig. 3b).

Field Tests The field trial was conducted during a period of high population levels of the insect, as evidenced by the captures in the pheromone traps (1:0 treatment; average of 314 males/wk and a total of 1,847 over the 6 wk of the experiment; Fig. 4). When the formates mixture was added to the septa, a dose-dependent decrease in male catches was observed (Fig. 4a). Factorial analysis showed no significant effect among blocks ($F_{3,130}$=0.75; P>0.5), but significant effects of time (sampling week; $F_{5,130}$=18.15; P<0.001) and treatment ($F_{5,130}$=217.68; P<0.001). All pheromone: formate blends captured significantly fewer males than pheromone-baited traps. When septa were loaded with a greater amount of formates than pheromone, only one insect was trapped (in 1:5 blend), even though the total amount of pheromone was the same (1 mg) for all treatments (Fig. 4a). The difference in captures was significant in the first week of the experiment, and this tendency was maintained throughout the whole experimental period (Fig. 4b).

Discussion

Our study demonstrates that the sensory system of *C. gnidiella* males can detect (*Z*)-9-tetradecenyl formate and (*Z*)-11-hexadecenyl formate, possibly due to their structural similarity to the sex pheromone components, (*Z*)-11-hexadecenal and (*Z*)-13-octadecenal, respectively. The structural change between each pheromone component and its corresponding analog is the replacement of a methylene group in the α-position to the carbonyl group of the aldehyde by an oxygen atom, with no change in the relative position of the double bond and chain length with respect to the C = O group. Although this substitution causes strong electronic and structural changes at the terminal (functional group) end of the molecule, other molecular characteristics may not be as strongly affected, and therefore may be recognized at the sensory level. This substitution did not result in the analogs being recognized as pheromone mimics but, rather, as antagonists of male attraction, as evidenced by both wind tunnel and field studies in which the formates were potent inhibitors of responses to synthetic or natural pheromone. It is possible that the antagonistic effect occurs at an integrative level affecting, for example, the perceived proportion of blend components, or at a peripheral level. In *Helicoverpa zea* (Noctuidae), for instance, Grant et al. (1989) showed that both the main sex pheromone component, (*Z*)-11-hexadecenal, and an analog, (*Z*)-9-tetradecenyl formate, elicited responses from the same class of receptor neurons on the male antenna. However, in the absence of further electrophysiological data, we cannot say whether the formates interact

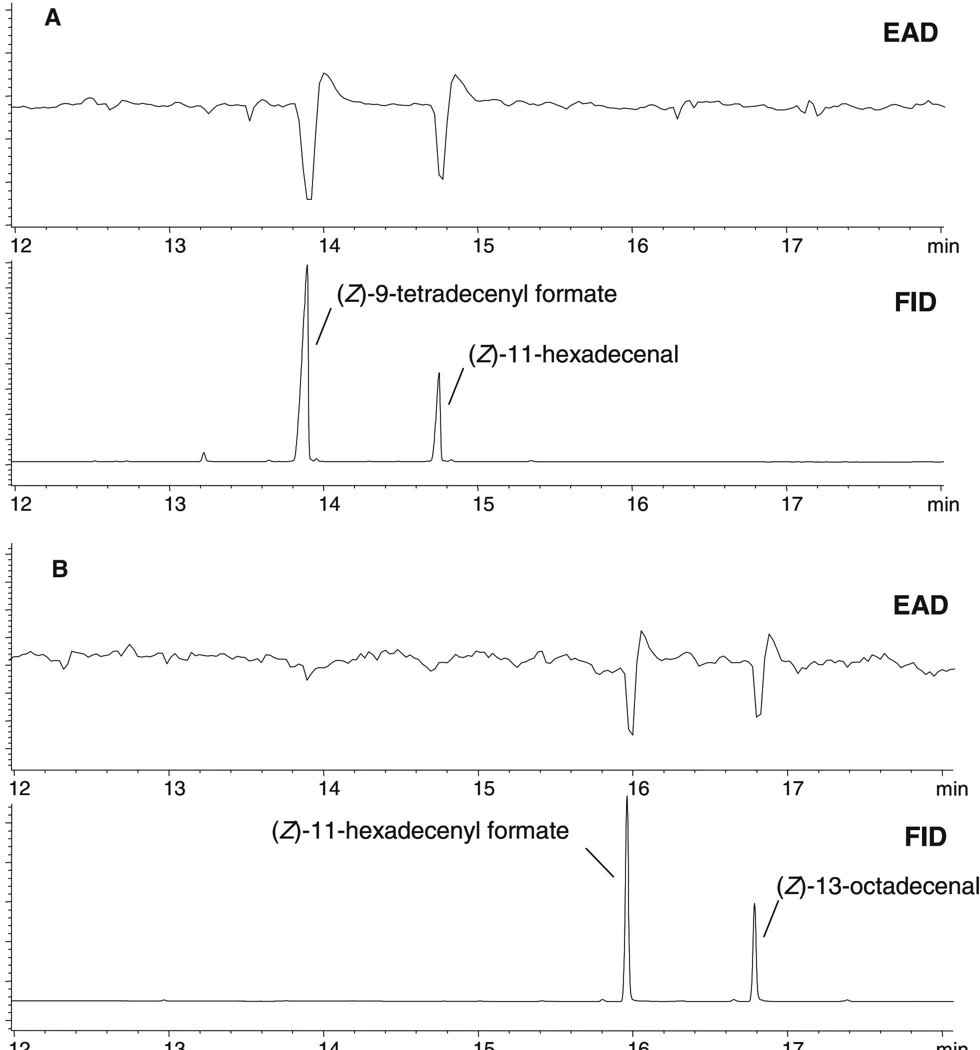

Fig. 1 Gas chromatography-electroantennogram detection responses of *Cryptoblabes gnidiella* male antennae to co-injected pheromone components and their corresponding formate analogs: (**a**) (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenyl formate, and (**b**) (*Z*)-13-octadecenal and (*Z*)-11-hexadecenyl formate. EAD = electroantennogram detector; FID = flame ionization detector

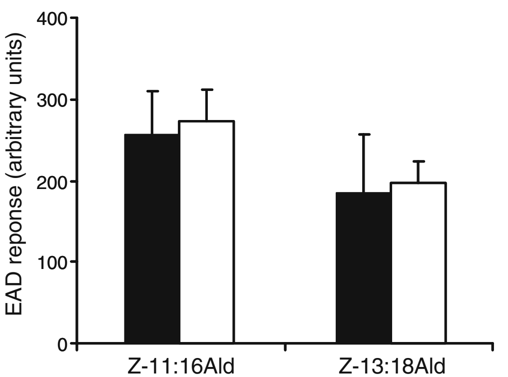

Fig. 2 Electroantennogram detection (EAD) responses of *Cryptoblabes gnidiella* male antennae to sex pheromone components, (*Z*)-11-hexadecenal (Z11-16:Ald) and (*Z*)-13-octadecenal (Z13-18:Ald), after pre-exposure to the vapors of formate pheromone analogs (*open bars*) or control treatment (*black bars*). Responses are expressed as the peak height of the antennal signal (in arbitrary units). N=10 antennae/treatment, excised from different males. Error bars indicate SEM, P>0.3 for both pheromone components (Mann-Whitney test)

with the same or different receptor cells as the pheromone components.

Our data also suggest that the formate analogs do not block the responses of the pheromone receptors to pheromone, with EAD responses from the male antenna being similar for both pheromone components, regardless of pre-exposure to formate. However, it is possible that blocking of the pheromone receptor responses by the analogs could occur with exposure to increased amounts of the formates or a shorter latency between exposure to the formates and the pheromone. A recent study (Giner et al., 2009), using (*E*,*E*)-8,10-dodecadienyl trifluoromethyl ketone, an analog of codlemone, (*E*,*E*)-8,10-dodecadien-1-ol, the sex pheromone of the codling moth, *Cydia pomonella*, found that the electrophysiological responses of antennae of *C. pomonella* males to codlemone were lower for analog-treated males, although only when higher dosages (>50 μg) of the analog were used.

Parapheromones generally are regarded as non-natural compounds, structurally related to natural pheromones, that

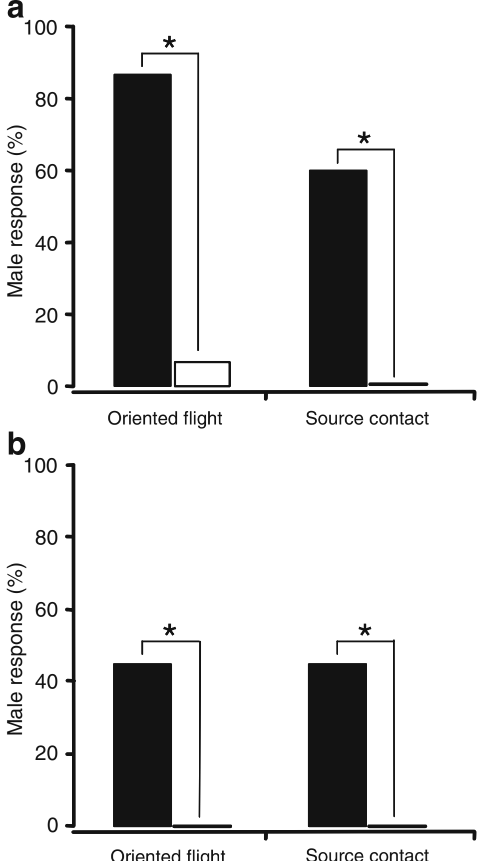

Fig. 3 Behavioral responses (oriented flight, source contact) of male *Cryptoblabes gnidiella* to different odor sources in wind tunnel tests: **a** 50 µg of pheromone (black bars; N=15) or 50 µg pheromone plus 50 µg formates (open bars if different from zero; N=15). **b** Calling virgin females (black bars, N=20) or calling virgin females plus a septum with 50 µg formates (zero males responded, N=15). The asterisks indicate responses of pairs of treatments that are different (χ^2 test; P<0.01)

affect a pheromone-based communication system (Renou and Guerrero, 2000). A large variety of parapheromones has been described and, according to their behavioral effect, are regarded as mimics, synergists, or agonists of the natural pheromone, or as antagonists or inhibitors (Renou and Guerrero, 2000 and references therein). Chemically, these analogs include modifications of the carbon chain or of a polar functional group. Among such compounds, the aldehyde group is replaced by more stable groups, such as formate, methyl, methylene, nitrile, oxime and isothiocyanate, resulting in compounds with either attractant or inhibitory activities (Ujvary et al., 1993).

Formates have been reported as mimics of moth aldehyde pheromones, but, to our knowledge, not conclusively as behavioral antagonists. In *Dichocrocis punctiferalis* (Pyralidae), a 10:1 mixture of (*E*)- and (*Z*)-8-tetradecenyl formate was as attractive as the natural pheromone, a 10:1 blend of (*E*)- and (*Z*)-10-hexadecenal (Mori et al., 1990). Likewise, (*Z,E*)-7,9-11-dodecatrienyl formate, the analog of the major sex pheromone component [(*Z,E*)-9,11,13-tetradecatrienal] of the carob moth, *Ectomyelois ceratoniae* (Pyralidae), was as attractive to males as the natural trienal (Todd et al., 1992). The case of *Heliothis virescens* (Noctuidae) is unclear. Tingle and Mitchell (1978) reported that males of *H. virescens* did not respond to the sex pheromone, a 16:1 blend of (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal, when it was released simultaneously with the analog (*Z*)-9-tetradecenyl formate. According to these authors, pre-exposure to the formate analog also reduced the behavioral responses of *H. virescens* males to the pheromone. However, another report (Mitchell et al., 1978) showed that a mixture of (*Z*)-9-tetradecenyl formate and (*Z*)-9-tetradecenal in a 16:1 ratio was as attractive as the natural pheromone in field trials.

Parapheromones have potential in integrated pest management strategies, especially when natural pheromones are difficult to synthesize or decompose rapidly under field conditions. While mixtures of pheromone and attraction antagonists have shown promising results in mating disruption

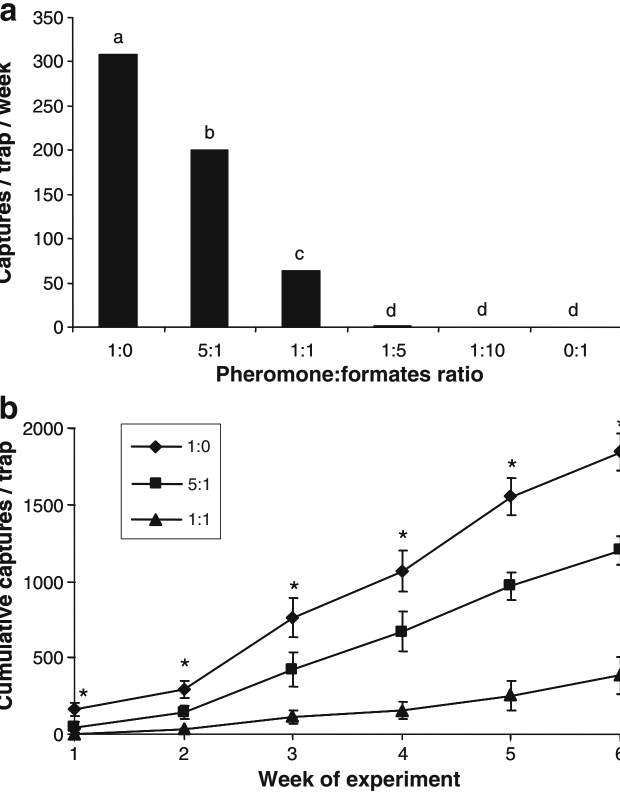

Fig. 4 Catches of *Cryptoblabes gnidiella* males during a 6-week field trial testing different ratios of a synthetic pheromone blend to a two-component blend of formate analogs. **a** Average weekly captures of males. Different letters above bars indicate means that are different (Tukey's test; family error rate <0.05). **b** Cumulative catches of males throughout the trial. Only the 1:0, 5:1, and 1:1 treatments are shown (week 1 = January 27, 2010; error bars indicate standard deviation, * indicates differences among treatments (Tukey's test; family error rate <0.05)

of several species, the potential of parapheromones as mating disruptants has been investigated less (Renou and Guerrero, 2000; Sole et al., 2008). In the case of *C. gnidiella*, there is a strong interest in developing a mating disruption strategy to produce pesticide-free wine with higher market value, especially in regions of the world in which pheromone-based control strategies are already in place for other grapevine pests. We will explore the use of these formate analogs for mating disruption of this pest.

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Three colonies were fed with 30% (w/w) sucrose syrup (hereafter "S-fed"), three fed with the same syrup containing 0.005% (w/w) quercetin (P1-fed), and the others fed the same syrup with 0.01% quercetin (P2-fed). Nectars contain about 0.0005% quercetin (Kenjerić et al., 2007), and honey contains about 2–12 mg phenolics/100 g (Yaoa et al., 2005). Here, we focused on the post-ingestive effect of nectar phenolics on workers and used quercetin concentrations within the typical range for natural honey. Each day, 200 g of test solution and 20 mg of pollen cake (corn pollen moulded into a doughy consistency using a 50% sucrose solution) were supplied to each colony. During the period of the feeding trial, water was available *ad libitum* for the caged colonies. The feeding trial lasted 15 days.

Colony Inspection and Worker Bees' Ovarian Examination Daily inspection of colonies was made twice a day, once in the morning (09:00–11:30) and again in the afternoon (12:00–14:30). At each time, each colony was observed for 10 min to record egg laying by the queen and in-hive worker bee activities such as building cells and tending brood. This procedure provides 'snapshot' data about the reproductive status in each colony.

After colonies were fed for 15 d, more than 300 foraging bees from each colony were captured at the hive entrance. The sampled bees were killed with 70% ethanol, and their ovaries were examined after fixing the insect to a wax board with an insect pin through the thorax. Under a microscope, the 3rd and 4th metasomal segments were separated using two pairs of forceps. The digestive tract was removed to reveal the ovaries. Ovaries were scored as: 0 = ovarioles not visible, 1 = ovarioles clearly visible, or 2 = small eggs or full-size eggs present. For each sample, the bees with each ovarian score were recorded as n_1 (score = 0), n_2 (score = 1), and n_3 (score = 2), and the weighted means of the ovarian scores were calculated as

Table 1 Effect of quercetin in nectar on ovarian development of worker bees

Colony[a]	Number of examined bees	Number of bees with each ovarian score[b]			Weighted mean of ovarian score
		0	1	2	
S-fed	306	176	117	13	0.47
S-fed	309	145	148	16	0.58
S-fed	302	180	115	7	0.43
P1-fed	301	128	162	11	0.61
P1-fed	309	157	137	15	0.54
P1-fed	316	132	171	13	0.62
P2-fed	318	96	183	39	0.82
P2-fed	306	82	183	41	0.89
P2-fed	301	89	177	35	0.82

[a] S-fed, P1-fed and P2-fed represent the colonies fed 30% sucrose syrup, the same syrup containing 0.005 and 0.01% quercetin, respectively.

[b] Ovaries were scored as: *0* ovarioles not visible, *1* ovarioles clearly visible, or *2* small eggs or full-size eggs present.

Fig. 1 Production of queen-like worker bees in colonies of *Apis mellifera* fed with quercetin-laced syrup. The colonies continuously built queen cells throughout the experiment when they were fed with 30% sucrose syrup containing 0.01% quercetin. Queen cells are typically concentrated in the bottom of the brood nest, but colonies fed quercetin-laced syrup built queen cells that were randomly distributed near the center and periphery of the brood nest. In contrast, control colonies fed a diet of pure 30% sucrose syrup with no phenolics did not produce any queen cells during the experiment. *Arrows* indicate the locations of queen cells in the quercetin-treated colonies

$(n_1\times0+n_2\times1+n_3\times2)/(n_1+n_2+n_3)$. The weighted means of the ovarian scores were compared between the S- and P-fed colonies using one-way ANOVA, followed by *post hoc* tests (least significant difference, LSD).

Results and Discussion

After the colonies were fed for 2 to 3 days, 3–11 queen cells emerged in each of the P2-fed colonies (Fig. 1). Some queen cells were torn down by worker bees, but new ones were re-built in the same or in different locations. The queen cells were not positioned in the bottom of the brood, but were randomly distributed throughout the brood nest (Fig. 1). Workers challenged their queens at least two times in each P2-fed colony during the first 3 days of the feeding trail. The S-fed and P1-fed colonies produced no queen cells during the experimental period.

Although workers in each colony were developed from a single mixed brood, experimental colonies had different levels of ovarian development 15 days after starting the feeding trial. In the P2-fed colonies, 12.4% of the worker bees had full-size eggs, and 58.7% had activated ovaries (Table 1). However, only 3.9% of the workers in the S-fed colonies and 4.2% in the P1-fed colonies contained small eggs in their ovarioles, and 41.4% and 50.8% had activated ovaries, respectively (Table 1). Ovarian scores were significantly different among three groups ($F_{2,8}$=25.52, P=0.001). The worker bees had higher ovarian scores in the P2-fed colonies than in the P1-fed and S-fed colonies (LSD, P<0.001 for comparisons between P2-fed and S-fed and between P2-fed and P1-fed). No differences were found between P1-fed and S-fed colonies (P>0.05). Thus, high levels of quercetin caused adult bees to become queen-like.

The excessive numbers of queen cells and well-developed ovarian workers suggested that high concentrations of quercetin in nectar disturbed the pheromonal communication system in honeybee colonies. Quercetin supplementation may affect the production of bee brood and queen pheromones. However, workers began to attack queens and built queen cells only a few days after the trial started. It is unlikely that queens and broods were affected by nectar phenolics during the very early stages of the feeding trial, as they did not directly feed on honey. Another possibility is that nectar phenolics altered worker responses to queen pheromone. The major components of QMP are not volatile, and are transmitted through worker-worker physical contact after workers lick QMP from the queen (Naumann et al., 1991). Worker gustatory receptors are involved in the QMP transmission, that nectar phenolics can modulate bee gustatory responsiveness (Liu et al., 2007; Liu and Liu, 2010). Thus, nectar phenolics such as quercetin might alter bee gustatory response thresholds, disturbing the effective transmission of QMP within a colony.

Although nest homeostasis and bee enzymes can partly detoxify or break down phenolics during the processing of nectar by worker bees (Mao et al., 2009), honey does contain phenolics especially when nectar and pollen were collected during extreme weather (Kenjerić et al., 2007). Extreme weather events are becoming more common due to global climate change (Easterling et al., 2000), suggesting that honeybee populations are at risk of altered social organization due to changes in plant composition in the future.

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grazing management. Within this grassland, *C. brevicollis* is distinguished by its strong tussocks with leaves that are rarely defoliated, in contrast to inflorescences and other accompanying plant species. In recent years, cattle farmers have reported the frequent occurrence of teratogenesis, mainly in the form of arthrogryposis, in early pregnant cows grazing in June–July in one of the main common grazings of Picos de Europa (*Puertos de Aliva*). In 2007, this type of teratogenesis occurred in 16% of the cows that were at risk the previous year while grazing in Aliva (Busqué et al., 2008). The scarcity of other known toxic plant species or teratogenic agents in Aliva, and the absence of similar diseases in cows grazing in nearby rangelands where *C. brevicollis* is not present, support the hypothesis of a causal link between the ingestion of this plant and the incidence of teratogenesis. Additionally, the similarity of this syndrome to the one known as Crooked Calf Disease in North America (Panter et al., 2009), where some plant species rich in certain alkaloids at specific phenological stages are ingested by early pregnant cows, strengthens this hypothesis.

The objectives of this study were to identify and quantify the existing alkaloids in the different plant parts (leaves, stems, and inflorescences) of *C. brevicollis*, and to analyze the relationship between phenology and their concentration. This information is fundamental to considering the possibility that this sedge, or certain parts of it, could be a teratogenic agent in grazing cattle, and to elucidating a hypothesis that explains the nature of this type of intoxication.

Methods and Materials

Study Area The valley of Aliva (43°10′24″N, 4°46′24″W) is a communal rangeland of glacier origin located within the Picos de Europa massif in northern Spain. Its grazing area ranges from 1,300 to 2,000 m above sea level (m.a.s.l), comprising a total of 1,500 hectares. Limestone is the predominant rock, and soils are highly permeable, with high levels of organic matter in the top horizon (12–16%) and moderately low pH values (5–6). Vegetation is mainly a mixture of different grassland types—species-rich *Nardus stricta*, *Mesobromion*, subalpine calcareous, and eutrophic grasslands- and *Genista*-*G. legionensis* and *G. occidentalis*—dominated shrubland. *Carex brevicollis* is abundant in the *Mesobromion* grasslands, which account for approximately 50% of the area of Aliva. Most of these plant communities are of conservation interest at the European level (European Commission 2007). In terms of grazing use, Aliva is an early summer rangeland for most livestock herds of the area. Stocking rate at the beginning of June is 1.5 Livestock Units (LU) per hectare, and in mid July 1.0 LU ha^{-1}. By the beginning of August, when available forage is usually very limited, most cattle and horse herds have already left for other nearby common grazing. Cattle are the predominant livestock type, with approximately 60% of the total LU and a total of 62 different herds. Horses (20%), sheep (16%) and goats (1%) also are present. Forage utilization is high in all grassland types (Bedia et al., 2009).

Plant Sampling Plant samples were collected periodically for the determination of alkaloids during the grazing periods of 2007 and 2008 in three zones within the rangeland at contrasted altitudes (1,350, 1,600, and 1,850 m; named Lower, Mid, and Upper zones henceforth). Sampling dates were 2nd June, 14th June, 28th June, 12th July, 27th July and 14th August in 2007, and 2nd June, 18th June, 3rd July, and 18th July in 2008. Sampling was performed at mid-day, collecting separately green leaves and reproductive stems-inflorescences from non-defoliated plants, and frozen to −8°C. Leaves were collected on all dates, but stems-inflorescences were not always present (Table 1). The total number of samples collected in the field was 68, of which 19 corresponded to inflorescences and stems, and 30 to leaves.

Determination of Alkaloids Frozen samples of leaves, stems, and inflorescences were freeze-dried and milled in a ball mill (Retsch, type MM2). Extraction of alkaloids was performed as described in Muzquiz et al. (1993, 1994). Finely ground samples (0.5 g) were homogenized in 5% trichloroacetic acid with an Ultra-Turrax for 1 min. The mixture was centrifuged for 5 min. at 12100 g, and the supernatant was decanted. The procedure was repeated twice. After centrifugation, 1 ml of 10 M NaOH was added to the combined supernatants. Alkaloids then were extracted with dichloromethane (3×5 ml). The dichloromethane extract was evaporated to dryness, and the alkaloids were dissolved in 1 ml of methanol.

For the quantification of alkaloids, a Perkin-Elmer gas chromatograph equipped with a nitrogen-phosphorous detector (NPD) and operated by a Turbochrom workstation was used. The column employed was a SPB-1 (30 m×0.25 mm id), and helium was used as carrier gas. The temperatures of the injector and detector were 240 and 300°C, respectively. The oven temperature was 150°C, increased by 5°C min^{-1} until reaching 235°C, the temperature that was maintained for 23 min.

As standards of brevicolline and brevicarine were not found, and considering that these alkaloids are derivative of harmane (Vember et al., 1967), their quantification was performed using the commercial standard harmaline hydrocloride dihydrate (Sigma). A stock solution of 1 mg harmaline/1 ml methanol was prepared to perform the calibration curve. Different dilutions from 0.1 to 1 mg/ml

Table 1 Concentration of alkaloids (% dry weight) in the samples of inflorescences, stems and leaves of *Carex brevicollis* collected at different dates and zones (lower: 1350; mid: 1600; upper: 1850 m.a.s.l.) in the Alpine Rangeland of Aliva (Picos de Europa National Park, Spain)

Year	Date	Plant part	Brevicolline			Brevicarine		
			Lower	Mid	Upper	Lower	Mid	Upper
2007	02-jun	Inflorescences	0.112	0.139	n.a.	0.125	0.183	n.a.
		Stems	0.233	0.158	n.a.	0.179	0.218	n.a.
		Leaves	0.461	0.304	0.391	0.068	0.129	0.150
	14-jun	Inflorescences	0.069	0.139	0.148	0.118	0.240	0.406
		Stems	0.185	0.247	0.283	0.107	0.218	0.144
		Leaves	0.462	0.503	0.404	0.054	0.129	0.090
	28-jun	Inflorescences	n.a.	0.092	0.162	n.a.	0.116	0.215
		Stems	n.a.	0.557	0.254	n.a.	0.104	0.090
		Leaves	0.381	0.448	0.479	0.040	0.116	0.106
	12-jul	Inflorescences	n.a.	0.054	0.108	n.a.	0.107	0.161
		Stems	n.a.	0.211	0.184	n.a.	0.062	0.091
		Leaves	0.4468	0.465	0.575	0.045	0.065	0.111
	27-jul	Inflorescences	n.a.	n.a.	0.070	n.a.	n.a.	0.159
		Stems	n.a.	n.a.	0.210	n.a.	n.a.	0.128
		Leaves	0.277	0.338	0.556	0.021	0.063	0.130
	14-aug	Inflorescences	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		Stems	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		Leaves	0.408	0.468	0.553	0.039	0.069	0.090
2008	02-jun	Inflorescences	0.178	0.223	0.145	0.205	0.351	0.219
		Stems	0.247	0.345	0.589	0.148	0.105	0.154
		Leaves	0.657	0.847	0.902	0.049	0.091	0.172
	18-jun	Inflorescences	0.117	0.145	0.140	0.105	0.024	0.180
		Stems	0.220	0.182	0.259	0.051	0.086	0.096
		Leaves	0.779	0.784	0.971	0.045	0.079	0.142
	03-jul	Inflorescences	0.118	0.053	n.a.	0.114	0.073	n.a.
		Stems	0.313	0.201	n.a.	0.096	0.057	n.a.
		Leaves	0.277	1.072	0.296	0.020	0.191	0.022
	18-jul	Inflorescences	n.a.	n.a.	0.082	n.a.	n.a.	0.116
		Stems	n.a.	n.a.	0.200	n.a.	n.a.	0.091
		Leaves	0.816	0.458	0.249	0.077	0.052	0.028

n.a.: sample not available due to the phenology of the plant.

produced a linear response with a correlation coefficient greater than 0.99. The amount of total alkaloids was determined as the addition of brevicolline and brevicarine concentrations.

A capillary GC-MS was applied for the identification of the alkaloids. A Perkin-Elmer Autosystem XL gas chromatograph (working with the same column and conditions as above) was coupled with a mass selective detector (Perkin-Elmer Turbomass Gold) that was combined with the Turbomass software for the identification of alkaloids in the samples.

Phenology In 2007, the reproductive phenology of *C. brevicollis* plants was measured in eight locations within Aliva at different altitudes (1,350, 1,450, 1,525, 1,600, 1,700, 1,775, 1,800, and 1,850 m), by using grazing enclosures, and in six consecutive weeks from the beginning of June to mid-July. Three of these locations corresponded to the Lower, Mid, and Upper zones where plant samples also were harvested. Reproductive phenology was quantified according to the state of the female flowers of the sedge by using the following scale: 1) flowers not yet visible, 2) stigmas just visible, 3) stigmas clearly visible and utricules starting to swell, and 4) utricules well developed. Phenological scores between 0 and 1 were assigned at each location and for each weekly measurement according to the proportion of spikes in each of the phenological states defined (e.g., 0 when all the spikes counted were in state 1, and 1 when all the spikes counted were in state 4).

Growing Degree Days (GDD) GDD were calculated for all sampling dates in the eight phenology locations in order to be used as indirect estimators of the reproductive phenol-

ogy of *C. brevicollis*. Their calculation involved integrating estimated average daily temperatures above 5°C from the 1st of April of each year until the measuring dates. Average daily temperatures were estimated from an exhaustive temperature database collected from a nearby valley (Riofrio, 16 km SSE of Aliva), where 12 temperature sensors (©Onset Hobo® Pro v2) were positioned at regular intervals in an altitudinal gradient from 700 to 2,500 m. These sensors were programmed to record hourly average air temperatures. The estimation of GDD involved the following steps. First, regressions of average daily temperature against altitude for each day between 1st April and 15th August of 2007 and 2008 were performed with the Riofrio dataset. Second, daily average temperature-altitude regressions for the Aliva sites were adjusted by relating the predicted daily average temperatures at 1,450 m in Riofrio (R) with the temperature data recorded by an additional sensor of the same type installed in Aliva at that altitude (A): A=1.06R -2.08 (R^2=0.93). Finally, and according to observed differences in Aliva, a daily correction of the average temperature values of +1°C was performed for the Aliva phenology locations with southern or eastern aspects.

The threshold daily average temperature of 5°C when calculating GDD was chosen after testing showed that it offered the best relationship with phenology and the highest values of explained variance in the statistical models performed.

Statistical Analyses We employed linear mixed-effects models to compare the concentration of each alkaloid (response variable) among the different plant parts (fixed effect), considering as random effect the sampling dates in each zone where samples of the three plant parts were collected (Table 1; a total of 19 subjects).

The existence of phenological changes in the concentration of alkaloids of *C. brevicollis* was approached by using the estimated GDD as surrogate for plant reproductive phenology after testing the robustness of their relationship through linear regression for the 2007 data (Fig. 3). GDD then was used as a continuous explanatory variable capable of capturing relevant phenological information from each of the observations collected in the experiment. The use of GDD instead of phenology in the models was justified by the advantages in its measurement and calculation.

The analyses of the concentration of alkaloids (brevicolline, brevicarine, and total alkaloids) in each plant part (inflorescences, leaves, and stems) were performed by constructing analysis of covariance (ANCOVA) models in which GDD was the covariate and Zone (Lower, Mid, and Upper) and Year (2007, 2008) were fixed effects. Starting from the more elaborate models with the three effects and their interactions, simpler models were developed to acquire finally the simplest models without significantly increasing the residual variance. Simplifications consisted mainly of initially removing non-significant interactions, and next removing non-significant main effects not involved in significant interactions. Some models also were simplified and improved by merging some levels within the Zone effect. Models in which the Zone and Year effects were removed were regression models in which GDD captured all the relevant information contained in the fixed effects (this was the case of the models represented in Figs. 4 a, b, c, and 5 c). Models in which some of the fixed effects or their interaction with GDD remained, were represented by different regression lines (models represented in Figs. 5 a and 6 a), as is characteristic of ANCOVA models.

Other ANCOVA models were performed to study the relationships between the concentrations of the two alkaloids in the same plant part (Figs. 4 d and 5 e) or different plant parts (Fig. 7). In these models, one alkaloid was used as response variable and the other as covariate, while Zone and Year again were used initially as fixed effects.

For certain models, some observations (maximum of two) were rejected if that improved significantly the explanatory power of the models. These rejections are mentioned explicitly in the text.

The final models selected in each analysis were diagnosed for their compliance with ANOVA assumptions through the observation of plots of standardized residuals against fitted values to discard heteroscedasticity, and of quantile-quantine normal error plots to check for normal distribution of the residuals. Transformation of the response variable or of the covariate was performed when that improved significantly the distribution of the residuals. All analyses were conducted in the R language and environment for statistical computing (R Development Core Team, 2009).

Results

Determination of Alkaloids The β-carboline alkaloids brevicolline and brevicarine were the major alkaloids present in *C. brevicollis*, showing distinct peaks in all samples analysed (Fig. 1). Their identity was confirmed by the comparison of their mass spectral degradation pattern (Fig. 2) with published results (Mahboobi et al., 1999; Terenteva et al., 1969).

Alkaloids in Different Plant Parts Brevicolline concentrations were significantly different among plant parts (P<0.001), following the order *leaves*>*stems*>*inflorescences* (mean values of 0.54, 0.24, and 0.11, respectively, *SE* of the log transformed values of 0.092 and 36 *df*). In

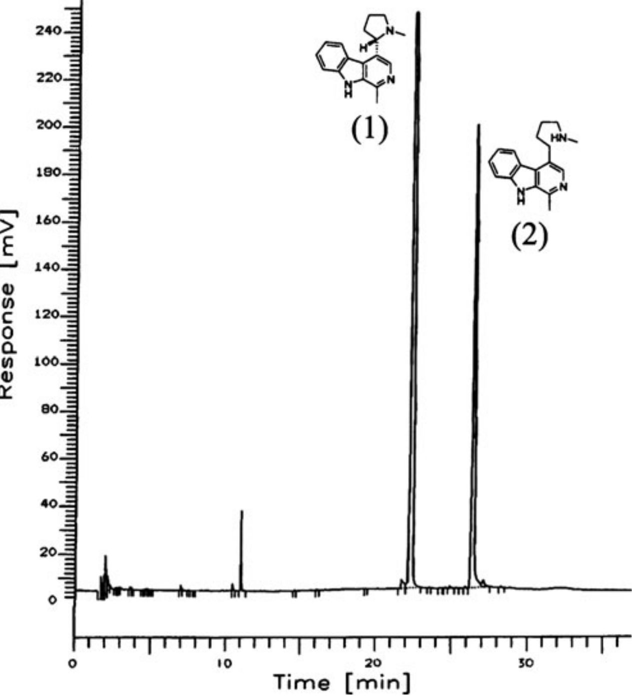


Fig. 1 Chemical structure of the β-carboline alkaloids brevicolline (1) and brevicarine (2) and an example of their profile in leaves of *Carex brevicollis* as obtained by capillary gas-liquid chromatography

the case of brevicarine, concentrations also were different ($P<0.001$), following the inverse order: *inflorescences> stems> leaves* (mean values of 0.16, 0.10, and 0.08, respectively, *SE* the log transformed values of 0.119 and 36 *df*).

Plant Phenology In 2007, GDD was a good predictor of *C. brevicollis* phenology measured by the state of female spikelets (Fig. 3). The regression obtained showed a window of phenological development between 250 and 550 degree days, below which reproductive stems were not yet totally formed, and above which the stems were starting to decay and fruits began to fall off.

Alkaloids in Inflorescences A strong negative logarithmic relationship between the concentration of the existing alkaloids in inflorescences and GDD was found (Fig. 4 a, b, and c). These models were built after rejecting the two observations with the lowest GDD values, as they diverted from the general trend. These outliers were located at values of GDD below those estimated to correspond with flower development (< 250°C; Fig. 3).

For brevicarine (Fig. 4 a), the resultant model was *log(carI)= log(GDD)+ Zone*, where the effect of the

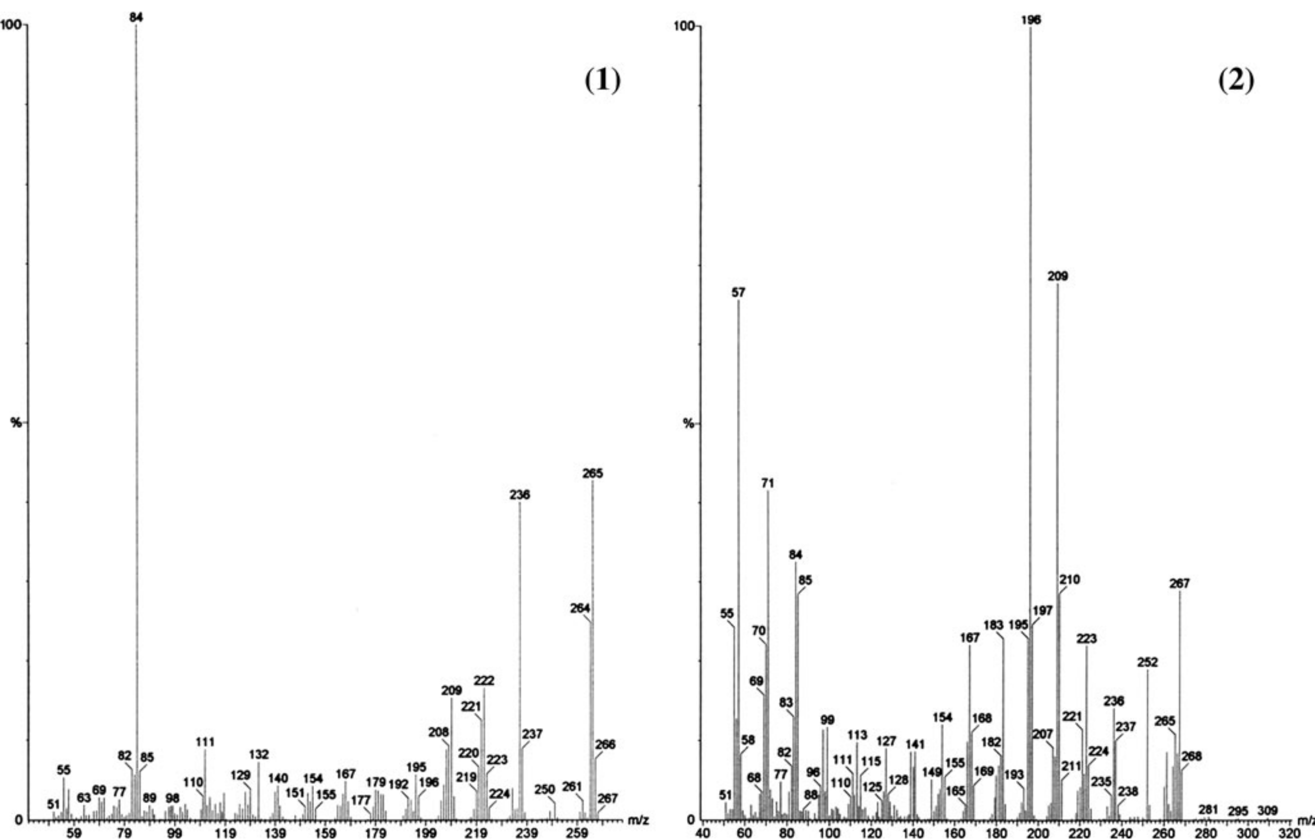


Fig. 2 Mass spectra of the alkaloids brevicolline (1) and brevicarine (2)

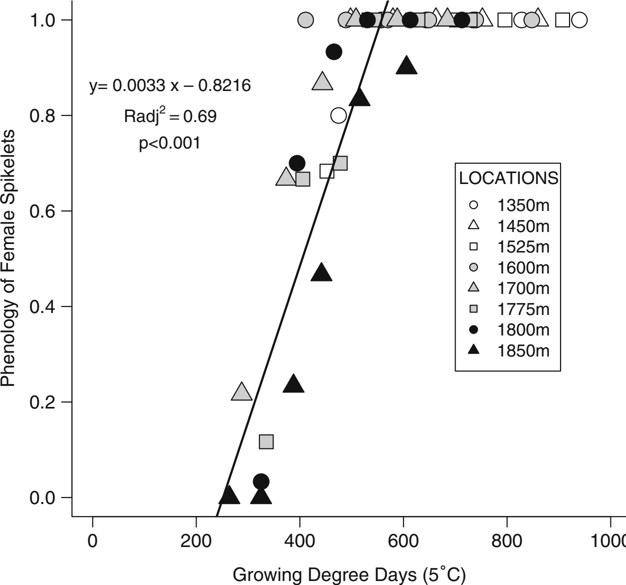


Fig. 3 Relationship between Growing Degree Days with a minimum threshold value of 5°C and an index of phenology of *Carex brevicollis* according to the state of female spikelets (0: all female spikelets with flowers not yet visible; 1: all female spikelets with utricules well developed). Regression performed considering only the observations with phenology values of less than 1

covariate *log(GDD)* was highly significant ($P<0.001$; slope of -1.21 ± 0.248 *SE*), while the *Zone* was not ($P=0.077$; but still kept in the model to reach high explanatory power). A regression model just considering the covariate showed an $R^2_{adj}=0.51$. For brevicolline (Fig. 4 b), the model used was *log(coll)= log(GDD)*. In this case, neither the fixed effects *Zone* and *Year*, nor their interactions, were significant, and could be removed. The covariate *log(GDD)* was highly significant ($P<0.001$; slope of -1.18 ± 0.208 *SE*). Finally, total alkaloids (Fig. 4 c) were fitted to the model *log(alkI)= log(GDD)*. As with brevicolline, neither the fixed effects nor their interactions were significant, and could be removed. The covariate *log (GDD)* was also highly significant ($P<0.001$; slope of -1.12 ± 0.210 *SE*).

A strong positive relationship was also found between the two main existing alkaloids in *C. brevicollis* (Fig. 4 d). In this case, two observations with high brevicarine values were discarded, one that clearly behaved as an outlier and the other because of its potential high influence on the regression. The resulting model was *carI= coli+ Zone*. In this model, the mid and upper zones were grouped together as a single level, as they behaved similarly, and this improved its statistical significance. The slope of the relation between brevicarine and brevicolline was 1.15

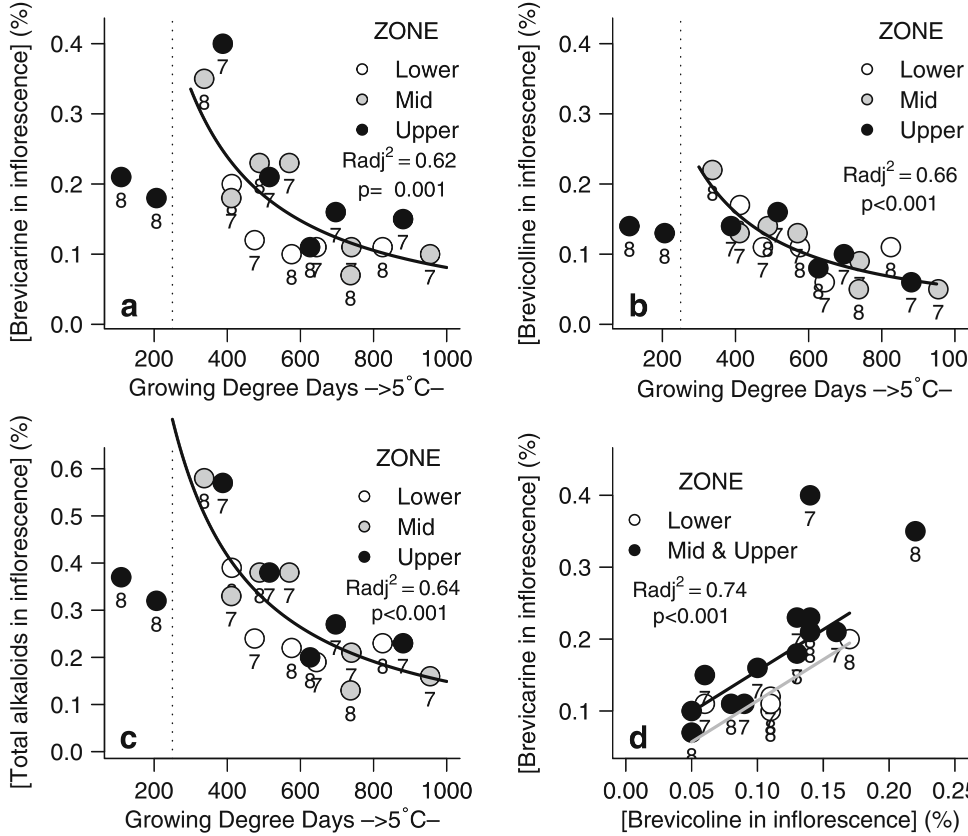


Fig. 4 Alkaloid concentrations in the inflorescences of *Carex brevicollis*. **a** Brevicarine against Growing Degree Days (GDD); **b** Brevicolline against GDD; **c** Total alkaloids against GDD; **d** Relationship between brevicolline and brevicarine. The coefficient of determination and probability in each figure correspond to those of each analysis of covariance. Lines correspond to the regressions between the response variable and the covariate (in 4D the black line refers to the mid and upper zones and the grey line to the lower zone). Labels next to each point indicate year. In figures A, B, and C the observations with GDD below 250, and in figure D the observations with brevicarine greater than 0.3, were not used in the generation of the models

(± 0.176 *SE*), the intercept for the lower zone was 0.00 (± 0.023 *SE*), and that for the mid and upper zones was 0.04 (± 0.014 *SED*) higher. A simple regression model without considering the zone effect reduced the R^2_{adj} to 0.61.

Alkaloids in Leaves Only the concentration of brevicarine showed certain trends related to *GDD*, though always with small absolute values (Fig. 5). The different behavior observed between 2007 and 2008 suggested that different models would be appropriate for each year. In 2007, brevicarine in leaves followed a model of the type *sqrt (carL)= GDD+ Zone*, where the mid and upper zones were grouped together due to their similar behavior (Fig. 5 a). Both *GDD* and *Zone* were highly significant ($P<0.001$). The slope of the regression was $-9.10\times10^{-5}\pm1.81\times10^{-5}$ *SE*, and the difference in intercept values between the lower and mid–upper zones was $+9.40\times10^{-2}\pm1.438\times10^{-2}$ *SED*. In 2008, a quadratic regression best fit the data after considering as an outlier the observation with the highest brevicarine value (Fig. 5 c). The model was *sqrt (carL)= GDD+ GDD²*, showing a minimum value of 0.025% of brevicarine at 703 *GDD*. The intercept was 0.54 ± 0.005 *SE*, the parameter for GDD $-1.09\times10^{-3}\pm2.02\times10^{-4}$ *SE*, and for GDD² $7.77\times10^{-7}\pm1.642\times10^{-7}$ *SE*.

Brevicolline in leaves for each year did not show any defined pattern with *GDD* or *Zone* (Fig. 5 b and d). In 2007, observations were closer to their mean value (0.44 ± 0.020 *SE*; $P<0.001$) than in 2008 (0.63 ± 0.082 *SE*; $P<0.001$). A model considering all observations and the year as the only effect, produced a significant difference between the 2 years (0.20 ± 0.068 *SED*; $P<0.007$). Due to the low brevicarine concentrations in leaves, total alkaloids showed a pattern similar to that of brevicolline.

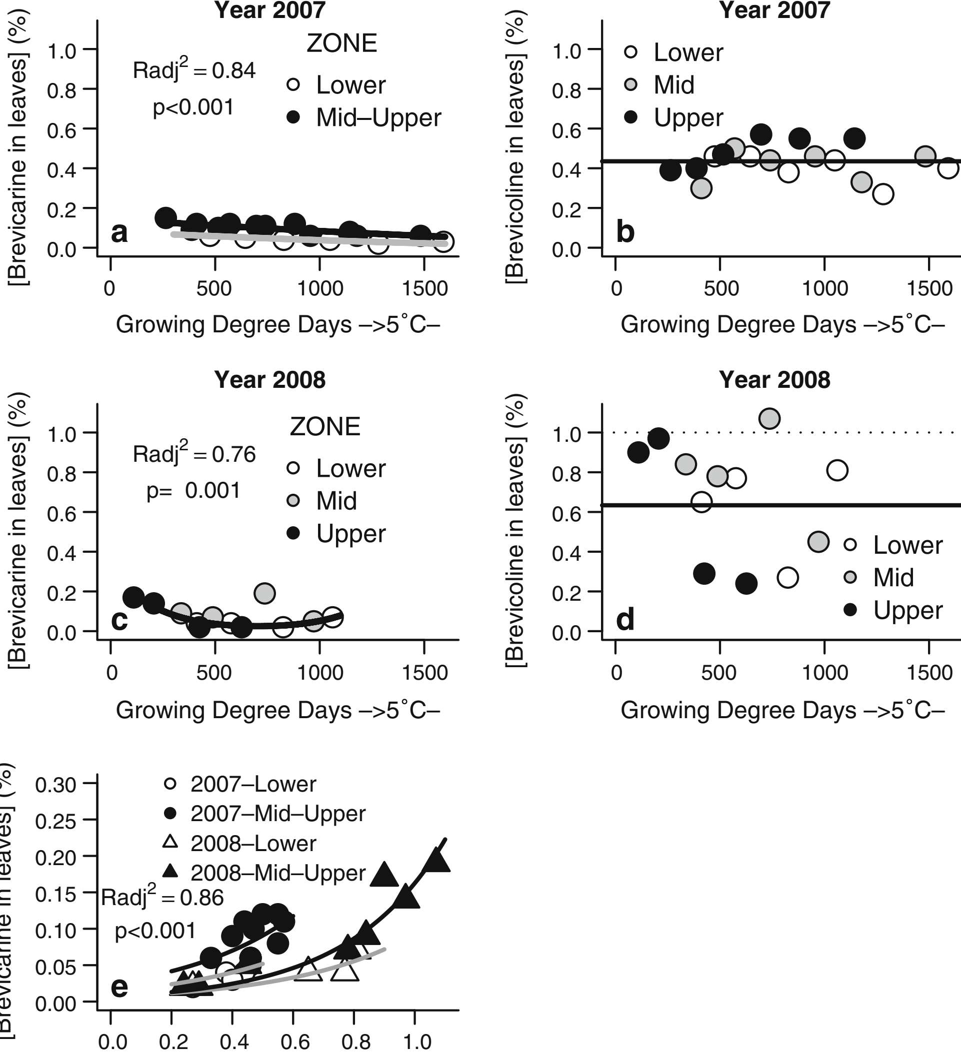
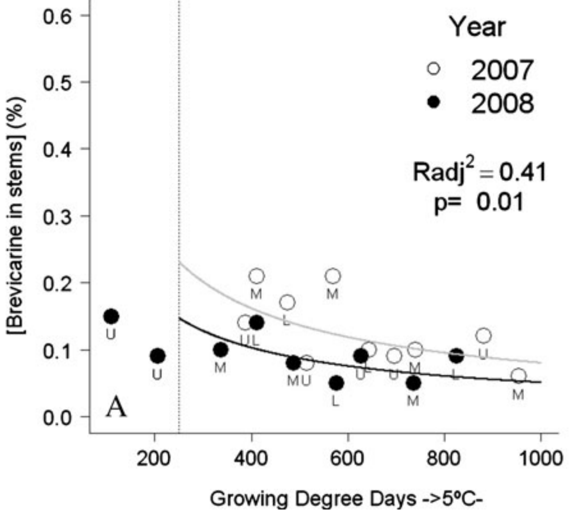
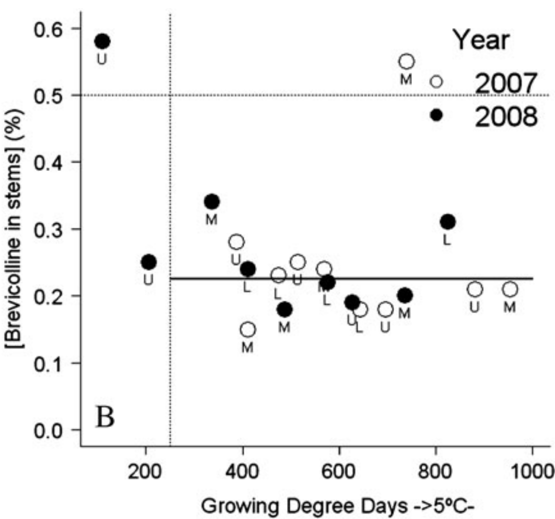


Fig. 5 Alkaloid concentrations in leaves of *Carex brevicollis*. **a** Brevicarine against Growing Degree Days **(**GDD) in 2007; **b** Brevicolline against GDD in 2007; **c** Brevicarine against GDD in 2008; **d** Brevicolline against GDD in 2008. **e** Relationship between brevicolline and brevicarine. The coefficient of determination and probability in each figure correspond to those of each analysis of covariance. Lines correspond to the regressions between the response variable and the covariate GDD (in 5A and 5E the black line refers to the mid and upper zones and the grey line to the lower zone). Observations that were not used in the generation of the models were the one with the highest brevicarine value in figure C, the one with the highest brevicolline value in figure D and two observations not represented in figure E





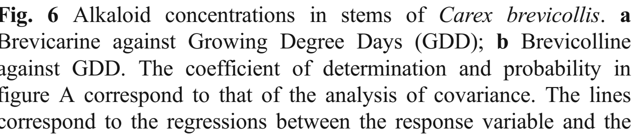


Fig. 6 Alkaloid concentrations in stems of *Carex brevicollis*. **a** Brevicarine against Growing Degree Days (GDD); **b** Brevicolline against GDD. The coefficient of determination and probability in figure A correspond to that of the analysis of covariance. The lines correspond to the regressions between the response variable and the covariate (black for the year 2008, and grey for the year 2007). Labels next to each point indicate site (Lower, Mid, and Upper). The two observations with brevicolline values greater than 0.5 in figure 6B were not considered in the model generation

Finally, a robust model was found that related brevicarine and brevicolline concentrations in leaves (Fig. 5 e): *log (carL)=colL+Year+Zone*. In this model, two observations corresponding to the first sampling date of 2007 in the mid and upper zones were omitted, as they behaved as outliers. In the case of the *Zone*, the mid and upper levels were merged as one in order to gain more explanatory power and parsimony. The three explanatory variables were highly significant ($P<0.001$): the slope for *colL* was 2.59±0.249 *SE*; the difference in the intercept between the lower and rest of zones was 0.56±0.098 *SED*; and the difference in the intercept between 2007 and 2008 was −0.71±0.109 *SED*.

Alkaloids in Stems Brevicarine in stems showed a pattern similar to that of inflorescences, although with lower values, less accentuated decrease with *GDD*, and with a weaker logarithmic relationship (Fig. 6 a). The resulting model was *log(carS)=log(GDD)+Year*. The effect of the covariate *log(GDD)* was significant ($P=0.032$; slope of −0.76±0.276 *SE*), and so was the fixed effect *Year* ($P=0.016$; difference in the intercept between years: −0.45± 0.166 *SED*). A model just considering the covariate was not significant ($P=0.066$).

Brevicolline in stems showed no clear pattern with any of the effects studied (Fig. 6 b). Withdrawing two observations with abnormally high values resulted in the rest of the observations close to a mean value of 0.23± 0.013 *SE*.

Finally, no clear relationship was found between the concentrations of brevicarine and brevicolline in stems (not represented).

Relations Between Alkaloids in Different Plant Parts The proportion of brevicarine to total alkaloids in inflorescences was related to the proportion of brevicolline to total alkaloids in leaves (Fig. 7). The model generated was: *prCarI=prColL+Zone+prColL x Zone*. Lower and Upper zones were grouped together. The significance of the interaction term in both models ($P<0.01$) was due to the different behavior of the Mid zone with respect to the Lower and Upper zones. *Zone* as fixed effect was not significant, and the covariate *prColL* was near the threshold of significance ($P=0.05$). The slope for the linear relationship between the continuous variables and for the Lower–

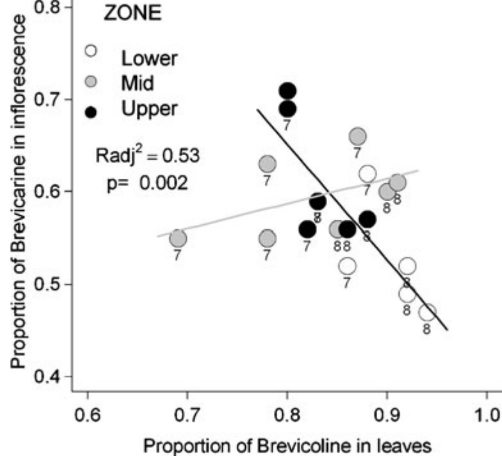


Fig. 7 Relationship between the proportion of total alkaloids in inflorescences being brevicarine and the proportion of total alkaloids in leaves being brevicolline. The black line corresponds to the linear regression for the lower and upper zones, and the grey line for the mid zone

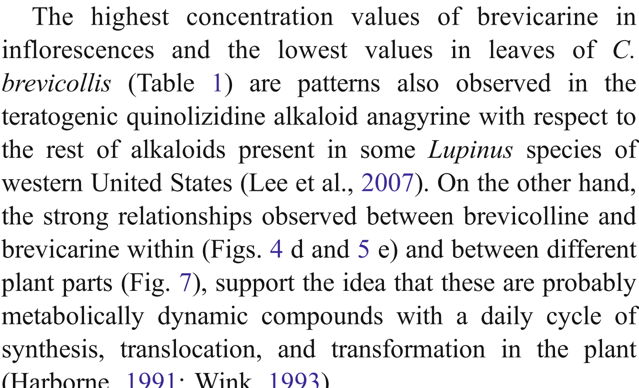
Upper observations was −1.25±0.272 *SE*. The change in slope for the Mid site was 1.52±0.351 *SED*.

Discussion

Flowering phenology of *Carex brevicollis* started early after snowmelt, and was of short duration (Fig. 3). This is characteristic of many sedges of temperate regions (Grime et al., 2007), and also is common in many subalpine and alpine plant species, allowing them to complete seed development before environmental conditions become adverse (Dunne et al., 2003; Inouye et al., 2003). This strategy also is seen in plants subjected to predictable disturbance events, such as strong defoliation by large herbivores during seasonal grazing periods (Pakeman, 2004; de Bello et al., 2005). The observed strong response of flowering phenology to growing degree days also reflects the lack of important additional sources of environmental control of the reproductive phase of *C. brevicollis*, such as water stress, which is frequent in this rangeland later in mid summer.

With respect to the determination of the existing alkaloids in *C. brevicollis*, the results of the mass-spectrometric investigation (Fig. 2) confirmed the existence of brevicolline, a derivative of harmane, with peaks of the molecular ion (M^+ 265) and fragment ions from the pyrrolidine ring. Brevicolline possesses the core nicotine structure (Wagner and Comins, 2006) and the base peak (m/e 84; Fig. 2.1) also is observed in the fragmentation of nicotine (Vember et al., 1967). The presence of brevicarine was supported by the findings of Terenteva et al. (1969), who located its molecular ion in the mass spectrum at m/e 267.

The concentrations of the β-carboline compounds found in *C. brevicollis* in Aliva reaffirm early findings on the presence of brevicolline and brevicarine in leaves of this species (Sharipov et al., 1975). β-Carbolines are a group of pharmacologically interesting and biologically active compounds (Cao et al., 2007). San Emeterio et al. (2008) found that the concentration of brevicolline in plants of *C. brevicollis* was related to the presence of endophytes, arguing that this alkaloid was produced by the plant as a defense against fungal infection. The presence of alkaloids in leaves of certain plant species of grasslands has been considered as a deterrent mechanism against herbivory. This is the case, for example, of *Merendera montana* (Gómez et al., 2003), a plant that frequently shares intensively grazed herbaceous communities with *Carex brevicollis.* With respect to inflorescences, herbivory and selectivity of pollinators also are mediated by the presence of alkaloids in different parts of the flowers and nectar of some herbaceous species (Adler et al., 2001; Adler and Irwin, 2005).



The highest concentration values of brevicarine in inflorescences and the lowest values in leaves of *C. brevicollis* (Table 1) are patterns also observed in the teratogenic quinolizidine alkaloid anagyrine with respect to the rest of alkaloids present in some *Lupinus* species of western United States (Lee et al., 2007). On the other hand, the strong relationships observed between brevicolline and brevicarine within (Figs. 4 d and 5 e) and between different plant parts (Fig. 7), support the idea that these are probably metabolically dynamic compounds with a daily cycle of synthesis, translocation, and transformation in the plant (Harborne, 1991; Wink, 1993).

Growing degree days explained most of the variation in the concentration of alkaloids linked to date and zone in the majority of models developed. The significance of the zone effect in some models, especially in those relating the concentrations of brevicolline and brevicarine within each plant part (Figs. 4 d and 5 e) could be a result of the increase of alkaloids concentration in plant tissues with altitude (Carey and Wink, 1994; Gómez et al., 2003). Year changes in the mean and deviation of brevicolline concentration in leaves (Figs. 5 b and d) also could be attributed to the influence of different environmental factors not explicitly considered in the study (Gay et al., 2007).

The decreasing concentration of brevicolline and brevicarine observed in inflorescences of *C. brevicollis* along its phenological development (Fig. 4) coincided with the pattern observed in other alkaloids from rangeland plant species, such as those of *Delphinium* spp. from western United States (Ralphs et al., 1997), but differed from others, such as anagyrine in *Lupinus leucophyllus*, with highest concentrations at seed maturation (Lee et al., 2007). The lack of a defined phenological pattern of brevicolline and brevicarine concentrations in leaves (Fig 5) also has been reported for other alkaloids in different plant species (Gómez et al., 2003; Lee et al., 2007). In the case of the reproductive stems of *C. brevicollis*, changes in their alkaloid concentrations with phenology were similar to those of inflorescences for brevicarine (Fig. 6 a) and to those of leaves for brevicolline (Fig. 6 b), underlining the main role of the stem in the translocation of these compounds between leaves and inflorescences.

Two factors suggest that the alkaloid brevicarine from the inflorescences of *C. brevicollis* could be the main teratogenic agent in cattle in the rangeland of Aliva. First, we observed frequent defoliation by livestock of inflorescences of this sedge, and at early phenological stages, when the concentration of brevicarine is highest (Fig. 4). In contrast, the ingestion of leaves usually is scarce. Unlike in Aliva, the ingestion of *C. brevicollis* in other rangelands has been related directly to the occurrence of abortions in cows, mares, and ewes (Sandwith and Montserrat, 1968). How-

ever, these abortions occur mainly at the end of summer and autumn (Ruiz de los Mozos et al., 2008), when no inflorescences remain on the plant, and so should be attributed only to the ingestion of leaves, possibly with higher concentrations in brevicolline (Fig. 5) and thus higher abortifacient capacity (Marcu, 1965; Yasnetso and Sizov, 1972). The second factor supporting our hypothesis on the nature of the teratogen in Aliva considers that the type of deformity produced, arthrogryposis, implies that the embryos are susceptible to the action of the teratogenic agent from 1 month of age, when their limbs start to develop (Curran et al., 1986). The data we have gathered on birth dates of deformed calves from cows that previously grazed in Aliva, although yet insufficient to draw definite conclusions, locates the period 30–60 days of gestation of most of these cows between June and July, generally coinciding with the presence of inflorescences of *C. brevicollis* in their home range areas.

The validation of this hypothesis, along with the described strong relationship of brevicarine concentration in inflorescences of *C. brevicollis* with growing degree days (Fig. 4 a), would enable the definition of the seasonal time window with maximum danger of toxicity, depending on the series of daily temperatures from snowmelt to summer and on the spatial distribution of each particular cattle herd within the rangeland. This could provide useful management information for farmers to minimize the risks of teratogenesis.

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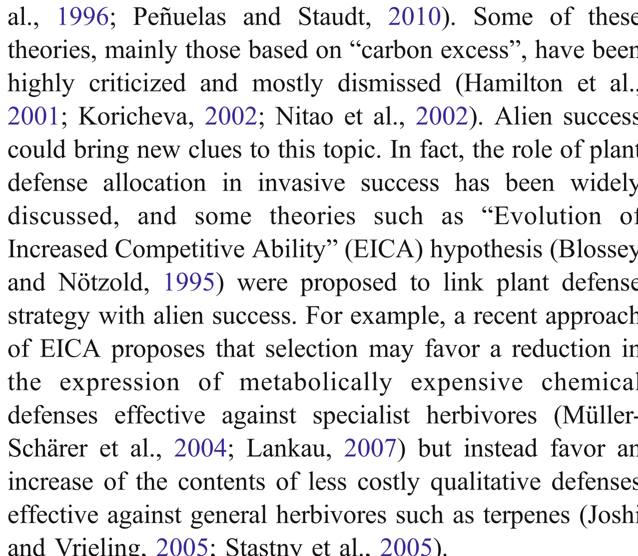
most isolated ecosystem of the Earth (Vitousek and Walker, 1989), are particularly vulnerable to invasions by non-indigenous species (Harrington and Ewel, 1997). Hawaiian native ecosystems and plant species are strongly affected by alien plant species (Mack and D'Antonio, 2003; Hughes and Denslow, 2005; Hughes and Uowolo, 2006). In the Hawaiian Islands, around 861 flowering plant species (47% of total Hawaiian angiosperm flora) are naturalized alien species (Wagner et al., 1999), that have been introduced mainly in the last 200 years (Smith, 1996).

The capacity to produce carbon based secondary compounds (CBSC) such as phenolics, tannins, and terpenes, can be involved in the competition between alien and native plant species by conferring chemical defenses against herbivores, allelochemical defenses against neighboring competing plants (Peñuelas et al., 1996; Peñuelas and Estiarte, 1998; Kursar and Coley, 2003; Mote et al., 2007; Cipollini et al., 2008; Khanh et al., 2008), or/and greater anti stress capacity (Peñuelas and Llusià, 2003, 2004; Filella and Peñuelas, 1999).

Phenolics, including low molecular mass phenolics and condensed polyphenolics such as tannins act by reducing digestibility rather than through direct toxicity (Eichhorn et al., 2007), but many of them also have toxic effects, and may be more toxic for some herbivores than for others (Mole et al., 1990; Cipollini et al., 2008). They are effective mainly against specialist herbivores (Beck and Schoonhoven, 1980; Coley et al., 1985; Bernays and Chapman, 1994; Bennet and Wallsgrove, 1994). Nevertheless, several studies also have demonstrated that they can inhibit the growth of generalist herbivores (Eck et al., 2001; Kouki and Manetas, 2002; Nomura and Itioka, 2002; Albrectsen et al., 2004; Boege and Dirzo, 2004; Kurokawa and Nakashizuka, 2008), although not always (Bi et al., 1997; Goverde et al., 1999; Mutikainen et al., 2000). Terpenes act as deterrents, toxins or modifiers of insect development (Bennet and Wallsgrove, 1994). They are effective against non adapted specialist herbivores (Sorensen et al., 2005), and generalist herbivores (Mihaliak et al., 1987; Landau et al., 1994; Mote et al., 2007). Moreover, terpenoids have many other protective properties such as defense against fungi and pathogens (Gershenzon and Duradeva, 2007), and against abiotic stresses such as high temperature, drought, ozone, or excess radiation (Peñuelas and Llusià, 2003, 2004).

Many hypotheses and theories have been proposed in the last few decades to explain plant defensive strategy against herbivores. They range from theories based on plant capacity to allocate resources in excess of growth demands into defenses, such as a "carbon excess" hypothesis (Bryant et al., 1983; Peñuelas and Estiarte, 1998), to theories with more evolutionary bases (Hamilton et al., 2001) that bypass theories on enhanced terpenoid production as a result of enhanced nutrient availability (Harley et al., 1994; Litvak et



al., 1996; Peñuelas and Staudt, 2010). Some of these theories, mainly those based on "carbon excess", have been highly criticized and mostly dismissed (Hamilton et al., 2001; Koricheva, 2002; Nitao et al., 2002). Alien success could bring new clues to this topic. In fact, the role of plant defense allocation in invasive success has been widely discussed, and some theories such as "Evolution of Increased Competitive Ability" (EICA) hypothesis (Blossey and Nötzold, 1995) were proposed to link plant defense strategy with alien success. For example, a recent approach of EICA proposes that selection may favor a reduction in the expression of metabolically expensive chemical defenses effective against specialist herbivores (Müller-Schärer et al., 2004; Lankau, 2007) but instead favor an increase of the contents of less costly qualitative defenses effective against general herbivores such as terpenes (Joshi and Vrieling, 2005; Stastny et al., 2005).

We aimed to study the link between the plant invasive success in the Oahu (Hawaii) flora and CBSC contents. With this purpose, we conducted an analysis of leaf total phenolic (TP), leaf total tannin (Tta), and leaf total terpene (TT) accumulation in 35 native and 38 alien woody plant species randomly chosen among the most representative current Hawaiian flora in order (i) to compare TT, Tta, and TP contents of native plants with those of alien plants after taking into account the phylogenetic effects, and (ii) to compare also the relationships of TT, Tta, and TP contents with the leaf traits linked to foliar economy and production capacity, such as photosynthetic capacity (Amass), leaf mass per unit area (LMA), and leaf N, P, and K contents. With these investigations, we additionally aimed to test the predictions of some of the CBSC hypotheses such as the "excess carbon" hypotheses (decreased phenolics and tannins in alien nutrient-rich fast-growing species), nutrient-enhanced terpene production, and the modified "competitive ability" hypotheses (increased less costly terpene accumulation against generalist herbivores and decreased more costly phenolic and tannins accumulation against adapted herbivore specialists in alien species).

Methods and Materials

Field Sites

The study was conducted in May 2007 on the island of Oahu (Peñuelas et al., 2010; Sardans et al., 2010), the third largest of the Hawaiian Islands. As typical of larger Hawaiian Islands, the climate is characterized by steep rainfall gradients over short distances (Müller-Dombois and Fosberg, 1998). While precipitation is distributed almost uniformly in lowland and mountain rain forests, lowlands at the leeward side have a pronounced dry summer season.

Due to the oceanic tropical climate, temperature oscillations are small with winters having on average 2–3°C cooler temperatures than summers. As large differences in composition of native and alien vegetation occur in response to rainfall gradients, four sites with distinct precipitation regimes were selected for plant sampling in the leeward lowlands of Oahu and at the leeward side of Koolau mountains (Appendix Table 1) (see details in Peñuelas et al., 2010).

The four key soil types found across the sites rank according to the state of weathering as *oxisols* > *ultisols* > *mollisols* > *inceptisols* (Uehara and Ikawa, 2000; Deenik and McClellan, 2007). More leached *oxisols* and *ultisols* with lower pH are among the soils with lowest fertility, and *Mollisols* exhibit the highest fertility (Uehara and Ikawa, 2000; Deenik and McClellan, 2007). *Inceptisols*, the youngest soils, typically show weak profile development, and exhibit tremendous variability in fertility depending on genesis (Deenik and McClellan, 2007). While the *inceptisols* in rocky soils and mountainous land are of low fertility, the Tantalus series *inceptisols* are of moderate to high fertility. Thus, in our study, the broad soil classes rank according to fertility as *mollisols* > *inceptisols* (Tantalus) > *oxisols* ≅ *ultisols* > *inceptisols* (mountainous soils).

Plant Sampling and Site Climate

For each species, 3 individual plants were sampled. Several twigs per plant were sampled in the morning hours between 8:00–12:00. Species coordinates and sampling altitude were noted for each site, and this information was used to link species locations to specific soil types and derive location-specific climatic data. We used ARCGIS 9.1 to determine long-term average monthly and annual precipitation, and precipitation of the 3 driest months and annual precipitation, and average, maximum and minimum temperatures from high resolution climatic grids (Giambelluca et al., 1986, T. Giambelluca unpublished data) (see details in Peñuelas et al., 2010).

Study Species

Altogether 73 species, randomly sampled in the 4 sites, were studied, 35 native and 38 aliens (Peñuelas et al., 2010). Out of the 73 studied species, 36 were trees, 29 shrubs, 3 woody vines to shrubs, 3 woody vines, one subshrub, and one mistletoe (Sardans et al., 2010) (Appendix Table 2). Once an individual of a given abundant species was randomly selected, then we were looking non-randomly for 2 more individuals of the given species. The event of species selection, however, was random. Also, in drier sites, dominated by a few species, we sampled essentially all woody species.

Species were classified according to site preference as dry, dry-mesic, mesic, dry-wet, mesic-wet, and wet forest species. The invasiveness of species was quantitatively scored by using a four-level scale as 0 (native species), 1 (low invasiveness), 2 (moderate-high), and 3 (very high). These scores were based on Australia/New Zealand weed risk assessment (WRA) system scores (Pheloung et al., 1999) modified to Hawaii and other Pacific Islands (Daehler et al., 2004) that are reported in Pacific Island Ecosystems at Risk (PIER) project online database maintained by U.S. Forest Service's Institute of Pacific Islands Forestry (http://www.hear.org/pier/), and on recent updates on species invasive potential in Oahu (Daehler and Baker, 2006). For species not quantitatively scored in these assessments, species invasiveness was based on authors' observations on species abundance, presence of seedlings, and capacity of vegetative reproduction throughout the leeward ecosystems in Oahu. Although the overall risk score assessment provides 32 scales for the scored species, we used a simplified scoring with 4 ranks to make it more robust.

Chemical Analyses

Total phenolic content (TP) of leaves was determined by the improved Folin-Ciocalteu assay (Singleton and Rossi, 1965; Marigo, 1973). The improvement relative to standard assay was the use of a blank of polyvinylpolypyrrolidone (PVPP). PVPP retains the phenolic compounds avoiding their reaction with Folin-Ciocalteu solution, thereby providing a true blank sample. For extraction of phenols, 15 mg of dried pulverized leaf sample with 10 ml methanol: water (70:30 v/v) extraction solution were incubated for 30 min in an ultrasound bath at 40°C. Thereafter, the mixture was centrifuged for 10 min at 10000 rpm. Two (A and B) aliquots of 1 ml were obtained from each sample and added to a 25 ml volumetric flask. One aliquot (B) was brought to pH 3.5 with 0.1 M HCl and mixed with 0.5 g of PVPP during 10 min. Sixteen ml of distilled water then were added to both samples A and B, and 1 ml of Folin-Ciocalteau reagent to each one of the flasks. After 3 min of incubation at 40°C, 2 ml of a saturated $NaCO_3$ (20%) solution was added to each one of the samples, and distilled water were added until 25 ml. The samples were incubated at 40°C for an additional 20 min, and the absorbances of the samples, A and B were determined at 760 nm using a spectrophotometer Helios Alpha (Thermo Spectronic, Cambridge, UK).

Total soluble tannins (Tta) were extracted from 20 mg of leaf powder with 12 ml of 70% acetone. Tubes containing the sample and the acetone were sonicated three times for 1 min allowing the tubes to cool for 3 min between successive sonications. After centrifugation, the extract was

assayed with the butanol/HCl method (Porter et al., 1986) modified as in (Makkar and Goodchild, 1996). Briefly, 0.5 ml of the extract was mixed with 3 ml butanol-HCl (95:5) and 0.1 ml of ferric reagent (ferric ammonium sulfate in 2 N HCl) and kept in a boiling water bath for 60 min. After cooling the tube, absorbance was measured at 550 nm by spectrophotometer Helios Alpha. Non-heated replicate tubes for each extract were used as anthocyanins blank, and its absorbance was substracted from the absorbance of the heated tubes. The Tta content on a dry weight basis was estimated by using a 1 cm wide cuvette (Porter et al., 1986; Makkar and Goodchild, 1996). Tta analyses were conducted in triplicate.

For terpene analysis, the leaves of each species were crushed in liquid nitrogen with a Teflon pestle in a Teflon tube until a homogeneous powder was obtained. After homogenization, 1 ml of pentane was added before the pulp defrosted. The tubs were maintained at 25°C during 24 h, and after this period a sample of each extract was put into a 300 µl glass vial. Samples were injected automatically into a GC-MS (HP 6800 series 2, Hewlett Packard, Palo Alto, CA, USA) following a split of 0.5:50. Solvent delay was 3 min. The initial temperature of 40°C was immediately increased with a ramp of 30°C$\,$min^{-1} to 60°C. The second ramp rate was 10°C$\,$min^{-1} to 150°C, which was held for 3 min. The third ramp rate was 70°C$\,$min^{-1} to 250°C, which was held for 5 min. The carrier gas was helium at 0.7 ml$\,$min^{-1}. A mass selective detector was used with an electronic impact of 70 ev. The identification of monoterpenes was conducted by GC-MS comparing with standards from Fluka (Buchs, Switzerland), literature spectra, and GCD Chemstation G1074A HP and Willey 7n spectra library. Frequent calibrations (once every 5 analyses) with α-pinene, 3-carene, β-pinene, β-myrcene, *p*-cymene, limonene, sabinene, and α-humulene standards were used for quantification. Terpene calibration curves (N=4 different terpene contents) were always highly significant (r^2>0.99 for the relationships between signal and the amount of terpene standard injected). The most abundant terpenes had similar sensitivity (differences were less than 5%). The quantification of the terpene peaks were conducted using the ion with mass 93 in the fractionation spectrum. The total GC run time was 23 min. All processes were performed in the same way for native and alien species.

