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COMMENTARY

New Synthesis: Exploring the Chemical Links in Ecological Food Webs

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For the past year, contributors to New Synthesis have explored conceptual and technological frontiers that promise to stretch the field of Chemical Ecology in new directions. One such opportunity is the integration of chemical signals and chemoreception into community ecology. Studies of "food web" networks have revealed the disproportionate importance of "keystone" species, the resiliency of certain interactions (e.g., generalized herbivores or pollinators with their hosts) to species turnover or disturbance, and the context dependence of network structure, with direct impacts on resource management and conservation as well as ecological theory.

At first glance, there would appear to be little common ground between chemical and community ecology, as scientists in these fields tend to employ different methodological approaches to their craft. For example, the chemical, physiological and behavioral assays used to study the pollination of sexually deceptive orchids by bees usually are not utilized by teams of ecologists measuring links between the same bee species and tens of other flowering plant species that they visit for nectar or pollen. Indeed, the null models used in some food web studies begin with the assumption that all species are equally likely to interact. Though heuristically useful, this assumption strikes many chemical ecologists and behaviorists as unreasonable. When "links" are found to be stronger or weaker than null expectations, biological explanations are sought. Until recently, community ecologists have been more likely to invoke habitat use, phenology, or population density than specific phenotypic or behavioral traits to explain how food webs are constructed.

How might chemistry explain link strength – or its absence - in ecological networks? Three recent or ongoing studies illustrate the dialectical potential between chemical ecology and community ecology. At the simplest (agricultural plot) scale, Poelman et al. (2009) have documented the effects of compositional variation in plant resistance compounds (glucosinolates) on herbivore diversity and abundance, using different cultivars of *Brassica oleracea* and wild *B. nigra* mustard plants in The Netherlands. Total glucosinolate concentration has no effect on insect diversity indices, whereas cultivars high in glucoiberin content but low in glucoraphanin host fewer aphids and caterpillars. Subsequent bioassays revealed at least two non-competitive mechanisms for this result: low oviposition and poor larval performance on high-glucoiberin cultivars by specialist (*Pieris rapae*) and generalist (*Mamestra brassicae*) lepidopterans. Additional work by the Wageningen group has revealed the bottom-up effects of specific glucosinolates on higher trophic levels in this agroecosystem network, including parasitoid and hyperparasitoid wasps. Thus, leaf chemistry has strong predictive power in the food web that stretches across a field of Brussels sprouts.

At a more complex (floral marketplace) scale, Junker and colleagues (2010) studied a network of 35 flowering plant species and 164 flower-visiting insect species in a German meadow. Most observed links deviated significantly from null model expectations, either more frequently ("hot links") or less frequently ("cold links") than expected, and several of these links were investigated using behavioral choice assays, through the use of a field-portable olfactometer. Interestingly, hot links between specific pollinators and flowers usually coincided with significant olfactory preference (either innate or learned) for that flower's scent, and the few testable cold links were reinforced by actual repellence, rather than indifference. This is the first community-level demonstration that floral volatile chemistry underlies both positive and negative network links, as an ecological filter.

At the most complex (landscape-ecosystem) scale, Zimmer and Ferrer (2007) suggest that toxins of microbial origin can propagate through food webs due to their powerful effects on predation and energy flow, and might profitably be considered "keystone molecules". Tetrodotoxin (TTX) and saxitoxin (STX) are potent neurotoxins that block sodium channels, often resulting in paralysis or death. Marine, riparian, and some terrestrial communities are heavily structured by the uptake and use of TTX as a defense compound (e.g., by newts), and the degree to which their predators (e.g., garter snakes) can tolerate such compounds. Similarly, marine food webs structured around kelp beds are strongly impacted by sea otter predation on urchins or butter clams, a decision that is directly impacted by the presence of STX in clams (and ultimately, on dinoflagellates as the source of STX).

Thus, diverse organic compounds ranging from simple ubiquitous volatiles (e.g., monoterpenes) to more complex defense compounds (glucosinolates and herocyclic alkaloids) do more than mediate the binary organismal interactions studied by chemical ecologists in the first half century of our field's history. They lend structure to the food webs through which abiotic sources of energy enter and flow through the biosphere.

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References

Poelman, E. H., van Dam, N. M., van Loon, J. J. A., Vet, L. E. M., and Dicke, M. 2009. Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. *Ecology* 90:1863–1877.

Junker, R. R., Höcherl, N., and Blüthgen, N. 2010. Responses to olfactory signals reflect network structure of flower-visitor interactions. *Journal of Animal Ecology* 79:818–823.

Zimmer, R. K. and Ferrer, R. P. 2007. Neuroecology, chemical defense and the keystone species concept. *Biological Bulletin* 213:208–225.

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Chemical Mediation of Ternary Interactions Between Marine Holobionts and Their Environment as Exemplified by the Red Alga *Delisea pulchra*

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Abstract The need for animals and plants to control microbial colonization is important in the marine environment with its high densities of microscopic propagules and seawater that provides an ideal medium for their dispersal. In contrast to the traditional emphasis on antagonistic interactions of marine organisms with microbes, emerging studies lend support to the notion that health and performance of many marine organisms are functionally regulated and assisted by associated microbes, an ecological concept defined as a holobiont. While antimicrobial activities of marine secondary metabolites have been studied in great depth *ex-situ*, we are beginning to understand how some of these compounds function in an ecological context to maintain the performance of marine holobionts. The present article reviews two decades of our research on the red seaweed *Delisea pulchra* by addressing: the defense chemistry of this seaweed; chemically-mediated interactions between the seaweed and its natural enemies; and the negative influence of elevated seawater temperature on these interactions. Our understanding of these defense compounds and the functional roles they play for *D. pulchra* extends from molecular interactions with bacterial cell signaling molecules, to ecosystem-scale consequences of chemically-controlled disease and herbivory. *Delisea pulchra* produces halogenated furanones that antagonize the same receptor as acylated homoserine lactones (AHL)—a group of widespread intercellular communication signals among bacteria. Halogenated furanones compete with and inhibit bacterial cell-to-cell communication, and thus interfere with important bacterial communication-regulated processes, such as biofilm formation. In a predictable pattern that occurs at the ecological level of entire populations, environmental stress interferes with the production of halogenated furanones, causing downstream processes that ultimately result in disease of the algal holobiont.

Keywords Holobiont · Seaweed · *Delisea pulchra* · Bacterial colonization · Disease · Halogenated furanones · Bleaching

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Introduction

Historically, the field of marine chemical ecology has focused mainly on binary intra- and interspecific chemical signal-mediated processes in marine animals and plants. Typically, studies have focused on the coordination of mate recognition, synchronization of reproduction, specific site selection of motile propagules, competition for space, detection of prey items, defense against predators and pathogens, and control of microbial colonization (reviewed in Hay, 2009). The need for animals and plants to control microbial colonization is particularly evident in the marine realm with its typically high densities of microscopic propagules, and seawater as an ideal mode of their dispersal (Harder, 2008). Unlike air, the average milliliter of seawater contains up to 10^7 viruses, 10^6 bacteria, 10^3 fungi and

microalgae, and 10–100 larvae and spores of marine invertebrates and plants, all of which exert significant selective pressures on marine animals and plants (reviewed in Davis et al., 1989; Engel et al., 2002; Paul et al., 2007).

Although marine microbes and higher eukaryotes have coevolved for millions of years, marine animals and plants have variable relationships with microbial propagules. Their interactions can be either beneficial or detrimental to the host organism (van Oppen et al., 2009). Past studies in the field of defense against marine microbes have led to a solid understanding that many marine microbes pose a risk, and that marine animals and plants use a range of secondary metabolites against microbial foulers and pathogens (Pawlik, 1993; Kushmaro et al., 1996; Engel et al., 2002).

In contrast to the traditional emphasis on antagonistic interactions of marine animals and plants with microbes, there is an emerging field of studies lending support to the notion that health, performance, and resilience of marine animals and plants are functionally regulated and positively assisted—at least in part—by microbial propagules such as bacteria, diatoms and viruses (Williams et al., 1987; Croft et al., 2005; Matsuo et al., 2005; van Oppen et al., 2009; Phelan et al., 2012). Such facilitative interactions have only recently emerged in consideration of the factors that impact populations and communities, although they may play a critical, but underappreciated role in ecological communities (Stachowicz, 2001). Functional assistance implies that epi- and endobiotic microbes form an associational entity with their host, an ecological concept coined "holobiont" (Rohwer et al., 2002). Only recently have the modern sequencing methods and metagenomic tools (Giovannoni and Rappe, 2000; Simon and Daniel, 2011), which made characterizing the holobiont possible, been employed systematically to study the associated microbes living in and on marine animals and plants, such as corals, sponges, and macroalgae (Thomas et al., 2010; Webster et al., 2010; Barott et al., 2011). In some cases, the astounding diversity of bacterial phyla associated with these marine organisms seems to be host-specific and well maintained in space and time (Longford et al., 2007; Lachnit et al., 2011). In others, the bacterial community composition is highly variable but instead functionally redundant (Burke et al., 2011a, b), i.e., different bacteria play equivalent roles. Besides these descriptive studies, all of which lend strong support to the functional holobiont concept, it is virtually unknown how mutualistic interactions between marine hosts and microbes are mechanistically established or regulated.

It is now recognized that many animals and plants not only live in close association with thousands of different microbial species, but also that a considerable percentage of bacterial genomes is dedicated to shaping the organisms' habitats and maintaining their community and niche in the ecosystem (Phelan et al., 2012). In many cases, the number of associated microorganisms and their combined genetic information far exceeds that of their host. Examples of well-studied functional holobionts span several marine phyla (corals, sponges, macroalgae, cephalopods), terrestrial insects, the rhizo- and phyllosphere, and the vertebrate gut (reviewed in Rosenberg and Zilber-Rosenberg, 2011).

All these findings support a novel and holistic perception of general species function and performance. The question arises then, what role does chemical ecology or chemical signaling play in the maintenance—or disruption—of the holobiont? The chemical ecology of the holobiont is maintained through a diverse chemical communication network. Not only does the host modulate and control its associated microbiota through bioactive metabolites, but on small scales, epibiotic bacteria coordinate and express their various traits through intercellular communication signals (autoinducers), a process known as quorum sensing (QS) (Miller and Bassler, 2001). This interactivity and communication extends beyond microbes on host interfaces. This research field has expanded drastically with the realization that bacterial QS signals can cross into the eukaryotic domain—a process dubbed inter-kingdom signalling (Rumbaugh, 2004). This has led to discoveries that eukaryotes eavesdrop on bacterial autoinducers to locate bacterial biofilms (Joint et al., 2007), or specifically interfere with QS-regulated bacterial traits, such as biofilm formation (Dobretsov et al., 2011), by modulating bacterial gene expression (Hughes and Sperandio, 2008).

Quorum sensing modulation between hosts and their associated microflora is only one means by which holobionts may regulate the association between the different players. Given the complex species composition of any holobiont and its sophisticated communication network, it is challenging to identify, trace, and categorize all the chemically mediated interactions and the underlying semiochemicals with classical concepts and definitions of chemical ecology. Instead, a more integrated perception is needed to account for these communication networks within an organism and their interaction with the environment, and vice versa.

The present article reviews two decades of research on arguably one of the best-investigated holobionts in the literature, the seaweed *Delisea pulchra*, with regard to chemically mediated interactions between the seaweed, epiphytic bacteria, and environmental parameters, which in turn are tightly linked to the state of chemical defense and a disease phenomenon in this seaweed.

Ternary Interaction Between Chemical Defenses, Environmental Influences, and Natural Enemies

To integrate the effect of environmental stress on host resilience and the pathogenicity of microbes, the ternary

interactions between marine hosts, host-associated microbiota, and environmental parameters need to be unraveled. This requires a better knowledge of the microbes, their identity and function, and the molecular signals exchanged between hosts and microbes. Our ability to look more closely into these interactions has drastically improved with the recent advent of modern and affordable sequencing methods and genomics.

The interaction between marine host surfaces and microbial pathogens represents an ideal site to study and integrate the chemical ecology of marine eukaryotes and microbial propagules, as these interactions often result in clearly observable, macroscopic changes of the host. Typical examples for such changes are disease phenotypes, such as wasting disease in seagrass (Short et al., 1987), the range of band diseases in corals (Bourne et al., 2009), and bleaching of crustose and fleshy marine algae (Campbell et al., 2011; Webster et al., 2011). Environmental influences, such as those linked to climate change (IPCC, 2007), have been suggested to play a major role in the increased frequency and severity of disease (Lafferty et al., 2004; Harvell et al., 2009; Harvell and Hewson, 2010), by either lowering the resistance of the host or by increasing the virulence of microbial pathogens (Konkel and Tilly, 2000). The impact and consequences of disease in natural systems are topical, given pathogens can cause the disappearance of entire ecosystems over large spatial scales (Short and Neckles, 1999).

Seaweeds

Seaweeds are the dominant habitat-forming organisms in temperate marine rocky reefs, and play similar important ecological roles as corals do in tropical reefs, or trees in rainforests. They are important primary producers and major foundation species, i.e., they structure a community by creating locally stable conditions for other species and thus stabilize fundamental ecosystem processes (Ellison et al., 2005). As seaweeds are in constant contact with abundant and ubiquitous marine microorganisms, lack an advanced immune system, and have no physical means to escape enemies, many seaweeds have evolved chemically-mediated processes that defend them against and also facilitate mutualistic associations with marine microorganisms (Matsuo et al., 2005).

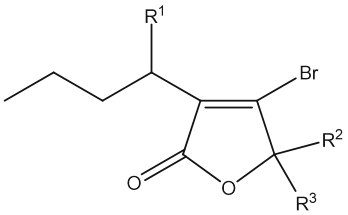
Research into the chemical ecology of seaweed holobiont functioning and maintenance is highly topical and timely given that evidence of seaweed decline is emerging from many parts of the world, and disease has been suggested as a potential cause (Vairappan et al., 2001; Edwards and Estes, 2006; Gachon et al., 2010; Vargas-Angel, 2010). However, these suggestions rarely have been confirmed or investigated further, and overall we have very limited knowledge of the chemical ecology, and the molecular mechanisms and interaction patterns of marine organisms with regard to a changing environment, stress, increased bacterial virulence, and the modes of molecular defense against these challenges.

A significant proportion of seaweed research has focused on characterizing and understanding chemical defenses (reviewed in Paul et al., 2011; Amsler, 2008; Goecke et al., 2010) and their ecological functions (e.g., Lane et al., 2009; Lachnit et al., 2010; Persson et al., 2011). Much of our own research has centered on *Delisea pulchra*, a chemically-defended foliose red seaweed that occurs commonly around temperate Australian, New Zealand, and Antarctic rocky reefs. This seaweed has been a model organism in our laboratory with respect to its defense chemistry and the interactions between this organism and its natural enemies, which are often chemically-mediated. Our growing understanding of these defense compounds and the functional roles they play for *D. pulchra* extends from molecular interactions with bacterial cell signal molecules, to the ecosystem-scale consequences of chemically-mediated disease and herbivory. This system, thus, provides a useful example to review in the context of marine chemical ecology, showcasing the ternary interaction pattern between algal defense, bacterial infection, and environmental stress.

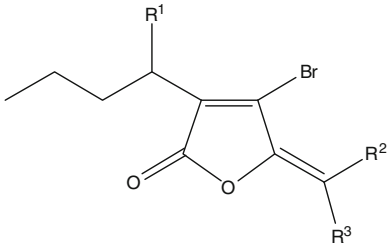
Halogenated Furanones—The Chemical Defense of *Delisea pulchra*

Delisea pulchra produces 21 derivatives of halogenated furanones (Fig. 1) that mediate a variety of ecological interactions for the alga (Pettus et al., 1977; De Nys et al., 1993). These compounds are enclosed in specialized gland cells (Dworjanyn et al., 1999), which are present internally and at the surface of the thallus. *Delisea pulchra* is a low preference food for local herbivores (Steinberg and Van Altena, 1992; Williamson et al., 2004; Wright et al., 2004) and often is unfouled *in situ* (Maximilien et al., 1998). Consistent with these observations, halogenated furanones have a broad range of biological activity against natural enemies, deterring local herbivores (Williamson et al., 2004; Wright et al., 2004), eukaryote fouling organisms (De Nys et al., 1995; Dworjanyn et al., 2006), marine epiphytic bacteria (Maximilien et al., 1998), and potential bacterial pathogens (Manefield et al., 1999, 2000).

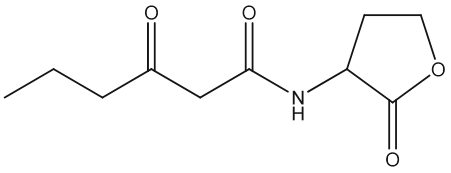
The mode of action of halogenated furanones present in *D. pulchra* against bacteria is a classical example of how eukaryotic organisms can interfere with prokaryotic communication signals. Halogenated furanones occupy the same specific receptor binding sites of acylated homoserine lactones (AHL, Fig. 1)—one class of many intercellular communication signals among bacteria that mediate quorum sensing (QS) (Fuqua et al., 1994)—thereby inhibiting



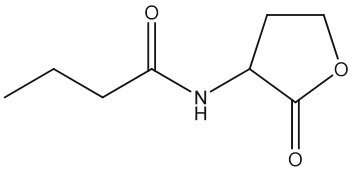
	R^1	R^2	R^3
1	OH	CH_3	OH
2	OH	OCH_3	CH_2I
3	OH	CH_2I	OCH_3
4	OAc	OCH_3	CH_2I
5	OAc	CH_3	OCH_3
6	OAc	OCH_3	CH_3
7	OAc	$CHBr_2$	OCH_3
8	OAc	OCH_3	$CHBr_2$



	R^1	R^2	R^3
9	H	Br	Br
10	H	H	Br
11	OH	H	Br
12	OH	Br	H
13	OH	H	I
14	OH	I	H
15	OH	H	Cl
16	OH	Cl	H
17	OH	Br	Br
18	OAc	H	Br
19	OAc	Br	H
20	OAc	H	I
21	OAc	Br	Br



Vibrio fischeri



Pseudomonas aeroginosa

Fig. 1 Halogenated furanones produced by the red alga *Delisea pulchra* and representative acyl-homoserine lactone autoinducers used by Gram-negative bacteria

AHL-mediated QS among bacteria (Givskov et al., 1996; Manefield et al., 1999). In bacteria, AHL-mediated QS controls the expression of many traits relevant to host interactions, such as surface colonization, biofilm formation, and disease (Rice et al., 2007; Fernandes et al., 2011). Thus, the ability of *D. pulchra* to specifically interfere with these bacterial traits via the production of halogenated furanones represents a powerful algal defense strategy.

Health and Disease

Globally, there is emerging evidence of the decline of kelp forests (Steneck et al., 2002; Thibaut et al., 2005; Edwards and Estes, 2006; Coleman et al., 2008; Connell et al., 2008). Large-scale die-offs have been observed in many seaweeds, and disease—often manifested as a bleaching phenomenon—has been suggested as a potential cause of massive declines of the habitat-forming *Ecklonia radiata* in New Zealand (Cole and Babcock, 1996), of *Laminaria religiosa* in Japan (Vairappan et al., 2001), and of coralline algae throughout the Pacific (Littler and Littler, 1995).

Natural populations of *D. pulchra* on the east coast of Australia undergo seasonal bleaching (Campbell et al., 2011), characterized by white patches of algal fronds that have lost the typical red pigmentation (Fig. 2). This bleaching phenomenon is prevalent in summer and correlates strongly with elevated seawater temperatures rather than increased levels of ultra-violet (UV) irradiation (Campbell et al., 2011). The direct consequences of bleaching include reductions in growth and fecundity of the seaweed (Campbell, 2011). Bleaching, however, also has indirect ecological effects, with bleached individuals attracting higher densities of herbivores and being consumed at greater rates (Campbell, 2011).

Seaweeds affected by bleaching characteristically have reduced concentrations of halogenated furanones (Campbell et al., 2011). In a long-term study over 3 years, levels of halogenated furanones were consistently the lowest when bleaching was most prevalent (Campbell et al., 2011). While

Fig. 2 Top panel: Diseased (bleached) and healthy thallus sections of *Delisea pulchra*. Bottom panel: Healthy *D. pulchra* in the natural environment (Botany Bay, Sydney). Photos: Alexandra Campbell

bleached individuals had significantly lower levels of halogenated furanones than healthy conspecifics, the reduction in chemical defenses was evident across the entire thallus of bleached seaweed, i.e., not limited to visibly bleached sections (Campbell et al., 2011). These observations were indicative that other, more complex mechanisms were involved in *D. pulchra* bleaching.

Protective Role of Halogenated Furanones Against Disease

To test if halogenated furanones were involved in the protection of *D. pulchra* from bleaching, we experimentally inhibited the production of these defense compounds in cultured sporelings (Dworjanyn et al., 2006; Case et al., 2011) and exposed these to natural seawater in the field. In this series of experiments, sporelings deficient in halogenated furanones bleached significantly more than unmanipulated conspecifics, demonstrating that these compounds play an important protective role against bleaching (Campbell, 2011; Campbell et al., 2011; Case et al., 2011).

Given concurrent observations of bleaching and furanone depletion and the experimentally demonstrated protective role of halogenated furanones against bleaching, we hypothesized that bleaching may be the result of an opportunistic bacterial infection in susceptible (i.e., furanone-depleted) seaweed. To further test if the early stages of bleaching were associated with shifts in the surface-associated microbial communities on the seaweed, we characterized these communities with the molecular fingerprinting technique of terminal restriction fragment length polymorphism. This analysis clearly revealed that bleached sections of *D. pulchra* supported a different community of associated bacteria than healthy conspecifics. This pattern was consistent across locations and depths and throughout time (Campbell et al., 2011). Unbleached sections adjacent to visibly bleached parts supported a ‘transition community’ that was statistically similar to both ‘bleached’ and ‘healthy’ microbial communities, suggesting that the microbial community composition shifted in response to the depletion of halogenated furanones in algal tissues, prior to visible bleaching.

The Role of Water Temperature and Bacterial Infection in the Outbreak of Disease in *D. pulchra*

To test the hypothesis that bleaching was a temperature-mediated bacterial infection that could be prevented by chemical defense mediated by halogenated furanones, we exposed defended (furanone-containing) and undefended (furanone-depleted) sporelings of *D. pulchra* to water temperatures representing low and high summer temperatures and to high and low bacterial abundances (Campbell et al., 2011). This experiment revealed that sporelings maintained in natural seawater containing ambient bacterioplankton bleached more frequently and severely than conspecifics maintained in sterile seawater. Moreover, bleaching was more severe in sporelings lacking halogenated furanones than in defended seaweed (Campbell et al., 2011). These results were consistent with the premise that bleaching was the result of a bacterial infection. This bacterially-mediated disease was more common and severe at elevated water temperatures.

We have isolated a large number of epiphytic bacteria from the surface of *D. pulchra* and screened them *in vitro* for their capability to induce a bleaching phenotype similar to that observed in the field. The bacterial strain *Nautella* sp. R11 (formally *Ruegeria* sp. R11) is to date the best studied bacterial pathogen capable of infecting *D. pulchra* (Case et al., 2011; Fernandes et al., 2011). *Nautella* sp. R11 colonizes and subsequently forms pronounced biofilms on chemically undefended sporelings. Subsequent to colonization and at elevated temperatures, Strain R11 has been seen to penetrate the algal tissue and invade individual algal cells (Case et al., 2011). Colonization and invasion coincides

with localized bleaching of the thallus, however, whether invasion occurs prior to bleaching is not yet known.

While details of the virulence mechanism of *Nautella* sp. R11 are yet to be elucidated, genome analysis of this strain suggests a role for QS-dependent regulation of virulence genes (Fernandes et al., 2011). Such regulation is not uncommon among bacterial pathogens where QS signals are used to control concerted bacterial traits, such as virulence gene expression to mount a co-ordinated attack on the host (von Bodman et al., 2003; Atkinson and Williams, 2009). While in this particular case, we have not yet determined a direct effect of elevated temperature on virulence and pathogenicity of *Nautella* sp. R11, a positive correlation between the expression of virulence genes and temperature has been observed in a broad range of other bacterial pathogens (Konkel and Tilly, 2000).

The extent to which *Natuella* sp. R11 causes bleaching in the field is yet to be determined. However, recent evidence, using *in vitro* infection assays with *D. pulchra* sporelings, suggests that there may be many more bacterial strains capable of causing disease in this alga. Indeed, we now have a collection of over six marine strains observed to induce disease symptoms ranging from bleaching to invasion of host cells (Fernandes, 2011). Interestingly, one of these strains appears capable of causing disease also on chemically defended sporelings, suggesting that in at least some cases furanone-mediated defense alone may not be sufficient to protect *D. pulchra* from disease (Fernandes, 2011).

To summarize our studies of the seaweed *Delisea pulchra,* we provide a comprehensive picture of the QS-based chemical ecology between marine bacteria and their seaweed host. The pool of *D. pulchra*-generic halogenated furanones competes with and inhibits bacterial cell-to-cell communication, and thus interferes with important QS-regulated processes ranging from biofilm formation and bacterial surface colonization to the expression of virulence genes in pathogenic bacteria. Thus, the lowering of furanone levels in the seaweed at elevated seawater temperatures results in a chemically mediated ternary interaction among algal defense, bacterial infection, and environmental stress (in this case elevated water temperature) (Fig. 3). This interaction pattern is highly predictable and occurs at the ecological level of populations and communities.

Due to the identified interaction mechanisms, the entire community of surface-associated bacteria on the seaweed host shifts in response to changes in levels of halogenated furanones. These changes have been demonstrated to be systemic, that is furanone levels and the bacterial assemblage of the entire diseased alga are the same and distinct from that of healthy algae, even though only a small portion of the seaweed shows signs of disease. While the QS-based interaction helps to mechanistically explain the ternary interaction pattern, potentially many other chemically-mediated influences of halogenated furanones and other seaweed metabolites will also influence these interactions.

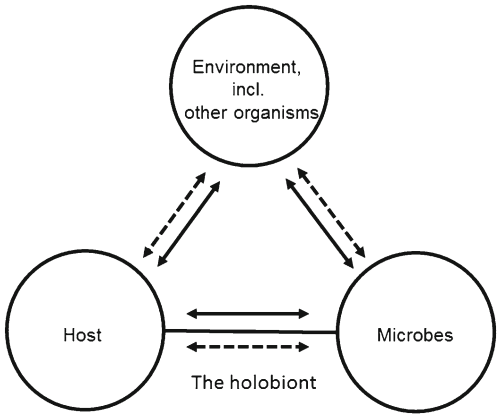


Fig. 3 Ternary interaction between the holobiont and microbial propagules. *Solid arrows* indicate chemically mediated interactions, *dashed arrows* indicate indirect effects on chemically mediated interactions

Other Marine Invertebrate Holobionts

Systemic changes of the entire community of associated microbiota on healthy vs. diseased marine invertebrates are common in marine invertebrate holobionts, such as corals, sponges, and bryozoans (Breitbart et al., 2005; Pantos and Bythell, 2006). Similar to our observations in the seaweed *D. pulchra*, these community changes often result from environmental stress caused by elevated seawater temperature, or changes in nutrient levels or UV irradiation, and can manifest themselves in disease and surface fouling (Glynn, 1996; Short and Neckles, 1999; Webster et al., 2008; Cebrian et al., 2011).

Similar to *D. pulchra*, where disease and fouling are controlled by host-produced QS-inhibitory compounds against pathogenic bacteria and fouling organisms, a range of other marine organisms is suggested to rely on a similar mode of action against potential pathogens. A screening of 284 extracts of marine organisms from the Australian Great Barrier Reef with a LuxR-derived QS reporter strain demonstrated that roughly a quarter of these species yielded organic solvent extracts with QS-inhibitory properties (Skindersoe et al., 2008).

The genes for quorum sensing signaling systems have been found in many bacterial genomes to date (Miller and Bassler, 2001). Given the important role of QS-regulated mechanisms in the beneficial or pathogenic outcomes of host-microbe associations, compounds that specifically mimic or interfere with bacterial QS are likely to be common in any form of eukaryote-prokaryote interactions. Beyond the marine realm, there is documented evidence for the presence of these compounds in freshwater algae (Teplitski et al., 2004) and higher plants (Teplitski et al.,

2000; Gao et al., 2003), although the identity of these compounds is still unknown.

In summary, holobiont functioning and maintenance is achieved through a communication network between the host and microbial mutualists, and among the microbial components. As outlined for the *Delisea pulchra* holobiont, many of these communication lines are maintained by chemical signals. In the seaweed example, we have mechanistically identified how environmental stress interferes with signal production, causing downstream processes that ultimately result in disease of the holobiont. It is currently unclear how other prominent marine holobionts, such as corals and sponges, maintain their synergy through chemical signaling. Since these phyla suffer from extreme disease phenotypes that result in drastic shifts of their associated microbiota, they will make ideal models to study the chemical ecology of holobionts.

References

Amsler, C. D. (ed.) 2008. Algal Chemical Ecology. Springer, Berlin.

Atkinson, S. and Williams, P. 2009. Quorum sensing and social networking in the microbial world. *J. R. Soc. Interface* 6:959–978.

Barott, K. L., Rodriguez-Brito, B., Janouskovec, J., Marhaver, K. L., Smith, J. E., Keeling, P., and Rohwer, F. L. 2011. Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral *Montastraea annularis*. *Environ. Microbiol.* 13:1192–1204.

Bourne, D. G., Garren, M., Work, T. M., Rosenberg, E., Smith, G. W., and Harvell, C. D. 2009. Microbial disease and the coral holobiont. *Trends Microbiol.* 17:554–562.

Breitbart, M., Bhagooli, R., Griffin, S., Johnston, I., and Rohwer, F. 2005. Microbial communities associated with skeletal tumors on Porites compressa. *FEMS Microbiol. Lett.* 243:431–436.

Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., and Thomas, T. 2011a. Bacterial community assembly based on functional genes rather than species. *Proc. Natl. Acad. Sci. U.S.A.* 108:14288–14293.

Burke, C., Thomas, T., Lewis, M., Steinberg, P., and Kjelleberg, S. 2011b. Composition, uniqueness and variability of the epiphytic bacterial community of the green alga Ulva australis. *ISME J.* 5:590–600.

Campbell, A. H. 2011. The ecology of bacterially-mediated bleaching in a chemically-defended seaweed. PhD dissertation. University of New South Wales, Sydney.

Campbell, A. H., Harder, T., Nielsen, S., Kjelleberg, S., and Steinberg, P. D. 2011. Climate change and disease: bleaching of a chemically defended seaweed. *Glob. Chang. Biol.* 17:2958–2970.

Case, R. J., Longford, S. R., Campbell, A. H., Low, A., Tujula, N., Steinberg, P. D., and Kjelleberg, S. 2011. Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. *Environ. Microbiol.* 13:529–537.

Cebrian, E., Uriz, M. J., Garrabou, J., and Ballesteros, E. 2011. Sponge mass mortalities in a warming mediterranean sea: are cyanobacteria-harboring species worse off? *Plos One* 6.

Cole, R. G. and Babcock, R. C. 1996. Mass mortality of a dominant kelp (Laminariales) at Goat Island, North-eastern New Zealand. *Mar. Freshw. Res.* 47:907–911.

Coleman, M. A., Kelaher, B. P., Steinberg, P. D., and Millar, A. J. K. 2008. Absence of a large brown macroalga on urbanised reefs around Sydney, Australia and evidence for historical decline. *J. Phycol.* 44:897–901.

Connell, S. D., Russel, B. D., Turner, D. J., Sheperd, S. A., Kildea, T., Miller, D., Airoldi, L., and Cheshire, A. 2008. Recovering a lost baseline: missing kelp forests from a metropolitan coast. *Mar. Ecol. Prog. Ser.* 36:63–72.

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., and Smith, A. G. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. *Nature* 438:90–93.

Davis, A., Targett, N., McConnell, O., and Young, C. 1989. Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth, *in* P. Scheuer (ed.), Bioorganic Marine Chemistry. Springer, Heidelberg, Berlin, New York.

de Nys, R., Wright, A. D., Konig, G. M., and Sticher, O. 1993. New halogenated furanones from the marine alga Delisea pulchra (Cf fimbrata). *Tetrahedron* 49:11213–11220.

de Nys, R., Steinberg, P. D., Willemsen, P., Dworjanyn, S. A., Gabelish, C. L., and King, R. J. 1995. Broad-spectrum effects of secondary metabolites from the red alga Delisea pulchra in antifouling assays. *Biofouling* 8:259–271.

Dobretsov, S., Teplitski, M., Bayer, M., Gunasekera, S., Proksch, P., and Paul, V. J. 2011. Inhibition of marine biofouling by bacterial quorum sensing inhibitors. *Biofouling* 27:893–905.

Dworjanyn, S. A., de Nys, R., and Steinberg, P. D. 1999. Localisation and surface quantification of secondary metabolites in the red alga Delisea pulchra. *Mar. Biol.* 133:727–736.

Dworjanyn, S. A., de Nys, R., and Steinberg, P. D. 2006. Chemically mediated antifouling in the red alga Delisea pulchra. *Mar. Ecol. Prog. Ser.* 318:153–163.

Edwards, M. S. and Estes, J. A. 2006. Catastrophe, recovery and range limitation in NE Pacific kelp forests: a large-scale perspective. *Mar. Ecol. Prog. Ser.* 320:79–87.

Ellison, A. M., Bank, M. S., Clinton, B. D., Colburn, E. A., Elliott, K., Ford, C. R., Foster, D. R., Kloeppel, B. D., Knoepp, J. D., Lovett, G. M., Mohan, J., Orwig, D. A., Rodenhouse, N. L., Sobczak, W. V., Stinson, K. A., Stone, J. K., Swan, C. M., Thompson, J., von Holle, B., and Webster, J. R. 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. *Front. Ecol. Environ.* 3:479–486.

Engel, S., Jensen, P. R., and Fenical, W. 2002. Chemical ecology of marine microbial defense. *J. Chem. Ecol.* 28:1971–1985.

Fernandes, N. 2011. Molecular studies on the role of bacteria in a marine algal disease. PhD dissertation. Sydney: University of New South Wales.

Fernandes, N., Case, R. J., Longford, S. R., Seyedsayamdost, M. R., Steinberg, P. D., Kjelleberg, S., and Thomas, T. 2011. Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga delisea pulchra. *PLoS One* 6:e27387.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. 1994. Quorum sensing in bacteria—the LuxR-LuxI family of cell density-responsive trabnscriptional regulators. *J. Bacteriol.* 176:269–275.

Gachon, C. M. M., Sime-Ngando, T., Strittmatter, M., Chambouvet, A., and Kim, G. H. 2010. Algal diseases: spotlight on a black box. *Trends Plant Sci.* 15:633–640.

Gao, M. S., Teplitski, M., Robinson, J. B., and Bauer, W. D. 2003. Production of substances by Medicago truncatula that affect bacterial quorum sensing. *Mol. Plant Microbe Interact.* 16:827–834.

GIOVANNONI, S. and RAPPE, M. 2000. Evolution, diversity, and molecular ecology of marine prokaryotes, *in* D. Kirchman (ed.), Microbial Ecology of the Oceans. Wiley-Liss, New York.

GIVSKOV, M., DE NYS, R., MANEFIELD, M., GRAM, L., MAXIMILIEN, R., EBERL, L., MOLIN, S., STEINBERG, P. D., and KJELLEBERG, S. 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. *J. Bacteriol.* 178:6618–6622.

GLYNN, P. W. 1996. Coral reef bleaching: Facts, hypotheses and implications. *Glob. Chang. Biol.* 2:495–509.

GOECKE, F., LABES, A., WIESE, J., and IMHOFF, J. F. 2010. Chemical interactions between marine macroalgae and bacteria. *Mar. Ecol. Prog. Ser.* 409:267–299.

HARDER, T. 2008. Marine epibiosis: concepts, ecological consequences and host defence, pp. 219–232, *in* H. Flemming, R. Venkatesan, and S. Murthy (eds.), Marine and Industrial Biofouling. Springer, Heidelberg, New York.

HARVELL, C. D. and HEWSON, I. 2010. Climate change and invertebrate microbial interactions. *Integr. Comp. Biol.* 50:E69.

HARVELL, D., ALTIZER, S., CATTADORI, I. M., HARRINGTON, L., and WEIL, E. 2009. Climate change and wildlife diseases: When does the host matter the most? *Ecology* 90:912–920.

HAY, M. E. 2009. Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annu. Rev. Mar. Sci.* 1:193–212.

HUGHES, D. T. and SPERANDIO, V. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* 6:111–120.

IPCC. 2007. Climate change 2007: the physical science basis, *in* S. Solomon, D. Qin, M. Manning (eds.), Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel Onclimate Change. Cambridge.

JOINT, I., TAIT, K., and WHEELER, G. 2007. Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed Ulva. *Philos. Trans. R. Soc. B* 362:1223–1233.

KONKEL, M. E. and TILLY, K. 2000. Temperature-regulated expression of bacterial virulence genes. *Microbes Infect.* 2:157–166.

KUSHMARO, A., LOYA, Y., FINE, M., and ROSENBERG, E. 1996. Bacterial infection and coral bleaching. *Nature* 380:396.

LACHNIT, T., WAHL, M., and HARDER, T. 2010. Isolated thallus-associated compounds from the macroalga Fucus vesiculosus mediate bacterial surface colonization in the field similar to that on the natural alga. *Biofouling* 26:247–255.

LACHNIT, T., MESKE, D., WAHL, M., HARDER, T., and SCHMITZ, R. 2011. Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. *Environ. Microbiol.* 13:655–665.

LAFFERTY, K. D., PORTER, J. W., and FORD, S. E. 2004. Are diseases increasing in the ocean? *Annu. Rev. Ecol. Syst.* 35:31–54.

LANE, A. L., NYADONG, L., GALHENA, A. S., SHEARER, T. L., STOUT, E. P., PARRY, R. M., KWASNIK, M., WANG, M. D., HAY, M. E., FERNANDEZ, F. M., AND KUBANEK, J. 2009. Desorption electrospray ionization mass spectrometry reveals surface-mediated antifungal chemical defense of a tropical seaweed. *Proc. Natl. Acad. Sci. U.S.A.* 106:7314–7319.

LITTLER, M. M. and LITTLER, D. S. 1995. Impact of CLOD pathogen on Pacific coral reefs. *Science* 267:1356–1360.

LONGFORD, S. R., TUJULA, N. A., CROCETTI, G. R., HOLMES, A. J., HOLMSTROM, C., KJELLEBERG, S., STEINBERG, P. D., and TAYLOR, M. W. 2007. Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes. *Aquat. Microb. Ecol.* 48:217–229.

MANEFIELD, M., DE NYS, R., KUMAR, N., READ, R., GIVSKOV, M., STEINBERG, P., and KJELLEBERG, S. A. 1999. Evidence that halogenated furanones from Delisea pulchra inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiol-UK* 145:283–291.

MANEFIELD, M., HARRIS, L., RICE, S. A., DE NYS, R., and KJELLEBERG, S. 2000. Inhibition of luminescence and virulence in the black tiger prawn (Penaeus monodon) pathogen Vibrio harveyi by intercellular signal antagonists. *Appl. Environ. Microbiol.* 66:2079–2084.

MATSUO, Y., IMAGAWA, H., NISHIZAWA, M., and SHIZURI, Y. 2005. Isolation of an algal morphogenesis inducer from a marine bacterium. *Science* 307:1598.

MAXIMILIEN, R., DE NYS, R., HOLMSTROM, C., GRAM, L., GIVSKOV, M., CRASS, K., KJELLEBERG, S., and STEINBERG, P. D. 1998. Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga Delisea pulchra. *Aquat. Microb. Ecol.* 15:233–246.

MILLER, M. B. and BASSLER, B. L. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55:165–199.

PETTUS, J. A., WING, R. M., and SIMS, J. J. 1977. Marine natural products.12. Isolation of a family of multi-halogenated gamma-methylene lactones from red seaweed Delisea fimbriata. *Tetrahedron Let.* :41–44.

PANTOS, O. and BYTHELL, J. C. 2006. Bacterial community structure associated with white band disease in the elkhorn coral Acropora palmata determined using culture-independent 16S rRNA techniques. *Dis. Aquat. Org.* 69:79–88.

PAUL, V. J., ARTHUR, K. E., RITSON-WILLIAMS, R., ROSS, C., and SHARP, K. 2007. Chemical defenses: From compounds to communities. *Biol. Bull.* 213:226–251.

PAUL, V. J., RITSON-WILLIAMS, R., and SHARP, K. 2011. Marine chemical ecology in benthic environments. *Nat. Prod. Rep.* 28:345–387.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911–1922.

PERSSON, F., SVENSSON, R., NYLUND, G. M., FREDRIKSSON, N. J., PAVIA, H., and HERMANSSON, M. 2011. Ecological role of a seaweed secondary metabolite for a colonizing bacterial community. *Biofouling* 27:579–588.

PHELAN, V., LIU, W., POGLIANO, K., and DORRESTEIN, P. 2012. Microbial metabolic exchange—the chemotype-to-phenotype link. *Nat. Chem. Biol.* 8:26–35.

RICE, S. A., MCDOUGALD, D., GIVSKOV, M., and KJELLEBERG, S. 2007. Detection and inhibition of bacterial cell–cell communication, pp. 55–68, *in* F. DeLeo and M. Otto (eds.), Bacterial Pathogenesis Methods and Protocols. Springer, New Jersey.

ROHWER, F., SEGURITAN, V., AZAM, F., and KNOWLTON, N. 2002. Diversity and distribution of coral-associated bacteria. *Mar. Ecol. Prog. Ser.* 243:1–10.

ROSENBERG, E. and ZILBER-ROSENBERG, I. 2011. Symbiosis and development: the hologenome concept. *Birth Defects Res. Part C-Embryo Today-Rev.* 93:56–66.

RUMBAUGH, K. 2004. The language of bacteria … and just about everything else. *Scientist* 18:26–27.

SHORT, F. T. and NECKLES, H. A. 1999. The effects of global climate change on seagrasses. *Aquat. Bot.* 63:169–196.

SHORT, F. T., MUEHLSTEIN, L. K., and PORTER, D. 1987. Eelgrass wasting disease—cause and recurrence of a marine epidemic. *Biol. Bull.* 173:557–562.

SIMON, C. and DANIEL, R. 2011. Metagenomic analyses: past and future trends. *Appl. Environ. Microbiol.* 77:1153–1161.

SKINDERSOE, M. E., ETTINGER-EPSTEIN, P., RASMUSSEN, T. B., BJARNSHOLT, T., DE NYS, R., and GIVSKOV, M. 2008. Quorum sensing antagonism from marine organisms. *Mar. Biotechnol.* 10:56–63.

STACHOWICZ, J. J. 2001. Mutualisms, positive interactions, and the structure of ecological communities. *BioScience* 51:235–246.

STEINBERG, P. D. and VAN ALTENA, I. 1992. Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temparate Australasia. *Ecol. Monogr.* 62:189–222.

STENECK, R. S., GRAHAM, M. H., BOURQUE, B. J., CORBETT, D., ERLANDSON, J. M., ESTES, J. A., AND TEGNER, M. J. 2002. Kelp forest ecosystems: biodiversity, stability, resilience and future. *Environ. Conserv.* 29:436–459.

TEPLITSKI, M., ROBINSON, J. B., and BAUER, W. D. 2000. Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol. Plant Microbe Interact.* 13:637–648.

TEPLITSKI, M., CHEN, H. C., RAJAMANI, S., GAO, M. S., MERIGHI, M., SAYRE, R. T., ROBINSON, J. B., ROLFE, B. G., and BAUER, W. D. 2004. Chlamydomonas reinhardtii secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol.* 134:137–146.

THIBAUT, T., PINEDO, S., TORRAS, X., and BALLESTEROS, E. 2005. Long-term decline of the populations of Fucales (*Cystoseira* spp. and *Sargassum* spp.) in the Alberes coast (France, North-western Mediterranean). *Mar. Pollut. Bull.* 50:1472–1489.

THOMAS, T., RUSCH, D., DEMAERE, M. Z., YUNG, P. Y., LEWIS, M., HALPERN, A., HEIDELBERG, K. B., EGAN, S., STEINBERG, P. D., and KJELLEBERG, S. 2010. Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *ISME J.* 4:1557–1567.

VAIRAPPAN, C. S., SUZUKI, M., MOTOMURA, T., and ICHIMURA, T. 2001. Pathogenic bacteria associated with lesions and thallus bleaching symptoms in the Japanese kelp Laminaria religiosa Miyabe (Laminariales, Phaeophyceae). *Hydrobiologia* 445:183–191.

VAN OPPEN, M. J. H., LEONG, J. A., and GATES, R. D. 2009. Coral-virus interactions: A double-edged sword? *Symbiosis* 47:1–8.

VARGAS-ANGEL, B. 2010. Crustose coralline algal diseases in the US-Affiliated Pacific Islands. *CORAL REEFS* 29:943–956.

VON BODMAN, S. B., BAUER, W. D., and COPLIN, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* 41:455–482.

WEBSTER, N. S., COBB, R. E., and NEGRI, A. P. 2008. Temperature thresholds for bacterial symbiosis with a sponge. *ISME J.* 2:830–842.

WEBSTER, N. S., TAYLOR, M. W., BEHNAM, F., LUCKER, S., RATTEI, T., WHALAN, S., HORN, M., and WAGNER, M. 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ. Microbiol.* 12:2070–2082.

WEBSTER, N. S., SOO, R., COBB, R., and NEGRI, A. P. 2011. Elevated seawater temperature causes a microbial shift on crustose coralline algae with implications for the recruitment of coral larvae. *ISME J.* 5:759–770.

WILLIAMS, W. M., VINER, A. B., and BROUGHTON, W. J. 1987. Nitrogen-fixation (acetylene reduction) associated with the living coral Acropora variabilis. *Mar. Biol.* 94:531–535.

WILLIAMSON, J. E., CARSON, D. G., DE NYS, R., AND STEINBERG, P. D. 2004. Demographic consequences of an ontogenetic shift by a sea urchin in response to host plant chemistry. *Ecology* 85:1355–1371.

WRIGHT, J. T., DE NYS, R., POORE, A. G. B., AND STEINBERG, P. D. 2004. Chemical defense in a marine alga: Heritability and the potential for selection by herbivores. *Ecology* 85:2946–2959.

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Trade-Offs in Defensive Metabolite Production But Not Ecological Function in Healthy and Diseased Sponges

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Abstract Diseases of marine organisms, and sponges in particular, are increasingly reported worldwide. Prior research indicates that the survival of sponges on reefs is due largely to their production of biologically active secondary metabolites that provide protection from a diversity of stressors. *Aplysina* Red Band Syndrome (ARBS) is an emerging disease affecting Caribbean rope sponges (*Aplysina* spp.), but it is not known whether secondary metabolites play a role in disease susceptibility and resistance. To investigate whether differences in secondary metabolites may explain variability in susceptibility to ARBS in *Aplysina cauliformis*, we used high performance liquid chromatography (HPLC) to generate chemical profiles from healthy tissue in both healthy and diseased sponges, and quantified peak areas for 15 metabolites. Analyses of healthy and diseased sponges revealed qualitative and quantitative differences in their chemical profiles. Aplysamine-1 and fistularin-3 were produced in significantly higher concentrations by healthy sponges, whereas aerothionin and 11-oxoaerothionin were found only in diseased sponges. At natural concentrations, extracts from both healthy and diseased sponges deterred feeding by an omnivorous reef fish. Fistularin-3 deterred feeding at concentrations found in healthy sponges, but not at concentrations found in diseased sponges. Aerothionin deterred feeding at concentrations found in diseased sponges, and may at least partially replace the loss of fistularin-3 as a feeding deterrent compound following pathogenesis, suggesting a trade-off in the production of feeding deterrent compounds. Extracts from healthy and diseased sponges inhibited bacterial growth, and both aplysamine-1 and fistularin-3 displayed selective antibacterial activity. Despite differences in secondary metabolite production between healthy and diseased sponges, the stress associated with ARBS does not appear to compromise the ability of *A. cauliformis* to maintain defenses against some of its natural enemies.

Keywords Sponge disease · Chemical defense · Antimicrobial · Inducible defense · *Aplysina cauliformis*

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Introduction

Emerging diseases of marine invertebrates are among the major causes of the accelerating destruction of Caribbean coral reef systems (Hughes, 1994; Aronson and Precht, 2001; Porter et al., 2001; Miller et al., 2009). Diseases affecting natural populations threaten biodiversity, resilience, and the ecological balance of communities, as well as the ecological services they provide (Knowlton, 2001). To date, most disease reports have focused on scleractinian corals, however, reports of sponge diseases also have increased across the globe (Webster, 2007). In only a few cases have the etiologic agents of sponge diseases been identified. For example, tissue necrosis in *Geodia papyracea*

from Belize resulted from the sponge's own symbiotic cyanobacteria multiplying faster than the host could control their numbers under thermal stress (Rützler, 1988). A sponginboring alpha-proteobacterium was identified as the etiologic agent of disease in *Rhopaloeides odorabile* from the Great Barrier Reef through the fulfillment of Koch's postulates (Webster et al., 2002). Various other bacteria (Vacelet et al., 1994; Cervino et al., 2006), fungi (Galstoff et al., 1939), and cyanobacteria (Olson et al., 2006) also have been postulated as sponge pathogens.

Aplysina Red Band Syndrome (ARBS) affects Caribbean branching rope sponges of the genus *Aplysina*. First reported affecting *Aplysina cauliformis* in the Bahamas in 2004 (Olson et al., 2006), ARBS has since been observed at sites throughout the wider Caribbean basin (Gochfeld et al., 2007; Wulff, 2007). Visible signs of ARBS include rust-colored leading edges, with a following area of necrotic tissue. The lesion eventually forms a continuous band around the sponge branch and then progresses along the length of the branch. Recovery of lesions rarely has been observed, although lesion growth may stop and the red band may disappear. ARBS reduces sponge growth and weakens the sponge skeleton, which often breaks at the site of the lesion (Olson et al., 2006; Gochfeld et al., 2012).

Allocation of limited resources to multiple physiological processes is one of the basic tenets of optimal defense theory (Rhoades, 1979; Zangerl and Bazzaz, 1992). The underlying assumption is that there are energetic costs associated with producing or maintaining defenses that compete with metabolic requirements for growth or reproduction, and therefore, defenses should be allocated preferentially towards tissues that are more likely to be consumed and/or represent the greatest fitness potential. Under stressful conditions (e.g., pathogenesis), the costs of defense may be magnified, and the ability to produce defenses may be compromised. Costs of defense may be direct (e.g., the biosynthetic cost of producing a particular defensive metabolite), or indirect (e.g., reduced investment in growth), and are notoriously difficult to measure (Zangerl and Bazzaz, 1992). Inducible defenses are theorized to provide a cost-saving alternative to the constant (constitutive) production of defensive metabolites, being produced only when the risk of attack (i.e., by predators, pathogens, fouling organisms, or competitors) is high (Karban and Myers, 1989; Tallamy and Raupp, 1991; Karban and Baldwin, 1997; Tollrian and Harvell, 1999; Gochfeld, 2004).

Like plants, sessile soft-bodied marine organisms rely heavily on chemical defenses to provide protection from predators, competitors, pathogens, and fouling organisms (Hay, 1996; McClintock and Baker, 2001; Paul et al., 2007), and such compounds are necessary for the survival of these species. Sponges, in particular, produce a diversity of secondary metabolites that play important roles in their survival (Paul et al., 2006; Pawlik, 2011). Sponges of the genus *Aplysina* produce a large number of bromotyrosine-derived metabolites that serve multiple ecological functions. In *A. cauliformis*, secondary metabolites exhibit feeding deterrence (Pawlik et al., 1995; Waddell and Pawlik, 2000), antimicrobial activity (Kelly et al., 2005), and have allelopathic effects on corals and other sponges (Engel and Pawlik, 2000; Pawlik et al., 2007). In plants, exposure to pathogens can induce the production of secondary metabolites, which may provide protection from the pathogens themselves, and from other natural enemies (Fritz and Simms, 1992; Tollrian and Harvell, 1999; Restif and Koella, 2004). In the marine environment, few studies have examined chemical responses to pathogenesis; however, there is evidence from hard corals (Gochfeld et al., 2006; Gochfeld and Aeby, 2008; Mydlarz et al., 2009), octocorals (Slattery, 1999; Kim et al., 2000; Dube et al., 2002), and sponges (Webster et al., 2008; Angermeier et al., 2011) that secondary chemistry varies with colony condition. Intraspecific variability in disease susceptibility also has been observed, with diseased colonies found immediately adjacent to unaffected conspecifics (Gochfeld et al., 2006), thus suggesting variability in resistance to infection. Differences in levels of defenses within and between genotypes or phenotypes may help explain these local patterns of disease occurrence on reefs.

ARBS currently affects approximately 10% of *A. cauliformis* on reefs near Lee Stocking Island, Exuma Cays, Bahamas. To assess whether this patchy distribution is due to differential resistance by individual sponges, we tested the hypothesis that healthy and diseased *A. cauliformis* produce different types or concentrations of secondary metabolites. Such differences could result either from pathogen-induced defenses in the diseased sponges or because healthy sponges have differential chemical resistance to pathogenesis. We also investigated whether differences in chemical profiles alter the susceptibility of diseased sponges to predators or pathogens. Since induced defenses are most likely to occur adjacent to the region of damage, and intra-colony variation in chemical defenses is known to occur in sponges (Schupp et al., 1999), we also predicted that there would be a gradient of response in chemical defenses to ARBS infection with distance along the affected sponge branch.

Methods and Materials

Sample Collection Healthy and ARBS-affected (diseased) *A. cauliformis* were collected using SCUBA from a depth of 5 m at North Norman's Reef (N 23° 47.388, W 76° 08.273) near Lee Stocking Island (LSI), Exuma Cays, Bahamas. Sponges were collected into individual resealable plastic bags underwater and immediately frozen upon return to the lab at the Caribbean Marine Research Center/Perry

Institute for Marine Science. Frozen sponges were transported to the National Center for Natural Products Research (NCNPR) at the University of Mississippi. To obtain volumetric concentrations, displacement volume was measured for each sponge piece prior to lyophilization. Following extraction (see below), the crude extract was dried, weighed, and a volumetric concentration (g extract/ml sponge) was calculated. Overall extract yields (g extract/g sponge dry weight) for organic and aqueous extracts from healthy and diseased sponges were compared using unpaired *t* tests.

Chemical Profiles Differences in chemical profiles between healthy and diseased *A. cauliformis* ($N=10$ of each) were examined by comparing chemical fingerprints generated by high performance liquid chromatography (HPLC). For the diseased sponges, only tissue that appeared healthy was used. Frozen sponges (10–15 cm in length) were freeze-dried, and extracted using an Accelerated Solvent Extractor (Dionex) with 1:1 methanol:methylene chloride (MeOH:CH_2Cl_2), followed by 80% MeOH:water, to produce organic and aqueous extracts, respectively. Aqueous extracts were used only in feeding assays, as described below. The organic extracts were concentrated under vacuum, redissolved to a concentration of 5 mg/ml, and filtered through a 0.45 μm PolyTetraFluoriEthylene (PTFE) filter. Chemical fingerprints of the organic extracts were generated by injecting 10 μl of the filtered solution onto an analytical HPLC system (Alliance 2695, Waters, Milford, MA, USA) coupled to a Waters 2998 Photodiode Array detector. Analysis was achieved on a Phenomenex 5 μ C18 250×4.6 mm column using a gradient system consisting of 0.05% trifluoroacetic acid (TFA) in water and 0.05% TFA in acetonitrile. Chromatograms were extracted at 254 nm from the data collected across the 210–400 nm wavelength range. Areas under the curve for each of 15 peaks were integrated and compared using multivariate analysis of variance (MANOVA), followed by unpaired t tests for each peak to assess differences between sponge conditions (healthy vs. diseased). Overall chemical constituent profiles in healthy and diseased sponges were compared using analysis of similarity (ANOSIM).

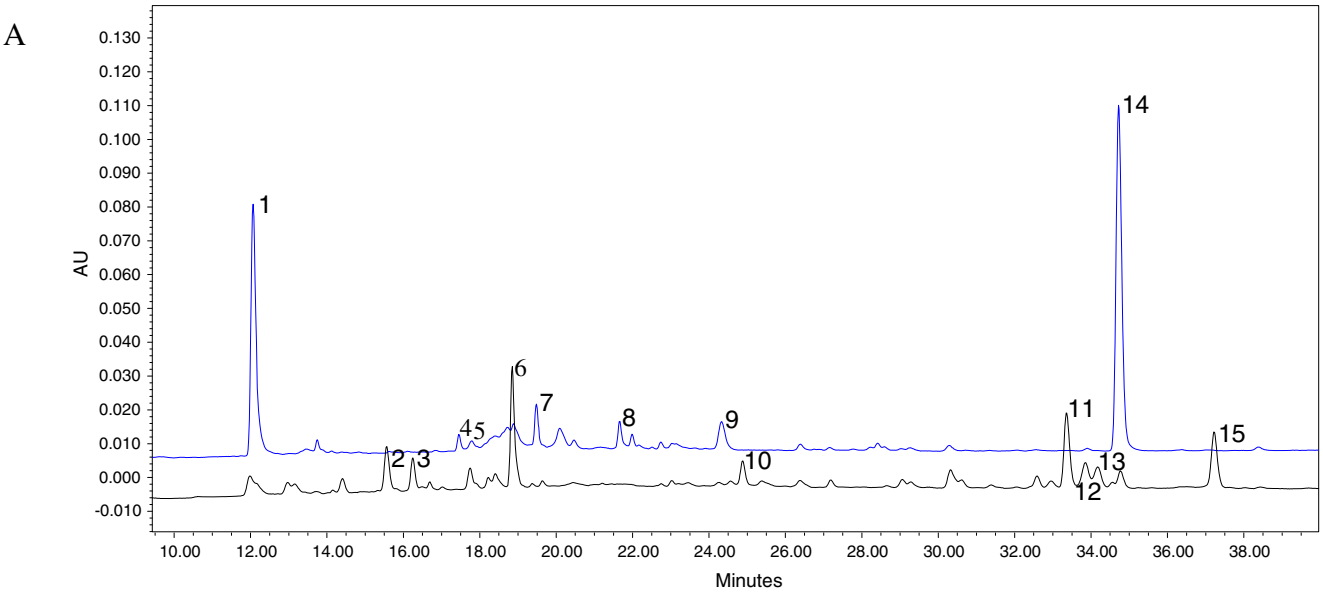
To determine whether chemical defenses are induced in tissues adjacent to the ARBS lesion or systemically, frozen diseased sponges ($N=10$) were cut into 1 cm sections, starting immediately adjacent to the active band, and progressing away from the lesion. Organic extracts from the 1st, 2nd, 4th, and 8th cm of healthy tissue in either direction from the lesion were chemically fingerprinted using the HPLC technique described above. For comparison, a similar analysis was performed on healthy sponges ($N=10$), starting at the tip of the sponge. In healthy sponges, we quantified the amount of each peak in the 1st, 5th, 10th, and 14th cm sections from the tip. Areas under the curve for each of 15 peaks were integrated, and one-way analyses of variance (ANOVA) were used to assess differences in the concentration of each peak with distance along the length of the sponge.

Isolation and Quantification of Pure Compounds The secondary metabolites in *Aplysina* spp. sponges are well characterized (Ciminiello et al., 1999; Putz et al., 2009; Sacristán-Soriano et al., 2011a). Thus, most of the peaks that we quantified are probably known compounds. We isolated and identified four of the largest and most well-separated peaks for use as marker compounds. From healthy sponges, peaks 1 and 14 were identified as aplysamine-1 and fistularin-3, respectively (Fig. 1). We also isolated two compounds found only in the diseased sponges, 11-oxoaerothionin (peak 11) and aerothionin (peak 12; Fig. 1).

For isolation of aplysamine-1 and fistularin-3, a bulk sample of healthy sponges was lyophilized and extracted 3× with 1:1 MeOH:CH_2Cl_2. The combined extracts were concentrated under vacuum leaving a gummy organic residue. The extract was fractionated with reversed-phase vacuum liquid chromatography using 0.05% TFA in water and 0.05% TFA in MeOH. Fraction III was subjected to reversed-phase HPLC (RP-HPLC) to yield aplysamine-1. Fraction V was subjected to RP-HPLC to yield fistularin-3. For isolation of aerothionin and 11-oxoaerothionin, extract from a pooled sample of healthy portions from diseased sponges was injected directly into RP-HPLC. Compound identification was verified by comparison of NMR and mass spectrometry data with published values (aplysamine-1: Xynas and Capon, 1989; fistularin-3: Gopichand and Schmitz, 1979; Rogers et al., 2005; aerothionin: Fattorusso et al., 1970; 11-oxoaerothionin: Acosta and Rodriguez, 1992). Chemical structures of these compounds are shown in Fig. 1.

For each of the pure compounds, we developed a standard curve to calculate actual concentrations in the healthy and diseased sponge extracts and in specific sections along the length of healthy and diseased sponges. Overall concentrations of the pure compounds in healthy and diseased sponges were compared using unpaired *t* tests. Concentrations of the pure compounds along the length of the sponge were analyzed using one-way ANOVAs.