Statistical and Phylogenetic Analyses

The program Phylomatic (Webb and Donoghue, 2005) was used to build a phylogenetic tree of the species studied (Fig. 1). Briefly, this program assembles a phylogeny for the species of interest employing a backbone plant megatree based on a variety of sources involving primarily DNA studies. Our phylogenetic hypothesis was based on the conservative megatree, where unresolved nodes were included as soft politomies. We employed programs in the PDAP package (Garland et al., 1993) to transform the phylogenetic tree into a matrix of phylogenetic distances, and assessed whether the studied traits showed significant phylogenetic signal—i.e., the tendency of closely related species to resemble each other due to shared ancestry—employing the randomization procedure in the PHYSIG module developed by Blomberg et al. (2003). This test consists in comparing the variance in phylogenetic independent contrasts observed in the real dataset against a null distribution obtained after the phenotypic data were randomized across the tips of the phylogeny (i.e., breaking any pattern of phylogenetic resemblance between relatives). Phylogenetic signal was considered significant if the variance in contrasts of the real dataset was lower than the variance in 95% of the permuted datasets. To perform comparisons across traits, we employed the k statistic that estimates how much phylogenetic signal is present in the phenotypic data compared against the expectation from a random walk model of phenotypic evolution (Blomberg et al., 2003). If k=1, then the phenotypic trait has exactly the amount of signal expected for the phylogenetic tree employed and a model of evolution of random walk (Brownian motion); k>1 indicates a stronger phylogenetic resemblance than expected; and k<1 the opposite pattern. These analyses were performed to determine if phylogenetic correction was necessary in subsequent regression analyses. We employed generalized linear models (GLM) to analyze how total leaf phenolics, tannins, and terpenes varied as a function of species origin (native or alien) that was included as independent categorical variable.

The sampling altitude (relative to sea level) correlated significantly with the main climate variables of each respective site (total annual precipitation, the precipitation of the three driest months, mean annual temperature, annual mean of the daily minimum temperature, annual mean of the daily maximum temperature, annual mean of the monthly temperature fluctuation, and annual mean of the coldest temperature of each month) (data not shown). Because of this, altitude was used as covariate in all the conducted general linear models in order to take into account the effects that climate could have on the analyzed leaf variables.

To analyze the sources of variation in total phenolic, total tannin, and total terpene contents, we conducted a general linear model (GLM) with site (4 different sample sites), species origin (native and alien), and soil type (5 different soil types) as independent categorical variables, altitude as independent continuous variable, and in the case of variables with phylogenetic fingerprinting, phylogenetic distances also included as continuous independent factor. To conduct these analyses we used Matlab 7.6.0 with REGRESSIONV2 module (Lavin et al., 2008).

Fig. 1 Phylogenetic tree of the Hawaiian plant species studied. The phylogenetic tree was constructed with the program PHYLOMATIC (Webb and Donoghue, 2005). The figure also shows a map of the four sampling areas

Tantalus
St. Louis Heights (SLH)
Williwillinui ridge (WR)
Hahaione valley (HV)
Native
Alien

Acacia confusa
Acacia koa
Senna surattensis
Desmodium incanum
Falcataria moluccana
Haematoxylum campechianum
Casuarina equisetifolia
Ficus macrophylla
Ficus microcarpa
Trema orientalis
Rubus rosifolius
Antidesma platyphyllum
Bischofia javanica
Ochna thomasiana
Passiflora suberosa
Elaeocarpus bifidus
Eucalyptus robusta
Melaleuca quinquenervia
Metrosideros macropus
Metrosideros polymorpha
Metrosideros rugosa
Metrosideros tremuloides
Syzygium cumini
Syzygium sandwicensis
Pimenta dioica
Psidium cattleianum
Psidium guajava
Heliocarpus americanus
Hibiscus arnottianus
Sida fallax
Wiskstroemia oahuensis
Mangifera indica
Schinus terebinthifolius
Melicope clusiifolia
Melicope peduncularis
Ageratina adenophora
Pluchea carolinensis
Scaevola gaudichaudiana
Clermontia oblongifolia
Cheirodendron trigynum
Ilex anomala
Ilex paraguariensis
Alyxia stellata
Labordia tinifolia
Bobea elatior
Coffea arabica
Coprosma longifolia
Hedyotis acumiata
Hedyotis fosbergii
Hedyotis terminalis
Buddleja asiatica
Citharexylum caudatum
Clerodendrum macrostegium
Lantana camara
Stachytarpheta cayennensis
Jasminum fluminense
Tabebuia rosea
Cestrum nocturnum
Carmona retusa
Ardisia elliptica
Myrsine lessertiana
Myrsine sandwicensis
Diospyros sandwicensis
Pouteria sandwicensis
Vaccinium calycinum
Broussaisia arguta
Korthalsella complanata
Santalum freycinetianum
Pisonia umbellifera
Cinnamomum burmannii
Persea americana
Freycinetia arborea
Smilax melastomifolia
300 200 100 0

We employed the same rationale to analyze potential differences between native and alien species in the ratios of total phenolics, tannins, and terpene contents relative to leaf chemical, physiological and anatomical traits: foliar N and P contents, foliar photosynthetic capacity (A_{mass}), and LMA reported in a previous study of these species (Peñuelas et al., 2010). We also conducted discriminant analyses with pairwise combinations of leaf total phenolics with leaf N and P contents, A_{mass}, and LMA as continuous independent variables and species origin (alien or native) as a grouping variable. As described above, ordinary least squares (OLS) GLM or Phylogenetic general least square (PGLS) GLM analyses were selected depending on the significance of the phylogenetic fingerprinting (see above). We conducted a principal component analysis (PCA) with leaf economics spectrum (LMA, A_{mass}, [N], and [P]) as variables, and then correlated the factor scores (PC1 and PC2) with the leaf contents of phenolics, tannins, and terpenes. We also conducted a PCA with the leaf contents of phenolics, tannins, and terpenes to investigate whether native and alien species were significantly separared by these variables. Thereafter, we used the PC1 and PC2 scores of this PCA to correlate with LMA, A_{mass}, [N], and [P]. To test whether the observed frequency of alien species that accumulated terpenes was equal in the different continents of origin we conducted a *Chi-square* analysis.

An ANOVA test also was conducted to test for different terpene content depending on the continent of origin. We used Matlab 7.6.0 with REGRESSIONV2 module (Lavin et al., 2008) and Statistica 6.0 software (StatSoft, Inc. Tulsa, OK, USA).

Results

Total Leaf Phenolic, Tannin, and Terpene Contents

Appendix Table 3 shows the species values of total phenolics (TP), total tannins (Tta), and total terpenes (TT). They ranged between 8 and 159 $mg g^{-1}$ DW, 1 and 36 $mg g^{-1}$ DW, and 0 and 13 $mg g^{-1}$ DW, respectively (Appendix Table 3). All analyzed species exhibited detectable contents of TP and Tta, whereas only 25 out of the 73 species analyzed had detectable contents of terpenes (Appendix Table 3). Neither TP nor Tta nor TT contents presented significant phylogenetic fingerprinting. Neither soil type nor sampling site had significant effects on leaf total phenolics (TP) (P=0.12 and P=0.62, respectively), total tannins (Tta) (P=0.45 and P=0.74, respectively), or total terpenes (TT) (P=0.31 and P=0.70, respectively).

Terpenes were detected in 13 alien species and in 12 native species. The frequencies observed were not different from the frequencies expected under the hypothesis of equal frequency of leaf terpene accumulation in the different continents of origin (observed X^2=2.12 that was within the range of X^2=7.81 that includes the 95% of the distribution at $d.f$=3). Overall, alien species had higher (135%; P=0.039) leaf TT contents compared with native species, but did not have significantly different total leaf phenolic and tannin contents (Fig. 2). The different levels of species invasiveness had no significant effect on TP and Tta contents either, but TT contents increased with increasing species invasiveness, considering only terpene-containing species (Fig. 2). There were no differences in leaf terpene content among invasive species of different continent origins (one-way ANOVA, P=0.67). The PCA for all the CBSCs did not separate alien from native species (data not shown). Only terpene contents were different between alien and native species (Fig. 2). The CBSC PCA scores did not correlate either with the PCA scores for physiological foliar economic spectrum traits (data not shown).

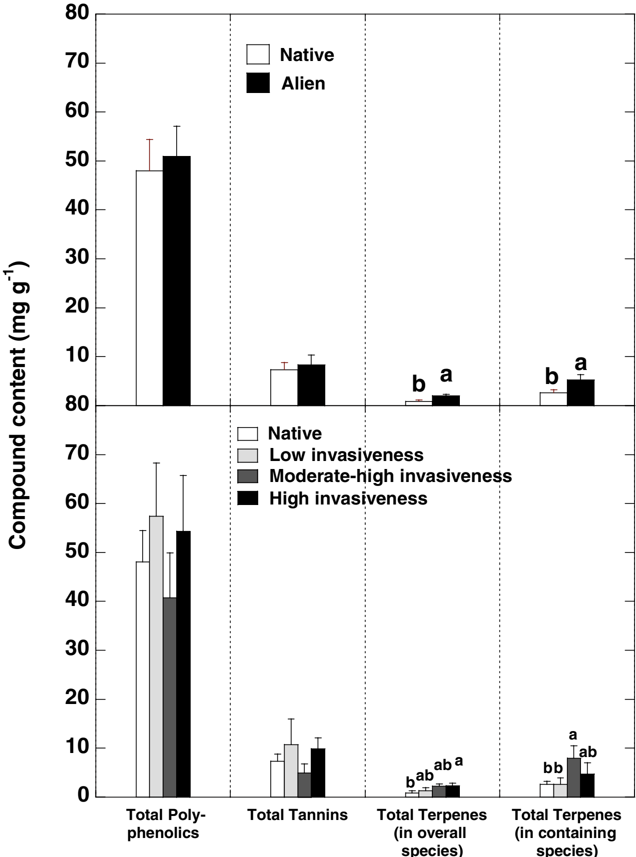


Fig. 2 Total leaf phenolics ($mg g^{-1}$), total leaf tannins ($mg g^{-1}$) and total leaf terpenes ($mg g^{-1}$) in native and alien species, and depending on the invasiveness index. Significant differences (P<0.05) between native and alien species are indicated by different letters.Total terpenes are shown calculated for the overall set of species studied and also only for the species containing terpenes

Relationships with Foliar Nutrient Contents, A_{mass}, and LMA

Appendix Table 3 shows the species values of the TP:N, TP:P, Tta:N, Tta:P, TT:N, and TT:P ratios. The ranges of these ratios among the studied species set were broad, showing great differences among species. The ranges of TP:N and TP:P ratios were 0.4–18.2 $g g^{-1}$ and 2.9–394 $g g^{-1}$, respectively. The ranges of Tta:N and Tta:P ratios were 0.05–3.11 $g g^{-1}$ and 0.8–74.5 $g g^{1}$, respectively. The ranges of TT:N and TT:P ratios were 0–15 $g g^{-1}$ and 0–428 $g g^{-1}$, respectively (Appendix Table 3). None of those ratios exhibited significant phylogenetic fingerprinting.

Despite having higher foliar N content (Peñuelas et al., 2010), aliens had higher leaf TT:N content ratio (80%; P= 0.03) compared with native plants (Fig. 3), reflecting much higher terpene content. No significant effects of sampling site or soil type were observed on the ratios between leaf TT content and leaf economic traits (data not shown).

The ratio between phenolic contents and photosynthetic capacity was 50% lower in alien species compared with native species (P=0.011). There was no other effect of species origin, sampling site, or soil type on the ratios of leaf TP and Tta contents relative to the leaf economic traits (Fig. 4).

In native species, TP was negatively correlated with leaf N, and leaf P contents and A_{mass}. In alien species, TP was

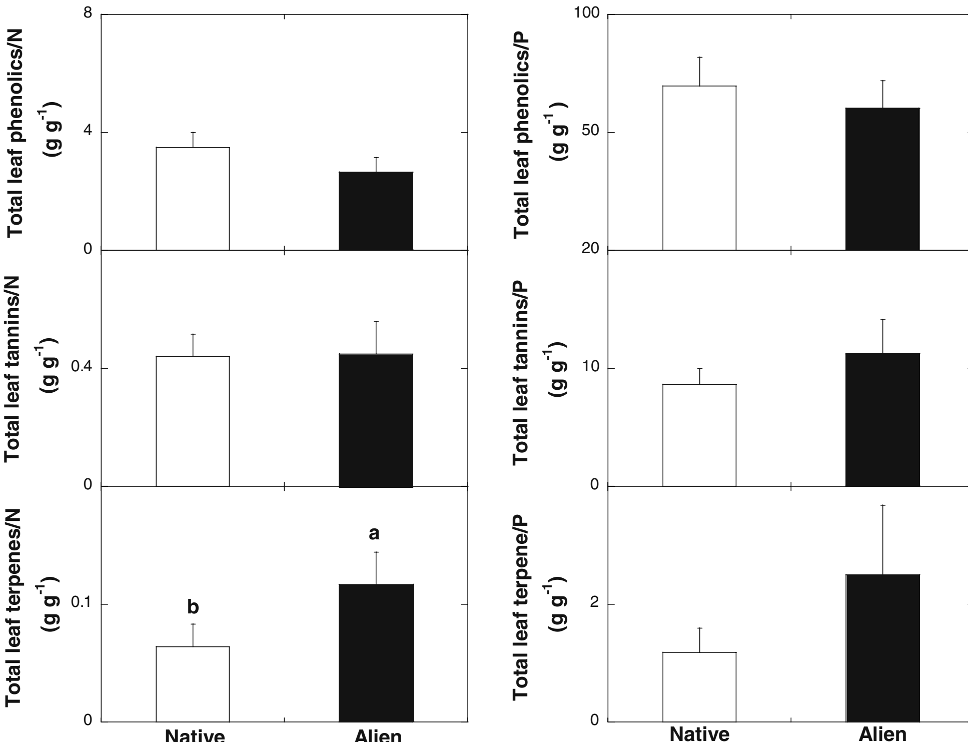


Fig. 3 Ratios of Total leaf phenolics, total leaf tannins and total leaf terpenes relative to leaf N and P contents ($g g^{-1}$). Significant differences ($P<0.05$) between native and alien species are indicated by different letters

significantly correlated only with leaf P content (Fig. 5). In contrast, we did not observe any significant relationships of Tta and TT contents with nutrient contents or with A_{mass} (data not shown).

The discriminant analyses of TP and leaf [N] (Wilk's Lambda=0.85, P=0.003), TP and leaf [P] (Wilk's Lambda= 0.84, P=0.008), and TP and Amax (Wilk's Lambda=0.84, P=0.006) separated alien from native species due to their higher N and P contents and A_{mass}.

Discussion

Alien species had similar total leaf phenolic (TP) and tannin (Tta) contents but higher leaf total terpene (TT) contents than native plants, with no phylogenetic signal in the differences. Moreover, alien species had higher leaf N and P contents, and Amass than native species, and, as a result, alien species had a lower leaf phenolics content/photosynthesis ratio than native species, thus suggesting a lower relative effort on accumulating leaf phenolics. Conversely, the total terpene content relative to N and P contents was larger in alien than in native species. These greater contents of terpenes, providing more qualitative (more dependent on their toxicity than on their quantity) lower cost chemical defenses against generalist herbivores, may counterbalance the lower leaf structural defenses and higher leaf nutritional quality of alien plants. Alien plants thus would be able to deter herbivore attacks using a different strategy of defense. On the other hand, the higher terpene accumulation was independent of the continent of origin of the alien species (Asia, Australia, the Americas, Africa), which indicates that being alien either selects for higher terpene contents post-invasion, or that species with high terpene contents are pre-adapted to invasiveness.

Another possibility to explain these results is that the Hawaiian environment is unusual in some sense that selected for lower terpene levels in the past, and so the real pattern is not that invasive plants have higher terpenes than natives, but Hawaiian plants have lower terpenes that continental species. In that case, the higher terpene levels of the alien species may not have any functional role in invasion, but simply be a result of Hawaiian plants having low concentration of terpenes. With our current knowledge, however, this possibility does not seem likely. Terpene contents have proven not to be determined phylogenetically, at least in this study, suggesting that this capacity is widespread throughout the entire plant taxa spectrum. Certainly, terpenes have been proven to accumulate in response to some climatic stresses (Peñuelas and Llusià, 2003, 2004) but in this regard, the climate conditions of the natural habitats of alien species are similar in most cases to Hawaiian climate. Moreover, the percentage of species that accumulate terpenes was not different between native and aliens showing that the capacity to produce and accumulate terpenes is not different although alien species accumulate greater amounts of terpenes. The number of studies that have conducted a terpene screening in an extensive number

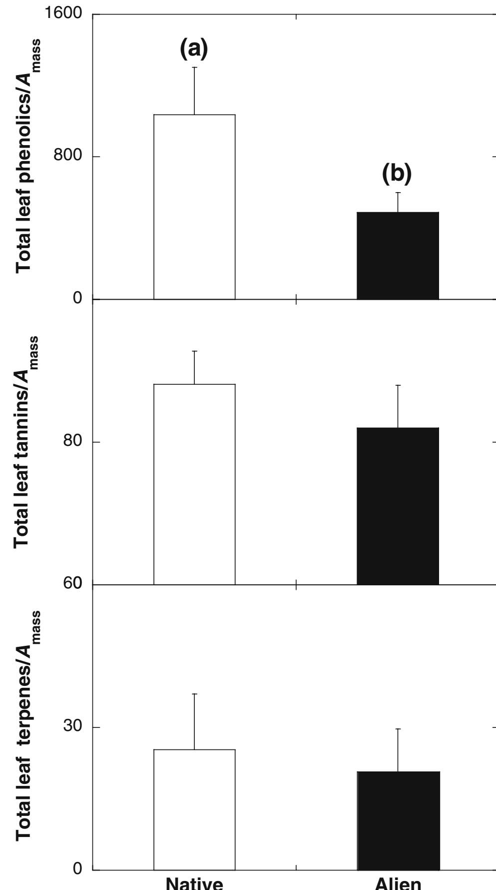


Fig. 4 Total leaf phenolics (mg g^{-1})/A_{mass} (μmols g^{-1} s^{-1}), total tannins (mg g^{-1})/A_{mass} (μmols g^{-1} s^{-1}) and total leaf terpenes (mg g^{-1})/A_{mass} (μmols g^{-1} s^{-1}) ratios. Significant differences (P<0.1) between native and alien species are indicated by different letters between brackets

of species in different regions of the world is, however, scarce, and to clearly discern whether or not terpene accumulation capacity is differently distributed throughout the world is not possible at this moment.

Some recent studies of the changes in chemical defenses in alien populations relative to non-alien populations of the same species have reported that invasive populations evolve a greater resistance to generalist herbivores (e.g., Legar and Forister, 2005). In temperate grassland systems, higher concentrations of toxic secondary compounds also have been found in invaders. The explanation seems to be that these compounds are not only defensive against generalists but also attractive to specialists. Thus, in the native range, the plants are constrained from evolving higher levels due to increased attraction of specialist enemies. In the introduced range where specialist herbivores are absent, the plants can invest more resources to defenses such as terpenes against generalist herbivores (Joshi and Vrieling, 2005). Previous studies also have observed no loss of defenses against generalist herbivores in alien species populations (Leger and Forister 2005; Hull-Sanders et al., 2007). The allocation to lower cost defenses such as terpenes should be more compatible with a low LMA and high photosynthetic capacity (Lankau, 2007). This strategy can be especially successful for alien plants in environmentally rich sites, such as wet tropical environments where higher growth and production provides a greater ability to compete for space occupation and resources uptake. Higher terpene contents are compatible with a fast return strategy of "leaf economics spectrum"

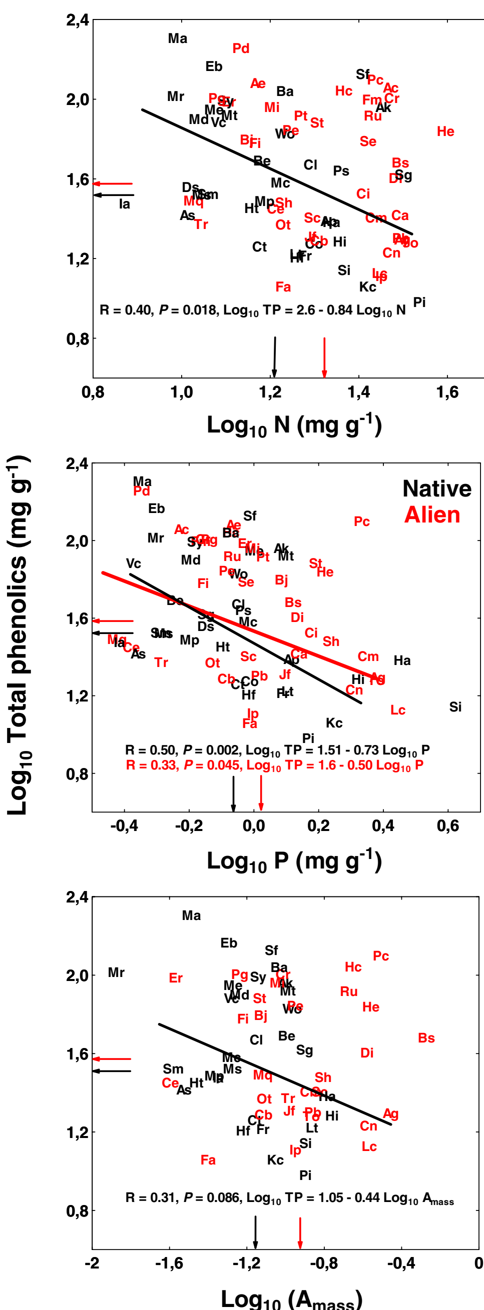


Fig. 5 Relationships between total log$_{10}$ leaf phenolics (mg g^{-1}) and log$_{10}$ leaf N (mg g^{-1}), log$_{10}$ leaf P (mg g^{-1}) contents, and log$_{10}$ A_{mass} (μmols g^{-1} s^{-1})

based on a higher photosynthetic capacity, higher nutrient content, lower LMA, and faster return on investment in foliage (Wright et al., 2004). These traits help to explain the success of invasive plant species (Pattison et al., 1998; Baruch and Goldstein, 1999; Funk and Vitousek, 2007) since they can contribute to faster growth rates for invaders and confer a competitive advantage over native species (Reich et al., 1997; Blumenthal and Hufbauer, 2007). Peñuelas et al., (2010) have observed these faster returns from their investments in nutrients and dry mass in leaves, and these higher contents of most nutrients in these alien species in Oahu (Hawaii).

The higher terpene contents and higher nutrient contents fit better the hypotheses based on higher nutrient availability translating into higher carbon fixation and activity of the enzymes involved in terpene production (Harley et al., 1994; Litvak et al., 1996; Peñuelas and Staudt, 2010) than the hypotheses based on carbon nutrient balance (Bryant et al., 1983; Peñuelas and Estiarte, 1998). The data for native species fits the latter hypotheses but the data for alien species does not. Total phenolic contents were inversely correlated with foliar N and P contents and A_{mass} in the native species, but this did not happen in alien species, except for foliar P content. Phenolics are especially active defenses against specialist herbivores (Coley et al., 1985; Bennet and Wallgrove 1994). Thus, in native plants, the presence of specialist herbivores placed species along a continuous gradient between a high allocation to defense (high phenolics content) and low leaf production capacity (low leaf nutrient content) to a low allocation to defense (low phenolics content) and high allocation to leaf production capacity (high nutrient content). Conversely, aliens with no specialist herbivores did not present this trend. Our results also provided partial support (in aliens) for the hypothesis that leaf phenolic contents would be affected by N and P in different ways because of their different cellular metabolic processes (Wright et al., 2010).

Foliar Tta and TT contents were not related to any of those leaf economic traits in any of both groups of species nor to any of the main nutrients, N or P. Regarding the more evolutive hypotheses, the results partially support the modified EICA-related hypothesis (Joshi and Vrieling, 2005; Stastny et al., 2005), which suggests that alien species have a larger content of low cost defenses such as terpenes against generalist herbivores. These results also demonstrate, however, that aliens had not lost the capacity for formation of high-carbon cost phenolics or tannins.

The different results of alien vs. native comparisons for TT, Tta, and TP contents also suggest a set of different possible physiological, ecological, and evolutionary roles of these different types of CBSC. The higher or lower content of different chemical compounds does not depend only on a defense strategy. Other environmental factors such as temperature or drought could explain changes of secondary metabolites contents such as terpenes since they are also involved in protection mechanisms in the face of abiotic (Peñuelas and Llusià, 2003, 2004; Peñuelas and Munné-Bosch, 2005; Lewis et al., 2006) and biotic (Viiri et al., 2001; Gershenzon and Dudareva 2007) stressors. Volatile terpenes also are involved in plant defense by multitrophic signalling (Dicke and Baldwin, 2010).

In summary, the results show that alien species invest less in structural defenses (they have lower LMA) but more in terpenes, which are low cost chemical defenses, effective at lower contents than other chemical defenses (Mote et al., 2007), and which strongly deter generalist herbivore attacks (Landau et al., 1994; Mote et al., 2007). Thus, the results are partly in accordance with the most recent "Evolution of Increased Competitive Ability" (EICA) hypothesis, which proposes that lower cost defensive compounds are effective in deterring generalist herbivores in the introduced range. However, the results are not in accordance with the expected decreases in other high cost defense components such as TP and Tta. These higher contents of lower cost defenses together with higher nutrient content and growth rates fit well with the fast-growing strategy of the alien species in resource rich areas such as the tropical islands with old weathered soils where the plant invasive success is an emerging phenomenon.

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Appendix

Table 1 Description of the study sites

Site	Coordinates	Average±SD[a] altitude (m)	Average±SD precipitation (mm)		Average±SD annual temperature (°C)		Species number		
			Annual	Three driest months	Minimum	Maximum	Total	Native	Alien
Hahaione Valley	21°19'N, 157°43'W	390±140	1268±22	157±7	17.1±0.6	25.7±0.5	14	2	12
St. Louis Heights	21°18'N, 157°48'W	171±65	1430±210	197±45	18.7±0.5	26.9±0.5	12	0	12
Tantalus	21°N, 20'157°49'W	441±24	3670±440	705±41	16.2±0.6	24.1±0.6	22	11	11
Wiliwilinui	21°19'N, 157°45'W	660±120	2100±150	413±60	15.2±0.9	23.8±0.8	25	22	3

[a] averages are based on the number of species sampled and species-specific locations. In statistical analyses, exact species-specific environmental data were used

Table 2 Family, plant growth form, sampling sites in Oahu, Hawaii, characteristic ecological distribution, continent of origin and invasiveness of all studied species

Species[a]	Family	Growth form	Sampling site[b]	Species ecology	Continent of origin	Invasi-veness[c]
Acacia confusa	Fabaceae	Tree	St. Louis Heights	dry-mesic	Asia	3
Acacia koa	Fabaceae	Tree	Wiliwilinui	mesic-wet		0
Ageratina adenophora	Asteraceae	Herb	Tantalus	mesic-wet	Americas	2
Alyxia stellata	Apocynaceae	Vine/Shrub	Wiliwilinui	mesic-wet		0
Antidesma platyphyllum	Euphorbiaceae	Tree	Tantalus	mesic-wet		0
Ardisia elliptica	Myrsinaceae	Shrub	Hahaione Valley	mesic-wet	Asia	3
Bischofia javanica	Euphorbiaceae	Tree	Tantalus	mesic-wet	Asia	2
Bobea elatior	Rubiaceae	Tree	Wiliwilinui	wet		0
Broussaisia arguta	Hydrangeaceae	Shrub	Wiliwilinui	wet		0
Buddleja asiatica	Scrophulariaceae	Shrub	Wiliwilinui	mesic-wet	Asia	2
Carmona retusa	Boraginaceae	Shrub	St. Louis heights	dry-mesic	Asia	1
Casuarina equisetifolia	Casuarinaceae	Tree	St. Louis heights	dry-mesic	Australia	3
Cestrum nocturnum	Solanaceae	Shrub	Tantalus	wet	Americas	3
Cheirodendron trigynum	Araliaceae	Tree	Wiliwilinui	wet		0
Cinnamomum burmannii	Lauraceae	Tree	Tantalus	mesic-wet	Asia	2
Citharexylum caudatum	Verbenaceae	Shrub	Tantalus	mesic-wet	Americas	3
Clermontia oblongifolia	Campanulaceae	Tree	Tantalus	wet		0
Clerodendrum macrostegium	Verbenaceae	Shrub	Tantalus	wet	Asia	1
Coffea arabica	Rubiaceae	Shrub	Tantalus	mesic-wet	Africa/Asia	2
Coprosma longifolia	Rubiaceae	Shrub	Wiliwilinui	mesic-wet		0
Desmodium incanum	Fabaceae	Shrub	Hahaione Valley	dry	Americas	2
Diospyros sandwicensis	Ebenaceae	Tree	Tantalus	dry-mesic		0
Elaeocarpus bifidus	Elaeocarpaceae	Tree	Tantalus and Wiliwilinui	mesic-wet		0
Eucalyptus robusta	Myrtaceae	Tree	St. Louis heights	dry-mesic	Australia	1
Falcataria moluccana	Fabaceae	Tree	St. Louis heights	dry-mesic	Asia	2
Ficus macrophylla	Moraceae	Tree	Hahaione Valley	mesic-wet	Australia	2
Ficus microcarpa	Moraceae	Tree	St. Louis heights	dry-mesic	Asia	3
Freycinetia arborea	Pandanaceae	Vine/Shrub	Tantalus	wet		0
Haematoxylum campechianum	Fabaceae	Tree	St. Louis heights	dry	Americas	2
Hedyotis acuminata	Rubiaceae	Vine/Shrub	Tantalus	mesic-wet		0
Hedyotis fosbergii	Rubiaceae	Shrub	Wiliwilinui	wet		0

Table 2 (continued)

Species[a]	Family	Growth form	Sampling site[b]	Species ecology	Continent of origin	Invasiveness[c]
Hedyotis terminalis	Rubiaceae	Shrub	Wiliwilinui	mesic-wet		0
Heliocarpus americanus	Malvaceae	Tree	Hahaione Valley	dry-mesic	Americas	1
Hibiscus arnottianus	Malvaceae	Tree	Tantalus	wet		0
Ilex anomala	Aquifoliaceae	Shrub	Wiliwilinui	wet		0
Ilex paraguariensis	Aquifoliaceae	Tree	Tantalus	mesic-wet	Americas	1
Jasminum fluminense	Oleaceae	Vine	St. Louis Heights	dry-mesic	Africa	2
Korthalsella complanata	Santalaceae	Mistletoe	Wiliwilinui	wet		0
Labordia tinifolia	Loganiaceae	Shrub	Tantalus	wet		0
Lantana camara	Verbenaceae	Shrub	Hahaione Valley	dry-mesic	Americas	3
Mangifera indica	Anacardiaceae	Tree	Tantalus	mesic-wet	Asia	1
Melaleuca quinquenervia	Myrtaceae	Tree	St. Louis heights	dry-mesic	Australia	2
Melicope clusiifolia	Rutaceae	Shrub	Wiliwilinui	wet		0
Melicope peduncularis	Rutaceae	Shrub	Wiliwilinui	wet		0
Metrosideros macropus	Myrtaceae	Tree	Wiliwilinui	wet		0
Metrosideros polymorpha	Myrtaceae	Tree	Hahaione Valley and Tantalus	mesic-wet		0
Metrosideros rugosa	Myrtaceae	Shrub	Wiliwilinui	wet		0
Metrosideros tremuloides	Myrtaceae	Tree	Tantalus	mesic-wet		0
Myrsine lessertiana	Myrsinaceae	Shrub	Wiliwilinui	wet		0
Myrsine sandwicensis	Myrsinaceae	Shrub	Wiliwilinui	wet		0
Ochna thomasiana	Ochnaceae	Shrub	Hahaione Valley	dry	Africa	1
Passiflora suberosa	Passifloraceae	Vine	Wiliwilinui	dry-mesic	Americas	2
Persea americana	Lauraceae	Tree	Tantalus series	mesic-wet	Americas	1
Pimenta dioica	Myrtaceae	Tree	St. Louis heights	dry	Americas	1
Pisonia umbellifera	Nyctaginaceae	Tree	Tantalus	mesic-wet		0
Pluchea carolinensis	Asteraceae	Shrub	Hahaione Valley	dry-mesic	Americas	2
Pouteria sandwicensis	Sapotaceae	Tree	Tantalus	mesic-wet		0
Psidium cattleianum	Myrtaceae	Tree	Hahaione Valley	mesic-wet	Americas	3
Psidium guajava	Myrtaceae	Tree	Hahaione Valley	dry-mesic	Americas	3
Rubus rosifolius	Rosaceae	Shrub	Wiliwilinui	wet	Americas	2
Santalum freycinetianum	Santalaceae	Tree	Wiliwilinui	dry-mesic		0
Scaevola gaudichaudiana	Goodeniaceae	Shrub	Wiliwilinui	wet		0
Schinus terebinthifolius	Anacardiaceae	Tree	Hahaione Valley	dry-mesic	Americas	3
Senna surattensis	Fabaceae	Tree	St. Louis heights	dry	Asia/Australia	1
Sida fallax	Malvaceae	Shrub	Hahaione Valley	dry-mesic		0
Smilax melastomifolia	Smilacaceae	Vine	Wiliwilinui	mesic-wet		0
Stachytarpheta cayennensis	Verbenaceae	shrub	Hahaione Valley	dry-mesic	Americas	3
Syzygium cumini	Myrtaceae	Tree	Tantalus	mesic-wet	Asia	1
Syzygium sandwicensis	Myrtaceae	Tree	Wiliwilinui	wet		0
Tabebuia rosea	Bignoniaceae	Tree	St. Louis heights	dry-mesic	Americas	1
Trema orientalis	Ulmaceae	Shrub	Hahaione Valley	dry-mesic	Africa/Asia	2
Vaccinium calycinum	Ericaceae	Shrub	Wiliwilinui	wet		0
Wikstroemia oahuensis	Thymelaeaceae	Shrub	Wiliwilinui	mesic-wet		0

[a] Species nomenclature follows ARS/GRIN online database (USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)*, National Germplasm Resources Laboratory, Beltsville, Maryland, http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl) and for Hawaiian native species missing from this database (mainly *Rubiaceae* and *Rutaceae*) species nomenclature follows the Manual of the flowering plants of Hawai'i (Wagner et al., 1999)

[b] Table 1 for the description of the study sites

[c] 0 – native species, 1 – low, 2 – moderate-high, 3 –high invasiveness

Table 3 Foliar contents of total phenolics (TP), total tannins (Tta) and total terpenes (TT) and their respective ratios relative to leaf N and P content for the studied native and alien species

Species[a]	Species code	Origin	Total Phenolics (TP) ($mg g^{-1}$)	Total Tannins (Tta) ($mg g^{-1}$)	Total Terpenes (TT) ($mg g^{-1}$)	TP:N ($g g^{-1}$)	TP:P ($g g^{-1}$)	Tta:N ($g g^{-1}$)	Tta:P ($g g^{-1}$)	TT:N ($g g^{-1}$)	TT:P ($g g^{-1}$)
Acacia confusa	Ac	A	101±12	20.9±9.9	n.d.	3.4±0.5	168.5+18.8	0.706±0.352	35.0±11.8	n.d.	n.d.
Acacia koa	Ak	N	80.5±10.0	35.7±2.7	n.d.	2.82±0.10	66.4±1.3	1.26±0.18	29.5±6.9	n.d.	n.d.
Ageratina adenophora	Ag	A	17.4+4.0	n.a.	12.7±1.8.	0.553±0.040	7.2±5.4	n.a.	n.a.	4.03±0.11	52.7±0.09
Alyxia stellata	As	N	23.0+5.3	2.09±0.71		2.23±0.12	48.1±3.4	0.203±0.011	4.8±0.2	n.d	n.d
Antidesma platyphyllum	Ap	N	21.6±3.5	2.50±0.28	n.d.	1.00+0.03	16.5+2.4	0.054±0.007	0.897±0.199	n.d.	n.d.
Ardisia elliptica	Ae	A	106±12	9.6±1.8	n.d.	7.13±0.6	123+11	0.647±0.015	11.1±1.7	n.d.	n.d.
Bischofia javanica	Bj	A	55.3±19.9	7.77±1.12	n.d.	3.94±2.23	45.4±13.0	0.554±0.037	6.39±1.02	n.d.	n.d.
Bobea elatior	Be	N	43.3±9.1	5.24±1.8	n.d.	2.85±0.24	75.7±7.7	0.389±0.110	10.3±3.9	n.d.	n.d.
Broussaisia arguta	Ba	N	96.9±8.0	15.8±4.2	n.d.	5.67±0.11	114±13	0.864±0.242	17.4±4.6	n.d.	n.d.
Buddleja asiatica	Bs	A	42.3±3.4	n.a.	n.d.	1.36±0.20	32.0±6.2	n.a.	n.a.	n.d.	n.d.
Carmona retusa	Cr	A	89.3±5.0	2.10±0.17	n.d.	2.99±0.22	128±65	0.069±0.009	2.97±1.07	n.d.	n.d.
Casuarina equisetifolia	Ce	A	24.9±5.8	9.98±1.17	n.d.	1.52±0.13	59.4±7.4	0.611±0.072	23.8±2.7	n.d.	n.d.
Cestrum nocturnum	Cn	A	15.0+4.1	2.39±0.01	n.d.	0.504±0.201	7.35±2.20	0.080±0.009	1.17±0.03	n.d.	n.d.
Cheirodendron trigynum	Ct	N	16.0±1.4	1.78±0.20	5.94±1.60	1.07±0.20	18.0±2.6	0.117±0.020	1.96±0.30	3.96±0.88	67.0±9.0
Cinnamomum burmannii	Cb	A	17.1±1.4	3.45±0.44	2.73±1.31	0.838±0.012	20.8±2.6	0.169±0.011	4.19±0.69	1.33±0.33	33.0±20.0
Citharexylum caudatum	Ci	A	29.6±1.8	1.94±0.12	n.d.	1.15±0.01	19.7±5.3	0.073±0.008	1.26±0.42	n.d.	n.d.
Clermontia oblongifolia	Co	N	16.6±0.8	n.a.	n.d.	0.835±0.060	17.1±0.3	n.a.	n.a.	n.d.	n.d.
Clerodendrum macrostegium	Cm	A	22.4±9.1	2.03±0.05	n.d.	0.816±0.422	9.96±5.61	0.073±0.013	0.885±0.195	n.d.	n.d.
Coffea arabica	Ca	A	23.0+1.4	n.a.	n.d.	0.740±0.041	16.7±1.7	n.a.	n.a.	n.d.	n.d.
Coprosma longifolia	Cl	N	41.3±7.1	5.33±2.02	n.d.	2.11±0.33	46.1±5.2	0.269±0.058	5.87±1.40	n.d.	n.d.
Desmodium incanum	Di	A	35.5±13.3	n.a.	n.d.	1.17±0.08	26.1±12.3	n.a.	n.a.	n.d.	n.d.
Diospyros sandwicensis	Ds	N	31.9±8.1	15.2±2.9	n.d.	3.04±0.09	45.0±13.0	1.36±0.24	20.0±4.0	n.d.	n.d.
Elaeocarpus bifidus	Eb	N	129±75	1.45±0.33	n.d.	11.0±0.7	240±170	0.038±0.020	0.90±0.09	n.d.	n.d.
Eucalyptus robusta	Er	A	85.6±14.6	4.13±0.54	1.06±0.21	6.69±0.59	91.3±10.2	0.243±0.022	3.32±1.01	0.831±0.050	11.3±1.3
Falcataria moluccana	Fm	A	87.4±26.9	14.4±6.3	n.d.	3.25±0.37	128±43	0.576±0.258	22.7±9.31	n.d.	n.d.
Ficus macrophylla	Fa	A	10.1±0.7	1.71±0.01	n.d.	0.596±0.050	10.4±2.0	0.101±0.011	1.76±0.08	n.d.	n.d.
Ficus microcarpa	Fi	A	53.0±1.7	21.7±0	n.d.	3.61±0.31	76.1±3.5	1.48±0.34	31.2±6.9	n.d.	n.d.
Freycinetia arborea	Fr	N	14.4±3.9	2.05±0.24	n.d.	7.59±0.30	11.7±4.5	0.104±0.019	1.61±0.41	n.d.	n.d.
Haematoxylum campechianum	Hc	A	97.3±24.8	8.66±0.80	n.d.	4.19±0.59	115±22	0.112±0.007	3.06±0.43	n.d.	n.d.
Hedyotis acuminata	Ha	N	21.3±4.3	15.0±13.2	n.d.	0.978±0.205	7.41±1.20	0.687±0.550	5.21±3.51	n.d.	n.d.
Hedyotis fosbergii	Hf	N	14.2±2.4	1.90±0.30	n.d.	0.776±0.080	14.7±1.3	0.105±0.020	1.98±0.62	n.d.	n.d.
Hedyotis terminalis	Ht	N	25.0±3.0	2.11±0.12	n.d.	1.74±0.04	31.2±11.7	0.146±0.013	2.62±0.53	n.d.	n.d.
Heliocarpus americanus	He	A	61.0±6.6	3.42±0.62	0.123 ±0.020	1.54±0.01	36.7±5.6	0.090±0.022	2.14±0.54	0.031±0.007	0.741±0.168
Hibiscus arnottianus	Hl	N	16.9±2.6	n.a.	n.d.	0.745±0.042	8.07±1.16	n.a.	n.a.	n.d.	n.d.
Ilex anomala	Ia	N	26.4±6.0	1.68±0.22	n.d.	3.56±0.90	68.9±20.3	0.218±0.042	4.22±0.65	n.d.	n.d.
Ilex paraguariensis	Ip	A	11.4±3.0	2.00±0.32	n.d.	0.402±0.120	11.5±5.3	0.070±0.011	1.98±0.17	n.d.	n.d.
Jasminum fluminense	Jf	A	18.0±2.1	2.02±0.41	n.d.	0.920±0.279	14.4±2.4	0.102±0.026	1.60±0.20	n.d.	n.d.

Korthalsella complanata	Kc	N	10.1±2.6	1.50±0.22	n.d.	0.384±0.011	5.72±2.61	0.055±0.006	0.819±0.097	n.d.	n.d.
Labordia tinifolia	Lt	N	14.7±1.0	n.a.	n.d.	0.813±0.036	11.6±3.2	n.a.	n.a.	n.d.	n.d.
Lantana camara	Lc	A	11.8±3.0	15.1±7.9	3.76±0.60	0.421±0.022	4.24±1.62	0.39±0.0	3.44±0.05	1.34±0.39	13.5±3.1
Mangifera indica	Mi	A	80.5±7.6	3.78±0.19	0.138±0.001	5.03±0.03	81.3±0.2	0.239±0.005	3.86±0.24	0.087±0.015	1.40+0.01
Melaleuca quinquenervia	Mq	A	27.4±0.9	6.59+2.32	16.2±1.1	2.58±0.20	72.4±12.9	0.609±0.171	17.1±6.0	15.2±0.0	428+1
Melicope clusiifolia	Mc	N	33.6±3.2	2.59±0.13	5.12±2.50	2.01±0.41	35.1±4.6	0.154±0.023	2.69±0.28	3.06±3.21	53.4±49.9
Melicope peduncularis	Mp	N	27.1+0.3	2.80±0.53	2.87±0.39	1.76±0.20	42.8±2.8	0.176±0.040	4.28±0.89	1.87±0.08	45.4±9.3
Metrosideros macropus	Ma	N	178±1	2.21±0.33	0.504±0.202	18.2±1.4	394±30	0.229±0.013	4.96±1.05	0.514±0.401	11.2±1.2
Metrosideros polymorpha	Me	N	78.2±16.3	3.16±0.18	3.09±0.90	6.59±0.69	77.9±25.8	0.273±0.003	3.22±0.56	2.60±0.20	30.7±3.9
Metrosideros rugosa	Mr	N	91.3±9.0	6.25±0.51	4.53±2.00	9.41±3.21	183±23	0.654±0.008	12.7±2.4	4.67±2.38	91.0±50.0
Metrosideros tremuloides	Mt	N	73.5±6.6	1.99±0.20	0.240±0.090	5.73±0.49	58.5±5.6	0.157±0.002	1.61±0.17	0.187±0.070	1.92±1.29
Myrsine lessertiana	Md	N	70.1±11.0	12.2±3.9	4.03±1.40	6.41±0.10	110±13	1.12±0.03	19.1±4.4	3.69±0.29	63.2±7.7
Myrsine sandwicensis	Ms	N	29.3±8.4	5.00±0.49	2.44±1.22	2.64±0.19	55.7±26.8	0.514±0.010	10.9±2.1	2.20±0.31	46.3±3.2
Ochna thomasiana	Ot	A	20.7±5.1	n.a.	n.d.	1.22±0.30	27.8±11.5	n.a.	n.a.	n.d.	n.d.
Passiflora suberosa	Pb	A	17.7±2.1	2.40±0.33	n.d.	0.566±0.197	17.0±6.3	0.076±0.002	2.27±0.66	n.d.	n.d.
Persea americana	Pe	A	61.5±20.1	47.4±0.2	0.831±0.101	3.48±1.10	74.4±22.6	3.11±0.80	66.5±6.5	0.470±0.089	10.1±0.8
Pimenta dioica	Pd	A	159±13	33.5±7.4	5.14±2.41	11.7±1.0	354±48	2.46±0.38	74.5±12.0	3.59±2.60	114±26
Pisonia umbellifera	Pi	N	8.4±0.5	2.04±0.05	n.d	0.245±0.019	5.74±0.79	0.058±0.001	1.37±0.08	n.d	n.d
Pluchea carolinensis	Pc	A	111±26	n.a.	0.192±0.097	4.04±0.61	51.4±1.4	n.a.	n.a.	0.070±0.036	0.891±0.374
Pouteria sandwicensis	Ps	N	38.5±11.4	3.92±0.72	n.d	1.68±0.22	41.6±17.6	0.174±0.033	4.30±1.11	n.d	n.d
Psidium cattleianum	Pt	A	72.7±6.3	9.70±6.50	6.66±0.60	3.92±0.53	68.2±5.1	0.518±0.182	9.02±4.19	3.59±0.20	62.5±9.4
Psidium guajava	Pg	A	89.4±5.0	12.5±2.9	2.11±0.69	7.43±0.18	123±13	0.996±0.130	16.5±3.7	1.75±1.11	29.0±26.5
Rubus rosifolius	Ru	A	73.0±4.9	2.54±0.52	2.35±1.00	2.70±0.80	85.1±18.9	0.042±0.003	1.34±0.40	0.868±0.311	27.4±21.2
Santalum freycinetianum	Sf	N	118±4	14.6±8.1	n.d	4.60±0.42	121±33	0.570±0.289	15.0±12.5	n.d	n.d
Scaevola gaudichaudiana	Sg	N	36.8±3.0	1.87±0.40	n.d	1.16±0.04	51.7±5.0	0.063±0.020	2.81±0.53	n.d	n.d
Schinus terebinthifolius	St	A	67.2±4.6	n.a.	12.8±1.1	3.32±0.20	43.2±4.0	n.a.	n.a.	6.30±1.82	82.1±15.5
Senna surattensis	Se	A	54.2±22.0	6.79±2.33	n.d	2.05±1.03	57.1±13.2	0.276±0.053	7.69±1.10	n.d	n.d
Sida fallax	Si	N	12.2±3.5	n.a.	n.d	0.526±0.071	2.92±0.81	n.a.	n.a.	n.d	n.d
Smilax melastomifolia	Sm	N	29.5±8.1	3.43±1.37	n.d	2.58±1.22	57.3±23.7	0.296±0.101	6.58±2.23	n.d	n.d
Stachytarpheta cayennensis	Sh	A	26.6±9.0	n.a.	n.d	1.56±0.03	15.4±5.0	n.a.	n.a.	n.d	n.d
Syzygium cumini	Sc	A	22.4±3.9	2.80±0.32	7.95±0.82	1.13±0.10	23.3±6.7	0.138±0.013	2.83±0.55	4.03±0.05	82.6±7.7
Syzygium sandwicensis	Sy	N	86.7+17.1	6.21±1.90	0.284±0.040	6.92±0.67	132±24	0.496±0.141	9.48±2.67	0.226±0.050	4.32±0.93
Tabebuia rosea	Tr	A	20.8±3.3	2.88±0.29	n.d	1.88±0.09	40.2±8.6	0.260±0.028	5.56±0.70	n.d	n.d
Trema orientalis	To	A	16.8±1.7	n.a.	n.d	0.513±0.051	7.05±2.50	n.a.	n.a.	n.d	n.d
Vaccinium calycinum	Vc	N	67.5±8.3	9.78±1.83	n.d	5.58±0.70	159±15	0.802±0.121	22.8±3.6	n.d	n.d
Wikstroemia oahuensis	Wo	N	59.3±3.1	8.98±1.01	n.d	3.47±0.46	66.4±3.1	0.527±0.074	10.1±0.8	n.d	n.d

[a] Species nomenclature follows ARS/GRIN online database (USDA. ARS. National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)*. National Germplasm Resources Laboratory. Beltsville. Maryland. http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl) and for Hawaiian native species missing from this database (mainly Rubiaceae and Rutaceae) species nomenclature follows the Manual of the flowering plants of Hawaii (Wagner et al., 1999)

n.d not detected. *n.a* no available data

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preserve vegetative growth and reproduction, yet minimize fitness costs to the plant (Koornneef and Pieterse, 2008; Dicke and Baldwin, 2010; Heil, 2010; Mooney et al., 2010). These complex biochemical and physiological responses are dynamic and often result in a local or systemic resistance to further challenge (De Vos et al., 2006; De Vos and Jander, 2009).

Plants deploy defense-signaling pathways regulated by salicylic acid (SA), jasmonic acid (JA), ethylene, and reactive oxygen species (ROS) in response to herbivores and pathogens (Glazebrook, 2005; Walling, 2009). These pathways are interconnected and can act antagonistically, additively, or synergistically (Mur et al., 2006). The convergence of these pathways at key signaling nodes (NPR1, WRKY70, and glutaredoxin) may enable prioritization of the signaling pathways allowing deployment of the most effective local and systemic defenses against an intruder (Koornneef and Pieterse, 2008). In addition, these defense pathways communicate with abscisic acid (ABA), auxin, gibberellic acid, and brassinosteroid signaling pathways to coordinate the magnitude and quality of the defense response activated (López et al., 2008; Spoel and Dong, 2008). There is accumulating evidence for additional defense-signaling pathways that are modulated after herbivore attack; however the identities of these pathways have yet to be revealed (van de Ven et al., 2000; Glazebrook, 2005; De Vos et al., 2006; De Vos and Jander, 2009; Bhattarai et al., 2010).

Plant defense responses to insects in the order Hempitera are distinct, as these insects use their stylets and salivary secretions in disparate manners to recover nutrients from their host plants. For example, mirid bugs, pyrrhocorids, lygaeids, planthoppers, and leafhoppers are destructive, since they lacerate cells, solubilize cell contents, and consume the cellular slurry (Miles, 1999; Walling, 2009). At the cellular level, these Hemiptera can be as damaging as caterpillars or beetles that cut and tear foliage. At the other end of the spectrum are aphids and whiteflies (Tjallingii, 2006; Walling, 2008). Aphids will puncture numerous mesophyll cells in their search for phloem sieve elements, while whiteflies rarely damage cells on their stylet's path to the phloem.

Over 1,555 whitefly species have been described, although most studies have focused on polyphagous whiteflies, such as the *Bemisia tabaci* species complex and *Trialeurodes vaporariorum* (Westwood), that interfere with agricultural and horticultural productivity (De Barro et al., 2005; Martin and Mound, 2007). *Trialeurodes vaporariorum* is a common pest of greenhouses worldwide and fields in temperate climates, while *B. tabaci* (Gennadius) biotype B, also known as *Bemisia argentifolii (*Bellows and Perring), is more widespread, destructive, and invasive (van Lenteren and Noldus, 1990; Inbar and Gerling, 2008). The success of *B. tabaci* biotype B likely is due to a variety of strategies including: ability to adapt to a wide range of plant habitats, broad host range, voracious feeding that may allow it to infest plants with phloem sap of different nutritional values, increased fecundity, breeding strategies that promote invasiveness, and rapid emergence of insecticide-resistant strains (Liu et al., 2007; Inbar and Gerling, 2008; Walling, 2008).

The destructiveness of *B. tabaci* biotype B is correlated with depletion of photosynthates, deposition of large amounts of excreta (honeydew) that supports sooty mold growth, and its ability to vector over 111 virus species (Inbar and Gerling, 2008). In addition, this whitefly causes plant developmental disorders that can contribute to agricultural losses including tomato fruit irregular ripening and the curcurbit leaf-silvering disorder, the characteristic used in the common name of this biotype (the silverleaf whitefly) (Schuster et al., 1990; De Barro and Khan, 2007). Recently, two newly identified biotypes (MS, Ug6) also were shown to cause silvering, thus demonstrating that this trait has been acquired several times (Delatte et al., 2005; Sseruwagi et al., 2005).

Several studies have evaluated the response of crop and model plants to *B. tabaci* feeding. In the model plant *Arabidopsis thaliana* (L. Heynh), *Bemisia tabaci* biotype B causes a profound reprogramming of gene expression (Kempema et al., 2007). *B. tabaci* causes increases in SA-regulated pathogenesis-related (*PR*) protein gene transcripts and decline in JA-regulated defense RNAs in infested leaves (locally) and systemically in apical, non-infested leaves (Zarate et al., 2007). Use of defense-response mutants and transgenic lines that alter the activity of the SA- and JA-regulated defense-signaling pathways (*coi1, npr1, cev1, cim10*, and NahG) and methyl jasmonate (MeJA) treatments show that *B. tabaci* induces decoy defenses (Zarate et al., 2007). The suppressed JA-regulated defense pathway, not the induced SA-regulated defense pathway, controls the resistance traits that retard nymph development.

The role of the JA, SA, and ethylene defense-signaling pathways in regulating the expression of the resistance traits in crop plants that deter *B. tabaci* nymph development is less well characterized. Studies in squash show that plants can discriminate the elicitors/effectors (chemical signals) introduced by two different *B. tabaci* biotypes (van de Ven et al., 2000). *SLW1* (a M20b peptidase-like gene) and *SLW3 (*a leaf-specific β-glucosidase-like gene) RNAs accumulate preferentially in response to *B. tabaci* biotype B feeding but not *B. tabaci* biotype A. Both *SLW1* and *SLW3* appear to be regulated by SA-independent pathways (van de Ven et al., 2000). *SLW1* RNAs accumulate after JA and ethylene treatments, while *SLW3* transcripts do not accumulate in response to known defense signals.

Changes in peroxidase, chitinase, and β-1,3-glucanase activities have been noted after *B. tabaci* infestations of

cassava, squash, and tomato (Jiménez et al., 1995; Mayer et al., 1996; Antony and Palaniswami, 2006). In addition, *B. tabaci* biotype B infestation of *Phaseolus vulgaris* (L.) causes the release of volatiles that are recognized by its parasitoid *Encarsia formosa* (Gahan) (Birkett et al., 2003). However, in cotton, no changes in volatile bouquets were detected (Rodriguez-Saona et al., 2003). The levels of two pathogenesis-related (*PR*) protein gene RNAs (PR69B and P6) are known to accumulate during *B. tabaci* biotype B infestation (Sánchez-Hernández et al., 2006). Furthermore, 244 differentially regulated genes were identified using spotted cDNA arrays after *B. tabaci* infestation of tomato (Estrada-Hernandez et al., 2009).

Given the ability of plants to discriminate between *B. tabaci* biotypes, *Macrosiphum. euphorbiae* (Thomas) biotypes, and *Tetranychus urticae* (Koch) lines (van de Ven et al., 2000; Hebert et al., 2007; Kant et al., 2008), it was of interest to develop a more comprehensive understanding of the changes in plant defense-response gene expression in response to two species of whiteflies with distinctive host ranges and abilities to induce developmental disorders and vector viruses. To this end, the temporal and spatial expression of nine tomato genes that respond to wound/ defense signals, including systemin, JA, ethylene, ABA, and SA, were monitored after feeding by *B. tabaci* biotype B or *T. vaporariorum*. In addition, the roles of wound signals in the tomato-*Bemisia* interaction were investigated using a transgenic tomato line expressing a *LapA1:GUS* (*Leucine aminopepetideA1*:β-glucuronidase) reporter gene construct, which is a sensitive monitor for wounding.

Methods and Materials

Plant Growth, Insect Colonies and Whitefly Infestations Tomato plants (*Solanum lycopersicum cv.* Rutgers and cv. Pto238R) were grown in UC Soil Mix III in a growth chamber at 25°C with a 16/8-h L:D. At planting, soil was supplemented with osmocote. At 4 wk, tomato plants were transferred to insect cages in the greenhouse. Plants were fertilized once per week with Miracle-Gro. Seven-wk-old plants (cv. Rutgers) were used for the temporal and spatial RNA studies. These plants had 15 leaves, and leaves were numbered beginning at the base of the plant. Greenhouse temperatures averaged 29°C (day) and 16°C (night). Silverleaf whitefly [*B. tabaci* (Gennadius) biotype B; also known as *Bemisia argentifolii* (Bellows and Perring)] and greenhouse whitefly [*Trialeurodes vaporariorum* (Westwood)] colonies were raised on *Phaseolus vulgaris* (L.) plants. *Bemisia tabaci* cultures were assayed periodically for isozyme variants to ensure culture purity.

When plants were 7-wk old, the 7th and 8th leaves were encased individually in nylon-mesh bags, and 250 whiteflies adults (200 females and 50 males) were placed inside each bag. Control plants were encased in a similar manner, except no insects were added (bagged control; Control B). A second set of control plants was neither bagged nor infested to assess the impact of bagging on wound- and defense-response gene expression (unbagged control; Control U). After 9 d of feeding, adult whiteflies were removed by aspiration, and the infested tomato leaves (leaves 7 and 8) and apical, non-infested leaves (leaf 9) were harvested directly in liquid nitrogen. In time-course experiments, infested and control leaves were harvested at 0, 1, 3, 5, 7, and 9 d post infestation. Experiments were repeated once and in each experiment each sample was a pool of leaves from three different plants. This infestation protocol resulted in ca. 2,000 nymphs per leaf. Similar nymph densities have been noted in field-grown tomatoes (Gusmão et al., 2006).

Excised shoots from 3- to 4-wk-old tomato (Peto 238R) plants were treated with 10 μM MeJA (24 hr), 100 μM ABA (24 hr), 29 ppm ethylene (24 hr), 1 pmol systemin (12 hr), or 0.1, 0.25 or 0.5 mM salicylic acid (24 hr). Treatments and controls have been described previously (Gu et al., 1996; Chao et al., 1999).

RNA Extractions and RNA Blot Hybridizations Leaves (2.5 g) were ground to a fine powder in liquid N_2. After N_2 evaporation, 5 ml extraction buffer (100 mM LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at 80°C and 5 ml of water-saturated phenol (80°C) were added. After vortexing for 30 sec, 6 ml chloroform:isoamyl alcohol (24:1) were added. The sample was vortexed for 30 sec and centrifuged at 3,200 × *g* at 4°C for 60 min. The aqueous layer was removed and mixed with one volume of 4 M LiCl. After 1 hr at −80°C, total RNA was recovered by centrifugation at 3,200 × *g* at 4°C for 60 min, washed with 70% EtOH, resuspended in water, and stored at −80°C.

Total RNAs (15 μg) were size fractionated on 1.0% formaldehyde-agarose gels, blotted to nylon membranes (Hybond-N, Amersham, Piscataway, NJ, USA), hybridized with ^{32}P-labeled probes, and washed under conditions of high stringency as described in Pautot et al. (1991). RNA blots for the whitefly infestation experiments were used once to assure maximum sensitivity. RNA blots with defense chemical treatments were reused once. Probes were labeled using [^{32}P]-dCTP (Amersham). Autoradiographic signals were quantitated using a phosphoimager (Molecular Dynamics).

The *PR-1* (P6 protein, pP6), *PR-4* (P2 protein, pPR-P2), *Chi3* (acidic chitinase, pChi3), *Chi9* (basic chitinase, pChi9), *GluB* (basic β-1,3-glucanase, pGluBAS), and *GluAC* (acidic β-1,3-glucanase, pGluAC) cDNA clones were provided by Dr. Pierre de Wit (Wageningen Agricultural University, Wageningen, The Netherlands) (van Kan et al., 1995). The proteinase inhibitor 2 (*pin2;* pT2-47*)* cDNA clone was provided by Dr. C.A. Ryan (Washington

State University, Pullman, WA, USA) (Graham et al., 1985). The *LapA1* partial cDNA clone (pDR57) was previously described (Pautot et al., 1993). There are two genes (*LapA1* and *LapA2*) encoding LAP-A that are 98% identical at the nucleotide level (Gu et al., 1996); transcripts for both genes are detected under the hybridization conditions utilized. The *PAL* gene family in tomato (cv. Craigella) consists of 26 family members (Chang et al., 2008). RNAs for only two *PAL* genes (*PAL1* and *PAL5*) have been detected in tomato (Lee et al., 1992). The *PAL1* RNA is not abundant, whereas the *PAL5* RNA accumulates during development and in response to abiotic and biotic stresses (Chang et al., 2008). The tomato *PAL5* cDNA clone pPal1-16 was 1.6 kb in length and was isolated as a whitefly-induced cDNA by using differential RNA display from cv Rutgers (D.P. Puthoff and L.L. Walling, unpublished results). Partial sequence of this clone showed 97% nucleotide identity with *PAL5* of tomato cv. BonnyBest (Accession P26600) (Lee et al., 1992).

GUS Histochemical and Fluorometric Assays The *LapA1:GUS* line U78 was characterized previously (Chao et al., 1999). Homozygous *LapA1:GUS* or UC82b (wild-type control; WT) tomato plants were planted per 1-gallon pot (2 plants/pot) and grown in the greenhouse in insect cages. At 3 wk, pots were encased in nylon mesh bags, and plants were infested with 100 *B. tabaci* biotype B or served as non-infested controls. After 21 d of infestation, second, third and early fourth instar nymphs were feeding on the abaxial leaf surfaces. At this time, developmentally matched leaves from the control and infested plants were harvested for GUS staining. Leaves from 6-wk-old control (WT) and *LapA1:GUS* plants grown in insect-free cages were wounded by pricking the abaxial surface of leaflets with a straight pin. Pin pricks were administered to reflect the density of feeding whitefly nymphs (10 pricks per cm^2). Six-wk-old control (WT) and *LapA1:GUS* plants were mechanically wounded by crushing of the leaf lamina as previously described Chao et al. (1999). Pin-pricked and crushed leaves, as well as developmentally matched leaves from non-damaged WT (control) and *LapA1:GUS* plants, were harvested 24 hr later. All tissues were stored at −80°C until use.

Extraction and measurement of GUS activity in leaves was according to Jefferson (1987) with the following modifications. Leaves were ground to a fine powder in liquid nitrogen, and 30 mg of tissue were homogenized in GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). Extracts (10 µl) were assayed in a 500-µl reaction with GUS assay buffer (2 mM 4-methylumbelliferyl-β-D-glucuronide in GUS Extraction Buffer) with 20% (v/v) methanol to reduce endogenous GUS activity (Kosuge et al., 1990). After 0, 30, and 60 min, 100-µl aliquots were removed and added to 1.9 ml 0.2 M Na_2CO_3. Methlyumbelliferone (MU) concentrations were determined by using a Dyna Quant 200 fluorimeter using MU standards. Each plant extract was assayed in triplicate. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) using bovine serum albumin standards. Protein samples were pretreated with 0.1 M iodoacetamide in 0.1 mM Tris-HCl (pH 8.0) at 37°C for 20 min prior to the BCA assay to reduce interference caused by β-mercaptoethanol (Hill and Straka, 1988).

For GUS histochemical staining, 3-wk-old, greenhouse-grown *LapA1:GUS* tomato seedlings were infested or wounded. Infested plants were monitored for appearance of first, second, and third instar nymphs. Feeding sites of immature insects in each instar were evaluated on at least three different plants after 20 d of feeding. Infested leaves were harvested and stained for GUS activity (Jefferson, 1987). In brief, tomato leaves were harvested into GUS staining buffer (100 mM $NaPO_4$, 0.5 mM $K_3[Fe(CN_6)]$, 0.5 mM $K_4[Fe(CN_6)]$, 10 mM EDTA, 0.5 mg/ml X-gluc, 0.1% Triton X-100, 20% methanol). X-gluc (5-bromo-4-chloro-3-indoyl β-D-glucuronic acid) was purchased from Research Products International (Mt. Prospect, IL, USA). After vacuum infiltration for 5 min at 20–25 mm Hg, tissue was incubated at 37°C overnight. Tomato leaves were cleared with repeated changes of 95% ethanol and viewed in a dissecting scope. While Arabidopsis gene-trap lines demonstrated that the GUS histochemical assay was working (data not shown), GUS activities were not detected histochemically in infested or wounded *LapA1:GUS* plants. Increasing infiltration times, increasing number of 5-min infiltrations, pretreating leaves by dipping in hexane for 10 sec to 1 min, or performing GUS staining reactions in the presence of 1%–4% DMSO or 0.05%–0.5% Silwet (a wetting agent) did not enhance histochemical detection of GUS activity in the mature *LapA1:GUS* leaves.

Results

Whiteflies Do Not Increase Levels of LapA or pin2 RNAs Whiteflies do not cause extensive damage to plant tissues since their stylets follow an intercellular path to reach phloem sieve elements. Therefore, it was important to determine if (a) the occasional epidermal cell piercing, (b) passage of the whitefly stylet through the leaf cell's extracellular matrix, and (c) the puncturing of phloem elements would be perceived as a wound response in tomato. To this end, the levels of RNAs for two well-studied wound-response genes—leucine aminopeptidase (*LapA)* and proteinase inhibitor 2 *(pin2)*—were used as

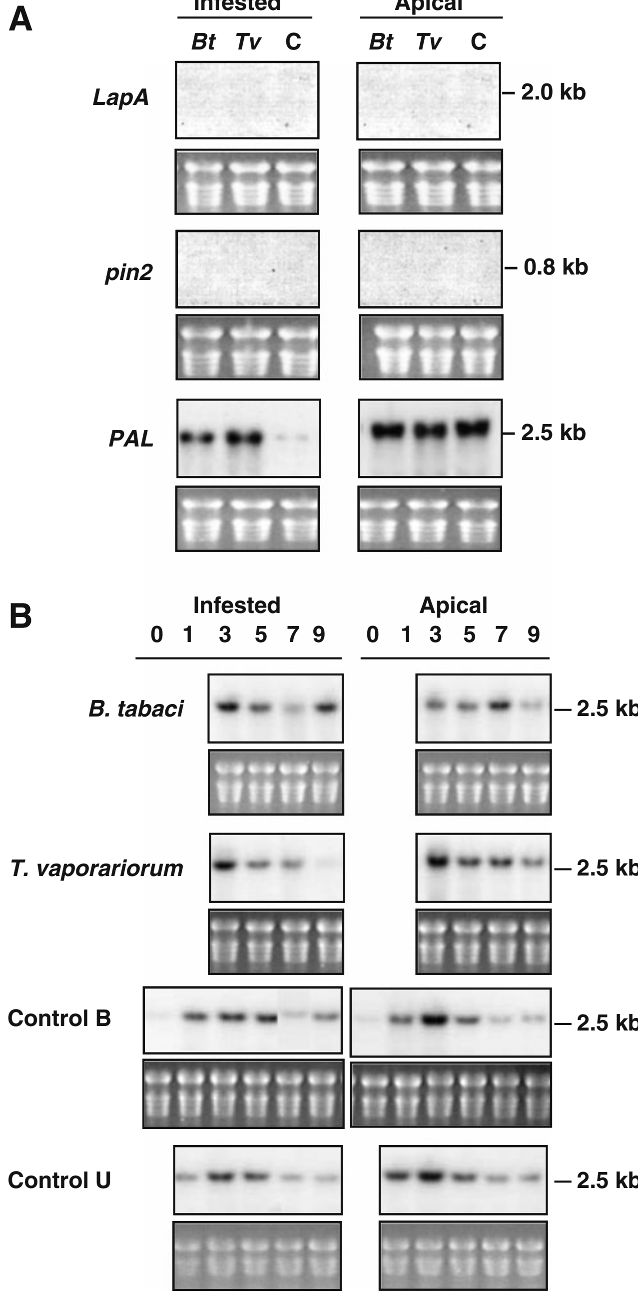


Fig. 1 Changes in *LapA*, *pin2*, and *PAL* gene expression after whitefly feeding. **A** Leaves 7 and 8 of 8-wk-old tomato plants were infested with 250 adult *Besimia tabaci* biotype B (*Bt*) or *Trialeurodes vaporariorum* (*Tv*). Leaves from non-infested control plants (C) were encased in nylon-mesh bags but no insects were added. After 9 d of infestation, adult whiteflies were removed and infested leaves and apical, non-infested leaves (leaf 9) were harvested. Total RNAs isolated and RNA blots (15 μg/lane) were hybridized with ^{32}P-labeled probes for leucine aminopeptidase (*LapA*), proteinase inhibitor 2 (*pin2*), and phenylalanine ammonia lyase (*PAL*). Film exposure times were 3 (*PAL*) or 6 (*LapA*, *pin2*) days. **B** Plants were infested with *B. tabaci* biotype B (*Bt*) or *T. vaporariorum* (*Tv*) as described in Panel **A**. Infested and apical, non-infested leaves were harvested 0, 1, 3, 5, 7, and 9 d after infestation. Leaves from control plants that had leaves 7 and 8 encased in insect bags (Control B) and untreated plants (Control U) were harvested at 0, 1, 3, 5, 7, and 9 d. Total RNA blots were hybridized with a ^{32}P-labeled *PAL* probe. Exposure times were 24 hr. Stained gels visualizing rRNAs are shown as a loading controls. Results from infested leaf 7 and apical leaf 9 are displayed (**A, B**). Infested leaf 8 was also analyzed and results were similar to that of leaf 7 (data not shown)

sentinels to monitor the activation of the tomato wound-signaling pathway.

The 7th and 8th leaves of 8-wk-old tomato plants were enclosed in fine-mesh nylon bags and each leaf was infested with 250 adult whiteflies/leaf (4 females:1 male). To control for possible responses to the inadvertent mechanical damage that occurred during the infestation procedures and plant handling, two sets of controls were included. Some control plants had their leaves encased in nylon bags but insects were not added (Control B). A second set of control plants were not bagged or infested (Control U). The levels of *LapA* and *pin2* RNAs were determined in the infested and apical non-infested leaves at 0, 1, 3, 5, 7, and 9 days after whitefly infestation (Fig. 1A).

Throughout the 9-day infestation, adults fed, mated, and oviposited. During the first 3 days of infestation, only whitefly adults were feeding (500 per plant). During this period, no increases in *LapA* or *pin2* RNAs were detected (data not shown). Between days 3 and 5, eggs began to hatch and first-instar nymphs established feeding sites. The numbers of feeding nymphs increased over the 9-day period. At the time of harvest, there were approximately 2,000 immature insects and 250 adult insects associated with each infested tomato leaf. Most nymphs were in their second and third instars. Despite the large numbers of whitefly nymphs (10 nymphs/cm^2 leaf area), neither the *LapA* nor *pin2* transcripts were detected in the infested leaves at 9 days post infestation (Fig. 1A) or at earlier times (data not shown).

Since many wound-response genes are expressed systemically and local and systemic responses can be distinct, the levels of *LapA* and *pin2* RNAs in apical, non-infested leaves were determined. *LapA* and *pin2* RNAs were not detected in apical leaves from control or infested plants after 9 days (Fig. 1A) or at earlier times (data not shown). This is in sharp contrast to the rapid increase in *LapA* or *pin2* mRNA levels that have been previously reported after mechanical wounding or caterpillar feeding (Pautot et al., 1993; Howe et al., 1996).

Although visual damage is not observed after whitefly feeding, wound-response genes (*LapA* and *pin2*) might be expressed in a subset of leaf cells (i.e., the epidermal cells that were pierced), and RNA signals may be diluted by the majority of non-responding cells. For this reason, it was of interest to determine if wound-response genes were activated in leaf cells immediately surrounding the stylet path or at the whitefly feeding sites. To this end, transgenic tomato plants expressing the wound-activated *LapA1* gene

promoter fused to the β-glucuronidase (GUS) coding region (*LapA1:GUS*) were used in whitefly infestation studies. Due to the exceptional stability of GUS in plant cells, GUS was the reporter gene of choice in these studies. GUS activity can be detected for several days after promoter activation (Weinmann et al., 1994). Three-wk-old *LapA1:GUS* and WT plants were infested with 100 *B. tabaci*. After 21 d of infestation, leaves had second, third, and early fourth-instar nymphs. As positive controls, leaves from developmentally matched plants were wounded by crushing or pin pricking. Both histochemical and fluorometric assays were performed.

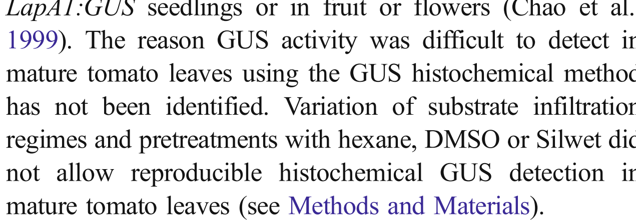
Using the GUS histochemical detection method, no GUS activity was detected in controls or, more surprisingly, in wounded or JA-treated samples; however, GUS activities were readily detected using a GUS fluorometric assay (Table 1). The inability to detect GUS staining in older leaves in this study contrasted to previous reports that readily detected GUS histochemically after JA treatments in 7- to 10-day-old

Table 1 Fluorometric analysis of GUS activity in transgenic *LapA1:GUS* tomato plants in response to wounding and whitefly feeding

Treatment	GUS activity[a] (pmol 4-MU/min/mg protein)	Average[b]
Untreated	195.1 ± 9.5	248.9±81.42
	342.6 ± 8.5	
	209.1 ± 2.0	
B. tabaci	430.8 ± 26.4	459.1±265.80
	296.6 ± 17.5	
	843.1 ± 13.3	
	266.0 ± 31.8	
Crushing	5585.5 ± 118.4	**6325.2±3328.47 ***
	10123.7 ± 774.5	
	1850.0 ± 38.4	
	3372.7 ± 1460.4	
	9541.7 ± 919.2	
	7477.3 ± 360.2	
Pin pricks	2416.6 ± 215.3	1293.3±806.76
	1334.7 ± 38.8	
	638.2 ± 6.3	
	783.5 ± 101.9	

[a] GUS activity in leaf extracts from 6-week-old *LapA1:GUS* control plants (untreated) or *LapA1:GUS* plants infested with *Bemisia tabaci* biotype B (20 d), wounded by leaf lamina crushing (24 hr), or wounded by pricking the leaf with a straight pin (24 hr). GUS activities were measured in triplicate from individual plants and therefore reflect plant-to-plant variation in treatment responses. GUS and protein levels were determined as described in Methods and Materials. One representative experiment is displayed

[b] Overall average for each treatment (+/− standard deviation). Bold numbers are statistically different from the "Untreated" sample determined by a *t*-test (2-tailed, unequal variance, 0.05 level). Numbers designated with an asterisk are significantly different from the "*B. tabaci*" treatment determined by a *t*-test (2-tailed, unequal variance, 0.05 level)



LapA1:GUS seedlings or in fruit or flowers (Chao et al., 1999). The reason GUS activity was difficult to detect in mature tomato leaves using the GUS histochemical method has not been identified. Variation of substrate infiltration regimes and pretreatments with hexane, DMSO or Silwet did not allow reproducible histochemical GUS detection in mature tomato leaves (see Methods and Materials).

For the reasons above, GUS activities in leaf extracts were monitored fluorometrically. These assays showed plant-to-plant variability in GUS activity levels in all treatments and controls; however, trends in GUS activities in the treated and control plants were readily discerned. Control, non-infested *LapA1:GUS* plants had low levels of GUS activity ranging from 195 to 342 pmol 4-MU/min/mg protein (Table 1) similar to that observed by Chao et al. (1999). *Bemisia tabaci* feeding caused no significant changes of GUS activity (Table 1). *LapA1:GUS* leaf lamina that were pin pricked to mimic mechanical damage (similar to that inflicted during feeding by some species of aphids) had higher levels of *LapA* promoter activity (2.6- to 9.7-fold); however, due to plant-to-plant variation, these values were not statistically significant from untreated leaves. Larger and statistically significant increases in *LapA1* promoter activity (7 to 40-fold) were detected after the more severe mechanical wound (leaf crushing; Table 1), which is known to activate *LapA1* (Chao et al., 1999).

PAL is Not a Good Molecular Sentinel for Whitefly Feeding Increases in *PAL* transcripts have been correlated with wounding, pathogen infection and aphid feeding (Lee et al., 1994; Moran and Thompson, 2001). Therefore, the changes in *PAL* RNAs in response to *B. tabaci* and *T. vaporariorum* feeding were monitored from 0 to 9 days after infestation of tomato plants. Analysis of temporal and spatial RNA blot studies showed that *PAL* RNA levels increased transiently (days 1–5) and then declined (days 7–9) in both bagged (Control B) and unbagged (Control U) leaves and in apical leaves of control, non-infested plants (Fig. 1B). These data indicated that the small mechanical stresses that occurred during movement of the plants within the insect cages and not the process of encasing leaves in nylon bags were sufficient to increase *PAL* transcript levels. In some experiments, large increases in *PAL* transcripts were detected in infested leaves after 9 days of feeding by *B. tabaci* or *T. vaporariorum* relative to controls (Fig. 1A). In other experiments, *PAL* RNAs were only slightly elevated after *B. tabaci* feeding relative to controls on day 9 (Fig. 1B). While it appears that tomato *PAL* genes respond to whitefly feeding, *PAL* was not a reliable molecular marker for whitefly feeding responses.

Whiteflies Cause PR RNA Accumulation To determine if tomato *PR* RNAs accumulated after whitefly feeding, the

levels of *GluB* (basic β-1,3-glucanase), *Chi9* (basic chitinase), *PR-1* (P6 protein), *PR-4* (P2 protein), *GluAC* (acidic β-1,3-glucanase), and *Chi3* (acidic chitinase) transcripts were determined at 9 days after *B. tabaci* or *T. vaporariorum* infestation. Overall, similar gene expression patterns were noted after infestations with both species of whiteflies. *GluB*, *Chi9*, and *PR-1* transcripts accumulated to high levels in infested leaves relative to the controls (Fig. 2A). Smaller increases in *PR-4* and *Chi3* RNAs were detected in infested leaves and *GluAC* transcripts were not detected (Fig. 2A).