Feeding Assays To determine whether pathogenesis changes the efficacy of chemical defenses, we compared the effects of natural concentrations of extracts from healthy and ARBS-affected sponges in feeding assays using the omnivorous pufferfish *Canthigaster rostrata*. Other species of *Canthigaster* have been used as model predators to test feeding deterrence in Pacific benthic organisms (Pennings et al., 1994; Slattery et al., 2008; Rohde and Schupp, 2011), and *C. rostrata* exhibits similar behavioral patterns and feeding ecology (Gochfeld, unpubl. data). Individual pufferfish were maintained in separate 9.5 L flow-through tanks and fed



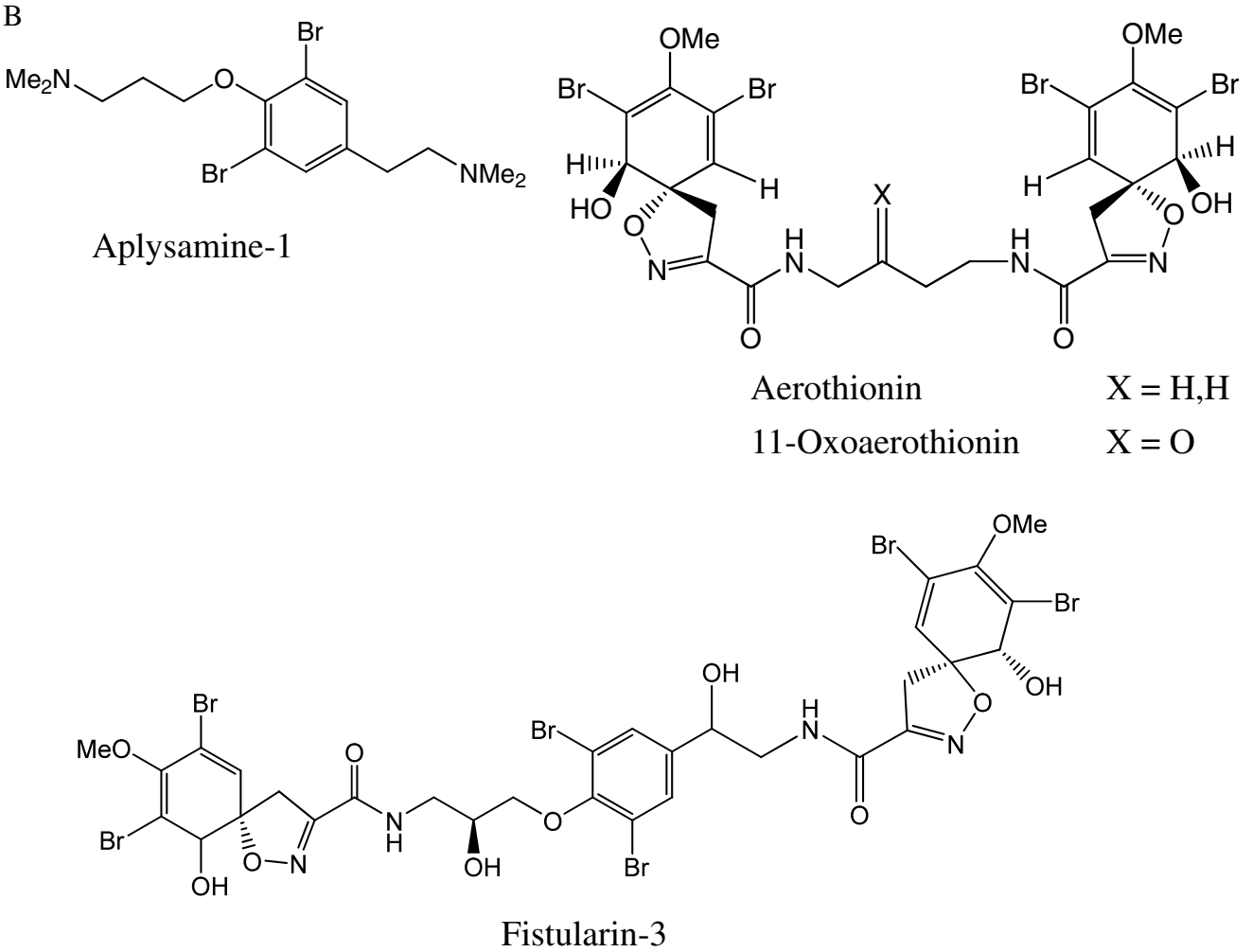


Fig. 1 **a** Representative HPLC chromatogram of healthy (upper line) and diseased (lower line) *Aplysina cauliformis* extracts at 254 nm. **b** Pure compounds isolated from extracts of *A. cauliformis*

a maintenance diet of squid paste for several days prior to the feeding assays. Squid paste consisted of a puree of 1:1 by volume of cleaned, skinned squid mantle and distilled water, to which 2% sodium alginate was added (Pawlik et al., 1995). Food pellets were produced by extruding the squid paste through a 100 µl Wiretrol pipette into a 0.25 M calcium chloride solution, where it hardened into a long strand. The strand then was removed and cut into small pellets. Crude organic and aqueous extracts from healthy and diseased sponges (N=5–7) were added to 300 µl of squid paste at natural volumetric concentrations. The mixture was stirred vigorously to homogenize and then pipetted into calcium chloride. After a few minutes, the food was removed and chopped into small pellets. Control pellets lacking extracts were prepared similarly, however, food coloring was added to match the color of extract-treated pellets. Although we recognize that target predators may be exposed to a subset of compounds within a crude extract, the use of crude extracts as a starting point to characterize active secondary metabolites is a standard method in chemical ecology (e.g., Pawlik, 1993, 2011; Hay, 1996). Each fish was fed 5–10 squid paste pellets prior to the assay to ensure that consumption of the treated pellets was representative of taste rather than hunger. A control and treated pellet then were offered sequentially to each *C. rostrata*. Pellets were either consumed (i.e., the pellet was swallowed) or rejected (i.e., the pellet was ingested and then spit out). Following rejection of the treated pellet, a second control pellet was offered to confirm that fish had not ceased feeding. A rejection was scored when both control pellets were eaten and the treated pellet was not; if the second control pellet was rejected, the fish was excluded from the assay. Trials were continued with additional fish to ensure that ten fish were used in each assay. McNemar's tests were used to test whether the palatability of treated pellets was significantly reduced compared to control pellets (Becerro et al., 2006).

To determine whether there were differences in feeding deterrence along the length of the sponges, both organic and aqueous crude extracts from tips vs. 11-cm away from the tips in healthy sponges, and from 1-cm and 5-cm away from lesions in diseased sponges, were added to squid-paste pellets at natural volumetric concentrations, and feeding assays were performed as described above. McNemar's tests were used to determine if the pure compounds were significantly deterrent relative to control pellets, and paired t tests were used to compare the relative palatability of extracts from different distances along the sponge for each extract type.

Feeding assays with the isolated pure compounds were conducted in the same way. Using the mean healthy and diseased concentrations of each compound calculated from the HPLC analysis, both volumetric concentrations of each compound were tested in the feeding assays. McNemar's tests were performed to determine the palatability of each compound relative to the control food.

Antimicrobial Assays In addition to predator deterrence, many secondary metabolites have antimicrobial activities, and in sponges, this is probably the major source of pathogen immunity (McClintock and Gauthier, 1992; Newbold et al., 1999). Disk diffusion assays were used to test the presence of antibacterial activity in the organic extracts of healthy and ARBS-affected sponges, and pure compounds of *A. cauliformis*, against a panel of 8 bacteria. Since the etiologic agent for ARBS has not yet been elucidated, we used known coral pathogens (*Aurantimonas coralicida, Serratia marcescens, Vibrio corallyticus*, *V. shiloi*), human enteric bacteria that have the potential to enter near-shore waters and can survive in the marine environment (*Yersinia enterocolitica*), and bacteria related to those that have been isolated from the surfaces of corals (*Klebsiella pneumoniae, Pseudomonas nautica, V. agarivorans*) (Gochfeld and Aeby, 2008). All organisms were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ, Braunschweig, Germany). Extracts of healthy and diseased sponges (N=4 of each) were added to 4-mm diameter paper disks at natural volumetric concentrations. Aplysamine-1 and fistularin-3 were added to disks at mean natural healthy concentrations. A 24-h culture of the test bacterium was plated onto marine agar (*A. coralicida*, *P. nautica, V. agarivorans,V. corallyticus, V. shiloi*), nutrient agar (*K. pneumoniae*), or trypticase soy agar (*S. marcescens*, *Y. enterocolitica*), and disks impregnated with the organic extracts and pure compounds were placed on the plate. A positive control (Ciprofloxacin; 1 mg ml^{-1}) was included in each assay. All assays were performed in triplicate. Following a 24-h incubation period, the zones of inhibition surrounding each disk were measured. Since extracts should diffuse through the same media at similar rates, we compared the zones of inhibition for organic extracts against the five bacterial strains grown on marine agar (*A. coralicida*, *P. nautica*, *V. agarivorans, V. corallyticus, V. shiloi*) using a two-way ANOVA, with sponge condition and bacterial strain as the factors. Although it is not possible to compare the activity of aplysamine-1 and fistularin-3 directly due to potential differences in diffusion rates through the media, the activity of each compound against the five strains grown on marine agar was compared using a one-way ANOVA.

Results

Overall yields of both aqueous and organic crude extracts were similar in healthy and diseased *A. cauliformis*. Aqueous extract yields were 0.148±0.018 g g^{-1} sponge dry

weight for healthy sponges, and 0.145±0.016 g g^{-1} sponge dry weight for diseased sponges (unpaired *t* test, P=0.90). Organic extract yields were 0.228±0.066 g g^{-1} sponge dry weight for healthy sponges, and 0.225±0.0053 g g^{-1} sponge dry weight for diseased sponges (unpaired *t* test, P=0.73).

Chemical fingerprints differed significantly between healthy and diseased sponges, with qualitative differences easily observed when comparing HPLC chromatograms (Fig. 1a), and striking quantitative differences shown by ANOSIM (R=0.989, P=0.001), non-metric multidimensional scaling (Fig. 2), and MANOVA (P<0.001). When measuring relative peak areas at 254 nm, 7 out of 15 compounds occurred in higher concentrations in the healthy sponges (unpaired *t* tests, P<0.05), and 6 occurred in higher concentrations in diseased sponges (unpaired *t* tests, P< 0.05; Fig. 3). Only peaks 3 and 6 occurred in similar concentrations in healthy and diseased sponges (unpaired *t* tests, P=0.16 and 0.051, respectively). Some compounds were present only in either healthy (e.g., peaks 8, 9) or diseased (e.g., peaks 10, 11, 12, 13, 15) sponges, indicating not only quantitative but also qualitative differences in chemical constituents. In healthy sponges, peaks 2, 3, 4, and 14 (fistularin-3) showed a significant effect of distance from the tip of the sponge (one-way ANOVAs, P<0.003), and in all of these cases, the tip had the highest concentration (Fig. 4a). In diseased sponges, there was no significant effect of distance from the lesion on concentrations for any of the peaks (one-way ANOVAs, P>0.05; Fig. 4b).

Overall, aplysamine-1 and fistularin-3 occurred in significantly higher concentrations in healthy sponges (healthy: 22± 3 μg mg^{-1} organic extract, diseased: 0.92±0.11 μg mg^{-1} organic extract for aplysamine-1; healthy: 124±14.6 μg mg^{-1} organic extract, diseased: 3.4±0.28 μg mg^{-1} organic extract for fistularin-3; unpaired *t* tests, P<0.001 for both compounds), although they did occur in diseased sponges as well.

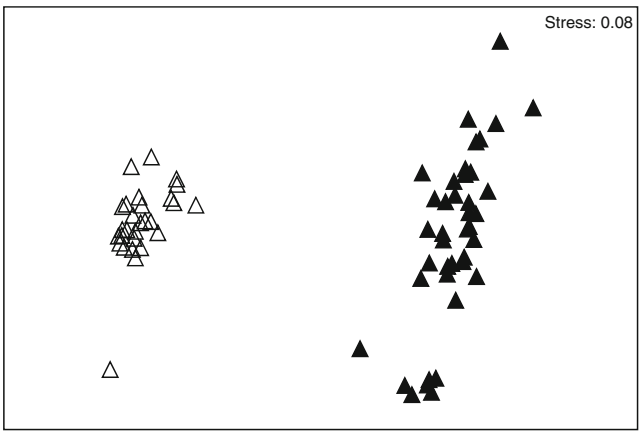


Fig. 2 Non-metric multi-dimensional scaling plot showing differences in chemical diversity in healthy (*open triangles*) and diseased (*filled triangles*) *Aplysina cauliformis*

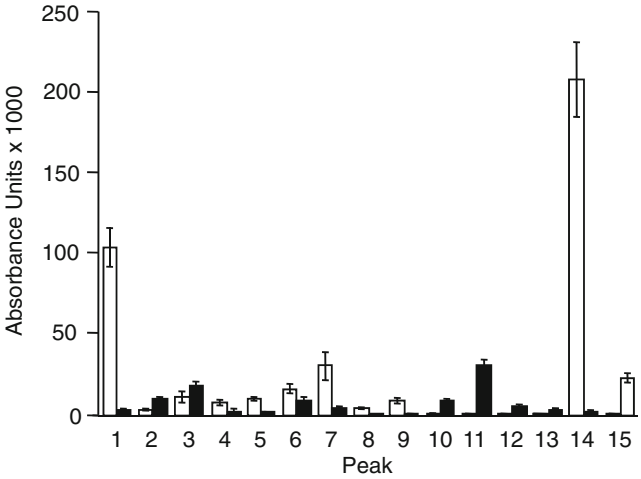


Fig. 3 Relative concentrations (mean±1 SE absorbance units) of 15 peaks from HPLC chromatograms of healthy (*white*) and diseased (*black*) *Aplysina cauliformis*. Significantly different concentrations of all peaks except peaks 3 and 6 were observed between healthy and diseased sponges (unpaired *t* tests, P<0.05)

In contrast, aerothionin (healthy: 0±0 μg mg^{-1} organic extract, diseased: 18.6±1.82 μg mg^{-1} organic extract; unpaired *t* test, P<0.001) and 11-oxoaerothionin (healthy: 0±0 μg mg^{-1} organic extract, diseased: 76±19.2 μg mg^{-1} organic extract; unpaired *t* test, P=0.003) were not found in the healthy sponges. Aplysamine-1 did not vary along the length of the sponge for either healthy (P=0.22) or diseased (P=0.58) sponges (Fig. 5a). The concentration of fistularin-3 declined with increasing distance from the tip of healthy sponges (one-way ANOVA, P=0.015; Fig. 5b), but did not vary with distance from the lesion in diseased sponges (one-way ANOVA, P=0.88). Aerothionin and 11-oxoaerothionin did not vary with distance from the lesion in diseased sponges (P=0.95 and 0.81, respectively; Fig. 5c, d).

Organic and aqueous extracts from both healthy and diseased sponges were deterrent to *C. rostrata* (McNemar's test, P<0.05; Fig. 6a). Healthy and diseased sponges did not differ in levels of feeding deterrence for either aqueous (unpaired *t* test, P=0.96) or organic (P=0.15) extracts. In healthy sponges, organic extracts from the tip were more deterrent than 11 cm from the tip (paired *t* test, P=0.008), although this was not the case for aqueous extracts (paired *t* test, P>0.05). There was no difference in feeding deterrence with distance from the lesion in diseased sponges (paired *t* tests, P>0.05).

When tested at mean concentrations found in the healthy and diseased sponges, aplysamine-1 was not feeding deterrent at either of the concentrations tested (McNemar's test, P>0.05; Fig. 6b), even though the healthy concentration was 25-fold higher than the diseased concentration. Fistularin-3 was feeding deterrent at the concentration found in the healthy sponges (McNemar's test, P=0.002), but not

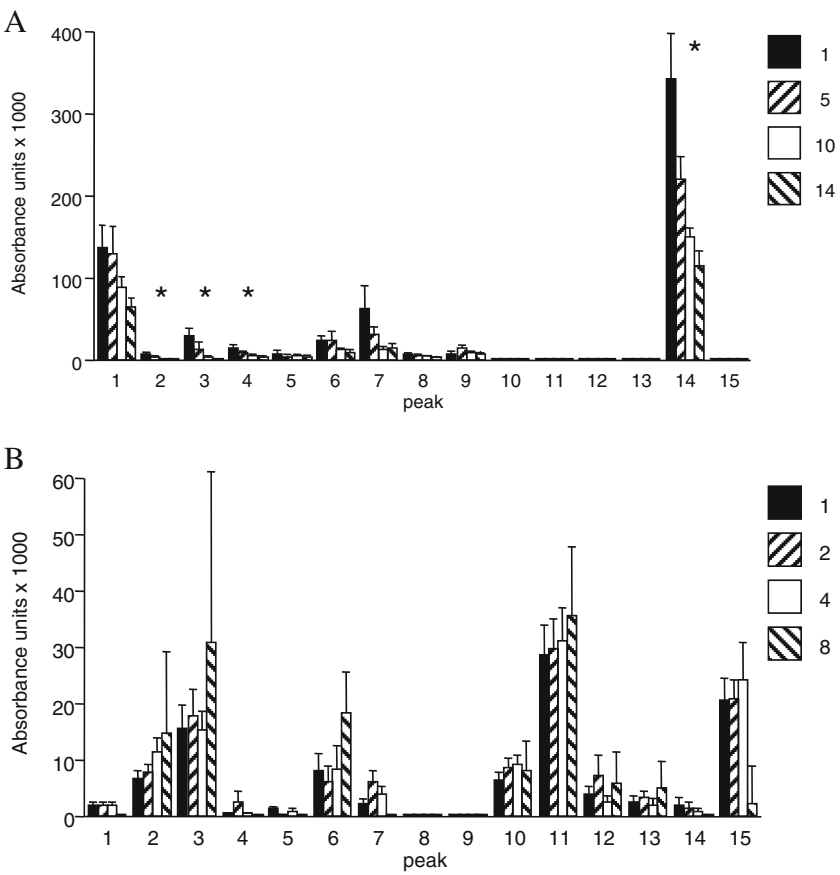


Fig. 4 Relative concentrations (mean±1 SE absorbance units) of 15 peaks from HPLC chromatograms of *Aplysina cauliformis* organic extracts with distance (cm) from **a**. the tip in healthy sponges, or **b**. the lesion in diseased sponges. *Asterisks* indicate peaks that exhibit significant variability with distance along the branch (one-way ANOVA, $P<0.05$)

at the much lower concentration found in the diseased sponges (McNemar's test, $P=0.19$; Fig. 6b). Aerothionin and 11-oxoaerothionin were not found in the healthy sponges, so those pellets were effectively the same as the controls, but aerothionin was feeding deterrent at the concentration found in the diseased sponges (McNemar's test, $P<0.001$; Fig. 6b).

In disk diffusion assays, the positive control ciprofloxacin produced large (>4.1 mm) zones of inhibition against all of the bacteria tested. Ciprofloxacin is omitted from Fig. 7 to enhance visibility of the extracts' activity. Natural volumetric concentrations of organic extracts from both healthy and diseased *A. cauliformis* produced zones of inhibition against the eight environmental bacteria tested (Fig. 7). Zones of inhibition against the five bacteria grown on marine agar did not vary significantly for organic extracts based upon either sponge condition (two-way ANOVA, $P=0.82$) or bacterial strain (two-way ANOVA, $P=0.18$). Aplysamine-1 and fistularin-3 exhibited selectivity in their activity at mean natural concentrations found in the healthy sponges (Table 1). Both compounds inhibited the growth of *V. corallyticus* more than the other bacteria; this trend was not statistically significant for aplysamine-1 (one-way ANOVA, $P=0.072$), but was a significant difference for fistularin-3 (one-way ANOVA, $P<0.001$).

Discussion

Variability in Chemical Profiles with Sponge Health *Aplysina cauliformis* infected by ARBS exhibit both qualitative and quantitative differences in the production of secondary metabolites, relative to healthy sponges. These differences may be due to: (1) induced responses to invasion by pathogen(s) and/or opportunistic symbionts; (2) changes in the sponge-associated microbial community; or (3) trade-offs in defense production as a result of stress. The higher concentrations of certain compounds in healthy sponges suggest that some of these compounds may be involved in antimicrobial defense of these sponges. The presence of unique compounds in the diseased sponges suggests a possible inducible response to pathogenesis. Alternatively there may be genotypic differences in chemical profiles and, therefore, pathogen resistance among individual sponges.

The diverse secondary metabolites found in *Aplysina* spp. are hypothesized to be produced by the sponge, although the process may involve some collaboration from microbial constituents (Sacristán-Soriano et al., 2011a). Variability in both secondary metabolite production and the composition of *Aplysina*-associated bacterial communities has been observed across a variety of spatial and temporal scales (Friedrich et al., 2001; Gerçe et al., 2009; Sacristán-Soriano et al., 2011a, b).

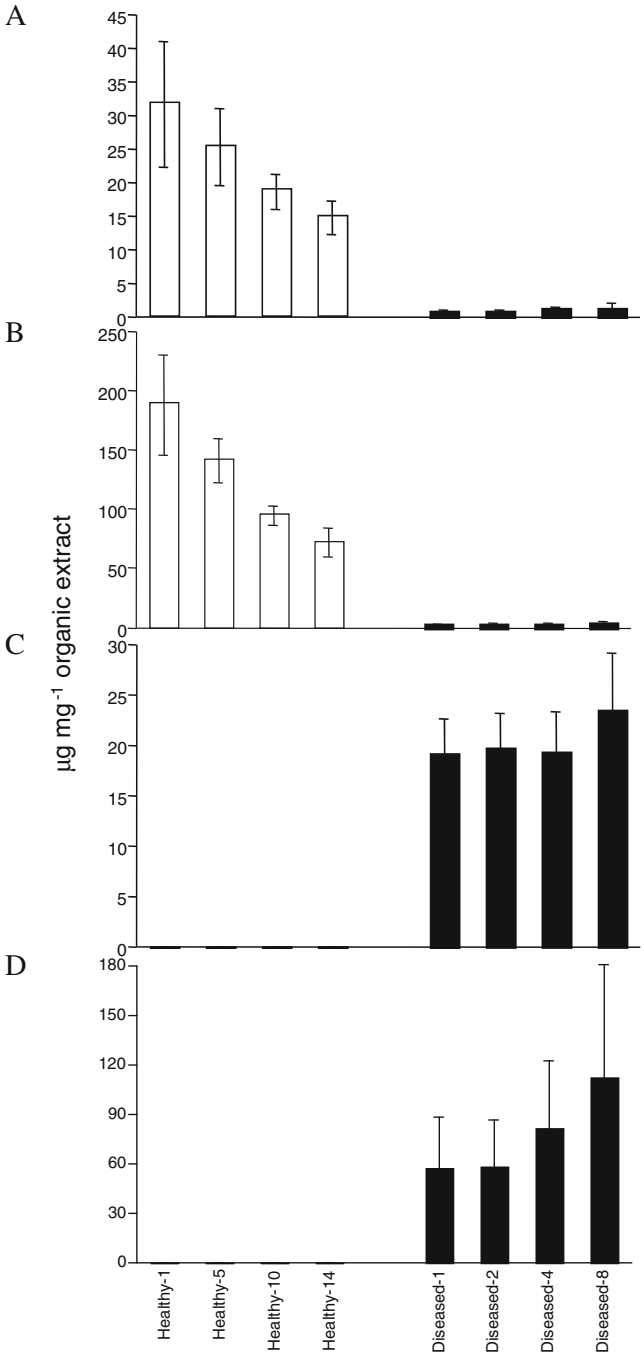


Fig. 5 Concentrations (µg mg^{-1} organic extract) of pure compounds isolated from *Aplysina cauliformis* with distance from the tip in healthy sponges (*white*) or the lesion in diseased sponges (*black*). **a** aplysamine-1, **b** fistularin-3, **c** aerothionin, **d** 11-oxoaerothionin

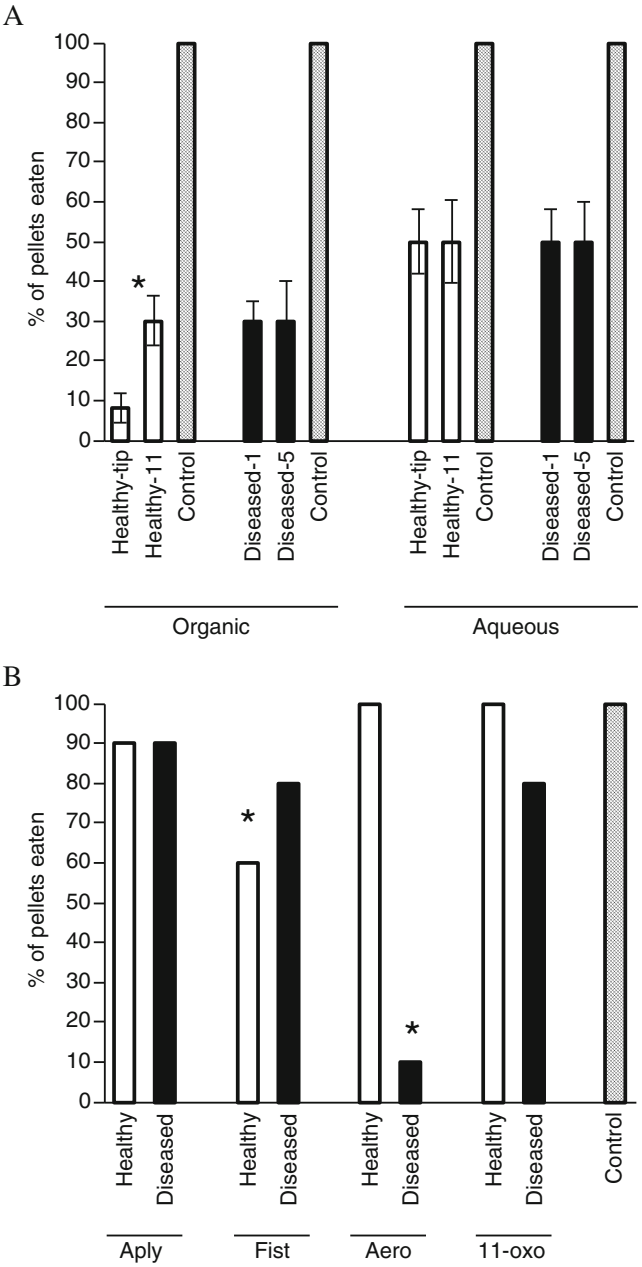


Fig. 6 Percent of pellets consumed in feeding assays against the omnivorous pufferfish *Canthigaster rostrata*. **a** Mean (± 1 SE) percent of pellets consumed containing aqueous or organic crude extracts from healthy and diseased *Aplysina cauliformis*. All extracts were significantly deterrent when compared to controls (McNemar's test, $P<0.05$). *Asterisk* indicates significant difference between distances along the sponge (paired *t* test, $P=0.008$). **b** Percent of pellets consumed containing mean healthy or diseased concentrations of pure compounds. Only one control bar is shown for simplicity. *Asterisks* indicate compounds that were significantly deterrent when compared to controls (McNemar's test, $P<0.05$). *Aply* aplysamine-1, *Fist* fistularin-3, *Aero* aerothionin, *11-oxo* 11-oxoaerothionin

Differences in chemical profiles and microbial communities between healthy and diseased individuals have been demonstrated previously for *Aplysina aerophoba* (Webster et al., 2008). In contrast, apparently healthy tissue adjacent to lesions in *Xestospongia muta* affected by Sponge Orange Band (SOB) disease showed quantitative but not qualitative changes in secondary metabolites (Angermeier et al., 2011). However, to date, a cause-and-effect relationship has not been demonstrated between SOB pathogenesis and secondary metabolite production.

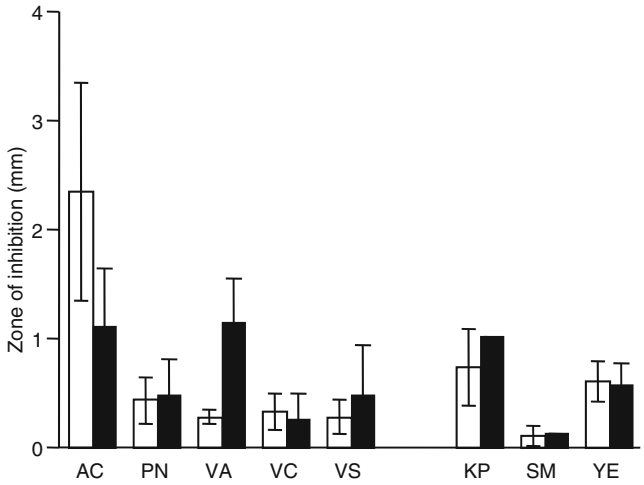


Fig. 7 Zones of inhibition (mean±1 SE mm) surrounding disks containing organic extracts from healthy (*white*) and diseased (*black*) *Aplysina cauliformis* in disk diffusion assays against eight environmental bacteria. *AC Aurantimonas coralicida*, *PN Pseudomonas nautica*, *VA Vibrio agarivorans*, *VC V. corallyticus*, *VS V. shiloi*, *KP Klebsiella pneumoniae*, *SM Serratia marcescens*, *YE Yersinia enterocolitica*

The presence of activated defenses in response to tissue wounding in *Aplysina* spp. has been reported (Teeyapant and Proksch, 1993; Ebel et al., 1997; Thoms et al., 2006, although see Puyana et al., 2003 for a dissenting view). This conversion is due likely to the breakdown of cellular compartmentalization as a result of tissue maceration (Teeyapant and Proksch, 1993; Thoms et al., 2006). Wounding of *A. aerophoba* catalyzes the production of aeroplysinin-1 and dienone to concentrations known to be bioactive (Weiss et al., 1996; Ebel et al., 1997), and it has been suggested that this response might protect injured cells that have an increased probability of penetration by pathogens (Thoms et al., 2006). However, this process is unlikely to provide any beneficial defensive advantage to the sponges since extensive tissue damage is required for this activation process to occur. Nonetheless, we standardized our extraction techniques and minimized damage to fresh tissue so that any potential chemical changes due to handling should be similar in all samples. Thus, differences in chemical profiles and biological activity observed in this study are due to inherent differences in the sponges themselves, rather than to sampling artifacts.

Optimal defense theory predicts that there should be a trade-off between defense production and other processes, including wound healing (Zangerl and Bazzaz, 1992). ARBS infection results in localized tissue necrosis that can spread along the branch of the sponge, causing partial or complete mortality. Stress to the sponge may result from direct effects of ARBS or other opportunistic pathogen(s) on cellular function, or from the loss of biomass available for filter feeding and photosynthesis. *Aplysina cauliformis* contains high numbers of photosymbiotic cyanobacteria that contribute to host sponge nutrition (Freeman and Thacker, 2011). Resource allocation trade-offs have been demonstrated for chemical defense in sponges with respect to wound healing (Walters and Pawlik, 2005), growth (Becerro et al., 1997; Leong and Pawlik, 2010), recruitment (Pawlik et al., 2008), and reproduction (Becerro et al., 1997). Thus, in addition to defense against its natural suite of enemies, a diseased sponge must expend energy fighting off the pathogen(s), as well as healing the wound. In diseased sponges that we have monitored over time, we have seen lesions in which the active bands have disappeared and the lesion remains, but does not expand (Gochfeld, unpubl. data). In these cases, it is possible that the ARBS pathogen(s) senesced, the sponge was successful in its defense against the pathogen(s), or another environmental factor limited the infection. On rare occasions, we have also seen lesions heal completely (Gochfeld, unpubl. data), indicating that *A. cauliformis* has the potential to mount an effective defense against ARBS.

Intracolony Variation in Secondary Metabolites Variation in secondary metabolite concentration occurs within *A. cauliformis*, particularly in the tips of healthy sponges. *Aplysina cauliformis* does not contain spicules and derives its structure from a fibrous skeleton along with sponge and microbial cells. The growing tips of healthy *A. cauliformis* have fewer structural fibers (Thacker, pers. obs.) and are softer than the rest of the sponge, so it is possible that the sponge invests in greater chemical defense at the tip to make up for the lack of structural defense, although the utility of structural components of sponges as defenses has been questioned (Chanas and Pawlik, 1996). Additionally, the tips of *A. cauliformis* contain a greater proportion of ectosome relative to choanosome due to their tapered morphology,

Table 1 Zones of inhibition (mean±1 SE mm) surrounding disks containing mean natural concentrations of aplysamine-1 and fistularin-3 found in healthy *Aplysina cauliformis* when tested against environmental bacteria in disk diffusion assays

	Aurantimonas coralicida	*Pseudomonas nautica*	*Vibrio agarivorans*	*V. corallyticus*	*V. shiloi*	*Serratia marcescens*	*Yersinia enterocolitica*
Aplysamine-1	0.61±0.61	0±0	0±0	1.03±0.006	0±0	0.16±0.029	0±0
Fistularin-3	0±0	0±0	0.013±0.013	1.26±0.19	0±0	0.27±0.27	0.095±0.068

and other studies have demonstrated differences in chemical constituents between these tissue types in other *Aplysina* spp. (Freeman and Gleason, 2010; Sacristán-Soriano et al., 2011a). Webster et al. (2008) reported changes in the production of certain secondary metabolites in *A. aerophoba* tissue adjacent to lesions caused by another disease, although other compounds only differed in the diseased tissue itself. In ARBS-affected *A. cauliformis*, there was not a simple gradient in concentration of compounds with distance from the lesion. Instead, all of the samples from diseased sponges produced similar chemical profiles, indicating that this may represent an example of systemic induced immunity. *Aplysina cauliformis* individuals can become very large, with complex branching structures over 1 m in length. Thus, it is possible that the scale of 8 cm in either direction from the lesion may be insufficient to see a localized induced response.

Ecological Consequences of Chemical Variation: Feeding Deterrence Ecological activity of the extracts from healthy and diseased sponges was similar, suggesting that while there may be different compounds involved, the net effect on ecological interactions remains the same. *Aplysina cauliformis* extracts were previously found to be feeding deterrent to the bluehead wrasse *Thalassoma bifasciatum* (Pawlik et al., 1995), and to the hermit crab *Paguristes puntaceps* (Waddell and Pawlik, 2000). The present study confirms feeding deterrence of *A. cauliformis* extracts to the omnivorous pufferfish *C. rostrata*, which includes sponges as a measurable portion of its diet (Gochfeld, unpubl. data). Extracts from both healthy and diseased sponges were feeding deterrent, although the compounds responsible for this activity varied with sponge condition. While other compounds also may play a role in feeding deterrence, fistularin-3 was deterrent to pufferfish on its own, at concentrations present in healthy *A. cauliformis*. Fistularin-3 was not deterrent at concentrations found in diseased sponges. Aerothionin, previously found to be feeding deterrent to a Mediterranean fish (Thoms et al., 2004), was found only in the diseased sponges, where it also exhibits feeding deterrence against *C. rostrata*. Both aqueous and organic extracts of *A. cauliformis* were feeding deterrent to *C. rostrata*, confirming the presence of multiple defensive compounds, but it does appear that the loss of at least one feeding deterrent compound is compensated for by the production of another, even under disease stress. This emphasizes the potential importance of predation as a selective pressure on sponge survival, and suggests that there may be little cost to the production of these defenses.

Ecological Consequences of Chemical Variation: Antimicrobial Activity Both healthy and diseased sponges have similar levels of antibacterial activity against the environmental bacteria that we tested, although there were minor differences in their activity against certain pathogens. This indicates that although diseased sponges are clearly susceptible to the ARBS and possibly associated opportunistic pathogens, they are not entirely immunocompromised. These sponges produce antibiotic compounds against a diversity of bacteria, but that activity is selective in nature. Newbold et al. (1999) also found that extracts from Caribbean sponges, including *Aplysina lacunosa*, exhibited selective antibacterial activity against the environmental strains that they tested, whereas Betancourt-Lozano et al. (1998) identified broad-spectrum and low temporal variability in antibacterial activity of *Aplysina fistularis* extracts. Comparable results have been characterized from other sessile marine invertebrates exposed to infectious disease. Healthy and aspergillosis-affected individuals of the sea fan *Gorgonia ventalina* did not differ in their levels of antifungal activity (Dube et al., 2002), although healthy and diseased tissue from the coral *Montipora capitata* exhibited variability in antibacterial activity towards *A. coralicida* and *Y. enterocolitica* (Gochfeld and Aeby, 2008), two pathogens tested in this study. The activity observed in the present study does not necessarily translate to the etiologic agent(s) or opportunistic pathogens associated with ARBS specifically, although the absence of disease in the healthy sponges suggests the potential inhibitory activity of one or more compounds found in healthy *A. cauliformis*. Aplysamine-1 and fistularin-3 exhibited selective antibacterial activity towards the strains tested at natural concentrations found in healthy sponges. Fistularin-3 has previously shown antiviral activity (Gunasekera and Cross, 1992), as well as antibacterial activity against *Mycobacterium tuberculosis* (De Oliveira et al., 2006), and clearly has multiple ecological functions as well. These compounds are at least partially responsible for the antibacterial activity observed. Unfortunately, we obtained limited amounts of the compounds from the diseased sponges and were, therefore, unable to test them in assays. The compounds aerothionin and homoaerothionin in *A. fistularis* are exuded into the water column, and are believed to play a role in inhibiting surface fouling (Walker et al., 1985). The ability of antimicrobial compounds to be released into the water column in the vicinity of the sponge surface, or at least to be bioavailable on the sponge surface likely is crucial to their efficacy in preventing pathogenesis.

Aplysina cauliformis exhibits phenotypic, and possibly genotypic, variability in the production of secondary metabolites with health status. In spite of this variability, the ecological function of chemical constituents in diseased *A. cauliformis* is maintained. These changes in chemical profiles, but not ecological function, suggest that infection by ARBS impacts the biosynthetic pathways associated with secondary metabolite production, but indicates that *A. cauliformis* produces a diverse arsenal of weapons to protect it from a variety of natural enemies.

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References

ACOSTA, A. L. and RODRIGUEZ, A. D. 1992. 11-Oxoaerothionin: a cytotoxic antitumor bromotyrosine-derived alkaloid from the Caribbean marine sponge *Aplysina lacunosa*. *J. Nat. Prod.* 55:1007–1012.

ANGERMEIER, H., KAMKE, K., ABDELMOHSEN, U. R., KROHNE, G., PAWLIK, J. R., LINDQUIST, N. L., and HENTSCHEL, U. 2011. The pathology of sponge orange band disease affecting the Caribbean barrel sponge *Xestospongia muta*. *FEMS Microbiol. Ecol.* 75:218–230.

ARONSON, R. B. and PRECHT, W. F. 2001. White-band disease and the changing face of Caribbean coral reefs. *Hydrobiol.* 460:25–38.

BECERRO, M. A., URIZ, M. J., and TURON, X. 1997. Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). *Hydrobiol.* 356:77–89.

BECERRO, M. A., STARMER, J. A., and PAUL, V. J. 2006. Chemical defenses of cryptic and aposematic gastropterid mollusks feeding on their host sponge *Dysidea granulosa*. *J. Chem. Ecol.* 32:1494–1500.

BETANCOURT-LOZANO, M., GONZALÉZ-FARIAS, F., GONZALÉZ-ACOSTA, B., GARCÍA-GASCA, A., and BASTIDA-ZAVALA, J. R. 1998. Variation of antimicrobial activity of the sponge *Aplysina fistularis* (Pallas, 1766) and its relation to associated fauna. *J. Exp. Mar. Biol. Ecol.* 223:1–18.

CERVINO, J. M., WINIARSKI-CERVINO, K., POLSON, S. W., GOREAR, T., and SMITH, G. W. 2006. Identification of bacteria associated with a disease affecting the marine sponge *Ianthella basta* in New Britain, Papua New Guinea. *Mar. Ecol. Prog. Ser.* 324:139–150.

CHANAS, B. and PAWLIK, J. R. 1996. Does the skeleton of a sponge provide a defense against predatory reef fish? *Oecologia* 107:225–231.

CIMINIELLO, P., DELL'AVERSANO, C., FATTORUSSO, E., MAGNO, C., and PANSINI, M. 1999. Chemistry of Verongida sponges. 9. Secondary metabolite composition of the Caribbean sponge *Aplysina cauliformis*. *J. Nat. Prod.* 62:590–593.

DE OLIVEIRA, M. F., DE OLIVEIRA, J. H. H. L., GALETTI, F. C. S., DE SOUZA, A. O., SILVA, C. L., HAJDU, E., PEIXINHO, S., and BERLINCK, R. G. S. 2006. Antimycobacterial brominated metabolites from two species of marine sponges. *Planta Medica* 72:437–441.

DUBE, D., KIM, K., ALKER, A. P., and HARVELL, C. D. 2002. Size structure and geographic variation in chemical resistance of sea fans *Gorgonia ventalina* to a fungal pathogen. *Mar. Ecol. Prog. Ser.* 231:139–150.

EBEL, R., BRENZINGER, M., KINZE, A., GROSS, H. J., and PROKSCH, P. 1997. Wound activation of protoxins in marine sponge *Aplysina aerophoba*. *J. Chem. Ecol.* 23:1451–1462.

ENGEL, S. and PAWLIK, J. R. 2000. Allelopathic activities of sponge extracts. *Mar. Ecol. Prog. Ser.* 207:273–281.

FATTORUSSO, E., MINALE, L., SODANO, G., MOODY, K., and THOMSON, R. H. 1970. Aerothionin, a tetrabromo-compound from *Aplysina aerophoba* and *Verongia thiona*. *J. Chem. Soc. D: Chem. Comm.* 1970:752–753.

FREEMAN, C. J. and GLEASON, D. F. 2010. Chemical defenses, nutritional quality, and structural components in three sponge species: *Ircinia felix*, *I. campana* and *Aplysina fulva*. *Mar. Biol.* 157:1083–1093.

FREEMAN, C. J. and THACKER, R. W. 2011. Complex interactions between marine sponges and their symbiotic microbial communities. *Limnol. Oceanogr.* 56:1577–1586.

FRIEDRICH, A. B., FISCHER, I., PROKSCH, P., HACKER, J., and HENTSCHEL, U. 2001. Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol. Ecol.* 38:105–113.

FRITZ, R. S. and SIMMS, E. L. 1992. Plant Resistance to Herbivores and Pathogens. University of Chicago Press, Chicago.

GALSTOFF, P. S., BROWN, H. H., SMITH, C. L., and SMITH, F. G. W. 1939. Sponge mortality in the Bahamas. *Nature* 143:807–808.

GERÇE, B., SCHWARTZ, T., VOIGT, M., RUHLE, S., KIRCHEN, S., PUTZ, A., PROKSCH, P., OBST, U., SYLDATK, C., and HAUSMANN, R. 2009. Morphological, bacterial, and secondary metabolite changes of *Aplysina aerophoba* upon long-term maintenance under artificial conditions. *Microb. Ecol.* 58:865–878.

GOCHFELD, D. J. 2004. Predation-induced morphological and behavioral defenses in a hard coral: implications for foraging behavior of coral-feeding butterflyfishes. *Mar. Ecol. Prog. Ser.* 267:145–158.

GOCHFELD, D. J. and AEBY, G. S. 2008. Antibacterial chemical defenses in Hawaiian corals provide possible protection from disease. *Mar. Ecol. Prog. Ser.* 362:119–128.

GOCHFELD, D. J., OLSON, J. B., and SLATTERY, M. 2006. Colony versus population variation in susceptibility and resistance to dark spot syndrome in the Caribbean coral *Siderastrea siderea*. *Dis. Aquat. Org.* 69:53–65.

GOCHFELD, D. J., SCHLÖDER, C., and THACKER, R. W. 2007. Sponge community structure and disease prevalence on coral reefs in Bocas del Toro, Panama, pp. 335–343, *in* M. R. Custódio, G. Lôbo-Hajdu, E. Hajdu, and G. Muricy (eds.), Porifera Research: Biodiversity, Innovation, and Sustainability. Série Livros 28. Museu Nacional, Rio de Janeiro.

GOCHFELD, D. J., EASSON, C. G., FREEMAN, C. J., THACKER, R. W., and OLSON, J. B. 2012. Disease and nutrient enrichment as potential stressors on the Caribbean sponge *Aplysina cauliformis* and its bacterial symbionts. *Mar. Ecol. Prog. Ser.* doi:10.3354/meps09716

GOPICHAND, Y. and SCHMITZ, F. J. 1979. Marine natural products: fistularin-1, -2 and -3 from the sponge *Aplysina fistularis* forma *fulva*. *Tetrahedron Lett.* 20:3921–3924.

GUNASEKERA, S. P. and CROSS, S. S. 1992. Fistularin 3 and 11-ketofistularin 3. Feline leukemia virus active bromotyrosine metabolites from the marine sponge *Aplysina archeri*. *J. Nat. Prod.* 55:509–512.

HAY, M. E. 1996. Marine chemical ecology: what's known and what's next? *J. Exp. Mar. Biol. Ecol.* 200:103–134.

HUGHES, T. P. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. *Science* 265:1547–1551.

KARBAN, R. and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago, Chicago.

KARBAN, R. and MYERS, J. H. 1989. Induced plant responses to herbivory. *Am. Nat.* 20:331–348.

KELLY, S. R., GARO, E., JENSEN, P. R., FENICAL, W., and PAWLIK, J. R. 2005. Effects of Caribbean sponge secondary metabolites on bacterial surface colonization. *Aquat. Microb. Ecol.* 40:191–203.

KIM, K., HARVELL, C. D., KIM, P. D., SMITH, G. W., and MERKEL, S. M. 2000. Fungal disease resistance of Caribbean sea fan corals (*Gorgonia* spp.). *Mar. Biol.* 136:259–267.

KNOWLTON, N. 2001. The future of coral reefs. *Proc. Natl. Acad. Sci. USA* 98:5419–5425.

LEONG, W. and PAWLIK, J. 2010. Evidence of a resource trade-off between growth and chemical defenses among Caribbean coral reef sponges. *Mar. Ecol. Prog. Ser.* 406:71–78.

MCCLINTOCK, J. B. and GAUTHIER, J. J. 1992. Antimicrobial activities of Antarctic sponges. *Antarctic Sci.* 4:179–183.

MCCLINTOCK, J. B. and BAKER, B. J. 2001. Marine Chemical Ecology. CRC Press, Boca Raton.

MILLER, J., MULLER, E., ROGERS, C., WAARA, R., ATKINSON, A., WHELAN, K. R. T., PATTERSON, M., and WITCHER, B. 2009. Coral disease following massive bleaching in 2005 causes 60% decline in coral cover on reefs in the US Virgin Islands. *Coral Reefs* 28:925–937.

MYDLARZ, L. D., COUCH, C. S., WEIL, E., SMITH, G., and HARVELL, C. D. 2009. Immune defenses of healthy, bleached and diseased Montastraea faveolata during a natural bleaching event. *Dis. Aquat. Org*. 87:67–78.

NEWBOLD, R. W., JENSEN, P. R., FENICAL, W., and PAWLIK, J. R. 1999. Antimicrobial activity of Caribbean sponge extracts. *Aquat. Microb. Ecol.* 19:279–284.

OLSON, J. B., GOCHFELD, D. J., and SLATTERY, M. 2006. *Aplysina* red band syndrome: a new threat to Caribbean sponges. *Dis. Aquat. Org.* 71:163–168.

PAUL, V. J., PUGLISI, M. P., and RITSON-WILLIAMS, R. 2006. Marine chemical ecology. *Nat. Prod. Rep.* 23:153–180.

PAUL, V. J., ARTHUR, K. E., RITSON-WILLIAMS, R., ROSS, C., and SHARP, K. 2007. Chemical defenses: from compounds to communities. *Biol. Bull.* 213:226–251.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911–1922.

PAWLIK, J. R. 2011. The chemical ecology of sponges on Caribbean reefs: Natural products shape natural systems. *BioSci.* 61:888–898.

PAWLIK, J. R., CHANAS, B., TOONEN, R. J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish. I. Chemical Deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

PAWLIK, J. R., STEINDLER, L., HENKEL, T. P., and BEER, S. 2007. Chemical warfare on coral reefs: Sponge metabolites differentially affect coral symbiosis *in situ*. *Limnol. Oceanogr.* 52:907–911.

PAWLIK, J. R., HENKEL, T. P., MCMURRAY, S. E., LOPEZ-LEGENTIL, S., LOH, T.-L., and ROHDE, S. 2008. Patterns of sponge recruitment and growth on a shipwreck corroborate chemical defense resource trade-off. *Mar. Ecol. Prog. Ser.* 368:137–143.

PENNINGS, S. C., PABLO, S. R., PAUL, V. J., and DUFFY, J. E. 1994. Effects of sponge secondary metabolites in different diets on feeding by three groups of consumers. *J. Exp. Mar. Biol. Ecol.* 180:137–149.

PORTER, J. W., DUSTAN, P., JAAP, W. C., PATTERSON, K. L., KOSMYNIN, V., MEIER, O. W., PATTERSON, M. E., and PARSONS, M. 2001. Patterns of spread of coral disease in the Florida Keys. *Hydrobiol.* 460:1–24.

PUTZ, A., KLOEPPEL, A., PFANNKUCHEN, M., BRÜMMER, F., and PROKSCH, P. 2009. Depth-related alkaloid variation in Mediterranean *Aplysina* sponges. *Z. Naturforsch* 64c:279–287.

PUYANA, M., FENICAL, W., and PAWLIK, J. R. 2003. Are there activated chemical defenses in sponges of the genus *Aplysina* from the Caribbean? *Mar. Ecol. Prog. Ser.* 246:127–135.

RESTIF, O. and KOELLA, J. C. 2004. Concurrent evolution of resistance and tolerance to pathogens. *Am. Nat.* 164:E90–E102.

RHOADES, D. F. 1979. Evolution of plant chemical defense against herbivores, pp. 3–54, *in* G. A. Rosenthal and D. H. Janzen (eds.), Herbivores: Their Interaction with Secondary Plant Metabolites. Acad. Press, New York.

ROGERS, E. W., DE OLIVEIRA, M. F., BERLINCK, R. G. S., KONIG, G. M., and MOLINSKI, T. F. 2005. Sterochemical heterogeneity in Verongid sponge metabolites. Absolute stereochemistry of (+)-fistularin-3 and (+)-11-epi-fistularin-3 by microscale LCMS-Marfey's analysis. *J. Nat. Prod.* 68:891–896.

ROHDE, S. and SCHUPP, P. J. 2011. Allocation of chemical and structural defenses in the sponge *Melophlus saranisorum*. *J. Exp. Mar. Biol. Ecol.* 399:76–83.

RÜTZLER, K. 1988. Mangrove sponge disease induced by cyanobacterial symbionts: failure of a primitive immune system? *Dis. Aquat. Org.* 5:143–149.

SACRISTÁN-SORIANO, O., BANAIGS, B., CASAMAYOR, E. O., and BECERRO, M. A. 2011a. Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*. *Appl. Env. Microbiol.* 77:862–870.

SACRISTÁN-SORIANO, O., BANAIGS, B., and BECERRO, M. A. 2011b. Relevant scales of chemical variation in *Aplysina aerophoba*. *Mar. Drugs* 9:2499–2513.

SCHUPP, P., EDER, C., PAUL, V., and PROKSCH, P. 1999. Distribution of secondary metabolites in the sponge *Oceanapia* sp. and its ecological implications. *Mar. Biol.* 135:573–580.

SLATTERY, M. 1999. Fungal pathogenesis of the sea fan *Gorgonia ventalina*: Direct and indirect consequences. *Chemoecol.* 9:97–104.

SLATTERY, M., KAMEL, H. N., ANKISETTY, S., GOCHFELD, D. J., HOOVER, C. A., and THACKER, R. W. 2008. Hybrid vigor in a tropical Pacific soft-coral community. *Ecol. Monog.* 78:423–443.

TALLAMY, D. W. and RAUPP, M. J. 1991. Phytochemical Induction by Herbivores. John Wiley and Sons, New York.

TEEYAPANT, R. and PROKSCH, P. 1993. Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba*—evidence for an induced chemical defense? *Naturwissenscheften* 80:369–370.

THOMS, C., WOLFF, M., PADMAKUMAR, K., EBEL, R., and PROCKSCH, P. 2004. Chemical defense of Mediterranean sponges *Aplysina cavernicola* and *Aplysina aerophoba*. *Z. Naturforsch* 59c:113–122.

THOMS, C., EBEL, R., and PROKSCH, P. 2006. Activated chemical defense in *Aplysina* sponges revisited. *J. Chem. Ecol.* 32:97–123.

TOLLRIAN, R. and HARVELL, C. D. 1999. The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton.

VACELET, J., VACELET, E., GAINO, E., and GALLISSIAN, M.-F. 1994. Bacterial attack of spongin skeleton during the 1986–1990 Mediterranean sponge disease, pp. 355–362, *in* R. W. G. van Soest, T. M. G. van Kempen, and J. C. Braekman (eds.), Sponges in Time and Space. Balkema, Rotterdam.

WADDELL, B. and PAWLIK, J. R. 2000. Defenses of Caribbean sponges against invertebrate predators. II. Assays with hermit crabs. *Mar. Ecol. Prog. Ser.* 195:125–132.

WALKER, R. P., THOMPSON, J. E., and FAULKNER, D. J. 1985. Exudation of biologically-active metabolites in the sponge *Aplysina fistularis*. II. Chemical evidence. *Mar. Biol.* 88:27–32.

WALTERS, K. D. and PAWLIK, J. R. 2005. Is there a trade-off between wound-healing and chemical defenses among Caribbean reef sponges? *Integr. Comp. Biol.* 45:352–358.

WEBSTER, N. S. 2007. Sponge disease: a global threat? *Env. Microbiol.* 9:1363–1375.

WEBSTER, N. S., NEGRI, A. P., WEBB, R. I., and HILL, R. T. 2002. A spongin-boring α-proteobacterium is the etiological agent of disease in the Great Barrier Reef sponge *Rhopaloeides odorabile*. *Mar. Ecol. Prog. Ser.* 232:305–309.

WEBSTER, N. S., XAVIER, J. R., FRECKELTON, M., MOTTI, C. A., and COBB, R. 2008. Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. *Env. Microbiol.* 10:3366–3376.

WEISS, B., EBEL, R., ELBRACHTER, M., KIRCHNER, M., and PROKSCH, P. 1996. Defense metabolites from the sponge *Verongia aerophoba*. *Biochem. Syst. Ecol.* 24:1–12.

WULFF, J. L. 2007. Disease prevalence and population density over time in three common Caribbean coral reef sponge species. *J. Mar. Biol. Ass. U.K.* 87:1715–1720.

XYNAS, R. and CAPON, R. J. 1989. Two new bromotyrosine-derived metabolites from an Australian marine sponge, *Aplysina* sp. *Aust. J. Chem.* 42:1427–1433.

ZANGERL, A. R. AND BAZZAZ, F. A. 1992. Theory and pattern in plant defense allocation, pp. 363–391, *in* R. S. Fritz and E. L. Simms (eds.), Plant Resistance to Herbivores and Pathogens: Ecology, Evolution and Genetics. University of Chicago Press, Chicago.

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Spatial Variability in Secondary Metabolites of the Indo-Pacific Sponge *Stylissa massa*

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Abstract Chemical diversity represents a measure of selective pressures acting on genotypic variability. In order to understand patterns of chemical ecology and biodiversity in the environment, it is necessary to enhance our knowledge of chemical diversity within and among species. Many sponges produce variable levels of secondary metabolites in response to diverse biotic and abiotic environmental factors. This study evaluated intra-specific variability in secondary metabolites in the common Indo-Pacific sponge *Stylissa massa* over various geographic scales, from local to ocean basin. Several major metabolites were quantified in extracts from sponges collected in American Samoa, Pohnpei, Saipan, and at several sites and depths in Guam. Concentrations of several of these metabolites varied geographically across the Pacific basin, with American Samoa and Pohnpei exhibiting the greatest differences, and Guam and Saipan more similar to each other. There were also significant differences in concentrations among different sites and depths within Guam. The crude extract of *S. massa* exhibited feeding deterrence against the omnivorous pufferfish *Canthigaster solandri* at natural concentrations, however, none of the isolated compounds was deterrent at the maximum natural concentrations observed, nor were mixtures of these compounds, thus emphasizing the need for bioassay-guided isolation to characterize specific chemical defenses. Antibacterial activity against a panel of ecologically relevant pathogens was minimal. Depth transplants, predator exclusion, and UV protection experiments were performed, but although temporal variability in compound concentrations was observed, there was no evidence that secondary metabolite concentration in *S. massa* was induced by any of these factors. Although the reasons behind the variability observed in the chemical constituents of *S. massa* are still in question, all sponges are not created equal from a chemical standpoint, and these studies provide further insights into patterns of chemical diversity within *S. massa*.

Keywords Porifera · Chemical defense · Geographic variability · *Stylissa* · Antimicrobial activity

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Introduction

Marine organisms from many phylogenetic groups produce secondary metabolites that have multiple ecological functions (Paul, 1992). Secondary metabolites have evolved under the pressure of natural selection and serve as ecological responses of organisms to their environment (Paul, 1992; Hay, 1996). Intra-specific spatial and temporal variation in secondary metabolites is widely distributed in benthic organisms, including algae (Wright et al., 2000; Martí et al., 2004), sponges (Martí et al., 2004; Page et al., 2005), cnidarians, (Slattery et al., 2001; Martí et al., 2005; Gochfeld et al., 2006; Slattery et al., 2008), tunicates and bryozoans (Martí et al., 2005), and worms (Fielman and Targett, 1995). Chemical variation at a geographic scale can be essential to understanding the evolution and function of secondary metabolites, as well as revealing large scale patterns that are poorly understood (Paul et al., 2006).

Marine benthic organisms provide unique opportunities to investigate patterns in secondary chemistry. Sessile organisms without effective escape mechanisms are likely to be chemically defended from predators (Ianora et al., 2006). Sessile organisms also are under intense pressures to settle and maintain space on which to live (Connolly and Roughgarden, 1999), to protect themselves from ultraviolet (UV) radiation (Stachowicz and Lindquist, 1997; Lotze et al., 2002), or to prevent fouling of their surfaces by other organisms (Wahl, 1989). Defensive traits can occur either in a constitutive form (i.e., permanently expressed at a constant level) or can be generated "on demand" (Karban and Baldwin, 1997; Harvell and Tollrian, 1999). Saving resources when defenses are not needed is proposed as a major advantage of inducible defenses. At the community level, phenotypic plasticity, like inducible defenses, may dampen population fluctuations and stabilize the system (Verschoor et al., 2004). The ecological (Miner et al., 2005) and evolutionary (Pigliucci, 2005) importance of these mechanisms have recently been highlighted.

Sponges are major components of benthic communities and among the richest sources of secondary metabolites isolated from marine organisms (Blunt and Munro, 2009). Many sponge secondary metabolites are known to deter feeding, confer competitive advantages, and protect against fouling, among other ecological roles (Paul et al., 2006). *Stylissa massa* is a widely distributed sponge, occurring in the Indo-Pacific from the Arabian Sea to the Great Barrier Reef (Van Soest et al., 2010). *Stylissa massa* belongs to the family Dictyonellidae, which is one of the better investigated groups of marine invertebrates from the perspective of natural products chemistry, and some of the identified compounds have known ecological roles (Becerro et al., 2003). The most prominent natural products isolated from *S. massa* include the alkaloids oroidin, debromohymenialdisine, hymenialdisine (Tasdemir et al., 2002), sceptrin (Mohammed et al., 2006), hymenidin (Kobayashi et al., 1986), and palau'amine (Kinnel et al., 1993). The aim of this study was to assess the variation in secondary metabolite production in *S. massa* at various spatial scales, including local, regional, and biogeographic. Recognizing that biotic and abiotic factors of a site could influence the production of these compounds, we used field and laboratory experiments to assess the role of phenotypic variation in *S. massa* compounds. Specifically, we examined the role of predation and pathogenesis, and light (with depth as a proxy), to explain variation in the concentrations of the most common secondary metabolites produced by *S. massa*.

Methods and Materials

Collection Sites and Sample Preparation Samples were collected at five reefs around Guam (Fig. 1). Piti (August 2009; 13°28.1′ N; 144°42.0′ E), Gab Gab (July 2009; 13°26.4′ N; 144°38.4′ E), Haps Reef (August 2009; 13°23.4′ N; 144° 39.1′ E), Marine Lab (13°25.3′ N; 144°47.5′ E), Double reef (July 2009; 13°33.3′ N; 144°49.0′ E), and Toguan (August 2009; 13°17.4′ N; 144°39.4′ E). At all sites, samples were collected using SCUBA at depths from 7 to 15 m. Replicates (6–12 per site) were collected at least 5 m apart to avoid collection of clones. With a scalpel, tissue was cut out of the sponge (ca. 5 ml volume) and transferred to a 15 ml plastic tube filled with ambient seawater. Samples were placed on ice in coolers for transportation to the University of Guam Marine Laboratory (UOGML), where they were frozen (−20 °C) until further processing.

Samples also were collected at three additional sites across the Pacific basin (Fig. 1) in April/May in the following years: Saipan (Commonwealth of the Northern Mariana Islands) in 2002 and 2003, Pohnpei (Federated States of Micronesia) in 2007, and American Samoa in 2004, spanning a distance of 5,746 km. These were compared to samples from Guam collected in May 2003. At these remote sites, fewer samples were collected from a single reef (N=2–5), and over a more narrow depth gradient (3–6 m depth). The sponges were placed into individual resealable plastic bags, and immediately frozen for transport back to the University of Mississippi.

All samples were lyophilized and ground to a fine powder with a mortar and pestle. 150 mg of each sample were extracted in 10 ml methanol:ethyl acetate (1:1) for 3 h. The sponge-solvent mixture was centrifuged at 15,000 rpm for 10 min, and the supernatant extract was carefully decanted. This process was repeated x 3, and the extracts were combined, resulting in 40 ml extract that was filtered and evaporated under reduced pressure.

Isolation of Marker Compounds 155 g of freeze-dried powdered sponge material were extracted with (2 L×4) 1:1 dichloromethane:methanol. The extract was filtered and evaporated to dryness under reduced pressure to produce a crude extract (11.5 g yield). This extract was subjected to various chromatographic procedures, including LH-20 and C_{18} column chromatography, and high performance liquid chromatography (HPLC) using a Waters 2695 HPLC with PDA detector (Waters, Milford, MA, USA), and a C_{18} column [250×20 mm], using 0.05 % TFA in acetonitrile/water gradient system. This resulted in the isolation of five pure compounds (Fig. 2a) representing approximately 90 % of the HPLC peak area: hymenialdisine, sceptrin, hymenidin, oroidin, and palau'amine, which were characterized by NMR, IR, UV, and MS spectral data, and identified based on published data.

HPLC Chemical Fingerprinting and Quantification of Alkaloids All quantitative analyses were performed on a Waters Acquity UPLC system using a proprietary BEH Shield RP18 column (100 mm×2.1 mm I.D., 1.7 μm) (Fig. 2b).

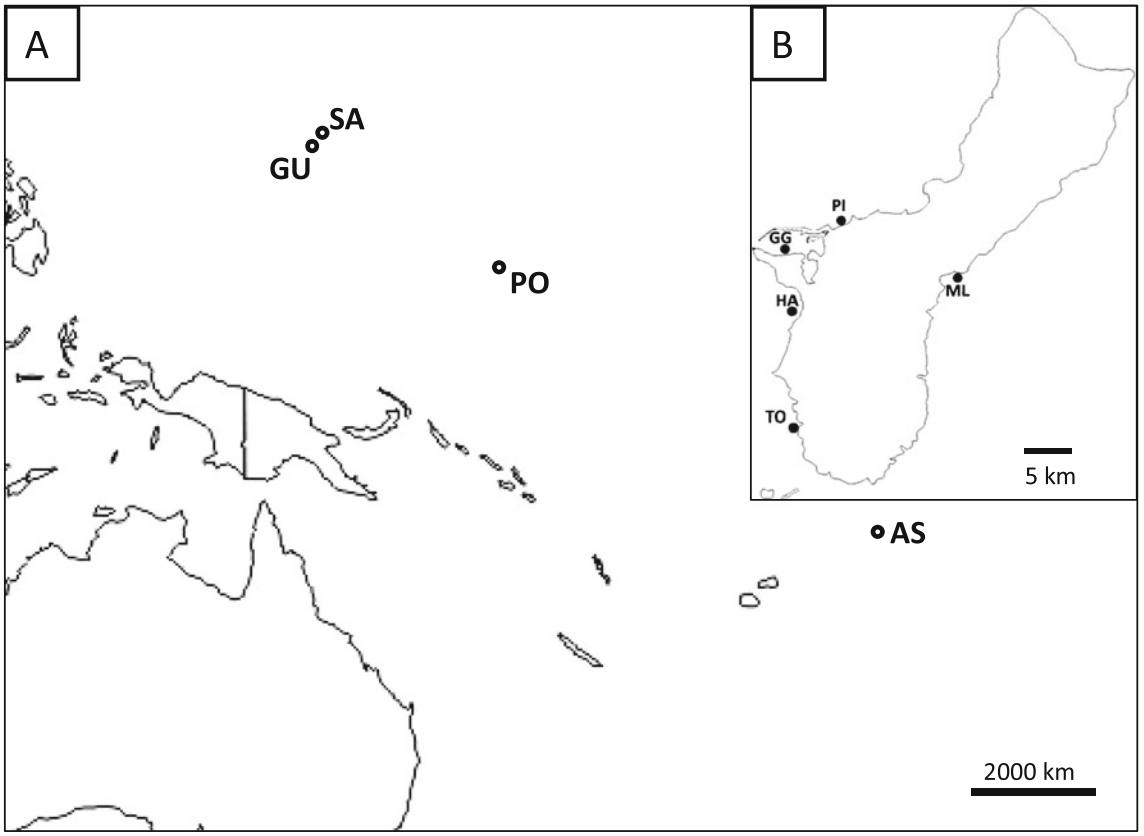

Fig. 1 Location of all sample sites of this study. **a**. Locations of the biogeographical scale, SA = Saipan, GU = Guam, PO = Pohnpei, AS = American Samoa. **b**. locations on Guam, PI = Piti, GG = Gab Gab, HA = Haps Reef, TO = Toguan, ML = Marine Lab

The column and sample temperature were maintained at 40 °C and 25 °C, respectively. The column was equipped with a LC-18 guard column (Vanguard 2.1×5 mm, Waters). The mobile phase consisted of water with 0.05 % formic acid (A), and acetonitrile with 0.05 % formic acid (B) at a flow rate of 0.2 ml/min, with gradient elution as follows: 0 min, 98 % A/2 % B, hold for 1 min, in next 7 min to 70 % A/30 % B, in next 1 min to 100 % B, and finally, reconditioning the column with 98 % A/2 % B for 3.5 min after washing the column with 100 % B for 2 min. The composition of the mobile phase was changed linearly (Waters curve type 6). The total run time for analysis was 8 min. All extracts were prepared at 2 mg/ml, and the injection volume was 2 μl. Peaks were detected using UV at 270 nm. The effluent from the LC column was directed into an ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The source temperature and the desolvation temperature were maintained at 150 and 350 °C, respectively. The probe voltage (capillary voltage), cone voltage, and extractor voltage were fixed at 2.5 kV, 40 V, and 3 V, respectively. Nitrogen was used as the source of desolvation gas (650 L/h) and drying gas (25 L/h). Alkaloids were confirmed in selected ion recording (SIR) mode at 621, 310, 324, 390, 420, and 246 $[M+H]^+$ ions for sceptrin, hymenidin, hymenialdisine, oroidin, palau'amine, and debromohymenialdisine, respectively. Mass spectra were obtained at a dwell time of 0.1 sec in SIR and 500 Da/sec of scan rate. Standard curves of all six compounds were produced using concentrations from 0.1 to 100 μg/ml to estimate compound concentrations in the crude extracts. All concentrations were subsequently calculated as mg per ml sponge volume. For statistical analyses, data were arcsin-transformed and multivariate analyses of variance (*ANOVA*) were used to determine whether the concentration of each compound varied by location. To estimate the magnitude of variance attributable to sites or regions, the coefficients of variation were calculated for the Guam sites and different regions. Analyses of similarity (*ANOSIM*) and non-metric multidimensional scaling plots (*MDS*) also were used to visualize differences among locations.