The levels of *PR* RNAs from infested and control plants were evaluated in infested and apical, non-infested leaves on days 0, 1, 3, 5, 7, and 9 after *B. tabaci* and *T. vaporariorum* infestation. While six *PR* gene RNAs were evaluated in this study (Table 2), only the *GluB* and *PR-1* data are presented here (Fig. 2). *GluB* transcripts were first detected 5 days after infestation with *B. tabaci* and *T. vaporariorum. GluB* RNA levels peaked 7 days post infestation (Fig. 2B). *GluB* RNAs also were detected in the apical, non-infested leaves on day 7. *GluB* transcripts were not detected in the leaves from the two sets of control plants that had either had their leaves encased in insect-proof bags (Control B) or were not manipulated (Control U) (Fig. 2B).

The spatial and temporal patterns of *PR-1* RNA accumulation were more complex. Over the 9-day infestation period, *PR-1* RNA levels were modulated by developmental signals. This conclusion was based on the fact that *PR-1* RNAs were detected at low levels in the non-infested leaves from both controls on days 1–3 and increased between days 5 and 7 (Fig. 2C). In addition, low levels of *PR-1* RNAs were detected in apical leaves on days 5–9, reflecting the younger age of these leaves and the beginning of the *PR-1* developmental program (Fig. 2C). It should be noted that these leaves showed no signs of senescence, and therefore *PR-1* transcript levels could not be attributed to initiation of the senescence program, as has been previously reported (John et al., 1997). Despite this developmental regulation, it was clear that both *B. tabaci* and *T. vaporariorum* feeding enhanced *PR-1* transcript accumulation in infested leaves relative to control plants. Differences in *PR-1* transcript levels in apical leaves from infested or non-infested plants also were observed in this experiment (Fig. 2C). In other replicate experiments, the systemic accumulation of *PR-1* RNAs was not as pronounced (Fig. 2A; data not shown).

Changes in Chi3, Chi9, GluAC, and PAL—Transcripts in Response to Wound and Defense Signals The responses of the tomato *PR* genes encoding the *GluB*, *PR-4*, and *PR-1* to wound and defense signals (i.e., MeJA, systemin, ABA, ethylene, and SA) in the tomato cultivar UC82b were reported previously (Chao et al., 1999) and are summarized in Table 2. To understand the potential defense signals important in the regulation of other tomato defense genes used in this study, the accumulation of transcripts for *Chi3*, *GluAC*, *Chi9*, and *PAL5* was determined after SA, MeJA, systemin, ABA, and ethylene treatments (Fig. 3; Table 2). Like previously characterized *PR* genes (Chao et al., 1999), *GluAC*, *Chi9*, and *Chi3* RNAs did not accumulate in response to systemin or ABA treatments.

Both *Chi9* and *GluB* encode proteins with a vacuolar location (van Kan et al., 1995). *Chi9* transcripts accumulated with a pattern similar to *GluB* RNAs; *Chi9* and *GluB* RNAs were most abundant after MeJA and ethylene treatments (Fig. 3; Chao et al., 1999). The dependence of *GluB*, *Chi9*, and *PR-1* RNA accumulation on ethylene signal transduction also has been supported by the analysis of mutant tomatoes expressing the *Nr* mutation, over-expressing the wild type NR, or down-regulating tomato *ETR* genes (Ciardi et al., 2001). *Chi9* RNA levels also were elevated in control treatments, as previously reported for *GluB* and *PR-1* (Chao et al., 1999). This may be due to a volatile released by control plants that accumulates in the enclosed environments used for the ethylene and MeJA treatments. Notably, *LapA*, *Chi3* and *GluAC* were not modulated by this additional defense signal.

Chi3 and *GluAC* encode apoplastic proteins (van Kan et al., 1995). Similar to the pattern previously reported for *PR4* (Chao et al., 1999), *Chi3* and *GluAC* transcripts were most abundant after ethylene treatments (Fig. 3). Of the three *PR* genes examined in this study, only *Chi3* transcripts accumulated in response to exogenous SA. These data contrast to studies by van Kan et al. (1995) who showed increases in *Chi3* and *GluAC* RNAs after treatment with 1 mM SA. This may be due to the facts that van Kan et al. (1995) used a different tomato cultivar, applied SA to petioles (vs. seedling shoots in this paper), and/or used a higher concentration of SA (1 mM). In the excised seedling assay used here, 1 mM SA caused necrosis in the UC82b cultivar and could not be evaluated (Chao et al., 1999).

Surprisingly little is known about *PAL5* expression after biotic stress in tomato plants (Gorlach et al., 1995). Figure 3 shows that tomato *PAL5* transcripts were detected in all control and treated leaf samples. *PAL5* RNAs increased in response to MeJA (3-fold) and smaller increases (1.4- to 2.0-fold) were seen with ethylene and ABA treatments (Fig. 3). In contrast, *PAL5* RNA levels declined after treatments with 0.1, 0.2 and 0.5 mM SA relative to control.

Discussion

The ability of plants to quickly and accurately perceive their biotic attackers is essential for mounting an effective defense. Plants must identify and respond to the chemical

Fig. 2 Changes in *PR* gene RNA levels in response to whitefly feeding. **A** Tomato plants were infested with *Besimia tabaci* biotype B (*Bt*) or *Trialeurodes vaporariorum* (*Tv*) or served as non-infested controls (C). Control leaves were encased and nylon bags but insects were not added as described in Fig. 1A. Total RNA blots were hybridized with ^{32}P-labeled basic β-1,3-glucanase (*GluB*), basic chitinase (*Chi9*), *PR-1* (P6), *PR-4*, acidic chitinase (*Chi3*), or acidic β -1,3-glucanase (*GluAC*) cDNA probes. Experiments were repeated once and each lane is pooled leaf material from three plants. Film exposure times were: 3 d for *Chi9*, *GluB*, and *PR-4*; 2 d for *PR-1*, 4 days for *Chi3*; 6 d for *GluA*, **B, C** Plants were infested as in Fig. 1B. Infested and apical, non-infested leaves were harvested 0, 3, 5, 7, and 9 d after *B. tabaci* biotype B (*Bt*) or *T. vaporariorum* (*Tv*) infestation. Controls were leaves from bagged but non-infested (Control B) or untreated (Control U) plants. RNA blots were hybridized to each ^{32}P-labeled *PR* gene probe in Panel **A**; only data from the *GluB* (Panel **B**) *or PR-1* (Panel **C**) probes are shown in Panel **B**. Stained gels visualizing rRNAs are shown as a loading control. Film exposures time were 2 d

Table 2 Tomato wound- and defense-response gene expression

Gene	Protein	Relative RNA increases in response to wound and defense signals[a]					
		WF	MeJA	Eth	ABA	SYS	SA
LapA[b]	Leucine aminopeptidase	o	+++	+	+	+++	−
pin2[c]	Proteinase inhibitor II	o	+++	−	+	+++	−
PAL[d]	Phenylalanine ammonia lyase	+/++	+	+	+	o	−
GluB[b]	Basic β-1,3-glucanase	++	+[e]	++[e]	o	−	+
Chi9[d]	Basic chitinase	++	++[e]	+[e]	o	o	o
PR-1[b]	P6 protein (PR-1-like protein)	++	++[e]	+++[e]	o	o	+++
GluAC[d]	Acidic β-1,3-glucanase	o	+	+++	o	−	o
Chi3[d]	Acidic chitinase	+	+	+++	−	o	++
PR-4[b]	P2 protein (win-like protein)	+	o	+++	o	o	o

[a] Fold increase in RNAs relative to control samples was determined by phosphoimager quantitation of RNA blot signals. RNAs that were unchanged or declined relative to control are indicated with a zero (o) or minus symbol (−), respectively. RNAs that increased are indicated with plus symbols: from 1.5 to 3.0 fold (+), 3.1 to 10 fold (++), and greater than 10 fold (+++). Treatments included silverleaf or greenhouse whitefly infestations (WF), 100 μM methyl jasmonate (MeJA), 29 ppm ethylene (Eth), 100 μM abscisic acid (ABA), 1 pmol systemin (Sys), and 0.25 mM salicylic acid (SA)

[b] Defense chemical treatment data are from Chao et al. (1999). Whitefly-response data appear in this paper

[c] The *pin2* probe was hybridized to a RNA blot similar to that shown in Fig. 3 and autoradiographic signals quantitated (data not shown). Whitefly response data appear in this paper

[d] Defense chemical treatment and whitefly-response data appear in this paper

[e] Control samples had elevated levels of these transcripts relative to other genes, therefore increases in these RNA levels may be underestimated. Discussion of these results are in Chao et al. (1999)

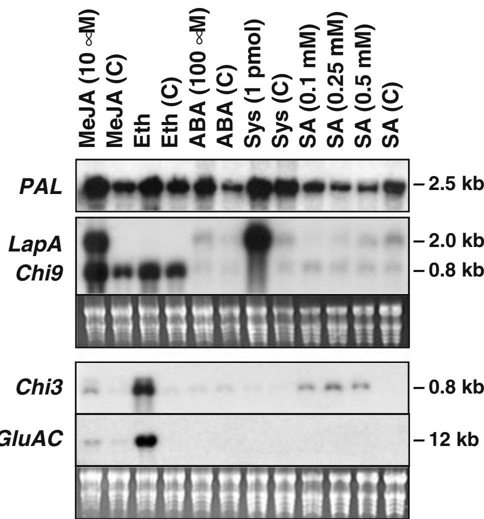


Fig. 3 RNA blot analyses of wound- and defense-response RNAs in tomato plants treated with defense signals. Excised tomato seedlings were treated with 10 μM MeJA, 29 ppm ethylene (Eth), 100 μM ABA, 1.0 pmol systemin (Sys), or 0.1, 0.25, or 0.5 mM SA. For each treatment, the corresponding control is shown (C). Total RNA blots were hybridized to ^{32}P-labeled *PAL*, *LapA*, *Chi9*, *Chi3*, and *GluAC* probes. Transcript sizes are shown in kb and were determined using a RNA marker run in a parallel lane. Stained gels visualizing rRNAs are shown as a loading control. The stained gel for the *PAL5* blot is not shown. Exposure times were 21–23 hr

(effectors) and mechanical signals that accompany pest attack (Felton and Tumlinson, 2008; Howe and Jander, 2008; Walling, 2009; Wu and Baldwin, 2009). Previous studies have indicated that plants can discriminate signals introduced by closely related arthropods, such as whitefly, aphid and spider mite biotypes (van de Ven et al., 2000; Hebert et al., 2007; Kant et al., 2008). For example, squash plants can perceive differences in the signals delivered by *B. tabaci* biotypes A and B. Only biotype B causes the squash leaf silvering disorder and preferentially induces the defense-response genes *SLW1* and *SLW3* (van de Ven et al., 2000). It is presumed that the *B. tabaci* biotype B effectors, which elicit these biotype-specific changes in gene expression and leaf development programs, are of insect or endosymbiont origin and introduced via the rapidly gelling or watery salivas of the whitefly (van de Ven et al., 2000).

For the reasons above, it was of interest to understand if two distinct whitefly species—*B. tabaci* and *T. vaporariorum*—would provoke similar or distinct defense gene expression programs in tomato. While *B. tabaci* and *T. vaporariorum* both have broad host ranges and use similar mechanical strategies to recover nutrients from the phloem, their ability to vector viruses and cause developmental disorders are distinct (Inbar and Gerling, 2008). *Bemisia tabaci* biotype B, but not *T. vaporariorum*, causes the tomato irregular fruit-ripening disorder (Schuster et al., 1990), but neither of these whiteflies induces leaf silvering in tomato. The data presented here showed that *B. tabaci*

and *T. vaporariorum* feeding on tomato leaves induce similar temporal and spatial patterns of defense- and wound-response gene expression. These results indicate that these whiteflies must introduce effectors with similar chemical structure(s) or activities to regulate wound- and defense-response gene expression. Furthermore, these whiteflies must also introduce species-specific effectors to cause the irregular-ripening disorder.

This study used nine tomato wound- and defense-response genes to assess defense responses to *B. tabaci* and *T. vaporariorum*. These genes included two well-characterized wound-response genes (*Pin2* and *LapA*) that are known to be JA-responsive and dependent on the jasmonate receptor JAI1 (COI1) (Chao et al., 1999; Li et al., 2004). *PAL5*, which encodes a rate-limiting enzyme for the shikimate pathway that provides the chemical precursors for phenolic compounds used for cell wall strengthening and as anti-nutritive compounds, also has been studied (Dixon et al., 2002). *PAL5* RNAs accumulate in response to a wide variety of biotic stresses; however, little was known about tomato *PAL5* responses to defense signals (Chang et al., 2008; Guo and Wang, 2009). The studies presented here show that tomato *PAL5* RNAs decreased in response to SA and increase after MeJA, ethylene, and ABA treatments (Table 2). *PAL5* RNA accumulation patterns reported here are consistent with the results of Lee et al. (1994). These investigators reported two *PAL5* RNA forms; the long *PAL5* RNA is detected constitutively, while the short *PAL5* RNA accumulates in response to wounding and pathogen infection.

The third group of tomato genes studied included six *PR* genes (*GluB, Chi9, PR-1, GluAC, Chi3*, and *PR-4)* that respond to pathogen attack (van Kan et al., 1995). Treatments of excised seedlings with MeJA, ABA, SA, systemin, and ethylene indicate that these genes display four basic expression programs (Table 2; Chao et al., 1999). All *PR* genes were ethylene responsive, and their response to exogenous MeJA and/or SA distinguished the *PR* genes further. *GluB, Chi9*, and *PR-1* (P6) transcripts increased in response to exogenous MeJA. However, their regulation was distinctive from *Pin2* and *LapA*, since *GluB*, *Chi9*, and *PR-1* RNAs did not increase in response to exogenous ABA or systemin (Table 2; Chao et al., 1999). Unlike *GluB* and *Chi9, PR-1* RNAs increased after SA treatments (Table 2). In contrast, *Chi3* and *PR-4* transcripts accumulated to low levels and *GluAC* RNAs were barely detected in response to exogenous MeJA; only *Chi3* RNA levels were modulated by SA (Table 2; Chao et al., 1999).

One prevailing theme from the studies presented here is that the tomato response to phloem-feeding whiteflies has a compelling similarity to pathogen infection (van Loon et al., 2006). *PR* RNAs and proteins accumulate after infection with both biotrophic and necotrophic pathogens in tomato including avirulent and virulent *Cladosporium fulvum* (Cooke.) (van Kan et al., 1992), *Fusarium oxysporum* (Schlecht) (Rep et al., 2002), *Phytophthora infestans* (Mont. de Bary) (Christ and Mosinger, 1989), and *Botrytis cinerea* (Pers.) (Benito et al., 1998). Both *B. tabaci* and *T. vaporariorum* infestations resulted in similar patterns of *PR* gene expression. *PR* RNAs were not detected at 1 or 3 days after whitefly infestation, indicating that a total of 500 adult whiteflies per plant (250 adults/leaf × 2 leaves) were insufficient to cause *PR* RNAs to accumulate locally or systemically. Five days after infestation, *GluB, Chi9*, and *PR-1* transcripts became abundant in the whitefly-infested leaves, and *GluB* RNAs accumulated systemically by day 7. *Chi3* and *PR-4* RNAs also increased but were less abundant and *GluAC* RNAs were barely detected after whitefly feeding.

The increases in *PR* RNAs correlated with the time of crawler emergence from eggs and the initiation of nymph feeding. Therefore, it is not clear if adults lacked the salivary effectors to activate *PR* gene expression, or if the changes in *PR* gene expression were dependent on the larger number of insects feeding during days 5–9. It is noteworthy, that *B. tabaci* biotype B leaf silvering of squash plants was thought initially to be a nymph-specific disorder, since as few as three feeding nymphs can cause squash leaf silvering (Costa et al., 1993). Recently, it was shown that large numbers of male *B. tabaci* biotype B infest squash are capable of inducing leaf silvering (De Barro and Khan, 2007). These data suggest that large numbers of adult whiteflies may be needed for tomato or squash plants to perceive whitefly effectors to induce defense genes and developmental disorders, respectively. This may reflect differences in the potency or quantities of effectors in adult whitefly saliva or the amount of time adults spend salivating relative to nymphs.

When the responsiveness of different *PR* genes to whitefly feeding and exogenous hormone treatments were examined collectively, the role of the defense-signaling pathways in the tomato-whitefly interactions could be proposed (Table 2, Fig. 3, Chao et al., 1999). This was based on the presumption that exogenous hormone treatments accurately report the roles of defense hormone signaling pathways after whitefly infestation. It is clear that all six *PR* genes examined in this study were complexly regulated, since these *PR* genes responded to two or more defense-hormones (i.e., MeJA, ethylene, and/or SA). As developed below, it is clear that no one defense hormone can explain *PR* gene regulation after whitefly infestation. Therefore, it is likely that multiple hormones act together to orchestrate this defense response; the possibility of a novel signal transduction pathway involvement cannot be excluded at this time.

It appears unlikely that a rise in ethylene can explain the induction of *PR* genes after whitefly feeding. This

conclusion is based on the facts that all six *PR* RNAs accumulated in response to exogenous ethylene, but *GluAC* RNAs did not accumulate after whitefly feeding. Increases in ethylene and induction of ethylene pathway genes have been noted during aphid infestations of both susceptible and resistant tomato, barley and melon plants (Argañdona et al., 2001; Mantelin et al., 2009; Anstead et al., 2010). In the *M. eurphorbiae*-tomato interaction, the ability to perceive ethylene is important for basal resistance (innate immunity) but not gene-for-gene resistance (Mantelin et al., 2009).

PR RNA levels after whitefly feeding were not correlated with responsiveness to SA, for only three of the five *PR* RNAs that accumulated were SA-regulated. This is a fundamental difference in the regulation of *PR* genes in tomato vs. Arabidopsis. Most *PR* genes in Arabidopsis are SA-regulated and suppressed by whitefly feeding (van Loon et al., 2006; Zarate et al., 2007).

Finally, the *PR* RNAs that were most abundant after whitefly feeding also increased markedly after MeJA treatments. This suggests that the JA-pathway may be activated after whitefly feeding. However, it should be noted that there was not a strict correlation of MeJA and whitefly responsiveness, since *GluAC* RNAs accumulated in response to MeJA treatments but not after whitefly infestation, and *PR-4* was whitefly induced but did not respond to exogenous MeJA. Several other genes induced by whitefly infestation are known to be regulated by MeJA. This includes a whitefly-induced tomato gene (*Wfi1*) that encodes a subunit of the NADPH oxidase (D.P. Puthoff and L.L. Walling, unpublished results) and the squash *SLW1* gene that is preferentially induced by the *B. tabaci* biotype B (van de Ven et al., 2000). These observations in tomato are in marked contrast to the changes in gene expression observed after the *B. tabaci* interactions in Arabidopsis, where SA-regulated *PR* RNAs accumulate and JA-regulated wound/defense response genes are suppressed (Kempema et al., 2007; Zarate et al., 2007). In addition, *B. tabaci* infestations of Lima bean (Zhang et al., 2009) and Arabidopsis (Zarate, Navarre and Walling, unpublished results) cause increases in SA levels; the changes in SA and JA levels after whitefly infestation of tomatoes has not yet been assessed.

While JA-regulated *PR* RNAs are abundant after whitefly feeding, this was not the case for JA-regulated wound-response genes. Two sets of data indicate that heavy infestations (up to 10 insects per cm^2) by *B. tabaci* and *T. vaporariorum* did not activate the wound-signaling pathway in tomato. First, feeding by over 500 *B. tabaci* adults (day 3) and over 2,000 *B. tabaci* nymphs and 500 adults (day 5) per tomato leaf were not sufficient to increase *LapA* or *pin2* transcripts to detectable levels locally or systemically. Second, *B. tabaci* infestation of transgenic *LapA1:GUS* tomato plants showed that the *LapA1* promoter activity was similar in leaves of *B. tabaci*-infested and non-infested control plants. These data suggest that the whitefly stylet's piercing of epidermal cells, movement between cells (which disrupts essential cell-to-cell contacts), puncturing of the sieve elements, and consumption of phloem nutrients were not perceived as sufficient physical or mechanical stresses to activate the tomato JA-dependent wound-response pathway (Walling, 2008).

These data also indicate that tomato responses to whitefly adult and nymph feeding were distinct from responses to tissue-damaging herbivores. Caterpillars, beetles, and cell-content feeding spider mites and thrips all induce JA-regulated wound-response gene expression in tomato (Pautot et al., 1993; Li et al., 2002; Ament et al., 2004). This regulation occurs at the transcriptional level, since *Pin2* and *LapA* promoter:reporter genes are activated by wounding and caterpillar feeding in transgenic tomato, potato, tobacco, silver birch, Arabidopsis, and white spruce (Thornburg et al., 1990; Keinonen-Mettälä et al., 1998; Chao et al., 1999; Godard et al., 2007).

Pin2 genes also are induced in some, but not all, aphid-plant interactions (Thornburg et al., 1990; Fidantsef et al., 1999; de Ilarduya et al., 2003). Consistent with aphids piercing mesophyll cells in their search for a phloem-sieve element, the potato aphid (*M. euphorbiae*) and green peach aphid [*Myzus persicae* (Sulzer)] infestations of tomato plants cause *Pin1* and *Pin2* RNA levels to increase transiently (6–12 hr post infestation), and prior to the accumulation of *PR* RNAs (12–48 hr post infestation) (de Ilarduya et al., 2003). Consistent with this result, *Pin2* RNAs were not detectable 7 days after *M. euphorbiae* feeding (Fidantsef et al., 1999). Interestingly, the *Pin2* promoter was not activated after a non-synchronous aphid infestation of transgenic *Pin2*:CAT tobacco plants in the field (Thornburg et al., 1990).

Whiteflies may avoid activation of wound-responses due to their refined feeding behaviors that avoid puncturing mesophyll cells, which distinguishes feeding by whiteflies from aphids (Tjallingii, 2006; Walling, 2008). It is also possible that chemical constituents of the watery and gelling salivas of whitefly adults and nymphs do not activate the JA-dependent wound signaling pathway. Alternatively, whitefly salivary factors may directly or indirectly antagonize the tomato wound-response pathway. Since whiteflies are known to increase SA levels during infestation of Arabidopsis and Lima beans (Zhang et al., 2009; Zarate, Navarre, and Walling, unpublished results), it is possible that the increases in SA promote the antagonistic cross-talk between the JA- and SA-defenses. Cross-talk, which is known to occur in the *B. tabaci*-Arabidopsis and in other biotic interactions, may suppress the JA-regulated traits that slow nymph development (Thaler et al., 2002;

Zarate et al., 2007; Koornneef et al., 2008). In addition, there is substantial evidence for constituents of hemipteran saliva influencing insect-plant interactions (Miles, 1999; Felton and Tumlinson, 2008; Walling, 2009). Some aphid salivary proteins appear important in chelating calcium to influence wound-induced protein coagulation at stylet punctures of the phloem (Will et al., 2007). Saliva constituents also contribute to aphid fitness, activate/suppress plant gene expression, induce a local resistance to further aphid infestations, and induce infestation symptoms (Lapitan et al., 2007; Mutti et al., 2008; De Vos and Jander, 2009). Some caterpillars and beetles have oral or salivary secretions that antagonize activation of wound-response genes. These interactions include: the corn earworm [*Helicoverpa zea* (Boddie)] with tobacco [(*Nicotiana tabacum* (*L.*)], the beet armyworm [*Spodoptera exigua* (Hübner)] with *Medicago truncatula* (Gaertn), the Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] with tomato, and the small white butterfly larvae [*Pieris rapae* (L.)] with Arabidopsis (Reymond et al., 2000; Musser et al., 2002; Bede et al., 2006; Lawrence et al., 2007).

Avoiding activation of wound-responses may contribute to the success of *B. tabaci* and *T. vaporariorum* on their hosts (Walling, 2008), and this appears to be the case for *B. tabaci* interactions in Arabidopsis (Kempema et al., 2007; Zarate et al., 2007). However, in tomato, the role of JA-regulated defense traits against whiteflies is not as well understood. Sánchez-Hernández et al. (2006) showed that the tomato *spr2* (*suppressor of prosystemin-mediated responses2*) mutant and transgenic line that ectopically expresses Prosystemin (*35S:ProSys*) influences whitefly-tomato interactions. The *spr2* mutant blocks JA biosynthesis and alters volatile emission profiles (Sánchez-Hernández et al., 2006). The *35S:ProSys* line over-expresses the wound peptide precursor Prosystemin and has constitutively activated wound-response genes (McGurl et al., 1994). *Bemisia tabaci* adults prefer to oviposit on *spr2* plants, indicating that JA is essential for the production of volatiles that deter *B. tabaci* acceptance of tomato plants; however, *35S:Prosys* plants did not display the expected reciprocal phenotype (i.e., repellency) (Sánchez-Hernández et al., 2006). When adult eclosion from fourth-instar nymphs was monitored, fewer adults emerged from *35S:Prosys* plants than wild-type or *spr2* plants (Sánchez-Hernández et al., 2006). These data suggest that ectopic expression of the tomato wound-response pathway may impede *B. tabaci* development at the level(s) of nymph settling or development. Therefore, avoiding activation of the wound-response pathway of tomato would be advantageous to whitefly success, as it is in the model plant Arabidopsis (Zarate et al., 2007).

The importance of *PR* gene products in defense against phloem-feeding insects is not understood at the present time. In tomato, the *PR* genes that responded most strongly to whitefly feeding encoded proteins with either a vacuolar (*Chi9* and *GluB*) or an apoplastic (*PR-1*) location (van Kan et al., 1995). Aphid feeding also causes *GluB* and *PR-1* RNAs to increase in tomato (de Ilarduya et al., 2003). Unlike aphids, it is anticipated that whiteflies would have limited contact with the vacuolar *PR* proteins, since their mouthparts rarely pierce mesophyll cells during the search for a feeding site in the phloem. It seems likely that whiteflies would contact the apoplastic proteins, such as *PR-1* protein. The exterior of whitefly mouthparts would have limited physical contact with *PR-1* due to the salivary sheath that encases the flexible whitefly stylet. In contrast, whiteflies may consume minute quantities of apoplastic proteins and chemicals, as they secrete and then ingest, a watery saliva to “taste” the chemical environment of the apoplast (Lei et al., 1998a). It is thought that whiteflies use these gustatory cues as indicators of host-plant suitability and as signals to guide the stylet’s route to the phloem sieve element. Consistent with this is the fact that the tomato resistance gene *Mi-1*, which mediates a modest resistance to whiteflies, appears to influence resistance traits that act prior to phloem feeding (Jiang et al., 2001).

Since whiteflies recover their nutrients from the phloem and occasionally drink water from xylem (Lei et al., 1998b), the phloem and xylem are additional sites for proteins/chemicals that can influence whitefly-plant interactions. For example, some aphid-resistance (*R*) genes control a phloem-localized resistance traits (Kaloshian et al., 1995; Klingler et al., 1998). It is not clear whether the tomato *PR* proteins accumulate in the phloem or xylem after hemipteran feeding, although some *PR* proteins have been reported in xylem sap of *Fusarium oxysporum*-infected tomato plants (Rep et al., 2002). Furthermore, the transcriptome of phloem sap, phloem tissue and vascular-enriched tissue have shown that some stress-related RNAs, including some *PR* RNAs, are detected in the phloem (Le Hir et al., 2008). The role of phloem- or xylem-localized PRs or other phloem-localized proteins that deter aphids (Yoo et al., 2000), on whitefly host acceptance, longevity, fecundity, and nymph development has yet to be tested.

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Letter from the Editor-in-Chief

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At a recent meeting of the Editorial Board of the Journal of Chemical Ecology, the decision was made to compile information and guidelines for authors whose chemical ecological papers contain a considerable molecular component. Previously, The Journal published two other sets of guidelines: one for the Identification and Syntheses of Compounds (August 2008—JCE 34:984–986); and another for SPME Techniques (December, 2009—JCE 35:1383).

The following guidelines were prepared by Associate Editor Steven J. Seybold with critical assistance from an *ad hoc* committee of Editorial Board Members and contributors that included Jonathan Gershenzon (Max Planck Institute for Chemical Ecology, Jena), Dezene Huber (University of Northern British Columbia), Patricia Nagnan-Le Meillour (INRA, Université de Lille), Julien Pelletier (University of California, Davis), Erika Plettner and Carlos Castillo (Simon Fraser University), Ada Rafaeli (The Volcani Center, Bet Dagan), Claus Tittiger (University of Nevada), and Linda Walling (University of California, Riverside).

The intent of these new "molecular" guidelines is not to daunt or discourage authors from submitting molecular work. Indeed, much of the information provided here is well-known to molecular contributors. The guidelines in this respect simply bring together useful information that can serve as a quick reference. For others, however, they should be useful for determining appropriateness of content of submission as well as accepted methods of presentation. Researchers should use them as they prepare submissions to The Journal.

John T. Romeo
December, 2010

3', 5'-monophosphate (cAMP), complementary deoxyribonucleic acid (cDNA) (= copy DNA), complementary ribonucleic acid (cRNA) (=copy RNA), cytidine 5'-mono-, di-, or triphosphate (CMP, CDP, or CTP), deoxyadenosine 5'-mono-, di-, or triphosphate (dAMP, dADP, or dATP), deoxycytidine 5'-mono-, di-, or triphosphate (dCMP, dCDP, or dCTP), deoxyguanosine 5'-mono-, di-, or triphosphate (dGMP, dGDP, or dGTP), deoxynucleotide triphosphate (dNTP), deoxyribonucleic acid (DNA), double stranded ribonucleic acid (dsRNA), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), guanosine 5'-mono-, di-, or triphosphate (GMP, GDP, or GTP), genomic deoxyribonucleic acid (genomic DNA, gDNA), messenger ribonucleic acid (mRNA), micro ribonucleic acid (miRNA), nicotinamide adenine dinucleotide (NAD, NAD+), nicotinamide adenine dinucleotide phosphate (NADP, NADP+), nicotinamide adenine dinucleotide phosphate, reduced (NADPH), nicotinamide adenine dinucleotide, reduced (NADH), ribonucleic acid (RNA), ribosomal ribonucleic acid (rRNA), short interfering (silencing) ribonucleic acid (siRNA), transfer ribonucleic acid (tRNA), uridine 5'-mono-, di-, or triphosphate (UMP, UDP, or UTP).

3) **Buffers:** 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES), *N,N'*-bis(2-hydroxyethyl)glycine (Bicine), 2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-1-propane-1,3-diol (BisTris), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), ethylenediaminetetraacetate (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(*N*-morpholilino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1,4-piperazinediethanesulfonic acid (PIPES), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), tris(hydroxymethyl)-aminomethane (Tris), tris-buffered saline (TBS).
4) **Related Terms:** base pair (bp), kilobase pair (kb), megabase pair (Mb), nucleotide (nt), Coenzyme A and its derivative (CoA), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), diethylaminoethyl (DEAE), bovine serum albumin (BSA), GTP-binding protein (G protein), gene of interest (= target gene) (GOI), quantification cycle (in PCR) (C_q).

For terms not present in the lists above, contributors should consult the recommendations of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/)

B. Abbreviations and Descriptions of Molecular Assays

1) **Gel Electrophoresis Assays**: polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), Southern blotting (gel electrophoresis with blotting and detection of DNA, capitalized because it is named after E.M. Southern = DNA blotting), northern blotting (gel electrophoresis with blotting and detection of RNA, not capitalized = RNA blotting), western blotting (gel electrophoresis with blotting and detection of proteins, not capitalized = protein blotting), two-dimensional polyacrylamide gel electrophoresis (2D-electrophoresis, 2-DE, or 2D-PAGE), electrophoretic mobility shift assay (EMSA).
2) **Polymerase Chain Reaction Assays**: polymerase chain reaction (PCR), rapid amplification of cDNA ends (RACE), real-time polymerase chain reaction (real-time PCR or qPCR), reverse transcription polymerase chain reaction (RT-PCR), RNA interference (RNAi).
3) **Microarray Assays**: expressed sequence tag (EST), genome survey sequence (GSS), high-throughput genome sequence (HTGS), sequence-tagged site (STS), tentative unique genes (TUGs).
4) **Protein/Peptide Assays**: dithiothreitol (DTT), electrospray ionization mass spectrometry (ESI-MS), enzyme-linked immunosorbent assay (ELISA), fast-protein liquid chromatography (FPLC), high-performance liquid chromatography (HPLC), immunoglobulin G, M, etc. (IgG, IgM, etc.), isoelectric point (pI), liquid chromatography tandem mass spectrometry (LC MS-MS), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), nuclear magnetic resonance (NMR), radioimmunoassay (RIA), tryptic digestion.

III. Presentation of Molecular Biological Content

A. PCR and PCR Primers

In the **Methods and Materials** include sequences of all forward and reverse PCR primers in one table and subdivide the table by the function of the primers. Functions might include generating probes for a northern blot experiment; primers for real-time PCR analysis; genomic or cDNA isolation; 3'- or 5'-RACE; or tissue localization assays. Examples of these tables can be found in: Lu et al. 2007, p. 1360; Xiu and Dong 2007, p. 950; Saltzmann et al. 2008, p. 1404; Li et al. 2008, p. 1594; Xiu et al. 2008, p. 489; Shivaji et al. 2010, p. 182. Primers should be written from 5' to 3', without any space between the abbreviations for the bases.

Provide the quantity of nucleic acid template used in the reaction; the quantities (molar concentrations) of all reagents (primers, dNTPs, and the polymerase); and the reaction volume. Also provide cycling conditions including temperatures and times, number of cycles, as well as the brand and model of the thermocycler and the method of isolation of the PCR products. Optimally, isolated PCR products (or products that have been cloned into plasmids) should be sequenced in both senses (5' to 3' and 3' to 5'), however, sequencing one strand of the product at least twice to confirm the identity of each base is also acceptable. The commercial entity or center responsible for the sequencing should also be indicated in the manuscript.

B. Real-time PCR (qPCR)

For guidelines to proper experimental design, calculation of gene expression, and statistical analysis in real-time PCR experiments, contributors should refer to Bustin et al. (2009) and Rieu and Powers (2009). Bustin et al. (2009) provide a checklist (in their Table 1) of all of the information necessary to consider and include when describing a high-quality real-time PCR experiment for publication. For the Journal, background data on real-time PCR should be included in the supplemental data section. Because real-time PCR is a quantification method highly dependent on the quality of the template (RNA or cDNA) and the efficiency of the amplification (C_q), authors should include tables that show the efficiencies of the amplification reaction for each gene monitored, including the reference gene(s). An example of this can be found in Zhang et al. (2009) (Supplemental Data Tables 1 and 2). R-squared values from the standard curves for genes analyzed by real-time PCR (including the reference gene(s) and the no-treatment controls) should also be included in these tables.

Authors are strongly encouraged to characterize the expression of multiple (typically three) reference genes for these experiments. Authors should determine the stability of the expression of their reference genes under their experimental conditions (validation of the reference genes) and then compare the normalized data from their treatments and provide statistical information on those comparisons that they view as most relevant. The expression of the reference gene should not differ significantly among the tissues, life stages, castes, etc. that are under investigation (based on the same experimental procedures used for the genes of interest). This cannot necessarily be predicted *a priori,* so multiple reference genes should be checked and then the gene that best fits the criterion of "no significant change" should be used as the reference.

C. Genomics

Papers that use genomic, proteomic, or metabolomic approaches to study problems relevant to chemical ecology are appropriate for the Journal, provided that they are focused on chemical ecological themes (Tittiger 2004; Liu et al. 2007; McLean et al. 2007; Lawrence et al. 2008; Leiss et al. 2009). Manuscripts describing studies that utilize these approaches should go beyond cataloging and provide unique insights into regulatory networks, mechanisms, or demonstrations of ecological function.

For guidelines on genomics content, the Journal follows the conventions adopted by the journal *Molecular Endocrinology* and those of the Functional Genomics Data Society (http://www.mged.org/). Authors submitting expression or tiling microarray datasets must clearly identify in the **Methods and Materials** the platform, which includes the name of the vendor or array source, the name of the genechip or array and its version (e.g., Affymetrix Murine Genome U74v2 Set). Authors must thoroughly describe the filtering criteria used to evaluate the raw data and provide references for the statistical methods used to analyze the data. Filtered gene lists provided as supplemental data must be provided as Excel spreadsheets and not PDFs. Specific examples of supplemental data accompanying gene expression profiling manuscripts can be found in Ohlsson Teague et al. (2009); supplemental data available at: (http://mend.endojournals.org/cgi/content/full/me.2008-0387/DC1).

Experiments must be described according to minimum information about a microarray experiment (MIAME) guidelines (Brazma et al. 2001). Papers based on unreplicated gene expression profiling experiments will not be accepted for publication. Supporting experiments (i.e., real-time PCR or RT-PCR) are not a substitute for the biological replications of a genomics gene expression profiling experiment. However, microarray data must be verified by using an independent RNA sample; not with the RNAs used in the microarray experiments. The Journal recommends consultation with a statistician regarding experimental design and data analysis strategies for all gene expression profiling experiments.

Upon acceptance of a paper, authors of microarray datasets are required to have submitted the complete dataset to the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), which is the public gene expression archive of National Institute of Biotechnology Information (NCBI); or to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/), which is the corresponding database of the European Bioinformatics Institute (EBI). Submission requirements can be found at either website. Datasets can be submitted confidentially to NCBI GEO

prior to publication and held there until the paper is in press or published.

D. Proteomics

For guidelines on proteomics content, the Journal generally follows the conventions adopted by the journal *Molecular and Cellular Proteomics* (http://www.mcponline.org/site/misc/itoa.xhtml or http://www.mcponline.org/site/misc/PhialdelphiaGuidelinesFINALDRAFT.pdf).

When presenting protein identifications based on a single peptide sequence, authors should provide precursor mass, charge, and mass error (Carletti et al., 2008, see Table 1 on p. 809). Papers that use proteomic methods and report molecular structures, whether based on x-ray crystallography, NMR, or computational modeling, will be accepted only after the structural coordinates have been deposited in the Worldwide Protein Data Bank: http://rcsb-deposit.rutgers.edu/ and http://pdbdep.protein.osaka-u.ac.jp/. Whenever possible, peptide sequences should be identified with matching accession numbers (see below).

E. GenBank and other Database Accessions for Manuscripts Reporting New Amino Acid or Nucleotide Sequences

Here, the Journal generally follows the conventions and publication guidelines adopted by *Insect Biochemistry and Molecular Biology* (http://www.elsevier.com/wps/find/journaldescription.cws_home/390/authorinstructions#). The GenBank sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced at NCBI at the U.S. National Library of Medicine as part of the International Nucleotide Sequence Database Collaboration. The data are organized by accession numbers, which are unique identifiers in bioinformatics allocated to nucleotide and protein sequences to allow tracking of different versions of that sequence record and the associated sequence in GenBank or the Worldwide Protein Data Bank.

The Journal requires that authors deposit any novel nucleic acid sequences described in their paper in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/ or http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) or in the EMBL data library (http://www.ebi.ac.uk/embl/) and report the accession number in the **Methods and Materials** of the manuscript. Deposition of such data should be made at the time of submission. Papers will be accepted only after these data have been deposited in one of these databases. Note that the information provided in the GenBank accessions (nucleotide sequence, deduced protein sequence, etc.) is extensive and often makes the presentation of a DNA sequence and its protein translation unnecessary as a figure in the manuscript.

New amino acid sequences should be deposited in the Worldwide Protein Data Bank: http://rcsb-deposit.rutgers.edu/ and http://pdbdep.protein.osaka-u.ac.jp/.

There are different types of accession numbers in use based on the type of sequence cited, each of which uses a different code. Authors should explicitly mention the *type of accession number together with the actual number*, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article. Please use the following format: accession number type ID: xxxx (e.g., MMDB ID: 12345; PDB ID: 1TUP).

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been demonstrated. Liu et al. (2010) reported that GOBPs of the navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), bind with high affinity to the major component of the sex pheromone, (11*Z*, 13*Z*)-16Ald.

In the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae), 44 genes that encode putative OBPs have been identified from the genome sequence (Gong et al., 2009). The genes that encode pheromone binding protein 1 (BmorPBP1) and general odorant binding protein 2 (BmorGOBP2) are clustered close together on chromosome 19 in the same orientation. They have the same intron insertion sites between codons and splice sites within codons. The transcripts for *BmorPBP1* and *BmorGOBP2* are expressed at an extremely high level in adult antennae compared to other tissues (Zhou et al., 2009). The *Bmor-GOBP2* transcript is higher than *BmorPBP1* in the brains of both sexes (Gong et al., 2009). Immunocytochemical studies showed that both proteins are expressed in the antennae of males and females (Steinbrecht, 1998; Maida et al., 2005).

Pheromones are blends of volatile chemicals that typically are specific to a species, and, for *B. mori,* the pheromone blend comprises bombykol, bombykal, and (10*E*,12*E*)-hexadecadien-1-ol (Kaissling et al., 1978). However, only bombykol is able to induce mating behavior at a physiological concentration, and bombykal acts as an antagonist to bombykol (Kaissling, 2009). BmorPBP1 and its binding to pheromone components has been the subject of intense study in recent years, as a model system for understanding the functions that OBPs may play in insect olfaction *in vivo*. Cells that express BmorPBP1 are closely associated with the cells that express BmorOR-1 and BmorOR-3 in the long trichoid sensilla (Krieger et al*.,* 2005; Nakagawa et al*.,* 2005; Forstner et al., 2006), and co-expression of BmorPBP1 with BmorOR-1 in an ‘empty’ neuron of *Drosophila melanogaster* increased the sensitivity of the receptor to bombykol (Syed et al., 2006). BmorPBP1 also has been shown to mediate a response to bombykol, but not to bombykal in cultured HEK293 cells expressing BmorOR-1 (Grosse-Wilde et al., 2006) and in moth sensilla (Pophof, 2004). Recently, a highly sensitive receptor to bombykol has been identified in *D. melanogaster* (Syed et al., 2010). This raises the question on the selectivity and specificity that olfactory receptors alone contribute in insect olfaction.

Binding of BmorPBP1 to bombykol has been demonstrated by X-ray crystallography (Sandler et al., 2000); NMR structural characterization (Damberger et al., 2000; Horst et al., 2001; Lee et al., 2002); electrospray ionization-mass spectrometry (ESI-MS) (Oldham et al., 2000; Hooper et al., 2009); and other biochemical methods (Wojtasek and Leal 1999; Leal et al., 2005a, b). The binding is both pH and ligand dependent. Thus, at an acidic pH, or in the absence of bombykol, the C-terminus of the PBP forms an α-helix and occupies the ligand binding pocket, and a conformational change, brought about by a change in pH to more acid near the ORs, is thought to be the ligand release mechanism when the BmorPBP1-bombykol complex reaches the ORs (Wojtasek and Leal 1999; Leal et al., 2005a, b). However, there is some evidence that BmorPBP1 can bind to both bombykol and bombykal (Gräter et al., 2006; Zhou et al., 2009). A recent binding study that used a high-throughput ESI-MS analysis showed that BmorPBP1 bound much more strongly to (10*E*,12*Z*)-hexadecadienoic acid and (10,12)-hexadecadiyn-1-ol than to bombykol (Hooper et al., 2009).

BmorGOBP2 is expressed in larval sensilla (Laue, 2000) and in the long trichoid sensilla of female moths (Steinbrecht, 1998; Maida et al., 2005), which respond specifically to linalool and benzoic acid (Kaissling, 2009). However, the binding of BmorGOBP2 to these two plant volatiles has not been elucidated, whereas the binding of BmorGOBP2 to both bombykol and bombykal has been demonstrated. Furthermore, X-ray crystallography has shown that, when bombykol is bound to BmorGOBP2, a different conformation is adopted from that found when bombykol binds to BmorPBP1. In the former, a hydrogen bond is formed with Arg110 rather than with Ser56, as is the case with BmorPBP1. Furthermore, a second hydrogen bond is formed with Glu98, which leads to an overall stronger affinity of BmorGOBP2 for bombykol (Zhou et al., 2009).

Ligand specificity and selectivity, and, in fact, the functional roles of insect OBPs in olfaction, have been challenged consistently, even more so since insect olfactory receptors have been characterized (Krieger et al., 2005). Zhou et al. (2009) showed differential binding of BmorGOBP2 to bombykol and bombykal by X-ray crystallography. In the present study, we provide further evidence of the possible involvement of the BmorGOBP2 gene in moth olfaction by examining the tissue expression patterns throughout the developmental stages of *B. mori*, and through a comparative binding study of recombinant BmorGOBP2 to plant volatiles, as well as to the sex pheromone components. We employed molecular ligand docking to predict the binding interactions of BmorPBP1 and BmorGOBP2 with the ligands. Two synthetic pheromone analogs were found to bind better than the sex pheromone components. Electroantennogram (EAG) recordings from the antennae of male moths were made with the analogs to investigate how the binding data and the molecular docking predictions might relate to biological significance.

Methods and Materials

Real-time PCR Bombyx mori cocoons were provided by Prof. Y. –P. Haung (Institute of Plant Physiology and

Ecology, CAS, China), and adult moths were dissected, immediately after eclosion, into antennae, heads (without antennae), and bodies. Heads and bodies also were collected from 2nd, 3 rd, 4th, and 5th instar larvae. Detailed protocols for total RNA extraction with the RNAqueous kit (Ambion, Huntingdon, UK) and two step real-time PCR on each tissue with the ETROscript kit (Ambion) on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) have been described previously (Zhou et al., 2009). The sequences of *BmorGOBP2* (GenBank No. NP_001037498) and *BmorPBP1* (GenBank No. NP_001037494) were downloaded from NCBI GenBank (http://www.ncbi.nlm.nih.gov/). The *B. mori* reference gene, actin A4 (GenBank No. U49644), was used in each real-time PCR experiment to check loading, reverse transcriptase efficiency, and the integrity of the transcripts. For each tissue, real-time PCR analyses were conducted with two tissue preparations. The reactions were hot-started for 2 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec. The PCR primers (Table 1) were designed with Primer3 (http://frodo.wi.mit.edu/).

Relative tissue expression was quantified as described in Zhou et al. (2009). Briefly, after PCR, Ct values were exported into the LinRegPCR program to correct the amplification efficiencies for each reaction. The relative expression levels (Pfaffl ratio) of each OBP gene to the reference gene then were calculated in each tissue from [(E,obp$^{\Delta Ct,obp}$]/[(E,ref$^{\Delta Ct,ref}$], where E,obp and E,ref are the corrected amplification efficiencies for OBP and the reference gene, respectively. ΔCt,obp is calculated from [Ct,obp of heads or antennae—Ct,obp of body], and ΔCt, ref is calculated from [Ct,ref of heads or antennae—Ct,ref of body]. The results are presented as the mean fold change of two biological samples combined from both male and female tissues.

Protein Expression and Purification of Recombinant OBPs Full-length cDNAs that encode mature BmorGOBP2 and BmorPBP1 were cloned into the bacterial expression vector pET17b (Novagen, Darmstadt, Germany) between the *Nde*I and *Eco*RI restriction sites, and verified by sequencing. Plasmids with the correct inserts were transformed into BL21(DE3)pLysS *E. coli* cells, and protein synthesis was induced at OD_{600} of 0.5–0.8 with IPTG (4 mM) for 3 h. All proteins were found to be expressed as inclusion bodies, and solubilization was performed by denaturation in urea/DTT, renaturation, and extensive dialysis, by using a protocol applied successfully to other OBPs. The recombinant proteins were purified by two rounds of anion-exchange chromatography with a HiPrep 16/40 column (GE Healthcare, Hatfield, UK) filled with DE-52 resin (Whatman, Kent, UK), followed by gel filtration on a Sephacryl S-200 HiPrep 26/60 column (GE Healthcare). A MonoQ column also was used at the final stage of purification. The purified proteins were stored at −20°C in 20 mM Tris-HCl pH 7.4 buffer.

Chemical Synthesis The seven analogs of bombykol, including bombykal, were synthesized in-house at Rothamsted Research. Nuclear magnetic resonance spectroscopy (NMR) and gas chromatography with flame ionization detection (GC-FID) analyses confirmed the structure and purity of the synthesized compounds. ^{1}H- and ^{13}C-NMR spectroscopy were performed by using a Bruker 500 Advance NMR spectrometer with ^{1}H referenced to $CDCl_3$ (7.25 ppm) and ^{13}C to $CDCl_3$ (77.0 ppm). Purified compounds were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph (GC), fitted with a nonpolar HP-1 capillary column (40 m, 0.32 mm i.d., 0.52 µm film thickness), a cool-on-column injector, and a flame ionization detector (FID) (Agilent Technologies, West Lothian, UK). The GC oven temperature was maintained at 40°C for 1 min and then raised by 10°C /min to 200°C, and the carrier gas was nitrogen at 80 cm/sec. Benzoic acid (99%), (±)-linalool (97%), and N-phenyl-1-naphthylamine (NPN) (98%) were purchased from Sigma-Aldrich (Dorset, UK).

Fluorescence Competitive Binding Assay To measure the binding of the fluorescent probe NPN to OBPs, a 2 µM protein solution (1 ml) in 20 mM Tris-HCl, pH 7.4 and 4.5, was titrated with aliquots of 1 mM NPN dissolved in methanol to final concentrations of 0.05–16 µM. The protein/NPN complex was excited at 337 nm, and emission spectra were recorded between 300 and 450 nm on a luminescence spectrometer LS50B (Perkin-Elmer, Cambridge, UK) at 25°C in a right angle configuration with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The competitive binding of ligands was measured by using NPN (4 µM) as the fluorescent reporter and 0.05–7 µM concentrations of each ligand

Table 1 The PCR primers used in real-time PCR for quantification of transcript levels in the silkworm, *Bombyx mori*

OBP gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
BmorGOBP2	ATCATATGACCGCCGAGGTGATGAGCCACG	GGAATTCTCAGTATTTTTCGATAACTGCTT
BmorPBP1	CAGTGGATGCGTCTCAAGAA	GTCTCATCGGCTCCATGTTT
Actin	CGTTCGTGACATCAAGGAGA	ACAGGTCCTTACGGATGTCG

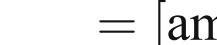
dissolved in methanol, which gave molar ratios from 0.01 to 1.61 (bombykol:NPN). The total volume of methanol in the protein solution was maintained at 21 µl (2.1%) by adding methanol without ligands. Bound ligand was evaluated from the values of fluorescence intensity assuming that the protein was 100% active, with a stoichiometry of 1:1 protein:ligand at saturation.

Partitioning Binding Assay In the partitioning binding assay (Danty et al., 1999), the protein concentration was calculated from the OD_{280} values and the extinction coefficient of the protein solution assuming all pairs of Cys residues form disulfide bridges. In practice, 20 µl of protein (0.5 mM) in 20 mM Tris buffer (pH 7.4) were added to the 100-µl v-shaped vial (Wheaton Scientific, Millville, NJ, USA), and then 20 µl of hexane containing a mixture of ligands (14 µM each), including bombykol, were layered on top [higher concentrations up to 10 mM of protein were used and similar results obtained (data not shown)]. The two phases were mixed gently, centrifuged at 13,000×*g* for 5 min, and then incubated at room temperature for at least 1 h. After incubation, 2 µl of the top phase containing the ligands were injected into the GC, and the amount of ligand that had gone into the protein phase was determined from the GC trace by using Equations 1 and 2 (below), and through comparison with the results obtained before incubation and without protein.

Each experiment was performed with at least three replicates and repeated at least twice for each sample. The binding of ligands to the OBPs was quantified by GC-FID on an HP Agilent 6890 Series GC system (Hewlett Packard, Wokingham, Berkshire, UK) with a wax column (HP-5MS, 25 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies) and a cool-on-column injector, and a flame ionization detector (FID). The system was operated under the following temperature program: 100°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 10 min. The carrier gas was nitrogen at a linear flow rate of 80 cm/sec. The amount of each ligand in the hexane fraction was calculated from the chromatograms by using a single point internal standard (IS). The internal standard used was tridecane (99% chemical purity) (Sigma-Aldrich, Dorset, UK). The first analysis contained a known amount of internal standard and the compounds of interest, and the binding of the ligand was calculated from:

$$\text{Internal Response Factor (IRF)} = [\text{areaIS} \times \text{amountSC}]/[\text{amountIS} \times \text{areaSC}], \quad (1)$$

$$\text{amount of specific compund} = [\text{amountIS} \times \text{IRF}]/\text{areaIS}, \quad (2)$$



Where, IS is the internal standard and SC is the specific compound of interest.

Docking Experimental Procedure Flexible docking of the ligands to BmorPBP1 and BmorGOBP2 was performed by using a genetic search algorithm and a semi-empirical force field of the AutoDock (v.4.2) program (Morris et al., 2009). The crystallographic structures of BmorPBP1 (1DQE) (Sandler et al., 2000) and of BmorGOBP2 (2WC6) (Zhou et al., 2009) were used. Water molecules were removed, and polar hydrogens were added to the proteins and ligands. For the ligands, all torsions were released except the ones around the conjugated double and triple bonds. The default AutoDock force field was applied (Huey et al., 2007). The whole proteins were covered by grid maps with a spacing of 0.374 Å. For the genetic algorithm, default parameters were used starting from random positions and orientation of the ligands. Each ligand was subjected to 100 Lamarkian genetic algorithm runs, with 25×10^6 evaluations in each and with the rest of the parameters set to the default values of the AutoDock GUI, AutoDock Tools. The root mean square deviation (rmsd) tolerance of the resulting docked structures was ≤2 Å. The binding pocket topologies (area and volume) were calculated by using the CASTp server (http://sts.bioengr.uic.edu/castp/calculation.php) (Dunda et al., 2006).

Electrophysiology Insects for electrophysiological studies either were obtained as pupae from Prof. Y. –P. Haung (see above) or purchased from Warwick Insect Technologies (Coventry, UK). Pupae were sexed and kept at 20°C, 16:8 h L:D, until adults emerged. Electroantennogram (EAG) recordings were made by using Ag-AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969), but without glucose]. An antenna of an adult male adult *B. mori* was excised and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure a good contact. Signals were passed through a high impedance amplifier (UN-06, Syntech, Hilversum, The Netherlands) and analyzed by using a customized software package (Syntech).

The stimulus delivery system, which employed a filter paper in a disposable glass Pasteur pipette cartridge, has been described (Wadhams et al., 1982). The stimulus (2 sec duration) was delivered into a purified airstream (1 l/min) flowing continuously over the preparation. Standard solutions (1 mg/ml) of test compounds were applied (10 µl) to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the strip was placed in the cartridge. The control stimulus was hexane (10 µl). Fresh cartridges were prepared immediately prior to each stimulation (N=5).

Responses to control and test solutions were compared for significant differences by using Student's *t*-test.

Results

Expression of Bombyx mori OBPs The levels of transcripts of *BmorGOBP2* and *BmorPBP1* were very low in heads at all larval stages and in adult heads without antennae (Fig. 1). They were very high in the adult antennae, indicating that both genes are highly up-regulated in adult antennae and could be involved in olfaction and detection of semiochemicals. These results are consistent with high levels of the moth proteins found in antennae by Western blot analysis and immunocytochemical labeling experiments (Steinbrecht, 1998; Maida et al., 2005), and also with the developmental profile of OBP *Aaeg-OBP10* mRNA isolated from the intact yellowfever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae) (Bohbot and Vogt, 2005).

The *B. mori* transcripts also were observed in the earlier stages. We cannot exclude the possibility that two OBPs may have a different function in the larva than in the adult. Larvae are the stage that feeds on plant materials, and they may use this OBP to detect plant volatiles, whereas adults are the stage that reproduces and disperses, and no longer needs to feed. Thus, they may use this OBP to detect pheromone components.

Fluorescence Competitive Binding Assay In the binding assay with BmorGOBP2, attempts to displace NPN by the sex pheromone component bombykol and the plant volatiles [(±)-linalool and benzoic acid] showed that bombykol gave the most displacement, whereas the other two ligands showed much less displacement (Fig. 2). The binding of bombykol to BmorGOBP2 also was pH-dependent, as demonstrated for BmorPBP1 (Leal et al., 2005b), with displacement of NPN greatest at pH 7.5, but displacement of NPN greatly decreased at pH 4.5 over a range of concentrations (Fig. 3).

We next measured the displacement of NPN from recombinant BmorGOBP2 and BmorPBP1 by bombykol and bombykal (Fig. 4). For BmorPBP1, there was no difference in binding between bombykol and bombykal, with the reductions in NPN fluorescence of 41.1±19.4% (N=30) and 34.3±14.2% (N=12), respectively, at 6 μM concentration. Interestingly, BmorGOBP2 showed a similar binding to bombykol as BmorPBP1, but BmorGOBP2 unexpectedly showed a difference in binding of bombykol from bombykal 41.1±15.1% (N=8) and 15.0±3.7% (N=3) (P<0.01, d.f.=10) at 6 μM concentration, respectively. However, the displacement at lower concentrations was low (10–20%). This could be due to non-specific binding and could be affected by other compounds that might bind during protein expression in *E. coli* cells (Oldham et al., 2000).

Partitioning Binding Assays We deployed a partitioning binding assay (Danty et al., 1999) to avoid using the fluorescent probe and the possibility of non-specific displacement. In this assay, the protein in buffer is incubated with a mixture of ligands dissolved in hexane in two phases, (see Methods and Materials), so that each

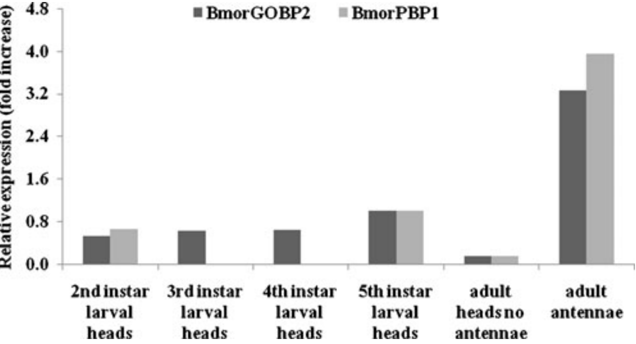


Fig. 1 Transcript levels of *Bombyx mori* OBP genes *BmorPBP1* and *BmorGOBP2*. The transcript levels in adult antennae (mixed sexes) and heads at different larval developmental stages were measured by real-time PCR (see Methods and Materials). The fold-changes are normalized with the actin gene and relative to the transcript level in the body of each stage. The values are the mean of two biological samples and three replicates of each sample

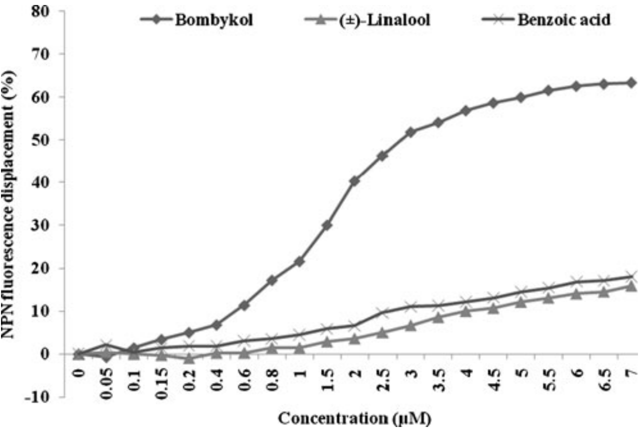


Fig. 2 Fluorescence displacement of N-phenyl-1-naphthylamine (NPN) from *Bombyx mori* BmorGOBP2 by bombykol (♦); benzoic acid (**X**); and (±)-linalool (▲). The protein and NPN were both at 2 μM. The protein/NPN complexes were excited at 337 nm and the fluorescence emission at 395 nm was measured before and upon titration with methanol solutions of the competitors to final concentrations of 0.5–7 μM. The reductions in NPN fluorescence emission at 395 nm were normalized to the NPN fluorescence before titration. The decrease in NPN fluorescence intensity at the emission maximum (395–400 nm), at increasing concentrations of competitors, is presented as (Fligand/Fnpn) with Fligand=the peak fluorescence intensity at a distinct competitor concentration. Fnpn=the peak fluorescence intensity of the OBP/NPN complex

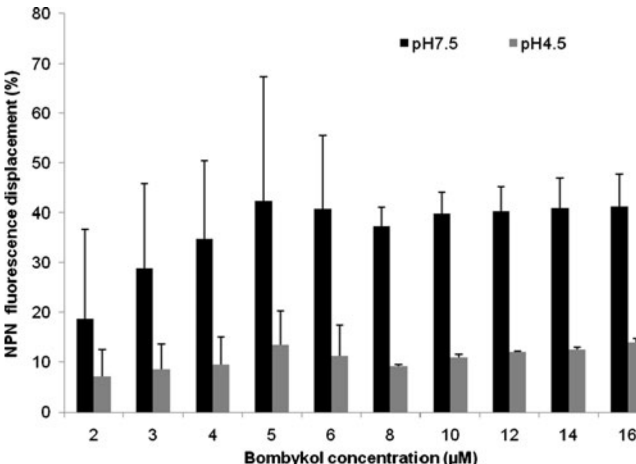


Fig. 3 Fluorescence displacement of NPN (2 μM) by bombykol from *Bombyx mori* BmorGOBP2 (2 μM) at different pHs using a mean of three or four replicates with standard deviations

compound competes for binding. The depletion of the compound from the hexane phase by the protein in the buffer can be measured by GC-FID analysis under equilibrium condition as compared with a control (with no protein). The advantage of this assay is that a mix of ligands (Fig. 5) can be used, and the high concentration of protein in the buffer resembles the high concentration of OBP within the antennal lymph. Both BmorGOBP2 and BmorPBP1 bound to bombykol and most of its analogs, except those with their double bonds replaced by triple bonds (Figs. 6, 7 and 8). Again, there was no difference between the binding of bombykol and bombykal to BmorPBP1, which is consistent with the fluorescence displacement binding results (Fig. 4) and with those of others (Gräter et al., 2006). For BmorGOBP2, bombykol binding was slightly better than bombykal, indicating the effect of changing of the functional group from an alcohol in bombykol to an aldehyde in bombykal to form two hydrogen bonds in BmorGOBP2 (Fig. 7) (Zhou et al., 2009). Interestingly, (10*E*,12*Z*)-hexadecadienyl acetate (**3**) bound best, and (10*E*,12*Z*)-octadecadien-1-ol (**4**) and bombykol (**1**) bound equally well. Moving the position of the double bonds of the pheromone (**6**) reduced the binding to the proteins, and changing from a double bond in bombykol to a triple bond in (10*E*)-hexadecen-12-yn-1-ol (**7**) and (10,12)-hexadecadiyn-1-ol (**8**) further reduced the binding to both BmorGOBP2 and BmorPBP1. Overall, BmorGOBP2 displayed better differential binding to the analogs than did BmorPBP1.

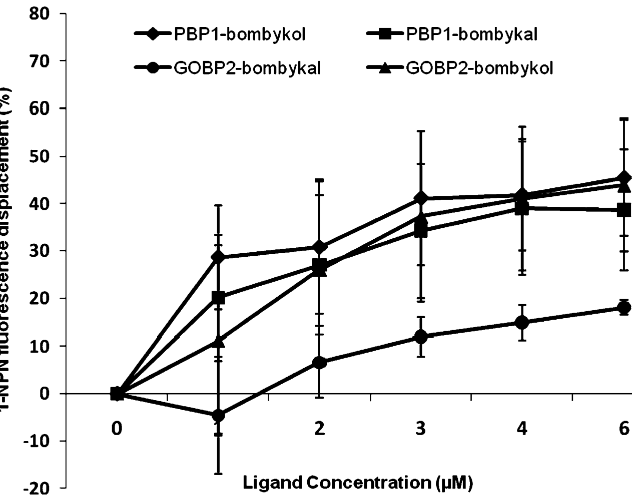


Fig. 4 Displacement of the fluorescent probe NPN by bombykol and bombykal. The protein and NPN were both incubated at a concentration of 2 μM. The OBP/NPN complexes were excited at 337 nm and the fluorescence emission at 395 nm was measured before and upon titration with bombykol or bombykal at a concentration of from 1–6 μM. The reductions in NPN fluorescence emission at 395 nm were normalized to the NPN fluorescence before titration. Each point is the average of at least two replicates (*N*) with the standard error (*N*=30 for BmorPBP1/bombykol, *N*=12 for BmorPBP1/bombykal, *N*=8 for BmorGOBP2/bombykol, *N*=3 for BmorGOBP2/bombkal)

Molecular Docking The previously published X-ray crystallography structures of BmorPBP1 (Sandler et al., 2000) and BmorGOBP2 (Zhou et al., 2009) provide a unique opportunity for molecular modeling and ligand docking. AutoDock 4.2 was used to predict blind docking of bombykol and the analogs to BmorPBP1 (1DQE) and BmorGOBP2 (2WCK), and to estimate the free energy of binding of the protein-ligand complexes. The docking algorithm successfully placed the analogs into the same binding cavity as those of the models obtained from crystal structures. Each ligand was subjected to 100 genetic algorithm runs (see Docking Experimental Procedures in the Methods and Materials). AutoDock performs cluster analysis or “structure binning” based on all-atom mean square deviation (RMSD). The resulting families of docked conformations are ranked in the order of increasing energy (rank 1 is the lowest energy cluster) (Table 2). In very few cases, less populated clusters were observed with lower energy. However, these were not selected as representative of the predicted bound ligand structure as their mean energy difference was 2.5 kcal/mol less than that of the most populated cluster. This is within the range of error of the AutoDock force field (±2.5 kcal/mol). In these cases, the largest cluster was taken as the predicted bound ligand structure (Table 2). Molecular docking showed that in the conformation of bombykol with the lowest binding energy, there are two hydrogen bonds with Glu98 and Arg110 (Fig. 7). The energy minima reached for the BmorGOBP2/ligand complexes are considerably lower than the corresponding ones for BmorPBP1 (Table 2), with most of the lower binding energy conformations having their hydroxyl group oriented towards Arg110 and away from Ser56 without any van der Waals interaction between Ser56 and bombykol (Fig. 7), which is consistent with the crystal

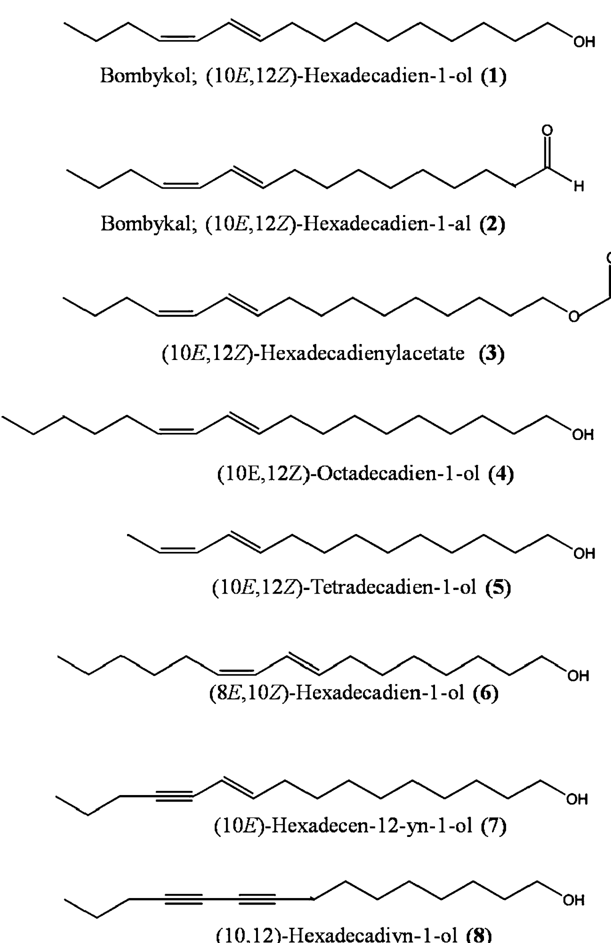


Fig. 5 Structures of the *Bombyx mori* sex pheromone components and their analogs used in this study

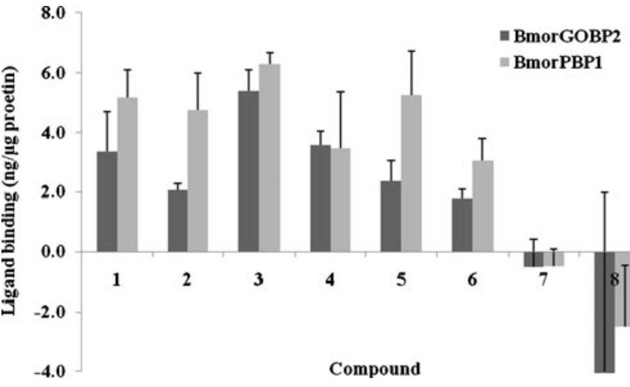


Fig. 6 Binding of bombykol and its analogs to *Bombyx mori* odorant-binding proteins (OBPs). The ligand binding (ng compound per μg protein) was measured with the partitioning binding assay (see Methods and Materials) as the depletion of compound from the top hexane phase into the bottom protein phase relative to that without protein. Each compound is represented with its code as shown in Fig. 5. Each column is the mean of at least three replicates (*N*) and the bar denotes the standard error (*N*=3 for OBP/compound 3, *N*=3 for OBP/compound 4, *N*=7 for OBP/bombykol, *N*=4 for OBP/bombykal, *N*=4 for OBP/compound 5, *N*=4 for OBP/compound 6, *N*=4 for OBP/compound 7, *N*=3 for OBP/compound 8)

structures of BmorGOBP2/ligand complexes (Fig. 8) (Zhou et al., 2009). Furthermore: 1) The docked ligand conformations show tighter clustering (fewer clusters) for BmorGOBP2 than for BmorPBP1. The lowest energy clusters are invariably more populated for all ligands (higher N values) for BmorGOBP2 than for BmorPBP1. This may be explained by a smaller binding cavity in BmorGOBP2 (volume=616.5 Å^3) as compared to that of BmorPBP1 (volume=888.8 Å^3) that may restrict the ligand(s) from attaining multiple conformations within the binding pocket; 2) The ligands rotate more freely in BmorPBP1 than in BmorGOBP2, as shown by greater RMSD differences between the best binding conformers; 3) Both bombykol and bombykal reach the same energy minimum (−7.87) with BmorGOBP2, showing that they have a similar binding affinity. These data provide further evidence and support the binding results (Figs. 2, 4 and 6) in showing that BmorGOBP2 can bind sex pheromones and discriminate between the pheromone analogues better than can BmorPBP1.

The analog (10*E*,12*Z*)-hexadecadienyl acetate (**3**) has a lower E_{min} value (i.e., the free energy of binding of the most favorable conformation within a given cluster). Thus, the analog is predicted to bind better than bombykol or bombykal to both BmorGOBP2 and BmorPBP1, consistent with the results obtained with the partitioning binding assays (Fig. 6) and by the ESI-MS analysis (Hooper et al., 2009). This indicates that the functional group of the analog may interact with Glu98 and Arg110 to form two hydrogen bonds as observed in the crystallographic structures of BmorGOBP2/bombykol (Zhou et al., 2009), and that it, together with bombykol, are better ligands than bombykal. The molecular docking also predicts the lowest binding energy for (10*E*,12*Z*)-octadecadien-1-ol (**4**)/OBP complexes (Table 2).

Electrophysiological Responses Electroantennogram (EAG) recordings showed that bombykol (**1**) and bombykal (**2**) are the most active in triggering an antennal response (Table 3, Fig. 9). (10*E*,12*Z*)-Hexadecadienyl acetate (**3**) and (10*E*,12*Z*)-octadecadien-1-ol (**4**) were shown, for the first time, to elicit significant responses from the male *B. mori* antenna ($P<0.01$ and $P<0.05$, respectively), whereas changing double bonds to triple bonds (**7**) and (**8**) reduced the EAG responses, respectively. This was consistent with the partitioning binding results (Fig. 6), as well as the ligand docking predictions (Table 2). The biological activity thus correlates with the binding data and molecular docking prediction for these compounds. Shortening the length of the alkyl carbon chain by two carbons (**5**) or moving two double bonds (**6**) further reduced the EAG activity as demonstrated by earlier work (Kaissling et al., 1978).

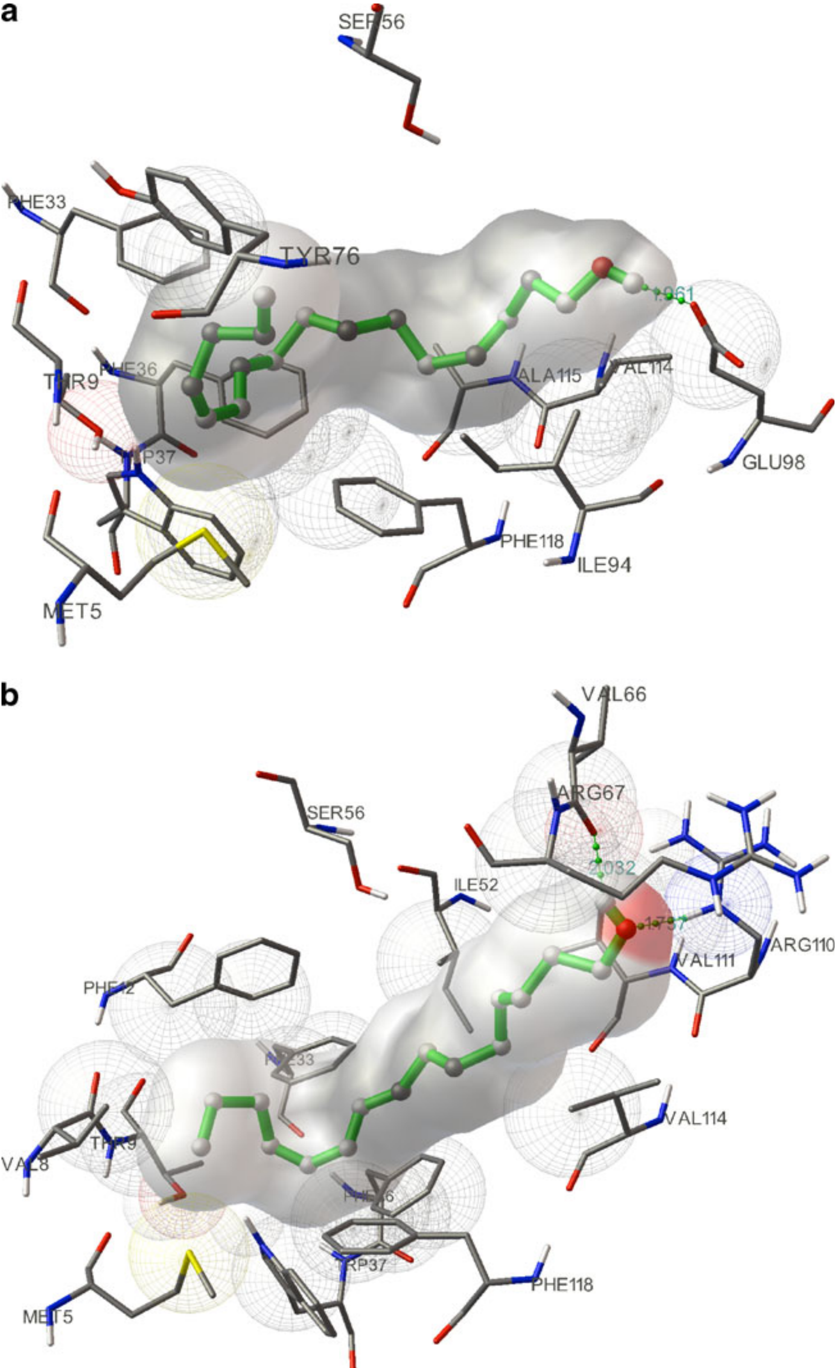


Fig. 7 Molecular contacts of bombykol in the binding pocket of *Bombyx mori* BmorGOBP2 as predicted by molecular docking. The figures were drawn with AutoDockTools (ADT)Python (Sanner, 1999). The spheres and the cloud around the docked bombykol molecule (green stick) are the electrostatic potential (red=oxygen, blue=nitrogen, grey=carbon) for van der Waals (vdWs) contacts and vdW's volume over the whole molecule, respectively. The hydroxyl group of the docked bombykol molecule, with the lowest binding energy, forms hydrogen bonds with Arg110 and Val66 (**a**) and Glu98 (**b**) as in crystal structures. Ser56 forms no vdWs interactions with bombykol

Discussion

Our understanding of the molecular and biochemical mechanisms that mediate chemoreception in insects has been improved greatly by the discovery of olfactory and taste receptor proteins. However, 50 years after the discovery of the first insect sex pheromone from *B. mori*, it is still unclear how such hydrophobic compounds selectively reach the dendrites of sensory neurons *in vivo* across aqueous space to interact with the sensory receptors. The presence of soluble polypeptides in high concentration in the lymph of chemosensilla still poses unanswered questions. More than two decades after their discovery and despite the wealth of structural and biochemical information available, the physiological functions of odorant-binding proteins (OBPs) are still not well understood. It is possible that the combinatorial actions by different OBPs and ORs may provide the foundation for insects to discriminate various odorants (Pophof, 2004; Krieger et al., 2005; Xu et al., 2005; Syed et al., 2006;

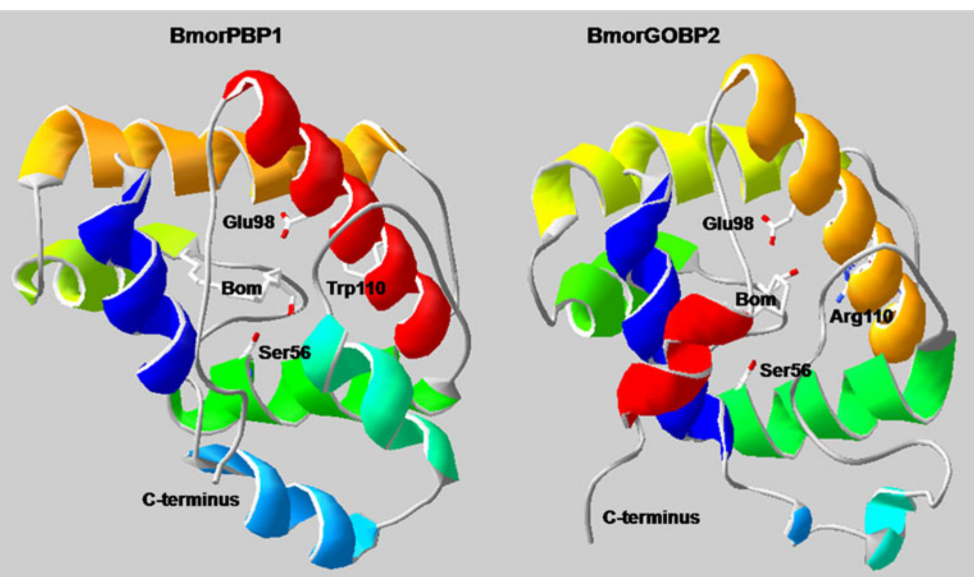


Fig. 8 Three-dimensional structure of *Bombyx mori* BmorPBP1 and BmorGOBP2 bound with the sex pheromone component (10*E*,12*Z*)-hexadecadien-1-ol (stick). The C-termini and the key amino acid residues, which form hydrogen bonds, are labeled. The BmorPBP1 structure 1DQE (Sandler et al., 2000) and BmorGOBP2 structure 2WC6 (Zhou et al., 2009) are downloaded from the protein data bank (http://www.rcsb.org/pdb/home/home.do) and displayed with Swiss-Pdb Viewer 3.7

Laughlin et al., 2008). The transcripts of *BmorGOBP2* and *BmorPBP1* were low in the larval stages, but very high in adult antennae, suggesting a role in adult moth olfaction related to mating and reproduction (adult moths do not feed). When the corresponding recombinant proteins were studied in partitioning binding assays, BmorPBP1 bound to the sex pheromone bombykol as well as its analogs (see also Sandler et al., 2000; Horst et al., 2001; Oldham et al., 2000; Hooper et al., 2009; Zhou et al., 2009). However, there was evidence from the binding assays that BmorGOBP2 can also bind the sex pheromone components bombykol and bombykal (Fig. 6; Gräter et al., 2006; Zhou et al., 2009). It is interesting that both BmorPBP1 and BmorGOBP2 bind to the sex pheromone components, suggesting that the specificity of binding to insect OBPs does not determine the specificity of the receptor neuron response (Kaissling, 2009). This prompted further investigations into the specific binding of BmorGOBP2 to other semiochemical analogs.

Both the fluorescence competition and the partitioning binding assays showed that BmorGOBP2 bound to bombykol more strongly than to bombykal, which is consistent with the results obtained by a cold binding assay (Zhou et al., 2009). The structural evidence indicates that the lack of a hydrogen bond to Glu98 in the BmorGOBP2/bombykal complex may be responsible for this difference (Fig. 8) (Zhou et al., 2009). On the other hand, although BmorGOBP2/ligand complexes have lower energy minimums than BmorPBP1/ligand complexes, the docking experiments failed to provide evidence for the discriminatory binding of

Table 2 Ligand binding energies for the *Bombyx mori* odorant-binding proteins Bmorgobp2 and Bmorpbp1 calculated from molecular modeling

		BmorGOBP2 (2wc6) (kcal/mol)					BmorPBP1 (1dqe) (kcal/mol)				
Ligand No	Compound	Cluster[a]	Rank	N	E_{min}	E_{mean}	Cluster	Rank	N	E_{min}	E_{mean}
4	(10*E*,12*Z*)-Octadecadien-1-ol	3	1	86	−8.48	−7.92	11	1	32	−7.10	−6.62
3	(10*E*,12*Z*)-Hexadecadienyl acetate	5	1	48	−8.24	−7.70	7	1	32	−7.33	−6.85
6	(8*E*,10*Z*)-Hexadecadien-1-ol	9	1	80	−7.90	−7.30	17	1	28	−6.69	−6.14
1	Bombykol	6	1	88	−7.87	−7.28	12	1	35	−6.83	−6.29
2	Bombykal	4	1	83	−7.87	−7.48	11	1[b]	20	−6.87	−6.55
7	(10*E*)-Hexadecen-12-yn-1-ol	6	1	67	−7.65	−7.29	15	1[c]	14	−6.62	−6.12
8	(10,12)-Hexadecadiyn-1-ol	9	1	44	−7.44	−7.01	17	1[d]	2	−6.54	−6.27
5	(10*E*,12*Z*)-Tetradecadien-1-ol	5	1	77	−7.22	−6.84	9	1	74	−6.43	6.09

[a] Cluster is the number of distinct multi-member conformational clusters found out of 100 runs. N is the number of conformations in the cluster. E_{min} is the free energy of binding of the most favorable conformation within a given cluster; E_{mean} is the cluster's mean energy (See Methods and Materials)

[b] Most populated cluster is Rank 4 comprising of 33 conformations

[c] Most populated cluster is Rank 5 comprising of 17 conformations

[d] Most populated cluster is Rank 12 comprising of 18 conformations

BmorGOBP2 between bombykol and bombykal (Table 2) as suggested previously (Zhou et al., 2009). It can be argued that BmorPBP1 could have higher affinity for bombykol than bombykal because of the difference in the energy minimum between BmorPBP1/bombykol and BmorPBP1/bombykal complexes (−6.83 kcal/mol vs −6.40 kcal/mol) (Table 2).

The specific expression of BmorGOBP2 in the female sensilla has been demonstrated (Steinbrecht, 1998; Maida et al., 2005), but neither binding to any semiochemicals nor the physiological role of BmorGOBP2 in *B. mori* has been reported. The specific and high up-regulation of the expression of BmorGOBP2 gene in adult antennae suggests that BmorGOBP2 may have a role in the perception of semiochemicals, most likely for plant volatiles. However, our data support the view that BmorGOBP2 can be used preferentially to capture the sex pheromone rather than common plant volatiles such as linalool and benzoic acid. Previous experiments that used an antibody raised against BmorGOBP2 have shown that only a small fraction of sensilla in male antennae contain BmorGOBP2, whereas only few sensilla of female antennae express BmorPBP1 (Steinbrecht, 1998; Maida et al., 2005). The perception of bombykol and bombykal occurs via male receptor neurons within sensilla containing only BmorPBP1. Furthermore, the female receptor neurons innervating the abundant long sensilla trichodea that contain only BmorGOBP2 are very sensitive to linalool and benzoic acid, and never have been found to respond to the pheromones (Kaissling, 2009). These results argue that there is a physiological role of BmorGOBP2 in sex pheromone perception *in vivo*. However, the moth GOBP2 also has been found expressed in male moth antennae (Vogt et al., 1991a) and co-expressed with PBPs (Nardi et al., 2003). The dramatic up-regulation of BmorGOBP2 gene in adult antennae and the binding to sex pheromone components of BmorGOBP2, as reported in this study and others (Gräter et al., 2006), warrant further investigation.

The binding data of BmorGOBP2 also suggest that the protonation of OBPs plays an important role in ligand binding and release, and support the mechanism of pH-dependent release of ligands as demonstrated previously for BmorPBP1 (Wojtasek and Leal 1999; Damberger et al., 2000; Horst et al., 2001; Kowcun et al., 2001; Lee et al., 2002; Leal, 2003) and other insect OBPs. For the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), PBP AmelASP1, ligand binding is activated at low pH and is dependent on protonation of Asp35, which locks down the C-terminus in a confirmation that closes the active site by a hydrogen bond to the main chain of Val118 (Pesenti et al., 2008). In the predicted BmorPBP1/bombykol structure, the C-terminus blocks the rear entry to the pocket, with Val135 coming within 4Å of bombykol (Sandler et al., 2000; Gräter et al., 2006). However, with BmorGOBP2, the blocking of the rear pocket by the bulge formed by amino acid 33-35 means that the C-terminus cannot sense the ligand directly and plays a role in ligand release (Zhou et al., 2009). The involvement of the C-terminus in BmorGOBP2 binding remains to be determined.

We used synthetic pheromone analogs to test the specificity in ligand binding of the two moth OBPs. (10*E*,12*Z*)-Hexadecadienyl acetate (**3**) showed better binding to BmorGOBP2 than did either bombykol or bombykal in the partitioning binding assay, consistent with the ligand docking experiments (Table 2). The ligand docking experiments also predict that BmorGOBP2 forms more energetically favorable complexes with this analog than does BmorPBP1, and that (10*E*,12*Z*)-octadecadien-1-ol (**4**) should have a good binding to both OBPs as supported by our binding experiments (Fig. 6) and to BmorPBP1 by previous ESI-MS analysis (Hooper et al., 2009). Our binding data for (10*E*,12*Z*)-tetradecadien-1-ol (**5**) is contradictory to the prediction of the docking results, showing good binding to BmorPBP1 and BmorGOBP2 (Fig. 6), but consistent with the poor binding obtained with a cold binding assay (Zhou et al., 2009). Our binding data confirmed the reduced binding of (8*E*,10*Z*)-hexadecadien-1-ol (**6**) compared with the sex pheromones, as measured by the ESI-MS analysis, but contradicted the better binding predicted by ligand docking (Table 2) and the cold binding assay (Zhou et al., 2009). The replacement of double bonds by triple bonds (**7**, **8**) significantly reduced the binding for both OBPs. The

Table 3 Electroantennographic response (± standard error) of male *Bombyx mori* antennae to semiochemical or analog ligands

Ligand No.	Compound[a]	Response (%)[b]	Significance (P)[c]
2	Bombykal	307±51	<0.01
1	Bombykol	234±43	<0.01
3	(10*E*,12*Z*)-Hexadecadienyl acetate	198±29	<0.01
4	(10*E*,12*Z*)-Octadecadien-1-ol	151±11	<0.05
7	(10*E*)-Hexadecen-12-yn-1-ol	125±8	<0.05
5	(10*E*,12*Z*)-Tetradecadien-1-ol	110±6	Not sig.
6	(8*E*,10*Z*)-Hexadecadien-1-ol	105±5	Not sig.
8	(10,12)-Hexadecadiyn-1-ol	67±13	Not sig.

[a] Ligands presented at 1 mg/ml

[b] Responses expressed as % of an artificial 0.1 mV signal (i.e., 0.01 mV=100%)

[c] P=significance of difference from hexane control (N=5)

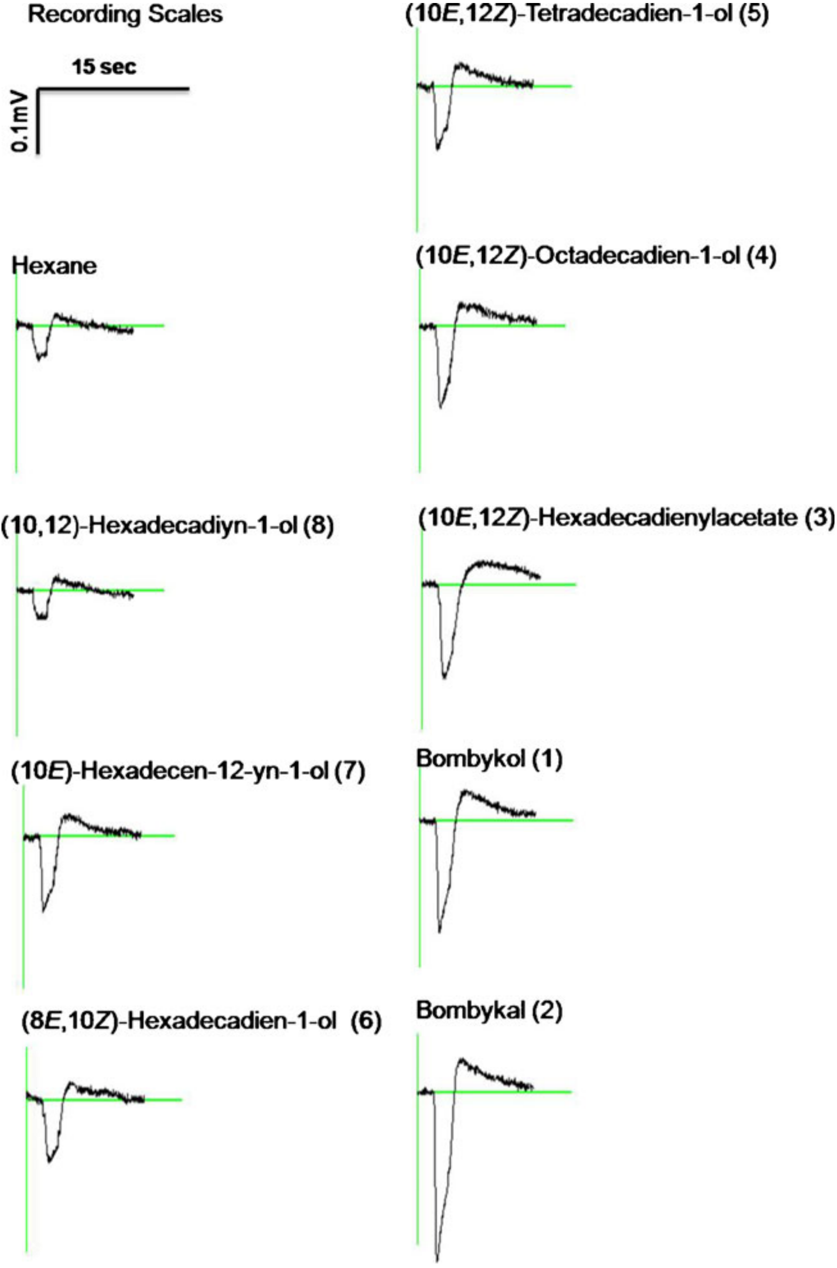


Fig. 9 Electroantennogram (EAG) recordings from male *Bombyx mori* antennae. The stimulus (2 sec duration) was delivered into a purified airstream (1 l/min) flowing c ontinuously over the preparation. The horizontal lines indicate a recording time of 15 sec in each case. Standard solutions (1 mg/ml) of test compounds were applied (10 µl) to filter paper strips and the solvent was allowed to evaporate (30 sec) before the strip was placed in a glass cartridge. The control stimulus was hexane (10 µl). Fresh glass cartridges were prepared immediately prior to each stimulation. Responses to control and test solutions were compared for significant differences by using Student's*t*-test. *N*=5

molecular docking also placed them as less favorable ligands for both OBPs. However, the ESI-MS analysis showed that (10,12)-hexadecadiyn-1-ol (8) bound twice as much as bombykol. In agreement with Hooper et al. (2009), bombykal did not bind to BmorPBP1. The negative values of the binding data to compound (**7**) and (**8**) are unexpected. Other compounds that bound to the OBPs during expression in *E. coli* (Oldham et al., 2000) were displaced by the ligands and measured by GC together with (**7**) and (**8**). Because of these surprising results, a measure of the biological activity was sought by recording overall EAG responses for the sex pheromones as well as the analogs (Table 3, Fig. 9). As expected, the EAG responses were significant for the pheromone components, bombykol (**1**) and bombykal (**2**). The monoyne analogue (**7**) elicited a small EAG response but the diyne analog (**8**) elicited no EAG response. The EAG activity relates well, not only to the results obtained by the partitioning binding assay, which showed significantly increased binding of (10*E*,12*Z*)-hexadecadienyl acetate (**3**) and (10*E*,12*Z*)-octadecadien-1ol (**4**), but also to the ligand docking results (Table 2) and to the ESI-MS analysis (Hooper et al., 2009). Unlike the data from *in vitro* binding and molecular docking, the higher EAG activity of sex pheromone components (**1**) and (**2**) over activity of the analogs (**3**) and (**4**) correlate better with the sexual behavior of adult male moths. Further behavioral studies are needed to

establish any physiological relevance of the analogs (**3**) and (**4**) in moth olfaction. Single sensillum recordings from sensillum types with known protein content could investigate precisely the effects of their binding to protein *in vivo*.

We previously reported the X-ray crystallography structures of BmorGOBP2 complexed with bombykol and four of its analogs that showed that the mode of bombykol binding to BmorGOBP2 differed from that found for BmorPBP1. In the BmorGOBP2 complex, the hydroxyl hydrogen of bombykol forms a hydrogen bond to Arg110, and the hydroxyl group of bombykol also is involved in forming a second hydrogen bond with Glu98, whereas in BmorPBP1, one hydrogen bond is formed with Ser56 (Fig. 8). This could explain why (10*E*,12*Z*)-hexadecadienyl acetate (**3**) appears to bind more strongly to BmorGOBP2 than other analogs. This chemical may be utilizing different molecular interactions when it binds to an OBP, which may influence the conformational properties of the OBP/ligand complex and thus signal transduction. EAG recordings derived from a response of the whole antenna and, at realistic physiological concentrations, as used here, usually correlate with behavior, which could be a positive or negative response. The differences in EAG activities and ligand binding between the sex pheromone components and the analogs necessitate further investigations into the physiological roles of OBPs *in vivo* and interaction between OBPs and sensory receptors. Such research has begun with electrophysiological approaches (Pophof, 2004) and modern molecular technologies such as RNA interference (RNAi) to control OBP gene expression in mosquitoes (Biessmann et al., 2010; Pelletier et al., 2010). Similar studies in crop pests, such as the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), which shares sex pheromone components with *B. mori*, could lead to an alternative pest control strategy by preventing sex pheromone detection, thus reducing moth populations (Zhou et al., 2010).

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(Couvillon et al., 2008). This suggested to us that guards might be responding to communication from an alerted nestmate.

A honey bee guard, upon encountering an intruder, frequently bites the intruder and raises her abdomen, extruding her sting. Both behaviors release pheromones (Free, 1987). The mandibular gland secretes a substance, of which 2-heptanone (2H) is important, which likely marks bitten individuals (Boch and Shearer, 1971), while an extruded sting shaft carries alarm pheromones that disperse (Free, 1987). The main component of this sting shaft alarm pheromone is iso-pentyl acetate (IPA); it alerts bees and induces them to sting (Boch et al., 1962). It has been hypothesized that these pheromones may be the mechanism by which guards communicate to other guards that there is an increased probability of intrusion (Couvillon et al., 2008).

We tested the hypothesis that alarm pheromones cause a rapid shift in guards so that they adopt a less permissive acceptance threshold. Such an induced shift should result in an increase in the rejection of both nestmates and non-nestmates, as a non-permissive threshold is less tolerant of differences between cues and colony templates.

Methods and Materials

We used six honey bee colonies of mixed European race, predominantly *Apis mellifera mellifera*, located in an apiary beside our laboratory. Housing and maintenance followed published protocols (Couvillon et al., 2008). Data were collected 12 November–5 December 2008, between 9 a.m. and 4 p.m., when it was warm enough for guarding to occur. Five colonies acted as discriminator hives, and the sixth as a non-nestmate source.

We used a standard behavior-recognition assay by natural entrance guards (Downs and Ratnieks, 2000). Each discriminator hive received one nestmate and one non-nestmate under each of eight chemical treatments (see below). Using forceps, we placed one bee at a time on the entrance platform and observed the guard reaction for 90 sec. An introduction was scored as rejected or accepted (Couvillon et al., 2008). A minimum of 30 min between treatments allowed residual odors to disperse. We made 960 introductions in total.

Chemicals were from Sigma Chemical Co (St Louis, MO, USA). We injected a known quantity into a 30-l plastic bag inflated with air to create the desired concentration, with air pumped from the bag via two plastic tubes to side holes on the entrance platform. Delivery of the air + chemicals (flow rate 1.5 l.min^{-1}) began 10 sec before the introduction of a bee and continued during the 90 sec of observation. Alarm pheromones release responses within seconds in recipients.

It was important to determine appropriate concentrations of treatments. For IPA and 2H, we established a minimum concentration that caused an increase in guard number. IPA induces these responses at lower concentrations than 2H (Boch and Shearer, 1971). Therefore, we tested IPA at 3, 10, and 30 µl per 30 liters of air (Treatments 1–3), and 2H at 10, 30, and 100 µl per 30 l of air (Treatments 4–6). Additionally, we included two controls, pentane (100 µl) and geraniol (100 µl), the latter the active component of honey bee Nasanov attraction pheromone (Boch and Shearer, 1962). These controls were important to test whether bees responded specifically to alarm pheromones or generally to chemicals (either pentane control or a non-alarm pheromone). Treatment sequence was randomized each day.

We used a binary logistic regression (BLR) model to analyze response variable (accept/reject by guard) against factors of day, colony, bee type (nestmate *vs.* non-nestmate), and treatment (1–8), as well as interactions.

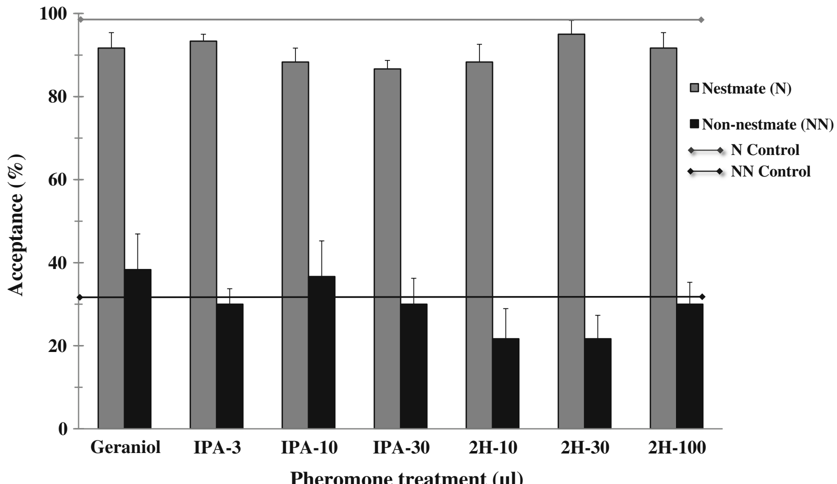

Fig. 1 Effect of chemical treatments on acceptance threshold by guard honey bees toward nestmates and non-nestmates. There was no effect (P=0.46) on guard acceptance threshold for either nestmates or non-nestmates under either iso-pentyl acetate (IPA) or 2-heptanone (2H) at any concentration. Acceptance (%) of nestmates (*grey*) and non-nestmates (*black*) did not differ from pentane control (*grey* and *black* lines, showing 98% nestmate and 32% non-nestmate acceptance, respectively). *Error bars* are S.E.M

Results

Guards discriminated nestmates (92% acceptance) from conspecific non-nestmates (30% acceptance) across all chemical treatments (BLR, Odds Ratio=0.08, P<0.001, Fig. 1). The 62% difference of acceptance demonstrates that normal guarding occurred and confirmed the suitability of the bioassay. There was no significant difference (BLR, P=0.97) in acceptance with (100% nestmate/50% non-nestmate) or without (91% nestmate/33% non-nestmate) the pump. Additionally, there was no significant difference (BLR, Odds Ratio=0.80, P=0.74) in acceptance under pure air (95% nestmate/25% non-nestmate) *vs*. pure air plus pentane control (95% nestmate/20% non-nestmate).

Neither IPA nor 2H affected guards (BLR, Odds Ratio=0.95, P=0.46, Fig. 1). This result indicated that no concentration of either chemical resulted in acceptance threshold shifts. Additionally, there was no effect of geraniol. Day and colony effects were significant (BLR, Day: Odds Ratio=1.116, P=0.004; Colony: Odds Ratio=1.37, P=0.011), so these were retained as factors. Neither is unusual, as it is normal to see day-to-day and inter-hive variation in acceptance (Couvillon et al., 2008, 2009).

There was no significant interaction between bee type and day (BLR, Odds Ratio=0.95, P=0.37), colony (BLR, Odds Ratio=0.84, P=0.23), or treatment (BLR, Odds Ratio=0.97, P=0.74).

Discussion

Our results show no significant effect of either IPA or 2H, which are major components of honey bee alarm pheromones. Therefore, we reject the hypothesis that alarm pheromone is the mechanism that causes rapid shifts in the acceptance threshold of guard honey bees. In rejecting this hypotheses, it is important to consider whether our experimental method may not have detected an effect. One possibility is that we did not administer the chemicals at appropriate levels. However, we performed a preliminary study to determine the minimum amounts of IPA or 2H that cause an increase in guard numbers (Maschwitz, 1964); we used a range of amounts that was lower than and above this threshold. Alternatively, the administered pheromone amounts may have been too high. While initial administration of IPA causes a dose-dependent increase in aggression, IPA amounts above a certain threshold cause guards to become hesitant (Boch and Shearer, 1971). At even our highest amount, we did not observe this change in behavior. Additionally, over the concentration range tested, if guards were non-permissive at low concentrations and hesitant/permissive at higher ones, this would give a higher order model fit for the acceptance threshold data. This was not the case. Therefore, it seems that our concentration range was behaviorally appropriate. Another possibility is that other components of the two alarm pheromones may be the cause of the acceptance threshold change. While we tested only the major alarm compounds, these did trigger the appropriate guarding responses, suggesting they were sufficient. Future work should examine the real-time chemical changes in the headspace that precede shifts in acceptance threshold, by using methods such as APCI-MS (atmospheric pressure chemical ionization-mass spectrometry; Goubault et al., 2006).

We have shown that guard honey bees rapidly respond to increased intrusion by becoming less permissive (Couvillon et al., 2008). The rejection of the alarm pheromone hypothesis strengthens the alternative hypothesis that each guard individually monitors intrusion levels and responds by shifting her threshold towards non-permissiveness following contact with intruders. This hypothesis could be tested by comparing the acceptance thresholds of guards who have and have not experienced increased intrusion before and after the increase.

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2009); *Anoplophora glabripennis* (Motschulsky) (Lamiinae), in which the male emits a 1:1 blend of the ethers 4-(*n*-heptyloxy) butanal and 4-(*n*-heptyloxy) butan-1-ol (Zhang et al., 2002); *Monochamus galloprovincialis* (Olivier) (Lamiinae), in which the male emits the ether 2-undecyloxy-1-ethanol (Pajares et al., 2010); and the brown spruce longhorn beetle, *Tetropium fuscum* (F.), and its congener, *Tetropium cinnamopterum* Kirby (Spondylidinae), in which males emit (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (geranyl acetol) (Silk et al., 2007).

Volatile isoprenoids are ubiquitous as semiochemicals in many insect herbivore–plant systems (Hick et al., 1999), but the *Tetropium* pheromone was the first homoterpenoid alcohol found in a cerambycid species. A terpenoid structural motif has also been identified in the subfamily Lamiinae, in the species *Hedypathes betulinus* (Klug) (Fonseca et al., 2010) and *Steirastoma breve* (Sulzer) (C. Liendo, pers. comm.). As the compound was first discovered in *T. fuscum*, Silk et al. (2007) named the pheromone "fuscol". We subsequently learned that this term had already been used to refer to a marine lobane diterpene isolated from the gorgonian, *Eunicea fusca* Duchassaing and Michelotti (Kosugi et al., 1998); hereafter we refer to the *T. fuscum* pheromone component as "fuscumol".

We have been developing tools for survey, detection, and control of *T. fuscum* since its discovery in Halifax, Nova Scotia, Canada in 1999 (Smith and Hurley, 2000). Native to Europe, *T. fuscum* was first discovered in Canada in mature, apparently healthy red spruce, *Picea rubens* Sarg., in Point Pleasant Park, next to a Halifax container port facility (Smith and Hurley, 2000). In Europe, *T. fuscum* breeds primarily in stressed or dying Norway spruce, *Picea abies* (L.) Karst, and is sympatric with *T. castaneum* (L.) (Juutinen, 1955); the latter is not known in North America. In Nova Scotia, *T. fuscum* has been detected in nine counties (Cunningham, 2010), and infests red spruce, white spruce, *P. glauca* (Moench) Voss, black spruce, *P. mariana* (Mill.) B.S.P., and Norway spruce (Smith and Humble, 2000). In its new range, *T. fuscum* overlaps with the transcontinental, Nearctic, *T. cinnamopterum*, which infests dying or recently felled spruce and occasionally pines (Furniss and Carolin, 1980).

Many cerambycids are attracted to host volatiles (Linsley, 1961; Allison et al., 2004). A synthetic "spruce" blend of monoterpenes plus ethanol was attractive to *T. fuscum* and its congeners, *T. cinnamopterum* and *T. castaneum* (Sweeney et al., 2004, 2006). Silk et al. (2007) found that racemic fuscumol was unattractive by itself but, when combined with the spruce blend and ethanol, it synergized capture of males and females of both *T. fuscum* and *T. cinnamopterum* in traps. As fuscumol synergizes attraction of both sexes, it functions as an aggregation pheromone (Wertheim et al., 2005), albeit with a significant bias to females, suggesting that it may also be involved in mating (Silk et al., 2007).

Fuscumol is a secondary alcohol with a chiral center, so two enantiomers, *R*- and *S*-, are possible. Chirality affects behavioral and olfactory responses to pheromones and host volatiles in many insect species (Mori, 2007). In most species, and particularly sex/aggregation pheromone systems studied to date, the pure, naturally occurring pheromone enantiomer is more attractive than the antipode (opposite enantiomer), but the effect of the antipode in racemic blends differs among species: it has no inhibitory effect on response to the active enantiomer in roughly 60% of species studied, but can reduce attraction in other species (Mori, 2007). Presence of the antipode in racemic blends of synthetic sex or aggregation pheromone inhibits attraction of the cerambycid species, *Neoclytus mucronatus mucronatus* (F.) (Lacey et al., 2007), *Hylotrupes bajulus* (L.) (Reddy et al., 2005a), and *Megacyllene caryae* (Gahan) (Lacey et al., 2008).

Our first objective was to determine which enantiomer(s) of fuscumol was produced by male *T. fuscum* and *T. cinnamopterum* and to test whether fuscumol chirality affected antennal response [determined by electroantennography (EAG)] and trap capture of *T. fuscum* and *T. cinnamopterum* in the field; our Canada field experiments were also replicated in Poland in order to test the effect of fuscumol chirality on a different population of *T. fuscum*, as well as *T. castaneum*. Our second objective was to test the relationship between release rate of fuscumol and attraction of *Tetropium* spp., as measured by capture in traps baited with racemic fuscumol and host odors. Our third objective was to compare the relative responses of males and females of each *Tetropium* spp. to fuscumol and host odors, so as to explore the compound's roles in sex attraction and aggregation.

Methods and Materials

Collection of Beetles and Volatiles Adult *T. fuscum* and *T. cinnamopterum* were reared from spruce bolts (35 cm long × 20–40 cm diam.) cut from infested trees in Halifax, Nova Scotia, and incubated at 20–22°C and 45–60% RH in a containment facility at the Atlantic Forestry Centre (AFC) of Natural Resources Canada in Fredericton, New Brunswick. Emergence was checked 5 days each week over 12 weeks. Adults were identified to species (Smith and Humble, 2000), sexed, and stored individually in glass vials at 3–4°C until used.

Volatiles were collected separately from male and female *T. fuscum* and male *T. cinnamopterum* by drawing air through activated charcoal, then through a glass chamber (2.8 cm diam. × 18 cm long) containing 5–6 beetles, and finally through Super-Q® (~200 mg), using a vacuum line

and a flow rate of 250–300 ml.min^{-1}. Two 9 cm diam. circles of Whatman® #5 filter paper were inserted in each glass chamber: one piece lined the inside of the chamber to provide purchase for the beetles, and the other was pleated to separate individuals. Beetles were acclimatized in the room (20–22°C, 40–60% RH, 15:9 (L: D) cycle) for 24 hr prior to volatile collection. Two identical systems were set up so that volatiles could be sampled simultaneously from separated male and female *T. fuscum* or *T. fuscum* and *T. cinnamopterum* males. Each pairing was replicated twice, with different beetles, for a total of 4 samples from *T. fuscum* males, 2 samples from *T. fuscum* females, and 2 samples from *T. cinnamopterum* males. Volatiles were collected for 24–96 hr per replicate. The glass chambers and lines were cleaned between each replicate, and the activated charcoal was replaced every second replicate. Dichloromethane (3×5 ml) was used to rinse the inside of the glass chambers and elute volatiles from the Super-Q®.

Chemical Analyses Extracts and synthetics were analyzed by gas chromatography/mass spectrometry (GC/MS) on a Hewlett-Packard 5890 GC coupled with a 5971 mass selective detector in the electron ionization (EI; 70 eV) mode. The GC was fitted with a SPB-5 capillary column (30 m×0.32 mm×0.25 µm film; Supelco, Bellefonte, PA, USA), and helium used as carrier gas. The splitless injection port was set at 220°C. The column oven temperature was programmed from 70°C (held for 3 min.) to 220°C at 15°C.min^{-1}, and held for 15 min.

NMR (^{1}H and ^{13}C) analysis was carried out on a Varian Inova® 300 MHz spectrometer in $CDCl_3$ with TMS as internal standard. IR spectra were recorded on a Perkin Elmer® 727B IR-spectrometer with KBr disks. Rotations were determined on neat samples or samples dissolved in methylene chloride, on a Perkin Elmer 241 polarimeter, using the 589 sodium D-line.

Electroantennography Extracts and synthetic compounds were tested for antennal stimulation of *T. fuscum* and *T. cinnamopterum* males and females, using a coupled gas chromatograph-electroantennogram detection (GC/EAD) and separate EAG systems. GC/EAD techniques have been described previously (Silk et al., 2007). Briefly, antennae from males and females were excised close to the head and used intact with Spectra®-60 gel (Parker Laboratories Inc., Fairfield, NJ, USA) for electrical contact. A Varian CP-3380 gas chromatograph with a flame ionization detector (FID) was modified for use with a GC/EAD signal-recording device (IDAC-232, Syntech, The Netherlands). The effluent from the capillary column (Supelco DB-5, 30 m×0.30 mm×0.25 µm) was split (50/50) between the FID and the antennal preparation. Injections were in the splitless mode with helium as carrier gas and the splitless injector at 220°C. The column oven was programmed from 60°C (held for 5 min.) to 220°C at 20°C.min^{-1}. Syntech GC/EAD software v.2.6 was used to analyze the data.

EAG responses of male and female *T. fuscum* to puffs of (*R*)-fuscumol, (*S*)-fuscumol, racemic fuscumol, and hexane (control), were recorded. For this, 1 µl of a 10 mg/ml dilution of a compound was placed on a strip of filter paper inside a disposable glass pipette. Each antenna was given a single puff of each treatment (including the hexane control) in random order, with 1 min between each puff. For each insect, a standardized relative response was calculated by dividing the amplitude of response to each treatment by the amplitude of response to the hexane control.

Chemical Syntheses Synthetic (racemic) fuscumol **(1)** (>99% pure; GC/MS) was furnished (Fig. 1) by lithium aluminum hydride (LAH) reduction of commercially available (*E*)-geranyl acetone **(2)** (Aldrich Chemicals, Milwaukee, WI, USA; <0.5% *Z*-isomer), and the structure of the secondary alcohol verified by EI mass spectrum, and ^{1}H and ^{13}C NMR (see Silk et al., 2007).

Enantioselective syntheses of (*S*)- and (*R*)-fuscumol (**3 & 5**, respectively) were first carried out via asymmetric reduction of (*E*)-geranyl acetone **(2)** with diethylaniline-borane (DEANB) and the stereospecific Corey-Bakshi-Shibata (CBS)-reduction catalysts (Salunkhe and Burkhardt, 1997), (*R*)-(+)-2-methyl-CBS-oxazoborolidine and (*S*)-(−)-2-methyl-CBS-oxazoborolidine (as 1M solutions in toluene; Aldrich Chemicals). Briefly, 0.13 ml of the (*R*)- or (*S*)-catalyst at 22°C (0.13 mmol), in 2 ml of toluene, was added to 424 mg (2.6 mmol) of DEANB, followed by 500 mg (*E*)-geranyl acetone (2.58 mmol) **(1)** over 1 hr. Following stirring for an additional hour, the reaction mixture was carefully quenched with MeOH (hydrogen evolution), followed by 5 ml of 1.0N HCl. The organic layer was separated, and the aqueous phase extracted with diethyl ether. The combined organic phases were washed with HCl, water, and brine, and dried over $MgSO_4$.

Fig. 1 Synthesis of racemic fuscumol. *LAH* lithium aluminum hydride

E-geranyl acetone (**2**) → LAH → racemic-fuscumol (**1**)

Second, we carried out a kinetic resolution of racemic (*E*)-fuscumol **(1)** by enantioselective syntheses using immobilized lipases: a thermostable acrylic resin-supported lipase from *Candida antarctica*, Novozyme® 435, and a Celite®-supported porcine pancreatic lipase (ppl) (Amano Pharmaceutical Co., Nagoya, Japan). Briefly, 71.1 mg of immobilized Novozyme 435 enzyme (1 M in toluene; 10,000 units/g, Aldrich Chemicals; Gries et al., 2006) were stirred with 600 mg (3.09 mmol) of racemic alcohol **(1)** and 0.53 ml of an acylating agent, vinyl acetate, (Aldrich Chemicals) in 3 ml of hexane. The mixture was warmed to 40°C, and stirred for 7 days. Progress of the reaction was monitored by TLC; the reaction was found to be complete [by GC/MS after derivatization with (*S*)-acetyl lactyl chloride; see below] after 7 days. The resin was filtered, and the solvent evaporated *in vacuo*. (*S*)-Alcohol **(3)** and (*R*)-acetate **(4)** were separated by flash chromatography using silica gel and diethyl ether/hexane (1:20) as eluent. (*R*)-Fuscumol **(5)** was furnished by hydrolysis of **(4)** with K_2CO_3/MeOH (Fig. 2). Optical purities of the alcohols, both synthetics and insect extracts, were determined by treatment with (*S*)-(−)-2-acetoxy propionyl chloride [(*S*)-acetyl lactyl chloride; pyridine/DMAP catalyzed] (Slessor et al., 1985), which gave baseline resolution of diastereomers by GC/MS on the SPB-5 column. In addition, some separation was effected by chiral β-cyclodextrin column chromatography (Supelco β-DEX™ 120, 30 m×0.25 mm×0.25 μm film) using the same temperature programming as for the SPB-5 column.

We also attempted a reduction of (*E*)-geranyl acetone **(2)** with fermenting Baker's yeast, *Saccharomyces cerevisiae*, (with D-glucose) and with the $NADP^+$-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*.

The absolute configuration of the Novozyme 435 lipase-produced fuscumol was determined as follows. Briefly, treatment of (−)-fuscumol acetate with catalytic OsO_4 and an oxidant gave a mixture of oxidized products (approximately a 1:1 mixture of each double bond oxidation product, as well as some oxidation of both double bonds) that was subjected to oxidative cleavage, using silica-supported $NaIO_4$, yielding three products, one of which was 4-acetoxypentanal, that were easily separated by silica gel chromatography using 10:1 hexanes:ethyl acetate as eluent. This aldehyde was oxidized to give the acid, which was treated with methanolic K_2CO_3 to give the corresponding lactone. Repeating the sequence, starting with the (+)-alcohol, which, after periodate cleavage, gave the hemiacetal, which was oxidized with PCC to give the lactone. Starting with the (−)-acetate (rotation −2.9), (*R*)-(+)-5-methyl-4,5-dihydrofuran-2(3H)-one {$[\alpha]_D$=+7.3 (c=3.7, CH_2Cl_2)} was obtained, whereas starting with (+)-alcohol ($[\alpha]_D$=+2.9), (*S*)-(−)5-methyl-4,5-dihydrofuran-2(3H)-one {$[\alpha]_D$=−6.7 (c=1.0, CH_2Cl_2)} was generated. Given this, (+)-fuscumol has the (*S*) configuration, whereas (−)-fuscumol has the (*R*) configuration. The spectral characteristics and optical rotation values fully agreed with an earlier report (Oritami and Yamashita, 1973). The Novozyme 435 lipase technique was then used to produce (**3**) and (**5**) (Fig. 2) in gram quantities for field testing.

Field Trapping Experiment—Chirality and Host Volatiles The effect of fuscumol chirality and host volatiles on attraction of *Tetropium* spp. was tested in a 4×2 factorial trapping experiment conducted in spruce-dominated forests on McNabs Island, Nova Scotia from 15 May to 27 June 2007, and in Białowieża, Poland, from 30 April to 25 June 2007 (sites described in Sweeney et al., 2006). There were

Fig. 2 Resolution of racemic fucumol, with the lipase Novozyme 435 producing (*S*)- and (*R*)-fuscumol

four levels of pheromone [(*S*)-fuscumol; (*R*)-fuscumol; racemic fuscumol; blank] and two levels of host volatiles (spruce blend + ethanol; blank) for a total of six different lure treatments. Fuscumol was dispensed from a lure, consisting of a 0.2 ml thin-wall, clear, dome-capped polymerase chain reaction (PCR) sample tube (Axygen Scientific Inc., CA, USA) containing ca. 15 mg of synthetic pheromone, with a 1 mm diam. hole drilled in the cap. Release rate of fuscumol was initially 800 μg/d, dropping to about 80 μg/d after 30 days at 23°C (determined by weight loss). Release rates of spruce blend (a blend of monoterpenes, Sweeney et al., 2006) and ethanol were about 2,000 mg/d, and 275 mg/d, respectively. The host volatile lures have a field life of about 90 days at 21–24°C, so were not replaced for the 8-wk duration of the experiment. The fuscumol lures were replaced once, after 4 weeks.

Treatments were replicated 15 times in Nova Scotia and nine times in Poland, using a randomized complete block design with 30 m between traps and blocks. Colossus® traps (ConTech Enterprises, Delta, British Columbia) were used in Nova Scotia and PT Intercept® traps (AlphaScents, Portland, OR, USA) were used in Poland (Sweeney et al., 2006). Each trap was suspended from a rope tied between two conifers, separated by at least 2 m, with the collecting bucket 10–20 cm above the ground. The bucket contained a 50:50 mixture of propylene glycol and deionized water plus 0.5 ml/l of Kodak Photo-Flo® 200 and 12.5 mg/L of Bitrex®. Traps were checked weekly and specimens preserved in 70% ethanol. All *Tetropium* spp. were sexed and identified to species, and voucher specimens retained at AFC, Fredericton, New Brunswick.

Field Trapping Experiment—Release Rate The effect of fuscumol release rate on capture of *Tetropium* spp. was tested in Nova Scotia and Poland using similar methods as described for the chirality experiment. There were five treatments: (1–3) low, medium, and high release rate, of racemic fuscumol (1 mg/d, 4 mg/d, or 32 mg/d, respectively, measured at 20–21°C), each combined with a spruce blend lure (2,000 mg/d) and ethanol lure (275 mg/d); (4) spruce blend and ethanol lures; and (5) unbaited trap. The fuscumol release devices (ConTech Enterprises, Delta, British Columbia) were a bubble cap (1 mg/d), or pouch design (4 mg/d and 32 mg/d) that released racemic fuscumol at a steady rate over roughly 60 days. The experiment was conducted from 27 May to 30 July 2008 on McNabs Island, Nova Scotia with 10 replicates, and from 7 May to 2 July 2008 at Białowieża, Poland, with 11 replicates. At both sites, treatments were laid out in a randomized complete block design.

Data Analysis Data for standardized antennal responses to treatment stimuli were first tested for differences between males and females using *t*-tests on log (y+1) transformed data; if antennal response did not differ between the sexes, data were pooled and analyzed by a repeated measures ANOVA with individual antennal preparations as blocks, and the Ryan-Einot-Gabriel-Welsch multiple range test (SAS Institute 2002–2003).

For trapping bioassays, data for total season catch per trap of each species were transformed by log (y+1) and subjected to ANOVA, using the model: mean catch = block + pheromone + host volatiles + pheromone × host volatiles + error. When there was significant interaction between pheromone and host volatiles, data were subjected to ANOVA using the model: mean catch = block + lure treatment + error. Means were compared using the Ryan-Einot-Gabriel-Welsch multiple range test. Loss of data because of trap disturbance was rare, but if a trap was found disturbed during a weekly check we did not include that week's catch in the season totals for all treatments in the affected block. Residuals from all ANOVAs were tested for significant deviations from normality using the Shapiro-Wilk test (Zar, 1999) in SAS (PROC Univariate) (SAS Institute, 2002–2003); if residuals were non-normal, data were rank transformed and subjected to ANOVA (Friedman's test) (Zar, 1999). We compared the relative responses of different *Tetropium* spp. to the treatments by testing for correlation in catch per trap of *T. fuscum* vs. *T. castaneum* in Poland and *T. fuscum* vs. *T. cinnamopterum* in Nova Scotia; traps that captured neither species were excluded.

Responses of Males vs. Females Separate ANOVAs were run for catch of males and females of each species in field-trapping bioassays. For each species and trapping bioassay, we tested the correlation between catch of males and females across all treatments, excluding traps with zero catch. For each species, the total numbers of males vs. females captured with each lure treatment (as well as all treatments pooled) were tested for significant deviation from 50:50 using chi square goodness-of-fit tests; treatments in which expected counts were <5 were excluded from analysis (Zar, 1999).

Results

Synthesis LAH reduction of (*E*)-geranyl acetone **(2)** gave racemic fuscumol **(1)** in high yield (>99%; Fig. 1) with spectra matching those previously reported (Silk et al., 2007).

Treatment of **(2)** with whole-cell-fermenting Baker's yeast failed to yield any reduced product, but a thermophilic enzyme, an $NADP^+$-dependant alcohol dehydrogenase from *Thermoanaerobium brockii*, did produce a small amount of (*S*)-(+)-fuscumol **(3)** after 7–8 days of reaction

[determined by (*S*)-acetyl lactate formation and GC/MS; ee >90%], but in very low yield (<1%); **(2)** is, therefore, a poor substrate for this enzyme.

Asymmetric reduction of (*E*)-geranyl acetone **(2)** with DEANB and CBS catalysts produced (*S*)-fuscumol **(3)** with the (*R*)-2-methyl-CBS-catalyst and (*R*)-fuscumol **(5)** with the (*S*)-2-methyl-CBS catalyst, at 87% ee and 74% ee, respectively, estimated by formation of the (*S*)-acetyl lactyl diastereomers followed by GC/MS analysis on the SPB-5 column with baseline resolution (Slessor et al., 1985). Both reactions occurred in good yield (>90%).

Enzymatic resolution of racemic fuscumol **(1)** with a ppl lipase produced (*S*)- and (*R*)-fuscumols, but only at ca. 60% ee. However, enzymatic resolution of racemic fuscumol **(1)** with a lipase from *C. antarctica* (Novozyme 435) produced (*S*)- and (*R*)-fuscumols (**3** and **5**, respectively; Fig. 2) with improved chiral (ee >95%) and overall purity (>98%). Spectroscopic data for the Novozyme 435 lipase-produced (*S*)- and (*R*)-fuscumols are reported. ^{1}H and ^{13}C assignments were based on extensive 1-D and 2-D NMR experiments, including 1-D ^{1}H and ^{13}C, DEPT, COESY, HMBC, HMQC, and NOESY. The ^{1}H and ^{13}C NMR chemical shifts are reported in ppm, and multiplicity terms used are: s (singlet), d (doublet), ht (heptet of triplets), st (sextet of triplets), and m (multiplet) (See Fig. 1 for C-numbering scheme).

^{1}H NMR (Fig. 1, $CDCl_3$, 300 MHz): 1.20 (d, *J*=6.1 Hz, C-1H_3), 1.45–1.54 (m, O*H* and C-3H_2), 1.60 (s, C-13H_3), 1.62 (s, C-12H_3), 1.68 (d, *J*=1.1 Hz, C-11H_3), 1.95–2.14 (m, C-4H_2, C-7H_2 and C-8H_2), 3.81 (sextet, *J*=6.2 Hz, C-2*H*), 5.08 (ht, *J*=1.3, 9.3 Hz, C-9*H*), 5.15 (st, *J*=1.3, 7.2 Hz, C-5*H*). ^{13}C NMR ($CDCl_3$, 75 MHz): 16.0 (C-12), 17.7 (C-13), 23.5 (C-1), 24.4 (C-4), 25.7 (C-11), 26.7 (C-8), 39.2 (C-3), 39.7 (C-7), 67.8 (C-2), 124.0 (C-5), 124.3 (C-9), 131.4 (C-10), 135.6 (C-6). IR (neat) cm^{-1}: 3323 (br, s), 2966 (s), 2921 (s), 2856 (m), 1669 (w), 1447 (s), 1375 (s), 1329 (w), 1303 (w), 1172 (w), 1125 (s), 1109 (s), 1083 (m), 1024 (w). $[\alpha]_D^{24}$=+24.9 (neat); = +7.3 (c=2.8; CH_2Cl_2); ee=95% {lit. Madyastha and Gururaja, (1994) (*S*)-fuscumol $[\alpha]_D$=+3.4 (c=5.0, $CHCl_3$)}. (*R*)-*fuscumol:* spectra as per *S*-fuscumol; $[\alpha]_D^{24}$=−24.7 (neat); −6.33 (c=1.4; CH_2Cl_2); ee=95%.

The absolute configuration of the Novozyme 435 lipase-produced fuscumol was initially assigned by comparison of spectra and optical rotation with the product previously published, in which (**3**) was produced by asymmetric reduction of (**2**) with cell-free systems from *Alcaligenes eutrophus* in the presence of $NADP^+$ (Madyastha and Gururaja, 1994). This allowed a tentative assignment of the *dextrorotatory* product (**3**) as having the (*S*)-configuration, with a rigorous confirmation of the absolute configuration of the alcohols by chemical degradation of optically enriched (*S*)- or (*R*)-fuscumol to the known (*S*)-(−)-5-methyl-4, 5-dihydrofuran-2(3H)-one and (*R*)-(+)-5-methyl-4, 5-dihydrofuran-2(3H)-one (Mori, 1975), respectively.

Pheromone Chirality Male-produced fuscumol from both *T. fuscum* and *T. cinnamopterum* was determined to have the (*S*)-stereochemistry by comparing mass chromatograms and spectra of the (*S*)-acetyl lactyl diastereomers from racemic fuscumol (Fig. 3a), male *T. fuscum* (Fig. 3b), male *T. cinnamopterum* (not shown), and (*S*)-(+)-fuscumol (Fig. 3c) produced by the lipase method whose absolute stereochemistry had been determined previously. Direct separation of fuscumol enantiomers using chiral capillary columns (β- or γ-cyclodextrin) was unsatisfactory, with poor resolution of the alcohols.

GC/EAD and EAG Analyses GC/EAD analysis of *T. fuscum* male volatiles collected on Super-Q® showed only one consistent EAD-active peak, with a retention time corresponding to that of fuscumol; male and female antennae were equally responsive.

EAG Analysis Antennal responses (Fig. 4) to (*R*)-fuscumol (*t*=−0.66, *df*=23, *P*=0.51), (*S*)-fuscumol (*t*=−0.3, *df*=23, *P*=0.77), or racemic fuscumol (*t*=0.03, *df*=23, *P*=0.98) did not differ between male and female *T. fuscum*, so data were

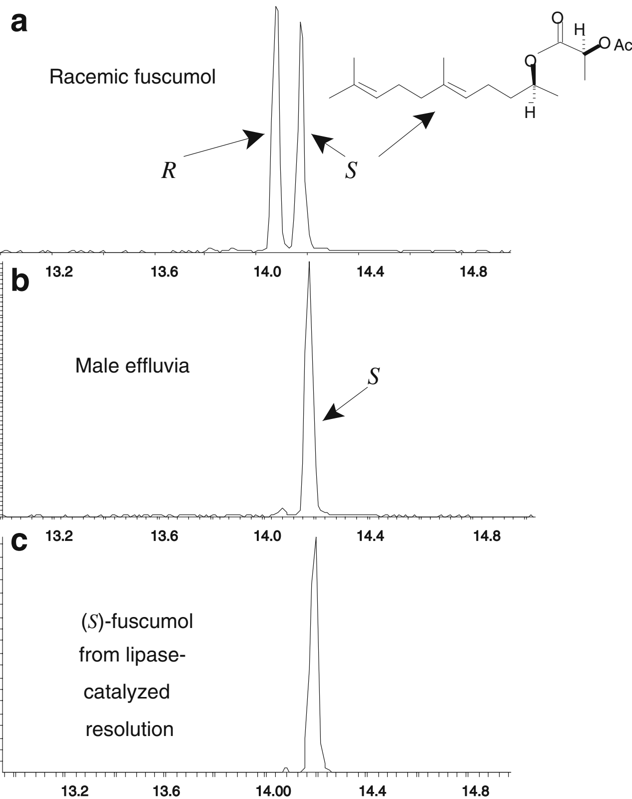

Fig. 3 Resolution of (***S***)-acetyl lactate diastereomers of racemic fuscumol shown by : total ion mass chromatograms of **a** racemic fuscumol diastereomers; **b** (*S*)-acetyl lactyl derivative of male *Tetropium fuscum* effluent, (*S*)-diastereomer, ~ 98% pure with the (*R*)-diastereomer as the earlier eluting peak; **c** (*S*)-(+)-fuscumol/(S)-acetyl lactyl derivative from Novozyme 435 resolution

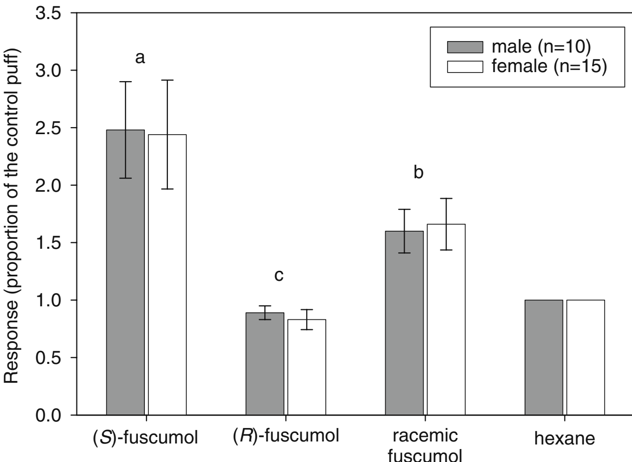

Fig. 4 Electroantennogram responses to the different enantiomers of fuscumol. For each antenna, the magnitude of each response was standardized as a percentage of response to a control (hexane) puff. Bars represent mean response ± SE. Males and females did not differ in response to any treatment (*t*-tests, $P>0.05$). *Different letters above the bars* indicate significantly different mean responses (sexes pooled), as determined by repeated measures ANOVA with REGW test on data transformed by log (y + 1)

pooled and analyzed by repeated measures ANOVA. Standardized antennal response differed among treatments (F= 57.4; df=2, 48; $P<0.001$), with the greatest response to (*S*)-fuscumol, then to the racemate, and (*R*)-fuscumol (Fig. 4).

Field Trapping Experiment—Chirality and Host Volatiles Mean catches of *T. fuscum, T. cinnamopterum*, and *T. castaneum* were affected by the presence of host volatiles (Table 1; Fig. 5a–d). Mean catches of *T. cinnamopterum* (Fig. 5b), *T. fuscum* in Poland (Fig. 5c), and *T. castaneum* in Poland (Fig. 5d) also were affected by fuscumol chirality and the interaction between chirality and host volatiles (Table 1). Host volatiles, therefore, had a strong effect on catches of *Tetropium* spp. to fuscumol. Without the spruce blend and ethanol lures, only pure (*S*)-fuscumol captured more *T. fuscum* (Fig. 5c) and *T. castaneum* (Fig. 5d) than the control, and this occurred in Poland only. In Nova Scotia, fuscumol by itself [pure (*S*)-, pure (*R*)-, or racemic] was not attractive to *T. fuscum* (Fig. 5a) or *T. cinnamopterum* (Fig. 5b). However, adding either (*S*)-fuscumol or racemic fuscumol to traps baited with host volatiles synergized attraction of all three species, increasing catch of *T. fuscum* some 1.7X in Nova Scotia and 6X in Poland, and increasing catch of *T. cinnamopterum* and *T. castaneum* by 5X, and 17X, respectively.

(*S*)-Fuscumol was the active enantiomer in all three *Tetropium* species. Mean catch in traps baited with (*R*)-fuscumol plus host volatiles did not differ from that in traps baited with host volatiles only, and was less than that in traps baited with (*S*)-fuscumol plus host volatiles for all species (Fig. 5). However, presence of the (*R*)-enantiomer did not affect response of beetles to the active (*S*)-enantiomer: mean catch in traps baited with (*S*)-fuscumol or racemic fuscumol did not differ for any *Tetropium* species (Fig. 5).

The catch of *T. castaneum* in traps baited with fuscumol and host volatiles was similar to that of *T. fuscum*, with significant correlations in catch per trap (r=0.79, N=46, $P<0.001$). There was a similar correlation between catches of *T. fuscum* and *T. cinnamopterum* (r=0.49, N= 73, $P<0.001$).

Field Trapping Experiment—Release Rate Addition of fuscumol, at any release rate tested, to traps baited with host volatiles increased catch of *T. fuscum* in Nova Scotia ($F_{4,36}$=18.6, $P<0.001$, Fig. 6a), *T. cinnamopterum* ($F_{4,36}$= 35.1, $P<0.001$, Fig. 6b) and *T. castaneum* ($F_{4,40}$=42.8, $P<$ 0.001, Fig. 6d). In Poland, only the combination of fuscumol at the medium release rate (4 mg/d) plus host volatiles captured more *T. fuscum* ($F_{4,40}$=35.4, $P<0.001$) than did host volatiles alone (Fig. 6c). However, mean catch in traps baited with racemic fuscumol plus host volatiles did not differ among the high, medium, and low release rates of fuscumol for any *Tetropium* species (Fig. 6), in spite of a 32-fold difference in release rate.

The response to fuscumol release rates was similar among *Tetropium* species, with significant correlations in catch per trap between *T. fuscum* and *T. castaneum* in Poland (r=0.46, N=46, P=0.001) and between *T. fuscum* and *T. cinnamopterum* in Nova Scotia (r=0.74, N=46; $P<0.001$).

Responses of Males vs. Females For each *Tetropium* species, males and females responded similarly to the treatments, with the highest catches of both sexes occurring in traps baited with the combination of host volatiles plus either racemic or (*S*)-fuscumol (Figs. 5 and 6). However, catches in these treatments were also often female biased (Tables 2 and 3). In the chirality experiment, catch of females was correlated ($P<0.001$) with that of males for *T. fuscum* (r= 0.90, N=44, Poland; r=0.77, N=70 Nova Scotia), *T. cinnamopterum* (r=0.69, N=49) and *T. castaneum* (r= 0.91, N=42). Results were similar in the release rate experiment, with correlations ($P<0.001$) between numbers of males and females captured of *T. fuscum* (r=0.70, N=43, Poland; r=0.62, N=45, Nova Scotia), *T. cinnamopterum* (r=0.73, N=39) and *T. castaneum* (r=0.88, N=45).

For traps baited with a combination of host volatiles, plus either racemic or (*S*)-fuscumol, the chi square goodness-of-fit tests indicated a bias toward female catch in about half of the cases (Tables 2 and 3). This was most apparent for traps baited with high release rates of racemic fuscumol plus host volatiles, in which catches were female-biased for all three *Tetropium* species (Table 3). In contrast,

Table 1 ANOVA tables showing the effect of pheromone [(*S*)-fuscumol, (*R*)-fuscumol, racemic fuscumol, blank], host volatiles (spruce blend plus ethanol) and their interaction on mean numbers of *Tetropium* spp. (sexes pooled) captured per trap [data transformed by log(y+1)]

Species–Site	Source	*df*	SS	*F*	*P* > *F*
T. fuscum Nova Scotia	Block	14	1.97	2.03	0.023
	Pheromone	3	0.41	1.98	0.122
	Host volatiles	1	20.45	294.46	<0.001
	P X H	3	0.47	2.28	0.084
	Error	98	6.80		
T. fuscum Poland	Block	8	1.69	2.53	0.020
	Pheromone	3	4.71	18.84	<0.001
	Host volatiles	1	15.71	188.47	<0.001
	P X H	3	0.72	2.88	0.044
	Error	56	4.67		
T. castaneum Poland	Block	8	1.27	2.17	0.044
	Pheromone	3	6.92	31.45	<0.001
	Host volatiles	1	10.02	136.48	<0.001
	P X H	3	1.87	8.50	<0.001
	Error	56	4.11		
T. cinnamopterum Nova Scotia	Block	14	2.36	3.19	<0.001
	Pheromone	3	1.25	7.86	<0.001
	Host volatiles	1	4.73	89.45	<0.001
	P X H	3	1.18	7.41	<0.001
	Error	98	5.19		

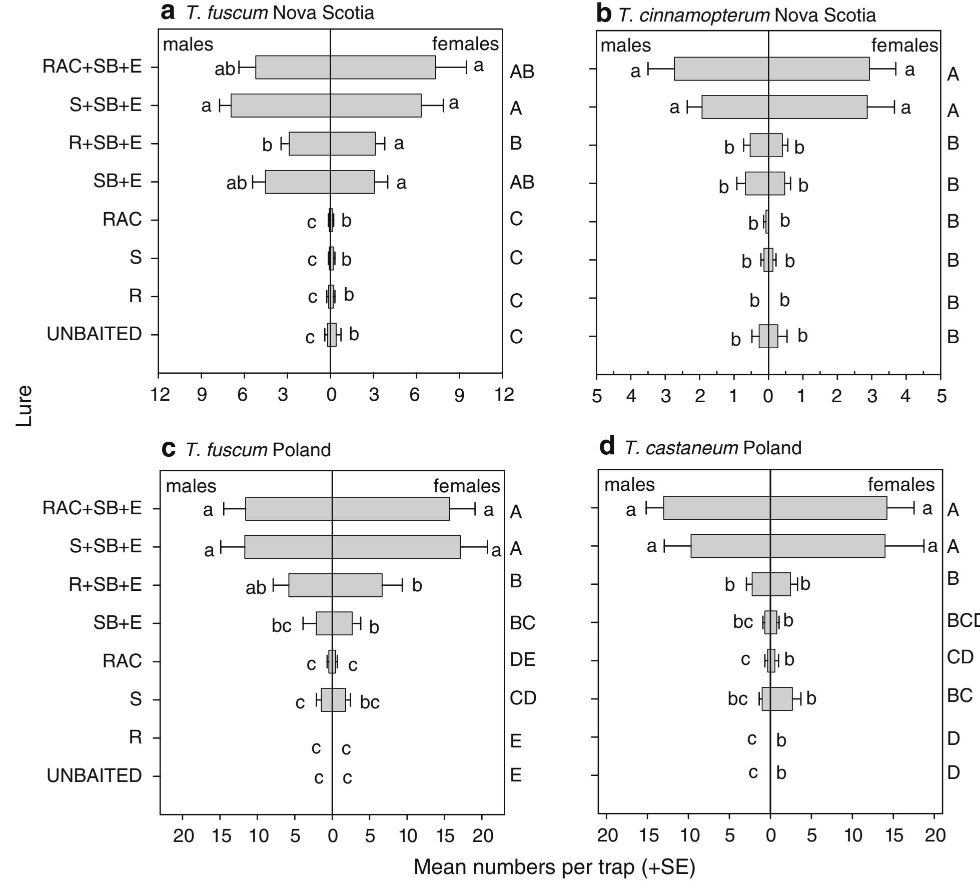

Fig. 5 Effect of fuscumol chirality on mean (+SE) total catch in intercept traps of: **a** *Tetropium fuscum* in Nova Scotia; **b** *T. cinnamopterum* in Nova Scotia; **c** *T. fuscum* in Poland; and **d** *T. castaneum* in Poland, in 2007. Traps were baited with fuscumol [racemic (RAC), S, or R enantiomers], either alone, or with spruce blend and ethanol (SB+E). Traps baited with SB+E only or left unbaited served as controls. *Lowercase letters* indicate significant differences in means within each species and sex; *uppercase letters* indicate significant differences in mean catch (sexes pooled) within species [ANOVA and Ryan-Einot-Gabriel-Welsh test on log(y+1) transformed data; P=0.05]

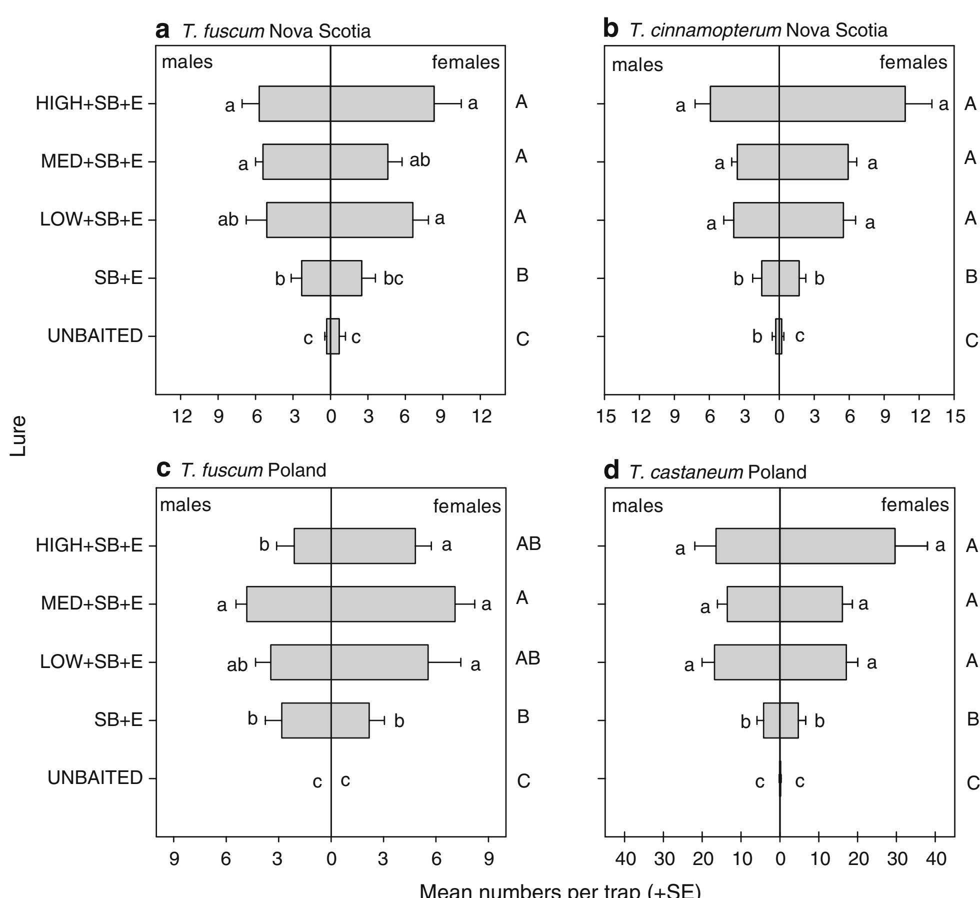

Fig. 6 Effect of racemic fuscumol release rate on mean (+SE) total catch in intercept traps of: **a** *Tetropium fuscum* in Nova Scotia; **b** *T. cinnamopterum* in Nova Scotia; **c** *T. fuscum* in Poland; and **d** *T. castaneum* in Poland, in 2008. Traps were baited with spruce blend and ethanol (SB+E) and fuscumol emitted at 32 mg/d (HIGH), 4 mg/d (MED), or 1 mg/d (LOW). Traps baited with SB+E only or left unbaited served as controls. *Lowercase letters* indicate significant differences within each species and sex; *uppercase letters* indicate significant differences in mean catch (sexes pooled) within species [ANOVA and Ryan-Einot-Gabriel-Welsh test on log(y+1) transformed data; P=0.05]

there was no female bias in traps baited with either host volatiles alone or (*R*)-fuscumol plus host volatiles (Tables 2 and 3). Catch of *T. fuscum* in Nova Scotia was male biased in traps baited with host volatiles alone (Table 2).

Discussion

We demonstrated that (*S*)-fuscumol [(2*S*,5*E*)-6,10-dimethyl-5,9-undecadienol], and not (*R*)-fuscumol, is emitted by males of the longhorn beetle species, *T. fuscum* and *T. cinnamopterum*, and that it synergizes long-range attraction of males and females of both species to host volatiles. Moreover, results from the trapping bioassays in Poland showed that the congener, *T. castaneum*, also is attracted to the combination of (*S*)-fuscumol and host volatiles, suggesting that males of this species also emit (*S*)-fuscumol; this requires chemical confirmation. Presence of the (*R*)-enantiomer did not, however, inhibit attraction of *T. fuscum, T. cinnamopterum*, or *T. castaneum* to the combination of (*S*)-fuscumol and host volatiles. Lack of an inhibitory effect of the antipode on the attractiveness of a natural pheromone enantiomer has been observed in several longhorn beetle species in the subfamily Cerambycinae (Lacey et al., 2004; Hanks et al., 2007).

The trapping bioassays confirm earlier findings (Silk et al., 2007) that fuscumol is only slightly attractive on its own, and that the presence of both host volatiles and fuscumol is necessary for increased and synergized attraction of *T. fuscum* and *T. cinnamopterum*, as well as *T. castaneum.* Synergy in attraction, to a combination of host volatiles and sex or aggregation pheromones, has been observed in other cerambycid species such as *H. bajulus* (Reddy et al., 2005b), *Anoplophora glabripennis* (Motschulsky) (Nehme et al., 2009), and *Monchamus galloprovincialis* (Pajares et al., 2010), as well as in many other phytophagous species in the Coleoptera, Diptera, and Lepidoptera (Landolt and Phillips, 1997).

The low level of attraction of females to fuscumol by itself and the synergistic effect in combination with host volatiles suggests that females should favor male *T. fuscum* that emit fuscumol (i.e., "call") on a suitable host tree. Indeed, we have evidence that *T. fuscum* males are induced to call by the presence of certain host volatiles (Silk et al., 2010). Lemay et al. (2010) observed <10% of *T. fuscum* males calling in clean laboratory Petri-dish bioassays, but

Table 2 Sex ratio of *Tetropium* species caught in traps baited with fuscumol [(*S*)-, (*R*)-, Racemic (Rac)], spruce blend (SB), and ethanol (E) or unbaited, in Nova Scotia (NS) and Poland (PL) in 2007. *Chi square* tests were run only when expected counts were 5 or greater

Species (*G* test)	Site	Lure	Total catch		Sex ratio	Goodness of fit	
			♂	♀	♂/♀	X^2	*P*
T. fuscum	NS	Rac+SB+E	78	110	0.71	5.45	0.02
		(S)+SB+E	104	95	1.09	0.41	0.52
		(R)+SB+E	43	47	0.91	0.18	0.67
		SB+E	68	46	1.48	4.25	0.04
		Rac	1	2	–	–	–
		(S)	1	3	–	–	–
		(R)	2	3	–	–	–
		Unbaited	3	6	–	–	–
		Overall	300	312	0.96	0.24	0.63
T. fuscum	PL	Rac+SB+E	104	141	0.74	5.59	0.02
		(S)+SB+E	105	154	0.68	9.27	<0.01
		(R)+SB+E	52	60	0.87	0.57	0.45
		SB+E	19	24	0.79	0.58	0.45
		Rac	4	4	–	–	–
		(S)	13	16	0.81	0.31	0.58
		(R)	0	0	–	–	–
		Unbaited	0	0	–	–	–
		Overall	297	399	0.74	14.95	< 0.01
T. cinnamopterum	NS	Rac+SB+E	41	44	0.93	0.11	0.74
		(S)+SB+E	29	43	0.67	2.72	0.10
		(R)+SB+E	8	6	1.33	0.29	0.59
		SB+E	10	7	1.43	0.53	0.47
		Rac	1	0	–	–	–
		(S)	2	2	–	–	–
		(R)	0	0	–	–	–
		Unbaited	4	4	–	–	–
		Overall	95	106	0.90	0.60	0.44
T. castaneum	PL	Rac+SB+E	117	128	0.91	0.49	0.48
		(S)+SB+E	87	126	0.69	7.14	< 0.01
		(R)+SB+E	20	22	0.91	0.09	0.76
		SB+E	6	7	0.86	0.08	0.78
		Rac	3	5	–	–	–
		(S)	9	24	0.38	6.81	< 0.01
		(R)	0	0	–	–	–
		Unbaited	0	0	–	–	–
		Overall	242	312	0.78	8.85	< 0.01

the frequency of calling increased in the presence of other males as well as the host volatiles, (*R*)-(−)-linalool and (3*Z*,6*E*)-α-farnesene (Silk et al., 2010).

The addition of fuscumol to traps baited with host volatiles synergized attraction of both sexes of *T. fuscum*, *T. cinnamopterum*, and *T. castaneum*, confirming that it functions as an aggregation pheromone. The most obvious function of such aggregation is for mating. By attracting females, males obviously increase their chances of finding mates. Responding males may benefit from increased encounters with responding females (Landolt and Phillips, 1997), while responding females may benefit by finding males, especially in low-density populations (Wertheim et al., 2005), as well as suitable hosts that may be scarce (Raffa et al., 1993). Other potential benefits of such aggregation to *T. fuscum* are "swamping" of natural enemies (Wertheim et al., 2005) or helping to overcome tree defenses and thereby increase colonization success, as shown for several Scolytine bark beetle-conifer systems (Borden, 1974). Similar to Scolytine bark beetles, *Tetropium* spp. are associated with ophiostomatoid fungi (Jacobs et al., 2003) and feed entirely in the phloem of

Table 3 Sex ratio of *Tetropium* species captured in traps baited with racemic fuscumol at three different release rates (HI=32 mg/d; MD=4 mg/d; LO=1 mg/d) plus the host volatile lures, spruce blend (SB), and ethanol (E), in Nova Scotia (NS) and Poland (PL) in 2008. Traps baited with host volatiles only and unbaited traps served as controls. *Chi square* tests were run only when expected counts were 5 or greater

Species (*G*-test)	Site	Lure	Total catch	Sex ratio		Goodness of fit	
			♂	♀	♂/♀	X^2	*P*
T. fuscum	NS	HI+SB+E	57	83	0.69	4.83	0.03
		MD+SB+E	54	46	1.17	0.64	0.42
		LO+SB+E	51	66	0.77	1.92	0.17
		SB+E	23	25	0.92	0.08	0.77
		Unbaited	3	7	0.43	1.60	0.21
		Overall	185	220	0.84	3.02	0.08
T. fuscum	PL	HI+SB+E	31	53	0.58	5.76	0.02
		MD+SB+E	53	78	0.68	4.77	0.03
		LO+SB+E	38	61	0.62	5.34	0.02
		SB+E	31	24	1.29	0.89	0.35
		Unbaited	0	0	–	–	–
		Overall	153	216	0.71	10.8	<0.01
T. cinnamopterum	NS	HI+SB+E	59	108	0.56	14.4	<0.01
		MD+SB+E	36	59	0.61	5.57	0.02
		LO+SB+E	39	55	0.71	2.72	0.10
		SB+E	15	17	0.88	0.13	0.72
		Unbaited	3	2	–	–	–
		Overall	149	239	0.62	20.9	<0.01
T. castaneum	PL	HI+SB+E	181	326	0.56	41.5	<0.01
		MD+SB+E	149	177	0.84	2.40	0.12
		LO+SB+E	185	188	0.98	0.02	0.88
		SB+E	46	52	0.88	0.37	0.54
		Unbaited	1	2	–	–	–
		Overall	561	743	0.76	25.4	<0.01

freshly felled trees or living trees that are weakened, stressed, or dying (Juutinen, 1955; Schimitschek, 1929). However, the effects of attack density and the role of associated fungi on colonization success of *T. fuscum* are unknown.

Although the combination of fuscumol plus host volatiles attracted both sexes, we observed a significant bias toward female catch for all three *Tetropium* spp. In contrast, catches in traps baited with host volatiles alone were either unbiased or, in one case, male-biased. Juutinen (1955) reported a natural 1:1 sex ratio for *T. fuscum*, and we have observed the same ratio in more than a thousand *T. fuscum* and *T. cinnamopterum* reared from infested spruce bolts (Sweeney et al., unpublished data). Female-biased attraction to male-produced aggregation pheromones has been observed frequently in Scolytine bark beetles, suggesting that male-produced aggregation pheromones may have evolved from initial roles as sex pheromones for female attraction (Raffa et al., 1993).

Positive dose–response relationships, with sex or aggregation pheromones, have been found for many insect species, including the longhorn beetle, *H. bajulus* (Reddy et al., 2005a). In bark beetles and their predators, a positive pheromone dose–response relationship may depend on the presence of host kairomones (Miller et al., 2005). We did not observe a dose–response relationship with fuscumol release rates. However, we found differences in trap catch at different fuscumol release rates: catch of male *T. fuscum* in Poland in host-volatile traps increased only when fuscumol was added at the medium release rate, while fuscumol, added at the high rate to host volatiles, captured all three *Tetropium* spp. with a strong female bias. These results suggest that higher densities (i.e., release rates) of males on host trees would increase the ratio of females to males and consequently the probability of male mating success. Both sexes of *T. fuscum* mate repeatedly (Schimitschek, 1929; Juutinen, 1955), and repeated copulation improves egg fertilization rate (Schimitschek, 1929).

The attraction of three different *Tetropium* spp. to the combination of (*S*)-fuscumol and host volatiles suggests that cross-attraction and aggregation of congeners on host trees may occur in areas of sympatry. *Tetropium fuscum* and *T. castaneum* are sympatric over much of Europe (Juutinen, 1955) and, since the introduction of *T. fuscum* to Canada, this species and *T. cinnamopterum* co-occur in several counties of Nova Scotia. The lack of response to (*R*)-fuscumol, indicates that any pre-mating species-specific

responses that might exist among the three *Tetropium* spp. are not mediated by enantiomer-specific responses to fuscumol. If cross-attraction is not costly, or reproductive isolation is mediated by other mechanisms, then there would be no selection pressure for divergence in pheromones (Symonds and Elgar, 2007). We cannot rule out the possibility that there are additional, as yet undetected, pheromone components emitted by *Tetropium* males that reduce attraction of congeners, however, it is likely that reproductive isolation among *Tetropium* spp. is mediated, at least in part, by close-range mechanisms. As shown for several species of longhorned beetles (Ginzel and Hanks, 2003), there is evidence that males of *T. fuscum* and *T. cinnamopterum* recognize conspecific females (i.e., are elicited to stop, mount, and copulate) by species-specific cuticular hydrocarbons or blends that act as contact pheromones (Silk et al., unpublished).