Transplantation Experiment To investigate whether depth/light had an effect on the chemical profile of *S. massa*, we conducted a transplantation experiment at Gab Gab Reef, Guam, starting in November 2009. From two depths (3–4 m=shallow; 23–24 m=deep), 20 specimens were collected by removing the substrate with hammer and chisel. The specimens, still attached to their substrata, were put into

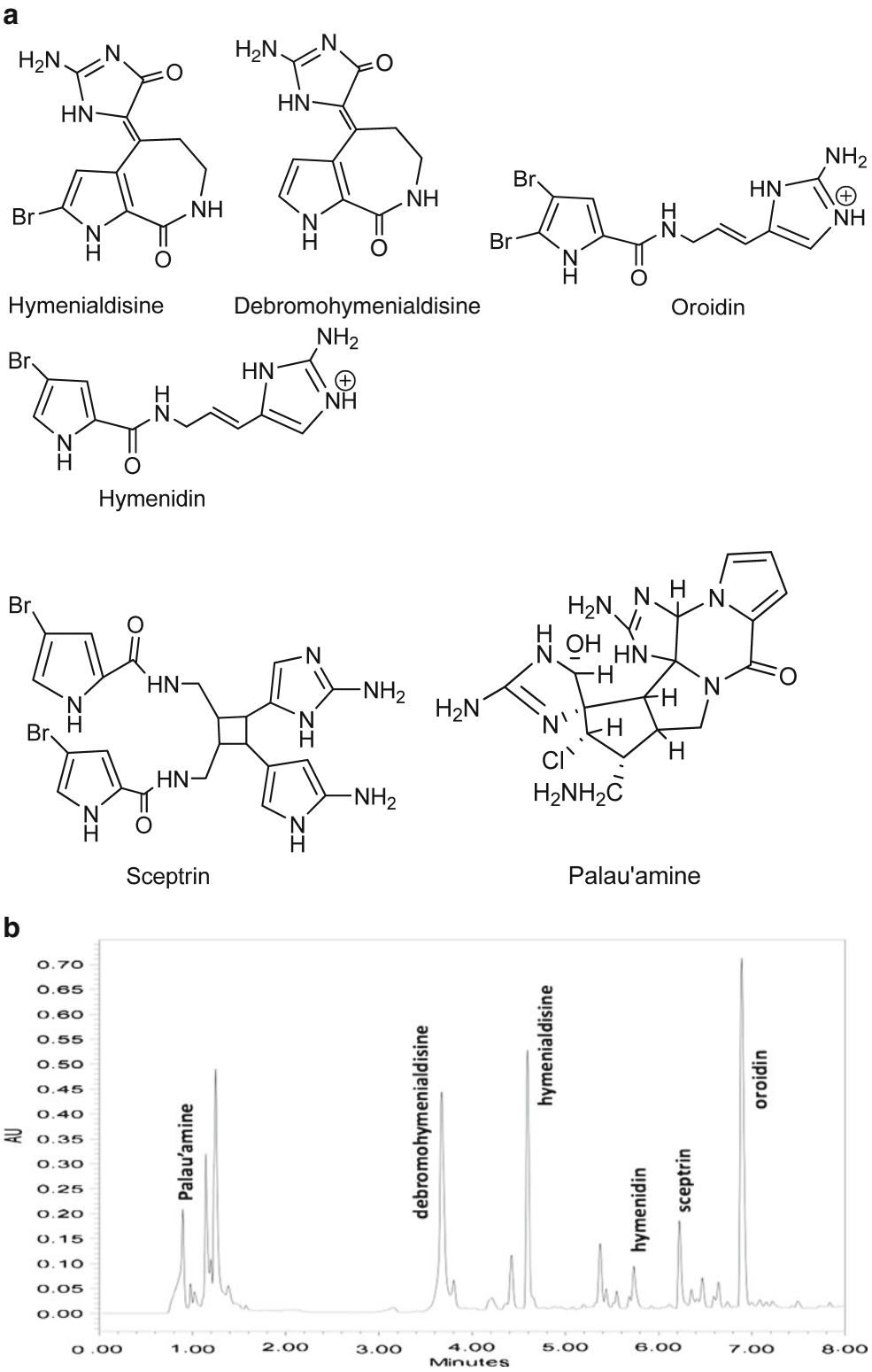

Fig. 2 **a**. Secondary metabolites identified from *Stylissa massa*. **b**. Representative UPLC chromatogram at 270 nm showing retention times of isolated compounds

individual 4-L plastic bags *in situ*, and then placed in a cooler filled with seawater for transportation to UOGML. At UOGML the sponges were placed in a 1500 L outdoor flow-through seawater tank. Using a scalpel, a small subsample (3 ml sponge tissue) was cut from each sponge and frozen for further analysis. The following day, the sponge substrata were glued to individually numbered concrete blocks (12×10×3 cm) using two-component epoxy (A-788 Splash Zone, Z-Spar), and sponges were redeployed at Gab Gab. Half of the specimens from each depth were deployed at

3 m depth, while the other half was placed at 24 m depth. The concrete blocks were attached with cable ties to 25 cm-long nails in the reef. To serve as handling controls, an additional 10 specimens of *S. massa* at each depth were tagged and tissue samples were collected according to the methods described above. After 3 mo, tissue samples from all sponges (5 ml volume) were collected and frozen to determine changes in the chemical profile over the course of the transplantation experiment. Changes in concentration over time were analyzed using repeated measure *ANOVA*s.

UV-Experiment To further determine the effects of UV exposure within the depth/light gradient on secondary metabolite production by *S. massa*, we collected 24 specimens of *S. massa,* attached to their substrate, from Gab Gab at 5–7 m depth. The sponges were transported to UOGML as described above, and placed in a 1500 L outdoor flow-through seawater tank. The following day, a tissue sample (3 ml sponge tissue) was collected from each sponge for chemical analysis, and their substrata were glued onto concrete blocks. Each sponge was placed individually in a round, clear 18 L plastic container, which allowed a water level of ca. 10 cm above the sponges. Ambient unfiltered seawater was pumped continuously in each tank at a flow rate of 11 Lh^{-1}, and tanks were placed outside without shading. Half of the containers were covered with UV-absorbing Lexan sheets (UV-transmission <3 %, General Electric, Pittsfield, MA, USA), while the other containers were covered with a plastic mesh (1 cm mesh size) to control for the slight loss in photosynthetically active radiation (PAR) under the PVC sheets. Light measurements (LICOR light meter, LI-250 A, with underwater sensor) in the containers revealed a 9.2 %±2.5 light reduction compared to ambient light under the PVC sheets and a 10.4 %±1.7 light reduction under the plastic mesh, resulting in comparable PAR regimes in all tanks (*t*-test, $P=0.91$). After 4 wk, another tissue sample of each sponge was collected for chemical analysis. Changes in concentration were analyzed using a repeated measure *ANOVA*. To compare the data from this UV-experiment with natural UV-exposed and non UV-exposed specimens of *S. massa*, we collected further tissue samples from Gab Gab in June 2010. Ten specimens were sampled in very shallow water (<1 m) and 10 specimens in deep water (20 m). Collection, transport, and fingerprinting of samples were conducted as described above. Differences in metabolite concentrations between shallow and deep samples were analyzed with an unpaired *t*-test.

Caging Experiment To estimate the effect of predation on the secondary metabolites of *S. massa*, a caging experiment was conducted on a reef in Apra Harbor, Guam (13°26.5′ N; 144° 40.0′ E). At a depth of 8–10 m, 36 specimens of *S. massa* were tagged by attaching numbered aluminum washers nearby. Tissue samples were collected as described above, and sponges were assigned randomly to the following three treatments: (1) Control: the sponges were left uncovered, allowing access to all potential predators; (2) Caged: plastic mesh cages (30×30×30 cm, 1 cm mesh size) were installed around the sponges to deny access to predators; (3) Cage Controls: cages with two adjacent sides removed were installed allowing predator access, but imitating light and water flow regimes inside the fully-closed cages. After 10 wk, another tissue sample from each sponge was collected. Changes in concentration were analyzed using a repeated measure *ANOVA*.

Feeding Assays To further investigate the potential mechanisms of feeding deterrence in *S. massa,* we examined chemical defenses against a fish predator, the omnivorous pufferfish *Canthigaster solandri* in laboratory feeding assays. *Canthigaster solandri* has been used previously as a model predator to test feeding deterrence in Pacific benthic organisms (Pennings et al., 1994; Slattery et al., 1998, 2008; Slattery and Paul, 2008; Rohde and Schupp, 2011). Ten individual pufferfish (7.1–7.6 cm total length) were maintained in separate 70 l flow-through tanks and fed for several days prior to the feeding assays to avoid the loss of preference patterns (Cronin and Hay, 1996b). The crude extract of 2 ml sponge tissue was mixed with 0.06 g alginic acid and 0.1 g of freeze-dried, powdered squid mantle. Distilled water was added to yield a final volume of 2 ml (Pawlik et al., 1995). The mixture was stirred vigorously to homogenize the extract with the squid powder, and then this was poured into a 0.25 M solution of calcium chloride. After a few minutes, the resulting pellet was rinsed in seawater, and chopped into 5×5 mm pellets with a scalpel. Control pellets were made the same way, but without the addition of extract; however, food coloring was added to match the color of extract-treated pellets. A control and treated pellet were offered sequentially to each *C. solandri*. Pellets were either consumed (i.e., the pellet was swallowed) or rejected (i.e., the pellet was spit out). Following rejection of the extract pellet, a second control pellet was offered to confirm that fish had not ceased feeding. A rejection was scored when both control pellets were eaten and the treated pellet was spat out at least three times. *Fisher's exact tests* were used to test whether the palatability of treated pellets was significantly reduced compared to control pellets.

Feeding assays with pure compounds also were conducted. Using the mean concentrations of each compound calculated from the HPLC analysis, both the highest and lowest volumetric concentrations of each compound were tested in the same pufferfish assay. In addition, following isolation of the pure compounds, the remaining extract was tested for feeding deterrence at natural concentrations.

Antibacterial Assays The antibacterial activity of *S. massa* and its secondary metabolites was assessed using disk diffusion assays, which tested the presence of antibacterial activity in the crude extract and pure compounds of *S.*

massa against a panel of 8 bacteria (Gochfeld and Aeby, 2008; Gochfeld et al., 2012, this issue), including known coral pathogens (*Aurantimonas coralicida, Serratia marcescens, Vibrio corallyticus*, *V. shiloi*), human enteric bacteria that have the potential to enter near-shore waters and can survive in the marine environment (*Yersinia enterocolitica*), and bacteria that have been isolated from the surfaces of Indo-Pacific corals (*Klebsiella pneumoniae, Pseudomonas nautica, V. agarivorans*). While this panel may be more appropriate for corals than sponges, the lack of any known sponge pathogenic bacteria necessitate this compromise (and many microbes have the capacity to "jump" between species). All organisms were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ, Braunschweig, Germany). Extracts were added to paper disks at natural concentrations, and the solvent was allowed to evaporate completely. A 24-hour culture of the test bacterium was plated onto the relevant growth medium, and disks impregnated with the mean natural concentrations of the crude extract and pure compounds were placed on the plate. Following a 24-h incubation period, the zones of inhibition surrounding each disk were measured. All assays were performed in triplicate. Disks impregnated with ciprofloxacin were used as positive controls.

Results

The concentrations of the major metabolites in *S. massa* were highly variable over all samples, and ranged from 0 % to 63 % by weight of the crude extract (Table 1). The sum of the five compounds analyzed typically accounted for over 95 % of the crude extract. On a local scale, samples from different reefs around Guam revealed great differences in metabolite concentrations. Multivariate *ANOVA* indicated significant differences in hymenidin ($P=0.03$), sceptrin ($P<0.01$), and oroidin ($P<0.01$) concentrations among reefs (Fig. 3a). Palau'amine was not found in any sponges from Guam, and dibromohymenialdisine and hymenialdisine did not differ between reefs ($P>0.05$). Sponges from Haps and Toguan reef were characterized by extremely low concentrations of all analyzed compounds, which only accounted for 4 and 10 % of the crude extract, respectively (Fig. 3a). Marine Lab sponges had the highest concentrations of hymenidin, sceptrin, and oroidin, while Double Reef was characterized by high debromohymenialdisine concentrations. The distributions of compounds in sponges from Piti, Marine Lab and Double reef sites were similar and tightly clustered, while sponges from Haps and Toguan showed significantly higher levels of variability in the concentrations of these compounds (*ANOSIM*, $P=0.001$; Fig. 4a).

Multivariate *ANOVA* demonstrated significant variability among locations across the Pacific for oroidin ($P=0.05$), sceptrin ($P=0.03$), palau'amine ($P<0.001$), and debromohymenialdisine ($P=0.029$), but not for hymenialdisine or hymenidin ($P>0.05$; Fig. 3b). *ANOSIM* confirmed significant overall variability in the distribution of these compounds among sponges from different locations ($P=0.001$). At this ocean basin scale, palau'amine was detected only in samples from American Samoa, and at low concentrations (<5 %). Samples from American Samoa contained the highest concentrations of oroidin. Sponges from Pohnpei were characterized by the highest concentrations of sceptrin and debromohymenialdisine. Hymenialdisine occurred in highest concentrations in sponges from Saipan. When all of the compounds are considered together in a *MDS* plot, sponges from Pohnpei and American Samoa segregated by site, and sponges from Guam and Saipan had overlapping distributions of chemical constituents (Fig. 4b). The coefficients of variation were used to compare the magnitude of variation between sites on Guam and across regions (Table 2). The variation of all compounds except dibromohymenialdisine was 2–3 time higher between Guam sites than across regions. Dibromohymenialdisine concentrations were the most consistent around Guam, but varied highly across regions.

Table 1 Concentrations of pure compounds in *Stylissa massa* from the Pacific. Data are calculated as mg/ml sponge volume

	Crude extract weights (mg/g dryweight)	Hymenidin	Sceptrin	Oroidin	DBH[a]	Hymenial-disine	Palau'amine[b]
Mean	488.86	2.403	2.184	3.196	3.728	0.704	0.635
Standard deviation	86.82	2.030	2.247	1.960	3.273	0.766	0.280
Variance	7536.89	4.121	5.049	3.842	10.716	0.587	0.078
Minimum	253.78	0.005	0.032	0.006	0.080	0.004	0.293
Maximum	836.56	13.123	13.752	11.879	14.524	4.633	1.061
n	162	152	153	154	154	160	7

[a] *DBH* debromohymenialdisine

[b] Palau'amine only found in samples from American Samoa

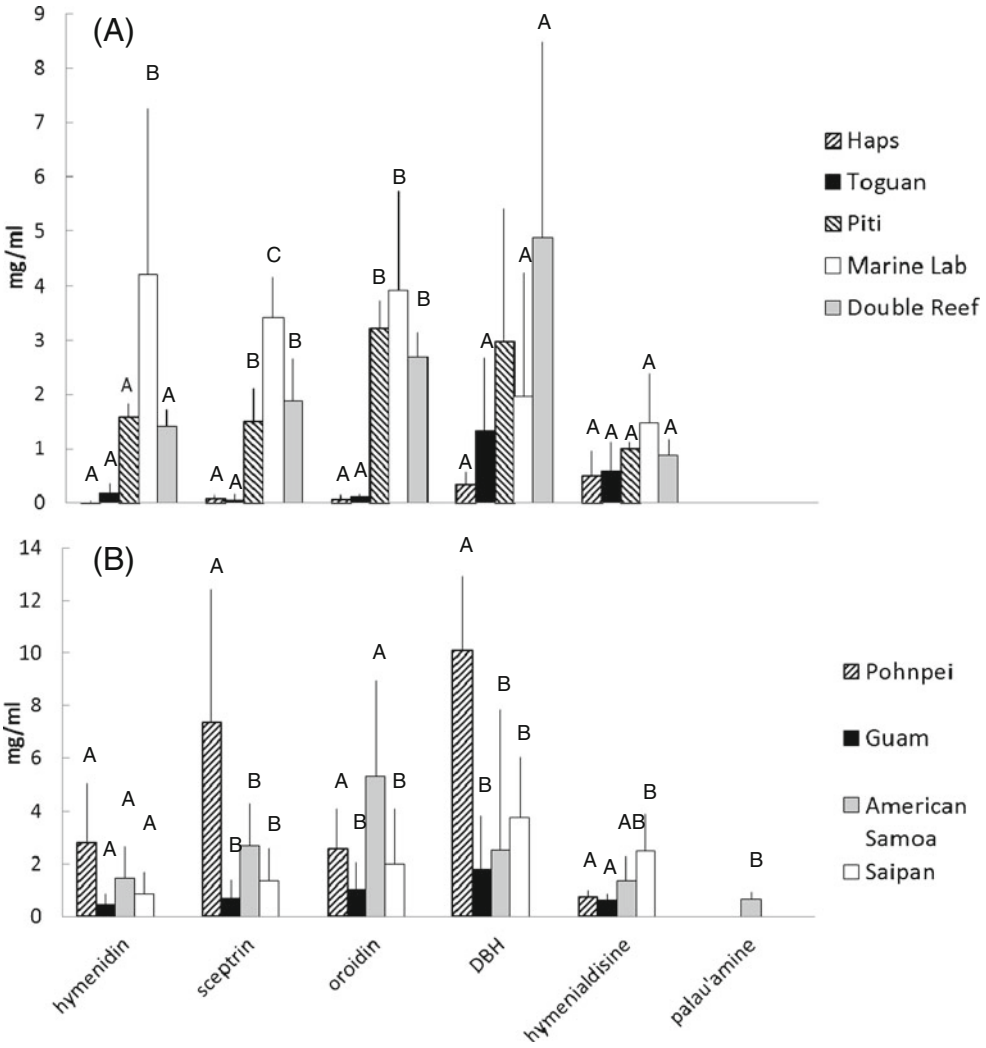

Fig. 3 Concentrations of pure compounds in *Stylissa massa* sponges from **a**. five sites in Guam, and **b**. four locations across the Pacific. Values are mean ± 1 SD mg/ml of sponge tissue. *Letters* above the bars indicate which sites are different from each other based on multiple comparisons for each compound (*LSD* test)

The initial samples from 2 to 24 m depth in the transplantation experiment showed no difference in content of hymenialdisine, sceptrin, oroidin, and debromohymenialdisine. However, the hymenidin concentrations were significantly higher in the sponges collected from the deep reef than from the shallow reef (deep: 15.6±2.6 %, shallow: 9.8±0.83 %; unpaired *t*-test, $P=0.04$). Consequently, only the change in hymenidin concentration over the course of the transplantation experiment was analyzed. During the transplantation experiment, the concentrations of hymenidin varied over time (repeated measure *ANOVA*, $P<0.01$), but transplantation depth had no significant effect on the concentrations (repeated measure *ANOVA*, $P=0.15$; Fig. 5). Even though the effect was not significant, the hymenidin concentrations decreased in sponges transplanted from shallow to deep. Interestingly, field samples from 1 to 20 m depth revealed higher concentrations of hymenidin at 20 m depth (unpaired *t*-test, $P<0.001$). During the UV experiment, hymenidin, sceptrin, and oroidin decreased in concentration over time (repeated measure *ANOVA*, $P<0.01$), while hymenialdisine and debromohymenialdisine did not change ($P>0.25$). However, sponge exposure to, or protection from, UV radiation had no effect on the concentrations of any compounds in this experiment (repeated measure *ANOVA*, $P=0.24$).

In the caging experiment, predation had no effect on metabolite concentrations. While concentrations of hymenialdisine, sceptrin, and debromohymenialdisine changed over time, there was no consistent pattern (repeated measure *ANOVA*, $P<0.01$), and caging had no effect (repeated measure *ANOVA,* P=0.09; Fig. 6).

The feeding assays revealed that the crude extract of *S. massa* was deterrent against feeding by *C. solandri* (3 out of 10 pellets consumed; *Fisher exact test*, $P<0.05$). However, none of the pure compounds, nor a mixture of the pure compounds combined, deterred feeding at either the highest or lowest natural concentrations (8–10 out of 10 pellets consumed; *Fisher exact test*, $P>0.05$).

Antimicrobial activity of *S. massa* was highly selective. Against the panel of potential marine pathogens, zones of inhibition were observed for the crude extract, oroidin, and palau'amine against *Klebsiella pneumoniae* and for the

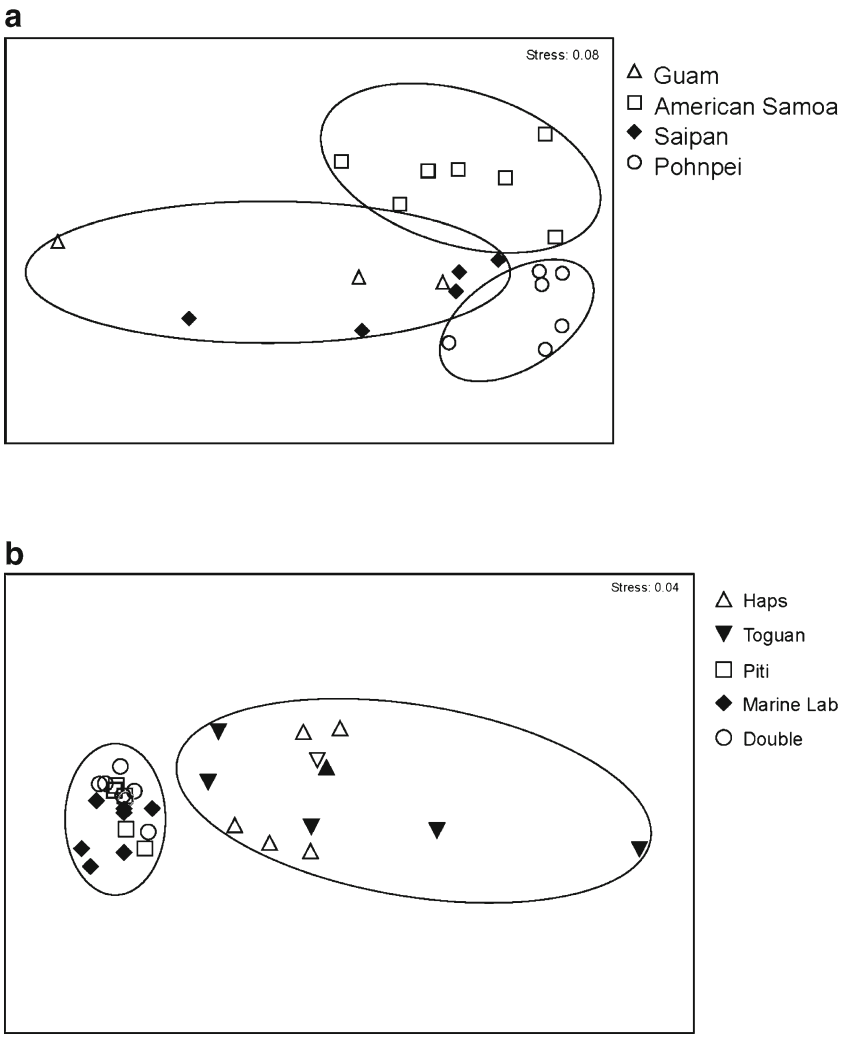

Fig. 4 Non-metric multidimensional scaling plots showing the similarity of chemical constituents for sponges from **a**. five sites in Guam, and **b**. four locations across the Pacific. *Circles* represent significant groupings by *ANOSIM*

crude extract, debromohymenialdisine and oroidin against *Yersinia enterocolitica* (Table 3). Neither the extracts nor the pure compounds exhibited activity against the 6 other bacteria tested in disk diffusion assays.

Discussion

Intraspecific Variability – From Local to Biogeographic Scale Stylissa massa is a common sponge in the Indo-Pacific region and due to its massive morphology and bright orange color, it is highly apparent on the reef. With a broad geographic distribution, *S. massa* is of both ecological and potential biotechnological importance on Indo-Pacific reefs. The variability of secondary metabolite production in sponges can be pronounced both on an inter- and intraspecific level (Turon et al., 1996; Betancourt-Lozano et al., 1998; Turon et al., 2009). Intraspecific production of secondary metabolites may vary among populations or even among individuals (Turon et al., 1996; Page et al., 2005). Many biological or environmental factors can affect these patterns. Marine organisms can modify their levels of secondary metabolites in response to predation (e.g., Cronin and Hay, 1996a; Rohde et al., 2004), but other factors including nutrient availability, light, UV radiation, and temperature can also influence this plasticity (Uriz et al., 1995; Becerro and Paul, 2004; Turon et al., 2009).

Table 2 Coefficents of variation as a measure of spatial metabolite variation

	Hymenidin	Sceptrin	Oroidin	DBH[a]	Hymenialdisine	All compounds
Between Guam sites	67.86	57.20	97.50	22.10	61.60	33.02
Across regions	19.65	27.66	41.36	57.92	24.95	11.53

[a]*DBH* debromohymenialdisine

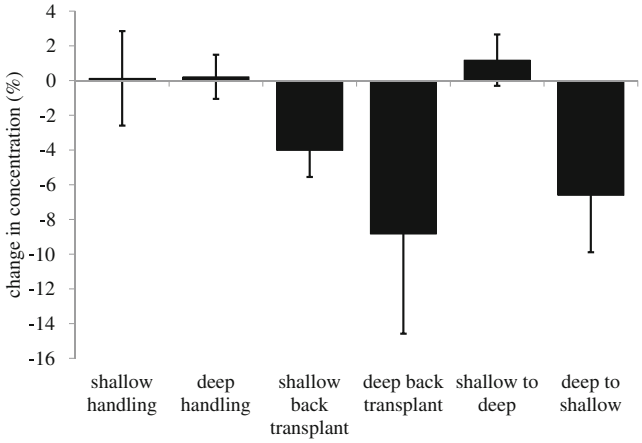

Fig. 5 Percent change in concentrations (mean ± 1 SE percent of crude extract) of hymenidin in *Stylissa massa* in the depth transplant experiment. Shallow=2 m; Deep=24 m

Investigations on the variability of secondary metabolites within a sponge species are scarce and controversial. Some studies have observed a low variability in sponge metabolite concentrations. The brominated pyrrole alkaloids hymenialdisine, debromohymenialdisine, dibromophakellin, and 3-bromohymenialdisin occur in similar concentrations within specimens of the sponge *Axinella carteri*, collected from sites over 2000 km (Supriyono et al., 1995). In contrast, a recent study on bromotyrosine derivatives from *Aplysina fulva* demonstrated chemical variability among specimens collected in multiple locations in Brazil and the United States (Nunez et al., 2008). The concentration of brominated compounds in specimens of the sponge *Aplysina aerophoba* collected around the Canary Islands varied significantly (Teeyapant and Proksch, 1993). In more detail, Soriano et al. (2011) showed that some brominated alkaloids of the same species vary most between sites less than 500 m apart, while others varied most between regions over 2500 km apart. This study follows a similar trend. Compounds varied most between sites, while only dibromohymenialdisine varied more across regions. Thus, small scale spatial variation seems to affect the metabolite concentrations of *S. massa* more profoundly than large scale variation. However, as discussed, other studies on sponges have reported controversial results, and studies on other species and phyla have produced a diverse pattern regarding metabolite concentrations as a function of location (Puglisi et al., 2000; Slattery et al., 2001; Fahey and Garson, 2002; Jumaryatno et al., 2007). Studies on biogeographical variation face the constraint that many factors such as water temperature, food availability, and light exposure vary highly between locations and could affect the production of secondary metabolites sponges, or other organisms. Moreover, temporal variation such as seasonal patterns can confound a spatial analysis (Turon et al., 1996; Page et al., 2005; Abdo et al., 2007). Within-species variation of secondary metabolites can be so high as to hinder the identification of extrinsic factors (Targett et al., 1992), and whether the variability in secondary metabolites is genetically or environmentally controlled is still unknown. The high overall variation of metabolite concentration in this study, even within sites, indicates at least partially a genetic control, while the changes over time in the transplant and caging experiments demonstrate an environmental effect, even though the specific controlling factors could not be resolved. However, so far nearly all studies on spatial and temporal variation of metabolite concentration are restricted to the identification and quantitative analysis of variation. The causing factors of spatial and temporal variation are virtually unstudied. Correlative studies will indicate potential relations of environmental or biotic factors with secondary metabolites,

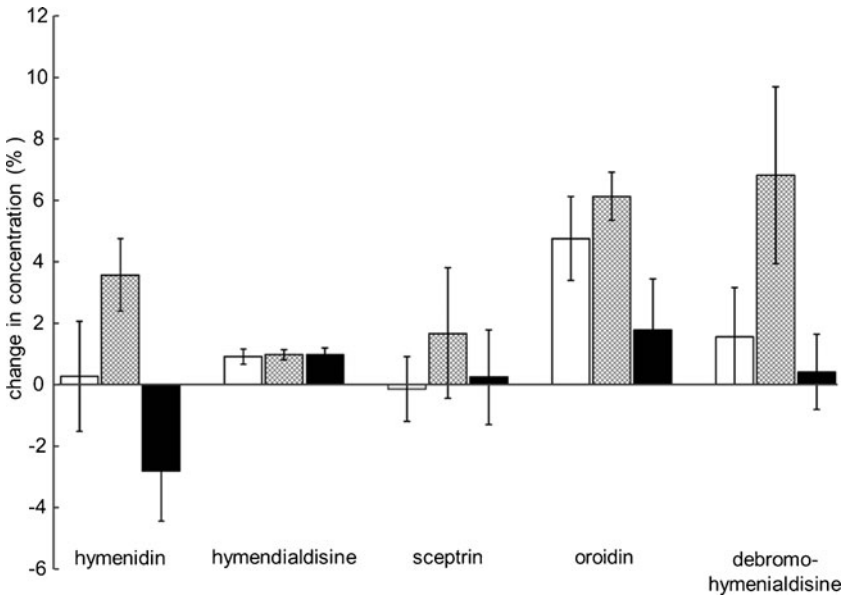

Fig. 6 Change in concentrations (mean ± 1 SE percent of crude extract) of each compound from *Stylissa massa* in the caging experiment on Guam. White bars are uncaged, *hatched bars* are caged controls, and *black bars* are caged

Table 3 Zone of growth inhibition (MM) of *Stylissa massa* crude extracts and pure compounds in disk diffusion assays

	Aurantimonas coralicida	*Klebsiella pneumoniae*	*Pseudomonas nautica*	*Serratia marcescens*	*Vibrio agarivorans*	*V. corallyticus*	*V. shiloi*	*Yersinia enterocolitica*
Ciprofloxacin	4.19±0.26	5.29±0.47	3.91±0.02	9.93±0.65	3.83±0.17	5.90±0.54	3.40±0.20	4.85±0.94
S. massa crude extract	0±0	0.07±0.07	0±0	0±0	0±0	0±0	0±0	0.35±0.35
Debromohymenialdisine	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0.32±0.32
Hymenialdisine	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Sceptrin	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Oroidin	0±0	0.03±0.03	0±0	0±0	0±0	0±0	0±0	0.76±0.76
Palau'amine	0±0	0.20±0.11	0±0	0±0	0±0	0±0	0±0	0±0

but more experimental studies are necessary to prove vigorously what the causes for metabolite variation are.

Defenses Against Abiotic Stressors The chemical profiles of *S. massa* from deep and shallow waters were quite similar. Although not quantified in this study, it was observed that environmental conditions such as light, water movement, or predator density vary between deep and shallow water habitats. Nonetheless, there were only slight changes in the chemical profiles with depth, indicating that the variability in environmental factors was small over the depth range studied, or that the expression of metabolite production is conserved over the range of environmental conditions experienced. Only hymenidin concentration was higher in deeper samples compared to shallow water sponges. Several previous studies found that secondary metabolite production did not vary significantly with depth in sponges (Becerro and Paul, 2004; Abdo et al., 2007; Putz et al., 2009). A transplantation experiment along a depth gradient with the sponge *Aplysina cavernicola* also revealed no changes in the metabolite profile in transplanted sponges (Thoms et al., 2003). However, the composition of diterpenes in *Rhopaloeides odorabile* varied with environmental factors such as light intensity and depth (Thompson et al., 1987). The present study suggests that hymenidin may behave similarly, although it was not induced over the 3-month duration of the transplantation study, suggesting that such metabolic changes may occur more slowly.

Some marine organisms produce metabolites that are suggested to provide protection against UV radiation (Pavia et al., 1997; Dunlap and Shick, 1998) and their levels could be adjusted to changing intensities of UV radiation. It is known that the photosymbionts of corals produce mycosporine-like amino acids (MAAs) (Shick et al., 1999), thus providing UV-protection for the coral host. Symbiotic cyanobacteria also are known to produce MAAs (Gröniger et al., 2000), and MAAs have been found in marine sponges (Bandaranayake et al., 1996, 1997; McClintock and Karentz, 1997). Additionally, Steindler et al. (2002) found a higher abundance of photosymbiotic sponge species in the intertidal compared to the subtidal and hypothesized that this may be related to a higher necessity for symbiont-derived substances that provide protection from UV radiation. However, no induced changes in UV absorbing compounds in response to an increased UV radiation have been found in sponges so far. We hypothesized that the metabolites hymenialdisne and debromohymenialdisine might serve similar purposes as MAAs, since their UVmax is between 350 and 355 nm. However, sponges from very shallow water (<1 m) had similar concentrations of hymenialdisine and debromohymenialdisine compared to sponges from deeper (20 m), UV-protected habitats. Additionally, the experimental removal of UV radiation did not affect the concentration of these metabolites, indicating that an induced defense against UV radiation is not expressed in *S. massa*. In concordance with the depth transplant experiment, the concentration of hymenidin was significantly higher in sponges from 20 m, however, the reason for difference remains unknown.

Defenses Against Biotic Stressors Not surprisingly, given its conspicuousness on the reef, *S. massa* is known to deter predation (Becerro et al., 2003). The major compounds found in *S. massa*'s crude extract have been described from other sponge species and have demonstrated feeding deterrent activities. Oroidin was isolated from the Caribbean sponge *Agelas clathrodes* and deterred feeding by the bluehead wrasse *Thalassoma bifasciatum* at natural concentrations of 0.7 mg ml^{-1} (Chanas et al., 1996). Slightly less deterrence against this species was shown by Lindel et al. (2000), who found that the deterrent concentration of oroidin was greater than 1 mg ml^{-1}. Sceptrin, isolated from the Caribbean sponge *Agelas conifera*, deterred feeding by *T. bifasciatum* at concentrations of 1 mg ml^{-1} (Assmann et al., 2000), and hymenidin from the Mediterranean sponge *Axinella verrucosa* deterred feeding by the shrimp *Palaemon elegans* at 3 and 5 mg ml^{-1} but not at 1 mg ml^{-1} (Haber et al., 2011). A combination of oroidin and sceptrin at natural concentrations found in the sponge *Agelas conifera* was also deterrent to the damselfish *Stegastes partitus* (Richelle-Maurer et al., 2003). Even though the crude extract from *S. massa* was highly deterrent in our feeding assays, none of the pure compounds had deterrent effects on feeding of the pufferfish *Canthigaster solandri*

when tested in isolation at either the highest or lowest natural concentrations found in our sponge samples. A combination of the major metabolites at natural concentrations also had no deterrent effect. *Canthigaster solandri* is a common omnivore on the reefs around Guam, feeding on sponges, ascidians, other invertebrates and benthic algae (Amesbury and Myers, 1982), and congruent results of feeding patterns with field feeding assays in a recent study corroborate the use of *C. solandri* as a bioassay organism for feeding preferences of benthic reef fishes (Rohde and Schupp, 2011). In contrast to these results from *C. solandri* in Guam, sceptrin deterred feeding by *Canthigaster rostrata* in the Bahamas, when tested using the same feeding assays at 1, 3, and 5 mg ml^{-1} (Gochfeld, unpublished data). The lack of deterrent activity in the analyzed metabolites in the present study suggests that either the metabolites are not generally active against all predators, they are active at a greater extreme of concentrations than typically found in *S. massa,* or they are active only in combination with other metabolites that were not isolated in this study. These results emphasize the importance of characterizing bioactive metabolites using bioassay-guided isolation rather than assuming the major compounds are of the ones of ecological relevance.

Many sponges produce chemicals that inhibit the settlement or growth of microorganisms that may foul their surfaces or act as pathogens (Slattery and Gochfeld, 2012). These same compounds often possess potential pharmacological activity when tested again microorganisms of biomedical relevance. When tested at natural concentrations against environmental bacteria, the crude extract of *S. massa* exhibited moderate antimicrobial activity against two strains. The observed activity against *K. pneumoniae* may be due, at least in part, to oroidin and palau'amine, which also inhibited bacterial growth. Activity against *Y. enterocolitica* may be due to oroidin and debromohymenialdisine. In contrast to our assays, using disk diffusion assays against human opportunistic pathogens, Richelle-Maurer et al. (2003) observed minor activity of sceptrin at 100–200 μg per disk, a concentration two orders of magnitude greater than that used in the present study. Haber et al. (2011) found that hymenidin exhibited antibacterial activity against certain laboratory and environmental bacterial strains, but not against several other strains tested, and the authors concluded that hymenidin plays a role in the antibacterial defense of the sponge in nature, but is not the sole compound responsible for its antibacterial defense. Oroidin and sceptrin inhibited bacterial attachment at natural concentrations, and sceptrin inhibited bacterial attachment at 50 % of natural concentration (Kelly et al., 2003). The limited activity observed in these assays suggests that antibacterial activity in *S. massa* is highly selective. This selectivity may serve to establish natural sponge-microbial associations, while inhibiting settlement or growth of potential pathogens. Similarly, the crude extract of *S. massa* and its pure compounds exhibited selective activity against a diversity of biomedical pathogens (unpublished data).

Although some temporal variability in concentrations of certain secondary metabolites was observed in our experiments, overall there was no evidence of inducible defenses in *S. massa* in response to changes in depth, UV, or predation levels. While the presence of inducible defenses in sponges has been postulated due to the complexity and presumed cost of the secondary metabolites that they produce, few studies have investigated the presence of inducible defenses in sponges by using manipulative experiments. Richelle-Maurer et al. (2003) found a rapid increase in oroidin and sceptrin in response to simulated predation, but there was no overall change in these compounds in a longer-term predation-exclusion experiment. Likewise, activated defenses have been postulated in *Aplysina* spp. (Teeyapant and Proksch, 1993; Ebel et al., 1997), however, longer predation studies have not been performed. There were no major differences in the abundance of spongivorous fishes at the locations sampled in the present study. The question of whether sponges produce inducible defenses in response to changing environmental conditions, thus, remains unanswered.

This study used chemical profiling to demonstrate that *S. massa* exhibits intra-specific chemical diversity over a variety of both temporal and spatial scales. Although chemical constituents were similar at the population scale, differences in concentrations were observed among sites on Guam. At the geographic scale, not only did concentrations vary, but there was greater variability in overall metabolite composition, with palau'amine being present only in samples from American Samoa. We tested the effects of depth, UV, predation, and microbial growth as potential selective factors on the pure compounds in *S. massa*, but did not find specific responses to these factors, indicating that other selective pressures may be acting on these compounds. These possibilities include water temperature, light conditions (as they relate to an energy source for photosymbionts), water quality (including both nutrient and pollution levels), and food availability. Alternatively, differences may represent population-specific constitutive defenses that represent the local defensive needs of *S. massa* on reefs throughout the Indo-Pacific. Manipulative experimental studies on metabolite variation are rare but needed to identify causing factors and generally valid ecological patterns.

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References

ABDO, D. A., MOTTI, C. A., BATTERSHILL, C. N., and HARVEY, E. S. 2007. Temperature and spatiotemporal variability of salicylihalamide A in the sponge *Haliclona* sp. *J. Chem. Ecol.* 33:1635–1645.

AMESBURY, S. S. and MYERS, R. F. 1982. pp. 1–141, Guide to the Coastal Resources of Guam Vol. 1. University of Guam Marine Lab Contribution No. 173, Mangilao.

ASSMANN, M., LICHTE, E., PAWLIK, J. R., and KÖCK, M. 2000. Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. *Mar. Ecol. Prog. Ser.* 207:255–262.

BANDARANAYAKE, W. M., BEMIS, J. E., and BOURNE, D. J. 1996. Ultraviolet absorbing pigments from the marine sponge *Dysidea herbacea*: Isolation and structure of a new mycosporine. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* 115:281–286.

BANDARANAYAKE, W. M., BOURNE, D. J., and SIM, R. G. 1997. Chemical composition during maturing and spawning of the sponge *Dysidea herbacea* (Porifera: Demospongiae). *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 118:851–859.

BECERRO, M. A. and PAUL, V. J. 2004. Effects of depth and light on secondary metabolites and cyanobacterial symbionts of the sponge *Dysidea granulosa*. *Mar. Ecol. Prog. Ser.* 280:115–128.

BECERRO, M. A., THACKER, R. W., TURON, X., URIZ, M. J., and PAUL, V. J. 2003. Biogeography of sponge chemical ecology: Comparisons of tropical and temperate defenses. *Oecologia* 135:91–101.

BETANCOURT-LOZANO, M., GONZALEZ-FARIAS, F., GONZALEZ-ACOSTA, B., GARCIA-GASCA, A., and BASTIDA-ZAVALA, J. R. 1998. Variation of antimicrobial activity of the sponge *Aplysina fistularis* (Pallas, 1766) and its relation to associated fauna. *J. Exp. Mar. Biol. Ecol.* 223:1–18.

BLUNT, J. W. and MUNRO, M. H. G. 2009. MarinLit. Marine Chemistry Group, Christchurch.

CHANAS, B., PAWLIK, J. R., LINDEL, T., and FENICAL, W. 1996. Chemical defense of the Caribbean sponge *Agelas clathrodes* (Schmidt). *J. Exp. Mar. Biol. Ecol.* 208:185–196.

CONNOLLY, S. R. and ROUGHGARDEN, J. 1999. Theory of marine communities: Competition, predation, and recruitment-dependent interaction strength. *Ecol. Monogr.* 69:277–296.

CRONIN, G. and HAY, M. E. 1996a. Induction of seaweed chemical defenses by amphipod grazing. *Ecology* 77:2287–2301.

CRONIN, G. and HAY, M. E. 1996b. Susceptibility to herbivores depends on recent history of both the plant and animal. *Ecology* 77:1531–1543.

DUNLAP, W. C. and SHICK, J. M. 1998. Ultraviolet radiation-absorbing mycosporine-like amino acids in coral reef organisms: A biochemical and environmental perspective. *J. Phycol.* 34:418–430.

EBEL, R., BRENZINGER, M., KINZE, A., GROSS, H. J., and PROKSCH, P. 1997. Wound activation of protoxins in marine sponge *Aplysina aerophoba*. *J. Chem. Ecol.* 23:1451–1462.

FAHEY, S. J. and GARSON, M. J. 2002. Geographic variation of natural products of tropical nudibranch *Asteronotus cespitosus*. *J. Chem. Ecol.* 28:1773–1785.

FIELMAN, K. T. and TARGETT, N. M. 1995. Variation of 2,3,4-tribromopyrrole and its sodium sulfamate salt in the hemichordate *Saccoglossus kowalevskii*. *Mar. Ecol. Prog. Ser.* 116:125–136.

GOCHFELD, D. J. and AEBY, G. S. 2008. Antibacterial chemical defenses in Hawaiian corals provide possible protection from disease. *Mar. Ecol. Prog. Ser.* 362:119–128.

GOCHFELD, D. J., OLSON, J. B., and SLATTERY, M. 2006. Colony versus population variation in susceptibility and resistance to dark spot syndrome in the Caribbean coral *Siderastrea siderea*. *Dis. Aquat. Org.* 69:53–65.

GOCHFELD D., KAMEL H., OLSON J., and THACKER R. 2012, this issue. Trade-offs in defensive metabolite production but not ecological function in healthy and diseased sponges. *J. Chem. Ecol*. doi:10.1007/s10886-012-0099-5

GRÖNIGER, A., SINHA, R. P., KLISCH, M., and HADER, D. P. 2000. Photoprotective compounds in cyanobacteria, phytoplankton and macroalgae—a database. *J. Photochem. Photobiol. B: Biol.* 58:115–122.

HABER, M., CARBONE, M., MOLLO, E., GAVAGNIN, M., and ILAN, M. 2011. Chemical defense against predators and bacterial fouling in the Mediterranean sponges *Axinella polypoides* and *A. verrucosa*. *Mar. Ecol. Prog. Ser.* 422:113–122.

HARVELL, C. and TOLLRIAN, R. 1999. Why inducible defenses? pp. 3–9, *in* R. Tollrian and C. Harvell (eds.), The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton.

HAY, M. E. 1996. Marine chemical ecology: What's known and what's next? *J. Exp. Mar. Biol. Ecol.* 200(1–2):103–134.

IANORA, A., BOERSMA, M., CASOTTI, R., FONTANA, A., HARDER, J., HOFFMANN, F., PAVIA, H., POTIN, P., POULET, S. A., and TOTH, G. 2006. New trends in marine chemical ecology. *Estuar. Coasts* 29:531–551.

JUMARYATNO, P., STAPLETON, B. L., HOOPER, J. N. A., BRECKNELL, D. J., BLANCHFIELD, J. T., and GARSON, M. J. 2007. A comparison of sesquiterpene scaffolds across different populations of the tropical marine sponge *Acanthella cavernosa*. *J. Nat. Prod.* 70:1725–1730.

KARBAN, R. and BALDWIN, I. T. 1997. Induced Response to Herbivory. University of Chicago Press, Chicago.

KELLY, S. R., JENSEN, P. R., HENKEL, T. P., FENICAL, W., and PAWLIK, J. R. 2003. Effects of Caribbean sponge extracts on bacterial attachment. *Aquat. Microb. Ecol.* 31:175–182.

KINNEL, R. B., GEHRKEN, H. P., and SCHEUER, P. J. 1993. Palauamine—a cytotoxic and immunosuppresive hexacyclic bisuguanidine antibiotic from the sponge *Stylotella agminata*. *J. Am. Chem. Soc.* 115:3376–3377.

KOBAYASHI, J., OHIZUMI, Y., NAKAMURA, H., and HIRATA, Y. 1986. A novel anatagonist of serotonergic receptors, hymenidin, isolated from the okinawan marine sponge *Hymeniacidon* sp. *Experientia* 42:1176–1177.

LINDEL, T., HOFFMANN, H., HOCHGURTEL, M., and PAWLIK, J. R. 2000. Structure-activity relationship of inhibition of fish feeding by sponge-derived and synthetic pyrrole-imidazole alkaloids. *J. Chem. Ecol.* 26:1477–1496.

LOTZE, H. K., WORM, B., MOLIS, M., and WAHL, M. 2002. Effects of UV radiation and consumers on recruitment and succession of a marine macrobenthic community. *Mar. Ecol. Prog. Ser.* 243:57–66.

MARTÍ, R., URIZ, M. J., and TURON, X. 2004. Seasonal and spatial variation of species toxicity in Mediterranean seaweed communities: Correlation to biotic and abiotic factors. *Mar. Ecol. Prog. Ser.* 282:73–85.

MARTÍ, R., URIZ, M. J., and TURON, X. 2005. Spatial and temporal variation of natural toxicity in cnidarians, bryozoans and tunicates in Mediterranean caves. *Sci. Mar.* 69:485–492.

MCCLINTOCK, J. B. and KARENTZ, D. 1997. Mycosporine-like amino acids in 38 species of subtidal marine organisms from McMurdo Sound, Antarctica. *Antarct. Sci.* 9:392–398.

MINER, B. G., SULTAN, S. E., MORGAN, S. G., PADILLA, D. K., and RELYEA, R. A. 2005. Ecological consequences of phenotypic plasticity. *Trends Ecol. Evol.* 20:685–692.

MOHAMMED, R., PENG, J. N., KELLY, M., and HAMANN, M. T. 2006. Cyclic heptapeptides from the Jamaican sponge *Stylissa caribica*. *J. Nat. Prod.* 69:1739–1744.

NUNEZ, C. V., DE ALMELDA, E. V. R., GRANATO, A. C., MARQUES, S. O., SANTOS, K. O., PEREIRA, F. R., MACEDO, M. L., FERREIRA, A. G., HAJDU, E., PINHEIRO, U. S., ET AL. 2008. Chemical variability within the marine sponge *Aplysina fulva*. *Biochem. Syst. Ecol.* 36:283–296.

PAGE, M., WEST, L., NORTHCOTE, P., BATTERSHILL, C., and KELLY, M. 2005. Spatial and temporal variability of cytotoxic metabolites in populations of the New Zealand sponge *Mycale hentscheli*. *J. Chem. Ecol.* 31:1161–1174.

PAUL, V. J. 1992. pp. 245, Ecological Roles of Marine Natural Products. Comstock Press, Ithaca.

PAUL, V. J., PUGLISI, M. P., and RITSON-WILLIAMS, R. 2006. Marine chemical ecology. *Nat. Prod. Rep.* 23:153–180.

PAVIA, H., CERVIN, G., LINDGREN, A., and ABERG, P. 1997. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. *Mar. Ecol. Prog. Ser.* 157:139–146.

PAWLIK, J. R., CHANAS, B., TOONEN, R. J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish.1. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

PENNINGS, S. C., PABLO, S. R., PAUL, V. J., and DUFFY, J. E. 1994. Effects of sponge secondary metabolites in different diets on feeding by 3 groups of consumers. *J. Exp. Mar. Biol. Ecol.* 180:137–149.

PIGLIUCCI, M. 2005. Evolution of phenotypic plasticity: Where are we going now? *Trends Ecol. Evol.* 20(9):481–486.

PUGLISI, M. P., PAUL, V. J., and SLATTERY, M. 2000. Biogeographic comparisons of chemical and structural defenses of the Pacific gorgonians *Annella mollis* and *A. reticulata*. *Mar. Ecol. Prog. Ser.* 207:263–272.

PUTZ, A., KLOEPPEL, A., PFANNKUCHEN, M., BRUMMER, F., and PROKSCH, P. 2009. Depth-related alkaloid variation in mediterranean aplysina sponges. *Z. Naturforsch. Sect. C* 64:279–287.

RICHELLE-MAURER, E., DE KLUJIVER, M. J., FEIO, S., GAUDENCIO, S., GASPAR, H., GOMEZ, R., TAVARES, R., VAN DE VYVER, G., and VAN SOEST, R. W. M. 2003. Localization and ecological significance of oroidin and sceptrin in the Caribbean sponge *Agelas conifera*. *Biochem. Syst. Ecol.* 31:1073–1091.

ROHDE, S. and SCHUPP, P. J. 2011. Allocation of chemical and structural defenses in the sponge *Melophlus sarasinorum*. *J. Exp. Mar. Biol. Ecol.* 399:76–83.

ROHDE, S., MOLIS, M., and WAHL, M. 2004. Regulation of anti-herbivore defence by *Fucus vesiculosus* in response to various cues. *J. Ecol.* 92:1011–1018.

SACRISTAN-SORIANO, O., BANAIGS, B., and BECERRO, M. A. 2011. Relevant spatial scales of chemical variation in *Aplysina aerophoba*. *Mar. Drugs* 9:2499–2513.

SHICK, J. M., ROMAINE-LIOUD, S., FERRIER-PAGES, C., and GATTUSO, J. P. 1999. Ultraviolet-B radiation stimulates shikimate pathway-dependent accumulation of mycosporine-like amino acids in the coral *Stylophora pistillata* despite decreases in its population of symbiotic dinoflagellates. *Limnol. Oceanogr.* 44:1667–1682.

SLATTERY M. and GOCHFELD D. J. 2012. Chemical interactions among marine competitors, and host-pathogens, *in*: E. Fattorusso, W. H. Gerwick, O. Taglialatela-Scafati (eds.), Handbook of Marine Natural Products: Springer.

SLATTERY, M. and PAUL, V. J. 2008. Indirect effects of bleaching on predator deterrence in the tropical Pacific soft coral *Sinularia maxima*. *Mar. Ecol. Prog. Ser.* 354:169–179.

SLATTERY, M., AVILA, C., STARMER, J., and PAUL, V. J. 1998. A sequestered soft coral diterpene in the aeolid nudibranch *Phyllodesmium guamensis*. *J. Exp. Mar. Biol. Ecol.* 226:33–49.

SLATTERY, M., STARMER, J., and PAUL, V. J. 2001. Temporal and spatial variation in defensive metabolites of the tropical Pacific soft corals *Sinularia maxima* and *S. polydactyla*. *Mar. Biol.* 138:1183–1193.

SLATTERY, M., KAMEL, H. N., ANKISETTY, S., GOCHFELD, D. J., HOOVER, C. A., and THACKER, R. W. 2008. Hybrid vigor in a tropical Pacific soft-coral community. *Ecol. Monogr.* 78: 423–443.

STACHOWICZ, J. J. and LINDQUIST, N. 1997. Chemical defense among hydroids on pelagic *Sargassum*: Predator deterrence and absorption of solar UV radiation by secondary metabolites. *Mar. Ecol. Prog. Ser.* 155:115–126.

STEINDLER, L., BEER, S., and ILAN, M. 2002. Photosymbiosis in intertidal and subtidal tropical sponges. *Symbiosis* 33: 263–273.

SUPRIYONO, A., SCHWARZ, B., WRAY, V., WITTE, L., MULLER, W. E. G., VANSOEST, R., SUMARYONO, W., and PROKSCH, P. 1995. Bioactive alkaloids from the tropical marine sponge *Axinella carteri*. *Z. Naturforsch. Sect. C* 50:669–674.

TARGETT, N. M., COEN, L. D., BOETTCHER, A. A., and TANNER, C. E. 1992. Biogeographic comparisons of marine algal polyphenolics—evidence against a latitudinal trend. *Oecologia* 89:464–470.

TASDEMIR, D., MALLON, R., GREENSTEIN, M., FELDBERG, L. R., KIM, S. C., COLLINS, K., WOJCIECHOWICZ, D., MANGALINDAN, G. C., CONCEPCION, G. P., HARPER, M. K., ET AL. 2002. Aldisine alkaloids from the Philippine sponge *Stylissa massa* are potent inhibitors of mitogen-activated protein kinase kinase-1 (MEK-1). *J. Med. Chem.* 45:529–532.

TEEYAPANT, R. and PROKSCH, P. 1993. Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba*—Evidence for an induced chemical defense. *Naturwissenschaften* 80:369–370.

THOMPSON, J. E., MURPHY, P. T., BERGQUIST, P. R., and EVANS, E. A. 1987. Environmentally induced variation in diterpene composition of the marine sponge *Rhopaleides odorabile*. *Biochem. Syst. Ecol.* 15:595–606.

THOMS, C., HORN, M., WAGNER, M., HENTSCHEL, U., and PROKSCH, P. 2003. Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation. *Mar. Biol.* 142:685–692.

TURON, X., BECERRO, M. A., and URIZ, M. J. 1996. Seasonal patterns of toxicity in benthic invertebrates: The encrusting sponge *Crambe crambe* (Poecilosclerida). *Oikos* 75:33–40.

TURON, X., MARTI, R., and URIZ, M. J. 2009. Chemical bioactivity of sponges along an environmental gradient in a Mediterranean cave. *Sci. Mar.* 73:387–397.

URIZ, M. J., TURON, X., BECERRO, M. A., GALERA, J., and LOZANO, J. 1995. Patterns of resource allocation to somatic, defensive and reproductive functions in the mediterranean encrusting sponge *Crambe crambe* (Demospongiae, Poecilosclerida). *Mar. Ecol. Prog. Ser.* 124:159–170.

VAN SOEST R. W. M., BOURY-ESNAULT N., HOOPER J. N. A., RÜTZLER K., DE VOOGD N. J., ALVAREZ DE GLASBY B., HAJDU E., PISERA A. B., MANCONI R., SCHOENBERG C., et al. 2010. World Porifera database. http://www.marinespecies.org/porifera.

VERSCHOOR, A. M., VOS, M., and VAN DER STAP, I. 2004. Inducible defences prevent strong population fluctuations in bi- and tritrophic food chains. *Ecol. Lett.* 7:1143–1148.

WAHL, M. 1989. Marine epibiosis.1. Fouling and ANTIFOULING—SOME BASIC ASPECTS. *MAR. ECOL. PROG. SER.* 58:175–189.

WRIGHT, J. T., DE NYS, R., and STEINBERG, P. D. 2000. Geographic variation in halogenated furanones from the red alga *Delisea pulchra* and associated herbivores and epiphytes. *Mar. Ecol. Prog. Ser.* 207:227–241.

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Are Leaf Glandular Trichomes of Oregano Hospitable Habitats for Bacterial Growth?

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Abstract Phyllospheric bacteria were isolated from microsites around essential-oil-containing glands of two oregano (*Origanum vulgare* subsp. *hirtum*) lines. These bacteria, 20 isolates in total, were subjected to bioassays to examine their growth potential in the presence of essential oils at different concentrations. Although there were qualitative and quantitative differences in the essential oil composition between the two oregano lines, no differences were recorded in their antibacterial activity. In disk diffusion bioassays, four of the isolated strains could grow almost unrestrained in the presence of oregano oil, another five proved very sensitive, and the remaining 11 showed intermediate sensitivity. The strain least inhibited by oregano essential oil was further identified by complete16s rRNA gene sequencing as *Pseudomonas putida*. It was capable of forming biofilms even in the presence of oregano oil at high concentrations. Resistance of *P. putida* to oregano oil was further elaborated by microwell dilution bioassays, and its topology on oregano leaves was studied by electron microscopy. When inoculated on intact oregano plants, *P. putida* was able not only to colonize sites adjacent to essential oil-containing glands, but even to grow intracellularly. This is the first time that such prolific bacterial growth inside the glands has been visually observed. Results of this study further revealed that several bacteria can be established on oregano leaves, suggesting that these bacteria have attributes that allow them to tolerate or benefit from oregano secondary metabolites.

Keywords Antimicrobial activity · Bacterial epiphytes · *Pseudomonas putida* · Phyllosphere · Essential oil · *Origanum vulgare* subsp. *hirtum*.

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Introduction

Bacteria are by far the most abundant dwellers of the phyllosphere, often found in numbers averaging 10^6 to 10^7 cells cm^{-2} leaf area (Lindow and Brandl, 2003). Epiphytic bacterial populations are unevenly distributed; they differ largely in size not only among plant species, individuals of the same species, and adjacent leaves of the same individual, but even among microsites of the same leaf (Hirano and Upper, 1989; Andrews, 1996; Yadav et al., 2004; Karamanoli et al., 2005). To a large degree, this variation is attributed to the rapid and pronounced alterations in the physical and nutritional characteristics of the phyllosphere (Lindow and Brandl, 2003; Beattie, 2007).

Temperature, water content, and solar radiation are prone to dramatic fluctuations within short time intervals. As a result, only strains easily adapted to contrasting environmental conditions have the ability to become successful epiphytes (Sundin and Jacobs, 1999). As leaf imprint studies suggest (Leben, 1988; Yadav et al., 2010) and leaf surface micrographs confirm (Lindow and Brandl, 2003; Monier

and Lindow, 2004; Baldotto and Olivares, 2008), bacterial epiphytes do not occur in a uniform pattern across leaf surfaces, yet they tend to be localized in particular microsites. Leaf surface structures such as stomata, veins, trichomes, and epidermal cell wall junctions are among them (Surico, 1993; Lindow and Brandl, 2003; Baldotto and Olivares, 2008). These potential habitats apparently constitute stress-protected and rich-in-water and nutrients sites, thus enabling bacterial growth (Leveau, 2006).

In this bacteria-plant interplay, the role of plant secondary metabolites has not yet been fully elucidated. It is widely accepted that such compounds, both constitutive and inducible, play an important role in plant life by mediating interactions between plants and other organisms (Bednarek and Osbourn, 2009). The role of constitutive metabolites (known as phytoanticipines) in particular, is believed to be rather important, since their accumulation on plant surfaces allows direct contact with pests and pathogens at the time of attack. In this context, plant appendages such as secretory cavities, ducts, or glandular trichomes (glands) are ideally positioned to provide toxic exudates as a first line of defense against attacking organisms, possibly offering time for the activation of more efficient induced defenses (Wagner et al., 2004).

Glandular trichomes are found on the surface of about 30 % of vascular plants. These epidermal protuberances are sites of secretion and accumulation of different compounds, such as Ca, Na, Mn, and Pb ions (Wagner et al., 2004), defensive proteins (Shepherd et al., 2005), and secondary metabolites. The latter are usually mixtures of various low molecular weight molecules, such as monoterpenoids, sesquiterpenoids, and phenylpropanoids (Hallahan, 2000; Duke et al., 2000). In the glandular trichomes of aromatic plants, essential oils are accumulated and further released from head cells in a species-dependent manner. Their contribution to leaf-bacteria interactions is not clear yet; they are considered both favorable sites for bacterial colonization, possibly due to local accumulation of nutrients (Lindow and Brandl, 2003), and hostile microenvironments for microbes due to the production of potent toxic metabolites (Wagner et al., 2004). The controversial role that glandular trichomes may play in microbial colonization could be of particular importance for determining the structure of epiphytic bacterial communities.

Previous studies by our research group have demonstrated that epiphytic bacterial populations of the aromatic plants *Lavandula angustifolia, Rosmarinus officinalis*, *Salvia fruticosa*, and *Origanum vulgare* subsp. *hirtum*, all growing at neighboring sites, are significantly different. Among them, *L. angustifolia* exhibited the highest bacterial colonization, being at the same time poor in drastic phenolic and isoprenoid constituents (Karamanoli et al., 2000). In contrast, when leaf bacterial populations of several perennial plants of a Mediterranean ecosystem were examined, the essential oil content of *Myrtus communis* L., *Lavandula stoechas* L., *Calamintha nepeta* (L.) Savi, and *Melissa officinalis* L. was not proven to be a major determinant of bacterial colonization (Yadav et al., 2005).

This study is a contribution to understanding the factors that determine bacterial colonization of the phyllosphere and, more specifically, those of aromatic plants that abound in the Mediterranean environment. It focuses on a widely used and well studied aromatic plant, the Greek oregano [*Origanum vulgare* subsp. *hirtum* (Link.) Ietswaart)] and examines (a) the importance of its dense leaf glandular trichomes in bacterial colonization, (b) whether minor differences in oregano essential oil generate different effects on microbes, (c) the predictability of bacterial colonization of oregano phyllosphere on the basis of microbial sensitivity to oregano essential oil, as evidenced in laboratory bioassays, and (d) features that may enable bacteria to become successful colonizers of aromatic plants.