In summary, we have shown that (*S*)-fuscumol, and not (*R*)-fuscumol, is emitted by males of *T. fuscum* and *T. cinnamopterum*, and this compound synergizes the attraction of *T. fuscum, T. cinnamopterum*, and *T. castaneum* to host volatiles. The presence of the (*R*)-enantiomer in synthetic racemic fuscumol did not inhibit attraction of any of the *Tetropium* spp. These results have practical benefits for trapping surveys of *Tetropium* spp., particularly surveys for early detection of the potential invasive species, *T. fuscum* and *T. castaneum* in North America, because a single trap can target more than one species and racemic fuscumol is easier and cheaper to synthesize than (*S*)-fuscumol. Indeed, synthetic fuscumol has been used in surveys for monitoring the spread of *T. fuscum* in Canada and the United States since 2007, and has enabled detection of the beetle in more than 40 new locations outside of the quarantine zone in Nova Scotia (Cunningham, 2010).

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Whitten (Givaudan, Switzerland) personal communication], suggesting that it may be a behaviorally active compound that is collected actively by males. This has remained speculative, though, since HHA failed to lure male orchid bees in previous bioassays (M. Whitten and G. Gerlach personal communication; T. Eltz unpublished data.). However, HHA is a chiral compound, and the tested synthetic product was a mix of unknown isomeric composition (R. Kaiser personal communication), leaving room for further investigation. In the present study, we determined the stereochemistry of HHA in tibial extracts of three species of *Euglossa*. Furthermore, we synthesized an optically pure RR-HHA [(6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-one] in addition to a mixture of the four possible optical isomers of HHA. Bioassays were conducted in southern Mexico and Panama to test behavioral attractiveness to male orchid bees.

Methods

Identification and Stereochemistry of 6,10,14-Trimethylpentadecan-2-one in Extracts of Euglossa Species Hexane extracts of pairs of hind legs of individual male *Euglossa imperialis*, *E. allosticta,* and *E. crassipunctata* (one each) were purified on a 500 mg Strata SI-1 Silica Teflon coated solid phase column with stepwise gradient elution using 1–11% ethyl acetate in pentane in steps of 1%. An elution and collection volume of 750 µl for each fraction was used, with twice the volume for fractions 4–6. These fractions, containing the ketone, were combined and evaporated to dryness under a stream of argon at room temperature, and then were redissolved in 1 ml of pentane and washed with 200 µl of 0.2 M KOH. The organic layer was collected and dried with sodium sulfate before it was evaporated to dryness under a stream of argon at room temperature and finally redissolved in 50 µl cyclohexane.

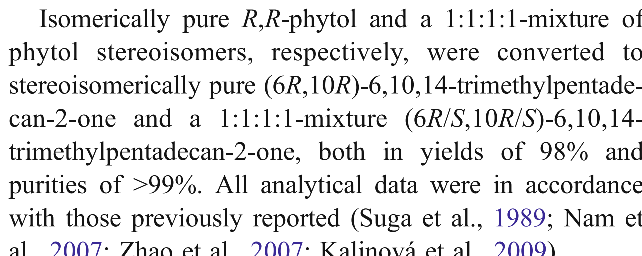
The ketone in the purified extract was reduced to alcohol with lithium aluminum hydride according to standard method, and then was derivatized with (R)-(+)-trans-chrysanthemoyl chloride (Brooks et al., 1973). The diastereomers were analysed with a Hewlett-Packard 6890N gas chromatograph (GC) with a polar Varian factorFOUR VF-23ms column (30 m×0.25 mm i.d., d_f=0.25 µm) and an HP 5973 mass spectrometer (GC-MS) in SIM mode (m/z=123, 124, 153, and 168). The carrier gas (1 ml/min) was helium; 1 µl of the sample was injected splitless, the injector temperature was 250°C, and the aux temperature was 280°C. The column temperature was increased from 50°C by 10°C/min up to 110°C, from 110°C by 0.01°C/min up to 115°C, and from 115°C by 10°C/min up to 230°C.

Identification was made by comparing the retention times and mass spectra of the unknown alcohol derivatives with a reduced and derivatized synthetic reference mixture of all four stereoisomers of 6,10,14-trimethylpentadecan-2-one and also with a derivatized synthetic reference mixture of (2*S*,6*R*,10*R*)- and (2*R*,6*R*,10*R*)-6,10,14-trimethylpentadecan-2-ol. Reduction of the ketone to the alcohol creates a new stereogenic center at the carbon with the formed hydroxyl group, which is disregarded when the stereochemistry of the original ketone is determined. Identification also was made by comparing the GC/infra red (GC/FT-IR) spectrum of the ketone in the extract with that of the synthetic ketone.

Chemicals Commercially available chemicals were used without further purification. Stereoisomerically pure (*R*,*R*)-phytol was purchased from TCI America. Preparative liquid chromatography was performed on normal-phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm, 10–50 g/g of product mixture) employing a gradient technique with an increasing concentration (0–10%) of distilled ethyl acetate in distilled cyclohexane. Progress of the reaction was monitored with thin layer chromatography on silica gel plates (Merck 60, precoated aluminium foil) using ethyl acetate (40%) in cyclohexane as an eluent. Plates were visualized with ultraviolet irradiation and/or by spraying with vanillin in sulfuric acid and heating at 120°C. Purity of the product was checked by GC on a Varian 3300 GC instrument using a capillary column (EC-1, 30 m×0.32 mm i.d, d_f=0.25 µm, with nitrogen as carrier gas and a split ratio of 1:20). The temperature was programmed for 2 min at 100°C followed by 10°C /min up to 300°C. GC-MS analyses were carried out on a Saturn 2000 GC/MS/MS instrument with a Varian 3800 GC instrument, using a capillary column (CP-Sil 5 CB, 30 m×0.32 mm i.d, d_f=0.25 µm, carrier gas helium, column flow = 1 ml/min, split ratio 1:20) and the temperature program described above. GC/FT-IR analysis of the natural and synthetic compounds was carried out on a Thermo Scientific Nicolet 6700 FT-IR spectrophotometer, coupled via an GC/FT-IR interface to an Agilent 7890A GC with a polar Varian factorFOUR VF-23ms column (30 m×0.25 mm i.d., d_f=0.25 µm). The carrier gas (1 ml/min) was helium; 1 µl of the sample was injected splitless, the injector temperature was 250°C, and the transfer line and flow cell temperature were both set at 250°C. The column temperature was increased from 50°C by 10°C/min up to 230°C, and kept at 230°C for 10 min. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 (500 MHz ^{1}H, 125.8 MHz ^{13}C) spectrometer using $CDCl_3$ as solvent and TMS as internal standard.

Oxidizing phytol with $NaIO_4$ and a catalytic amount of $RuCl_3$ (Sasaerila et al., 2003) gave pure (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecan-2-one in near quantitative yield. Synthesis of (6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-one was performed using the same method from stereoisomerically pure (*R*,*R*)-phytol.

Bioassays The *RR*-HHA and the 1:1:1:1 stereoisomeric mixture of HHA were dissolved at 100 mg/ml in pentane (Uvasol, Merck, Germany). Bioassays were conducted on 2 days in two different forest localities (near Palenque and near Lancanja, Chiapas) in southern Mexico in September 2008, and on 3 days in one forest locality in Central Panama (Barro Colorado Island) in May 2009. Aliquots of 50 µl of test solutions and solvent controls were pipetted onto filter papers (Whatman 1, 2.5 cm) pinned to trees at breast height. Filter papers were observed between 0900 to 1200 and refreshed once or twice during the morning with additional aliquots of 50 µl of test solution or solvent. We routinely exposed other synthetic chemical bait compounds (e.g., 1,8-cineole, methyl salicylate, *p*-dimethoxybenzene) in the vicinity. Those were of much higher absolute concentration than the HHA lures and at least 5 m away from them. Males were only counted as having visited a HHA lure when they had landed on the respective filter paper and performed characteristic volatile collecting behavior.



Isomerically pure *R*,*R*-phytol and a 1:1:1:1-mixture of phytol stereoisomers, respectively, were converted to stereoisomerically pure (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and a 1:1:1:1-mixture (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecan-2-one, both in yields of 98% and purities of >99%. All analytical data were in accordance with those previously reported (Suga et al., 1989; Nam et al., 2007; Zhao et al., 2007; Kalinová et al., 2009).

In bioassays both the (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and the stereoisomeric 1:1:1:1-mixture attracted male euglossine bees in southern Mexico and Panama (Table 1), whereas solvent controls were not attractive. Overall, the (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one attracted more individuals and species than the 1:1:1:1-mix. Generally, the HHA lures attracted relatively few bees in comparison with other synthetic compounds that were exposed at the same time (data not shown). On several occasions, a male *E. imperialis* first was attracted to a 1,8-cineole bait and subsequently visited a HHA filter paper.

Results

HHA in hind leg pouches of male *Euglossa imperialis*, *E. allosticta*, and *E. crassipunctata* is optically pure (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one, based on comparison with synthetic reference mixtures of known stereoisomeric composition (Figs. 1 and 2).

Discussion

Our analytical results demonstrate that HHA in male *Euglossa* hindleg pouches is (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one. This is in agreement with HHA being chemically derived from phytol [(2E,7*R*,11*R*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol] from source plants. We further show that (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-

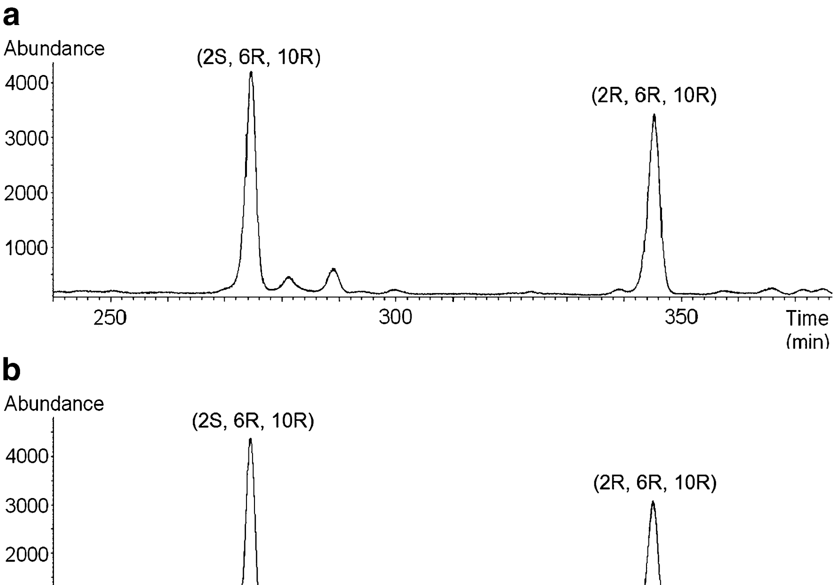


Fig. 1 **a** GC-MS chromatogram of a derivatized synthetic mixture of (2*S*,6*R*,10*R*)- and (2*R*,6*R*,10*R*)-6,10,14-trimethylpentadecan-2-ol. **b** GC-MS chromatogram of a reduced and derivatized extract from *Euglossa crassipunctata*. Two peaks appear due to an extra stereogenic centre created by the reduction of the ketone to alcohol. Extracts from *E. imperialis* and *E. allosticta* show the same result

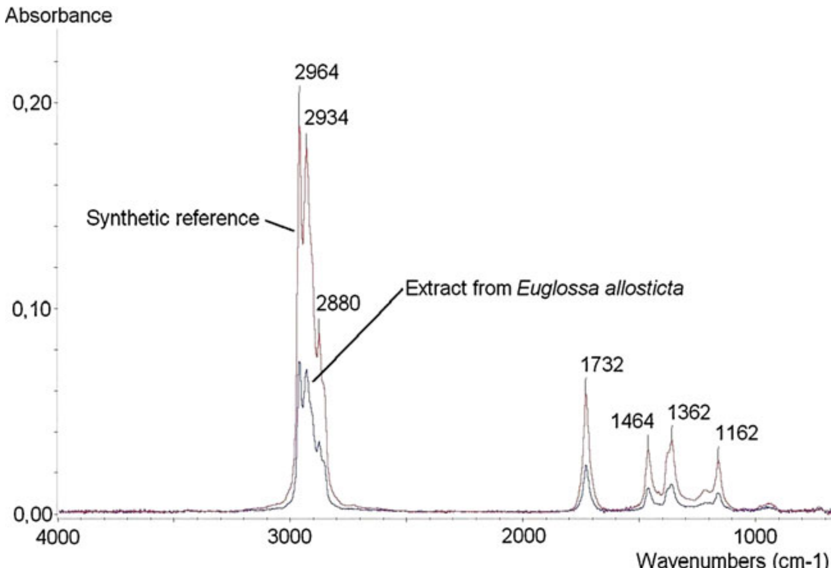


Fig. 2 GC/FT-IR spectra of synthetic (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and extract of *Euglossa allosticta*

one is a behaviorally active component for males of at least six species of euglossine bees that actively approached and collected the compound. Probably, extended bioassays would further extend the list of responsive taxa, especially with regard to rarer euglossine species. However, it should be noted also that our bioassays in Panama failed to attract *Euglossa despecta*, a species that has large quantities of HHA in its hindlegs (Zimmermann et al., 2009) and that was common at other chemical baits at the time of the study. Generally, HHA did not attract as many male bees as did some of the traditional synthetic bait compounds such as 1,8-cineole, methyl salicylate, or *p*-dimentoxybenzene. This may in part be explained by the relatively lower concentration of HHA in our bioassays, but probably also by its low volatility. HHA has a molecular weight of 268, whereas the mentioned other attractants have molecular weights around 150. In fact, HHA is the largest natural molecule known to date that attracts male orchid bees in pure form (Williams and Whitten, 1983; Ramírez et al., 2002). Its attractiveness to males, along with the recent discovery of other male-attracting semivolatiles in euglossine fragrances (Eltz et al., 2008; Ramírez et al., 2010), suggests that these larger and less volatile compounds are functionally important in the male signal. On one hand, they might act as a matrix or fixative for more volatile components such as mono- and sesquiterpenes (Eltz et al., 2007). On the other hand, they might represent the characteristic core of the fragrance signal, which is ornamented with more volatile but ubiquitous additives. In other words, the large and heavy components may represent the "base note" of a complex and multi-layered male odor.

Table 1 Number of males of different species of orchid bees visiting filter papers impregnated with either (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one or a 1:1:1:1-mixture of (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecane-2-one at equal (overall) concentration. Solvent (pentane) controls attracted no bees at all. Data were pooled across localities/dates for Mexican and Panamanian bioassays

	RR-isomer	1:1:1:1-mixture
Mexico		
Euglossa imperialis	22	13
Euglossa hemichlora	1	
Euglossa obtusa	1	
Panama		
Euglossa imperialis	22	3
Euglossa hemichlora	4	
Euglossa igniventris	1	
Exaerete frontalis	1	
Eufriesea corusca	1	

Little is known about the natural source(s) of HHA for male euglossine bees. Among the many floral scents and essential oils analyzed by R. Kaiser (personal communication), HHA is relatively widespread, but occurs normally only as a minor component. Notable exceptions are a small number of euglossophilous orchids, in which HHA is the dominant compound found in the floral headspace (>50% of peak area): *Polycycnis ornata, Kegeliella kupperi*, *Acineta alticola*, and *Soterosanthus shepheardii* (R. Kaiser and M. Whitten personal communication). However, it is doubtful whether any of these orchids would be present in sufficient population densities to represent a significant HHA source for orchid bees. The outstanding abundance of HHA in male fragrances (Zimmermann et al., 2009) suggests rather that HHA sources are highly available, i.e., not orchids. Euglossine-pollinated aroids seem more likely candidates. HHA represented about half of the

volatiles in the headspace of inflorescences of *Anthurium thrinax*, an euglossophilous aroid from French Guyana (Hentrich et al., 2010). Among the male *Euglossa* visiting this plant for fragrance collection, the majority were *Euglossa hemichlora* (Hentrich et al., 2010), a widespread species that also collected HHA in southern Mexico and Panama (Zimmermann et al., 2009, this paper). *Anthurium thrinax* is restricted to the Guianas; other euglossophilous congeners occur in Central America (Dressler, 1968), but their floral scent has not been investigated.

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attraction is so powerful that the lure has been used in the male annihilation technique to eradicate *B. dorsalis* (e.g., in Okinawa, Japan) (Steiner et al., 1965; Koyama et al., 1984). *Bactrocera dorsalis* males that have fed on ME sequester the metabolite phenylpropanoids, (*E*)-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol, in their rectal glands, and use them as a sex pheromone attractive to females, as well as for defense against predators (Nishida et al., 1988a; Shelly and Dewire, 1994; Tan and Nishida, 1998; Hee and Tan, 1998; Khoo et al., 2000; Wee and Tan, 2001). Although *B. correcta* males have a strong affinity to ME, nothing is known about the acquisition of ME in the rectal gland nor the ecological significance of the metabolites in this species. Our preliminary analyses of *B. correcta* revealed an entirely different composition of ME metabolites in the rectal gland from those found in *B. dorsalis*. This prompted us to examine rectal volatiles in *B. correcta*, as it may provide a clue for understanding the role of the sex pheromone in promoting reproductive isolation between sympatric populations of these species.

Methods and Materials

Insects Bactrocera correcta were raised on an artificial diet, developed for the Sterile Insect Technique mass rearing program, at 26±2°C and 65–70% RH, under a photoperiod of 12L:12D (light intensity 2000 Lux) at the Irradiation for Agricultural Development Sub-Division Bureau of Agricultural Production Quality Development, Department of Agricultural Extension, Bangkok, Thailand (Orankanok et al., 2007).

Instruments Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured in $CDCl_3$, with a Bruker Avance 400 FT-NMR, using TMS as an internal standard (J-values are given in Hz; letters s, d, t, m represent singlet, doublet, triplet, and multiplet, respectively). Electron ionization (EI), gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent 5975 inert XL mass spectrometer coupled with an Agilent 6890 gas chromatograph. The GC was fitted with an HP-5MS capillary column (28 m×0.25 mm, 0.25 μm film thickness), which was programmed from 60°C (1 min. holding) to 280°C at 10°C.min^{-1}. Helium was the carrier gas.

Feeding Test and Sample Preparation (1) Laboratory-reared males of *B. correcta* (11-d-old after eclosion, segregated from females) were fed on ME (Aldrich Chemicals Co., Milwaukee, WI, USA), impregnated (10 μl/disc) on small filter paper discs (Advantec, antibiotic test disc, thick, 8-mm diam.), from 15:30–16:00. Adults were then kept in small plastic cages with a sugar-yeast hydrolysate mixture and water. Rectal glands were dissected from males 6 or 24 hr after treatment, and each gland was extracted with ethanol (250 μl). After GC quantification of each individual sample, 10 male rectal gland extracts were combined and chromatographed on a silica gel column (Wako gel C-200, 0.3 g, Wako Pure Chemical Industries, Japan), eluting in sequence with mixtures containing increasing concentration of ethyl acetate in hexane. Major phenylpropanoids, **1** and **2**, were eluted with 25% ethyl acetate in hexane, and purified further by HPLC (YMC-pack SIL, 300×10 mm i.d., S-5 μm), with 66% ethyl acetate in hexane (2.5 ml.min^{-1}) as the mobile phase. The two compounds eluted at retention times of 13.8 and 14.4 min, respectively. (2) In order to examine the propensity to sequester sesquiterpene hydrocarbons by *B. correcta*, laboratory-raised males (15 d after eclosion, segregated from females) were exposed for several minutes to small filter paper discs (same as above) treated with a mixture of ME (1 mg/disc), β-caryophyllene (2 mg/disc), and α-humulene (2 mg/disc). The treated flies were kept with a sugar-yeast hydrolysate mixture for 24 hr and individuals were extracted with ethanol (500 μl). Amounts of compounds in each whole body extract were quantified by GC analysis.

Field Trapping of Male Flies (1) Male *B. correcta* were collected in Bangkok (Wireless Rd, Bangkok 10330, Thailand) on November 24, 2006, using ME traps. Rectal glands were dissected from males, and each gland extracted with ethanol (250 μl). (2) In a similar manner, males of *B. correcta* and *B. dorsalis* were collected in a mixed fruit orchard in Northern Bangkok on November 30, 2007, using ME as a lure (males could not contact the lure). Males were soaked individually in small vials, each with 250 μl ethanol, after identification to species. (3) In order to examine the attractiveness of sesquiterpene hydrocarbons, wild male flies were captured in sticky traps, either with ME (5 mg/rubber septum) or a sesquiterpene mixture (β-caryophyllene + α-humulene, 5 mg each/rubber septum) at a mango orchard (15°21′36″ North; 99°38′15.7″ East) on July 3, 2009, and at a guava orchard (14°07′55.559″ North; 100°48′28.762″ East) in Thailand on July 24 and August 24, 2009. Trapped flies were removed from the sticky board and soaked individually in 250 μl ethanol for species identification and chemical analysis.

Chemicals A geometry-selective Horner-Emmons reaction was employed for syntheses of (*Z*)-3,4-dimethoxycinnamyl alcohol and (*Z*)-coniferyl alcohol. An ylide was prepared from bis(2,2,2-trifluoroethyl)(methoxycarbonylmethyl) phosphonate (Aldrich Chemicals Co.) (3.3 g, 10.3 mmol) and 18-crown-6 (10.9 g, 41.6 mmol), dissolved in THF (50 ml), followed by mixing with 20.0 ml (10.1 mmol,

0.5 M in toluene) of potassium bis(trimethylsilyl)amide [KN(TMS)$_2$] (Aldrich Chemicals Co.) under a N_2 current (−78°C). 3,4-Dimethoxybenzaldehyde (2.0 g, 12.0 mmol, Wako Pure Chemical Industries) dissolved in THF was added to the above with a syringe and stirred for 30 min. After usual work-up and purification by silica gel column chromatography (Wako gel C-200, 10 g, eluting with 20% ethyl acetate in hexane), methyl (*Z*)-3-(3,4-dimethoxyphenyl)-2-propenoate 2.43 g (yield: 91%) was obtained as a solid. The methyl ester (960 mg, 4.32 mmol) was dissolved in anhydrous toluene (20 ml) in a three-necked flask, and DIBAL (40 mmol, 1.0 M in hexane, Wako Pure Chemical Industries) added slowly at 0°C; the reaction mixture was held at this temperature for 1 hr. The ethyl acetate-soluble reaction mixture was eluted with 30% ethyl acetate in hexane from silica gel (Wako C-200, 10 g) to yield 700 mg (yield: 83%, >96% *Z*) of (*Z*)-3,4-dimethoxycinnamyl alcohol as a colorless solid. Likewise, (*Z*)-coniferyl alcohol (>98% *Z*) was obtained as a crystalline mass by reacting the ylide with *O*-acetyl vanillin (prepared by acetylation of vanillin in acetic anhydride-pyridine), followed by reduction with DIBAL in toluene. Authentic samples of sesquiterpenes, (–)-β-caryophyllene (Sigma Chemical Co., St. Louis, MO, USA), and α-humulene and (–)-alloaromadendrene (Fluka Chemie AG, Buchs, Switzerland) were purchased.

Quantification of Rectal Volatiles Ethanolic extracts of rectal glands from *B. correcta* males were quantified using a Hewlett Packard 5890 Series II Plus GC, equipped with a flame ionization detector and an HP-5MS (15 m×0.25 mm i.d, 0.25 μm film thickness) column, and an Agilent 5975 inert/N MSD Chemstation. One microliter of each rectal gland sample from laboratory-raised males fed on methyl eugenol, containing an internal standard (1-hexadecanol; Wako Pure Chemical Industries, Japan), was analyzed by GC. For whole body samples of wild *B. correcta* males, and for laboratory-raised males fed on a mixture of methyl eugenol + β-caryophyllene + α-humulene, 1-pentadecanol (Aldrich Chemicals Co.) was used as an internal standard.

Preparation of a Sesquiterpene Fraction from Wild B. correcta Males Ethanolic extracts of field-trapped *B. correcta* males (9 specimens that contained sesquiterpenes), captured at the Bangkok site (2) on November 30, 2007, were combined, and the solvent was removed *in vacuo* (below 35°C). The residue was dissolved in a small amount of hexane and passed through a short column of silica gel (0.30 g of Wako gel C-200). The fraction eluting with hexane (3 ml) was used to obtain a ^{1}H-NMR spectrum.

Molecular Cloning and Sequence Analysis Total DNA extraction, PCR amplification, and sequence analysis were carried out as described in a companion paper (Tan et al., unpublished). PCR amplifications were performed for the rDNA containing a part of the 16S rRNA gene, the tRNA^{val} gene, and a part of the 12S rRNA gene, in male fly specimens obtained from two different outdoor locations, to compare with those from the laboratory strain (Tan et al., unpublished). The DNA sequence data have been deposited in Genbank under the following accession numbers: (1) AB569585 [Northern Bangkok City (Nov. 30, 2007)], and (2) AB569586 [Guava orchard (14°07′55.559″ North; 100°48′28.762″ East, August 24, 2009)].

Results

Sequestration of ME Metabolites in Rectal Glands Within 24 hr after feeding on ME, two phenylpropanoid volatiles, **1** and **2** (Fig. 1), increased in quantity in male rectal glands. These chemicals were in approximately equal amounts. After isolation of pure **1** and **2** by preparative HPLC, ^{1}H-NMR of both compounds showed a typical cinnamyl olefinic moiety with a coupling constant between H-2 and H-3 of J=11.6 or 11.7 Hz, confirming the geometry to be *Z*. These compounds were identified as (*Z*)-coniferyl alcohol (1, *cis*-4-hydroxy-3-methoxycinnamyl alcohol) and (*Z*)-3,4-dimethoxycinnamyl alcohol from the spectrometric data below and by comparison with the data of the authentic compounds.

(*Z*)-Coniferyl alcohol (**1**). GC relative retention index on HP-5MS: 1682. EI-MS: *m/z* (%): 180 (58, M$^+$), 162 (48), 147 (44), 137 (100), 124 (53), 119 (36), 103 (21), 91 (60), 77 (23), 65 (22). ^{1}H-NMR δ (ppm): 3.90 (3H, s, OCH_3), 4.44 (2H, double d, $J_{1\text{-}2}$=6.5, $J_{1\text{-}3}$=1.6, H-1), 5.63 (1H, s, OH), 5.79 (1H, double t, $J_{2\text{-}3}$=11.7, $J_{2\text{-}1}$=6.5, H-2), 6.52 (1H, d, $J_{3\text{-}2}$=11.7, H-3), 6.74 (1H, double d, $J_{6'\text{-}5'}$=8.1, $J_{6'\text{-}2'}$=1.9, H-6′), 6.77 (1H, d, $J_{2'\text{-}6'}$=1.9, H-2′), 6.89 (1H, d, $J_{6'\text{-}5'}$=8.1, H-5′).

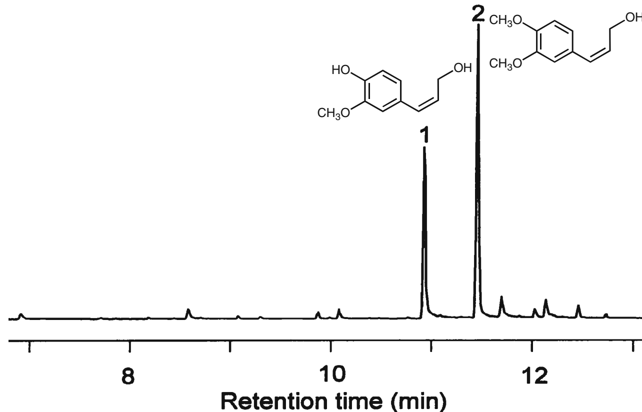

Fig. 1 Gas chromatogram of rectal gland volatiles in *Bactrocera correcta* 24 h after feeding on methyl eugenol [**1**: (*Z*)-coniferyl alcohol, **2**: (*Z*)-3,4-dimethoxycinnamyl alcohol]

(*Z*)-3,4-Dimethoxycinnamyl alcohol (**2**). GC relative retention index on HP-5MS: 1727. EI-MS: *m/z* (%) 194 (66, M^+), 176 (54), 165 (14), 161 (29), 151 (100), 138 (50), 115 (16), 105 (21), 91 (30), 77 (25). ^{1}H-NMR δ (ppm): 3.89 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 4.45 (2H, double d, $J_{1\text{-}2}$=6.5, $J_{1\text{-}3}$=1.6, H-1), 5.80 (1H, double t, $J_{2\text{-}3}$=11.6, $J_{2\text{-}1}$=6.5, H-2), 6.51 (1H, d, $J_{3\text{-}2}$=11.6, H-3), 6.78 (1H,dd, $J_{6'\text{-}5'}$=9.6, $J_{6'\text{-}2'}$=1.8, H-6′), 6.79 (1H, d, $J_{2'\text{-}6'}$=1.8, H-2′), 6.85 (1H, d, $J_{6'\text{-}5'}$=9.6, H-5′).

No other ME metabolites were detected in significant amounts in the rectal glands, although ME was present in a trace amount in glands 6 hr after treatment. Mean amounts (µg/male ± SD) of **1** and **2**, respectively, after ingestion of ME were 2.66±4.0 and 4.7±8.8, 6 hr after treatment, and 54.9±26.8 and 40.0±14.6, 24 hr after treatment (*N*=10 for each time). Thus, the total amount of **1** + **2** was roughly 10 µg/male, 6 hr after feeding on ME, and had increased to nearly 100 µg/male, 24 hr after feeding on ME.

Volatiles in the Rectal Glands of Wild Flies Whole body extracts of *B. correcta* males, captured at various sites in Thailand using ME-baited traps, were made and analyzed. Among 24 males captured in Bangkok (November 30, 2007), 16 (67%) contained both or either of **1** and/or **2** in varying quantities (Fig. 2). However, 9 of the 24 males (38%) possessed three major compounds, **3**, **4**, and **5**, in much larger quantities than compounds **1** and **2**, accompanied by several other related minor components, all of which exhibited a molecular ion at *m/z* 204, characteristic of sesquiterpene hydrocarbons. Compounds **3**, **4**, and **5** were identified as β-caryophyllene, α-humulene, and alloaromadendrene, respectively (Fig. 3), from the retention indices (on HP-5MS), mass fragmentation patterns, and diagnostic ^{1}H-NMR spectra of the sesquiterpene fraction (hexane eluate on silica gel chromatography) isolated from a whole body extract.

Compound **3** (β-caryophyllene). GC relative retention index on HP-5MS: 1435. EI-MS: *m/z* (%): 204 (5, M^+), 189 (20), 175 (10), 161 (34), 147 (29), 133 (90), 120 (42), 105 (58), 93 (100), 91 (80), 79 (69), 69 (79), 55 (30), 41 (60). ^{1}H-NMR δ (ppm): 0.98 (3H, s) 1.00 (3H, s), 1.61 (3H, s), 4.82 (1H, s), 4.94 (1H, s), 5.30 (1H, m).

Compound **4** (α-humulene). GC relative retention index on HP-5MS: 1470. EI-MS: *m/z* (%): 204 (4, M^+), 189 (3), 161 (3), 147 (15), 121 (28), 107 (16), 93 (100), 80 (32), 55 (9). ^{1}H-NMR δ (ppm): 1.06 (3H, s), 1.43 (3H, s) 1.64 (3H, s), 1.93 (2H, d, *J*=7.1), 2.51 (2H, d, *J*=7.4), 4.87 (1H, t, *J*=8.4), 5.16 (1H, d, *J*=15.9), 5.60 (1H, double t, *J*=15.9 and 7.4).

Compound **5** (alloaromadendrene). GC relative retention index on HP-5MS: 1477. EI-MS: *m/z* (%): 204 (29, M^+), 189 (34), 161 (95), 147 (56), 133 (71), 119 (64), 107 (74), 105 (89), 93 (81), 91 (100), 79 (69), 69 (66), 55 (35), 41 (58). ^{1}H-NMR δ (ppm): 0.25 (1H, double d, *J*=9.4 and 10.8), 0.55 (1H, double double d, *J*=6.2, 9.4, 10.9), 0.94 (3H, d, *J*=6.8), 0.96 (3H, s, H-1), 1.00 (3H, s), 2.67 (1H, q, *J*=8.1), 4.72 (2H, m).

The mean quantities of the three sesquiterpenes in the bodies of the nine males are given in Fig. 4, with total amounts of the three compounds as high as 250 µg/male. However, as shown in Fig. 2, the profiles of sesquiterpenes

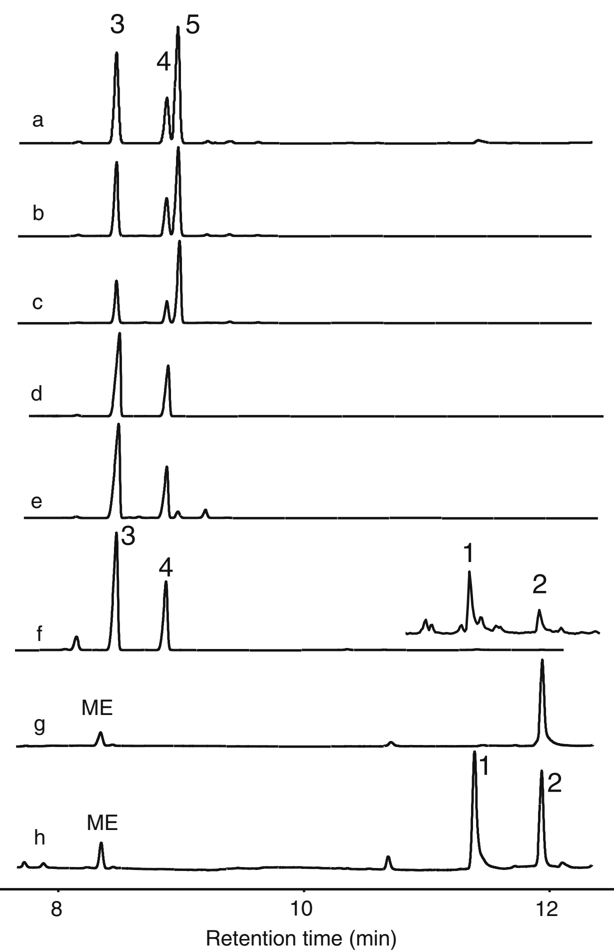

Fig. 2 Gas chromatogram of rectal gland volatiles in individual males (a-h) of *Bactrocera correcta* captured in Bangkok, Thailand (Nov. 30, 2007). All chromatograms show flame ionization detection only, except sample "f", for which mass spectral total ion current detection is also shown because of the trace amounts of compounds **1** and **2**. ME = methyl eugenol. ME-metabolites **1**: (*Z*)-coniferyl alcohol, **2**: (*Z*)-3,4-dimethoxycinnamyl alcohol. Sesquiterpenes **3**: β-caryophyllene, **4**: α-humulene, **5**: alloaromadendrene

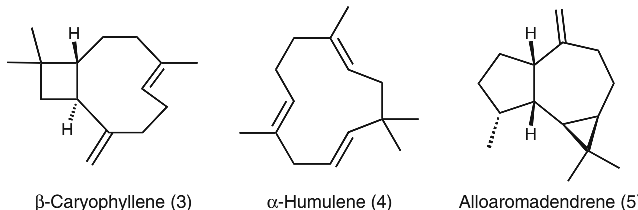

Fig. 3 Sesquiterpene hydrocarbons sequestered in the rectal gland of wild *Bactrocera correcta* males collected in Thailand (relative stereochemistry is shown for compounds **3** and **5**)

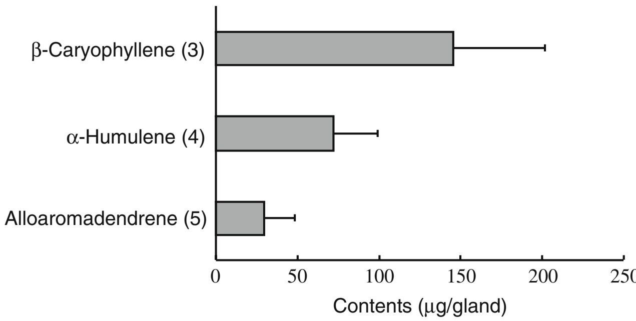

Fig. 4 Mean amounts (±SE) of rectal sesquiterpenoids in 9 *Bactrocera correcta* males captured in Bangkok, Thailand (November 24, 2006)

in each male differed from each other and could be classified roughly into two types: a "β-caryophyllene + α-humulene + alloaromadendrene (**3** + **4** + **5**)" type, in which **5** was frequently the most abundant component (e.g., a-c in Fig. 2), and a "β-caryophyllene + α-humulene (**3** + **4**)" type, in which **3** was greater in quantity (e.g., d-f in Fig. 2). In both types, trace amounts (<1.0 μg/male) of ME-metabolites **1** and **2** were detected. In contrast, some males (e.g., g and h in Fig. 2) possessed large quantities of **1** and/or **2**, but only trace amounts of sesquiterpenes. A small amount of unmetabolized ME was detected in gas chromatograms "g" and "h" (whole body extracts) (Fig. 2).

The above quantifications were carried out using whole body extracts of males. In order to determine the reservoir site of the sesquiterpenes, the rectal gland was dissected from 5 males of *B. correcta* captured in Bangkok (November 24, 2006), and analyzed by GC-MS. All rectal glands possessed ME metabolites **1** and **2** in varying quantities, ranging from 0.1 μg to 10 μg/gland, and sesquiterpenes **3**, **4**, and **5** in much larger quantities (over 250 μg total/gland; mean ± SD/gland: β-caryophyllene (**3**), 134.5±84.5 μg; α-humulene (**4**), 86.6±64.2 μg; alloaromadendrene (**5**), 41.8±42.5 μg; total, 262.1±115.5 μg, N=5].

Incorporation of ME Metabolites and Sesquiterpenes In order to determine a fly's ability for accumulating sesquiterpenes and ME-metabolites, laboratory-raised males of *B. correcta* were fed with a mixture of ME, β-caryophyllene (**3**), and α-humulene (**4**), in a ratio of 1:2:2. Males incorporated the sesquiterpenes (**3** and **4**), together with ME metabolites (**1** and **2**), in an approximate ratio of total ME metabolites: β-caryophyllene: α-humulene of 0.8: 2: 2 (Table 1). No other compounds derived from the sesquiterpenes were detected.

Field Traps with Sesquiterpenes and Rectal Volatile Composition of Wild Males A 1:1 mixture of β-caryophyllene and α-humulene was tested, along with ME-baited traps, in a guava orchard in Thailand (July 24 and August 24, 2009).

Table 1 Phenylpropanoid metabolites and sesquiterpenes in the whole body extracts of *Bactrocera correcta* males fed on a mixture of methyl eugenol, β-caryophyllene (**3**) and α-humulene (**4**) (N=7)

Compound	Content (μg/male) ± SD
(*Z*)-Coniferyl alcohol (**1**)	11.0±3.8
(*Z*)-3,4-Dimethoxycinnamyl alcohol (**2**)	1.3±1.1
β-Caryophyllene (**3**)	31.0±5.4
α-Humulene (**4**)	30.6±9.3

The catch (3) of male *B. correcta* in the sesquiterpene-baited traps was low relative to that (43) in the ME-baited traps. No males of *B. dorsalis* were caught in the sesquiterpene-baited traps, while 9 males of this species were caught in ME-baited traps. The same experiment was conducted in a mango orchard on July 3, 2009, but no *B. correcta* males were trapped, while 15 *B. dorsalis* males were captured only in ME-baited traps.

The whole body extract of individual flies was subjected to GC or GC-MS analysis. Among 19 *B. correcta* males captured alive in the ME-baited traps on July 24 and August 24, 2009, 17 possessed large quantities of compounds exhibiting either "**3** + **4**" or "**3** + **4** + **5**" type patterns; the remaining 2 flies contained only trace, but significant, amounts of "**3** + **4**" or "**3** + **4** + **5**" mixtures. Of two males collected alive in the sesquiterpene-baited traps on August 24, one possessed substantial quantities of compounds **1**, **3**, and **4**, and the other had only trace amounts of the "**3** + **4**" pattern. Among the flies captured in orchards, some males possessed minor components, tentatively identified as β-elemene and germacrene D by retention index values (1402 and 1495, respectively) and MS fragmentation patterns. In contrast, 7 males of *B. dorsalis* captured on July 3 at a mango orchard, and 9 males captured in a guava orchard on July 24 and August 24 possessed no sesquiterpenes.

Morphological and DNA Analyses There were no morphological differences among the 3 groups (i.e., laboratory strain and the two collections of wild flies in Bangkok and the guava orchard) of *B. correcta*. The DNA sequences of *B. correcta* captured in the field-trapping test were analyzed and compared to those from laboratory-raised *B. correcta* and *B. dorsalis* (Tan et al., unpublished). The alignment of rDNA fragments indicated that the 3 groups belonged to the same clade of *B. correcta* species and differed from the distinct clade of *B. dorsalis* (Fig. 5).

Discussion

In laboratory tests, we characterized the production of two phenylpropanoid volatiles, (*Z*)-coniferyl alcohol (**1**) and

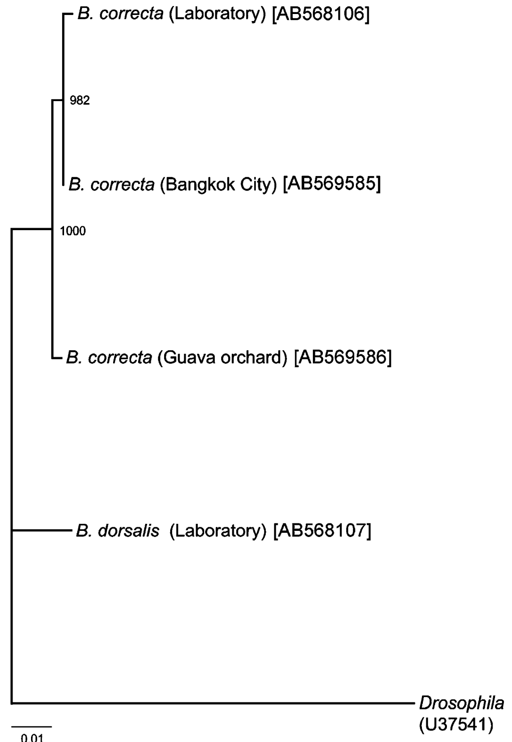

Fig. 5 Phylogenetic relationships resulting from the neighbor-joining analysis of rDNA sequences. The values shown at the nodes of the branches are the confidence levels from 1000 replicate bootstrap samplings. The scale bars indicate the evolutionary distance between the groups. GenBank accession numbers are given for the members reported in this paper together with those from laboratory-raised *B. correcta* (Tan et al., unpublished) and the mitochondrial genes of *Drosophila melanogaster*

(*Z*)-3,4-dimethoxycinnamyl alcohol (**2**), sequestered in the rectal glands of males of *B. correcta* after feeding on ME. The production of these compounds contrasts (Fig. 6) with the situation in *B. dorsalis*, in which (*E*)-coniferyl alcohol (ECF) and 2-allyl-4,5-dimethoxyphenol (DMP) are the two major metabolites (Nishida et al., 1988a), although (Z)-3,4-dimethoxycinnamyl alcohol (**2**) was detected in several specimens of wild *B. dorsalis* as a very minor component (Nishida et al., 1988b). The total quantity of **1** and **2** sequestered in the rectal gland of *B. correcta* is over 100 μg/gland, a quantity similar to that of total ECF and DMP sequestered by male *B. dorsalis* supplied with ME as an intact oil. It has been demonstrated that ME metabolites are transported selectively via hemolymph to the rectal gland after biotransformation of ME in the crop in *B. dorsalis* (Hee and Tan, 2006). Our work suggests that similar, but different, enzymatic processes function in these two sympatric species.

ECF and DMP, sequestered in the rectal gland and subsequently released during courtship as "smoke", act as a sex pheromone that attracts conspecific females in *B. dorsalis* (Nishida and Fukami, 1990; Tan and Nishida, 1996, 1998; Hee and Tan, 1998; Khoo et al., 2000). The function of **1** and **2** in mating of *B. correcta* is unknown. Tests should be conducted to determine their attractiveness to conspecific females as well as their deterrence/non-responsiveness to *B. dorsalis*, and similarly with *B. dorsalis* ME-sequestered metabolites toward *B. correct*a females. The sequestered metabolites in *B. dorsalis* also play a role in defense, deterring or poisoning predatory animals, such as birds, lizards, and spiders (Nishida and Fukami, 1990; Tan and Nishida, 1998; Wee and Tan, 2001, 2005). The amount of sequestered phenylpropanoids in our trapped wild males of both species was often large (>50 μg/body). Thus, a possible allomonal function of these metabolites needs to be investigated.

Further, a large proportion of males in the wild population of *B. correcta* contained large quantities of a series of sesquiterpene hydrocarbons, including β-caryophyllene and α-humulene. As we did not detect these compounds in our laboratory-reared males (raised on an artificial diet during larval and adult stages), this suggested that adults might seek out and sequester these compounds. β-Caryophyllene and α-humulene are common plant volatile constituents, particularly in fruits that are major hosts of these flies, such as mango and guava (Nishimura et al., 1989; Tamura et al., 2000; Sandoval et al., 2007). Our studies confirmed this, with the laboratory-feeding test that demonstrated the ability of *B. correcta* males to sequester these sesquiterpenes, and the field trial that showed that *B. correcta*, but not *B. dorsalis*, males were attracted to a mixture of β-caryophyllene + α-humulene.

Fig. 6 Comparison of methyl eugenol metabolites accumulated in the male rectal gland of *Bactrocera correcta* and *B. dorsalis*

Methyl eugenol (ME)
B. dorsalis
4,5-Dimethoxy-2-allylphenol (DMP) + (*E*)-Conferyl alcohol (ECF)
B. correcta
(*Z*)-Conferyl alcohol (**1**) + (*Z*)-3,4-Dimethoxycinnamyl alcohol (**2**)

The GC-profiles of the major sesquiterpenes found in *B. correcta* males sorted into two distinct types (**3** + **4** + **5** or **3** + **4**), suggesting two distinct sources of these sesquiterpenes (including or excluding alloaromadendrene) that the respective insects had fed on. Both β-caryophyllene and α-humulene have been described as smelling "sweet fruity" to humans (Shivashankara et al., 2006). Ripe guava fruits produce more β-caryophyllene than do immature fruits (Chyau et al., 1992; Soares et al., 2007). Therefore, the rectal gland sesquiterpenes released by males during courtship may convey the presence of ripe fruits to females.

β-Caryophyllene and α-humulene were detected in a male *Bactrocera unimacula* captured on a fruit fly-attracting orchid flower, *Bulbophyllum vinaceum*, which produces ME and other phenylpropanoid volatiles (but not sesquiterpenes), in Borneo (Tan et al., 2006). One of the flies captured (on August 24, 2009) by the sesquiterpene mixture in the guava orchard was identified tentatively as *B. unimacula*. Males of *B. dorsalis* occurring sympatrically with *B. correcta* in Thailand did not seem to accumulate sesquiterpenes **3**, **4** and **5**, suggesting a different role of the rectal sesquiterpenes during intra- and/or inter-specific interactions. Sesquiterpenes are known to be associated with other tephritid fruit fly species. β-Caryophyllene has been reported following aeration of calling males of Mediterranean fruit fly, *Ceratitis capitata*, together with other terpenoids and esters (Gonçalves et al., 2006). Males of Caribbean fruit fly, *Anastrepha suspensa*, release a multi-component volatile pheromone including sesquiterpenes (Nation, 1990). The quantity of sesquiterpenes in *B. correcta* males is extraordinarily high (often over 250 μg/gland), which also suggests a possible role in defense. Further work is needed to clarify the pheromonal and/or allomonal roles of rectal sesquiterpenoids in conjunction with the unique phenylpropanoids (**1** and **2**) in *B. correcta*.

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a deterrent, or an anti-nutritional factor, could be involved in plant part discrimination.

Glucosinolates, amino acid-derived secondary metabolites primarily found in the mustard order Brassicales, are crucial host plant recognition cues for pierid butterflies such as the cabbage whites (*Pieris* sp.) and related species (Renwick and Chew, 1994; Hopkins et al., 2009). Glucosinolate containing plants often contain a mixture of different structures, metaphorically known as the glucosinolate 'profile', which often differ between plant organs (e.g., Agerbirk et al., 2008).

Differential insect behavioral responses and their correlations with variation in individual glucosinolates have been observed (e.g., under field conditions, Rodman and Chew, 1980; Griffiths et al., 2001; Bidart-Bouzat and Kliebenstein, 2008; under laboratory conditions, Huang and Renwick, 1994; Giamoustaris and Mithen, 1995; Li et al., 2000; Gols et al., 2008; de Vos et al., 2008; Sun et al., 2009, but see also Reifenrath et al., 2005; Reifenrath and Städler, 2009; Badenes-Pérez et al., 2010). In one case that involves a monophagous insect and a glucosinolate of unusual proposed structure, host plant preference was linked to presence of a distinct glucosinolate (Larsen et al., 1992). Thus, there may be a chemosensory basis for evolution of host plant or plant part discrimination based on differential sensitivity to individual glucosinolates (Hopkins et al., 2009). Phytophagous butterfly larvae also have sensory organs for various primary metabolites (Schoonhoven et al., 2005), some of which could be correlated to total nitrogen contents of plant parts.

The purpose of this work was to search for evidence for a chemical basis of the assumed plant part preferences of orange tip larvae, and to develop a bioassay for a hypothetic semiochemical responsible for floral parts feeding. We pursued this purpose in two parallel ways: (1) by observation of caterpillar feeding choice on original host plants and after transfer to various positions on *A. petiolata*, and (2) by chemical analysis of plants collected during the period of orange tip larval occurrence.

Methods and Materials

Identification of Plants and Animals Flowering *C. pratensis* and *A. petiolata* were identified in the field from their characteristic morphologies. Eggs of the orange tip butterfly were tentatively identified in the field, and the identity confirmed from comparison of the morphology of the resulting larvae with the same illustrated descriptions as used previously (Agerbirk et al., 2006). Selected larvae were reared to pupation to confirm the identification based on pupal morphology. Photographs of the studied material including egg and the transition from larva to pupa have been published elsewhere (Agerbirk and Jørgensen, 2008). Four pupae produced four female butterflies the following spring, all of which were confirmed to be *A. cardamines* by morphology.

Collection of Plants Plant shoots of flowering plants (*C. pratensis* cut above the rosette and *A. petiolata* cut below a number of fresh leaves) were collected in plastic bags at natural growth sites in May 2008 and transported to the laboratory within a few hours. Unless otherwise noted, all plants and animals were from Lake Utterslev Mose (= locality DK1), Copenhagen, Denmark. *A. petiolata* was collected within 20 m of the lake rim (e.g. 55 42′ 57″ N, 12 30′ 22″ E), while *C. pratensis* was collected at the public lawns ("Gyngemosen") NE of the lake and NW of Highway 16 (e.g. 55 43′ 19″N, 12 29′ 43″E). We also sampled *C. pratensis* floral parts from two other, previously described localities (DK2, DK3) (Agerbirk et al., 2010a). The individual plants DK3a and DK3b were the same as described in that paper and were dried without lyophilization as described in that paper.

Observation of Egg Position and Caterpillar Feeding Behavior Flowering shoots of *C. pratensis* and *A. petiolata* with orange tip eggs or neonate larvae were collected as above and kept in individual, numbered beakers with tap water in the laboratory, and exposed to sunlight through an east facing glass window. If the orange tip egg could be located, the position was registered. Most L1/L2 larvae collected (N=26) were subject to detailed observations on the original host plants. (However, L1/L2 larvae collected on days with experimental manipulation of larval position were used directly in the behavioral experiments, and hence not observed on the original host plants). The position and behavior of each larva, as well as signs of feeding, were observed and noted at least daily between Monday and Friday, and representative specimens were photographed in order to illustrate characteristic feeding patterns. The observations on original host plants typically were carried out for 2–3 d for an individual larva, after which they were moved to fresh plants to be used for experiments. It was not meaningful to standardize the observation period because the material was collected at different stages, including eggs, newly hatched larvae, and slightly older larvae.

One intact *C. pratensis* plant (on the flower of which oviposition by an orange tip was observed the day before), with its root system and approximately 1 l of attached soil+grass, was removed from the locality, and was cultivated in the laboratory during the entire larval development including hatching and pupation (May 7–24). The spontaneous feeding behavior of the resulting larva, which was not manipulated in any way, was observed 5 d a week. When the remains of the host plant had been left, *A. petiolata* was offered for

completion of the 5th instar and pupation (pupal wt. 112 mg), to confirm identification.

Rearing of Larvae for Behavioral Experiments As soon as or before original plants started to deteriorate visibly, larvae were gently transferred (with a moist paint brush) to fresh bolting *A. petiolata* shoots, collected at the locality 0–2 d before, and kept in the laboratory immersed in beakers with tap water. They were exposed to reduced daylight through an east facing window until use. This stock of larvae, supplemented with newly collected larvae of various instars, served as the material for additional behavioral experiments.

Behavioral Experiments For behavioral experiments, *A. petiolata* side branches with a specific morphology and growth stage were used, with leaves immediately next to the lower 1–2 siliques (Fig. 1) and with flowers with fresh petals. As the natural availability of branches with this morphology (including fresh flowers) ceased late in the experimental period, we ended this type of experiment even though additional late instars were available.

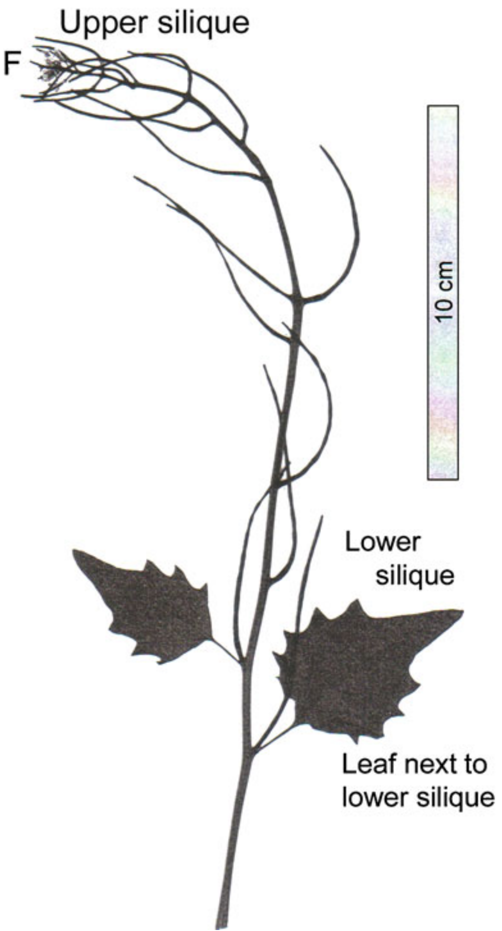


Fig. 1 *Alliaria petiolata* branch with siliques next to cauline leaves, as used for behavioral experiments with orange tip larvae. F=flowers

Two plant part preference experiments were carried out. The purpose of the 'leaf/silique choice experiment' was to test whether an absolute preference for silique feeding could be demonstrated in a choice situation, where both a silique and a leaf were within close proximity to the larva. Larvae of known instar were gently transferred to lower parts of *A. petiolata* branches (one larva per branch in separate beakers), with about half of the larvae of each instar placed on lower siliques and the other half placed on leaves immediately next to lower siliques (Fig. 1). The feeding during the 24 hr experimental period was registered by careful inspection of the branch for feeding traces at the end of 24 hr. Occasional observation of some larvae during the experiment also was carried out in order to qualify the subsequent inspection of the branch for feeding traces. Selected branches with larva and feeding traces were photographed with a background 5×5 mm grid after the experiment, and ingested amounts were estimated by comparison with un-touched leaves.

The purpose of the 'vertical position experiment' was to test whether the vertical position of the larva, either near the plant apex with flowers or at lower levels near the leaves, would influence the subsequent plant part feeding choice of the larva (as suggested by the outcome of the leaf/silique choice experiment). Larvae were placed either on the uppermost silique, near the flowers, or on the lower-most silique, next to a leaf (Fig. 1). Evaluation of the feeding choices was as described for the leaf/silique choice experiment.

Whether or not occurrence of leaf feeding depended on the initial position of the larva was tested by Rice's conditional binomial exact test (Rice, 1988) of 2×2 matrixes with leaf feeding vs. no leaf feeding as a function of larval position. (Fisher's exact test gave the same pattern of significance vs. non-significance). The significance level was set at 5% (two-tailed test), and results for L1-L4 were pooled because there was no indication of differences among these instars. Whether or not flower feeding depended on initial larval position was tested in the same way.

Plant Sampling and Dissection for Chemical Analysis - Plants for chemical analysis were sampled (in parallel to larvae and plants for behavioral experiments) at the main study locality (DK1) in May (2008), which was the oviposition and larval feeding period of orange tip butterflies at the locality. Plants for chemical analysis were dissected and lyophilized immediately after arriving in the laboratory; the time from collection in the field to dissection and lyophilization was 3 hr or less. In dissection for chemical analysis, upper stem was defined as the upper inflorescence (with flowers) and included flower stalks; middle stem was defined as the lower inflorescence (with

siliques) and included silique stalks; and lower stem was defined as the basal part of the stem, from the lowermost silique downwards. Leaves were cauline (stem) leaves, not rosette leaves. Siliques and flowers included only these organs, as the stalks attaching them to the stem were included with stems. For nitrogen analysis of specific plant organs, we aimed at pooling only from the same individual, but in the case of flowers, siliques, and upper stems, the samples in most cases had to be pooled from several individual plants to provide sufficient amounts for the analysis.

Determination and Identification of Glucosinolates Glucosinolates were determined by extraction of lyophilized plant parts in boiling 70% MeOH, binding to anion exchange columns, enzymatic desulfation, elution (in 5×1 ml H_2O) and subsequent HPLC with diode array detection of desulfo derivatives relative to an external standard of sinigrin (**15**) treated similarly in parallel (Agerbirk et al., 2007). The exact HPLC conditions were different for the two plant species: HPLC conditions for *C. pratensis* samples were optimized specifically to achieve separation of all glucosinolates known from this species (Agerbirk et al., 2010a). For *A. petiolata*, a Supelcosil LC-ABZ column, 25 cm× 4.6 mm, 5 µm, was used with flow rate 1 ml/min, and elution by 2 min of H_2O followed by a 48 min linear gradient from 0 to 60% MeOH, a brief wash with MeOH, and equilibration with H_2O. Peak identification by comparison with authentic reference compounds supplemented by LC-MS of selected samples was as previously described (Agerbirk et al., 2010a). Glucosinolate levels in *A. petiolata* were $\log_{10}$-transformed to remedy non-normal distribution of the original data, and subjected to statistical analysis. Due to unexpected occurrence of several chemotypes of *C. pratensis* with qualitative differences among them, the various chemotypes were reported separately, and quantitative statistical tests of glucosinolate levels in this plant were not considered meaningful.

Nitrogen Determination Total nitrogen (and carbon) contents of lyophilized samples from locality DK1 were determined on an elemental analyzer (Carlo Erba model NA 1500, Carlo Erba, Milan, Italy) in the laboratory of Jeffrey Dukes at University of Massachusetts, Boston, MA, USA. Two samples (*A. petiolata* leaves,%N 3.27, and upper stems,%N 5.99) deemed unreliable by the analysis-lab due to abnormal values for associated standards were excluded from the data and calculations. Numbers for %N were converted to proportions (0.00–1.00), which were arcsin transformed to remedy non-normal distribution of the original data.

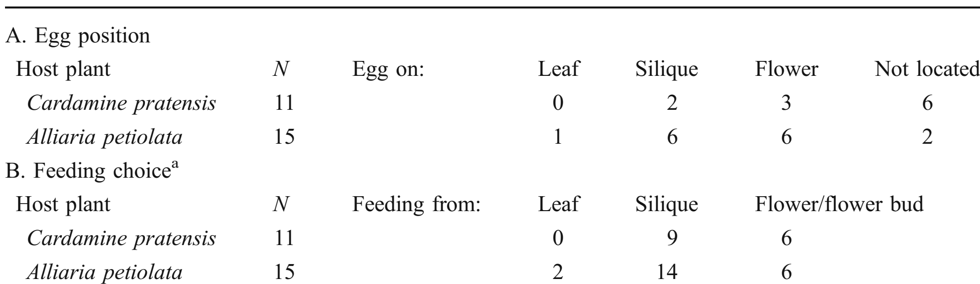
Statistical Analysis of Chemical Data Levene's test was used to confirm that transformed glucosinolate and nitrogen levels met the variance homogeneity assumptions of ANOVA. Differences between nitrogen and glucosinolate levels among plant parts within each species were examined by ANOVA. Where the ANOVA was significant, unplanned multiple comparison among plant parts was made using Scheffé *post-hoc* test with significance level set at 0.05. In one case with $N=1$ for one plant part, this plant part was excluded before ANOVA with *post-hoc* test.

Results

Oviposition Site Almost all eggs or egg shells located on *C. pratensis* or *A. petiolata* were on siliques or flowers, including their basal parts, while a single egg was observed on a leaf close to a silique (Table 1A). Usually, only a single egg or larva was observed per plant, but in some cases two (Fig. 2) or even three orange tip eggs or larvae were present naturally.

Plant Part Choice on Original Host Plants Feeding habits of L1/L2 instars on the original host plants nearly always (88%) included silique feeding. Flower feeding also was frequent (46%), while only few larvae ate leaves at all (Table 1B) (but one fed exclusively on a leaf during the first and the initial part of the 2nd instar). The flower feeding behavior by early instars on *C. pratensis* was quite stereotypic, with the feeding starting from the basal part of the flower (Fig. 2) whether or not the flower was open. Floral parts egg position and feeding by orange tips was obviously dominant for both host plant species, and it was

Table 1 Egg position and feeding choice by orange tip L1/L2 larvae hatched on original host plant shoots



A. Egg position						
Host plant	*N*	Egg on:	Leaf	Silique	Flower	Not located
Cardamine pratensis	11		0	2	3	6
Alliaria petiolata	15		1	6	6	2
B. Feeding choice[a]						
Host plant	*N*	Feeding from:	Leaf	Silique	Flower/flower bud	
Cardamine pratensis	11		0	9	6	
Alliaria petiolata	15		2	14	6	

[a] The observation period was restricted to the L1 and L2 instars and typically 2–3 days

Fig. 2 Second instar orange tip larvae ("L") feeding on flowers of *Cardamine pratensis*. The entire flower was usually eaten, starting from the outside of basal parts as illustrated ("Feeding damage")

not considered relevant to test statistically whether slight differences between patterns on the two plant species existed. For comparison of plant part distribution with published field data, see "Discussion".

The feeding behaviour of a single larva through all five instars on an intact *C. pratensis* plant complemented the observations. In the first three instars (L1-L3), the larva ate flowers and siliques. The 4th instar (L4) also ate upper stem, and 5th (final) instar (L5) ate the remaining stem except the lower 9 cm (ca. one third) as well as all cauline leaves and part of the rosette leaves.

Leaf/Silique Choice Experiment The probability of leaf feeding was not significantly different for larvae placed initially on a leaf vs. larvae placed initially on a nearby silique (P=0.21). Irrespective of their initial position, larvae frequently ingested leaves as well as siliques (Table 2A). The amounts of leaf ingested were typically substantial i.e., more than the minute test bites seen when another species of insect larvae probed unacceptable *Barbarea vulgaris* plants (Agerbirk et al., 2003). Flower feeding was infrequent for L2-L4 instars in this experiment. Apparently, the frequency of feeding on each plant part was a consequence of a limited mobility within 24 hr, and was sufficient to reach the leaf even when they were placed at the silique and vice-versa, but was generally insufficient to reach the more distant flowers.

A single L5 larva was included in the experiment, but was not included in the pooled results for statistical testing due to its atypical behavior: The individual was placed on the leaf next to the lower silique, from which approximately 3 cm^2 (ca. 1/5 of the entire leaf) was eaten. Rather than eating the remaining leaf or neighboring silique, it moved overnight to the flowers and upper siliques, which were ingested entirely. This observation led us to include as many L5 larvae as available in the vertical position experiment.

Vertical Position Experiment This experiment, in which larvae were placed on either lower or upper siliques, confirmed the importance of larval position inferred above. Young to intermediate instars (L1-L4) placed near leaves exhibited frequent leaf feeding (50%) and no flower feeding, while larvae placed near flowers exhibited frequent flower feeding (45%) and no leaf feeding (Table 2B). The probability of leaf feeding was significantly higher when larvae were placed on lower siliques than when placed on upper siliques (P=0.01). Likewise, the probability of flower feeding was significantly higher when larvae were placed on upper siliques compared to lower siliques (P=0.015). Based on the combined experiments, we concluded that any preference of L1-L4 larvae for floral parts (siliques and flowers) would be too weak to be of practical use in a bioassay for a hypothetic stimulant or deterrent. Indeed,

Table 2 Feeding choice by orange tip larvae during 24 hr after experimental manipulation of larval position

Instar	Placed at	*N*	Feeding from:	Leaf	Silique	Flower/flower bud
A. Leaf/silique choice experiment: larvae placed on either lower *Alliaria petiolata* silique or the leaf immediately next to it						
L2-L4	Lower silique	10		5	7	0
	Leaf at silique	6		5	4	1
L5	Leaf at silique	1		1	1	1[a]
B. Vertical position experiment: larvae placed on either lower *Alliaria petiolata* silique (at leaf) or upper silique (at flowers)						
L1-L4	Silique at leaf	10		5	8	0
	Silique at flowers	11		0	9	5
L5	Silique at leaf	4		1	4	3[a]
	Silique at flowers	3		0	3	3[a]

[a] Ate all flowers as well as flower-supporting part of stem

For statistical evaluation: See text

feeding choice by young and intermediate instars (L1-L4) seemed to be governed mainly by proximity, at least under our laboratory conditions.

Seven available L5 larvae also were included in the vertical position experiment, and all except one (from lower silique) moved to flowers and ingested the entire upper part of the inflorescence within 24 hr, while only a single had eaten from leaves (Table 2B). For this instar, the probability of flower feeding did not depend on the initial position of the larvae ($P=0.94$). Despite the low number of replicates, we interpret this result as a tendency for L5 instars to prefer floral parts.

Glucosinolate Profiles of Leaves and Floral Parts The two main hosts of orange tip butterflies at the DK1 locality, an urban lake habitat in the greater Copenhagen area, appeared to be *A. petiolata* and *C. pratensis*. There was no difference in the kinds of glucosinolates in vegetative or floral parts of *A. petiolata*, but when the levels were compared, there was a significant effect of the plant parts in statistical analysis by ANOVA. Flowers and upper stems contained higher levels of the major glucosinolate sinigrin (**15**) and the phenolic indole glucosinolate **12** (with proportional traces of **10**) relative to leaves (Table 3, Fig. 3).

In the other common host plant at the locality, *C. pratensis*, the mixture of glucosinolates was more complex. Plants from the main locality (DK1) contained three dominant glucosinolates – the phenolic glucosinolate sinalbin (**8**), the *O*-methyl derivative **9,** and the hydroxylated aliphatic glucosinolate **4** – and a number of minor glucosinolates including the indole glucosinolate **10** (Table 4, Fig. 3). The glucosinolate profiles of floral and vegetative parts were similar, with no obvious indications of a particular glucosinolate profile or higher glucosinolate level of floral parts.

As a hypothetic floral parts signature was expected to be of general nature, we included floral parts of the same plant

Table 3 Glucosinolate profiles (μmol/g dry wt., mean (s.d.)) of floral and vegetative parts of *Alliaria petiolata* in May

Plant part	**10**	**12**	**15**	*N*
Flowers	0.29 (0.05)	4.6 (0.6) a	73.8 (11.1) ab	4
Siliques	0.03 (0.03)	0.8 (0.8) b	31.6 (20.7) bc	3
Leaves	0.02 (0.02)	0.1 (0.1) b	35.0 (2.6) c	3
Upper stem	0.17 (0.02)	4.3 (0.9) a	124.9 (11.5) a	2
Middle stem	n.d.	0.3 (0.1) b	14.4 (4.4) c	3
Lower stem	n.d.	0.1 (0.0) b	0.9 (0.5) d	3
Significance	–	***	***	

n.d. not detected

Statistical significance of differences in major glucosinolates between plant parts were tested by ANOVA ($P<0.001$: ***, $P<0.01$: **, $P<0.05$: *, $P>0.05$: ns, not tested: -). Significant differences in a *post-hoc* Scheffé test ($P<0.05$) are indicated with different letters

species from other localities (DK2, DK3). Floral parts of individual *C. pratensis* plants from these localities showed qualitative differences from both floral parts and leaves at the main locality in terms of glucosinolate profiles. In a plant from locality DK2, *sec*-butylglucosinolate (**3**) rather than the hydroxy derivative **4** was the dominating aliphatic glucosinolate in flowers. At locality DK3, flowers of two individual plants had glucosinolate profiles that deviated even more from those at locality DK1, as the methylated aromatic glucosinolate **9** was nearly absent (Table 4). Plant DK3a accumulated benzylglucosinolate (**7**) and a short chain hydroxylated aliphatic glucosinolate (**2**) in flowers while both **3** and **4** were absent. In contrast, plant DK3b accumulated the non-hydroxylated **3** but not **7**. The distinctive profiles of floral parts from localities DK2 and DK3 were similar to leaf glucosinolate profiles of the same plants reported elsewhere (Agerbirk et al., 2010a).

Nitrogen Levels in Different Plant Parts In *A. petiolata*, total N content was higher in upper stems than in leaves, and there was also a tendency for high N levels in flowers. In the case of *C. pratensis*, however, there was no statistically significant difference in N content among floral parts and leaves (Table 5).

Discussion

An initial purpose of the behavioral experiments was to establish a bioassay for a hypothetic semiochemical (Agerbirk et al., 2003; Miles et al., 2005; Nielsen et al., 2010) responsible for floral parts feeding. Branches of *A. petiolata* (Fig. 1) with adjoining lower siliques and upper leaves (in contrast to the distance between these organs on the main stem), as well as a controlled laboratory environment, were used in an attempt to maximize the sensitivity for any larval preference. However, the larval feeding behavior seemed to be governed mainly by the position of the larvae in laboratory experiments with young and intermediate instar larvae (L1-L4). We believe that the laboratory test situation was relatively similar to the natural situation in terms of physical conditions and plant chemistry because relatively fresh *A. petiolata* branches were used. It can be argued that biochemical changes in the detached *A. petiolata* branches kept in the laboratory may have compromised a semiochemical responsible for plant part preference. Obvious consequences of the laboratory rearing at lower light intensity and significantly less UV light than in the field could be decreases in levels of photosynthetic products or UV induced metabolites with a significant turnover rate. Indeed, a recent experiment used for demonstrating specific movement to flowers of third

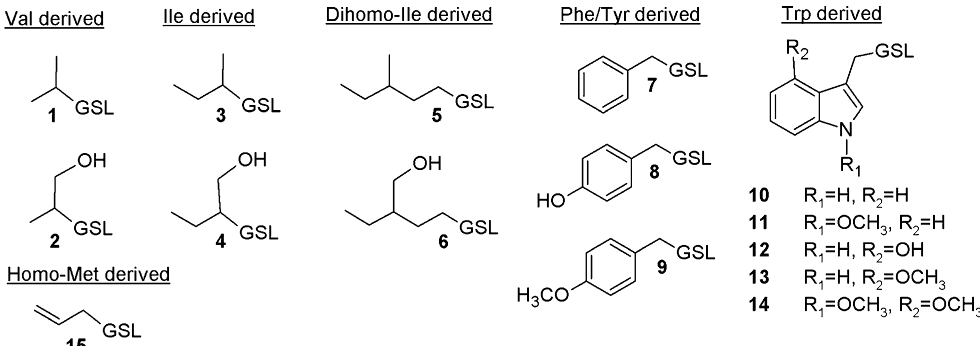


Fig. 3 Glucosinolates detected in *Cardamine pratensis* or *Alliaria petiolata* from the investigated localities, and two glucosinolates (**6** and **14**) known from other accessions of *C. pratensis* but not detected in this investigation. GSL: The constant part of the glucosinolate molecule, C (SGlc)$NOSO_3^-$. Systematic (and common names, if in general use) of the glucosinolates (GSLs) are: **1**, 1-methylethylGSL (isopropylGSL); **2**, 1-(hydroxymethyl)ethylGSL; **3**, 1-methylpropylGSL (*sec*-butylGSL); **4**: 1-(hydroxymethyl)propylGSL; **5**, 3-methylpentylGSL; **6**, 3-(hydroxymethyl)pentylGSL; **7**, benzylGSL (glucotropaeolin); **8**, 4-hydroxybenzylGSL (sinalbin); **9**, 4-methoxybenzylGSL; **10**, indol-3-ylmethylGSL (glucobrassicin, GB); **11**, 1-methoxy**10** (neoGB); **12**, 4-hydroxy**10** (4-hydroxyGB); **13**, 4-methoxy**10** (4-methoxyGB); **14**, 1,4-dimethoxy**10** (1,4-dimethoxyGB), **15**, 2-propenylGSL (sinigrin)

instar *Pieris brassicae* (Smallegange et al., 2007) involved intact plants (in a greenhouse). However, the feeding on detached original host plants kept in the laboratory was almost exclusively from floral parts, suggesting that even if plant biochemistry changed due to the laboratory conditions, the positional effect was sufficient to enable the larvae to behave as in the field. Hence, biochemical changes due to the laboratory conditions are not likely to have influenced the plant part choices of the larvae. In agreement with this argument, the spontaneous behavior of young larvae, when their position had not been manipulated, agreed well with published field observations (Wiklund and Åhrberg, 1978; Courtney, 1981; Dempster, 1997), except that Dempster (1997) described floral feeding to precede silique feeding in general on *C. pratensis*. This slight difference from our observations may depend on local conditions such as relative phenologies of insect and host plant (Wiklund and Friberg, 2009).

Table 4 Glucosinolate profiles (µmol/g dry wt.) of floral and vegetative parts of *Cardamine pratensis* plants from three localities in May

Locality:	DK1						DK2		DK3a	DK3b
Plant part:[a]	Flo.	Sil.	Lea.	Stem			Flo.	Sil.	Flo.	Flo.
			Upper	Upper	Middle	Lower				
Glucosinolate										
Aliphatics										
1	tr.	0.1	0.2	0.1	0.1	0.2	0.5	0.7	0.2	0.3
2	1.5	1.9	2.3	2.2	1.9	1.9	0.1	0.1	**4.7**	0.1
3	0.2	0.1	0.2	0.6	0.5	0.3	**9.4**	**12.1**	n.d.	**7.2**
4	**2.9**	**3.0**	**5.1**	**5.9**	**4.6**	**3.8**	n.d.	n.d.	n.d.	tr.
5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	tr.
Aromatics										
7	tr.	n.d.	tr.	n.d.	tr.	n.d.	tr.	n.d.	**13.7**	n.d.
8	**6.0**	**7.5**	**26.3**	**7.2**	**4.6**	**4.0**	**10.8**	**10.2**	**10.2**	**14.4**
9	**6.0**	**6.0**	**4.1**	**13.6**	**9.6**	**8.7**	**15.8**	**16.6**	0.2	0.1
10	0.6	0.6	0.2	0.7	0.5	0.6	0.7	0.9	0.6	0.4
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr.	n.d.	n.d.
12	n.d.	n.d.	n.d.	n.d.	tr.	tr.	tr.	tr.	0.1	0.1
13	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	tr.
Not identified	1.0	1.1	2.5	1.0	0.6	0.5	0.2	0.1	0.1	0.7
Total	18.3	20.3	40.9	31.3	22.5	19.9	37.7	40.9	29.8	23.4
SD of total	1.7	1.0	8.9	–	–	–	–	–	–	–
N	2	3	3	1	1	1	1	1	1	1

Bold: More than 10% of total glucosinolate level in that plant part. Glucosinolates **6** and **14**, known from other accessions of *C. pratensis*, were not detected in any sample

[a] Flo.: Flowers; Sil: Siliques; Lea.: Leaves

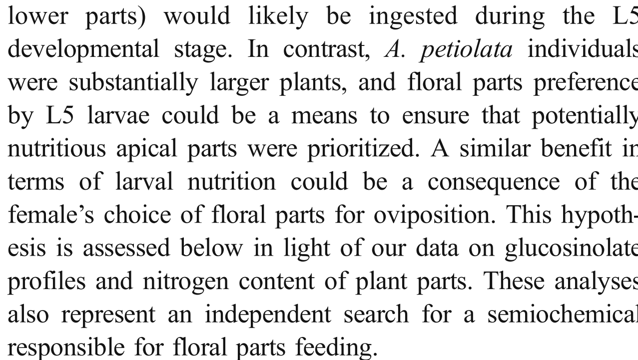
Table 5 Nitrogen contents (% n wt./dry wt.) of floral and vegetative parts of two common host plants in May

	Alliaria petiolata		*Cardamine pratensis*	
Plant part	Mean (SD)	*N*	Mean (SD)[a]	*N*
Flowers	5.10 (0.17) ab	3	2.76 (0.45) a	3
Siliques	4.37 (0.22) b	3	3.34 (0.30) a	3
Leaves	3.76 (0.73) b	2	3.00 (0.45) a	4
Upper stem	6.63 (0.58) a	2	3.10 (0.17) a	2
Middle stem	2.97 (0.64) c	3	2.15 (0.51) ab	3
Lower stem	1.54 (–) –	1	1.19 (0.05) b	2
Significance	***[a]		***	

Statistical significance of differences in major glucosinolates between plant parts were tested by ANOVA ($P<0.001$: ***, $P<0.01$: **, $P<0.05$: *, $P>0.05$: ns). Significant differences in a *post-hoc* Scheffé test ($P<0.05$) are indicated with different letters. [a] The level of significance was *** whether or not lower stem was excluded in ANOVA.

The feeding behavior of the single larva followed through all instars on the same plant immediately suggested a biological advantage for flexible feeding behavior. The inflorescence of the *C. pratensis* individual chosen by the female was too small to support full development of the larva if only siliques and flowers were ingested. Larval migration to other plants would be needed if only floral parts were accepted, but such movement to other, perhaps distant plants poses an obvious risk (Wiklund and Åhrberg, 1978; Dempster, 1997). Hence, utilization of vegetative parts would allow larvae to avoid or delay risky migration. Although this observation of a single larva can be dismissed due to lack of replication, we find it of interest and worthy of additional investigation as a complement to the traditional assumption of the existence of leaf avoidance (Wiklund and Åhrberg, 1978). The outcome of our experimental manipulations and the observation of occasional leaf feeding by young larvae on original host plant shoots (Table 1) confirm the observed spontaneous leaf acceptance by orange tip larvae.