Methods and Materials

Plant Material Greek oregano is a species of the Labiatae family, with a strong aromatic character. As holds true for many members of this family, its leaves are covered with trichomes, both glandular and non glandular (Bozabalidis, 2002). The major constituents of the essential oil accumulated in oregano glands are the monoterpenoid phenols, thymol and carvacrol (Vokou et al., 1993). These two isomers are potent antimicrobial agents, having both antifungal and antibacterial activity in laboratory bioassays (Karamanoli et al., 2000; Vokou, 2007; Kadoglidou et al., 2011). Plants from two different oregano lines, GrO and O-5, were used, kindly provided by Ecopharm Hellas S.A. and the research team of Professor Kalidas Shetty, respectively; O-5 is a phenolic-rich oregano clone originated from a single heterozygous seed (Eguchi et al., 1996; Shetty et al., 1996).

Essential Oil Analysis Essential oil analysis was performed as previously described (Karamanoli et al., 2000). In brief, leaves (100 g) from the two oregano lines were steam distilled in a Clevenger apparatus for 3 hr. The isolated essential oil was dried over sodium sulfate and analyzed in an HP 5890 gas chromatograph coupled with a VG Trio Mass Spectrometer. The column was an HP-5MS capillary column. Conditions were the following: linear velocity 31.9 cm sec^{-1}, oven temperature programmed from 60 to 240 °C at a 38 °C min^{-1} rate of increase, electron energy 70 eV, and split ratio 1:20.

Reference Bacterial Strains and Media Two bacterial strains, *Pseudomonas syringae* van Hall strain BPIC 5 and *Erwinia amylovora* strain BPIC 980 were kindly provided by the Benaki Phytopathological Institute, Athens Greece. They were used as reference epiphytic organisms in all bioassays. Nutrient Agar Medium (NAG) supplemented

with 0.3 % of the fungicide natamycin (Gist-Brocades, Delft, The Netherlands) was used as a standard growth medium. Rifampicin (100 μg ml^{-1}) was added in the NAG rif media (Karamanoli et al., 2005).

Isolation of Bacteria from the Vicinity of Oregano Glandular Trichomes To obtain bacteria with potential resistance to the oregano essential oil, samples from the area around active glandular trichomes on leaves of both oregano GrO and O-5 lines were collected under the stereoscope by using sterile wooden sticks. Samples were aseptically transferred on NAG-containing plates and incubated for 48 to 72 hr at 22–24 °C. Bacterial colonies grown on the plates were then transferred to fresh agar plates to obtain pure cultures.

Characterization of the Isolated Bacteria The morphology (shape and color) of the isolated bacterial colonies as well as their biochemical characteristics (Gram stain, oxidase and catalase response) were determined. The morphology of single colonies on NAG media was observed under a light microscope. Gram staining of bacterial isolates was implemented according to Vincent (1970). Oxidase and catalase tests were performed as described by Steel (1961) and MacFaddin (1980), respectively.

The bacterium isolate that exhibited the highest resistance to essential oils was sub-cultured on NAG plates. After 72 hr of growth at 22–24 °C, bacterial cells were harvested with 1 ml sterile dd H_2O and pelleted by centrifugation. Their DNA was then extracted using the Nucleospin Tissue kit (Macherey-Nagel, Germany). The 16S rRNA gene of the bacterial isolates was amplified using the universal bacterial primers 8 f – 1512 r as described previously (Karpouzas et al., 2010). The product obtained was purified (Nucleospin II clean-up kit, Macherey-Nagel, Germany), cloned into plasmid vector pGEM®-T (Promega, Madison, USA), and transformed into *Escherichia coli* (DH5a Competent Cells, Invitrogen, USA) following standard procedures (Sambrook et al., 1989). Three clones were sequenced according to manufacturers' instructions using a PRISM BigDye Terminator Cycle Sequence reaction kit (Applied Biosystems, UK). In all cases, the products were sequenced in both directions to derive the full length sequence of the 16S rRNA gene. Sequences were deposited in the European Molecular Biology Laboratory (EMBL) database under accession number FR725962. Similarity comparisons to known 16S rRNA gene sequences were performed using the Basic Local Alignment Search Tool (BLAST).

Evaluation of Bacterial Isolate Resistance to Oregano Essential Oils—Disk Diffusion Bioassays To initially evaluate the ability of bacterial isolates to grow in the presence of oregano essential oils, disc diffusion bioassays were used. Essential oils from GrO and O-5 oregano plants were applied (5 μl per disc) as previously described (Karamanoli et al., 2000). *Pseudomonas syringae* strain BPIC 5 and *Erwinia amylovora* strain BPIC 980 also were examined in the bioassays as reference microorganisms.

MIC and MBC Assessment of Oregano Essential Oil The bacterial isolate showing the highest resistance to oregano essential oil in the disc diffusion bioassay was further examined for its response to a range of essential oil concentrations by applying a microwell dilution bioassay. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of the essential oils from the two oregano lines were determined. The bioassays were performed in 96-well microtiter plates where oregano essential oils at different concentrations were applied. Fresh overnight bacterial cultures in NG broth (Karamanoli et al., 2000) were inoculated in each well. The essential oil concentrations tested were 0, 0.625, 1.250, 1.875, 2.5, 3.125, 3.75, 4.375, 5.0, and 10.0 μl ml^{-1} broth. Bacterial inhibition was recorded by measuring the optical density (OD) at 600 nm. The MIC value was assigned to the lowest essential oil concentration that totally inhibited bacterial growth. The MBC value was determined as follows: From the content of the wells where zero OD was recorded, 30 μl were inoculated onto NAG-containing plates. The MBC value was assigned to the lowest concentration of the essential oil at which no bacterial growth was recorded in the plates following a 48-hr incubation at 22–24 °C. Each treatment had six replicates, and each bioassay was run twice. *P. syringae* strain BPIC 5 and *E. amylovora* BPIC 980 also were examined in these bioassays as reference microorganisms.

Evaluation of the Colonization Ability of Selected Bacteria on Oregano Plants Two bacterial strains were tested for their ability to colonize intact plants: (i) the isolated strain that exhibited the highest resistance to oregano oil in the bioassays described above, and (ii) the least tolerant reference strain. Rifampicin resistant strains of these two bacteria were prepared as described previously (Andersen et al., 1991; Karamanoli et al., 2005), and each was inoculated on five O-5 oregano plants. Inoculation of oregano plants was performed by spraying leaves to run off with aqueous suspensions of each bacterial culture. Following inoculation, plants were placed in a moist chamber, and bacterial colonization was evaluated an hour after spraying (0 time) and at 24 hr intervals thereafter over a 72 hr period, as described elsewhere (Karamanoli et al., 2005). Recovery of bacteria from non-inoculated oregano plants was evaluated as well on rifampicin containing plates.

Visualization of Bacterial Colonization Topography Bacterial topography on oregano leaves, especially in the vicinity of glandular trichomes was studied by scanning electron

microscopy (SEM) 48 hr after plant inoculation. Two samples (0.5–1.0 cm^2) from each of 5 oregano plants were scanned. Both the abaxial and adaxial leaf blade surfaces were examined. Preparation of leaf segments for SEM was performed as described by Karamanoli et al. (2005). In brief, small pieces of oregano leaves were initially fixed for 3 hr with 5 % glutaraldehyde in 0.05M phosphate buffer (pH 7.0). Post-fixation was performed with 1 % osmium tetroxide, similarly buffered (5 °C, 4 hr). After dehydration in an alcohol series, the specimens were critical point dried on a Balzers CPD 030 device, coated with carbon in a JEE-4X vacuum evaporator and observed with a JSM 840-A scanning electron microscope at 20 kv.

Biofilm Formation Assay The ability of the bacterial isolate with the highest resistance to oregano oil to form biofilms was evaluated. The reference bacteria *Pseudomonas syringae* strain BPIC 5 and *Erwinia amylovora* strain BPIC 980 also were included in the study for comparison purposes. The method applied was the one described by Nakhamchik et al. (2008) with minor modifications: Fresh overnight bacterial cultures grown in NG broth were inoculated into 96-well microtiter plates (10 μl broth per culture). Essential oils were added to each well at concentrations of 0, 0.625, 2.5, and 5 μl ml^{-1}. Following a 48 hr incubation at 22–24 °C, bacterial growth was recorded by measuring optical density (OD cells) at 600 nm. The broth was removed carefully and the wells were washed (× 3) with phosphate buffer. The wells then were air-dried, and stained with a 0.3 % crystal violet (CV) solution. The plate was subsequently washed once more with phosphate buffer, and the wells were air-dried. Ethanol (200 μl) was added to the wells to dilute the dye, and the OD (OD CV) was recorded at 600 nm. The OD CV:OD cells ratio was calculated to determine the relative level of biofilm formation. Six replicates per treatment were used and the bioassay was performed twice.

Statistical Analysis Statistical analysis was conducted with SPSS Statistics 19.0 software package (SPSS, Inc. Chicago, IL, USA). Bacterial growth in the presence of O-5 and GrO oregano oil, as evaluated in the disc diffusion and microwell dilution bioassays, was analyzed using a three-way analysis of variance, with oil concentration, oil origin, and bacteria as factors. Biofilm production from the different bacteria in the presence of oregano oil was analyzed using a two-way analysis of variance with oil concentration and strain as factors. In all statistical tests, significance was determined at $P<0.05$.

Results

Composition of Leaf Essential Oils The overall yield of essential oil (2.3±0.1 ml oil per 100 g dry leaf material) did not differ between the two oregano lines. GC-MS analysis revealed that the isomers carvacrol and thymol were the main essential oil constituents accounting for 77.3 and 65.8 % of the GrO and O-5 oil, respectively (Table 1). In both essential oils, oxygenated compounds were by far more abundant than hydrocarbons, their concentration in the O-5 was higher than in the GrO oil by approximately 20 %. The O-5 oil contained only monoterpenoids, whereas the sesquiterpenes β-caryophyllene, γ-murolene, and β-bisabolene were in addition, detected in the GrO oil.

Isolation of Bacteria from Glandular Trichomes—Initial Assessment of Resistance Forty bacterial colonies were obtained from microsites around glandular trichomes of oregano O-5 and Gr-O leaves. After incubation and subculturing, twenty were macroscopically recognized as different bacterial isolates and were further grown in monocultures. The morphological and biochemical characteristics of these isolates are summarized in Table 2. Half of the isolates were Gram positive and half were Gram negative.

Table 1 Qualitative and quantitative composition of essential oils from the two oregano lines, O-5 and GrO

Compounds	RI	O-5	GrO
α-thujene	930	0.35	0.98
α-pinene	939	–	0.53
camphene	954	–	0.19
sabinene	975	–	0.15
1-octen-3-ol	979	2.89	0.15
β-myrcene	991	0.20	1.50
3-octanol	993	0.55	–
α-phellandrene	1003	–	0.19
α-terpinene	1017	–	1.06
p-cymene	1025	7.71	13.20
γ-terpinene	1060	0.52	10.16
cis-sabinene hydrate	1070	0.66	0.54
linalool	1097	0.40	–
borneol	1159	0.91	0.58
terpinen-4-ol	1177	0.49	0.30
thymol methyl-ether	1235	3.40	–
thymoquinone	1252	0.67	–
thymol	1286	0.27	10.43
carvacrol	1295	76.98	55.38
β-caryophyllene	1412	–	0.81
γ-muurolene	1475	–	0.20
β-bisabolene	1501	–	2.98
Total (%)		96.00	99.33
Hydrocarbons (%)		8.78	31.95
Oxygenated compounds (%)		87.22	67.38

RI retention index

Table 2 Morphological and biochemical characteristics of the 20 isolates inhabiting glandular trichome sites of oregano leaves, and size of the inhibition zone in a disk diffusion bioassay following exposure to 5 μl of O-5 oregano essential oil

No of bacterial isolate	Gram stain	Cell shape	Oxidase	Catalase	Inhibition zone (mm)
1	−	coccus	−	+	6.0±0.3
2	+	rod	+	+	6.0±0.7
3	+	rod	−	+	6.0±0.1
4	+	rod	−	+	7.5±0.7
5	+	coccus	−	+	8.0±0.6
6	−	coccus	−	+	8.0±0.5
7	−	coccus	+	+	8.5±0.5
8	+	coccus	−	+	9.0±0.3
9	+	coccus	−	+	9.0±1.3
10	+	coccus	−	+	9.0±0.5
11	+	rod	+	+	9.0±0.5
12	−	irreg. coccus	+	+	9.0±0.7
13	−	rod	−	+	9.5±0.1
14	−	coccus	+	+	9.5±0.5
15	−	coccus	−	+	10.5±0.6
17	−	coccus	−	+	11.5±0.5
18	−	rod	−	+	11.0±0.7
19	+	rod	+	+	14.0±0.5
20	+	rod	−	+	21.5±1.5
21	−	rod	+	+	4.5±0.3
Pseudomonas syringae					6.0±0.3
Erwinia amylovora					20.0±0.5

All isolates were found catalase positive, and among the Gram negative isolates all but four were oxidase negative.

Disc diffusion bioassays were applied in order to test the ability of isolates to grow in the presence of the two oregano essential oils. At an application dose of 5 μl essential oil per disc (extracted from either oregano line), the inhibitory zone was negligible (<5 mm) for only one of the isolates (no 21), very narrow (6 mm) for three isolates, <10 mm for 11 isolates, and of considerable size (>10 mm) for the remaining five isolates (Table 2, exemplified with O-5 oregano oil). The two reference strains differed considerably in their sensitivity: the inhibitory zone for *P. syringae* was only 6 mm, whereas that for *E. amylovora* was 20 mm.

The isolate exhibiting the highest resistance to oregano essential oil was further identified. Sequencing of its 16S rRNA gene showed highest sequence homology to *Pseudomonas* strains and maximum sequence identity (99.7 %) to the 16S rRNA gene of a *Pseudomonas putida* strain (Accession Number EU275363).

MIC and MBC Determination of Oregano Essential Oils Resistance of the *P. putida isolate 21*, obtained from oregano leaves, to oregano oil extracted from O-5 and GrO plants, was further studied and compared to that of *E. amylovora* and *P. syringae*. A three-way ANOVA of the results obtained from the microwell dilution bioassay revealed that the level of essential oil concentration and the type of bacteria tested (*P. putida* isolate *21, E. amylovora* and *P. syringae*) had a significant effect on bacteria survival ($P<0.001$). In contrast, the origin of the essential oil (GrO or O-5 plants) did not significantly affect bacterial survival ($P>0.05$) and, therefore, respective data were pooled. Interactions between essential oil concentration and bacteria also were significant ($P<0.001$, in all cases).

Minimum inhibitory concentrations and MBC of oregano essential oils are presented in Fig. 1 (a–c). *Pseudomonas putida* isolate 21 was the most resistant bacterium: its MIC and MBC values were significantly higher than the corresponding values of the other two bacteria tested ($P<0.001$). MIC and MBC values were 5 and 10 μl of oregano essential oil ml^{-1} broth respectively for *P. putida* isolate 21 (Fig. 1a), 4.4 μl and 5.0 μl for *P. syringae* (Fig. 1b), and only 1.8 μl and 2.5 μl for *E. amylovora* (Fig. 1c).

Colonization of Oregano Plants by P. putida Isolate 21 The most resistant among the bacterial isolates, *P. putida* isolate 21 was selected, inoculated on O-5 oregano plants, and its growth studied. GrO plants were not used in this assay, since no differences in antibacterial activity between the essential oils of the two plant lines were detected in bioassays previously described. The colonization ability of the *P. putida* isolate 21 was compared to that of *E. amylovora*. *Pseudomonas putida* populations on oregano leaves 24 hr after inoculation were more than a hundred fold higher than those of *E. amylovora* (5.8×10^4 vs. 8×10^6 CFU $leaf^{-1}$), a difference persisting at 48 and 72 hr after inoculation. No bacteria were detected at any time on samples from non-inoculated oregano plants (controls).

Leaf segments of oregano plants were cut and subsequently prepared for SEM at 48 hr after inoculation with *P. putida* isolate 21. Examining the topography of bacterial growth in the area of glandular trichomes, bacterial cells almost attached to intact glandular trichomes could be seen (Fig. 2 a,b). Bacterial growth was even more pronounced in dissected glandular hairs (Fig. 3 a–d). No bacterial cells were detected in the vicinity of glandular trichomes of leaves inoculated with *E. amylovora*.

Biofilm Formation by P. putida Isolate 21 In order to explore possible mechanisms for the successful colonization and attachment of the *P. putida* isolate 21 cells on the glandular trichomes, the bacterium ability to form biofilms in the presence of different concentrations of oregano

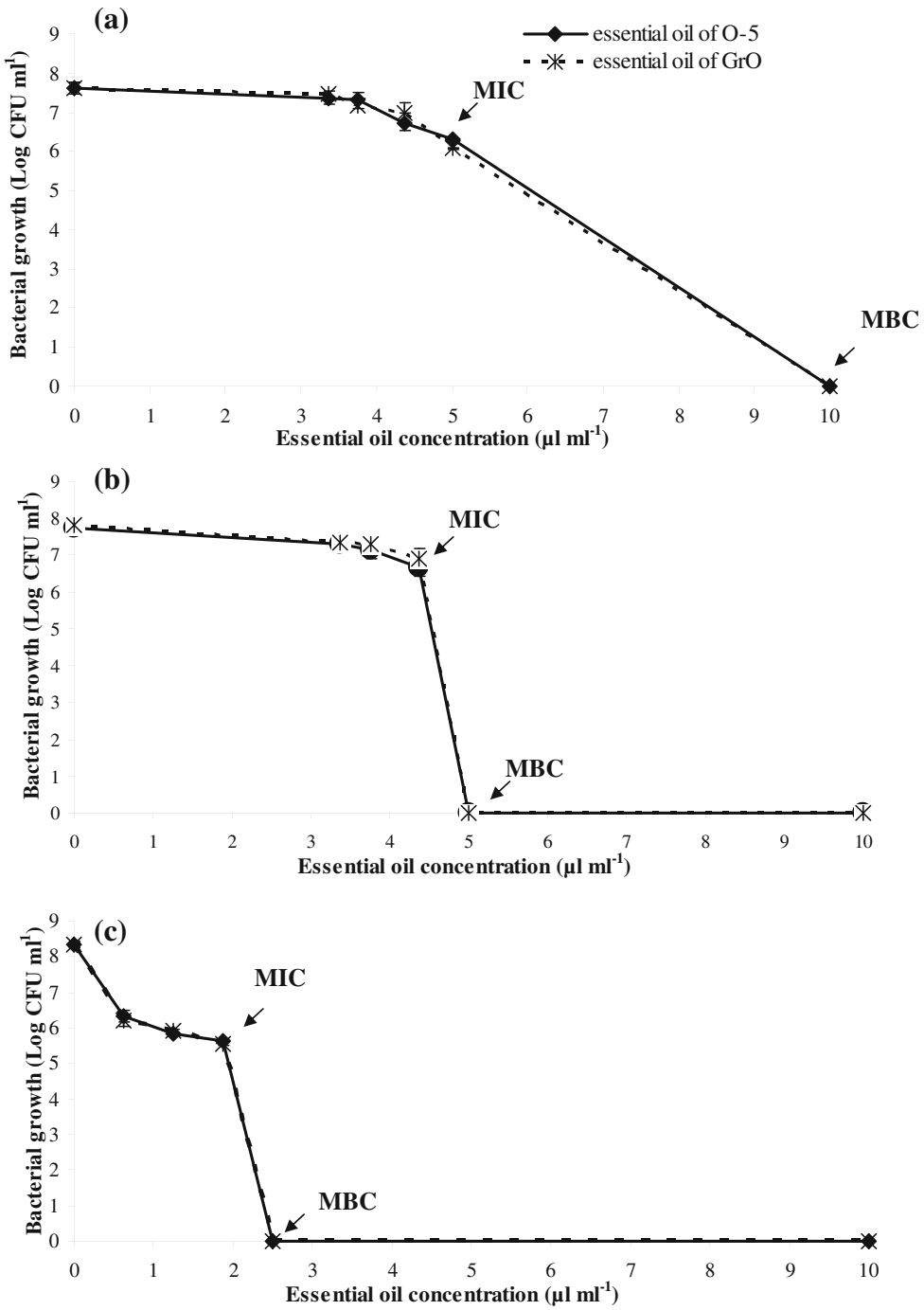

Fig. 1 Bacterial growth of *Pseudomonas putida* isolate 21 (**a**), *P. syringae* (**b**), and *Erwinia amylovora* (**c**) in the presence of essential oil extracted from leaves of O-5 and GrO oregano lines, at different concentrations. *Arrows* indicate the MIC and MBC values

essential oil was evaluated. Biofilm formation by the reference strains *P. syringae* and *E. amylovora* also was examined. Among the three bacteria tested, *P. putida* isolate 21 was the most efficient in biofilm formation ($P<0.05$). In the absence of essential oil, *P. putida* was able to produce 3.5× more biofilm compared to the other two bacteria tested (Fig. 4). In addition, *P. putida* was able to produce significantly more biofilm compared to the other strains at least at essential oil concentration approaching the MIC value (5 µl ml^{-1}) determined for this strain.

Discussion

Essential oils of the two oregano lines differed qualitatively. Participation of oxygenated compounds in the essential oil from O-5 plants was about 20 % higher than from GrO plants, whereas participation of sesquiterpenes was detectable only in GrO plants. Despite the differences in chemistry, there were no substantial differences in antibacterial activity. Both oils had a high content (>65 %) of the phenolic volatiles carvacrol and thymol, which are well known for

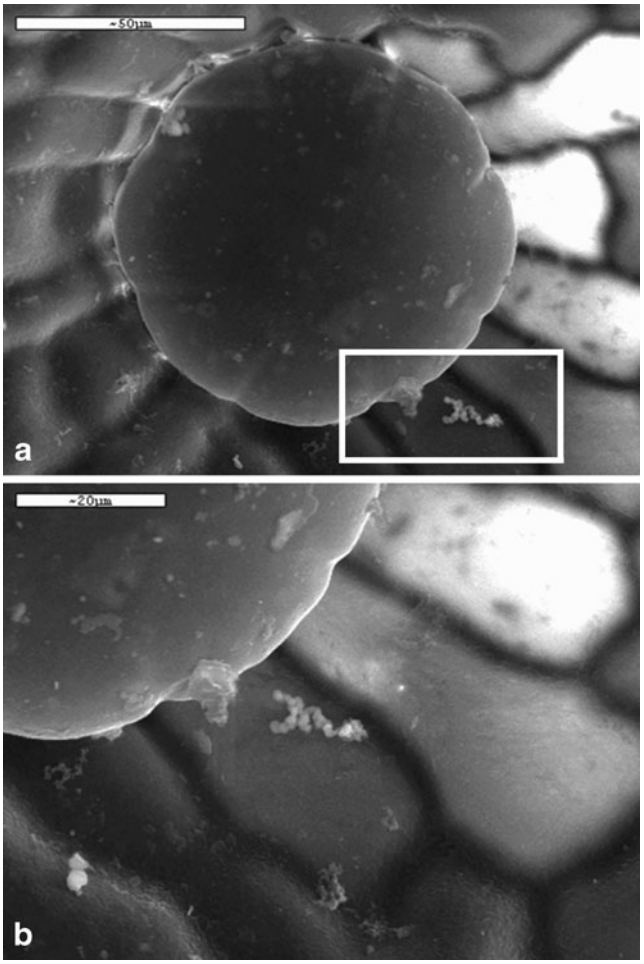

Fig. 2 Bacterial populations in the vicinity of an intact peltate glandular trichome (subcuticular space filled with essential oil) of oregano leaf. Scale bars: **a** 50 μm and **b** 20 μm

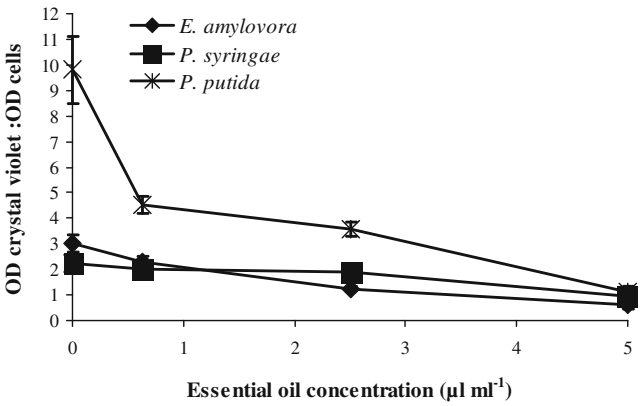

Fig. 4 Biofilm formation by *Pseudomonas putida* isolate 21, *Erwinia amylovora* strain 980 and *P. syringae* strain BPIC 5 as quantified by OD Crystal *Violet*: OD cells ratio following a 48 hr incubation in the presence of oregano essential oil at different concentrations

their biological potency (Kubo et al., 1992; Karamanoli et al., 2000; Cristani et al., 2007; Vokou, 2007). For example, the two isomers showed strong activity against *Erwinia* and *Pseudomonas* strains, as exemplified by their MIC and MBC values (Karamanoli et al., 2000). GrO oil had presently a lower content (65.8 %) of the two isomers than the O-5 oil (77.3 %) but a higher representation of the isomers' precursors, *p*-cymene and γ-terpinene. All together, the four highly related compounds made up the bulk of the two essential oils (85.5 % for O-5 and 87.4 % for GrO plants). They probably acted synergistically (Ultee et al. 2000, 2002), thus minimizing composition differences and leading to similar bioactivities of the two oils.

Several studies have suggested that glandular trichomes are included among the favorable sites for bacterial growth, probably because they provide protection from environmental

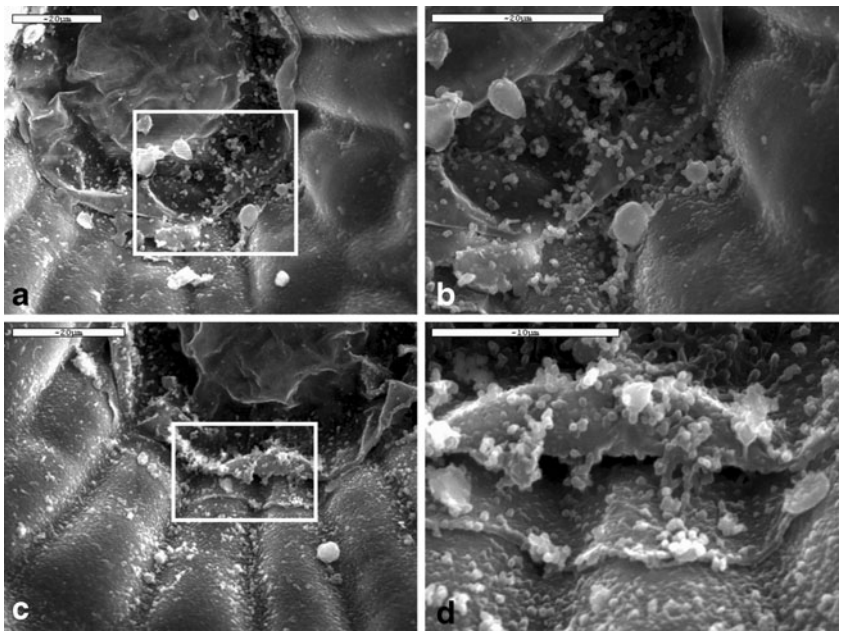

Fig. 3 **a–d** Intracellular colonization of bacteria observed within head cells of broken oregano peltate glandular trichomes. Periclinal cell wall of head cells is removed. Scale bars: **a**–**c** 20 μm, **d** 10 μm

stresses and/or food through nutrient release to bacteria. This has been reported for leaf glands of *Phaseolus vulgaris* (Lindow and Brandl, 2003), *Cucumis sativus* and *Leonurus sibiricus* (Baldotto and Olivares, 2008), i.e., for glands located on leaves of crop plants. In their case, most of the trichome-derived metabolites have been lost during domestication (McDowell et al., 2011). However, this is not the case for glands and metabolites of aromatic plants; the glandular trichome is a feature desired and preserved in domesticated aromatic plants, hence, the associated activity of their metabolites also is preserved.

In the present study, six out of the ten Gram-negative bacteria isolated from the vicinity of leaf glandular trichomes of both O-5 and GrO oregano lines were found oxidase negative, suggesting members of Enterobacteraceae as successful colonizers of sites around oregano glands. This is not surprising, since strains of Enterobacteraceae along with strains belonging to the family of Pseudomonadaceae are common members of the phyllosphere microbial community (Hunter et al., 2010; Vokou et al., in press). Regardless of Gram-staining, the 20 bacteria isolated from sites adjacent to grandular trichomes were all found catalase positive. This indicates that only bacteria armed with mechanisms that enable them to cope with oxidative stress may survive in sites around glands.

The bacterial isolate no 21 that was the most resistant to oregano oil was identified as a *P. putida* strain by sequencing its 16S rRNA gene. Bacteria belonging to *P. putida* are common saprophytes in well-aerated soil and water systems (Timmis, 2002) and can reach leaf surfaces through splashing or aerosols. Moreover, they are characterized as metabolically versatile microorganisms that can recycle different organic compounds including those with aromatic rings (Loh and Cao, 2008). For example, a *P. putida* strain, isolated from the soil, was capable of using essential oil constituents as carbon and energy sources (Vokou et al., 2002). Thus, the ability of *P. putida* isolate 21 to colonize sites inside or adjacent to essential-oil-containing glands, when inoculated on intact oregano plants, may be attributed to a large degree to its efficacy in utilizing essential oils as food sources. Regarding the intracellular colonization success of *P. putida*, it is not clear whether the strain could cause or trigger the rupture of the gland after its internal growth, or if it grew in large numbers in an already broken gland. Whatever the case, this is the first time that bacteria are shown to have such a prolific growth inside glandular hairs of aromatic plants.

The ability of bacterial isolates to form biofilms is of great importance for the colonization of solid surfaces. Few studies have addressed the characteristics and the particular function of biofilms on leaf surfaces (Warner et al., 2008). However, it is generally acknowledged that microbial cells within biofilms have better chances of adaptation and survival due to the protection from the biofilm matrix (Mah and O'Toole, 2001; Loh and Cao, 2008). In the present study, *P. putida* isolate 21 was able to form biofilms in the presence of oregano essential oil, even at high oil concentrations. This could be an additional attribute that allows this bacterium to efficiently colonize leaves and glands of oregano plants.

Sensitivity of the 20 strains isolated from glandular trichome sites to oregano oils was tested in disc diffusion bioassays. Not all 20 isolates were found tolerant, as one might have expected from the field observation: only one bacterial strain (no 21) grew almost normally, and another three strains tolerated oregano oil efficiently. The rest showed intermediate (11 strains) to high (5 strains) sensitivity. Unless most of these strains are not true colonizers, this suggests that the estimated sensitivity in lab experiments does not fully represent real conditions *in situ*; else, non-tolerant strains should not have survived on oregano leaves, particularly in the area of leaf glandular hairs.

It should be noted that previous studies regarding bacterial colonization of aromatic and non-aromatic Mediterranean plants led to inconclusive results; Yadav et al. (2004, 2005, 2008) showed that aromatic plants did not support less abundant or less diverse microbial communities in comparison to other co-existing plants, whereas Karamanoli et al. (2000) found the epiphytic microbial abundance of aromatic plants to be related, at least to a certain degree, to the concentration and activity of the leaf secondary metabolites that they contained. Such results raised the issue of how comparable laboratory experiments and field trials are, and how predictable the relationship between bacteria and aromatic plants *in situ* is. It is evident from the results of this study that the high antibacterial activity of oregano essential oil observed in laboratory bioassays, is not fully verified in the field. Apparently, several bacteria have attributes that allow them to tolerate these secondary metabolites or even benefit from them under field conditions. The ability to be protected from oxidative stress or to form biofilms possibly is among them, along with other yet unidentified attributes. On the other hand, differences in content and composition of essential oils, as well as in density of glandular trichomes may be important factors and should be taken into consideration when studying and interpreting bacterial colonization of the phyllosphere *in situ*.

The present study shows that bacterial epiphytes, identified as belonging to *P. putida*, are able to colonize sites adjacent to or even inside oregano leaf glands and to grow there prolifically. It also shows that oregano plants can be colonized in the field by bacteria that have been found sensitive to oregano oil in laboratory tests. These results demonstrate that the prediction of the identity and localization of bacteria in nature is not a simple task, and that laboratory sensitivity tests are not always adequate

predictors of the microbial colonization on aromatic plant phyllosphere.