The apparent preference for flowers by L5 larvae was reminiscent of a similar behavior reported for *Pieris brassicae* L3 and *Athalia rosae* L4 larvae (Smallegange et al., 2007; Bandeili and Müller, 2010), and suggests that proximity was not the only factor controlling orange tip L5 larval feeding. The most direct advantage of the tendency for feeding from the top would be to avoid accidental cutting of the main plant axis by the vigorously feeding L5 instars, which would lead to loss of the upper parts of the plant. As usual for late instar caterpillars (Theunissen et al., 1985), L5 larvae ingested a tremendous amount of material. In this case it was comparable to the remaining parts (after feeding by previous instars) of the relatively small *C. pratensis.* Thus, a strong plant part preference might have little effect on this plant species, because the majority of the plant (except tough


lower parts) would likely be ingested during the L5 developmental stage. In contrast, *A. petiolata* individuals were substantially larger plants, and floral parts preference by L5 larvae could be a means to ensure that potentially nutritious apical parts were prioritized. A similar benefit in terms of larval nutrition could be a consequence of the female's choice of floral parts for oviposition. This hypothesis is assessed below in light of our data on glucosinolate profiles and nitrogen content of plant parts. These analyses also represent an independent search for a semiochemical responsible for floral parts feeding.

A comparison of the glucosinolate profiles of vegetative and floral parts of two host plant species revealed no consistent floral parts glucosinolate profile. Based on the analyses of the chemically simple *A. petiolata*, a preliminary hypothesis for a hypothetic glucosinolate 'signature' or profile of floral parts could be a higher total level of glucosinolates, or a higher level of indole glucosinolates (**10**–**14**) or perhaps of aromatic (**7**–**14**) or phenolic (**8**+**12**) glucosinolates in general. Based on the literature, a different balance of aromatic vs. aliphatic glucosinolates (van Loon et al., 1992; Huang and Renwick, 1994) or of *O*-methylated vs. non-substituted aromatics (Sun et al., 2009) would be candidate signatures that could possibly be distinguished by insect sensory organs. Based on separately reported analytical chemistry research (Agerbirk et al., 2010a), we obtained reliable glucosinolate profiles of *C. pratensis* floral parts and leaves. However, none of the hypotheses of a floral parts 'signature' was supported when data for three populations of *C. pratensis* were considered. From the data in Table 4, a tendency for higher levels of **10** in floral parts than in leaves is suggested, but levels of **10** were higher in leaves than in floral parts in the individual plants from locality DK2 and 3 (Agerbirk et al., 2010a; unpublished results), so a hypothesis of a role of **10** was not generally supported. Indeed, the difference in glucosinolate profile between the two species and between individuals from different populations of *C. pratensis* appeared to be much greater than any systematic differences between vegetative and floral parts (Table 4). Consequently, there appears to be no basis for using glucosinolate profiles for larval distinction of floral parts from leaves.

A similar glucosinolate profile of flowers and vegetative parts, with a general tendency for higher levels in flowers of undamaged plants, also had been reported from *Arabidopsis thaliana*, *Raphanus sativus*, *Brassica nigra,* and *Sinapis alba* (Brown et al., 2003; Strauss et al., 2004; Smallegange et al., 2007; Bandeili and Müller, 2010). Glucosinolates were constitutively high but generally less inducible in radish flowers compared to leaves, as expected for an organ with high fitness value (Strauss et al., 2004). Our data do not exclude the possibility that floral parts could be deficient in the enzyme myrosinase (which converts glucosinolates to defensive products such as

isothiocyanates), but a recent report demonstrated high levels of myrosinase in floral parts of the crucifer *S. alba* (Bandeili and Müller, 2010), showing that floral parts high in glucosinolates may indeed also be high in myrosinase.

It was a surprise to discover that *C. pratensis* plants from different localities had different glucosinolate profiles; a more extensive investigation of glucosinolate variation in the species is published separately (Agerbirk et al., 2010a). Two subspecies of *C. pratensis* with different chromosome numbers had different probabilities of oviposition by orange tip butterfly females in Sweden (Arvanitis et al., 2007, 2008), but the *C. pratensis* populations at three Danish localities investigated here had identical chromosome numbers (Agerbirk et al., 2010a).

In *A. petiolata*, there was a tendency for higher N contents in upper parts, although the difference from leaves was statistically significant only for upper stems (which were frequently eaten by late instar orange tip larvae). Given the low number of replicates, this result should be interpreted with caution. However, the *A. petiolata* parts high in N were also high in glucosinolates and were both from the plant apex, suggesting that the measured tendency reflects a real biological phenomenon. As nitrogen is considered to be a limiting resource for herbivorous insects (Mattson, 1980), floral parts feeding may thus be a nutritional advantage in the case of feeding on *A. petiolata*. Position-dependent levels of nitrogen and glucosinolates also have been reported by Traw and Feeny (2008) for various leaf-positions of *Brassica nigra* and *B. kaber* (syn. *Sinapis arvensis*). However, in the case of *C. pratensis*, floral parts did not have higher total N than leaves (Table 4). Hence, our data do not support the hypothesis that floral parts feeding in general provide a nutritional advantage for orange tip larvae in terms of N content.

Nutritionally available N can only be approximated by total N if the majority of plant N is in a form available for protein or nucleotide biosynthesis; N in secondary metabolites may not be available for such biosynthesis. Intake of nitrogen in glucosinolates by orange tips and related species is balanced 1:1 by excretion of nitrogen in a nitrile (Agerbirk et al., 2006), a nitrile-derived functional group (Vergara et al., 2006; Agerbirk et al., 2007), or possibly inorganic ammonia from hydrolysis of nitrile groups (Agerbirk et al., 2010b). However, even a high glucosinolate level of 100 μmol/g dry wt. would correspond only to 0.14% N for non-indoles (and 0.28% N for the indoles **10**–**14**), so glucosinolate N was only a low fraction of the total N content of any plant part and total N could be regarded as a proxy of nutritionally available N. This result agreed with another investigation (Traw and Feeny, 2008).

In summary, we found a tendency for floral parts preference for L5 larvae, but no evidence for any L1-L4 larval behavioral preference for floral parts. For a classical example of a floral parts feeding butterfly, it was surprising that the feeding preferences of the larvae were so unspecific compared to the recently discovered within-plant selective foraging by intermediate to late instar *P. brassicae* and *A. rosae* (Smallegange et al., 2007; Bandeili and Müller, 2010). If the tendency of late instars to move to flowers is a real phenomenon, it still is not certain that a chemical signal is involved; we did not, for example, test the effect of positioning the inflorescences up-side down (Bandeili and Müller, 2010). Much of the natural tendency for young orange tip larvae for floral parts feeding can apparently be attributed to the choice of floral parts for oviposition by female butterflies (Wiklund and Åhrberg, 1978). Having established that floral parts are as rich and diverse in glucosinolates as vegetative parts, the wide range of host plants used by orange tip butterflies may imply that there is no basis for selection of a chemically less well defended plant part (Courtney and Chew, 1987). However, flower feeding recently has been demonstrated to lead to faster growth of two species of glucosinolate adapted larvae (Smallegange et al., 2007; Bandeili and Müller, 2010), supporting a hypothesized overall nutritional or micro-environmental benefit of feeding on floral parts.

Alternative explanations for flower oviposition in *Anthocharis* could be phylogenetic conservatism (the entire clade of anthocharines and euchloeines oviposits on floral and fruiting parts), perhaps due to mutual dependency with other behavioral patterns such as the “red egg syndrome” (Shapiro, 1981; Nomakuchi et al., 2001) or an optimized host plant search strategy: exclusive investigation of flowers may save valuable time during the oviposition period or allow the search for nectar source plants (Wiklund and Åhrberg, 1978) and plants for oviposition to be combined. The present demonstration of flexible but position-dependent larval plant part choice, glucosinolate diversity in floral parts and variable but relatively high N levels in floral parts underlines the importance of the positioning of eggs by ovipositing females, and shows that crucifer floral parts may be as diverse and well defended as crucifer foliage.

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soccata, but populations may survive on wild graminaceous plants when sorghum is not available (Padmaja et al., 2010).

In India, the losses due to *A. soccata* damage have been estimated to reach as high as 90% of grain, and 45% of fodder yield (Sukhani and Jotwani, 1980; Jotwani, 1982). The annual economic losses in sorghum due to this pest have been estimated at US$200 million (ICRISAT, 1992). The pest is especially serious in late-sown crops, but sometimes also appears with early sowing, when the preceding dry season is interrupted by frequent rain showers (Nimbalkar and Bapat, 1987). The levels of infestation may go up to 90–100% under delayed sowing (Hiremath and Renukarya, 1966). Although these are old estimates, similar yield losses occur even today because of the lack of acceptable levels of genetic tolerance/resistance to this insect pest in parental lines, compounded further by cost constraints that limit the use of insecticides by farmers in developing countries.

Odors emanating from crops play a role in the orientation of insect pests towards their host plants and in recognition of these plants as sites for feeding and oviposition (Visser, 1986; Bruce et al., 2005). Thus, knowledge of the volatile compounds emitted by *S. bicolor* and insect responses to them is needed in the study of insect pest-sorghum plant relationships. Field observations of insect trap catches have suggested that *A. soccata* females are attracted to volatiles emitted by shoot fly susceptible seedlings but not resistant seedlings, and it has been shown that trap catch varies according to the growth stage of the plant (Nwanze et al., 1998). However, the semiochemicals involved have not been identified previously. Therefore, the objective of the present study was to identify plant-derived attractants (kairomones) for *A. soccata.*

Methods and Materials

Insect Rearing The culture of *A. soccata* used in the experiments was established with field collected insects from the Directorate of Sorghum Research (Hyderabad, India). *A. soccata* females were released inside wiremesh screened cages (30×30×30 cm) onto 15–20-d-old sorghum seedlings (cultivar DJ 6514) and were provided with 20% sucrose solution on a cotton swab in a Petridish. The sucrose solution was changed daily. They were maintained under controlled conditions (27°C, 70% RH, 12:12 h L:D) in the laboratory on sorghum seedlings.

Plants Seeds of sorghum cultivars 'Swarna' and IS 18551 were planted individually in 7 cm (diam.)×6 cm (deep) plastic pots filled with compost. Plants were grown in a glasshouse under controlled conditions (25°C, 60% RH, 16:8 h L:D). Natural lighting was supplemented with sodium lamps (SON-T 600W) that gave photosynthetically active radiation of approximately 24600 lux at bench height. The seedlings used for experiments were 15–21 d after seedling emergence when they carried fifth leaves and the sixth leaf had just started to show.

Chemicals Authentic chemical standards used in behavioral studies were (*Z*)-3-hexen-1-yl acetate, octanal, methyl salicylate (all 99% purity, Avocado Research Chemicals), (-)-(*E*)-caryophyllene (85% purity, Pfaltz & Bauer inc., Stamford, CT, USA), (-)-α-pinene, (+)-α-pinene (98% purity, Sigma Aldrich, Gillingham, UK), 6-methyl-5-hepten-2-one, decanal (99% purity, Sigma Aldrich), and nonanal (95% purity, Sigma Aldrich).

Air Entrainment of Sorghum Plants Volatiles from the highly susceptible sorghum cultivar 'Swarna' and a resistant control, IS 18551, were collected with equipment that allows sampling of volatiles from live plants (Agelopoulos et al., 1999). Plants were 15–21 d after seedling emergence, the most susceptible growth stage. All equipment viz., glassware and aluminium plates, was washed with Teepol detergent (Herts County Supplies, Herts, UK), rinsed with acetone and distilled water, then dried in an oven at 180°C for 2 h. Porapak Q tubes were eluted with redistilled diethyl ether and heated at 132°C for 2 h under a stream of purified nitrogen to remove contaminants. Plants were enclosed individually in glass vessels (190 mm high×100 mm wide), open at the bottom and closed with a collection port at the top. The bottom was closed with two semicircular aluminium plates that fitted around the stem of the plant and were clipped to a flange on the open end of the glass vessel. One of the aluminium plates was drilled to accommodate an inlet port, and air, purified by passage through an activated charcoal filter, was pumped into the vessel through this (400 ml/min). Volatiles were collected on Porapak Q (50 mg, 60/80 mesh; Supelco, Bellefonte, PA, USA) in a glass tube (5-mm diam; Alltech Associates, Lancashire, UK) inserted into the collection ports on the top of the vessels. Further pumps drew air (300 ml/min) through these tubes. Rates were controlled so that more purified air was pumped in than was drawn out, ensuring that unfiltered air was not drawn into the vessel from outside and obviating the need for a tight seal around the stem, which would have caused damage to the plant. All connections were made with PTFE tubing (Alltech Associates, Lancashire, UK) with brass ferrules and fittings (North London Valve, London, UK) and sealed with PTFE tape (Gibbs & Dandy, Luton, UK). Plants were entrained for 2 d, and the Porapak Q filter was eluted with 0.5 ml of redistilled diethyl ether, providing a solution that contained the isolated volatile compounds. Samples were

stored in vials in a freezer (−20°C) until used for the experiments.

Gas Chromatography Analysis Volatiles were analyzed on a Hewlett-Packard 6890 GC equipped with a cold on-column injector, a flame ionization detector (FID), a non-polar HP-1 bonded-phase fused silica capillary column (50 m×0.32 mm i.d., film thickness 0.52 μm) and a polar DB-WAX column (30 m×0.32 mm i.d., film thickness 0.82 μm). The oven temperature was maintained at 30°C for 1 min, and programmed at 5°C min^{-1} to 150°C, and held for 0.1 min, then 10°C min^{-1} to 230°C. The carrier gas was hydrogen. A 2 μl aliquot of the headspace sample was injected onto a capillary GC column. Quantification was carried out by comparison with 100 ng of external standard using authentic samples of standards.

Stereochemistry of α-pinene and caryophyllene was determined by using an HP5890 GC (Agilent Technologies, UK) equipped with a cool on-column injector and a FID, fitted with a β-cyclodextrin chiral capillary column (30 m× 0.25 mm i.d., 0.25 μm film thickness). The GC oven was maintained at 30°C for 1 min and then raised by 5°C min–1 to 150°C where it was held for 30 min. The carrier gas was hydrogen. A 1-μl aliquot solution that contained equal quantities of both enantiomers of the compounds in redistilled hexane was injected onto the chiral GC to establish that successful separation of enantiomers took place. This was followed by co-injections of the air entrainment sample, first with an authentic standard of one enantiomer and then with the second enantiomer. Peak enhancement with either enantiomer confirmed the presence of that enantiomer in the air entrainment sample.

Coupled Gas Chromatography-Electroantennography (GC-EAG) Electroantennogram (EAG) recordings were made with Ag-AgCl glass microelectrodes filled with Ringer solution (7.55 g l^{-1} sodium chloride, 0.64 g l^{-1} potassium chloride, 0.22 g l^{-1} calcium chloride, 1.73 g l^{-1} magnesium chloride, 0.86 g l^{-1} sodium bicarbonate, 0.61 g l^{-1} sodium orthophosphate). The head of a female *A. soccata*, anaesthetized by chilling, was separated from the body with a microscalpel, and the tip of the arista of one antenna was removed. The indifferent electrode was inserted within the head capsule, and the recording electrode was placed over the cut arista. The coupled GC-EAG system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on a Hewlett-Packard 6890 gas chromatograph equipped with a cold on-column injector and a FID. The column used was 50 m×0.32-mm i.d. HP-1. The oven temperature was maintained at 30°C for 2 min, and then programmed at 15°C min^{-1} to 250°C. The carrier gas was helium. Electroantennogram signals were passed through a high-impedance amplifier (UN-06; Syntech, the Netherlands), and simultaneous recordings of the EAG and FID responses were obtained with specialized software (EAD version 2.3; Syntech, the Netherlands). Ten coupled runs were completed for each sample. FID peaks were considered to be active if they elicited an EAG response in two or more coupled runs.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) A 2 μl aliquot of the air headspace sample was injected onto a capillary GC (Agilent 6890) directly coupled to a mass spectrometer (Agilent 5973 MSD). Ionization was achieved by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°C min^{-1} to 250°C. Tentative identifications of compounds that elicited an EAG response were made by comparison of spectra with those of authentic samples in a database (NIST 2005). Tentative identifications were subsequently confirmed by coinjection of the natural headspace sample with authentic standards and showing peak enhancement on two GC columns of different polarity (HP-1 and DB-WAX).

Olfactometer Bioassays A Perspex four-arm olfactometer (Pettersson, 1970) was used to determine behavioral responses of *A. soccata* females to intact *S. bicolor* (var. 'Swarna') plants, and a synthetic volatile blend that comprised all identified compounds that elicited an EAG response at the same concentration (2.64 μg) and ratio as in the headspace sample. The natural ratio was 24.7: 1.0: 0.014: 2.8: 7.8: 3.9: 1.1: 3.3, respectively, for (*Z*)-3-hexen-1-yl acetate, α-pinene, (-)-(*E*)-caryophyllene, methyl salicylate, octanal, decanal, 6-methyl-5-hepten-2-one, and nonanal. Prior to each experiment, all glassware was washed with Teepol, rinsed with acetone and distilled water, and baked in an oven overnight at 160°C. Perspex components were washed with Teepol solution, rinsed with 80% ethanol solution and distilled water, and left to air dry. The olfactometer was fitted with a filter-paper base (Whatman No. 1, 11 cm diam) to provide traction for the walking insect, and was illuminated from above by uniform lighting from two 18W/35 white fluorescent light bulbs screened with greaseproof paper to make it diffuse. It was surrounded by black paper to remove any external visual stimuli. *A. soccata*, obtained from the laboratory culture (3–5-d-old mated females), were transferred individually from the rearing cage into the central chamber of the olfactometer. A single shoot fly was introduced through a hole in the top of the olfactometer. Air was drawn through the central hole at the rate of 400 ml min^{-1}, and subsequently was exhausted from the room. Each shoot fly was given 2 min

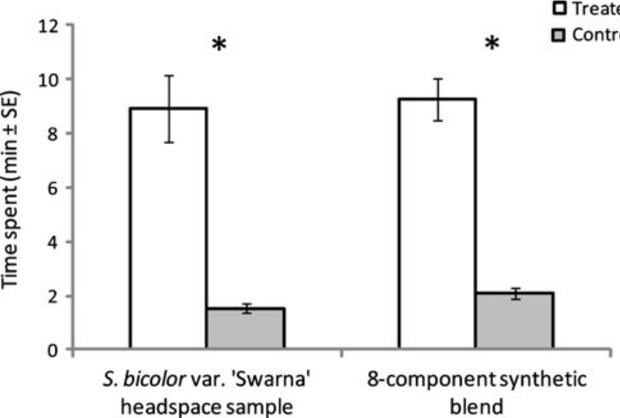


Fig. 1 Behavioral response of female *Atherigona soccata* to sorghum cv. 'Swarna' seedling volatiles in the olfactometer: time spent in treated and control arms (N=10). *Significantly different from control (P<0.01). The 8-component synthetic blend comprised (*Z*)-3-hexen-1-yl acetate, α-pinene, (-)-(*E*)-caryophyllene, methyl salicylate, octanal, decanal, 6-methyl-5-hepten-2-one, and nonanal in a 24.7: 1.0: 0.014: 2.8: 7.8: 3.9: 1.1: 3.3 ratio (2.64 μg total dose on filter paper)

to acclimatize in the olfactometer after which the experiment was run for 16 min for each replicate. The olfactometer was rotated 90° every 2 min to eliminate any directional bias in the room. It was divided into four regions corresponding to each of the four glass arms. Time spent and number of entries into each olfactometer arm was recorded with Olfa software (F. Nazzi, Udine, Italy). Ten replicates were carried out for each odor source tested. For each series of replicates, the mean time spent in treated and control regions were compared using a paired *t*-test (Genstat v. 12).

Initially, responses to headspace samples collected from sorghum seedlings were tested. Ten μl of the headspace sample were placed onto a piece of filter paper, 30 sec were allowed for the solvent to evaporate, and then it was placed in the treated arm. This dose was approximately the amount emitted by one sorghum plant over 1 h. The three control arms contained a piece of filter paper with 10 μl of redistilled diethyl ether. Synthetic compounds that elicited an EAG response were used as treatments in subsequent bioassays. Responses to individual compounds were tested by using 10 μl of 100 ng μl^{-1} solutions formulated in redistilled hexane in the treated arm and 10 μl of redistilled hexane in the control arms. Finally, compounds that elicited a positive behavioral response were formulated as a synthetic blend in redistilled hexane that comprised all the active compounds in the same concentration and ratio as in the headspace sample from which they were identified. A choice test between 10 μl synthetic blend and 10 μl headspace sample was carried out (Webster et al., 2008). These two treatments were assigned randomly to different glass arms in each replicate. The other two arms contained filter paper with 10 μl hexane, and were used as controls. Time spent in each region was recorded for 16 min. Ten replicates were carried out.

Results

Behavioral Responses to Headspace Samples In the olfactometer bioassay, female *A. soccata* spent significantly more time in the treated region of the olfactometer than the controls when a 10 μl aliquot of the *S. bicolor* headspace sample from the shoot fly susceptible cultivar 'Swarna' (growth stage: 15–21 days after emergence) was used (P= 0.001). Flies spent almost six times longer in the treated region of the olfactometer; mean time spent in the treated region (± S.E.) was 8.92 (± 1.22) min, whereas mean time spent in the control regions was 1.54 (± 0.18) min (Fig. 1). However, when volatiles collected from the shoot fly resistant cultivar IS 18551 were tested, attraction did not occur; mean time spent in the treated region was 1.99 (± 0.33) min, whereas mean time spent in the control regions was 4.49 (± 0.11) min.

Identification of Compounds that Elicited an EAG Response Coupled GC-EAG with female *A. soccata* revealed eight compounds eliciting an EAG response in the headspace sample of 'Swarna' (Fig. 2). By using coupled GC-MS and GC peak enhancement on two GC columns of different polarity, these compounds were identified as (*Z*)-3-hexen-

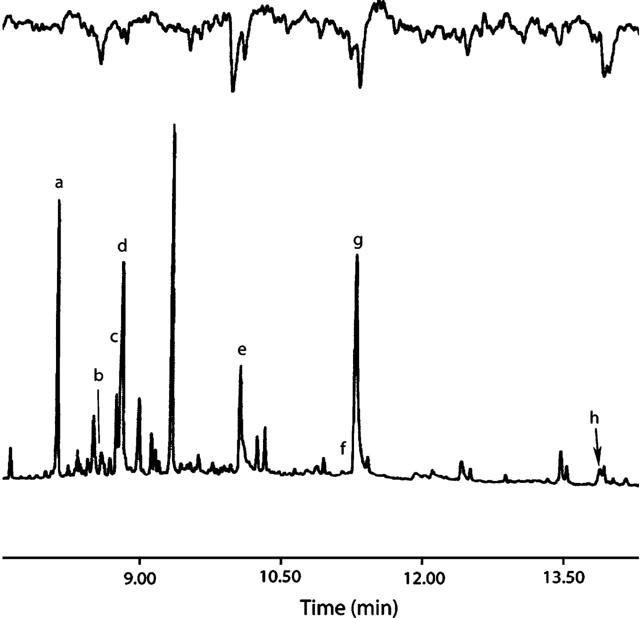


Fig. 2 Representative GC-EAG recording of female *Atherigona soccata* responses to sorghum cv. 'Swarna' seedling volatiles (HP1 column). The FID peaks marked are those that elicited responses in two or more coupled runs: *a* (-)-α-pinene, *b* 6-methyl-5-hepten-2-one, *c* octanal, *d* (*Z*)-3-hexen-1-yl acetate, *e* nonanal, *f* methyl salicylate, *g* decanal, *h* (-)-(*E*)-caryophyllene

Table 1 Compounds electrophysiologically active with *A. soccata* identified in *Sorghum bicolor* cv. 'Swarna' volatiles

Compound	Retention Index (HP-1, non-polar)	Retention Index (DB-WAX, polar)	Concentration (ng μl^{-1})
Green leaf volatiles and other aliphatic compounds			
6-Methyl-5-hepten-2-one	966	1,346	6.7
Octanal	980	1,298	46.3
(Z)-3-Hexen-1-yl acetate	988	1,317	146.0
Nonanal	1,084	1,393	19.4
Decanal	1,186	1,498	22.8
Aromatic compounds			
Methyl salicylate	1,175	1,796	16.3
Terpenoids			
(-)-α-Pinene	934	1,024	5.9
(-)-(*E*)-Caryophyllene	1,432	1,602	0.08

1-yl acetate, α-pinene, (-)-(*E*)-caryophyllene, methyl salicylate, octanal, decanal, 6-methyl-5-hepten-2-one, and nonanal. Chiral GC analyses showed that (-)-α-pinene and (-)-(*E*)-caryophyllene were present in the natural sample. The quantities of the EAG active compounds present in the attractive 'Swarna' headspace sample used in the bioassay are listed in Table 1.

Behavioral Responses to Synthetic Compounds All eight compounds that elicited an EAG response were behaviorally active when presented individually at a standard dose (1 μg on filter paper), with female *A. soccata* spending significantly longer in the treated region of the olfactometer (Table 2). When an eight-component synthetic blend of the EAG active compounds, at the same concentration and ratio as in the natural headspace sample (Table 1), was tested, a similar response to that obtained with the natural sample was observed, with the shoot flies spending more time in the treated region of the olfactometer than the controls ($P=0.001$) (Fig. 1). Furthermore, when this synthetic blend and the natural headspace sample were tested in a choice test, the shoot flies did not show any preference for either of the two treatments ($P=0.951$) (Fig. 3), thus demonstrating that the synthetic blend had activity similar to the natural sample. The total time spent in the two olfactometer treatment arms was greater than the total time spent in the two control arms ($P<0.001$). Further bioassays showed that a reduced component blend, based on a different headspace sample, comprising (*Z*)-3-hexen-1-yl acetate, (-)-α-pinene, and (-)-(*E*)-caryophyllene in the ratio (10.9: 1.0: 0.04) and concentration in that sample (0.42 μg) was highly attractive ($P<0.001$). When presented alone and in a choice test with a natural sample, it elicited a similar level of attraction ($P=0.763$).

Comparison of the Emission of Volatiles Between the Two Sorghum Varieties The shoot fly susceptible cultivar 'Swarna' and the shoot fly resistant control IS 18551 had different volatile profiles (Fig. 4). IS 18551 emitted fewer volatiles than 'Swarna' (overall emission was approx. 16 times less). In addition to the pronounced quantitative variation, we found substantial qualitative variation as IS 18551 did not emit α-pinene, (-)-(*E*)-caryophyllene, methyl salicylate, or octanal.

Table 2 Response of female *Atherigona soccata* to compounds (1 μg on filter paper) tested individually in olfactometer bioassay ($N=10$)

Compound	Treated mean (min)	SE	Control mean (min)	SE	*P* value (*t*-test)
(*Z*)-3-Hexenyl acetate	6.23	(± 0.61)	2.72	(± 0.21)	<0.001
Methyl salicylate	6.09	(± 0.63)	2.81	(± 0.26)	0.002
6-Methyl-5-hepten-2-one	5.05	(± 0.78)	3.18	(± 0.24)	0.048
(-)-α-Pinene	7.64	(± 0.69)	2.57	(± 0.20)	<0.001
Nonanal	5.61	(± 0.64)	3.04	(± 0.27)	0.01
Decanal	5.11	(± 0.44)	3.21	(± 0.21)	0.008
(-)-(*E*)-Caryophyllene	7.93	(± 0.98)	2.29	(± 0.29)	<0.001
Octanal	6.87	(± 0.72)	2.69	(± 0.21)	<0.001

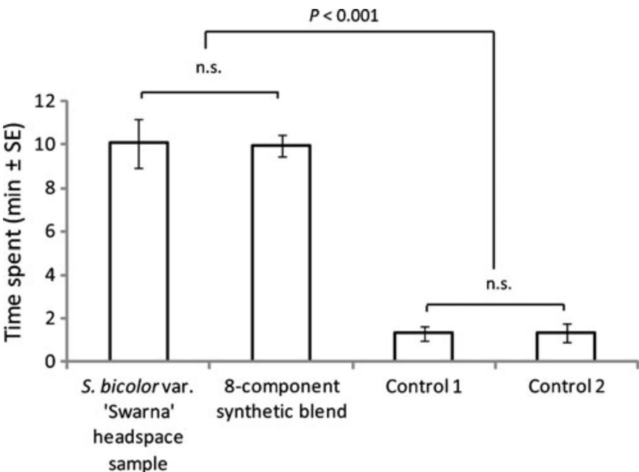
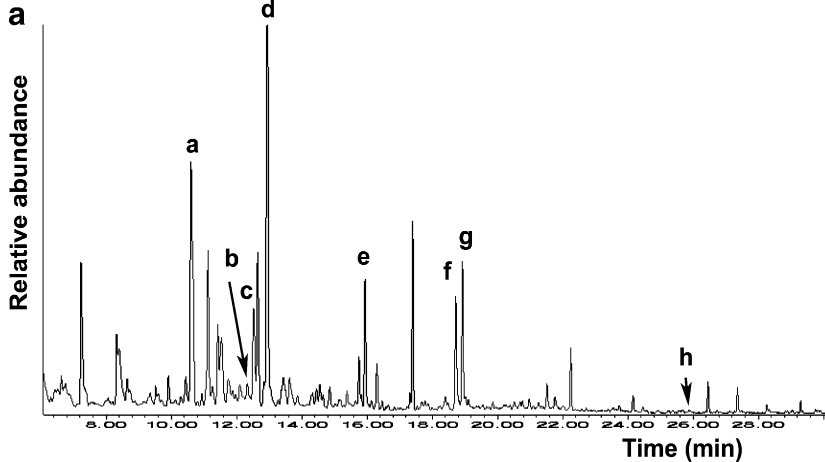


Fig. 3 Behavioral response of female *Atherigona soccata* to sorghum cv. 'Swarna' headspace sample and synthetic blend with both odour sources present in the same olfactometer (N=5)

Discussion

Host plant recognition and selection in insects is determined mainly by the ovipositing female since newly emerged larvae often are limited in their dispersal abilities. Choice of a suitable oviposition site is crucial to the survival of progeny. Visual factors such as shape, color, and size are involved in this process, but chemical cues unambiguously play a major, if not decisive, role in host selection (Renwick and Chew, 1994). This study combined GC-EAG detection of active compounds in headspace collections and bioassays of behavioral responses of sorghum shoot fly, *A. soccata*, to these volatiles. Through this dual approach, we were able to identify active compounds in complex headspace blends and confirm that these compounds attracted female *A. soccata*. To the best of our knowledge, this is the first identification of sorghum seedling volatiles functioning as kairomones in attracting *A. soccata* to hosts.

The four-arm olfactometer bioassay (Pettersson, 1970) was chosen because it gives sufficient time for the initial escape responses of insects after release from the holding chamber to wear off. Air was drawn from the center of the arena, which was divided into four discrete odor fields. A significantly longer period of time spent in the treated arm indicated a positive response that required attraction for movement into it, although there may have been an arrestment component to a positive response. *A. soccata*



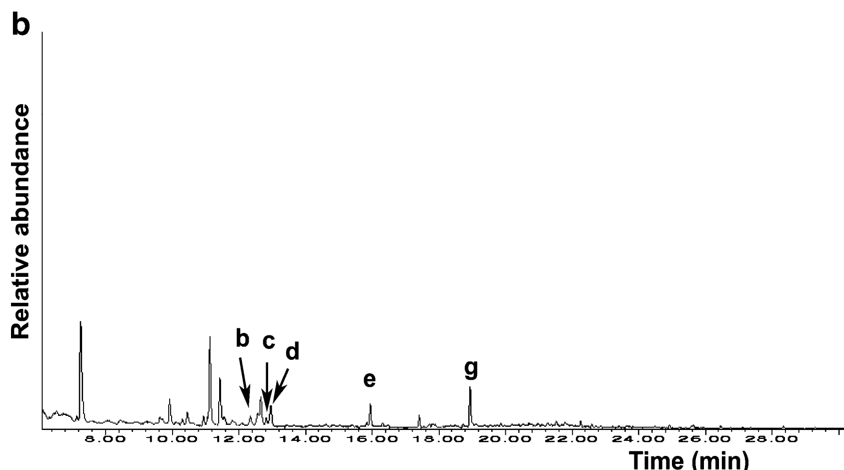


Fig. 4 Gas chromatogram (GC) traces of compounds identified in headspace samples of sorghum seedlings (HP5 column) **A** sorghum cv. 'Swarna'; **B** sorghum cv. IS18551. *a* (-)-α-pinene, *b* 6- methyl-5-hepten-2-one, *c* octanal, *d* (*Z*)- 3-hexen-1-yl acetate, *e* nonanal, *f* methyl salicylate, *g* decanal, *h* (-)-(*E*)-caryophyllene

females were attracted to the volatiles of susceptible sorghum seedlings in an earlier investigation, but the compounds responsible were not identified (Nwanze et al., 1998). In the present study, coupled GC-EAG revealed eight compounds that elicited an EAG response in a headspace sample collected from the susceptible sorghum cultivar, 'Swarna'. A synthetic kairomone blend that comprised all eight compounds in the same concentration and ratio (Table 1) elicited a similar behavioral response to the odor of headspace sample (Fig. 1). Furthermore, the shoot flies did not show any preference for the headspace sample over the synthetic blend when offered the choice, suggesting that the volatile compounds used in host location have been identified (Fig. 3). Laboratory bioassays where compounds are tested against clean air are a simplification of the more complicated situation that occurs in the field, where there are background volatiles emitted from other plants. In field situations, it is likely that blends of compounds are required for host recognition when ubiquitous compounds are used (Bruce et al., 2005; Bruce and Pickett, 2010). In the current study, individual host compounds were attractive, whereas, in other insects, positive behavioral responses are obtained only when a blend of volatiles is used (e.g., Webster et al., 2010).

Three compounds (nonanal, decanal, and (*Z*)-3-hexen-1-yl acetate) have been reported previously in volatiles from 4-wk-old *S. bicolor* (Serena cultivar) seedlings trapped on Tenax TA adsorbent (Lwande and Bentley, 1987), but the response of *A. soccata* to these compounds was not reported. Our study also revealed differences in the absolute quantity of odors emitted, as well as in the qualitative composition between the attractive susceptible cultivar 'Swarna' and a resistant control, IS 18551, which was not attractive. IS 18551 did not emit α-pinene and (-)-(*E*)-caryophyllene, which we found were key compounds responsible for the positive response to 'Swarna' volatiles in the olfactometer, even when released at relatively low rates. Trap crops could be developed by selection of plants that release higher amounts of the attractive compounds. Varietal or genotypic differences in volatile emission have been recorded for other plant species (Loughrin et al., 1995; Halitschke et al., 2000). Studies on different maize varieties revealed intraspecific variation both in quantity and quality of the odors released (Turlings et al., 1998; Gouinguene et al., 2001) within maize cultivated in temperate regions (Degen et al., 2004).

Host plant resistance is one of the most effective and preferred methods of controlling *A. soccata*. Considerable progress has been made in developing techniques to screen for *A. soccata* resistance, identifying the sources of resistance and the mechanisms of resistance. However, efforts to breed *A. soccata* resistant high yielding cultivars have not yet been successful. Thus, there is a need to look into the role of biochemical factors like plant volaties in ovipositional behavior of *A. soccata* as has been done successfully in the related cereal maize (Lupoli et al., 1990). Plant chemistry is probably the most important source of information contributing to the final decision by an insect to oviposit or not and depends on the balance of opposing positive and negative cues evoked by phytochemicals that determine whether a plant is accepted or rejected by a herbivore. Selection of sorghum genotypes that are not preferred for oviposition by *A. soccata* is valuable for the development of *A. soccata* resistant plants. This study, by identifying sorghum volatiles that elicit electrophysiological and positive behavioral responses with *A. soccata*, provides insight into the differential attraction/oviposition between susceptible and resistant genotypes observed in the field. Future work will analyze volatiles of other sorghum cultivars, and assess effects on attraction of *A. soccata* in the field.

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However, Ballhorn et al. (2008a) observed higher cyanogenic potential in some cultivars (CV-2116, CV-2357, and CV-2233) than in wild types. Low concentrations of linamarin are found in U.S. varieties such as Fordhook 242 and Henderson also known as butter beans.

Arthropods like some other insects have developed detoxification mechanisms for these compounds. For instance, *Heliconius* butterflies metabolize cyanogenic glycosides found in their *Passiflora* host plants, sequestering some of its metabolic derivates and utilizing nitrogen from the rest (Engler et al., 2000). Larvae of *Acraea horta* (L.) were found to feed selectively on *Kiggelaria Africana* L. plants with high content of cyanogenic glycosides, and accumulate these compounds in the body in all developmental stages (Raubenheimer, 1989). Some zygaenid butterflies require the presence of cyanogenic glycosides in the host plant for normal development. Larvae of the butterfly *Zygaena filipendulae* (L.) (Zygaenoidea) developed at a decelerated rate when feeding on young acyanogenic *Lotus corniculatus* Plenus host plants as compared to those feeding on host plants with accumulated cyanogenic glycosides (Zagrobelny et al. 2007). In some Lepidopterans such as *Heliconius melpomene* (L.) (Papilionoidea) and Zygaenidae, detoxification is accomplished *via* a β-cyanoalanine synthase located in the mitochondria (Zagrobelny et al., 2008). The cherry-oat aphid, *Rhopalosiphum padi* L. (Hemiptera: Aphididae) prefers plants with endogenous β-glucosidase, which is released when the insect punctures and destroys plant tissue (Leszczynski et al., 2003).

The two spotted spider mite, *Tetranichus urticae* (Koch) (Acari: Tetranychidae), feeds on more than 150 different plant species (Jeppson et al., 1975) and has a high acceptability for plants of the Fabaceae family. Agrawal et al. (2002) and Magowski et al. (2003) reported that host plant preference by the two-spotted spider mite was influenced by the presence of natural toxins, and these impacted mite reproductive performance. Negative response to those chemicals can be overcome by conditioning, i.e., increasing acceptability of the host plants to subsequent spider mite generations (Navajas, 1998; Yang et al., 2001). Host plants also induce physiological changes in the mites by activating detoxification enzymes such as general esterases and glutathione S-transferases; this affects the susceptibility of the mites to some pesticides. Increased spider mite tolerance to λ-Cyhalothrin, which contains the cyano functional group, has been observed when mites were reared on lima beans (Yang et al., 2001).

Linamarin is used as a translocation chemical of sugars and nitrogen to the fruit (Frehner et al., 1990). In bean plants, linamarin is stored in the leaf epidermis (Poulton, 1990) instead of leaf mesophyll protoplasts (Frehner and Conn, 1987), which has been reported in other plants. Mites feed on the cell contents of plant epidermis (Boutaleb et al., 2000), which could imply that they ingest significant quantities of linamarin.

Plant chemical defenses against herbivores may negatively affect the fitness of entomophagous natural predators (Ode, 2006). In fact, some herbivorous insects sequester plant chemicals (including cyanogenic glycosides) and use them to defend against predators (Nishida, 2002; Hartmann, 2004). Such compounds also may affect insect predators indirectly by reducing the health of the herbivore prey (Hunter, 2003), or the predator may be affected by the plant compounds indirectly when feeding on their herbivore host or prey (Ode, 2006). Evidence for indirect toxic effects of plant chemicals to parasitoids comes from studies of host-parasitoid interactions. Campbell and Duffey (1979, 1981) described the effects of α-tomatine on the parasitoid *Hyposoter exiguae* (Viereck) parasitizing *Heliothis zea* (Boddie), which were grown on artificial diets that simulated tomato plant contents including α-tomatine. Similarly, Thorpe and Barbosa (1986) and Barbosa et al. (1986, 1991) studied the effects of nicotine on *Cotesia congregata* (Sey) parasitizing *Manduca sexta* L., which were grown on artificial diet that simulated tobacco plant concentrations of nicotine. Increased mortality of the parasitoid occurred at high concentrations of nicotine. There is no evidence for toxic effects of plant chemicals on predatory arthropods, but the fecundity and movement of the predatory mite *P. persimilis* Athias-Henriot is significantly affected by the host plant species used to feed its prey, *T. urticae* (Skirvin and de Courcy Williams, 1999). Because *T. urticae* is capable of infesting many plant species, the use of predatory mites as biological control agents may be impacted by the host plant on which the prey feeds, particularly within the Fabaceae where the content of cyanogenic glycosides is well documented. Additionally, bean plants frequently are used in the mass rearing of Phytoseiid mites because these plants grow fast and are excellent host for *T. urticae*. It is important to determine whether some of these plants may indirectly affect the fitness of the predators and therefore their quality as biological control agents.

Our specific objectives were: (1) to determine the content of linamarin and lotaustralin in lima bean, in *P. lunatus,* primary and terminal trifoliate leaves, in adults and nymphs of *T. urticae* feeding on primary and trifoliate lima bean leaves, and in adult mite predators, *P. persimilis,* feeding on spider mites fed on primary and trifoliate lima bean leaves; and (2) to determine the impact of host plant on fecundity and sex ratio of *T. urticae* and *P. persimilis*.

Methods and Materials

Maintenance of Mite Cultures Colonies of *T. urticae* and *P. persimilis* were established from stocks provided by Syngenta Bioline, Oxnard, CA, USA. Spider mites were

reared on Shell bean, a dark red kidney bean, *Phaseolus vulgaris*, (The wax Co., LLC, Armony, MS, USA), in a greenhouse. The predatory mites were reared in cages by using a variation of the procedure described by Fournier et al. (1985) in an environmentally controlled rearing room.

Maintenance of Plants Bean plants of *P. vulgaris* and *P. lunatus* were grown by using the same method. Plants were grown in 60 long × 20 wide × 20 high cm plastic planters filed with a bottom layer of perlite and a top layer of top soil vermiculite and polyacrylamide mix.

A mixture of 20 g water absolving polymer (cross-linked potassium polyacrilate / polyacrylamide copolymer) (TeraGel®, T-400, The Terawet Corporation, San Diego, CA, USA) and 10 g water soluble fertilizer (All purpose, plant food, Miracle-Gro, Marysville, OH, USA) was added to 2.5 L of tap water. The polymer provided a consistent water balance to the soil mix and reduced water lost. The aqueous solution was left undisturbed for 24 hr to allow TeraGel's polyacrylamide crystals to become completely hydrated. The resulting gel was homogeneously incorporated into a 2:1 potting soil (Moist control, Miracle-Gro Marysville, OH, USA) and vermiculite (Coarse, Sunshine, SunGro Horticulture, Bellevue, WA, USA) mixture with the help of a gardening spatula. The bottom of the planters was filled with 2.5 L of perlite (coarse, Sunshine, SunGro Horticulture, Bellevue, WA, USA) as support and to allow drainage for maintaining a consistent soil moisture. The soil mixture then was added to the planters, and 70 seeds were planted in each. Five g of slow release fertilizer (vegetable and bedding, Osmocote, Marysville, OH, USA) were spread on top of the planted seeds. Planters were irrigated daily with tap water after seed germination.

Leaf Sampling for Chemical Extraction Leaf samples of two different ages were collected from un-infested red and lima bean plants. From lima beans, 21-d-old primary leaves and 5-d-old terminal leaves (trifoliate) and 21-d-old primary leaves from red beans were selectively cut and placed in plastic boxes (35.5×19.6×10.7 cm) for transport to the laboratory. Leaf samples were dried in a vacuum oven at a low temperature of 20°C and a negative pressure of 3.63 kPa (kilopascals) until they were completely dry. Dry leaf samples were placed in plastic Ziploc® bags, labeled, and stored in a refrigerator at minus 16°C until ready for chemical extraction and analysis.

Tetranychus urticae Rearing and Sample Preparation Red bean and lima bean plants were infested with spider mites 10 d after germination. Infestation of the planters was carried out by manually introducing spider mites (from infested red bean leaves) onto 20 bottom leaves from each planter. The infested red bean leaves were placed on top of clean leaves and left there for 8 hr, thus allowing adult mites to migrate to the new leaves. The dry red bean leaves were removed, and the spider mites were allowed to reproduce on the plants of study for 22 d under greenhouse environmental conditions at 26±3°C and 35±10% RH and constant artificial light.

Infested lima bean leaves of the two ages described above and infested primary red bean leaves were collected individually by corresponding group age and placed in 4 L round plastic containers (half full) to which 2 cm diam round holes covered with a 60 µm nylon screen were randomly distributed as a source of ventilation. The boxes were closed with lids, and then were placed into an environment control room at 27±1°C and 50±5% RH for a period of 24 hr. The lids of the containers were gently removed to collect the web with mites, and this web was transferred to a piece of tissue paper with the help of a spatula. The tissue paper pieces with spider mites from the different leaf samples then were placed flat in plastic containers and transferred immediately into a freezer at minus16°C for 15 min to freeze the spider mites. Finally, the paper towel pieces with the frozen mites were gently shaken inside a 100 ml plastic cup to separate the frozen mites from the web. One hundred mg samples of clean frozen mites from each leaf species and type sample were weighed (PB303-S Mettler Toledo, Fisher Sci., Suwanee, GA, USA) into 1.5 ml plastic centrifuge tubes (P. No. FX4264GG, A. Daigger & Co., Inc., Vernon, Hills, IL, USA) and stored at minus16°C until they were analyzed chemically.

Phytoseiulus persimilis Rearing and Sample Preparation The predatory mites were reared using modified Ziploc® screw cover cylindrical 473 ml containers (Ziploc Twist'n Loc, small, round, S.C. Johnson & Son, Inc. Racine, WI, USA) connected from top to bottom to allow vertical movement of the predators between containers as reported by Fournier et al. (1985). Different sets of rearing cages were provided with each leaf type described above, and were infested with spider mites from the greenhouse. Each starting cage was filled with 15 infested leaves placed vertically to allow mite movement. Approximately 100 *P. persimilis* females from the stock colony were introduced into each cage. New cages with infested leaves were attached to the top of the starting cages after 3 d to allow predators to move from old leaves onto new ones. This process was repeated every 3 d for a 3 wk period at 27±2°C, 65±5% RH and 14:10 h (L: D) photoperiod to ensure that more than 2 generations of *P. persimilis* feeding developed on spider mites from each of the 3 leaf types tested (primary and terminal trifoliate lima bean and primary red bean leaves).

Adults of *P. persimilis* were collected from each of the 3 treatments in 120 ml plastic sample containers, connected to the top of each cage series. These containers were transferred to a freezer at −16°C for 15 min to freeze the

predators. One hundred mg samples of clean frozen mites from each leaf species and age sample were weighed (PB303-S Mettler Toledo, Fisher Sci., Suwanee, GA, USA) into the 1.5 ml plastic centrifuge tubes and stored at −16°C until chemical analyzes.

Chemical Analysis The samples consisting of un-infested primary red bean leaves, primary and trifoliate lima bean leaves, *T. urticae* reared on these 3 different leaf types, and *P. persimilis* reared on *T. urticae* from these 3 leaf types were all analyzed for contents of linamarin and lotaustralin by using the same method. Determination of the content of linamarin and lotaustralin was done by high performance liquid chromatography (HPLC) following a modification of the method reported by Stochmal and Oleszek (1994) and Oomah et al. (1992).

Lima Bean Leaves Portions of 1 g of each of the dry leaf samples were placed individually into 20 ml scintillation glass vials (P. No. 3002-1, Fisher Scientific, Suwanee, GA, USA), and 10 ml 70% methanol were added to each vial. The mixture was blended with the use of a tissue tearor (Model 985-370, Biosperr products, Inn, Daigger, Vernon Hills, IL, USA) for 3 min. Vials were closed and placed in a Branson sonic water bath (Model 3210, Fisher Scientific, Suwanee, GA, USA) for 20 min at 25°C. Samples were transferred individually to 25 ml Corex glass centrifuge tubes (P. No. A-34520-25, Cole Palmer, Vernon Hills, IL, USA) and loosely closed with screw lids. The tubes with the samples were centrifuged (Model Br4i, Jouan, Thermo Fisher Scientific, Suwanee, GA, USA) at 8,000 RPM at 10°C for 10 min. The aqueous solution was transferred to clean scintillation glass vials and placed into a vacuum oven at 40°C for 2 hr to remove the methanol. Carbohydrates and other impurities were removed from the water extracts by solid-phase extraction passing them through a pre conditioned C18 Sep-Pak (WAT036905, Waters, Co., Franklin, MA, USA) with 3 ml aliquot 20% methanol. One ml of the water extract was placed on top of the Sep-Pak and pushed slowly into the C18 material with a soft stream of pure nitrogen; then 5 ml of the 20% methanol solution were also gently pushed through into clean 20 ml glass vials with the gentle stream of pure nitrogen. This procedure was repeated for the remaining extracts. Purified eluates were combined and dried in the vacuum oven at 40°C. The concentrated samples were dissolved in 0.450 ml of a methanol:water:phosphoric acid (85:15:0.05) solution, which was the solvent used as mobile phase during HPLC analysis. Before injection, the 0.450 ml sample was filtered throughout a Watman mini-uniprep, 45 μm nylon filter media (Cat. No. UN203NPUNYL, VWR Suwanee, GA, USA) to remove any precipitates.

Tetranychus urticae and Phytoseiulus persimilis One ml of the 70% methanol solution was added to each of the 100 mg frozen samples. The samples were centrifuged (Model No. 5410, Eppendorf, Fisher Scientific, Suwanee, GA, USA) at 14,000 rpm for 10 min; then transfered to the sonic water bath as described above. After sonication, the excess methanol was removed by blowing with a gentle stream of nitrogen, with a glass Pasteur pipette directly into the centrifuge tube. The contents of the tube were transfer to the filtering unit, and 100 μl of the methanol:water: phosphoric acid (85:15:0.05) were added. Finally, each sample was transferred to 12×32 mm glass screw neck, total recovery glass vials (P. No. 186999385c, Waters, Co. Milford, MA, USA) prior to HPLC analyses. Peak identification was done as described above.

The HPLC analysis was done on a Waters Alliance 2690 controller, equipped with a Waters 410 differential refractometer detector, by automatically injecting a 20 μl sample into an Eurospher 100-5, RP18 (Icon, Scientific, Inc., North Potomac, MD, USA) column (P. No. 25VE181ESJ, Knauer, 250 mm× 4.6 mm i.d. and 5 μm particle size) with an integrated pre-column. The column was eluted with an isocratic flow rate of the methanol:water:phosphoric acid (85:15:0.05) at 1 ml per min at room temperature. Data were collected, and peaks were identified against the retention times for linamarin and lotaustralin standards (P. No. L466000 and L471250, respectively, TRC, Inc. Ontario, Canada) dissolved in the methanol: water:phosphoric acid at a known concentration of 1 mg/ml, via Chromeleon V. 6.8 software, (Dionex, Co. Houston, TX, USA) (Fig. 1).

Bioassays To determine the effect of linamarin on *T. urticae*, young adult male and female pairs from the stock culture reared on shell kidney red bean leaves were transferred to un-infested leaves with the aid of a 00 soft bristle brush. These un-infested leaves were either one of 3 types, and one was treated with linamarin: (1) 5-d-old shell red kidney bean primary leaves as control (2) 5-d-old lima bean terminal trifoliate leaves, (3) 21-d-old primary lima bean leaves, and (4) 5-d-old primary leaves of red shell kidney beans sprayed with 200 μl of a 800 ppm solution of linamarin. Each mite pair was encased inside a 50×9 mm tight fit Petri dish (P. No. 351006, Falcon, Fisher Scientific), which also enclosed a portion of the leaf when closed. Each dish had a 5 cm diam window covered with a 60 μm nylon screen in the center of the lid for ventilation. Three of these dishes were placed inside of 100×100×30 mm square clear plastic boxes (P. No. 091-C, Pioneer Packaging, Dixon, KY, USA), and 1 g of the saturated polyacrylamide was added to maintain humidity. The boxes were closed with lids and placed inside an environmental chamber (Model I30VL, Percival, Perry, IA) at 26±1°C, 65±5% RH and 14:10 h (D:L) photoperiod for 8 d. Nine pairs of *T. urticae* per each treatment and control were placed in experimental dishes.

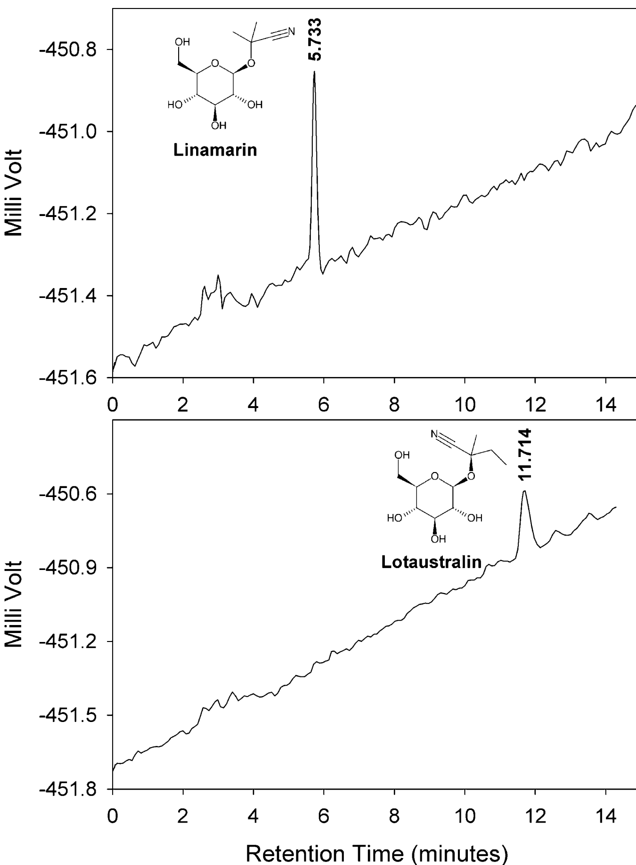
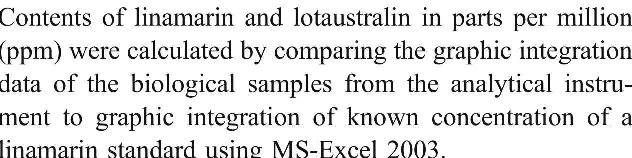


Fig. 1 Output graphs of HPLC data of standard solutions of linamarin and lotaustralin. Linamarin peak appears at 5.7 min and lotaustralin at 11.7 min. Baseline increased with retention time

At the end of the 8 d, period each dish was examined carefully under the stereo microscope to determine adult survival, total progeny including eggs, larvae, and nymphs. The sex of nymphs was determined, and the sex ratio within each dish was calculated as number of females per male (= females / males).

The effect of linamarin on the third trophic level was studied by a modification of the method described above. Pairs of male and female of *P. persimilis* were placed on leaves of the 3 types and were heavily infested with spider mites. These pairs and the corresponding infested leaves were encased in the tight fit Petri dishes as described. Dishes with predator pairs were placed inside boxes and allowed to reproduce for 8 d in an environmental chamber under the same conditions as described above. At the end of the 8 d period, dishes were examined carefully to determine adult survival, and to count total progeny produced including eggs, larvae, and nymphs. Twelve pairs of predators were set for each treatment.

Data Analysis Identification of the sample peaks was done by comparison of their retention times to those of standard solutions of linamarin by using the same procedures. 
Contents of linamarin and lotaustralin in parts per million (ppm) were calculated by comparing the graphic integration data of the biological samples from the analytical instrument to graphic integration of known concentration of a linamarin standard using MS-Excel 2003.

Data consisting of total progeny and progeny sex ratio in females per male of mite pairs of *T. urticae* and *P. persimilis* were compared among the different leaf type treatments using analysis of variance (ANOVA), and means were compared by the Tukey-Kramer HSD test using JMP statistical software (SAS Institute, 2007). Regression analysis was used to determine the impact of observed linamarin concentrations on the total progeny produced per female, and the progeny sex ratio was expressed as females per male.

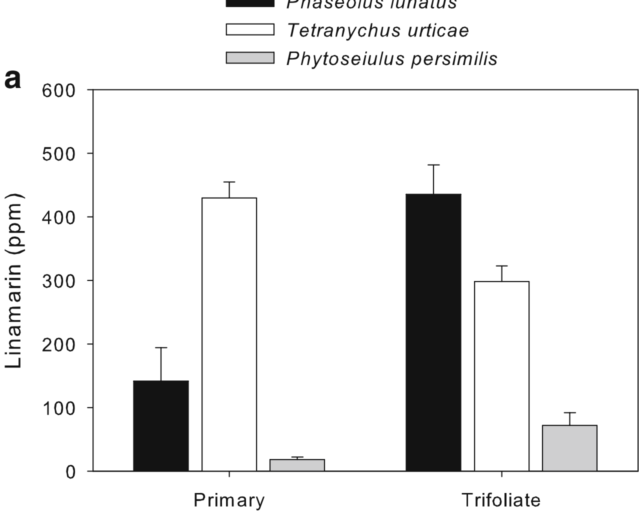
Results

Content of Linamarin and Lotaustralin Linamarin concentrations observed in the tissue of two types of lima bean leaves were not consistent with the concentration at the second trophic level. That is, chemical analyzes revealed that the content of linamarin was higher in terminal trifoliate leaves (435.5 ppm) than in primary leaves (142.1 ppm) of Henderson bush lima beans. However, the content of linamarin was higher in spider mites from primary leaves (429.8 ppm) than in those from terminal trifoliate leaves (298.2 ppm) of lima beans (Fig. 2). Lotaustralin concentrations were detectable only in the primary leaves of lima beans (103.12 ppm) and in the spider mites feeding on them (175.0 ppm). In terminal trifoliate Lima bean leaves and spider mites feeding on them lotaustralin was undetectable (Fig. 2). In addition, linamarin and lotaustralin were not detected in red bean leaves and spider mites feeding on them.

Eggs of spider mites feeding on primary Lima bean leaves had a concentration of linamarin of 26.32 ppm, approximately 6% of the concentration observed in adult mites, and there were no detectable quantities of lotaustralin.

The concentration of linamarin in mite tissue declined at the third trophic level. Predatory mites feeding on prey from terminal trifoliate and primary Lima bean leaves had a mean concentration of 71.9 and 18.42 ppm of linamarin, respectively. Analyses of predatory mite tissue did not detect lotaustralin in any of the samples (Fig. 2).

Effect of Host Plant on Reproduction and Sex Ratio The total progeny produced per female of *T. urticae* was not significantly affected by host plant species, but spider mites feeding on red bean primary leaves sprayed with a



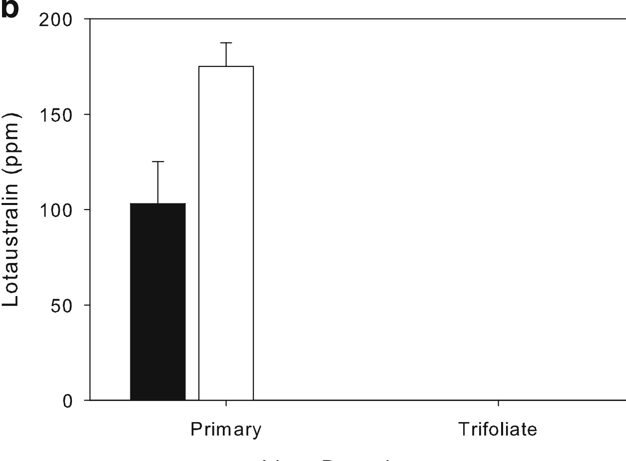


Fig. 2 Content of linamarin (**a**) and lotaustralin (**b**) in parts per million (ppm) in plant, spider mite, and predatory mite tissues of primary and terminal trifoliate leaves of Henderson lima bean plants. Brackets represent standard deviation

linamarin solution produced significantly less progeny than similar un-sprayed leaves (F=7.88; df=3, 36; P<0.001) (Table 1). The female:male sex ratio of the progeny of spider mites feeding on un-sprayed red bean primary leaves was significantly higher than that of mites feeding on both types of lima bean leaves and sprayed red bean leaves (F=12.72; df=3, 34; P<0.001) (Table 1).

Host plant linamarin effects were more noticeable on the predatory mites. Both, number of progeny per female (F=133.41; df=2, 33; P<0.001), and female:male sex ratio (F=24.68; df=2, 31; P<0.001) were significantly different among the 3 host plant leave types tested and were inversely proportional to linamarin content present in the prey tissue (Table 2). Linear regression analysis showed a significant negative impact of prey linamarin content on progeny per female (R^2=0.83; F=169.50; df=1, 34; P<0.001) and progeny female:male sex ratio (R^2=0.55; F=38.83; df=1, 32; P<0.001).

Table 1 Effect of host plant linamarin concentration on the reproduction and sex ratio of *Tetranychus urticae*

Host[a]	Linamarin[b]	N	Progeny[c]	Sex ratio[d]
RBL	0	9	43.22±13.62a	4.75±0.84a
HPL	142.08	9	31.00±7.53ab	2.48±0.32b
HTL	449.18	9	29.33±13.30ab	1.89±1.01b
RBS	1,000	13	17.23±13.58b	1.43±2.00b

Mean ± standard deviation. Means with the same letter are not significantly different after Tukey-Kramer HSD test at α=0.05

[a] Host keys: *RBL* red bean primary leaves; *HPL* Henderson lima bean primary leaves; *HTL* Henderson lima bean terminal trifoliate leaves; and *RBS* Red bean primary leaves sprayed with 200 µl of a 1,000 ppm solution of linamarin

[b] Concentration in parts per million (ppm) in host plant tissue

[c] During a 3d period at 27±1°C

[d] In females per male

Discussion

Our results showed that the concentration of linamarin was higher in terminal trifoliate leaves than in primary leaves in Henderson lima bean plants. This is consistent with results reported previously by Ballhorn et al. (2008b). However, the concentration of linamarin in spider mites feeding on Henderson lima bean leaves was reversed. Spider mites feeding on primary leaves had a higher concentration of linamarin than mites feeding on terminal trifoliate leaves. One possible explanation for this apparent inconsistency could be the lower nutritional value of primary leaves as compared to young (terminal) trifoliate leaves. Ballhorn et al. (2008b) reported higher concentration of soluble protein in young secondary leaves than in primary leaves of lima beans. Nitrogen in the form of protein is considered to be one of the critical elements in the growth of herbivores (Scriber, 1984; Panda and Khush, 1995). Spider mite fitness has been reported to correlate with host plant nutritional content,

Table 2 Effect of linamarin concentration in prey mites on the reproduction and sex ratio of *Phytoseiulus persimilis*

Host plant[a]	Linamarin[b]	N	Progeny[c]	Sex ratio[d]
RBL	0	12	16.25±2.14a	1.07±0.25a
HTL	298.25	12	9.92±1.73b	0.75±0.14b
HPL	429.82	12	2.75±2.18c	0.30±0.35c

Mean ± standard deviation. Means with the same letter are not significantly different after Tukey-Kramer HSD test at α=0.05

[a] Host plant keys: *RBL* red bean primary leaves; *HTL* Henderson lima bean terminal trifoliate leaves; and *HPL* Henderson lima bean primary leaves

[b] Concentration in parts per million (ppm) in spider mite prey tissue

[c] During a 3d period at 27±1°C

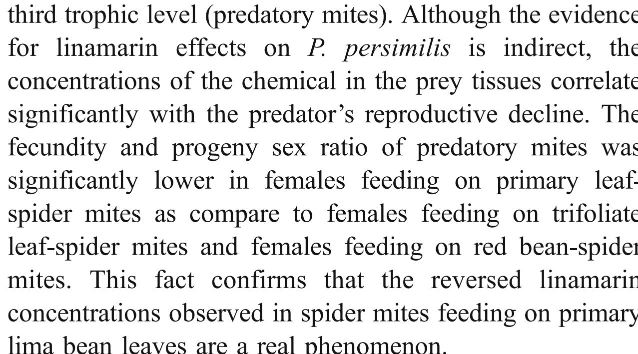
[d] In females per male

especially nitrogen in the form of protein (Wermelinger et al., 1991). Herbivores have the capacity to compensate for variations in plant nitrogen content by adjusting consumption (Panda and Khush, 1995; Felton, 1996). Spider mites growing on primary leaves must consume more tissue in order to maintain the same growth and reproductive activity, thereby increasing the amount of secondary compounds ingested. In most plants, aging leaves tend to go through changes in chemical composition, decreasing in water and nitrogen content, thereby reducing their nutritional value (Scriber, 1984). The fecundity and intrinsic rate of increase (r_m) of *Mononychellus tanajoa* (Bondar) increased when they were developing in young leaves of cassava plants as opposed to older leaves of these plants (Yaninek et al., 1989). Howeler and Cadavid (1983) reported that old leaves of cassava have reduced nitrogen content. In our study, we found no evidence of reduced fecundity in spider mites feeding on older primary leaves, which suggests that spider mites may be adjusting for the reduced nutritional value of the host leaves.

Fecundity and progeny sex ratio of *T. urticae* females feeding on primary vs. trifoliate lima bean leaves did not show significant differences despite the presence of lotaustralin in primary leaves and its absence in trifoliate leaves and the higher accumulation of linamarin observed in spider mites feeding on primary leaves. Spider mites feeding on red bean primary leaves did not produce more progeny, but, the sex ratio of their progeny was twice as high as that of spider mites feeding on Lima bean leaves. In a study of host plant suitability, Yano et al. (2001) observed that fecundity was not affected in spider mites feeding on unsuitable host; however, fitness of the population was impacted by reduced fertility of males. While female fecundity remained unaffected in lima bean leaves, the reduced sex ratio observed in their progeny could be explained by male infertility.

The sex ratio of spider mite progeny from females feeding on red bean leaves was reduced to the same levels as those observed in females feeding on Lima bean leaves when a solution of 1,000 ppm of linamarin was sprayed on the red bean leaves. The fecundity of female spider mites feeding on red beans was not statistically different from that of females feeding on Lima bean leaves. However, females feeding on linamarin-sprayed red bean leaves produced significantly fewer progeny. The concentration of linamarin in the spray solution was more than twice the levels observed in Lima bean leaves (1,000 vs. 449 and 142 ppm) producing a higher level of linamarin exposure in mites feeding on sprayed red bean leaves. This may be an indication of the existence of a linamarin concentration threshold for biological activity on spider mite females.

The impact of host plant linamarin accumulated at the second trophic level (spider mites) was amplified at the



third trophic level (predatory mites). Although the evidence for linamarin effects on *P. persimilis* is indirect, the concentrations of the chemical in the prey tissues correlate significantly with the predator's reproductive decline. The fecundity and progeny sex ratio of predatory mites was significantly lower in females feeding on primary leaf-spider mites as compare to females feeding on trifoliate leaf-spider mites and females feeding on red bean-spider mites. This fact confirms that the reversed linamarin concentrations observed in spider mites feeding on primary lima bean leaves are a real phenomenon.

Although *P. persimilis* feeds on spider mite eggs, which showed a much lower concentration of linamarin, fecundity and progeny sex ratio were both inversely proportional and negatively correlated to the linamarin concentrations present in the spider mite prey. Adults of *P. persimilis* do not eat eggs exclusively, and apparently these predators were considerably more sensitive to the concentrations of linamarin than *T. urticae*. Arthropod natural enemies, such as predators and parasitoids, often are more susceptible to plant secondary chemicals than herbivores (Ode, 2006). Direct evidence for linamarin impact on the third trophic level requires prey reared on artificial diet with artificial manipulation of linamarin concentrations. These studies will be conducted in the future.

The third trophic level effect of cyanogenic glycosides on *P. persimilis* may have implications for the relative effectiveness of this predator as a biological control agent against *T. urticae* in host plants with a high content of cyanogenic glycosides. The fitness of *P. persimilis* is reduced greatly by the presence of linamarin, while *T. urticae* appears to have significant tolerance to this chemical. The reduction in progeny sex ratio that *T. urticae* experience while feeding on lima bean leaves seems to be a small trade off for the protection that these plant semiochemicals provide against its predator *P. persimilis*. However, our data do not provide evidence for linamarin sequestration in *T. urticae*, as concentrations of this chemical in plant tissue do not correlate with the concentrations accumulated in the mite tissues. Increased linamarin concentrations in mites feeding on primary lima bean leaves can be explained by trophic interactions between herbivore and plant. Linamarin concentration in plant tissues also is an important consideration when choosing a host plant for *in vivo* mass rearing of phytoseiid mites on spider mite prey.

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herbivores elicits different quantities and blends of HIPVs than do jasmonates (Bruinsma et al., 2009).

Research on other feeding-induced plant responses often shows substantial seasonal or ontogenetic variation in their magnitude (Stout et al., 1996; Thaler et al., 1996; Karban and Baldwin, 1997). Such findings suggest that these induced responses may be best developed in, and perhaps limited to, relatively young plants or plant tissues (Karban and Myers, 1989).

In contrast to inducible direct defenses, the potential for seasonal variation in HIPV production has received minimal study. In maize (*Zea mays*), HIPV production in seedlings is inducible, but HIPV production in mature leaves apparently is not (Köllner et al., 2004). In soybean (*Glycine max*), feeding by the fall armyworm, *Spodoptera frugiperda*, induced ca. ten times the total HIPVs from leaves of 5-wk-old non-flowering vegetative plants compared to 10-wk-old flowering plants (Rostas and Eggert, 2008).

Although the study of induced HIPVs in cultivated species in the laboratory has logistic advantages, it is difficult to draw strong inferences on the evolution and maintenance of HIPV production in cultivated species because the interacting species often are far removed from their evolutionary context. One natural system in which such limitations can be overcome involves the native, undomesticated plant species, *Datura wrightii* (Solanaceae) and its native herbivores and natural enemies. In the laboratory, young plants of this species produce similar blends of HIPVs either when fed upon by the specialist chrysomelid beetle *Lema daturaphila*, or when treated with MeJA (Hare, 2007).

Unlike most of the rapidly-growing annual species in which HIPV production has been studied, *D. wrightii* has a long growing season in the sage scrub habitats of inland southern California. Seeds germinate in March or April after winter rains, but flowering usually is not initiated until June, after which plants continue to grow while also producing flowers and seed capsules through November. The plant is perennial, and plants may produce new sprouts from the root crown as early as February.

Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes, and aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam and Hare, 1998; van Dam et al., 1999). The 'velvety' phenotype is densely covered by short, non-glandular trichomes and feels velvety to the touch, whereas the 'sticky' phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids, and these leaves feel sticky to the touch. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al., 1999). Seedlings, new sprouts produced during the spring, and even new sprouts produced in mid-season after plants have been completely defoliated, all produce leaves that are covered with glandular trichomes (Elle et al., 1999). In all cases, however, the homozygous recessive genotype soon begins to produce leaves with a progressively greater proportion of nonglandular trichomes until differentiation to the velvety phenotype is completed (van Dam et al., 1999).

The herbivore community that attacks *D. wrightii* is relatively simple, being comprised of some five to ten species, depending upon season and location (Elle and Hare, 2000; Hare and Elle, 2002). The most abundant herbivore species overall is *Lema daturaphila*, a folivore whose adult and larval stages impose chronic, season-long herbivory on both trichome phenotypes. On sticky plants, the most abundant herbivore is the mirid bug, *Tupiocoris notatus*. This insect punctures and kills leaf cells while removing their contents. Both species are multivoltine and feed on *D. wrightii* throughout the plant's growing season. Other herbivores usually are less abundant and more limited seasonally in their occurrence (Elle and Hare, 2000).

A number of generalist natural enemies are associated with the herbivores of *D. wrightii*, but the most abundant is the predaceous western big-eyed bug, *Geocoris pallens* (Heteroptera: Lygaeidae). This is one of several generalist predators that is more effective against both *L. daturaphila* and *T. notatus* when the herbivores are on velvety rather than on sticky plants (Gassmann and Hare, 2005). Individual components of the HIPV blends of other plant species are known to attract *G. pallens* (Kessler and Baldwin, 2001; Halitschke et al., 2008), and this suggests that this predator may be attracted to some key compounds within a blend regardless of the blend's overall composition. This predator also inflicts higher mortality on eggs and young larvae of *L. daturaphila* on herbivore-damaged or MeJA-treated *D. wrightii* plants than on undamaged or untreated plants (J. D. Hare, pers. obs.).

In order to understand the potential for *D. wrightii* to release HIPVs that may attract natural enemies of its herbivores, this study analyzed the HIPV production of field-grown *D. wrightii* plants that were damaged by resident herbivore populations. Abundances of *L. daturaphila, T. notatus*¸ and *G. pallens* were monitored to determine how the seasonal production of HIPVs might relate to seasonal abundance patterns of the historically most abundant herbivores and their natural enemy. The goal was to test the null hypothesis of no qualitative or quantitative variation in induced HIPV production during the growing season. If the null hypothesis were rejected, then the variation in HIPV production might be caused either by variation in abiotic factors, such as temperature and relative humidity, that are known to vary over the growing season as well as to affect HIPV production (Gouinguene and Turlings, 2002), or by

ontogenetic changes in plants that are normally correlated with seasonal development. Seasonal variation in HIPV production indeed was observed. Therefore, additional experiments were performed on other plants using MeJA to control more precisely the process of induction to separate the effects of ontogenetic development of plants from seasonal variation in abiotic parameters on qualitative and quantitative variation in HIPV production.

Methods and Materials

Study Site Experiments were carried out in two adjacent fields at the experimental farm of the University of California, Riverside. In one field, plants were attacked naturally by a resident population of herbivores, whereas plants in the other field were induced by MeJA. To control for possible genetic variation in HIPV production, only plants of the highly inducible MVV6 and MVV8 lines (Hare, 2007) were used. All plants were backcross progeny of heterozygote sticky pollen acceptors and their original homozygous recessive velvety pollen parent for four (herbivory plot) or five (MeJA-induced plot) generations.

The collection of volatiles from herbivore-damaged plants, and herbivore and natural enemy abundances were monitored in a 2-yr-old field plot of plants that was maintained in a 'naturalized' condition with minimal weed control and no irrigation after the normal winter rains. Both herbivore and natural enemy populations had become established in this field, and surviving velvety plants were more abundant than sticky plants by the time this experiment was initiated.

For collection of volatiles from plants with better control of the timing and level of induction, a second plot was established 0.1 km away. Given the size of mature plants (1 m in height and 3 m in diam., Hare et al., 2003), it would have been impractical to attempt to induce these experimental plants to the same extent and at the same time with natural herbivory, so precisely-timed applications of MeJA were used instead. The separation of this plot from the herbivore-infested plot aided in keeping this plot free from herbivores and the plants uninduced prior to MeJA application.

For this plot, seeds were planted in a seedling flat on 24 January, 2009, stratified in a cold room (5.6 ° C) for 10 d, then placed in an incubator with alternating day and night temperatures (16:8 L:D, 30°:20 ° C). After 21 d, seedlings were transplanted to 10 cm pots and kept in the greenhouse (15°–30 ° C), with natural illumination extended to 16 h/d with sodium vapor lamps.

There were 10 blocks of 16 plants spaced 2.3 m apart within and between rows, and blocks were separated from each other by 8.4 m of bare ground. The area was treated with a pre-emergence herbicide (Trifluralin at the rate of 668 ml/ha). The plot was furrow-irrigated prior to transplanting for 24 h, then for 8 h 3 days after transplanting, and weekly for 8 h for three wk thereafter. The plants were not irrigated after 21 April, 2009, and no rainfall occurred during the experiment. The absence of any rainfall during the spring and summer is characteristic of the sage scrub habitat in which *D. wrightii* commonly occurs. Seedlings were transplanted to the field prior to differentiation of adult trichome type to facilitate establishment on 31 March, 2009, but this precluded having equal numbers of sticky and velvety plants in each block. After differentiation was complete, the observed frequency of sticky to velvety plants over all blocks was 82 sticky: 78 velvety, not significantly different from an expected 1:1 ratio for these backcross progeny. Plants were treated weekly, except during the weeks when volatiles were collected, with prophylactic applications of acephate (Orthene 97, Valent Chemical Co., Walnut Creek, CA, USA 1.2 g/l) in water to protect them from herbivory. Preliminary experiments showed that acephate alone did not induce HIPVs.

Induction and HIPV Collection in the Field Field procedures utilized aeration chambers, air lines, and traps of the same design as used previously in the laboratory (Hare, 2007). Aeration chambers were made from polyester cooking bags (unprinted 45 × 55 cm bags, Terinex, Bedford, U.K.). HIPVs were adsorbed on traps made of 4.6 mm O.D. glass tubing and filled with 25 mg of Super-Q (Alltech, State College, PA, USA). Air was introduced into the bag through PTFE tubing (4.6 mm O.D.) and was withdrawn from the bags through the traps through PVC tubing (4.6 mm I.D.) downstream of the traps. Air flow was provided by two 12-V portable air pumps (Model # MOA-P125-JH, Gast Manufacturing, Benton Harbor, MI, USA). One pumped air to the bags and the other withdrew it. A 12-V deep-cycle marine battery powered both pumps. A manifold with 16 pairs of flow meters (Aalborg Instruments, Orangeburg, NY, USA) was constructed so that inlet and exhaust flow to and from 16 sampling bags could be precisely and independently regulated at 1.0 l/min for each plant. Inlet air was drawn through a filter of activated charcoal placed between the pump and the manifold. Preliminary studies showed that the charcoal filter was sufficient to remove all ambient HIPVs.

Because the canopies of mid-season plants are comprised of several thousand leaves (Hare et al., 2003), it was not possible to sample HIPV production from the whole plant. An aeration bag was placed over the terminal leaves of a branch and secured with a twist-tie around the stem. The bag enclosed 4–6 full-sized leaves and a similar number of growing leaves. Branches with flowers were not used to avoid confounding floral volatiles with foliar

HIPVs. Volatiles were collected for 90 min. At the termination of the aeration, traps were wrapped in aluminum foil, labeled, returned to the laboratory, and stored at −20° C for extraction later that day. Two groups of 16 plants were aerated each day, usually from 08.30 h until 10.00 h, and from 11.00 h until 12.30 h.

Plants to be treated with MeJA were aerated twice, once prior to MeJA treatment and once afterward. As bags were removed from the aeration prior to MeJA treatment, the twist-ties were reattached to the branch where they sealed the bag to serve as a marker so that precisely the same leaves could be aerated after MeJA treatment. The upper surfaces of all leaves were sprayed with a solution of 8 mM MeJA in 0.1% Tween 20® in water just to run-off after the pretreatment aeration. This rate was chosen because it induced similar quantities of HIPVs as feeding by *L. daturaphila* in laboratory experiments (Hare, 2007). Plants were aerated a second time on the 3rd day following treatment. After post-treatment aeration, the aerated branches were clipped, brought to the laboratory, then all aerated leaves were removed and dried (80ºC for 48 h) to determine dry mass. A preliminary experiment showed that 0.1% Tween 20 alone did not induce HIPVs. After both aerations in a natural state, plants were "rejuvenated" by cutting them back to the root crown and allowing them to resprout and produce new foliage. After a month, plants were once again aerated before and after treatment with MeJA.

Herbivore-damaged plants were aerated only once. Because they were all being damaged, no pre-treatment aeration was possible. For both trichome phenotypes, branches were selected with actively feeding, 2nd or older instars of *L. daturaphila,* but larvae were removed prior to aeration. Sticky plants also were attacked by *T. notatus*, which also were removed prior to aeration. After aeration, the aerated leaves were removed and brought back to the laboratory. The percent defoliation averaged over all aerated leaves was estimated by a trained person before leaves were dried, as above, and the same person made all estimates throughout the experiment.

Five series of aerations were completed at 5wk intervals, starting the week of 27 April, 2009, and concluding the week of 14 September, 2009. Uninduced plants were aerated on Mondays and Tuesdays, herbivore-damaged plants were aerated on Wednesdays, and MeJA-induced plants were aerated on Thursdays and Fridays. All aerations took place under clear skies and full sunlight and were typical of inland southern California in all respects. Data on air temperature, relative humidity, and light intensity, recorded hourly from 09.00 h until 13.00 h, were obtained from records from the California Irrigation Management Information System (CIMIS) weather station located at the UC Riverside experimental farm (supporting information Table S1). A total of 736 aerations were planned over the season, but data from 18 were lost due to random accidents such as leaky or punctured bags, leaving a total of 718 aerations: 561 aerations from MeJA-treated and rejuvenated plants and 157 from plants naturally damaged by herbivores.

Volatiles were eluted from the traps with 150 µl of CH_2Cl_2 containing 4 ng/µl of 1-bromoheptane as an internal standard into autosampler vials with 250 µl glass inserts and sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography largely as described in Hare (2007) except that a shorter column (DB-5, 30 m, 0.25 mm ID, 0.25 µm film thickness, J & W Scientific, Folsom, CA, USA) and an autoinjector (Hewlett-Packard 7673) were used. To confirm identifications, selected samples were analyzed with a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass selective detector, and the mass spectra and retention times of compounds were matched with those of authentic standards as described in Hare (2007). Peak quantification in units of $ng{\cdot}g^{-1}$ leaf (dry wt.) h^{-1} was made using Agilent ChemStation® software based on comparison of their peak height with that of the internal standard.

Insect Abundances Insect censuses were taken at 2 wk intervals from 1 May through 25 September, 2009. For each census, up to 16 plants, 8 sticky and 8 velvety, were randomly selected, and a sleeve cage was placed around the base of each plant with the lower drawstring tied around the base of the plant. The cage remained open to allow recolonization of any arthropods that were disturbed by placing the cage around the plant. On the next day, the sleeve cage was quickly drawn around the plant, and the upper drawstring was tied to fully cage the plant, then the plant was cut at the base and returned to the laboratory. Plants were placed in plastic trash bags, and all arthropods were anesthetized with CO_2. Cages were opened, and all insects were removed, counted, and placed in labeled vials with 70% ethyl alcohol. Abundances of *L. daturaphila* and *G. pallens* are reported for each sampling date for both sticky and velvety plants, but abundances of *T. notatus* are reported only for sticky plants because *T. notatus* rarely occurs on velvety plants (Hare and Elle, 2002).

Statistical Analyses Analyses were carried out on total HIPVs, calculated as the sum of all individual HIPVs per plant, followed by multivariate analyses on the HIPV blend because the quantities of individual HIPVs were intercorrelated (van Dam and Poppy, 2008). I used PROC PRINCOMP of SAS (SAS Institute, 2008) to conduct an exploratory Principal Components Analysis (PCA) based upon the covariance matrix of all 718 samples to calculate a smaller number of uncorrelated components that were linear combinations of the original variables and to identify particular components contributing most to the patterns of variation in

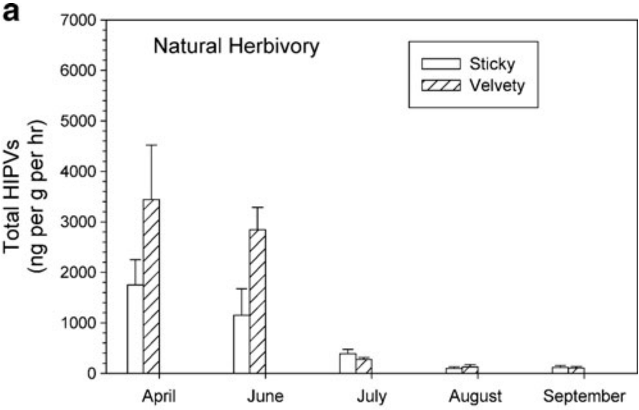
HIPV production. The factor loadings show how much each of the original variables contributes to each new component, and factor scores for the new components were calculated for each original observation. General patterns of variation illustrated by bivariate plots of the factor loadings and means of factor scores were explored further with restricted maximum likelihood (REML) analyses of variance (ANOVA) on the individual HIPVs with greatest factor loadings. Repeated measures ANOVAS using PROC MIXED of SAS were used to compare HIPV production of nonrejuvenated and rejuvenated plants before and after MeJA treatment. These models also included trichome type and aeration date, and all interactions as fixed effects. Where appropriate, two-way interactions were decomposed using the SLICE option of PROC MIXED. REML approaches to ANOVA are more appropriate than conventional approaches (e.g., PROC GLM of SAS) when sample sizes are unequal. In all models, 'block' (e.g., the group of 16 plants) was included as a random factor but was deleted from the final model when not statistically significant. For all HIPV analyses, the $\log_{10}$ (x+1) transformation was used to ensure normality of errors; the x+1 transformation was required because a few plants produced no detectable HIPVs, especially late in the season.

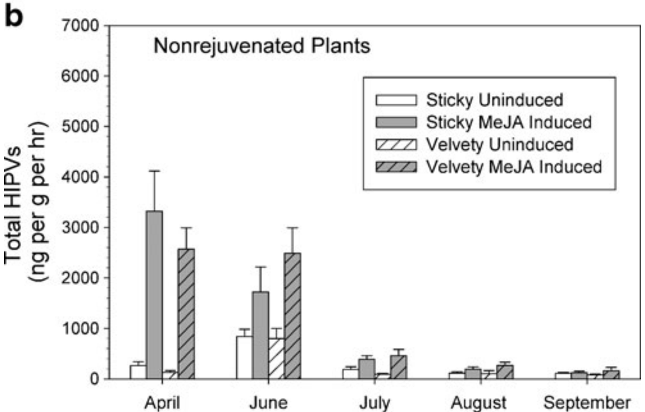
Analysis of covariance (ANCOVA) was used to analyze $\log_{10}$-transformed HIPV production of herbivore-damaged plants. Aeration date, trichome type, and their interaction were included as fixed effects, and percent defoliation was included as a covariate. Insect abundances also were analyzed by restricted maximum likelihood ANOVA after the $\log_{10}$ (x+1) transformation using a model that including sampling date, trichome phenotype, and their interaction as fixed treatment effects.

Results

HIPVs in the Field In general, the group of compounds detected was similar to those reported in a previous laboratory study (Hare, 2007). Abundant compounds included (*E*)-β-ocimene, (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, and (*E*)-4,8,dimethyl-1,3,7 nonatriene (hereafter DMNT). Other components of more limited abundance and occurrence included limonene, linalool, an isomer of bergamotene, α-humulene, β-selinene, nerolidol, and (*E*, *E*)-4,8,12 trimethyl-1,3,7,11 tridecatetraene (hereafter TMTT). In contrast to the laboratory studies, methyl salicylate, (*E, E*)-α-farnesene, and geranyl acetone were detected occasionally from plants aerated in the field. Basic statistics for all compounds are available in supporting information Table S2.

HIPV production by herbivore-damaged plants varied significantly over time (ANCOVA, date effect $F_{4,\ 132}$= 44.04, P<0.001, Fig. 1a). In April, defoliation averaging 7.3% and 8.3% on sticky and velvety plants, respectively, resulted in the production of over 2,500 ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1}. In June, defoliation of 4.9 and 10.5% on sticky and velvety plants resulted in the production only about 2,000 ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1} (Fig. 1a). Defoliation levels were higher in July, averaging 13.1 and 13.4% on sticky and velvety plants, respectively, but the production of HIPVs dropped to less than 350 ng $\cdot g^{-1}$





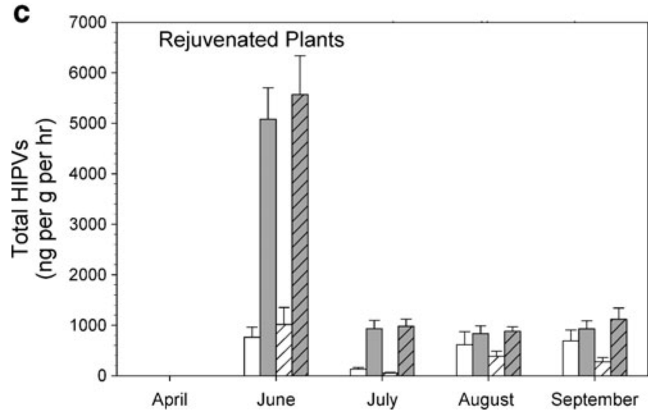


Fig. 1 Total HIPV production in the field for herbivore-damaged *Datura wrightii* plants (**a**), and for nonrejuvenated (**b**) and rejuvenated (**c**) plants before (Uninduced) and after application of MeJA (MeJA Induced). Mean (+ standard errors) production in $ng \cdot g^{-1}$ (dry wt) leaf wt h^{-1} are shown for plants expressing the sticky or velvety trichome phenotype

leaf tissue (dry wt.) h^{-1}. Plants produced only about 110 ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1} in August and September in response to herbivory averaging 8.5 and 7.3% on sticky and velvety plants in August and 8.8% and 8.4% in September. There was no linear relationship between percent defoliation and $\log_{10}$ HIPV production overall (P=0.14, Fig. 2), nor was there any heterogeneity of slopes between percent defoliation and total HIPV production within sampling dates (P=0.78). Neither did HIPV production differ significantly between trichome types (P=0.26). In summary, herbivory averaging 7–8% defoliation resulted in the production of nearly 25-fold more HIPVs when inflicted on plants in April than when inflicted in August or September.

MeJA treatment also significantly increased HIPV emission but the amount varied over the season (repeated measures ANOVA, treatment by date interaction $F_{4,\ 149}$= 22.16, P<0.001). In April, plants emitted nearly 2,700 more ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1} after MeJA treatment than before treatment, but the difference was cut substantially by June (Fig. 1b). The differences before and after MeJA treatment continued to decline throughout the summer, but were statistically significant from April through August (repeated measures ANOVA, SLICE decomposition of monthly differences, all $F_{1,\ 149}\geq$ 16.32, all P<0.001). The difference in HIPV production before and after MeJA treatment in September was not statistically significant (P=0.45), indicating that MeJA did not induce HIPV production at this time.

All sampled plants successfully resprouted, and the differences in total HIPV production of these rejuvenated plants before and after MeJA treatment always were statistically significant (repeated measures ANOVAs, all $F_{1,\ 120}\geq$19.59, all P<0.001) and ranged from a maximum difference of over 4,000 ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1} in the June aeration to a low of 365 ng total HIPVs g^{-1} leaf tissue (dry wt.) h^{-1} in the August aeration (Fig. 1c). Direct comparison of nonrejuvenated and rejuvenated plants showed that rejuvenated plants produced

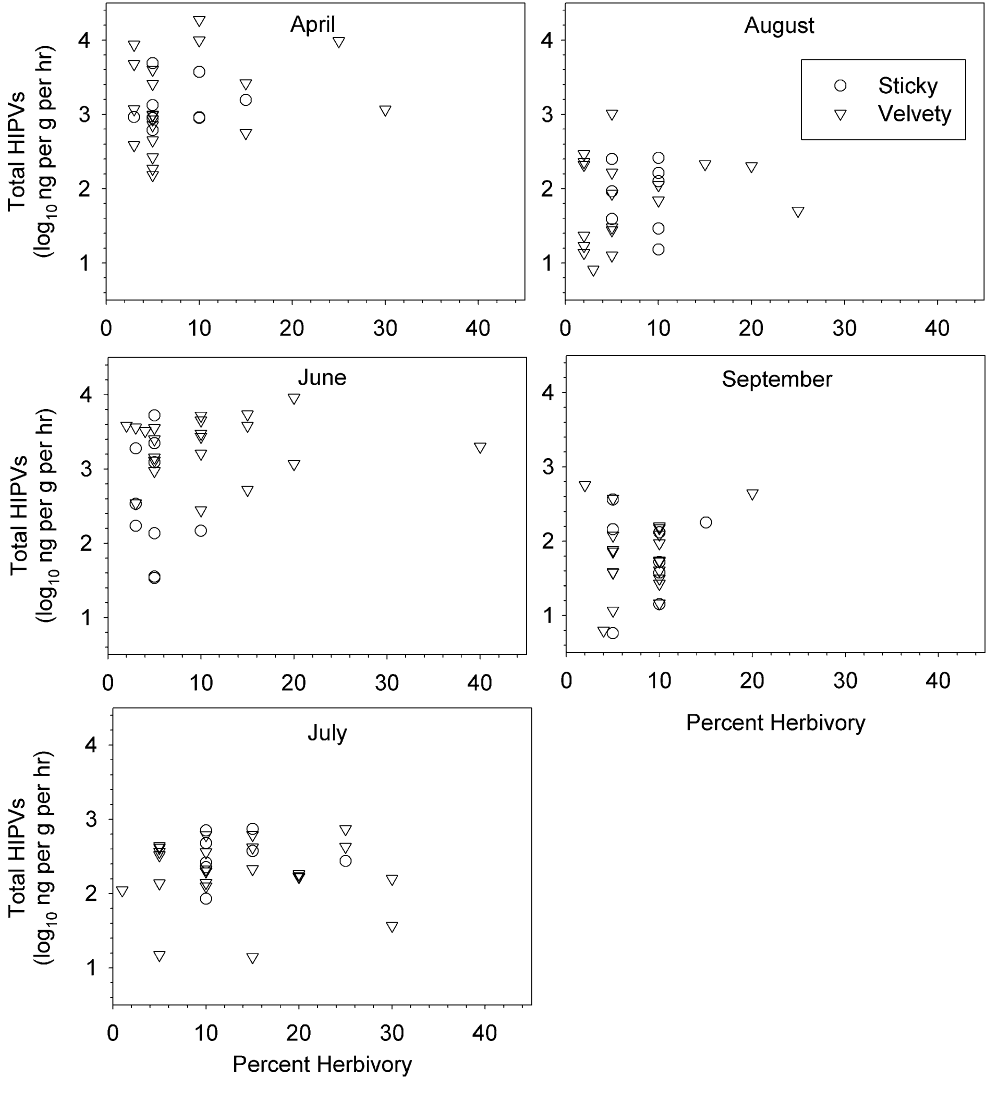


Fig. 2 Relationships between total HIPV production ($\log_{10}$ ng·g^{-1} (dry wt) leaf wt h^{-1}) and percent defoliation of *Datura wrightii* for each monthly aeration for herbivore-damaged plants expressing the sticky (*circles*) or velvety (*triangles*) trichome phenotype

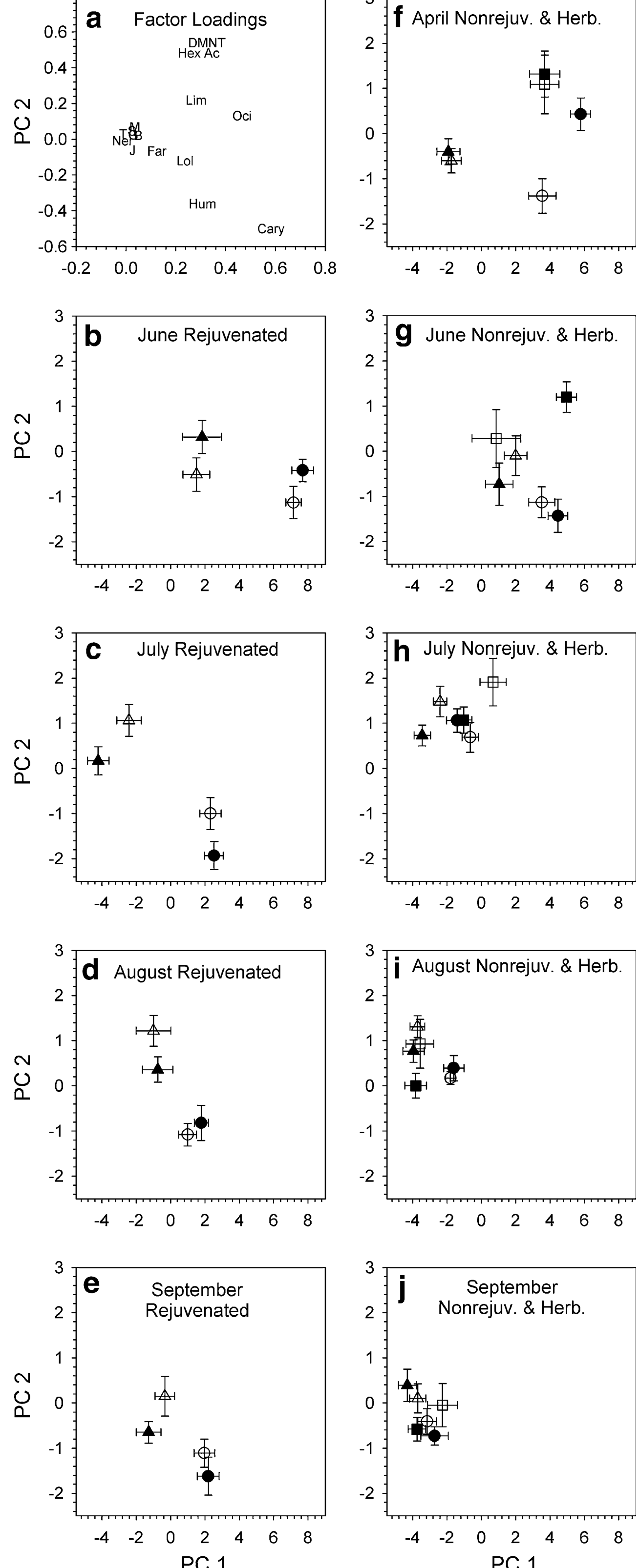


Fig. 3 Plot **a**: Factor loadings for individual herbivore-induced plant volatiles of *Datura wrightii* on PC1 and PC 2; Plots **b**–**e**: rejuvenated plants; Plots **f**–**j**: nonrejuvenated and herbivore-damaged plants. Mean (± standard error) factor scores for Principal Components 1 and 2 by trichome type, aeration date, and induction treatment. Triangles: uninduced plants; circles: MeJA-induced plants; squares: herbivore-damaged plants. Open symbols: sticky plants; filled symbols, velvety plants. Chemical abbreviations in Plot a: Cary: (*E*)-β-caryophyllene; DMNT: (*E*)-4,8,dimethyl-1,3,7 nonatriene; Far: (*E, E*)-α-farnesene; Hex Ac: (*Z*)-3-hexenyl acetate Hum: α-humulene; J: jasmone; Lim: limonene; Lol: linalool; Ner: nerolidol; Oci: (*E*)-β-ocimene; T: (*E*)-4-8,12 trimethyl-1,3,7,11 tridecatetraene. Overlapping near the origin: B: α-bergamotene; G: geranyl acetone; M: methyl salicylate; S: β-selinene

significantly greater quantities of HIPVs after treatment with MeJA, (repeated measures ANOVA rejuvenation effect $F_{1,\ 269}$=61.55, P<0.001), but the differences declined over the season (rejuvenation by date interaction $F_{3,\ 269}$= 9.85, P<0.001, Fig. 1b, c). For neither non-rejuvenated nor rejuvenated plants did total HIPV production vary with trichome phenotype (all P≥0.09).

PCA analysis yielded seven components whose eigenvalues were greater than 1.0, and the first component (PC 1) accounted for 50.3% of the total variance in the data. Principal Components # 2 through 7 accounted for 8.0, 7.1, 6.4, 5.7, 4.8, and 4.2% of the total variance, respectively, for a total of 86.5%. All original variables except nerolidol and TMTT had positive loadings on PC 1, indicating that this component can be interpreted largely as a “general production” factor much like a “general size” factor in morphological studies. The highest factor loadings were for (*E*)-β-ocimene and (*E*)-β-caryophyllene (Fig. 3a), and plants that emitted relatively greater quantities of (*E*)-β-ocimene and (*E*)-β-caryophyllene had relatively higher scores on PC 1.

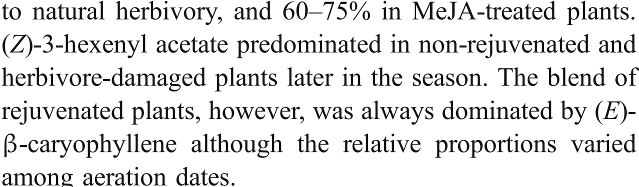
The original variables loaded positively or negatively on PC 2, indicating differences in the composition of the HIPV blends. PC 2 contrasts plants that, among other differences, had higher concentrations of (*Z*)-3-hexenyl acetate and DMNT with plants that produced higher concentration of the sesquiterpenes, α-humulene, or (*E*)-β-caryophyllene (Fig. 3a). The remaining principal components generally contrasted particular compounds of limited occurrence that were not easily interpreted, so detailed analyses were carried out only for PC 1 and PC 2.

Factor scores for PC 1 were greater for MeJA-treated plants or herbivore-damaged plants than uninduced plants to varying amounts, depending upon aeration date, induction method, and rejuvenation treatment (Fig. 3b–e for rejuvenated plants and Fig. 3f–j for nonrejuvenated and herbivore-damaged plants); scores for PC 1 reflected patterns similar to those for total HIPVs (Fig. 1). The changes in factor scores for PC 2 were more variable among aeration dates and treatments. There was relatively little change factor scores for PC 2 before and after MeJA treatment for nonrejuvenated plants in April and rejuvenated plants in June (Fig. 3f, b), indicating

that the blends were greater in concentration but similar in composition after induction. Substantial changes in scores for PC 2 were observed in the post-treatment aerations for rejuvenated plants in July, August, and September (Fig. 3c–e) and for herbivore-damaged plants in April (Fig. 3f). The relatively greater negatives scores for PC 2 post-induction in the rejuvenated plants reflect relatively higher production of (*E*)-β-caryophyllene and other compounds with high negative loadings for PC 2 in rejuvenated plants. The differences in composition of HIPV blends from nonrejuvenated plants before and after induction declined in the later aerations, however, as shown by similar scores for PC2 in July through September (Fig. 3h–j).

The high factor loadings for (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, DMNT, and (*E*)-β-caryophyllene (Fig. 3a) on PC1 and/or PC 2 justified more detailed analyses of these particular compounds. For herbivore-damaged plants, all of these major compounds were produced in significantly higher concentrations in April and June than in July through September (ANOVA; all date effects $P<0.001$, Fig. 4a, d, g, j). For MeJA-treated plants, (*Z*)-3-hexenyl acetate was generally the most abundant HIPV prior to MeJA treatment, but the ability of MeJA to induce higher levels of this compound was inconsistent in both non-rejuvenated and rejuvenated plants over time (repeated measures ANOVA, treatment by date interaction $F_{4,\ 149}=5.32$, $P<0.001$ for non-rejuvenated plants and $F_{3,\ 120}=7.23$, $P<0.001$, for rejuvenated plants, Fig. 4b, c). (*E*)-β-Ocimene and (*E*)-β-caryophyllene were induced consistently by MeJA in rejuvenated plants and relatively young nonrejuvenated plants, but less so in older nonrejuvenated plants [(*E*)-β-ocimene: MeJA treatment by date interaction $F_{4,\ 149}=21.55$, $P<0.001$ for nonrejuvenated plants and $F_{3,\ 120}=8.41$, $P<0.001$ for rejuvenated plants; (*E*)-β-caryophyllene: MeJA treatment by date interaction $F_{4,\ 149}=9.34$, $P<0.001$ for nonrejuvenated plants and $F_{3,\ 120}=13.90$, $P<0.001$ for rejuvenated plants, Fig. 4h, i, k, l]. More variable results were obtained for DMNT (MeJA treatment by date interaction $F_{4,\ 149}=28.09$, $P<0.001$ for non-rejuvenated plants and $F_{3,\ 120}=7.58$ for rejuvenated plants, Fig. 4e, f). Rejuvenated plants produced significantly more quantities of (*E*)-β-ocimene and (*E*)-β-caryophyllene than non-rejuvenated plants (all rejuvenation treatment effects $P>0.003$) although the magnitude of the differences varied over the season (all rejuvenation treatment by date interactions $P<0.004$).

In summary, the composition of the HIPV blends varied over the season as a result of quantitative differences in the induction of different HIPVs at different times. The induced blend of herbivore-damaged and nonrejuvenated plants was dominated by (*E*)-β-caryophyllene in April and June and comprised ca. 44–49% of all HIPVs, depending upon trichome phenotype and sampling date in plants subjected



to natural herbivory, and 60–75% in MeJA-treated plants. (*Z*)-3-hexenyl acetate predominated in non-rejuvenated and herbivore-damaged plants later in the season. The blend of rejuvenated plants, however, was always dominated by (*E*)-β-caryophyllene although the relative proportions varied among aeration dates.

Insect Abundances All sampled plants were infested with *L. daturaphila*, and all sampled sticky plants were infested with *T. notatus* in the first census, and the infestation rate of *L. daturaphila* never dropped below 85%. Similarly, the infestation rate of *T. notatus* never dropped below 67%. The infestation rate of *G. pallens* was more variable, ranging from 0 and 26% of sticky and velvety plants in the first census to between 57–100% of all plants irrespective of trichome phenotype through mid-September. After an initial peak following colonization of plants in the spring, densities of larvae of *L. daturaphila* declined to a range of from 3–35 per plant throughout the remainder of the season (ANOVA, census date effect $F_{10,\ 153}=6.25$, $P<0.001$, Fig. 5a). Abundances did not differ significantly between sticky and velvety phenotypes ($P=0.92$) nor did the census date by time interaction ($P=0.27$). Densities of *T. notatus* increased from ca. 28 per plant at the beginning of the season to over 200 per plant in mid-August, but because of the large variation within censuses, the differences in densities did not differ significantly among censuses ($P=0.21$, Fig. 5b). Densities of *G. pallens* also showed a seasonal peak in the spring but then declined to a range of from 1–5 per plant for the remainder of the season (census date effect $F_{10,\ 153}=7.91$, $P<0.001$, Fig. 5c). Surprisingly, abundances of *G. pallens* were significantly greater on sticky than velvety plants throughout the season (phenotype effect $F_{1,\ 153}=7.97$, $P=0.005$). Observations of egg masses of *L. daturaphila* showed evidence of predation by *G. pallens*, throughout the season, but predation was not quantified.

Discussion

The ability of *D. wrightii* to increase HIPV production in response to natural herbivory and treatment with MeJA is constrained seasonally and by ontogenetic stage. Greater quantities of induced HIPVs were produced by induced plants during the two aerations in the spring, when plants were growing vegetatively and had not yet begun to flower, than in the summer when plants continued to grow vegetatively but also began flowering and fruiting. The ability of plants to emit HIPVs in response to MeJA treatment during the three summer aerations was restored partially by cutting the plants back to ground level and allowing them to produce new foliage from the root crown.

Fig. 4 Production of (*Z*)-3-hexenyl acetate, DMNT, (*E*)-β-ocimene, and (*E*)-β caryophyllene in the field for herbivore-damaged *Datura wrightii* plants, and nonrejuvenated and rejuvenated plants before (Uninduced) and after application of MeJA (MeJA Induced). Mean (+ standard errors) in ng·g^{-1} (dry wt.) leaf hr^{-1} are shown for plants expressing the sticky or velvety trichome phenotype

Although the HIPV production of rejuvenated plants was significantly greater than nonrejuvenated plants during each of the three summer aerations, it always was lower than nonrejuvenated plants in the spring.

The relatively high proportion of (*E*)-β-caryophyllene in young plants is consistent with previous laboratory work with this system (Hare, 2007), and it suggests that this may be one compound that is differentially induced during the

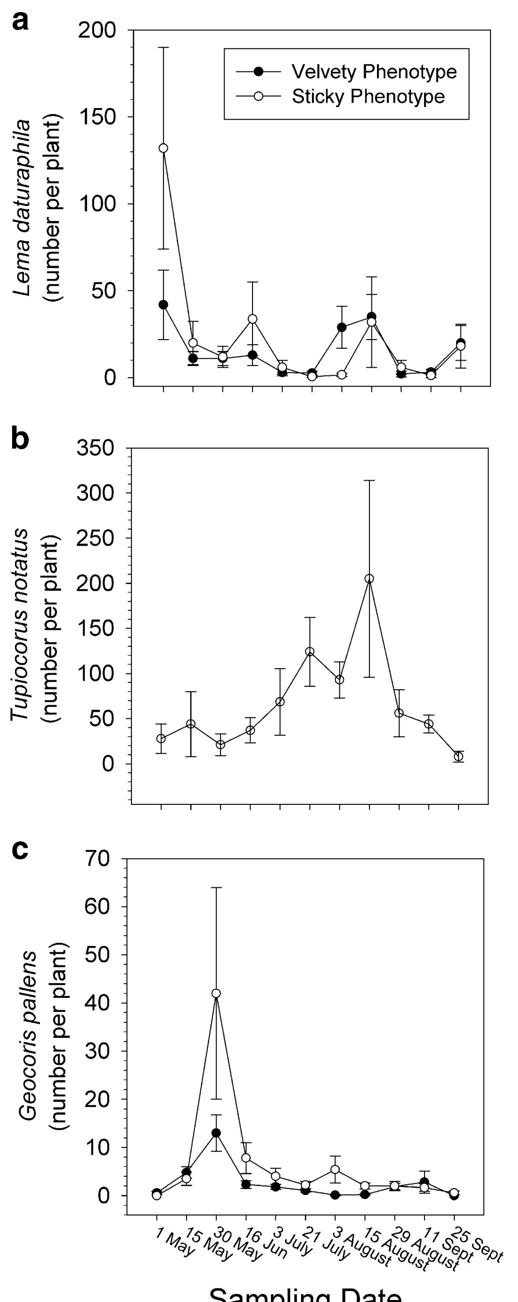


Fig. 5 Densities per plant of motile stages of the herbivores *Lema daturaphila* (**a**), *Tupiocoris notatus* (**b**), and the predator *Geocoris pallens* (**c**) sampled from *Datura wrightii* every 2 weeks. Means (± standard errors) are shown

earlier stages of plant ontogenetic development. Over time, however, the production of (*E*)-β-caryophyllene in older plants declined, leaving the blend of induced HIPVs relatively richer in (*Z*)-3-hexenyl acetate later in the summer.

Because of the potential for seasonal variation in the inducibility of HIPV production in response to natural herbivory, it was important to attempt to separate the effect of seasonally varying factors, such as temperature and humidity, from endogenous aspects of plant development that might have caused induced HIPV production to vary. One approach to separate seasonality from ontogeny might have been to transplant fresh seedlings to the field at different times during the summer (Thaler et al., 1996), but this would have been ecologically unrealistic with regard to natural germination of *D. wrightii,* as well as logistically difficult to establish transplants in the field during the heat of the summer. The observation that plants completely defoliated by herbivores resprout with juvenile foliage and then redifferentiate (Elle et al., 1999) suggested a different, more ecologically relevant approach toward separating seasonal from ontogenetic variation in HIPV production. This approach simply required that mature plants be cut back and allowed to resprout. This manipulation restored HIPV production, but to varying extents depending upon when during the season the plants were cut back. Thus, both seasonal and ontogenetic factors may constrain inducible HIPV responses in *D. wrightii*. The rejuvenated plants also served as a positive control and showed that the lower levels of HIPVs from unrejuvenated plants in the three summer aerations compared to the two spring aeration were not simply the result of any environmental factor that might preclude HIPV collection during the summer.

It is well-established that induced direct defenses are more pronounced in younger plant tissues (Thaler et al., 1996; Karban and Baldwin, 1997; van Dam et al., 2001), but relatively little is known as to the potential age-related variation in inducible HIPV production. In maize, HIPV production of leaves could be induced in 14-d-old seedlings but not in mature leaves (Köllner et al., 2004). HIPV production was an order of magnitude higher in vegetative stages of soybean than in flowering plants (Rostas and Eggert, 2008). This limited evidence, along with the present study, suggests that, as with inducible direct defenses, induced HIPV production may also be maximized in relatively young tissues.

Under natural conditions, *D. wrightii* is embedded in a community of plants, each with their own communities of herbivores and natural enemies. Plants occur in patches that persist for several years (Hare and Elle, 2004). Individual plants within patches quickly become colonized by over-wintering adults of *L. daturaphila*, *T. notatus*, and other herbivores in the early spring, then suffer chronic herbivory throughout the growing season. Plants also are colonized by *G. pallens* and other generalist natural enemies at about the same time (Gassmann and Hare, 2005). Only two species of parasitoid have been recovered from southern California *L. daturaphila* populations, and these are the tachinid fly, *Myiopharus infernalis,* and the chalcid wasp, *Conura delumbis*. The host range of both are poorly known but include, at least, other species of beetles from non-solanaceous plant species (Arnaud, 1978; Burks, 1979).

Both parasitoids also are most abundant in late summer (J. D. Hare, pers. obs., see also Puttler (1966)).

The original goal of this study was to understand the potential for *D. wrightii* to release HIPVs that may attract natural enemies of its herbivores. The temporal pattern of abundance of insects and HIPV production suggests several possibilities. In view of the rapid colonization of plants by both herbivores and natural enemies, it may not be beneficial for *D. wrightii* to produce any "long-range" cues except perhaps in the early spring, when some plants are colonized by herbivores but most are not. Using a modeling approach, Puente et al. (2008a) concluded similarly that "the utility of the volatile cues is consistently greater when a smaller proportion of plants is occupied by herbivores, indicating that their usefulness may be reduced to zero in fields saturated with volatiles," presumably when all plants are finally discovered and damaged by herbivores. On the other hand, if natural enemies can respond quantitatively as well as qualitatively to HIPVs, then plants producing stronger or more desirable cues may benefit over plants that produce weaker cues even when all plants are infested.

A possible explanation that would be consistent with the greater HIPV production by young *D. wrightii* plants is based upon the "optimal defense" theory of defense against herbivores. Optimal defense theory is based upon the premise that the allocation of resources is determined by the net result of the costs of that defense balanced against its benefit in terms of plant fitness (McKey, 1974). One consequence of this is the prediction that defensive traits should be maximally deployed during stages of high risk and/or low tolerance (Boege and Marquis, 2005). Although *L. daturaphila* densities were highest in the spring, those of *T. notatus* were not, and these species have shown different seasonal patterns of abundance in southern California; particularly a secondary peak usually occurred in late September and October in other years (Elle and Hare, 2000; Hare and Elle, 2002). It may be difficult to identify periods of differential risk of herbivory based upon the persistent abundance of these two herbivore species. A more likely application of the optimal defense theory in this system would be based upon ontogenetic variation in tolerance to herbivory. As *D. wrightii* grows, the plants produce a large, fleshy tap root that stores carbohydrate. Possibly, the ability of *D. wrightii* to tolerate herbivory increases with age such that continued allocation to defense, including HIPV production, becomes progressively less beneficial, possibly accounting for the seasonal decline in HIPV production.

Additionally, induced volatiles may be adaptive to *D. wrightii* for reasons other than, or in addition to, mediating herbivore-natural enemy interactions. In other systems, for example, some HIPVs confer resistance against herbivores or pathogens, and others mitigate abiotic stresses (Holopainen, 2004; Penuelas and Llusia, 2004; Dudareva et al., 2006; Gershenzon and Dudareva, 2007). Although no such studies have yet been undertaken in *D. wrightii* to evaluate these possibilities, they cannot be ignored. Such additional roles, of course, would introduce substantial complications into the study of the production of HIPVs by *D. wrightii* and in assessing how and when HIPVs might benefit plant fitness (Dicke and Baldwin, 2010). Finally, it also is possible that HIPV production is merely a genetically correlated consequence of other induced plant responses to herbivore damage and incidental to the interactions between *D. wrightii*'s herbivores and their natural enemies. Nevertheless, current results suggest the testable hypothesis that, by whatever combination of mechanisms, any benefits of HIPV production by *D. wrightii*, as well as any natural selection on *D. wrightii* genotypes that favors different quantities or blends of HIPVs (Hare, 2007), may be confined largely to the early periods of vegetative growth when HIPV production is greatest.

Most research on HIPVs has been performed in the laboratory and on cultivars of domesticated plant species (Dicke and Baldwin, 2010), and this is one of a few studies to investigate the seasonal variation in the production of induced HIPVs of an undomesticated plant species under field conditions. Results generally support previous laboratory studies on *D. wrightii* showing that young, vegetative plants can be induced to produce HIPVs. The laboratory studies, however, were performed only on young plants and were insufficient to capture both the dynamic change in the ability of plants to produce HIPVs over the course of the natural growing season and the changes in the composition of those HIPV blends. That the induced HIPV response of *D. wrightii* is largely confined to ontogenetically young tissues is consistent with studies in other systems showing that other induced plant responses are largely confined to young plants (Karban and Baldwin, 1997; Boege and Marquis, 2005). It is perhaps less obvious that HIPV production in *D. wrightii* is well-matched to the patterns of seasonal abundance of *D. wrightii*'s herbivores and their natural enemies.

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Several enzymes located inside and on the surface of plant cells are probably involved in the synthesis of H_2O_2 (Apel and Hirt, 2004). Membrane-bound NADPH oxidase (Levine et al., 1994; Bolwell, 1999), an apoplastic peroxidise (POD) (Bolwell et al., 1995), and peroxisomal xanthine oxidase (XO) (Sandalio et al., 1997) have been implicated in hydrogen peroxide production. Xanthine oxidase produces $O_2^{\bullet -}$, which is converted to H_2O_2 by superoxide dismutase (SOD) (Montalbini, 1992a). The oxidative purine catabolic pathway, which includes XO, is selectively induced in wheat plants infected with *Puccinia recondita* (Montalbini, 1992b). Increases in XO activity found in beans upon infestation with *Uromyces phaseoli* were successfully inhibited by allopurinol treatment, and the resultant hypersensitive cell collapse also was inhibited when infected with pathogens (Montalbini, 1992a).

Moloi and Van der Westhuizen (2006) demonstrated that NADPH-oxidase is involved in the defense response of wheat to the RWA. In this study, we demonstrated the importance of the xanthine/xanthine oxidase system for the induction of resistance to Russian wheat aphid infestation.

Methods and Materials

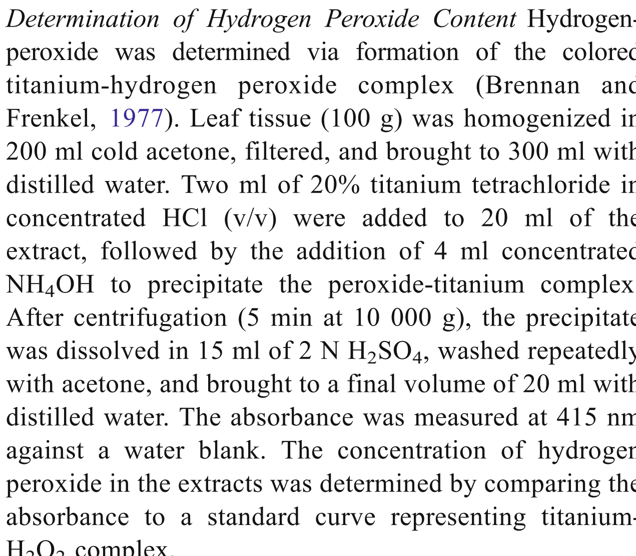
Plant and Aphid Material Wheat (*Triticum aestivum*) plants, resistant (cv. *Tugela DN*) and susceptible (close isogenic cv. *Tugela)* (Du Toit, 1989) to the RWA (biotype SA1) *Diuraphis noxia* (Mordvilko) were grown in a glasshouse at day and night temperatures of 25° C and 21° C respectively.

A colony of RWA was obtained from the Agricultural Research Station in Bethlehem, South Africa. The aphids were maintained on *Tugela* wheat until experimental wheat plants were infested. Plants were infested at the second-leaf growth stage with 25 fourth instar aphids per plant.

All the leaves of the plant were collected at specific time intervals after infestation and immediately frozen in liquid nitrogen. They were then stored at −20° C for subsequent analyses.

Enzyme Extraction Enzymes were extracted by grinding 1 g of frozen leaf tissue with 100 mg acid-washed sand in 10 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1% PVP (Rao et al., 1997). After centrifugation (25 000×g, 20 min), the supernatant was used for the enzyme assays.

Protein concentrations were determined with the Biorad Microplate method (Rybutt and Parish, 1982). The assay mixture consisted of 160 µl distilled water, 40 µl BioRad, and 10 µl standard (0.5 µg. μl^{-1} γ-globulin) or sample (Bradford, 1976).



Determination of Hydrogen Peroxide Content Hydrogen-peroxide was determined via formation of the colored titanium-hydrogen peroxide complex (Brennan and Frenkel, 1977). Leaf tissue (100 g) was homogenized in 200 ml cold acetone, filtered, and brought to 300 ml with distilled water. Two ml of 20% titanium tetrachloride in concentrated HCl (v/v) were added to 20 ml of the extract, followed by the addition of 4 ml concentrated NH_4OH to precipitate the peroxide-titanium complex. After centrifugation (5 min at 10 000 g), the precipitate was dissolved in 15 ml of 2 N H_2SO_4, washed repeatedly with acetone, and brought to a final volume of 20 ml with distilled water. The absorbance was measured at 415 nm against a water blank. The concentration of hydrogen peroxide in the extracts was determined by comparing the absorbance to a standard curve representing titanium-H_2O_2 complex.

Determination of Superoxide Dismutase Activity Following the method of Keppler and Novacky (1987), SOD activity was measured by measuring the inhibition of the nitroblue tetrazolium (NBT) reduction. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, and 50 µl sample. The reaction mixture was irradiated by placing it 30 cm below two fluorescent lamps (2×40 W) for 10 min. A non-irradiated duplicate was used as a control (reference cuvette). After irradiation, the absorbance of the reaction mixture was measured at 560 nm (Hitachi U-2000 double-beam spectrophotometer). In addition, a sample without crude extract was irradiated and used to measure the maximum attainable absorbance at 560 nm. The SOD activity is expressed as log [A560 (with crude extract) mg^{-1}/A560 (without crude extract)] (Keppler and Novacky, 1987).

Determination of Peroxidase Activity The peroxidase assay mixture consisted of 40 mM potassium buffer (pH 5.5), 5 mM guaiacol, and 8.2 mM H_2O_2. The change in absorbance was measured at 470 nm for 180 sec at 30°C (Hitachi U-2000 double-beam spectrophotometer) (Zieslin and Ben-Zaken, 1991).

Determination of Chitinase Activity Chitinase activity was measured according to the method of Wirth and Wolf (1990), with slight modifications. The method is based on the precipitability of carboxymethyl-chitin-remazol brilliant violet 5R (CM-Chitin-RBV) (Loewe Biochemica GmbH) from buffered solutions with HCl.

The reaction mixture for determining chitinase activity consisted of 66.6 mM Na-acetate buffer (pH 5.0), and 100 µl CM-chitin-RBV (2 $mg.ml^{-1}$) and sample. The reaction mixture was incubated at 37°C, and after 30 min

the reaction was stopped by the addition of 100 µl of 2 M HCl. The sample was put on ice for 10 min to allow complete precipitation and subsequently centrifuged at 12 000 g for 7 min. Before measuring the absorbence at 550 nm, the sample was diluted 4× with distilled water (Wirth and Wolf, 1990).

In Vivo Treatment with Allopurinol The effect of allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine) on the HR was evaluated according to the method described by Montalbini (1992b). Allopurinol was applied to plants as a soil drench (250 ml of 0.4 mM solution daily). Application was started 7 d after the wheat was planted, and continued until the last sampling date. Allopurinol is a purine analogue and a competitive inhibitor of XO. It tightly binds to the reduced molybdenum component of the enzyme, thus inhibiting activity and the production of oxygen radicals cannot take place (Massey et al., 1970; Hille and Massey, 1981).

In Vitro Treatment with Allopurinol Allopurinol was added to the respective reaction mixtures for SOD, POD and chitinase to test for *in vitro* inhibition. Final concentration of allopurinol in the reaction mixtures was 0.4 mM.

Statistical Analysis Six leaf samples of each treatment were collected. Enzyme activities were repeated four times for every sample. Statistical analyses were performed using STATISTICA, version 9, from StatSoft®. The normality of the data was checked using the Shapiro-Wilkes test. A one-way ANOVA, using the Turkey HSD test, was performed at a significance level of 95%. The purpose of the ANOVA was to check whether there is a statistical difference in the enzyme activities between the infested resistant wheat and the corresponding controls.

Results

Hydrogen Peroxide Content An increase ($P<0.05$) in H_2O_2 was detected in the infested, resistant wheat as early as 8 h after infestation (Fig. 1a). Significantly increased H_2O_2 levels were observed over a 48 h period (Fig. 1b) with the highest levels detected 15 h after infestation in the infested, resistant wheat. At 15 h post infestation, the H_2O_2 was 112% higher in the infested, resistant wheat than in the non-infested resistant wheat. After 48 h, the H_2O_2 was 85% higher in the infested, resistant wheat compared to the infested, susceptible wheat. There were no significant differences between the H_2O_2 content of the non-infested, resistant, and susceptible wheat leaves.

In Vitro Effect of Allopurinol on Enzyme Activities The *in vitro* effect of allopurinol was tested on the activities of SOD, POD, and chitinase. Allopurinol did not inhibit the activities of SOD, POD, or chitinase *in vitro* (results not shown).

Effect of Allopurinol on Superoxide Dismutase Activity In Vivo RWA infestation significantly increased ($P<0.05$) SOD activity in infested, resistant wheat (Fig. 2). SOD activity did not change during the investigated period in susceptible, infested, or resistant wheat. SOD activity reached a peak 10 h after infestation in infested, resistant wheat. The allopurinol drench significantly ($P<0.05$) inhibited the induced SOD activity by 70% in infested, resistant wheat (Fig. 2).

Effect of Allopurinol on Peroxidase Activity RWA infestation significantly ($P<0.05$) induced POD activity in infested, resistant wheat, peaking at 24 h. Little change occurred in the controls where the activity remained constant. The allopurinol drench significantly ($P<0.05$)

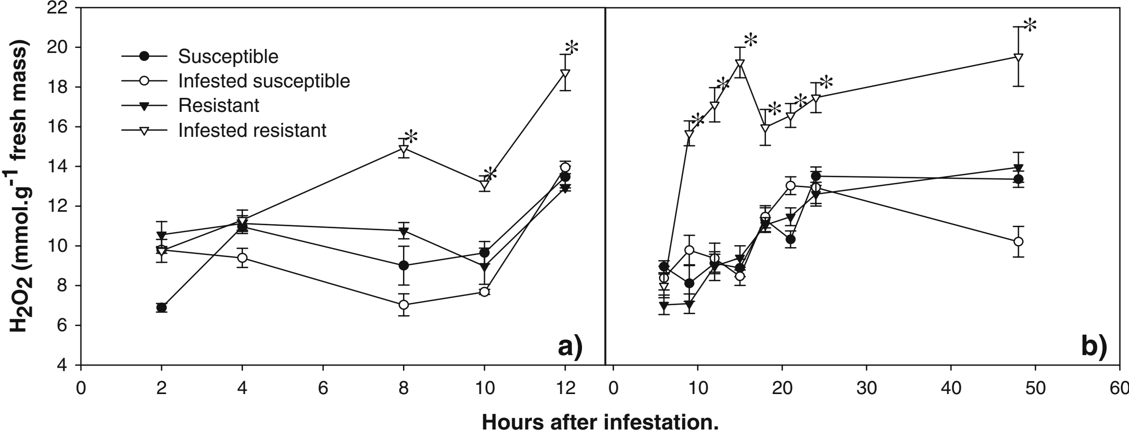


Fig. 1 Time course of H_2O_2 levels (**a**) for a 12 h period and (**b**) for a 48 h period. Error bars indicate standard deviation. Asterisks denote significant increases above the infested control ($P<0.05$)

inhibited the induced (24 h) POD activity by 80% (Fig. 3) in infested, resistant wheat.

Effect of Allopurinol on Chitinase Activity Aphid infestation induced chitinase activity ($P<0.05$) in infested, resistant wheat (Fig. 4). The selectively induced chitinase activity was significantly ($P<0.05$) inhibited by the allopurinol drench to levels similar to those of controls.

Discussion

H_2O_2 is responsible for induction of the defense genes as well as for cell death in the development of restricted lesions (Pellinen et al., 2002; Apel and Hirt, 2004). Felton et al. (1994, 1999) demonstrated differential increases in H_2O_2 in the incompatible interaction between plants and insects. During the incompatible interaction between wheat and the RWA, H_2O_2 elicited the accumulation of salicylic acid (Mohase and Van der Westhuizen, 2002), which is responsible for systemic acquired resistance.

A possible mechanism for the production of active oxygen species such as H_2O_2 during the HR is via the enzyme XO, which converts hypoxanthine xanthine, and xanthine to uric acid (Montalbini 1992a, b). Enhanced activity in XO and uricase preceded and accompanied the HR induced by *Uromyces phaseoli* infection in beans (Montalbini, 1991). Furthermore, active oxygen species drive the synthesis of cell-membrane structural proteins; oxidize cinnamyl alcohols to free radicals by peroxidase/ H_2O_2, leading to the formation of lignin (Karlsson et al., 2005); trigger programmed cell death; and diffuse to neighboring cells to induce defense genes (Mur et al., 2008).

In this study, the authors wanted to determine if oxygen radicals and, in particular, H_2O_2, are responsible for inducing the downstream defense response in wheat that is resistant to RWA. Allopurinol, which is an inhibitor of XO activity (Montalbini 1991, 1992a, b) was used to inhibit the production of O_2^- and consequently H_2O_2. The interaction between resistant wheat and the RWA was manifested as a differential increase in H_2O_2 that reached peak values quite soon after infestation (8 h).

The activity of SOD was dramatically reduced (Fig. 2a) in the infested, resistant wheat growing in the soil drenched with allopurinol, thus suggesting that no H_2O_2 was produced. The low SOD activity could be due to the

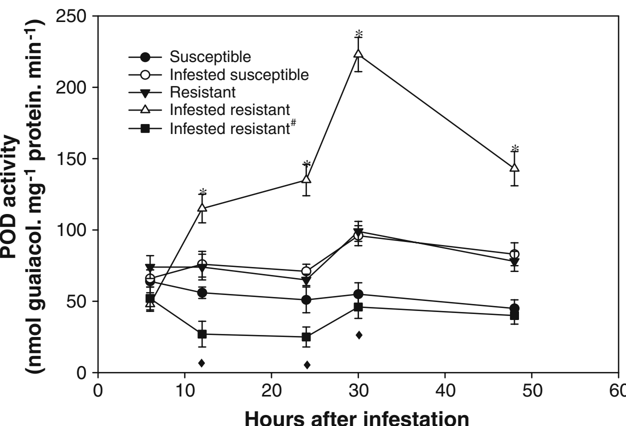


Fig. 3 Time course study of POD activity for a 48 h period and the inhibitory effect of drenching the plants with allopurinol (#) on POD activity. Error bars indicate standard deviation. Asterisks denote significant increases above the infested control ($P<0.05$), diamonds denote significant decreases ($P<0.05$) from the corresponding controls

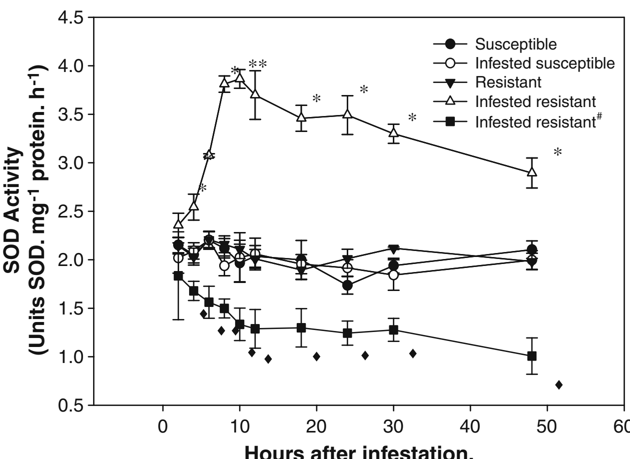


Fig. 2 Time course of SOD activity for a 48 h period and the inhibitory effect of drenching the plants with allopurinol (#) on SOD activity. Error bars indicate standard deviation. Asterisks denote significant increases above the infested control ($P<0.05$), diamonds denote significant decreases ($P<0.05$) from the corresponding controls

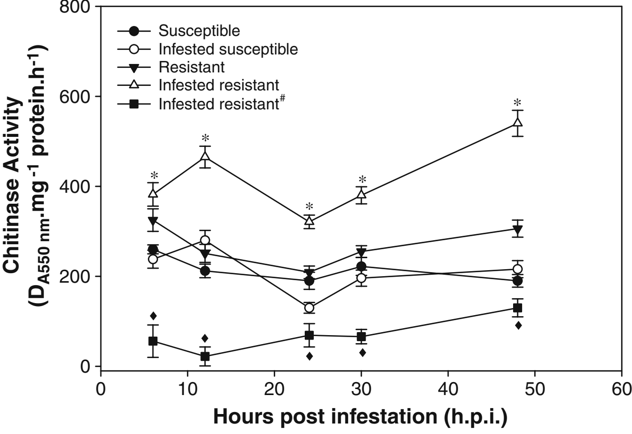


Fig. 4 Time course of chitinase activity for a 48 h and the inhibitory effect of drenching the plants with allopurinol (#) on chitinase activity. Error bars indicate standard deviation. Asterisks denote significant increases above the infested control ($P<0.05$), diamonds denote significant decreases ($P<0.05$) from the corresponding controls

disappearance of its substrate (O_2^-), or it may be that the genes encoding for SOD were not induced.

It is well established that POD (Van der Westhuizen et al., 1998a, b) and chitinase (Van der Westhuizen et al., 1998b) activities are involved in the downstream defense response of wheat against the RWA, and they were used in this study to indicate whether the downstream defense response is activated or not. This study confirmed the selective induction of POD (Fig. 3) and chitinase (Fig. 4) activities in the infested, resistant wheat upon infestation with the RWA. Allopurinol drenching prevented differential induction of POD (Fig. 3). Loss of POD activity may be the result of insufficient substrate (H_2O_2) and/or insufficient gene induction due to low H_2O_2 levels. For this reason, the inhibition of POD activity by allopurinol does not necessarily prove that the H_2O_2 was necessary to induce the downstream defense response. The authors, therefore, additionally investigated the effect of allopurinol inhibition on the chitinase defense response.

The expression of chitinase has a defensive role when plants are infected with pathogens. Some chitinases have lysozymal activity and can hydrolyze the peptidoglycans in bacterial cell walls, whereas others have exohydrolytic activity (Punja and Zhang, 1993). Wheat infested with the RWA showed differential increases in chitinase activity (Van der Westhuizen et al., 1998b). In this study, we confirmed the differential induction of chitinase activity (Fig. 4). Allopurinol drenching resulted in a 97% decline in activity (Fig. 4). The successful induction of chitinase is, therefore, dependent on the activity of xanthine oxidase. Allopurinol applied *in vitro* had no effect on the activity of chitinase in infested resistant wheat.

Surprisingly, the leaves of resistant wheat displayed signs of rolling when allopurinol was applied (results not shown), a phenomenon seen only in susceptible plants when infested with the RWA. This can be explained because of the suppressed defense responses that resulted from the allopurinol drenching. Montalbini (1992a, Montalbini and Torre, 1996) has shown that defense responses associated with the HR are suppressed after allopurinol treatment.

Resistant wheat infected with the RWA showed differential increases in H_2O_2 levels and SOD activity. The importance of H_2O_2 during the defense response to induce the downstream defense mechanism was demonstrated by inhibiting XO activity with allopurinol. Allopurinol drenching resulted in suppressed SOD, POD, and chitinase activities, thus indicating that the generation of H_2O_2 via XO is necessary for the activation of downstream defense responses.

Acknowledgements We wish to acknowledge the financial support from the National Research Foundation and the University of the Free State towards this study.

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(Rudman, 1965; Chang et al., 1999, 2000; Cheng et al., 2005, 2008), there are few reports concerning the chemical components of conifer cones and the antifungal properties of abietane-type diterpenes. Research into natural antifungal compounds from *T. distichum* cones may be beneficial to chemical ecology, phytochemistry, and agrochemistry.

In our previous study, we reported the isolation and identification of abietane-type diterpenes from *T. distichum* cones. We found a relationship between ferruginol oxidized compounds and termicidal and antifeedant activities against the subterranean termite, *Reticulitermes speratus* Kolbe (Kusumoto et al., 2009). In this study, we report on twelve diterpenoids including ten abietane-type components and their antifungal activities against white- and brown-rot fungi.

Methods and Materials

Plant Material Cones of *T. distichum* (Taxodiaceae class) that were not seriously damaged or weathered were collected at the Yamagata Field Science Center (Faculty of Agriculture, Yamagata University, Japan) located in Tsuruoka City in western Yamagata prefecture. Cones were gathered from the ground and identified by the Yamagata Field Science Center.

General Experimental Procedures GC-FID analysis was performed with an HITACHI G-3000 gas chromatograph under the following conditions: DB-1 capillary column (30 m×0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); column temperature from 100°C (1 min) to 320°C (10 min) at 5°C/min; injection temperature 230°C; detection temperature 250°C. GC-MS data were obtained with a SHIMADZU QP-5000 GC-MS: DB-1 capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); column temperatures were programmed to be equal to GC-FID analysis. The acquisition mass range was 50–450 amu using helium as carrier gas (3.6 ml/min). ^{1}H-NMR, ^{13}C-NMR, DQF-COSY, HMBC, and HMQC spectra were obtained to aid structural identification. 1D and 2D NMR spectra were measured with a JEOL JNM-EX400 (^{1}H 400 MHz/^{13}C 100 MHz) spectrometer.

Extraction and Isolation The air-dried *T. distichum* cones (800 g) were milled and extracted successively at ambient temperature for 7 d with *n*-hexane (*n*-C_6H_{14}), ethyl acetate (EtOAc), and methanol (MeOH). These extracts were tested as potential inhibitors of fungal growth as described below. The *n*-C_6H_{14} soluble fraction (84.9 g) was partitioned with saturated $NaHCO_3$, 10% Na_2CO_3, and 1% NaOH aqueous solutions in a separatory funnel, and was purified by silica-gel 60 N (spherical 63–210 μm, neutral, Kanto Chemical Co., Japan) column chromatography. The 12 compounds (Fig. 1) that were isolated have been identified previously (Kusumoto et al., 2009): 6,7-Dehydroferruginol (**1**), ferruginol (**2**), 6,7-dehydroroyleanone (**3**), sandaracopimaric acid (**4**), taxodione (**5**), taxodal (**6**), sugiol (**8**), xanthoperol (**9**), salvinolone (**10**), 5,6-dehydrosugiol (**11**), and 14-deoxycoleon U (**12**). The structures of were determined by comparison of NMR spectral data with published data. The amounts of pure isolated compounds **1**, **2**, and **8** were too small to use for antifungal tests; therefore, we isolated compounds **2** and **8** from *Cryptomeria japonica* as in previous studies (Ashitani et al., 2001). Compound **1** was synthesized from **8** by chemical conversion (Matsui et al., 2004). In this study, the novel compound taxodone (**7**) was isolated from the neutral fraction of *n*-C_6H_{14} extract, and the chemical structure was identified by comparison of 1D NMR spectral data with published data (Kupchan et al., 1969). The configuration of **7** was confirmed and determined by decoupling experiments using 2D NMR spectral data.

Taxodone (7) 7.8 mg. Pale orange amorphous solid. EI-MS *m/z*: 316 (M^+, $C_{20}H_{28}O_3$, 26%), 301 (7), 298 (8), 283 (9), 273 (12), 255 (9), 245 (13), 242 (12), 233 (19), 231 (21), 229 (24), 220 (43), 219 (37), 217 (24), 215 (30), 205 (33), 203 (27), 191 (25), 177 (28), 173 (21), 128 (27), 115 (33), 95 (21), 91 (35), 83 (31), 77 (28), 69 (50), 55 (100), 53 (26); ^{1}H-NMR ($CDCl_3$): δ 1.11 (3 H, *d*, *J*=6.8 Hz, 17-CH_3), 1.13 (3 H, *d*, *J*=6.8 Hz, 16-CH_3), 1.13 (3 H, *s*, 20-CH_3), 1.19 (3 H, *s*, 18-CH), 1.19 (3 H, *s*, 19-CH), 1.40 (2 H, *m*, 3-CH_2), 1.43 (2 H, *m*, 3-CH_2), 1.54 (1 H, *m*, 5-CH), 1.55 (2 H, *m*, 2-CH_2), 1.63 (2 H, *m*, 1-CH_2), 1.65 (2 H, *m*, 2-CH_2), 2.89 (2 H, *m*, 1-CH_2), 3.04 (1 H, *hept*, *J*= 6.8 Hz, 15-CH), 4.67 (1 H, *brd*, *J*=10.0 Hz, 6β-axial-CH), 6.52 (1 H, *d*, *J*=2.7 Hz, 7-CH), 6.79 (1 H, *s*, 14-CH), 7.46 (1 H, *s*, 11-OH); ^{13}C-NMR ($CDCl_3$): δ 18.8 (2-CH_2), 20.8 (16-CH_3), 21.4 (17-CH_3), 21.7 (20-CH_3), 22.8 (19-CH_3), 26.7 (15-CH), 34.1 (4-C), 36.7 (18-CH_3), 37.6 (1-CH_2), 40.7 (10-C), 43.2 (3-CH_2), 58.0 (5-CH), 70.0 (6-CH), 126.2 (9-C), 130.4 (13-CH), 135.7 (14-CH), 142.0 (11-C), 143.4 (8-C), 149.1 (7-CH), 181.7 (12-C).

Fungal Strains The wood-decay fungi *Trametes versicolor* (white-rot) (NBRC: 30340), and *Fomitopsis palustris* (brown-rot) (NBRC: 30339), were provided by the Natural Institute of Technology and Evaluation Biological Resource Center (NBRC; Tokyo, Japan). These fungi are chosen routinely for antifungal tests according to Japan Industrial Standard (JIS) K1571. Cultures of each fungus were maintained on potato dextrose agar (PDA) medium (Eiken Chemical Co., Japan) and stored at 4±1°C. Before antifungal tests, each strain was incubated on PDA medium

Fig. 1 Chemical structures of isolated diterpenes from n-C_6H_{14} extract of *Taxodium distichum* cones. 6,7-dehydroferruginol (**1**), ferruginol (**2**), 6,7-dehydroroyleanone (**3**), sandaracopimaric acid (**4**), taxodione (**5**), taxodal (**6**), taxodone (**7**), sugiol (**8**), xanthoperol (**9**), salvinolone (**10**), 5,6-dehydrosugiol (**11**), and 14-deoxycoleon U (**12**)

in a Petri dish at 26±1°C until the fungus covered most of the surface of the plate.

Antifungal Assays Antifungal assays were performed according to previously published methods (Kofujita et al., 2006; Sekine et al., 2009). Before fungal inoculation, test sample was dissolved in acetone or methanol (1 mg/ml), and 600 µl of each solution were applied to the surface of each 15 ml PDA medium in a Petri dish (88 mm diam) so that the final concentration was 10 µg/cm^2 in solid agar. After the application, each plate was air-dried on a clean bench for 1 hr. An equal amount of pure acetone or MeOH was spread on the control medium. An inoculum of each strain was obtained using a 5.5 mm diam cork-borer, and was placed on the center of the test medium. The dishes were cultured in the dark at 26±1°C and 70% relative humidity in an incubator. When mycelia reached the edge of the Petri dish of the control, the average mycelium diameter per treatment was calculated from the measurement of four radial directions. All results are expressed as means±SE. The antifungal assay was repeated three times for each sample and six times for the control. The antifungal activity was evaluated as a percentage of relative growth rates, and it was calculated as follows:

$$\text{Average growth rate}\ (\%) = 100 \times \text{Da}/\text{Db}$$

Da : Total average of the mycelium diam of each sample
Db : Average diam of control mycelium

Results and Discussion

Yields and Antifungal Properties of Successive Extracts The material extracted successively by n-C_6H_{14}, EtOAc, and MeOH extracts from 800 g of air-dried *T. distichum* cones ranged from 10.8% of the tissue mass (n-C_6H_{14}) to 3.53% (EtOAc) or 1.56% (MeOH). Both the n-C_6H_{14} and EtOAc extracts were potent mycelial growth inhibitors for *F. palustris* (Fig. 2). Similarly, both n-C_6H_{14} and EtOAc

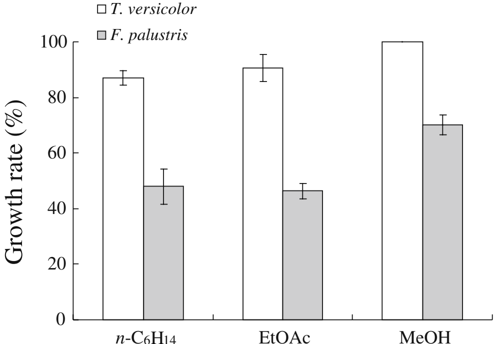

Fig. 2 Antifungal activities of successive extracts from *Taxodium distichum* cones against two wood-rot fungi

extracts were active against *T. versicolor*. On the other hand, the MeOH extract revealed mycelium growth inhibition for *F. palustris*, but not for *T. versicolor*.

Clearly the n-C_6H_{14} and EtOAc extracts had higher antifungal activities against both *T. versicolor* and *F. palustris* than the MeOH extract. Because the yield of the *n*-C_6H_{14} extract was about three times higher than that of EtOAc extract, we chose to characterize the hexane extract.

Chemical Compositions of n-C_6H_{14} Extract A quantitative analysis by GC-FID showed that 70% of *n*-C_6H_{14} extract was composed of diterpenes (Fig. 3). The percentage content of each component was calculated from the ratio between each component and the total diterpenoid peak area, which was measured by GC-FID (Table 1). The isolated compounds, excluding sandaracopimaric acid (**4**) and taxodal (**6**), were diterpenes with an abietane-type structure (Kusumoto et al., 2009). The amounts of 6,7-dehydroferruginol (**1**) and ferruginol (**2**) were remarkably high in the *n*-C_6H_{14} extract (Table 1). In addition, the amounts of taxodione (**5**), taxodone (**7**), salvinolone (**10**), and 14-deoxycoleon U (**12**) also were high. The GC-FID peak areas of compounds **4**, **6**, and **11** were too small to calculate their percentage content.

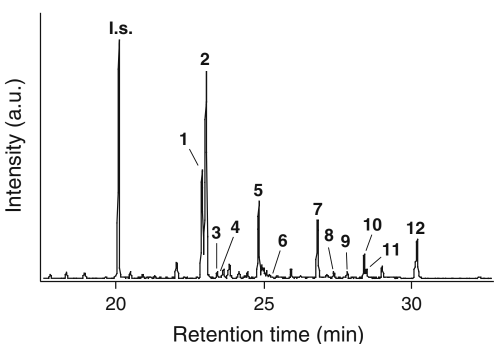

Fig. 3 GC-MS chromatogram of diterpene composition in *n*-C_6H_{14} extract of *Taxodium distichum* cones. I.s.; Internal standard (heneicosane). 1-12; refer to Fig. 1

Table 1 Constituents of diterpenes in *n*-C_6H_{14} extract of *Taxodium distichum* cones

Compound	Retention time (min)	Contents (%)[a]
6,7-dehydroferruginol (1)	22.9	20.4
ferruginol (2)	23.0	39.4
6,7-dehydroroyleanone (3)	23.4	0.88
sandaracopimaric acid (4)	23.5	t[b]
taxodione (5)	24.8	12.3
taxodal (6)	25.5	t[b]
taxodone (7)	26.8	6.19
sugiol (8)	27.4	0.74
xanthoperol (9)	27.8	0.79
salvinolone (10)	28.4	4.80
5,6-dehydrosugiol (11)	28.5	t[b]
14-deoxycoleon U (12)	30.1	2.08

[a] Contents (%)=100×Each component peak area/Total diterpenoids peak area (GC-FID)

[b] Trace

The amounts of compound **1** and **2** were higher than any other compounds in the *n*-C_6H_{14} extract, suggesting that **1** and **2** were the major compounds in all extracts of this tissue. In order to evaluate the antifungal properties of *T. distichum* cones, it was necessary to confirm the relationship between the quantities and the activities of isolated compounds.

Antifungal Properties of Isolated Compounds The antifungal activities of isolated compounds **1** to **12** were tested at 10 μg/cm^2 against two wood-decay fungi (Fig. 4). 14-Deoxycoleon U (**12**), taxodione (**5**), and salvinolone (**10**) were strong mycelium growth inhbitors in *T. versicolor*. These three compounds also had antifungal activity against *F. palustris*, with mycelium growth inhibitions by **5** or **12**

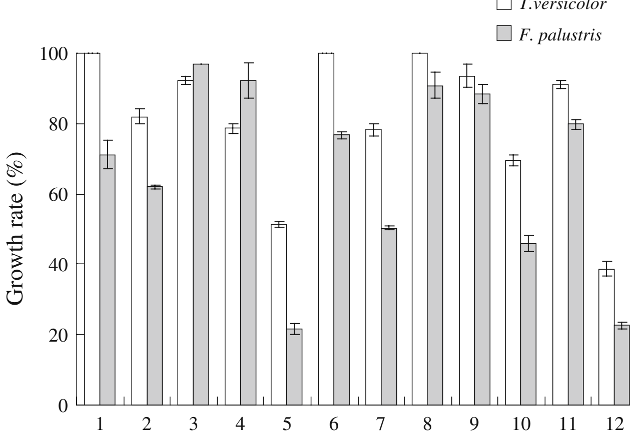

Fig. 4 Antifungal activities of isolated diterpenoids against two wood-rot fungi. 1-12; refer to Fig. 1

two times stronger than **10**. Taxodone (**7**) and ferruginol (**2**) also had antifungal activity against *F. palustris*.

6,7-Dehydroferruginol (**1**), taxodal (**6**), and sugiol (**8**) did not inhibit mycelial growth of *T. versicolor*; but they reduced growth rate of *F. palustris* (Fig. 4). Isolated compounds, except for **3** and **4**, had much stronger antifungal activities against brown-rot fungi (*T. versicolor*) than against white-rot fungi (*F. palustris*).

We evaluated the relationship between antifungal activity and chemical structure. Taxodione (**5**), which showed strong antifungal properties, is a hydroxyquinone. Some of the abietane-type diterpene quinones have antifungal properties (Kofujita et al., 2002) but 6,7-Dehydroroyleanone (**3**) is a hydroxybenzoquinone that does not have activity against either fungus. Quinones must not be the only chemical group required for antifungal activity. A comparison of taxodione (**5**) and taxodone (**7**) revealed that both compounds contain a ketone group, but that its position differs. The ketone group on **5** was at C-6, and several lines of evidence suggest that substitution at this position is associated with antifungal activity against wood decay fungi. Ferruginol (**2**) has more antifungal activity than 6,7-dehydroferruginol (**1**), which comprises a C-6 and C-7 dehydration of **2**. Sugiol (**8**), which is a hydroxyabietatrien structure with a C-7 carbonyl group, has no activity against either fungus; but 5,6-dehydrosugiol (**11**), which includes a C-5 and C-6 dehydration of **7**, is more growth inhibitory than **8**. Compounds **11**, **10**, and **12** reduced growth rates in both *T. versicolor* and *F. palustris*. Growth inhibition by compounds **11**, **10,** and **12** increased with an increasing number of hydroxyl groups at C-6 and C-11; the number of hydroxyl groups at these positions appeared to be related to the antifungal activities against *T. versicolor* and *F. palustris*. Only compounds **5**, **7**, **10,** and **12** had a hydroxyl group at C-11 suggesting that a strong relationship between antifungal activity and this chemical structure exists. In conclusion, several specific abietane-type antifungal compounds were investigated from *T. distichum* cones. Taxodione (**5**), salvinolone (**10**), and 14-deoxycoleon U (**12**) had strong antifungal properties. The activities of **5** and **12** against both wood-decay fungi were especially apparent. The n-C_6H_{14} extract contained high levels of 6,7-dehydroferruginol (**1**) and ferruginol (**2**), which had antifungal activity against *F. palustris*, but not against *T. versicolor*. Ferruginol (**2**) has been reported to be effective against wood-decay fungi (Rudman, 1965); but against *F. palustris*, the antifungal activity of **2** was only one third that of **5** and **12**, which were oxidized compounds of **2**. These results suggest that the quantity of abietane-type compounds is not the only factor influencing the antifungal activities of *T. distichum* cones, but that there is also an effect of oxidized abietane-type compounds. This illustrates that it is important to investigate the antifungal properties of both major compounds and minor oxidized compounds when evaluating potentially active compounds. We believe that identifying the notable antifungal abietane-type compounds from *T. distichum* cones could be meaningful for developing chemical defenses of coniferous species.

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temperature, pH, alkalinity, mixing regime, and light, many of which show significant relationships to cyanobacteria biomass and dominance (Watson et al. 1997; Dokulil and Teubner 2000).

Among the bloom-forming cyanobacteria, particular attention has been paid to *Microcystis*, a colonial, buoyancy controlling K-strategist (Benndorf and Henning 1989). Species of *Microcystis* are widely distributed in eutrophic (Reynolds 2006) and hypereutrophic lakes (Kotak et al. 1995) and rivers (Krogmann et al. 1986), where they can develop dense, potentially toxic blooms. Extensive research has focussed on the ecophysiology of these cyanobacteria, and several key mechanisms have been proposed that may provide these taxa with a competitive edge over other species. These studies have focused primarily on bottom-up factors related to light, mixing and nutrient uptake; others have recognized the potential importance of size-selective grazing (Hyenstrand et al. 1998). However, there is a growing body of evidence to suggest that more subtle grazer interactions controlled by allelochemicals play important but poorly understood roles (DeMott and Tessier 2002; Hansson et al. 2007).

Under natural conditions, the cells of *Microcystis* are embedded in a matrix of mucilage and form colonies with sizes up to 8 mm in diameter (Joosten 2006). Large colonies often exceed the maximum ingestible size range of even large freshwater crustaceans that is in the range of 75 μm for an adult *Daphnia magna* (Burns 1968). This provides little protection, however, for smaller *Microcystis* colonies and single cells, which remain within the food spectrum even of small sized crustaceans (Fulton and Paerl 1987a; Jarvis et al. 1987; Rohrlack et al. 1999a; Ghadouani et al. 2004). In addition to providing the colonial matrix, evidence suggests that a second protective function may be involved by the externally produced mucilage. *Daphnia* showed lower ingestion and increased its labral rejection rate of small colonies and single cells covered with robust mucilage compared to mucilage poor cells (Rohrlack et al. 1999a; Ghadouani et al. 2004). Indirect chemical interactions that promote grazer selectivity also may influence some top–down processes. For example, sterols are essential membrane constituents of animal grazers, and can only partially be synthesized by crustaceans (von Elert et al. 2003). A form of non-specific biochemical defense may be accomplished by the extremely low production of these sterols by all cyanobacteria including *Microcystis,* which may lead to reduced grazing losses as herbivores may prefer ingestion of food items of higher quality (Koski et al. 2006)

More direct chemical defense may be provided by the production of toxic microcystins, which are potent inhibitors of eukaryote protein phosphatase 1 and 2A. Comparisons of microcystin-producing and non-producing *Microcystis* strains as sole food for crustaceans have reported both toxic and non-toxic effects (Wilson et al. 2006). However, these experiments have failed to address some important confounding factors: Notably, i) differences in *Microcystis* chemotypes among studies or in field populations; ii) among- and within-strain variance in production of multiple microcystin congeners of different toxicity; iii) the co-production of other bioactive oligopeptides by *Microcystis* (Welker et al. 2006; Jüttner and Lüthi 2008); iv) differences in the genetic capacity for toxin production and its activation, even within the same bloom (Rinta-Kanto et al 2009); and v) the development of grazer resistance (DeMott et al. 1991). More definitive evidence has been obtained by using a mutant strain of *Microcystis* PCC 7806 that lacks the microcystin synthetase gene *mcy B*, but that does not affect synthesis of other oligopeptides (Rohrlack et al. 1999b). Only the wild type exhibited toxicity to *Daphnia galeata* and clearly showed the efficiency of microcystins. Other *Microcystis* secondary metabolites also have a direct inhibitive or toxic effect. The oligopeptides aeruginosins and cyanopeptolins are effective serine protease inhibitors (Ishida et al. 1999; Bister et al. 2004), microviridin J disrupts moulting (Rohrlack et al. 2003), and aerucyclamides are bioactive (Portmann et al. 2008) but the molecular targets are not yet known. Serine protease inhibitors severely affect the hydrolytic cleavage of serine proteases isolated from *Daphnia* gut, likely having a similar effect under natural conditions (von Elert et al. 2004).

As known in many examples from higher plants, unsuitable food organisms produce warning signals that alert grazers. Volatile organic compounds (VOCs) are widely used by different taxa to locate and evaluate other organisms in the environment by a form of 'remote sensing'. These allelochemicals include pheromones, attractants, and repellents (Watson 2003; Fink 2007). In terrestrial ecosystems, volatiles are airborne, and both primitive and highly advanced organisms have developed extremely sensitive odor receptors that detect trace concentrations of them. In aquatic ecosystems, the matrix for VOC distribution is water or biofilm mucilage (Jüttner 1999). Many of the allelochemicals identified to date are lipophilic, but they can induce behavioral responses in target organisms at trace levels. Hence, their signalling function is not impaired by their low water solubility—as seen, for example, with marine phaeophyte spermatozoids, which are attracted to mature eggs by pheromones (alkenes) at picomolar concentrations (Boland et al. 1995).

While VOCs may reduce ingestion and may have toxic properties, there has been little convincing evidence to suggest that they function in a more subtle manner, by influencing grazer behavior towards *Microcystis*. However, intriguing preliminary evidence has implicated a VOC derived from photosynthetic pigments that may, in fact,

function in this indirect manner. This was based on early studies, which demonstrated clearly that for all *Microcystis* strains tested, cell disruption activates the rapid cleavage of β-carotene by an unique carotene dioxygenase, forming two major derivatives, β-cyclocitral, a volatile that is released into the water, and crocetindialdehyde, a non-volatile product which remains associated with the cell debris (Jüttner 1984; Jüttner and Höflacher 1985). β-Cyclocitral is a well known odor compound that affects drinking water supplies, and gives *Microcystis* blooms a characteristic hay-tobacco odor, but its role in aquatic chemical defense against grazers has only recently been examined (Watson et al. 2007). As a lipophilic VOC, β-cyclocitral is well suited as a chemical signal. In recent experiments, we observed a strong and immediate increase in swimming velocity of *Daphnia magna* at low concentrations of this compound (Watson et al. 2007). In the present study, we elucidated this reaction in more detail, by testing the hypothesis that β-cyclocitral serves as a signal for food avoidance and induces a phobic response of *D. magna*. We also quantified this process on a molecular level, thus providing the basis for calculating this microscale biochemical interaction that occurs during *Daphnia*–*Microcystis* interactions.

Methods and Materials

Microcystis Microcystis NRC-1 (*Microcystis* sp. Zehnder 1954 / NRC-1, identical with SAG B14.85) was obtained originally from the Institute of Botany, Culture Collection of Autotrophic Organisms, Třěboň, Czechoslovakia in 1988, and since, it has been held at the Univerity of Zürich, Department of Limnology where it has been maintained in batch culture (120 ml) in 300-ml Erlenmeyer flasks at 20°C under continuous light of 7 $\mu mol\, m^{-2} sec^{-1}$ from fluorescent tubes in cyanobacterial mineral medium (Jüttner and Lüthi 2008). Experiments were carried out by using 16-d-old culture material with average cell diameter of 4.1 µm and cell volume 35 μm^3 (as determined by light microscopy).

Daphnia Magna Standardized, 1- to 2-d-old *D. magna* were raised from 3-wk-old mothers from a single genetic strain (Watson et al. 2007). Each behavior experiment was performed with individuals of equivalent size, taken from a single age-class cohort of *Daphnia*. Eight animals were held in each of two parallel flow-through glass cells (36 ml) of a bbe© *Daphnia* Toximeter (bbe Moldaenke, Kiel-Kronshagen, Germany), continually supplied with an inflow of untreated bank filtrate from the Limmat River, Zürich, at a constant flow rate and temperature (9 $ml\, min^{-1}$ at 20°C±0.2°C). The *Daphnia* were fed on a controlled supply of algal food (*Scenedesmus acuminatus*) added continually to the cell inflow. Animals were replaced each week, and acclimated for 2–3 days in the flow-through cells before experiments were carried out. The bbe© *Daphnia* Toximeter is a commercial instrument developed for biomonitoring of toxic compounds in raw water of drinking water industries. Detailed descriptions of the instrument setup, measuring and data system and a flow chart outlining the experimental protocol used here and in previous work are published by Lechelt et al. (2000) and Watson et al. (2007).

Determination of Chlorophyll a One-ml of the *Microcystis* suspension was applied in triplicate to a glass fiber filter (GF 6, Schleicher & Schuell, Dassel, Germany) as small spots under low vacuum. The spots were cut out, and each was extracted with 3 ml methanol overnight in a fridge. The full absorption spectrum of the extracts was measured on a spectrophotometer (Cary 3), and the absorption of the maximum at 666 nm was used to determine the amount of chlorophyll *a* (Ogawa and Vernon 1971).

Determination of Cell Counts by Flow Cytometry Twenty-ml suspensions of *Microcystis* first were preserved with 100 µl Lugol's solution (0.5%) and then with 2 ml 20% formaldehyde. After decolorization with 100 µl 0.1 mol/l sodium thiosulphate, 10 µl Sybr Green I solution (No 7563, Invitrogen, Basel, Switzerland) that had been diluted 1:100 with dimethylsulfoxide were added to 1 ml diluted cell suspension. After staining for 15 min, the cell concentration was determined with a CyFlow space (Partec GmbH, Münster, Germany). The excitation at 488 nm gave strong signals in the green emission range.

β-Cyclocitral in Live Microcystis Cells The concentration of β-cyclocitral in live *Microcystis* cells was determined by minimizing the activation of carotene oxygenase by addition of trifluoroacetic acid. A volume of 250 µl from a 16-d-old *Microcystis* suspension was suspended in fresh growth medium (3.5×10^7 cells / ml) and transferred into a 250 ml round bottom stripping vessel containing 250 µl of 0.05% (v/v) trifluoroacetic acid to immediately kill the cells. Ten ml of water then were added, and the suspension was neutralized with $NaHCO_3$. The volume was increased to 50 ml with water, 2 µl of a *R*-pulegone solution (11.2 ng / µl methanol) were added as the internal standard, and β-cyclocitral was analyzed by using the protocol described below.

Quantitative Analysis of β-Cyclocitral and Hydrocarbons β-Cyclocitral and other volatile hydrocarbons were extracted from the samples onto Tenax cartridges by using a closed-loop stripping technique, and analyzed quantitatively on a GC-MS as described previously (Durrer et al. 1999; Jüttner 2005) but at a capillary column temperature of 50°C during thermal desorption. The temperature program of the oven was 4 min at 50°C, then ramped 10°C / min up to 200°C.

Under these conditions, β-cyclocitral and pulegone eluted at 12.24 and 12.55 min, respectively. Single ion chromatograms (m/z 152 [M^+]) were extracted from the full mass spectra, and the peak areas were integrated. Calibration was performed with β-cyclocitral (BASF, Ludwigshafen, Germany) and *R*-pulegone (Fluka, Buchs, Switzerland). Straight chain hydrocarbons were obtained as reference compounds from Fluka. 7-Methylheptadecane was synthesized according to Han et al. (1968).

Age-Dependant Production of β-Cyclocitral by Microcystis Cells Batch cultures of *Microcystis* were grown for 16 to 207 days and used to investigate age-dependant production of β-cyclocitral. To remove cell clumps and allow quantitative centrifugation, *Microcystis* suspensions were passed through a 50 µm net and then pressurized in a stainless steel pressure cylinder (16 cm high, 10 cm i.d.) with 2 MPa argon to collapse gas vesicles. GC-MS analysis verified that β-cyclocitral formation was not initiated by this procedure. The treated suspensions were centrifuged, and the pellets were resuspended in 50% diluted cyanobacterial medium to a typical cell count of 2×10^7 cells / ml. A volume of 10 ml then was transferred into a 90-ml test tube and frozen by dipping into liquid nitrogen. After 15 min at −196°C, the pellet was melted 3 min under running tap water and transferred into a glass vial, and closed with a screw cap leaving a small headspace. The subsequent carotene oxygenase reaction was carried out in these tubes for 90 min at 23°C. Then 1 ml of the suspension was transferred into an Eppendorf vial with a cap and centrifuged for 1 min. A supernatant volume of 250 µl was removed immediately and added below the surface to a rotating 50 ml 20% NaCl solution containing *R*-pulegone as an internal standard. The quantitative determination of β-cyclocitral was done as outlined above. To determine the potential loss of β-cyclocitral by the centrifugation process, we analyzed a 100 µmolar solution under the same conditions. The integration values of the peak areas of m/z 152 were 0.63±0.10 before and 0.57±0.17 (mean±SD) after centrifugation. The concentrations were not statistically different (t-test for dependent samples, t=0.585; P= 0.590. The data met the assumptions of normality (Shapiro-Wilk W test) and homogeneity of variance (Levene's test). We therefore conclude that opening of the samples and subsequent centrifugation in closed Eppendorf vials did not result in a loss of β-cyclocitral.

Kinetics of β-Cyclocitral Formation The kinetics of β-cyclocitral formation were measured by using the above protocol. A volume of 20 ml of a 15-d-old culture of *Microcystis* was subjected to a freeze-thaw cycle. One ml-volumes were removed after different periods, transferred into an Eppendorf that was closed with a lid, and centrifuged. Further analysis was performed as outlined above. To obtain kinetics for osmotic shock conditions, 5 ml of a *Microcystis* suspension were mixed with 200 ml 20% (w/v) NaCl solution, and 50 ml-samples were removed after defined incubation times for quantitative analysis by stripping analysis.

Determination of β-Cyclocitral Odor Threshold for Daphnia The movement of each of the eight individuals of *D. magna* was tracked with a camera in the cell (36 ml net volume) of a toximeter. Mean values of the swimming velocity were recorded to a database every 50 sec by an intrinsic program. These data were used to determine the odor threshold of β-cyclocitral for this crustacean, using changes in swimming velocity as an index of compound detection, according to the basic protocol outlined in our previous work (Watson et al. 2007), but with the following modifications. The response to β-cyclocitral was tested at three different concentrations, 250 $nmol l^{-1}$, 750 $nmol l^{-1}$, and 2.5 $\mu mol l^{-1}$. To avoid changes in background water quality during an experiment, a total bank filtrate volume of 40-l of bank filtrate water was first collected at the beginning of each day and stored in 10-l glass bottles (using a supply flow rate of 72 ml / min). The water was supplemented with 5 µl ethanol per liter (= 86 µmol/l) to control for the solvent used in the β-cyclocitral treatment. Our earlier work had shown that this ethanol concentration caused no adverse effects or significant change in the swimming velocity of *D. magna* (Watson et al. 2007). To have the same age class of *Daphnia*, only one experiment was conducted per week.

The mean of four baseline measuring periods, two before and two after switching to β-cyclocitral spiked water in the inflow, was used to calculate the baseline swimming velocity of ca. 0.5–0.6 $cm s^{-1}$. Due to the large void volume of the system, the measurements at that time were actually performed with reference water. The response of swimming velocity of *Daphnia* was observed 15–18 min after switching to β-cyclocitral spiked water. This lag period was necessary to attain a detectable β-cyclocitral concentration in the toximeter cell. The lag period increased slightly with decreasing concentrations of β-cyclocitral.

Daphnia Behavioral Response to Live Microcystis Cells To show the effect on *D. magna* swimming velocity elicited by live *Microcystis* cells, the same experimental setup was used as for odor threshold concentration measurement. A 22-d-old culture of *Microcystis* was pressurized, and after centrifugation the cells were resuspended in fresh medium (4.7 nmol chl *a* / ml). A volume of 2 ml of this suspension was centrifuged in an Eppendorf vial and added to 1 ml of the same *Scenedesmus* suspension that was supplied continuously as food source in the toximeter. A volume of 0.35 ml of this mixed cell suspension was injected with a

syringe through the wall of the Tygon inflow tube directly before the peristaltic pump to avoid any pressure pulse in the cuvette. The time lag for the reaction was ca. 9 min.

Liberation of β-Cyclocitral by Daphnia The liberation of β-cyclocitral by feeding *Daphnia* was measured by using a young culture of *Microcystis* (5-d-old). To reduce sedimentation of the cells to a minimum, the cell suspensions were not pressurized before centrifugation (Sorvall SLA-1500, 5 min at 5,000 rpm). The cell pellets were resuspended in half strength cyanobacterial medium to a volume of 500 ml to achieve a cell abundance and chlorophyll *a* concentration of 1.7×10^{6} cells / ml and 188±13 pmol / ml, respectively. For the first experiment (A), we used 155 individuals of a mixed age class of *D. magna* with all stages of development. For the second experiment (B), 325 individuals of a more uniform age class (3.5-wk-old) were used. Incubations were carried out in 1-l glass bottles closed with a screw cap (to avoid loss of the liberated volatile β-cyclocitral) at 20°C and constant illumination of 2 $\mu mol m^{-2} s^{-1}$ (near the light compensation level of *Microcystis*). Liberation of β-cyclocitral was measured over 20.5 hr following the addition of *D. magna*. At different time intervals, the bottles were opened, allowing a short air exchange and retrieval of 5 ml of *Microcystis* suspension, from which the cells were removed by filtration through a glass fiber filter under low vacuum. The filtrate was added to 45 ml of water containing 2 µl internal standard solution and 10 g NaCl. Volatiles were recovered and analyzed by using closed-loop stripping and GC-MS analysis, as described above.

Liberation of β-Cyclocitral in a Microcystis Culture To determine the liberation of β-cyclocitral in a culture of *Microcystis*, 20 ml of a *Microcystis* suspension were centrifuged, and the cell pellet was resuspended in half strength cyanobacterial medium. The suspension was transferred into 1-l glass bottles and incubated under the same conditions as in the *Daphnia* experiment (see above). After different time intervals, 10 ml samples were removed, and the cells were separated by filtration through a glass fiber filter GF 6. The filtrate containing the liberated β-cyclocitral was stripped and analyzed by GC-MS. Single ion monitoring (*m/z* 152 for measurement and *m/z* 137 for control) was used for detection of β-cyclocitral and pulegon that was added as an internal standard. The filter with the *Microcystis* cells was transferred into a stripping vial, covered with 10 ml of 1/2 strength medium and frozen at −20°C. On the next day, the suspension was melted, amended with 40 ml of medium, and incubated for 1 hr to complete the activation of the β-carotene oxigenase. After stripping, full spectra were obtained by GC-MS analysis from which the molecular ion (*m/z* 152) was extracted to calculate the amount of β-cyclocitral.

Statistical Analysis We tested for effects of different concentrations of β-cyclocitral by pairwise comparison of the swimming velocity before and after the addition of the respective concentration of β-cyclocitral. As the same animals were used for the measurement of velocity before and after addition of β-cyclocitral, both values were regarded as dependent. Accordingly, *t*-tests for dependent samples were used to test for significant effects of a given concentration of β-cyclocitral. Data were tested for normality (Shapiro-Wilk *W* test) and for homogeneity of variance (Levene's test). A level of significance of P= 0.05 was applied. Analyses were performed using the software package Statistica 6.0.

Results

Microcystis NRC-1 produced a limited number of VOCs when activated by salt-induced cell rupture. The VOCs were dominated by β-cyclocitral and to a lesser extent by the long-chain hydrocarbons heptadecane, octadecane, 7-methylheptadecane (characteristic fragment ions at *m/z* 168 and *m/z* 112), and 6-methylheptadecane (*m/z* 182 and *m/z* 98). Isopropylthiol and its derivatives, known to be produced by other *Microcystis* strains (Jüttner 1984), were not detected. When *Microcystis* cells were killed by adding trifluoroacetic acid to minimize the activation of the carotene oxygenase by denaturation of the proteins, the per capita β-cyclocitral production was extremely low (2.6 amol / cell), indicating that minimal amounts of this VOC—less than 3.4% of the total production capacity—were present in intact cells.

When added to the toximeter inflow, a cell suspension containing *Microcystis* NRC-1 initiated an increase in *D. magna* swimming velocity from an average baseline level below 0.6 to 0.8 cm / sec (Fig. 1). In contrast, the injection of a pure *Scenedesmus* suspension did not cause a similar increase of swimming velocity

To investigate whether β-cyclocitral production by *Microcystis* is responsible for this grazer response, the toximeter inflow of riverbank filtrate was replaced by riverbank filtrate spiked with this VOC. Again, we observed an increase in swimming velocity following this treatment. However, with continued addition of β-cyclocitral a rapid acclimation also was apparent (as similarly observed in our earlier work; Watson et al. 2007). With prolonged application of spiked water, *Daphnia* swimming velocity finally decreased to the initial value before treatment. Based on the above results, we evaluated the odor threshold concentration for this grazer by exposing the animals to a series of different β-cyclocitral concentrations (Fig. 2). Upon the addition of 2.5 µmol / l β-cyclocitral, the swimming velocity of *Daphnia* increased

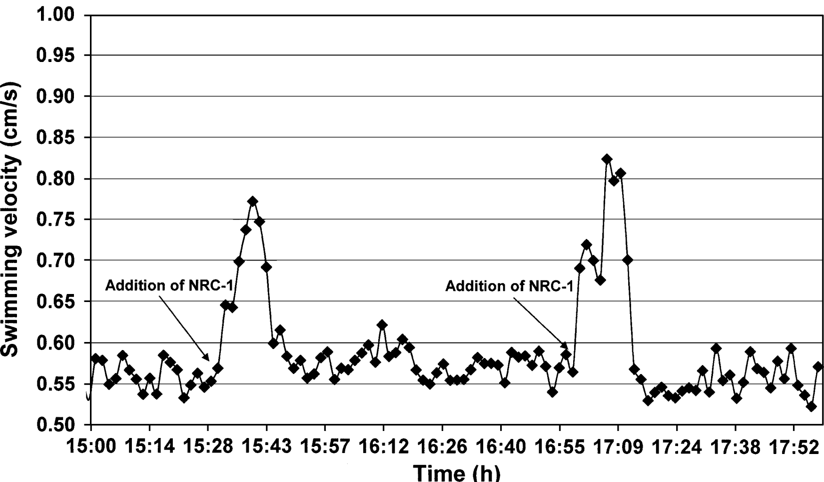


Fig. 1 Swimming velocity of *Daphnia magna* in the toximeter cell. Individual points represent mean values of measures from eight individuals, measured at 100 sec intervals. *Arrows* indicate addition of 0.35 ml of a *Microcystis* NRC-1 suspension to the inflowing water-*Scenedesmus* stream

significantly from 0.48 to 0.65 cm/sec (Fig. 2, Table 1). A similarly significant increase from 0.60 to 0.72 cm/sec was observed upon addition of 750 nmol / l β-cyclocitral (Fig. 2, Table 1). However, 250 nmol / l of β-cyclocitral did not affect *Daphnia*'s swimming velocity (Fig. 2, Table 1).

Experiments conducted to measure β-cyclocitral liberation from *Microcystis* by *Daphnia* grazing showed that initially, virtually no β-cyclocitral was detectable in the medium, but after a lag phase the concentration of this VOC increased continuously (Fig. 3). In experiment A, an older population of *Daphnia magna* was used that included individuals in all stages of development, while for experiment B, 3.5-wk-old mothers and their neonates were chosen. Following 20.5 hr of feeding on the *Microcystis* suspensions, the crustaceans liberated 65 and 55 fmol β-cyclocitral / *Daphnia* / min in experiment A and B, respectively. This cor6responded to an activation of 14 and 12 *Microcystis* cells / *Daphnia* / s, respectively, assuming the same activation efficiency of the carotene oxygenase as observed by a freeze-thaw cycle. The liberation of β-cyclocitral in a *Microcystis* culture exposed to the same environmental conditions is shown (Fig. 4). The data demonstrate that most cell production of β-cyclocitral occurs with cell rupture and activation of carotene oxygenase, while the amount liberated by intact cells and dissolved in the medium is extremely small. The relative proportion of liberated β-cyclocitral to the total maximum cell production capacity of this compound was 0.04% at the beginning and 0.15% at the end of the 42 hr incubation period.

To quantify the kinetics of β-cyclocitral production following cell damage, we applied two different techniques to activate the carotene oxygenase responsible for the formation of this VOC: a freeze-thaw cycle that used liquid nitrogen; and an application of a 20% NaCl solution. Both treatments caused rapid β-cyclocitral formation, but the activation by an osmotic shock required an incubation period considerably longer than 80 min to achieve the maximum potential yield per cell (Fig. 5). A more efficient activation of carotene oxygenase was achieved by using a freeze-thaw process with liquid nitrogen. This procedure was more effective than the activation by NaCl addition. About 48% of the final concentration of β-cyclocitral

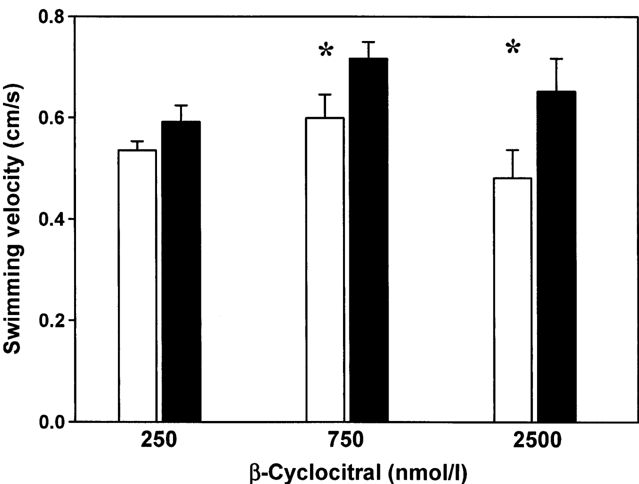


Fig. 2 Mean swimming velocity (+SE, $N=3$) of *Daphnia magna* before (*white columns*) and after (*black columns*) addition of different concentrations of β-cyclocitral. Mean values are based on independent experiments performed at different days. Statistically significant differences in swimming velocity between pre- and post-β-cyclocitral addition are indicated by an *asterisk*

Table 1 Results of pair-wise comparisons of swimming velocities of *Daphnia magna* before and after the addition of known concentrations of ß-cyclocitral (*t*-test for dependent samples)

ß-cyclocitral	*df*	*t*-value	*p*
250 nM	2	−2.6250	0.120
750 nM	2	−4.4900	**0.046**
2.5 μM	2	−11.798	**0.007**

P-values given in bold indicate significant differences

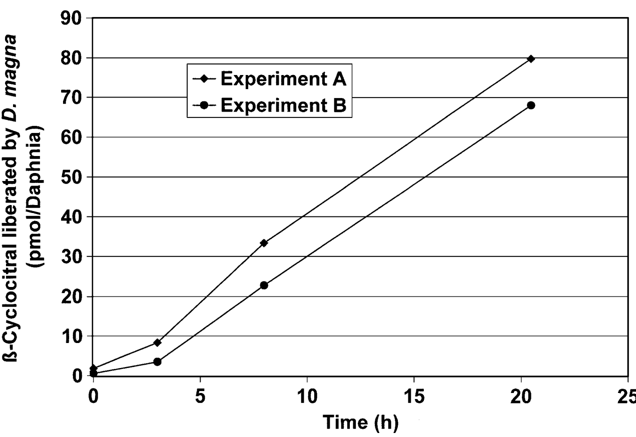


Fig. 3 Liberation of β-cyclocitral from *Microcystis* by *Daphnia magna* feeding (pmol / *Daphnia*). A—155 individuals of *Daphnia* at all developmental stages, B—3.5-wk-old mothers and neonates (325 individuals)

already was present by ~5 min (Fig. 6) using the freeze-thaw process. The completion of the oxygenase reaction occurred in less than 90 min with only a marginal further increase in β-cyclocitral formation in the total observation period of 1,440 min when the experiment was terminated. Although the first concentration measurements of β-cyclocitral in both Figs. 5 and 6 are labelled at 0 min, this in fact represents 5 min following the initial *Microcystis* cell activation by the osmotic shock or thawing process and subsequent centrifugation separation process.

Age-dependent changes in β-cyclocitral production capacity by *Microcystis* were investigated by using batch cultures of *Microcystis* NRC-1 grown over periods between 16 and 207 days after transfer into new growth medium. With the exception of the 16-d-old culture, the cells were at a stationary growth phase. Activation was initiated by a liquid nitrogen freeze-thaw cycle, and β-cyclocitral production was measured over a 90 min incubation period. The results showed no consistent age-dependant change of the β-cyclocitral production capacity between cultures, but a significant increase in cellular chlorophyll *a* content with increasing age, translating to a lower β-cyclocitral production capacity per unit of chlorophyll *a*. (Table 2).

Discussion

The experiments provide new insight into grazer-cyanobacterial/algal interactions and show that chemical ecology may play a significant, taxon-specific role in facilitating grazer selectivity against bloom forming cyanobacteria. Our results showed a marked behavioral change in the common crustacean grazer *Daphnia magna* in response to the appearance of detectable levels of β-cyclocitral in the swimming medium. This implies that β-cyclocitral may provide a significant and exclusive competitive advantage to *Microcystis* among planktonic cyanobacterial/algal communities. However, while unique to this cyanobacterial genus, our related work indicates no apparent differentiation at the species level or relationship to toxicity. The specific β-carotene 7,8 (7′,8′) oxygenase involved in this process (Jüttner and Höflacher 1985) appears to be coded for by all *Microcystis* species, with β-cyclocitral production documented in all species tested to date (Jüttner 1984). These findings support our central hypothesis that β-cyclocitral acts as a genus-specific grazer repellent for these bloom forming cyanobacteria and facilitates optimal grazer foraging by acting as an effective signal of an unsuitable food source.

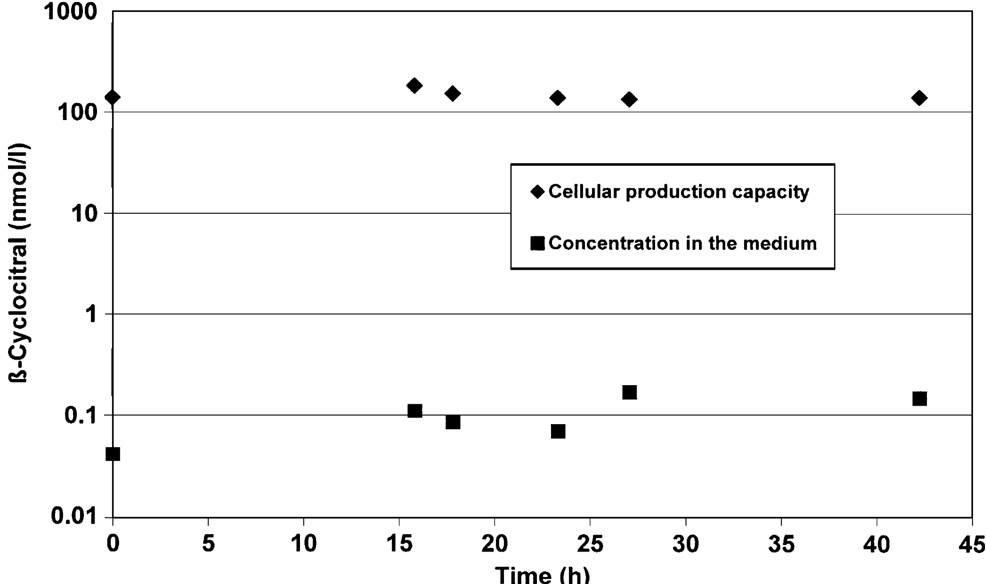


Fig. 4 Liberation of β-cyclocitral in a culture of *Microcystis*. *Diamonds*: formation capacity of the living cells; *squares*: dissolved fraction in the medium

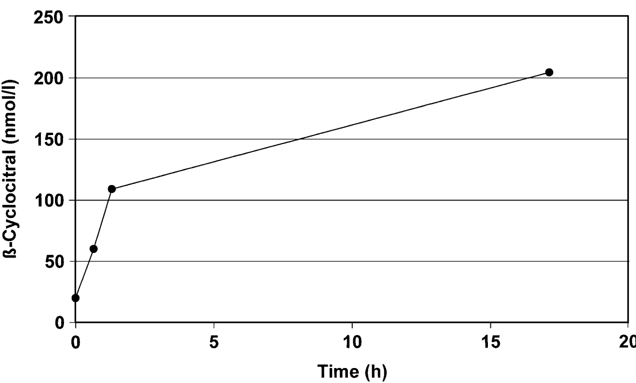
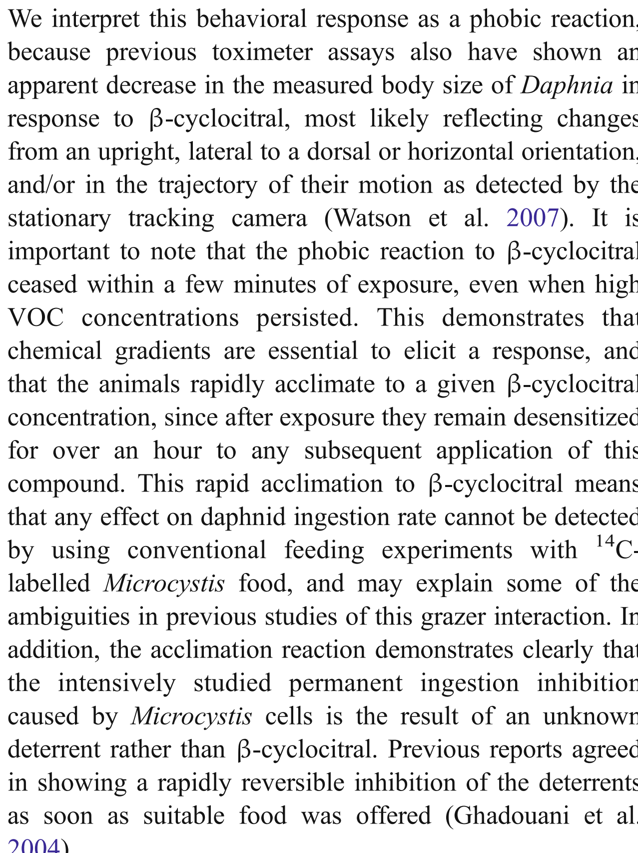


Fig. 5 Formation kinetics of β-cyclocitral (integration units / ml suspension) upon activation of *Microcystis* with an osmotic shock (20% NaCl). The time-scale (X-axis) starts at the point of complete solution of the solid NaCl and separation of the cell matter from the aqueous phase by centrifugation

The tenet that some cyanobacteria are a poor food source and/or selectively avoided by crustaceans is as old as the study of Limnology (Birge 1898). The later development of quantitative measurements that used ^{14}C-labelled food organisms, showed clearly that compared to algae from other taxonomic groups, *Microcystis aeruginosa* strongly inhibited crustacean ingestion rates (Sorokin 1968). This selective prey uptake was manifested as strong changes in the beat and movement rates of the thoracic appendages, mandibules, maxillules, etc. (Lampert 1982; Fulton and Paerl 1987b; Rohrlack et al. 1999a; Ghadouani et al. 2004). To date, however, studies have failed to implicate the primary toxins produced by many *Microcystis* (microcystin, MC) with this behavioral reaction, and no other chemicals have been linked with this grazer response. For example, MC-producing and non-producing *Microcystis* strains exhibit the same inhibitory reaction among grazers, and MCs have been ruled out as the causative agents in the avoidance reaction (Rohrlack et al. 1999b; Ghadouani et al. 2004). Similarly, this has been shown for isopropylsulfide producing and deficient *Microcystis* strains (Jungmann et al. 1991).

Unlike calanoid copepods, which are highly cued raptoral grazers, *Daphnia* are not considered to be selective feeders (Friedman and Strickler 1975; Poulet and Marsot 1978; DeMott 1986; Fulton and Paerl 1987b). Nevertheless, daphnids may have developed mechanisms to select particles based on morphological, physical (Hartmann and Kunkel 1991), and chemical cues (Jensen et al. 2001). Our experiments demonstrate clearly that *Daphnia* can detect the presence of trace levels of β-cyclocitral and respond to this with a behavioral change. We propose that a phobic reaction is initiated in these crustaceans when they encounter sufficient concentration differences of β-cyclocitral near damaged *Microcystis* cells, or when release of this VOC has occurred through grazing attack or by disruptive agents of some cells of the *Microcystis* colony.



We interpret this behavioral response as a phobic reaction, because previous toximeter assays also have shown an apparent decrease in the measured body size of *Daphnia* in response to β-cyclocitral, most likely reflecting changes from an upright, lateral to a dorsal or horizontal orientation, and/or in the trajectory of their motion as detected by the stationary tracking camera (Watson et al. 2007). It is important to note that the phobic reaction to β-cyclocitral ceased within a few minutes of exposure, even when high VOC concentrations persisted. This demonstrates that chemical gradients are essential to elicit a response, and that the animals rapidly acclimate to a given β-cyclocitral concentration, since after exposure they remain desensitized for over an hour to any subsequent application of this compound. This rapid acclimation to β-cyclocitral means that any effect on daphnid ingestion rate cannot be detected by using conventional feeding experiments with ^{14}C-labelled *Microcystis* food, and may explain some of the ambiguities in previous studies of this grazer interaction. In addition, the acclimation reaction demonstrates clearly that the intensively studied permanent ingestion inhibition caused by *Microcystis* cells is the result of an unknown deterrent rather than β-cyclocitral. Previous reports agreed in showing a rapidly reversible inhibition of the deterrents as soon as suitable food was offered (Ghadouani et al. 2004).

We could only detect trace amounts of β-cyclocitral in intact *Microcystis* cells, and even these likely were the result of procedural artefacts caused by a small number of damaged cells. The formation of β-cyclocitral is achieved rapidly by activation of the β-carotene oxygenase pathway that cleaves β-carotene into crocetindial and two molecules of β-cyclocitral. Experimentally, this activation can be caused by any mechanism that disintegrates the physiological integrity of the cells, such as a freeze-thaw cycle,

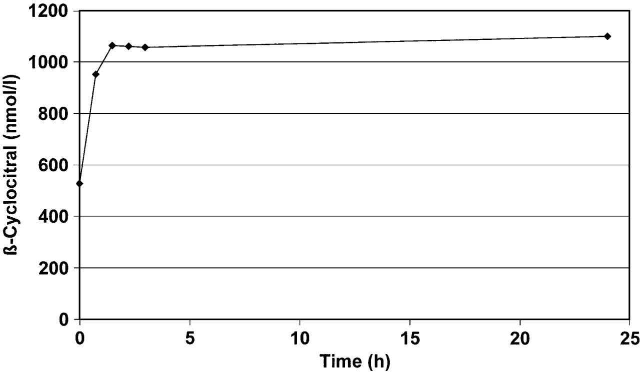


Fig. 6 Formation kinetics of β-cyclocitral upon activation of *Microcystis* (pmol β-cyclocitral / ml suspension) by a freeze-thaw cycle and subsequent reaction time at ambient temperature (23°C). The time-scale (X-axis) starts at the point of complete melting of the cell suspension and separation of the cell matter from the aqueous phase by centrifugation

Table 2 Age-dependant (days) capacity of *Microcystis* NRC-1 to form ß-cyclocitral (amol ß-cyclocitral per cell, SD in brackets, N=3). The chlorophyll *a* concentration per cell (amol chl *a* per cell) and the ratio amol chlorophyll *a* per amol ß-cyclocitral are also given

Culture age (d)	amol ß-cyclocitral/ cell	amol Chl *a* / cell	amol chl *a* / amol ß-cyclocitral
16	77(±5.5)	127(±4.3)	1.6
20	61(±17)	108(±1.6)	1.8
52	49(±7.6)	109(±1.7)	2.2
141	47(±5.9)	191(±7.8)	4.1
207	65(±2.2)	201(±2.5)	3.1

osmotic shock, mechanical damage, or addition of organic solvents. Under natural conditions, the formation of β-cyclocitral may be caused by *Daphnia* appendage bristles (Fryer 1991), which may puncture *Microcystis* cells and thus initiate β-carotene oxygenase activation. This activation may be continued and induced during ingestion and gut passage. Our feeding experiments with *Daphnia* and *Microcystis* NRC-1 under simulated natural conditions indicate that the activation of ~12–14 *Microcystis* cells per second per grazer is sufficient to elicit the behavioral response. In many lakes, a colonial matrix can contain up to several hundred *Microcystis* cells, and wounding one or several cells could produce a β-cyclocitral odor plume at sufficient concentrations to be detected by grazers. The adaptive function of this compound as a repellent would be afforded largely to *Microcystis* colonies, where a limited number of sacrificed cells can provide chemical protection to the majority. That is even more important because the growth rate of cells in colonies is comparable to that of single cells, and the energetic drawback of colonial growth is small (Nielsen 2006).

Our quantitative measurements estimate that the maximum theoretical intracellular β-cyclocitral concentration in an activated *Microcystis* cell (for a young cell, estimated to be 2.2 mM) would be some 3,000 times higher than the odor threshold concentration for *Daphnia magna* (750 nmol/l). Even where β-cyclocitral is rapidly diluted from the microzone around the activated cell(s), the concentration would be sufficient to initiate a phobic reaction by *Daphnia*, particularly where more than one cell is activated. This represents an effective chemical defense mechanism for *Microcystis*, since detection of the compound leads to a change in grazer orientation and an increase in swimming velocity, which we interpret as an avoidance reaction. In fact, olfactory receptors are widely distributed among invertebrate grazers including *Daphnia*, which may use the initial grazing-induced release of β-cyclocitral and other VOCs as a cue to reduce their ingestion of poor cyanobacterial and algal food.

The β-cyclocitral formation kinetics showed that the oxygenase reaction required some time to reach the maximum potential yield. Because the reaction mechanism is unclear, it is difficult to speculate which reaction step is rate-limiting. The ecological implication is that β-cyclocitral production continues for some time following cell damage, thus allowing the formation of a transient odor plume or a microzone around a *Microcystis* colony (Blackburn et al. 1998) and thus reducing the probability of further grazer attack. Furthermore, it is feasible that sequestration of the liberated β-cyclocitral by the colonial mucilage could occur, which would effectively prolong the persistence of the VOC plume.

The defense reaction that initiates β-cyclocitral formation is constitutively present in *Microcystis*, and minimal differences in this capacity were observed as a function of age. Cells at late stationary phase (207 days) still exhibited this capacity, although an increase in their cell chlorophyll *a* content translated to a decrease in β-cyclocitral production per chlorophyll *a*. Thus, under natural conditions, cells and colonies at all stages of growth maintain this grazer defense mechanism.

The formation of β-cyclocitral controls the residual amount of β-carotene in ingested *Microcystis* cells. Since two molecules of β-cyclocitral are formed from one molecule of β-carotene (Jüttner and Höflacher 1985), at least 30.5 amol β-carotene are required to produce 61 amol β-cyclocitral in a *Microcystis* cell. This is consistent with measured pigment content, assuming that most of the available β-carotene is oxidatively cleaved and that *Microcystis* NRC-1 has a chlorophyll *a* to β-carotene molar ratio comparable to that of other *Microcystis* strains (e.g., 2.4 and 4.0 in SAG 1450-1 and PCC 7806, respectively; Stransky and Hager 1970; Jüttner and Höflacher 1985). It can be hypothesized further that the above-described grazer defense mechanism also may act to reduce the food value of the *Microcystis* cells themselves. Photoreceptors are known for *Daphnia*, and constituents of vitamin A (retinal and opsin) are likely key components of these receptors (Kashiyama et al. 2009). The grazing-activated cleavage of β-carotene by 7,8 (7′,8′) carotene oxygenase yields a product that cannot be used as a precursor for retinal. Once *Microcystis* cells are attacked, this catalytic process hypothetically might contribute to vitamin A deficiency by decreasing available levels of the precursor in a food supply. Depletion of β-carotene supply, however, could only be expected in those cases where *Microcystis* is the major food component of a *Daphnia* population.

Effective defense strategies in predator-prey interactions often involve multi-tiered responses that are difficult to fully characterize. In this study, we demonstrated that the production of β-cyclocitral by *Microcystis* acts as a front-line chemical signal of an unsuitable food organism. It is triggered by grazer attack and is the first such infochemical identified in these cyanobacteria. From the grazer's perspective, this warning signal is justified by the presence of potent oligopeptides and other bioactive toxins in the cyanobacterial cells (Trubetskova and Haney 2006).To reinforce grazer recognition of *Microcystis* as an unsuitable food source, a logical next step in the signalling process would be through the production and intracellular storage of other chemical deterrents which, when present in the ingested food material, would act to reduce grazer ingestion rate. In fact, suppressed ingestion activity has been observed in a number of grazer feeding studies with *Microcystis* (e.g., Rohrlack et al. 1999a, b), but the chemical agent(s) have not been identified (although microcystins have been implicated by some authors, Kim et al. 2003). It is unlikely, however, that microcystins play a major role in grazer defense since microcystin-deplete colonies are frequently found in natural assemblages. On the other hand, digestive protease inhibitors are far more widely produced and found in almost every cyanobacterial bloom (Agrawal et al. 2005; Schwarzenberger et al. 2010), and it is possible that these compounds may act to suppress grazing. Future research in this area would contribute significant insight into the role of chemical ecology in top down regulation of noxious blooms.

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volatiles emitted from the roots have not been as thoroughly explored (Lin et al., 2007; Ens and French, 2008; Ens et al., 2009b). 1,8-cineol is one of the few volatile organic compounds (VOCs) emitted from the roots of of *Arabidopsis thaliana*, and its root-specific terpene synthase have been characterized (Chen et al., 2004; Steeghs et al., 2004).

Recently, volatile terpenoids, especially monoterpenes, were identified by solvent and dynamic headspace extraction of roots and the rhizosphere of *Pinus* spp. (Lin et al., 2007). Ens and his co-workers (2009a) extracted volatiles from the roots and soil surrounding the invasive plant known as the bitou bush (*Chrysanthemoides monilifera*), analyzed them using gas chromatography and mass spectrometry (GC-MS), and demonstrated allelopathic effects of the detected sesquiterpenes. They also invented a novel technique to capture hydrophobic substances, including volatile sesqui- and diterpenes, in the rhizosphere of plants (Ens et al., 2009b). Some of the earliest work on the detection of volatile terpenoids in the soil was done by Halligan (1975) who distilled the leaves, leaf litter, and soils surrounding the shrub zones of *A. californica* in a "Clevenger orange still," and subjected the resulting essential oils to a germination bioassay against *Madia sativa* seeds. Camphor and 1,8-cineol were the most toxic substances, while isothojone, the major compound in the soil, had intermediate toxicity. A full decade before Halligan's work, Muller and colleagues examined the allelochmaical volatile terpenoids emitted from the aerial parts of sagebrush and sage plants, *Artemisia californica* and *Salvia leucophylla* (Muller et al., 1964). The selective phytotoxicity of the volatiles from *A. californica* against some species of the co-existing grasses together with the grazing of animals were both considered important reasons for the bare area between the grasses and the shrubs in the chaparral in California (Halligan, 1973).

Artemesia tridentata is an important shrub of western North America; it ranges from British Columbia to Mexico. The plant's ability to inhibit the establishment of competitors has been attributed to allelochemical volatile terpenoids emitted from its aerial parts; these terpenoids include camphor, 1,8-cineol, and α-pinene (Weaver and Klarich, 1977). A leaf litter extract of *A. tridentata* significantly inhibited the seed germinations of three native grasses in the sagebrush grass zone: *Stipa thurberiana, Sitanion hystrix*, and *Agropyron spicatum*; however, the litter did not have an inhibitory effect after the seeds had germinated (Schlatterer and Tisdale, 1969). Several authors have shown the allelopathic effects of volatiles and aqueous extracts from leaves or litter of big sagebrush, including the inhibition of seed germination and shoots and the radicle growth of test plants such as *Elymus cinereus, Agropyron cristatum*, and *Cucumis sativa* (Schlatterer and Tisdale, 1969; Weaver and Klarich, 1977; Groves and Anderson, 1981). The non-volatile sesquiterpene lactones from the shoots of *A. tridentata,* namely, arbusculin-A, achillin, desacetoxymatricarin, viscidulin-B, and viscidulin-C inhibit the growth of radicles and hypocotyls in cucumber (*Cucumis sativus*) and increase the plant's respiration (McCahon et al., 1973). In addition to the sesquiterpenes, the coumarin, esculin, isolated from the aerial parts of the sagebrush inhibits the growth of *C. sativus* especially of its radicles, but does not affect germination (McCahon et al., 1973). The essential oils from the aerial parts of different species of sagebrush, including *A. tridentata,* have been investigated (Kelsey et al., 1983; Epstein et al., 1984; Preston et al., 2001; Gunawardena et al., 2002), but thorough analyses of root volatiles of this genus have not been carried out (Kennedy et al., 1993; Blagojević et al., 2006; Goel et al., 2007).

Methyl jasmonate (MeJA), the volatile form of the endogenous plant hormone, is produced in allelopathic quantities from the aerial parts of *A. tridentata* (Preston et al., 2002) and can elicit the production of defensive compounds of *Nicotiana attenuata* plants growing nearby (Baldwin, 1998; Preston et al., 2002; Karban et al., 2000, 2003, 2006; Kessler et al., 2006). The germination of *N. attenuata* seeds is delayed by both airborne and water-transported MeJA released by *A. tridentata* (Preston et al., 2004). Moreover, when the seeds of *N. attenuata* were buried in soil near *A. tridentata*, or when the soil underneath the plant was used to germinate *N. attenuata* seeds, germination was delayed significantly (Preston et al., 2002). From 16 to 60% of the inhibitory activity of original litter extract of sagebrush could be attributed to MeJA (Preston et al., 2002). Therefore, it was concluded that in addition to the MeJA emitted from the shoots of big sagebrush, other compounds also play an allelopathic role in the soil near the plant (Preston et al., 2002). Although previously it was suggested that the toxic volatiles from the leaves of some plants such as *A. californica*, *S. leucophylla*, and *S. apiana* could be deposited by dew on seedlings growing nearby (Muller et al., 1964), this would require dissolving the hydrophobic substances in water, which seems an unlikely mechanism.

Here, we tested the hypothesis that root volatiles, in addition to airborne volatiles, affect the growth of *N. attenuata* seedlings growing adjacent to *A. tridentata* plants. We describe a novel approach for analyzing the VOCs released from the roots of intact *A. tridentata* plants [dynamic headspace extraction, headspace solvent-microextraction (HSME) and headspace solid phase-microextraction (HSPME) followed by GC-MS analysis] and examine the phytotoxicity of the indentified compounds against seedlings of the naturally co-occurring plant, *N. attenuata*.

Methods and Materials

Plant Material, General Procedure and Reagents *Artemisia tridentata* (Natt.) subspecies *tridentata* and *Nicotiana attenuata* were grown as described previously (Preston et al., 2002). ^{1}H- and ^{13}C NMR spectral experiments were measured on a Bruker Avance DRX 500 NMR. The test chemicals, camphor, 1,8-cineol, nerol, geraniol, α-thujone, and camphene, were purchased from Sigma and Aldrich. Neryl and geranyl isovalerates were synthesized according to the process described by Hassner and Alexanian (1978). Briefly, an equimolar solution of isovaleric acid, geraniol or nerol, N,N-dicyclohexylcarbodiimide, and catalytic amounts (0.1 mol) of 4-pyrrolidinopyridine in dry tetrahydrofurane (THF) was stirred at room temperature until the reaction was completed. The esters were purified by column chromatography over silica gel using hexane and dichloromethane (DCM) as the mobile phase.

Extraction of VOCs from the Roots of Big Sagebrush and the Growth Bioassay of N. Attenuata on Plant Agar The roots of *A. tridentata* (108 g) were extracted in 80% methanol (MeOH), and the extract (5.45 g) was partitioned between water and DCM. The DCM extract (1 g) was divided into methanol (MeOH/DCM ext.) and DCM-soluble fractions (DCM/DCM ext.). The DCM, MeOH, and water layers of the aqueous methanol extract (H_2O/80%MeOH ext.) were used in the root growth bioassay for *N. attenuata*.

Seeds of *N. attenuata* after treatment with liquid smoke were germinated on GB5 plant agar according to the process described previously (Krügel et al., 2002). After 4 d, seedlings were transferred to freshly prepared agar containing 0.25 µg/ml of the above extracts dissolved in 250 µl dimethylsulfoxide (DMSO). For the control, 250 µl DMSO were mixed well with 40 ml agar media. Root lengths were measured 6 d after transferring seedlings to the agar.

Dynamic Headspace Extraction of the Roots of Big Sagebrush Volatiles were collected from the head space of the sagebrush's roots with a procedure similar to those reported previously with some modifications (Karban et al., 2000; Preston et al., 2001). Roots were removed from the surrounding soils or sand and put into a desiccator. The aerial parts of the plant were separated by a plastic sheet from the roots in the desiccator, and the shoots were enclosed in a 1.5 l plastic bottle as shown (Fig. 1). The headspace of the roots and shoots was trapped for 2 h by pulling air using a vacuum diaphragm pump at a flow rate of approximately 500 ml/min through 30 mg Super-Q (Alltech, Deerfield, IL, USA) adsorbent in a Pasteur pipette. Two 1 ml plastic samplers were filled with 500 mg activated charcoal to filter the incoming air into the headspace of the plants' shoots and roots. After the adsorbent was eluted with DCM, the solution was analyzed by GC-MS to identify its constituents. Five µg geranyllinalool/10 µl hexane as an internal standard were added to the adsorbent prior to its elution with 250 µl DCM.

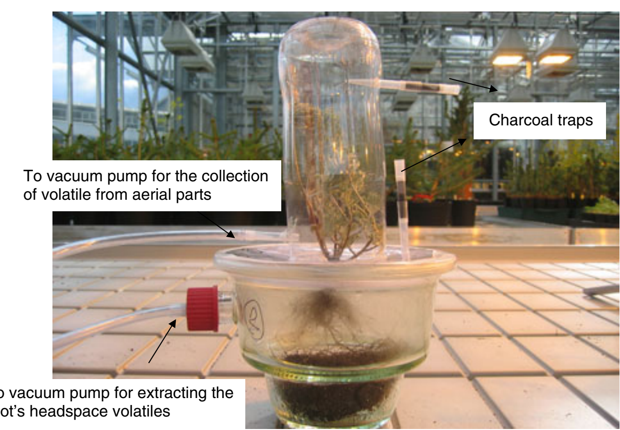


Fig. 1 A homemade apparatus for the simultaneous extraction of headspace volatiles from roots and shoots; the roots' volatiles are adsorbed on a Super-Q trap in a glass tube and the incoming air is filtered by a column of ground charcoal. The trapped volatiles adsorbed on the solid phase are then eluted by dichloromethane (DCM) containing geranyllinalool as the internal standard and subjected to gas chromatography-mass spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) GC-MS analysis was carried out on a Varian 3800 gas chromatograph, coupled to a Varian Saturn 2000 mass spectrometer operating in EI mode at 70 ev. The GC was equipped with a DB-5 MS (J & W Scientific column, 30 m×0.25 mm i.d., 0.25 µm film thickness). The oven temperature was programmed from 60°C to 220°C at 5°C/min and kept for 10 min at 220°C. The carrier gas was helium (He) with a flow rate of 1 ml/min, and the injector temperature was set at 260°C in splitless mode. The injection volume was 1 µl for all samples. Volatiles were identified by comparing their retention times, indices, and mass spectra with those of the authentic samples (from Fluka, http://www.sigmaaldrich.com, and Sigma) or those published in the literature (Adams, 2007).

Identification of the Major Constituents by ^{13}C NMR Spectroscopy The fractions collected from the column chromatography, after analysis by GC-MS, were subjected to NMR experiments in $CDCl_3$ using TMS as the internal standard. For identification of some of the compounds (Table 1), ^{13}C NMR spectral data were compared to those published in the literature (Uchio et al., 1981; Formacek and Kubeczka, 1982; Martínez et al., 1987).

Headspace Solvent Micro-Extraction (HSME) of the Roots of Big Sagebrush Headspace solvent micro-extraction (HSME) was performed using a 10 µl Hamilton syringe

Table 1 Peak area percentage of volatiles detected in the root extracts and head space of sagebrush roots

Compounds	RRI[a]	Area% roots DCM extract[b]	Area% active Fraction[b]	Area% *A. tridentata* roots[c]	Area% *A. tridentata* shoots[c]	Identification[a]
Monoterpenoids						
Santolina triene	909	–	–	1.3±0.6	0.2±0	GC-MS
Artemisia triene	927	–	–	8.4±2.4	4.3±1.2	GC-MS
α-thujene	934	–	–	–	0.1±0	GC-MS
α-pinene[d]	950	–	–	0.9±0.3	–	GC-MS, Co-inj.
camphene[d]	966	–	–	0.2±0.1	–	GC-MS, Co-inj.
1,8-cineol[d]	1021	5.5	–	6.4±2.0	3.9±0.8	GC-MS, Co-inj.
Chrysanthenone	1133	10.0	–	–	–	GC-MS
Camphor[d]	1158	8.0	–	27.5±3.0	31.3±9.5	GC-MS, Co-inj.
Pinocarvone	1174	1.2	–	–	–	GC-MS
Geranyl isobutanoate[d]	1531	0.9	2.6	–	–	GC-MS, Co-inj.
Geranyl butanoate[d]	1579	1.5	10.9	–	–	GC-MS, Co-inj.
Neryl isovalerate[d]	1591	16.2	24.7	0.8±0.5	–	GC-MS, Co-inj.
Geranyl isovalerate[d]	1599	trace	2.3	–	–	GC-MS, Co-inj.
Total		43.3	40.5	45.5	39.8	
Non-terpenoids volatiles						
3-(*E*)-Octen-2-ol	983	–	–	–	3.0±0.5	GC-MS
2-octanone	987	–	–	–	2.0±0.7	GC-MS
2-heptyl acetae	1040	–	–	–	1.8±0.3	GC-MS
3-(*Z*)-hexenyl-oxy-acetaldehyde	1067	–	–	–	4.2±1.6	GC-MS
5-(*Z*)-octenol	1073	–	–	–	3.8±1.5	GC-MS
n-undecane	1096	–	–	–	2.4±1.0	GC-MS
2-(*E*)-nonenol acetate	1305	–	–	–	3.2±1.4	GC-MS
(9*E*)-undecen-1-al	1314	–	–	–	3.6±1.6	GC-MS
(9Z)-undec-en-1-al	1322	–	–	–	2.5±1.1	GC-MS
(*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.5]dec-3-ene	1953	8.8	46.8	–	–	GC-MS, NMR
Total		8.8	46.8	trace	26.5	
Sesquiterpenoids						
Silphinene	1358	1.0	–	–	–	GC-MS
α-longipinene	1362[e]	2.0	–	1.5±0.6	–	GC-MS
Cyclosativene	1361[e]	–	–	3.5±1.6	–	GC-MS
Longicyclene	1386	2.3	–	–	–	GC-MS
β-elemene	1400	2.6	–	–	–	GC-MS
Longifolene	1422[e]	1.2	–	1.9±0.8	–	GC-MS
α–himachalene	1463[e]	1.2	–	trace	–	GC-MS
β-chamigrene	1486	0.9	–	–	–	GC-MS
γ-himachalene	1492[e]	13.7	–	trace	–	GC-MS, NMR
β-selinene	1479[e]	–	–	0.5±0.2	–	GC-MS
β-himachalene	1494[e]	–	–	1.4±0.4	–	GC-MS
α-bulnesene	1521	1.4	–	–	–	GC-MS
Total		26.3	–	8.8	trace	GC-MS

[a] Identification of compounds was performed by calculated relative retention index (RRI) on a DB-5 capillary column and mass spectrum (GC-MS), for the major compounds in the dichloromethane extract (DCM) the identification was further confirmed by ^{13}C NMR spectral data in $CDCl_3$ at 125 MHz (NMR) and in some cases ([d]) with co-injection of authentic (co-inj.) samples on the capillary GC. [b] GC-MS area normalized percentage of the volatiles identified in the DCM extract and in the phytotoxic active fraction obtained from the DCM extract. [c] Means of GC peak area%±*SE* of three biological replicates of the volatiles from the sagebrushe's roots-headspace adsorbed on SPME fiber. [e] The calculated RRI are different in various experiments, but they have the same elution sequence as those reported in the literature.

model 1701 N. A hole (diam. 3×10 cm) was dug in the soil approximately 10 cm from the plant's stem. By inserting in the hole, a 50 ml-open end Falcon-tube, the headspace of the rhizosphere was captured in the tube (Fig. 2). The syringe was inserted through a narrow hole on the cap of the tube and sealed with parafilm. Two µl hexadecane were extruded from the tip of the syringe as a single drop to adsorb the volatiles in the head space of the rhizosphere of the plant (Fig. 2). After overnight exposure, the drop was retracted in the micro-syringe and injected directly into the GC-MS.

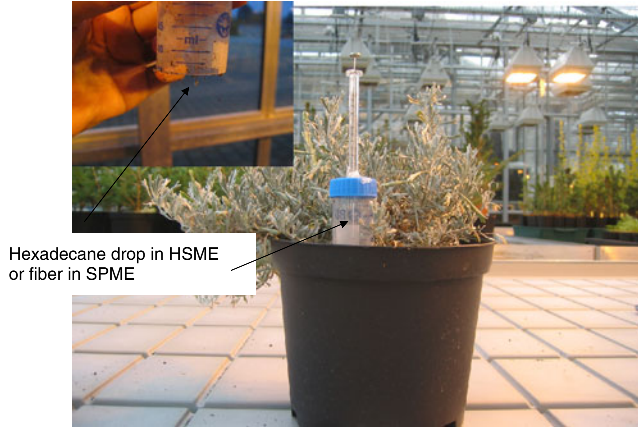


Fig. 2 Headspace solvent micro-extraction (HSME) for detecting volatiles emitted from plant roots; the volatiles are adsorbed onto a single drop of hexadecane overnight, retracted into a syringe, and injected into the GC-MS for analysis of the constituents. The same arrangement was used for solid-phase micro-extraction (SPME) of the volatiles replacing the syringe by a SPME apparatus

Solid Phase Micro-Extraction-GC-MS Analyses (SPME-GC-MS) The experimental design for the adsorption of the volatiles was the same as for the HSME experiment, but instead of the micro-syringe a stableFlex Divininylbenzene/Carboxen/PDMS (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte PA, USA) was used to adsorb the volatiles from the rhizosphere of the plant (Fig. 2). Volatiles emitted from below-ground parts of the plants were adsorbed on the SPME fiber for 1 h; after retracting the fiber, it was injected into the injector of the GC-MS. The fiber was conditioned about 15 min before each injection at 220°C.

Germination and Post-Germination Bioassay Nerol, geraniol, their isovalerates esters, camphor, 1,8-cineol, camphene, methyl jasmonate, *E*-caryophyllene, and α-thujone were dissolved in 50 μl acetone and applied to filter paper (Macherey- Nagel, MN 85/70 diam. 25 mm: www.mn-net.com) at 50 to 0.1 μmol amounts in two-chambered Petri dishes (diam. 10 cm). After treatment with liquid smoke, 12–13 seeds of *N. attenuata* were placed on GB5 agar (15 ml) poured into one compartment of the Petri dish; after 6 d at 28/24°C 16/8 hL/D, the number of germinated seeds was counted. To determine the toxicity of the volatiles on *N. attenuata* seedlings, seeds were germinated on agar and after 4 d, they were exposed to the volatiles applied to a paper disc in the divided Petri dish. The appearance and development of the leaves of the seedlings were evaluated at d 10.

Statistical Analyses Student's*t-tests* were performed with the algorithms embedded in the StatView.

Results

Root Extracts from Big Sagebrush Added to Plant Agar Inhibit the Growth of N. Attenuata Seedlings To test for phytotoxic effects of extracts, we extracted the roots of big sagebrush with different solvents and added the extracts to the agar media (0.25 μg/ml) used to grow the seedlings of *N. attenuata*. Only the methanolic layer of the dichlromethane (DCM) extract at 0.25 μg/ml agar inhibited the growth of *N. attenuata* seedlings significantly (56% of the control, unpaired *t-test*, $P<0.001$).

The active extract was subjected to silica gel-column chromatography fractionation. The hexane-dominant fractions were active at a concentration of 3.75 μg/ml agar in the root growth bioassay, and inhibited seedling growth rates to 74% of that of control plants (unpaired *t-test*, $P<0.001$). All fractions collected from column chromatography were analyzed by GC-MS and ^{13}C NMR spectroscopy. In addition to an acetylenic spiroether compound previously isolated from some species of *Artemisia* and *Chrysanthemum* (Uchio et al., 1981; Martínez et al., 1987), geranyl- and neryl-esters were found as the major phytotoxic constituents in the active fractions eluted with hexane (Table 1).

Dynamic Headspace GC-MS Analyses of the Roots of A. tridentata To determine whether or not the phytotoxic volatiles are emitted from the roots of growing plants, the root headspace was collected from intact plants grown under different conditions (Fig. 1) and analyzed by GC-MS. Camphor, the most abundant monoterpene in the roots' headspace, was emitted at 1846.2±474.0, 344.5±147.7, and 231.1±126.4 ng$L^{-1}h^{-1}$ for plants grown in soil, sand, or under hydroponic conditions, respectively (Table 2). The other major compounds were 1,8-cineol, which was emitted at 398.7±220.9, 41.3±12.0, and 25.9±13.4 ng$L^{-1}h^{-1}$, respectively, and the monoterpene hydrocarbon, camphene, emitted at 508.2±189.8, 84.3±28.6, and 46.9±26.8 ngL^{-1} h^{-1}, respectively. From the roots of a 1-yr-old plant cultured in soil, γ-himachalene (34.8%; peak area normalized%) and other sesquiterpenes were detected as the major emitted volatiles, while neryl isovalerate (6.3%) and geranyl esters were the minor volatile constituents. The head space of the roots of a 2-mo-old plant composed of neryl isovalerate (46.9%) while γ-himachalene (6.7%) was detected as a minor compound.

Headspace Solvent Micro-Extraction (HSME) of the Roots of Big Sagebrush In order to determine whether or not volatiles were emitted from the roots of the plant, we used headspace solvent micro-extraction (HSME) with a single 2 μl drop of hexadecane hanging from a micro-syringe to adsorb the volatiles; the syringe was placed above a hole

Table 2 Volatiles emitted from the roots of *Artemisia tridentata,* cultured in different media and analyzed by dynamic HS-GC-MS

Compounds	Volatiles from-soil-grown roots ng L^{-1} h^{-1}	Volatiles from sand-grown roots ng L^{-1} h^{-1}	Volatiles from hydroponically grown roots ng L^{-1} h^{-1}
α-pinene	150.2±40.7	36.1±12.0	13.2±7.8
camphene	508.2±189.8	84.3±28.6	46.9±26.8
β-pinene	72.2±25.5	16.1±5.0	11.8±6.9
1,8-cineol	398.7±220.9	41.3±12.0	25.9±13.4
α-thujone	19.0±12.8	11.4±9.7	6.3±0.0
camphor	1846.2±474.0	344.5±147.7	231.1±126.4
nerol	3.7±1.3	15.1±7.5	1.8±0.7
longifolene	4.8±0.7	9.1±3.1	0.5±0.1
β-caryophyllene	1.4±0.0	–	0.2±0.0
neryl isovalerate	5±2.0	60.1±28.1	4.8±1.2

The mean value (ng L^{-1} h^{-1}) of the emitted compounds from each plants' root±SE of 5 different biological replicates were calculated for some selected compounds by measuring their response factors compared to the internal standard

(diam. 3×10 cm) excavated in the soil near the plant (Fig. 2). Camphene, 1,8-cineol, camphor, neryl isovalerate, and γ-himachalene were detected by GC-MS in the headspace.

Headspace Solid Phase-Microextraction GC-MS Analysis (HSPME-GC-MS) of the Volatiles Emitted From the Rhizosphere of A. tridentata In addition to HSME, we used SPME to reconfirm the emission of volatiles from the roots of intact plants and to identify the emitted volatiles more precisely. We placed the SPME fiber in a hole near the roots to collect the VOCs emitted from the rhizosphere of the plants, exactly as we had done for the HSME (Fig. 2). After the roots' volatiles were adsorbed on the SPME fiber and subjected to GC-MS analysis, the analysis revealed that the roots of *A. tridentata* produced different types of secondary metabolites compared with those released by the shoots (Table 1). Monoterpenes constituted 39.8% and 45.5% of the volatiles emitted from the shoots and roots of the plant. Of these, camphor was the most abundant constituent in roots (27.5±3.0%) and in shoots (31.3±9.5%). In the headspace of the shoots, different green leaf alcohols and their esters, constituted 26.5% of the total volatiles, while none of these green leaf volatiles were detected in the roots or in the rhizosphere; however, sesquiterpenes were detected at 8.8% of the total volatiles emitted from the roots, while only traces of these were detected in the shoots' headspace. The other major compounds detected in the roots but not in the shoots were the geranyl and neryl esters and γ-himachalene: these compounds were never detected in the shoots' volatiles. The headspace of the roots and shoots of *Artemisia annua* was analyzed by SPME-GC-MS to determine if the same pattern of volatile distribution that was detected in *A. tridentata* could be observed; sesquiterpenes were the major constituents in the roots, and monoterpenes and green leaf volatiles were the major constituents in the shoot volatiles of *A. annua* (unpublished data).

Effects of the Chemical Constituents of VOCs from the Roots of Big Sagebrush on Seed Germination in N. attenuata After identifying the main VOCs emitted from the roots of big sagebrush grown under differing conditions, the following compounds, which the literature suggested might be phytotoxic, were tested for their ability to influence seedling growth and seed germination of *N. attenuata*. Nerol, geraniol, their isovalerate esters, camphor, 1,8-cineol, camphene, methyl jasmonate, and α-thujone were applied to paper discs at 50 to 0.1 μmol dose amounts in two-chambered Petri dishes (see Fig. S1), and their effects on the germination of 12–13 seeds growing on GB5 agar were evaluated after 6 d. Among the tested compounds, MeJA completely inhibited seed germination at all concentrations tested (Fig. 3). In the presence of nerol, α-thojone, and camphor, at 10 μmol/disc, 51.9±8.5, 46.2±5.7, and 49.2±8.6% of the seeds, respectively, germinated. By applying geraniol at 1, 5, and 10 μmol/disc, 65.4±11.9, 23.1±8.3, and 1.9±1.9% of the seeds germinated. Camphene did not inhibit seed germination in any concentrations applied. When geranyl isovalerate was applied at 25 and 50 μmol/disc 30.8±17.4 and 2.4±1.6% of the *N. attenuata* seeds germinated, but neryl isolvalerate was nearly inactive (Fig. 3). The oxygenated monoterpene 1,8-cineol was active only at 50 μmol/disc, which allowed 36.5±5.8% of the seeds to germinate.

Effect of Some of the Chemical Constituents of VOCs from the Roots of Big Sagebrush on Seedlings of N. attenuata after the Seeds Germinated Four days after the seeds of

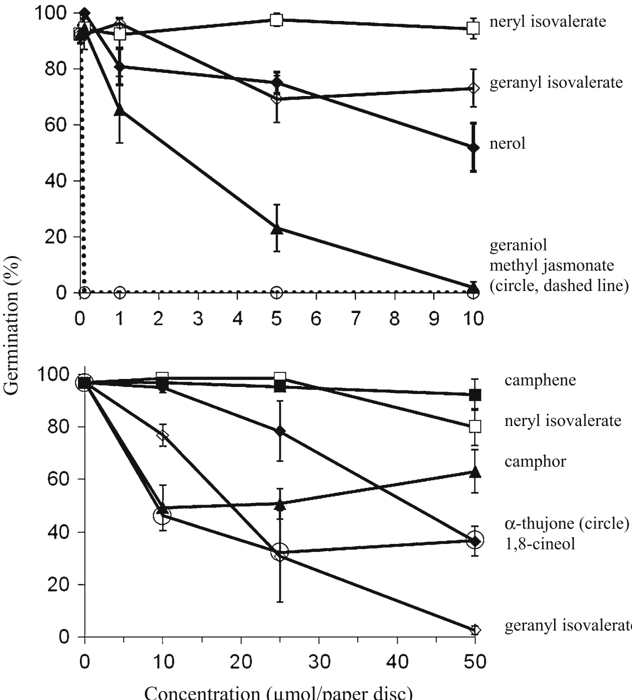
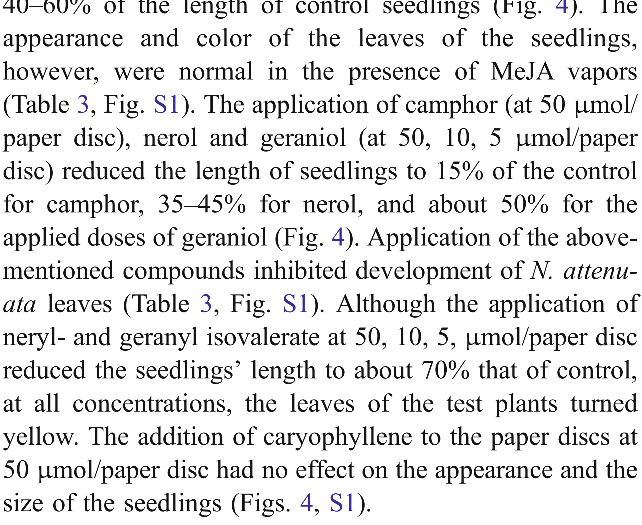


Fig. 3 Germination of the seeds of *Nicotiana attenuata* are inhibited by the presence of volatiles detected in the roots of *Artemisia tridentata* at different concentrations applied to paper discs in two-chambered Petri dishes. The germination% mean value [(number of germinated seeds/total number of seeds)×100]±*SE* of five biological replicates for each concentration of the volatiles in 50 µl acetone were compared with those for the control treatments in which 50 µl acetone was applied on the paper disc

N. attenuata germinated, seeds were transferred to fresh agar in one compartment of a two-chambered Petri dish; in the other, the selected volatiles were applied to paper discs and their effects on the seedlings were evaluated by measuring the seedlings' length on day10 after the beginning of tests (Figs. 4, S1). MeJA in all doses applied at 50, 10, 5, and 1 µmol to the paper discs reduced the seedlings' length to



40–60% of the length of control seedlings (Fig. 4). The appearance and color of the leaves of the seedlings, however, were normal in the presence of MeJA vapors (Table 3, Fig. S1). The application of camphor (at 50 µmol/paper disc), nerol and geraniol (at 50, 10, 5 µmol/paper disc) reduced the length of seedlings to 15% of the control for camphor, 35–45% for nerol, and about 50% for the applied doses of geraniol (Fig. 4). Application of the above-mentioned compounds inhibited development of *N. attenuata* leaves (Table 3, Fig. S1). Although the application of neryl- and geranyl isovalerate at 50, 10, 5, µmol/paper disc reduced the seedlings' length to about 70% that of control, at all concentrations, the leaves of the test plants turned yellow. The addition of caryophyllene to the paper discs at 50 µmol/paper disc had no effect on the appearance and the size of the seedlings (Figs. 4, S1).

Discussion

The release of metabolites into the environment is the first requirement for a compound to play a role as an allelochemical (Inderjit and Nilsen, 2003). Therefore, volatilization from aerial parts was considered as one of the modes of release of allelochemicals into the media in addition to the exudation of water-soluble metabolites from the roots (Inderjit and Duke, 2003). Direct volatilization from roots has not been well studied.

Here, we demonstrated that the *A. tridentata* roots release volatiles deep into the rhizosphere and showed that some of the roots' volatiles have phytotoxic effects on seed germination and seedling growth of the co-occuring plant, *N. attenuata*. The different volatiles detected in the shoots and roots, and the fact that the emitted compounds are found in the root extracts, demonstrated that the compounds detected in the rhizosphere originate from the roots and not transferred from aerial parts of the plant. As dry soil can better adsorb toxic volatile terpenes than moist soil (Muller

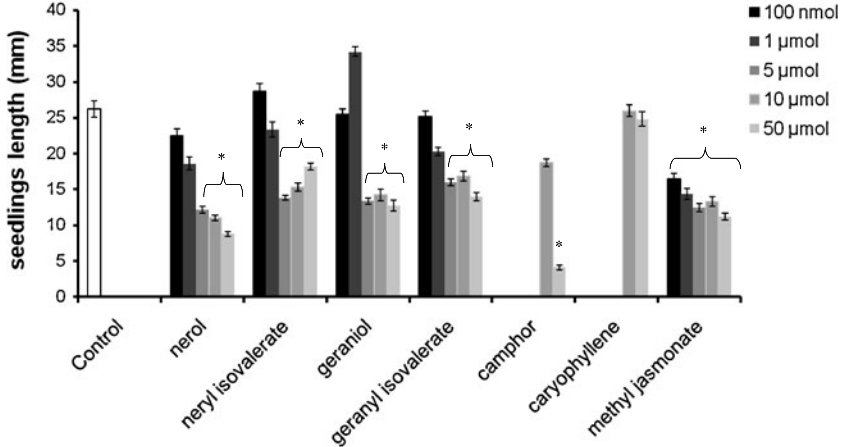


Fig. 4 Growth inhibition potential expressed as means±*SE* of the seedlings length of the germinated seeds in the presence of the volatile compounds identified in the headspace of roots of *Artemisia tridentata* on *Nicotiana attenuata* seedlings in five different replicates (each replicate contained 12–13 seedlings) of the post-germination bioassay after 10 d. * significantly different from control seedlings; $P<0.05$

Table 3 The appearance of the leaves and shoots (L&S) of *Nicotiana attenuata* seedlings after six days of exposure to root volatiles applied to paper discs on plant agar media in the post-germination tests (Figs. S1, 4)

Compound	L&S (50 μmol/PD)	L&S (10 μmol/disc)	L&S (5 μmol/disc)	L&S (1 μmol/disc)	L&S (0.1 μmol/disc)
Nerol	ND[a]	ND	Yellow	Yellow	Normal[b]
Neryl isovalerate	Yellow	Yellow	Yellow	Yellow	Normal
Geraniol	ND	ND	ND	ND	Normal
Geranyl isovalerate	Yellow	Yellow	Yellow	Yellow	Normal
Camphor	ND	Normal	–	–	–
Caryophyllene	Normal	Normal	–	–	–
Methyl jasmonate	Normal-small[c]	Normal-small	Normal-small	Normal-small	Normal-small

[a] leaves did not develop, [b] the leaves were green as they were observed in the control, [c] the leaves were green but the seedlings were smaller than those of the control

and del Moral, 1966), this form of release may be particularly effective in making soils phytotoxic to competitors. The phytotoxicity of soils exposed to the volatile terpenoids of the leaves of *Salvia leucophylla* was sustained up to four months (Muller and del Moral, 1966).

Previously, several methods have been used to detect allelochemicals in soils, mostly based on the extraction of chemicals from the soil (Inderjit and Nilsen, 2003; Lin et al., 2007; Ens and French, 2008; Ens et al., 2009b; Mohney et al., 2009), but by using organic or inorganic solvents, the non-volatile metabolites and those which do not originate from roots or those which are transformed by soil microbes, may also be extracted. Here, with the assistance of headspace solvent microextraction (HSME) and solid phase microextraction (SPME), we confirmed that live roots introduce phytotoxic substances into the soil; these may last for a long time or may be microbially transformed and subsequently inhibit the germination or the growth of competing plants.

Quantification of allelochemicals in the soil is one of the main tasks in the allelopathic studies (Inderjit and Nilsen, 2003). To accomplish this, we used dynamic HS-GC-MS analyses; the calculated total emitted substances per day from a single plant's roots were used to load the test compounds onto paper disc in the germination bioassays with the seeds of *N. attenuata* on agar. Lin and his coworkers (2007) recently used a similar apparatus to quantify the volatiles from the roots of different *Pinus* species. The two methods of dynamic headspace extraction differed in the shape of apparatus and the mode of adsorption-desorption of the volatiles. Both methods, however, disturb the roots by removing the adjacent soil and, therefore, the roots may release different types of volatiles when wounded (Inderjit and Nilsen, 2003). In our experiment, we extracted the headspace of the plants' roots as quickly as possible to avoid this problem. Analyzing the roots' volatiles by both HSME- and SPME-GC-MS allowed us to address a disadvantage of the HSME method namely that the solvent peak in the GC chromatogram overlaps with a relatively broad area of the chromatogram and, therefore, some compounds may be missed.

Our results demonstrate that the roots of *A. tridentata* release a rich source of different secondary metabolites (Tables 1 and 2). Depending on the method of extraction, age of the plants, and the media used for the culturing, different kinds and quantities of volatile substances were identified in the roots' headspace (Tables 1, 2). Some of the volatiles identified in the shoots of *A. tridentata,* such as camphor, 1,8-cineol, α-pinene, and nerol, are phytotoxic (Asplund, 1968; Romagni et al., 2000; Kordali et al., 2007). Among these, the phytotoxicity of the monoterpene alcohols is higher than that of the ketones, and the monoterpene hydrocarbons are the least toxic (Asplund, 1968; Kordali et al., 2007). These previous results are consistent with our finding that the monoterpene alcohols nerol and geraniol are more active than their isovalerate esters, and even more active than the monoterpenes with a ketone functionality, namely camphor and α-thujone. The monoterpene and sesquiterpene hydrocarbons, camphene and caryophyllene, were inactive on *N. attenuata* seedlings and seeds at all concentrations tested. The higher toxicity of the oxygenated-monoterpenes compared to their hydrocarbon derivatives purportedly is due to the higher solubility of oxygenated-monoterpenes in the media (Weidenhamer et al., 1993; Kordali et al., 2007). However, as we have shown here in the paper disc-agar bioassay, the vapor of the hydrocarbon- and oxygenated-monoterpenes can be transferred to the seedlings of the test plants on agar, but the oxygenated monoterpenoids have a greater phytotoxic effect on the seedlings than do the hydrocarbon terpenoids. Thus, we conclude that the phytotoxicity of the monoterpenes is likely due to their chemical structures rather than to their water solubility.

In summary, this research suggests that big sagebrush uses below-ground volatile terpenoids as phytotoxic substances against competing plants including *N. attenuata* in

addition to its well-studied shoot-derived volatiles. Because the conditions under which plants are grown are different in the field and in the glasshouse, and because the natural soil contains microorganisms that are absent in the agar or are different from those in the potting soil that we used in our experiments, additional bioassays and analytical studies will be required to demonstrate conclusively the allelochemical potential of the compounds emitted from the roots and shoots of field-grown plants.

SPME-GC-MS analyses showed that the plant's roots emitted types of volatiles different from those released by the shoots. For absolute quantification, it is necessary to perform the experiments under standard analytical conditions (Romeo, 2009). In addition, new germination and growth bioassays that use the field soils, and that quantify the potential allelochemicals in the soil and rhizosphere surrounding the sage brush are essential to confirm allelopathy originating from root volatiles.

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