References

ANDERSEN, G. L., MENKISSOGLU, O., and LINDOW, S. E. 1991. Occurrence and properties of copper-tolerant strains of *Pseudomonas syringae* isolated from fruit trees in California. *Phytopathology* 81:648–656.

ANDREWS, J. H. 1996. Phyllosphere ecology: past, present and future, pp. 285–294, *in* C. E. Morris, P. C. Nicot, and C. Nguyen (eds.), Aerial Plant Surface Microbiology. Plenum, New York.

BALDOTTO, L. and OLIVARES, F. 2008. Phylloepiphytic interaction between bacteria and different plant species in a tropical agricultural system. *Can. J. Microbiol.* 54:918–931.

BEATTIE, G. A. 2007. Plant associated bacteria: survey, molecular phylogeny, genomics and recent advances, pp. 1–56, *in* S. S. Gnanamanickan (ed.), Plant associated bacteria. Springer, Dordrecht.

BEDNAREK, P. and OSBOURN, A. 2009. Plant–microbe interactions: Chemical diversity in plant defense. *Science* 324:746–748.

BOZABALIDIS, A. M. 2002. Structural features of *Origanum* sp, pp. 11–64, *in* S. E. Kintzios (ed.), Oregano. The genera Origanum and Lippia. Taylor & Francis, London.

CRISTANI, M., ARRIGO, M., MANDALARI, G., CASTELLI, F., SARPIETRO, M. G., AND MICIELI, D. 2007. Interaction of four monoterpenes contained in essential oils with model membranes: Implications for their antibacterial activity. *J. Agric. Food Chem.* 55:6300–6308.

DUKE, S. O., CANEL, C., RIMANDO, A. M., TELLEZ, M. R., DUKE, M. V., AND PAUL, R. N. 2000. Current and potential exploitation of plant glandular trichome productivity. *Adv. Bot. Res.* 31:121–141.

EGUCHI, Y., CURTIS, O. F., and SHETTY, K. 1996. Interaction of hyperhydricity-preventing *Pseudomonas*sp. with oregano (*Origanum vulgare*) and selection of high rosmarinic acid-producing clonal lines. *Food Biotechnol.* 10:191–202.

HALLAHAN, D. L. 2000. Monoterpenoid biosynthesis in glandular trichomes of Labiate plants. *Adv. Bot. Res.* 31:77–111.

HIRANO, S. S. and UPPER, C. D. 1989. Diel variation in population size and ice nucleation activity of *Pseudomonas syringae* on snap bean leaflets. *Appl. Environ. Microbiol.* 55:623–630.

HUNTER, P. J., Hand, P., PINK, D., WHIPPS, J. M., AND BENDING, G. D. 2010. Both leaf properties and microbe–microbe interactions influence within-species variation in bacterial population diversity and structure in the Lettuce (Lactuca Species) phyllosphere. *Appl. Environ. Microbiol.* 76:8117–8125.

KADOGLIDOU, K., LAGOPODI, A., KARAMANOLI, K., VOKOU, D., BARDAS, G. A., MENEXES, G., and CONSTANTINIDOU, H.-I. A. 2011. Inhibitory and stimulatory effects of essential oils and individual monoterpenoids on growth and sporulation of four soil-borne fungal isolates of *Aspergillus terreus, Fusarium oxysporum, Penicillium expansum*, and *Verticillium dahlia*. *Eur. J. Plant Pathol.* 130:297–309.

KARAMANOLI, K., MENKISSOGLU-SPIROUDI, U., VOKOU, D., and CONSTANTINIDOU, H.-I. A. 2000. Bacterial colonization of the phyllosphere of the phyllosphere of Mediterranean aromatic plants. *J. Chem. Ecol.* 26:2035–2048.

KARAMANOLI, K., MENKISSOGLU-SPIROUDI, U., BOSABALIDIS, A. M., VOKOU, D., and CONSTANTINIDOU, H.-I. A. 2005. Bacterial colonization of the phyllosphere of nineteen plant species and antimicrobial activity of their leaf secondary metabolites against leaf associated bacteria. *Chemoecology* 15:59–67.

KARPOUZAS, D. G., NTOUGIAS, S., ISKIDOU, E., ROUSIDOU, C., PAPADOPOULOU, K. K., ZERVAKIS, G., and EHALIOTIS, C. 2010. Olive mill wastewater affects the structure of soil bacterial communities. *Appl. Soil Ecol.* 45:101–111.

KUBO, I., MUROI, H., and HIMEJIMA, M. 1992. Antimicrobial activity of green tea flavor components and their combination effects. *J. Agric. Food Chem.* 40:245–248.

LEBEN, C. 1988. Relative humidity and the survival of epiphytic bacteria with buds and leaves of cucumber plants. *Phytopathology* 78:179–185.

LEVEAU, J. 2006. Microbial communities in the phyllosphere, pp. 334–367, *in* M. Riederer and C. Muller (eds.), Biology of the plant cuticle. Annual plant reviews Vol. 23. Blackwell, Oxford.

LINDOW, S. E. and BRANDL, M. T. 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69:1875–1883.

LOH, K. C. and CAO, B. 2008. Paradigm of biodegradation using *Pseudomonas putida*—A review of proteomics studies. *Enzyme Microb. Technol.* 43:1–12.

MACFADDIN, J. F. 1980. pp. 51–54, Biochemical tests for identification of medical bacteria. Williams and Wilkins, Baltimore.

MAH, T. F. and O'TOOLE, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9:34–39.

MCDOWELL, E. T., KAPTEYN, J., SCHMIDT, A., LI, C., KANG, J. H., DESCOUR, A., SHI, F., LARSON, M., SCHILMILLER, A. A., AN, L., JONES, A. D., PICHERSKY, E., SODERLUND, C. A., and GANG, D. R. 2011. Comparative functionally genomic analysis of S*olanum* glandular trichome types [1] [W] [OA]. *Plant Physiol.* 155:524–539.

MONIER, J. M. and LINDOW, S. E. 2004. Frequency, size and localization of bacterial aggregates on bean leaf surfaces. *Appl. Environ. Microbiol.* 70:346–355.

NAKHAMCHIK, A., WILDE, C., and ROWE-MAGNUS, D. A. 2008. Cyclic-di-GMP regulates extracellular polysaccharide production, biofilm formation and rugose colony development by Vibrio vulnificus. *Appl. Environ. Microbiol.* 74:4199–4209.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York.

SHEPHERD, R. W., BASS, W. T., HOUTZ, R. L., and WAGNER, G. J. 2005. Phylloplanins of tobacco are defensive proteins deployed on aerial surfaces by short glandular trichomes. *Plant Cell* 17:1851–1861.

SHETTY, K., CURTIS, O. F., and LEVIN, R. E. 1996. Specific interaction of mucoid strains of Pseudomonas spp. with oregano (*Origanum vulgare*) clones and the relationship to prevention of hyperhydricity in tissue culture. *J. Plant Physiol.* 149:605–611.

STEEL, K. J. 1961. The oxidase reaction as a toxic tool. *J. Gen. Microbiol.* 25:297–306.

SUNDIN, G. W. and JACOBS, J. L. 1999. Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogeae* L.). *Microb. Ecol.* 38:27–38.

SURICO, G. 1993. Scanning electron microscopy of olive and oleander leaves colonized by *Pseudomonas syringae* subsp. *savastanoi*. *J. Phytopathol.* 138:31–40.

TIMMIS, K. N. 2002. *Pseudomonas putida*: a cosmopolitan opportunist par excellence. *Environ. Microbiol.* 4:779–781.

ULTEE, A., SLUMP, R. A., STEGING, G., and SMID, E. J. 2000. Antimicrobial activity of carvacrol on rice. *J. Food Prot.* 63:620–624.

ULTEE, A., BENNIK, M. H. J., and MOEZELAAR, R. 2002. The phenolyc hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Appl. Environ. Microbiol.* 68:1561–1568.

VINCENT, J. M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell, Oxford.

VOKOU, D. 2007. Allelochemicals, allelopathy and essential oils: a field in search of definitions and structure. *Allelopathy J.* 19:119–135.

VOKOU, D., KOKKINI, S., and BESSIERE, J.-M. 1993. Geographic variation of Greek oregano (*Origanum vulgare* ssp. *hirtum*) essential oils. *Biochem. Syst. Ecol.* 21:287–295.

VOKOU, D., CHALKOS, D., YANGOU, M., and KARAMANLIDOU, G. 2002. Activation of soil respiration and shift of the microbial population balance in soil as a response to *Lavandula stoechas* essential oil. *J. Chem. Ecol.* 28:755–768.

VOKOU, D., VARELI, K., ZARALI, E., KARAMANOLI, K., CONSTANTINIDOU, H.-I. A., MONOKROUSOS, N., HALLEY, J. M., and SAINIS, I. In press. Exploring biodiversity in the bacterial community of the Mediterranean phyllosphere and its relationship with airborne bacteria. *Microb. Ecol.*

WAGNER, G. J., WANG, E., and SHEPHERD, R. W. 2004. New approaches for studying and exploiting an old protuberance, the plant trichomes. *Ann. Bot.* 93:3–11.

WARNER, J. C., ROTHWELL, S. D., and KEEVIL, C. W. 2008. Use of episcopic differential interference contrast microscopy to identify bacterial biofilms on salad leaves and track colonization by *Salmonella* Thompson. *Environ. Microbiol.* 10:918–925.

YADAV, R. K. P., HALLEY, J. M., KARAMANOLI, K., VOKOU, D., and CONSTANTINIDOU, H.-I. A. 2004. Bacterial populations on the leaves of Mediterranean plants: Quantitative features and testing of distribution models. *Environ. Exp. Bot.* 52:63–77.

YADAV, R. K. P., KARAMANOLI, K., and VOKOU, D. 2005. Bacterial colonization of the phyllosphere of Mediterranean perennial species as influenced by structural and chemical leaf features. *Microb. Ecol.* 50:185–196.

YADAV, R. K. P., PAPATHEODOROU, E. M., KARAMANOLI, K., CONSTANTINIDOU, H.-I. A., and VOKOU, D. 2008. Abundance and diversity of the phyllosphere bacterial communities of Mediterranean perennial plants that differ in leaf chemistry. *Chemoecology* 18:217–226.

YADAV, R. K. P., KARAMANOLI, K., and VOKOU, D. 2010. Estimating bacterial population on the phyllosphere by serial dilution plating and leaf imprint methods. *Ecoprint* 17:47–52.

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Seasonal Changes in *Undifilum* Colonization and Swainsonine Content of Locoweeds

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Abstract Locoweeds (*Astragalus* and *Oxytropis*) are leguminous plants that are toxic due to a symbiotic association with the endophytic fungus *Undifilum oxytropis*. The fungus produces the alkaloid swainsonine, an α-mannosidase-inhibitor that causes serious damage to mammals when consumed. A real-time PCR technique was developed to quantify the colonization extent of *Undifilum* in locoweeds and to compare it to the swainsonine concentration in the plants. Amplification of the endophyte nuclear ITS region allowed reliable quantification of *Undifilum* DNA from field plants and *in vitro* cultures. Swainsonine concentration was highly correlated (ρ=0.972, $P<$ 0.001) with the proportion of *Undifilum* DNA during the first 4 weeks of *in vitro* culture growth. Species of *Astragalus* and *Oxytropis* were sampled seasonally in New Mexico and Colorado for two years. High swainsonine concentration in plant samples was associated with high levels of endophyte DNA, except in plant reproductive tissues.

Keywords *Undifilum* · Quantitative PCR · Swainsonine

Introduction

Locoweeds are toxic legumes in the genera *Astragalus* and *Oxytropis* (Marsh, 1909; Patterson, 1982; James and Panter 1989). They are toxic because of a mutualistic association with the endophytic fungus *Undifilum oxytropis* (Braun et al., 2003; Ralphs et al., 2008; Pryor et al., 2009). The fungus produces swainsonine, an indolizidine alkaloid that inhibits α-mannosidases causing serious damage to mammals when consumed. Animals often develop behavioral changes after repeated consumption of locoweed, as they become more intoxicated. Different types of physiological damage result from swainsonine consumption, including changes in serum enzyme levels and intracellular vacuolation in various tissues, which results in neurological disorders and reproductive complications (Ralphs et al., 1993; McLain-Romero et al., 2004; Stegelmeier et al., 2005).

Astragalus species tend to be annuals depending on precipitation, whereas *Oxytropis* species tend to have a perennial life cycle (Ralphs et al., 2003). Locoism outbreaks of major economic importance caused by *A. lentiginosus*, *A. emoryanus*, *A. wootonii*, *A. whawhaepensis*, and *A. pubentissimus* have been recorded in North America (Williams et al., 1979; Molyneaux and James, 1982; Davis et al., 1984; Pfister et al., 2003; Ralphs et al., 2003), some of which occur after heavy rainfall years. Locoweed seeds can remain viable for decades and currently are the only known dispersal mechanism for both the plant and the endophyte (Ralphs et al., 2003). *Undifilum* colonizes the seed coat while in the ovary (Oldrup et al., 2010), and embryo colonization occurs later on, during germination. Although *U. oxytropis* does not appear to induce disease to North American locoweeds, recent reports describe a related fungus, *Embellisia astragali*, causing disease in *A. adsurgens* and *A. sinicus* in China (Li and Nan, 2007a, 2007b).

Rangeland management and exposure avoidance have been crucial in decreasing the economic impact of locoism on the cattle industry (Ralphs et al., 1984, 1993; James et al., 2005). Swainsonine concentration is measured using liquid chromatography-mass spectrometry (LC-MS) and/or gas

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chromatography–mass spectrometry (GC-MS) (Gardner et al., 2001). *Undifilum* can be detected by isolation or PCR (Ralphs et al., 2008), but these techniques do not produce quantitative information on how much endophyte biomass is present in locoweed tissues. Quantitative PCR could be used to better explain the processes that link endophyte colonization with seasonal fluctuations in the swainsonine content of locoweeds.

Quantitative PCR or real-time PCR (qPCR) has been useful to describe the extent of colonization of toxigenic fungi in plant tissues. Targeting single or low copy number genes through qPCR makes it possible to quantify *Alternaria* DNA in plant tissue, having a lower detection limit of about one picogram of pathogen DNA or one genome copy per reaction (Gachon and Saindrenan, 2004; Andersen et al. 2006). By targeting two different single copy genes in the fescue endophyte *Neotyphodium*, the lower detection limit of qPCR assays was established at 20 genome copies per reaction (Rasmussen et al., 2007); this quantitative data was used to study the relationship between the extent of endophyte colonization and the synthesis of different alkaloids in the plant-endophyte system.

Here, we present the development of qPCR assays to assess the colonization level of *Undifilum* in locoweeds. We compare the extent of endophyte colonization to the swainsonine content in plants grown *in vitro*, as well as plants collected from wild populations in New Mexico and Colorado.

Methods and Materials

In Vitro Grown Plants. *Oxytropis sericea* plants infected with *U. oxytropis* were cultured *in vitro* as described in Oldrup et al. (2010), on baseline, acidified and PEG ULT-0 media. Groups of 6 plants were grown in 25 mm deep Petri dishes. Eighteen plants coming from three dishes were pooled and considered an individual sample. Five replicates were used per media type per week during 4 wk. Three independent trials of the experiment were performed. Plants were incubated in a growth chamber at 28°C and 550 μmoles m^{-2} s^{-1} continuous light. Samples were ground to a fine powder with a mortar and pestle using liquid nitrogen. A subsample was immediately processed for DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Inc., Germany), and the rest was desiccated at 50°C for 48 h in a drying oven. The dried material was ground a second time with a mortar and pestle to homogenize the particle size prior to swainsonine extraction.

Field Plant Collections A minimum of 7 healthy-looking locoweed plants were sampled from wild populations in New Mexico and Colorado (Table 1). Plants were flagged and coded for non-destructive sampling throughout eight seasons, from the summer of 2006 to the summer of 2008. Each sample consisted of 5 leaves, 5 inflorescences, or 5 infrutescences collected at the midpoint of each season. Stem samples were gathered only when plants were senescent and were not expected to be found alive the next season. Taxonomic identification was done according to Fox et al. (1998) and under the guidance of Dr Kelly Allred (Department of Animal and Range Sciences, NMSU).

DNA was extracted from a composite mixture of leaflet and petiole fragments, pooled from 5 leaves per each plant. The same procedure was used for inflorescences and infrutescences. The rest of each sample was desiccated as mentioned before. Dry samples were ground with a Wiley® mini-millgrinder, sieved through a 0.45 mm mesh screen, and processed for swainsonine extraction.

Real-Time PCR Assays The DNeasy Plant Mini Kit was used to extract DNA from *Undifilum oxytropis* isolated from *A. mollissimus* and *O. sericea*. Primers ITS5 (GCAAGTAAAAGTCGTAACAAGG) (White et al. 1990), OR1 (GTCAAAAGTTGAAAATGTGGCTTG), EqR4 (CTGACGCTGATTGCAATTACA), SARF4 (GAGAACTCCAGGAGAACTTG), and SARR3 (GTGGCAAGATCCTATCCTTC) were used in qPCR assays as follows. Primer set ITS5-OR1 targets the ITS region and produces a 580 bp fragment and was used for field plants

Table 1 Locoweed populations sampled

Field site - County, State (Habitat)	Coordinates and Elevation	Sampled area (m^2)	Species
Jornada del Muerto - Doña Ana Co., NM (Chihuahuan Desert)	32 °31'46.02"N 106 °46'04.94"W 1333 m	2400	*Astragalus mollissimus var. bigelovii*
City of Rocks - Grant Co., NM (Chihuahuan Desert/Grassland)	32 °34'42.05"N 107 °58'21.35"W 1581 m	4000	*Astragalus mollissimus var. bigelovii*
Capulin - Union Co., NM (Short grass prairie)	36 °38'14.55"N 103 °56'25.63"W 2062 m	290	*Astragalus mollissimus var. mollissimus*
Black Range - Grant Co., NM (Montane Conifer Forest)	32 °53'03.97"N 107 °51'48.34"W 2075 m	300	*Oxytropis lambertii* var. *bigelovii*
Capulin - Union Co., NM (Short grass prairie)	36 °38'14.55"N 103 °56'25.63"W 2062 m	290	*Oxytropis sericea*
Virginia Dale - Larimer Co., CO (Short grass prairie)	40 °54'16.82"N 105 °18'23.15"W 2042 m	400	*Oxytropis sericea*

Table 2 Weather stations in New Mexico and Colorado

Field site	Weather station	Coordinates		Data[a]	Distance (km)	Source
Jornada	J. Exp. Range	32 °37'00.00"N	106 °43'60.00"W	Temp/Ppt	10.43 NE	NOAA
City of Rocks	Faywood	32 °37'60.00"N	107 °52'00.00"W	Temp/Ppt	12.28 NE	NOAA
Capulin	Capulin	36 °43'60.00"N	103 °59'60.00"W	Temp/Ppt	12.23 NW	NOAA
Black Range	McKnight cabin	33 °00'00.00"N	107 °52'01.20"W	Temp	13.42 N	NMCC
	Mimbres Ranger STN	32 °55'60.00"N	108 °01'00.00"W	Ppt	15.41 NW	NOAA
Virginia Dale	Virginia Dale 7 ENE	40 °58'00.00"N	105 °13'00.00"W	Temp/Ppt	10.13 NE	NOAA

[a] Temperature (Temp); Precipitation (Ppt)

only. ITS5 is a universal forward primer and OR1 is a reverse primer that amplifies both *Undifilum* and *Embellisia*. Since the ITS5/OR1 amplicon is larger than is commonly used for qPCR, we designed the reverse primer EqR4 to be paired with ITS5. This primer set produces a 234 bp amplicon from *Undifilum* DNA and can also amplify templates from other ascomycetes. Since ITS5/EqR4 was used for *in vitro* plants only, specificity was not a concern. The primer set SARF4/SARR3 was designed to target the *U. oxytropis saccharopine reductase* gene (*sr*) (GeneBank HQ010362; Mukherjee and Creamer, unpublished data), producing a 96 bp amplicon under the same qPCR conditions as ITS5/EqR4.

Twenty microliter reactions were prepared using the DyNAmo SYBR® Green qPCR kit (Finnzymes, Finland), containing

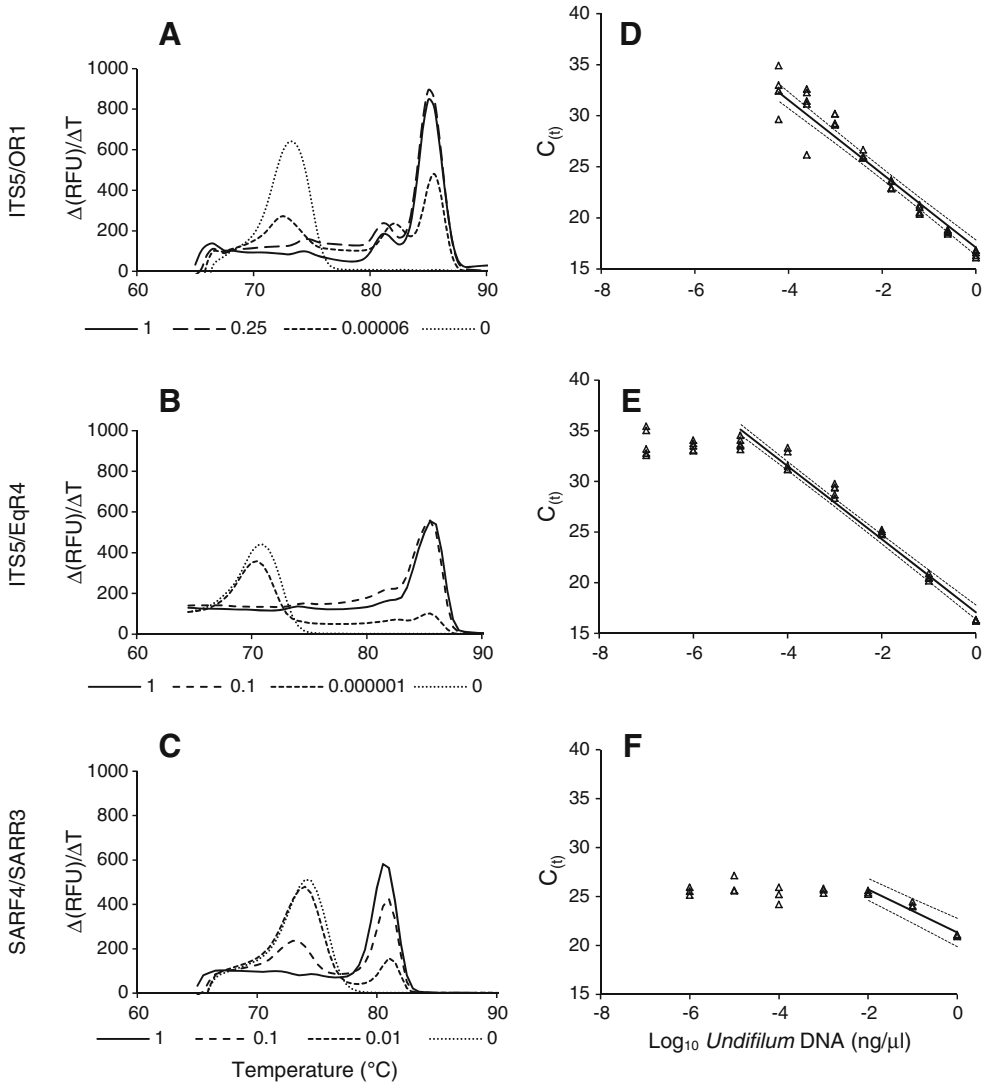

Fig. 1 Real-time PCR quantification of *Undifilum* DNA standards using primer sets ITS5/OR1 (A,D), ITS5/EqR4 (B,E) and SARF4/SARR3 (C,F): Melting curves (**a**, **b** and **c**) produced by the two highest working concentrations (in ng/µl), the lowest one at which quantification was attainable and from a control with no template. Quantification range (**d**, **e**, and **d**) and regression curves (*solid line*) used to calculate *Undifilum* DNA concentration. Confidence intervals (*dashed lines*) for quantification were estimated with α=0.05

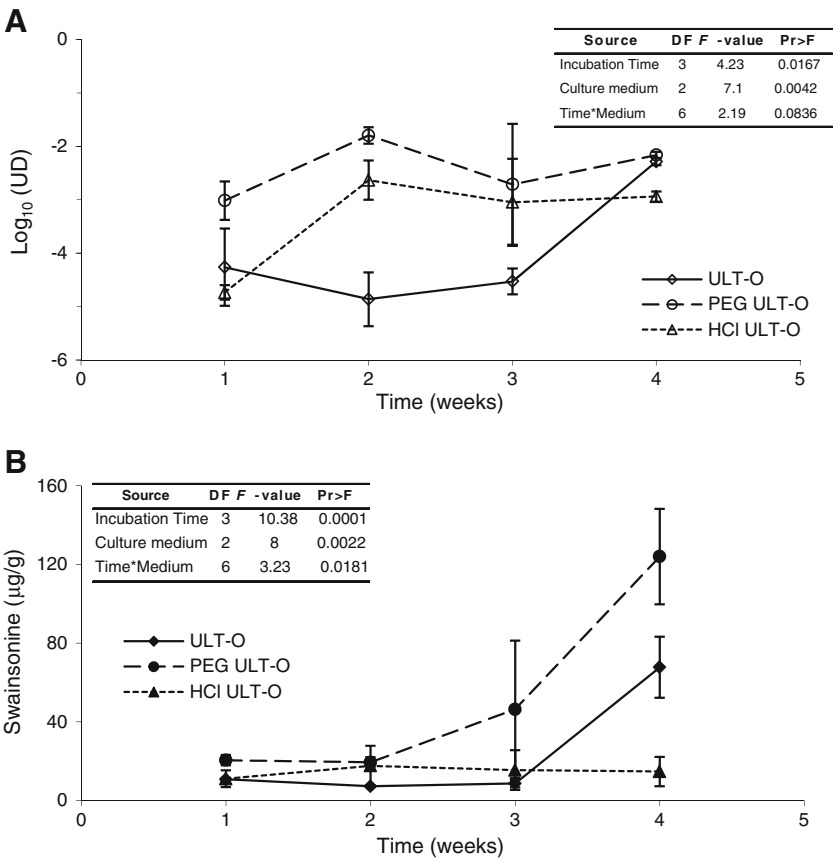

Fig. 2 Endophyte colonization (**a**) and swainsonine content (**b**) in *Oxytropis sericea* grown *in vitro*. Two-way ANOVA results in the insets express the effect of treatments on *Undifilum* colonization and swainsonine content

2.5 mM $MgCl_2$, 0.625 µM primers, and 1 µl of DNA extract. Assays were carried out in an iQ5 thermal cycler (BioRad, USA). The temperature profile for the reactions consisted of 10 min at 95°C for initial denaturation, followed by 45 cycles of 94°C/10 sec, 54.5°C/20 sec, 72°C/25 sec, and fluorescence acquisition. This was followed by an extension step at 72°C/10 min, and melting curve acquisition from 65°C to 95°C, reading fluorescence every 0.5° with a hold time of 0.5 sec. A final extension step at 72°C/10 min completed the program. The fluorescence threshold was automatically adjusted by the iQ5 Optical System Software 2.0 for every plate. DNA extracted from *Undifilum* cultures was used to prepare concentration standards; a four-fold dilution series from 1 ng/µl to 61 fg/µl for primer set ITS5/OR1 and a ten-fold dilution series from 1 ng/µl to 100 ag/µl for primer sets ITS5/EqR4 and SARF1/SARR3. The relationship between the *Undifilum* DNA concentration in the standards and their $C_{(t)}$ was used to build a linear equation relating both variables. The $C_{(t)}$ from locoweed DNA samples was used in the equation to solve for *Undifilum* DNA concentration in each sample. Since the total DNA concentration is variable between extracts, the *Undifi-*

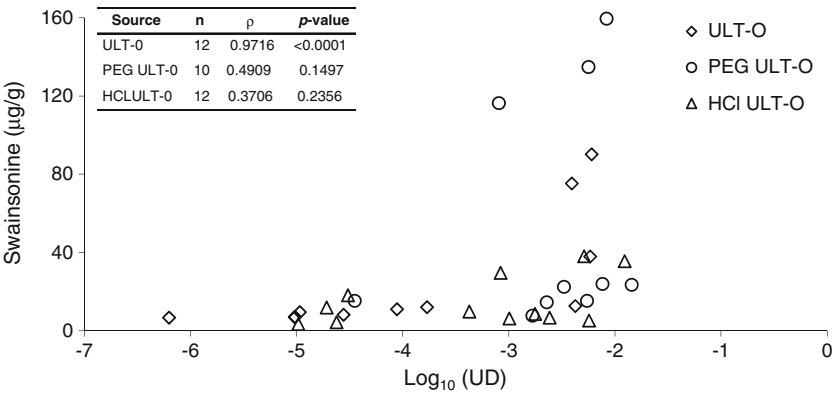

Fig. 3 *Undifilum* DNA (UD) and swainsonine concentration in *in vitro* grown *Oxytropis sericea* plants. Type of media is indicated by different shaped symbols as described in the key. Correlation statistics are given in the inset

Table 3 Phenological composition of locoweed populations

Species (location)	Season	n	Phenological composition (%)			
			V[a]	Fl[b]	Fl/Fr	Fr[c]
Astragalus mollissimus var. *bigelovii*	Summer 06	12	100	-	-	-
(Jornada del Muerto, NM)	Fall 06	10	100	-	-	-
	Winter 07	10	90	10	-	-
	Spring 07	10	50	-	20	30
	Summer 07	2	100	-	-	-
Astragalus mollissimus var. *bigelovii*	Summer 06	14	100	-	-	-
(City of Rocks, NM)	Fall 06	14	100	-	-	-
	Winter 07	13	92	8	-	-
	Spring 07	9	-	11	67	22
	Summer 07	4	100	-	-	-
	Fall 07	1	100	-	-	-
Astragalus mollissimus var. *mollissimus*	Summer 06	7	43	-	29	29
(Capulin, NM)	Fall 06	7	100	-	-	-
	Winter 07	5	100	-	-	-
	Spring 07	5	-	100	-	-
	Summer 07	7	100	-	-	-
	Fall 07	1	100	-	-	-
	Winter 08	2	100	-	-	-
Oxytropis lambertii var. *bigelovii*	Summer 06	8	25	75	-	-
(Black Range, NM)	Fall 06	8	25	-	-	75
	Winter 07	8	100	-	-	-
	Spring 07	8	100	-	-	-
	Summer 07	8	-	75	25	-
	Fall 07	7	100	-	-	-
	Winter 08	6	100	-	-	-
	Spring 08	6	100	-	-	-
Oxytropis sericea	Summer 06	7	100	-	-	-
(Capulin, NM)	Fall 06	7	100	-	-	-
	Winter 07	7	100	-	-	-
	Spring 07	7	-	100	-	-
	Summer 07	7	100	-	-	-
	Fall 07	7	100	-	-	-
	Winter 08	5	100	-	-	-
	Spring 08	2	50	50	-	-
Oxytropis sericea	Summer 06	4	100	-	-	-
(Virginia Dale, CO)	Fall 06	8	100	0	-	-
	Spring 07	8	100	-	-	-
	Summer 07	8	100	-	-	-
	Fall 07	7	100	-	-	-
	Winter 08	6	100	-	-	-
	Spring 08	7	71	29	-	-

[a] Vegetative (V), [b] Flowering (Fl), [c] Fruiting (Fr)

lum DNA concentration was divided by the total DNA concentration for each individual extract resulting in a ratio we call "UD" for simplicity. We use the UD ratio as an estimation of the extent of *Undifilum* colonization in plant tissue.

Swainsonine Extraction and Quantification Swainsonine was extracted by cationic exchange chromatography (CEC) and quantified by LC-MS (Gardner et al., 2001). Two hundred milligrams of ground plant material were used for the extractions. Two hundred microliters of swainsonine extract were evaporated in HPLC vials and rehydrated prior to LC-MS analysis. Swainsonine concentration in the extracts was transformed to "μg of swainsonine per gram of dry plant matter".

Weather Data Precipitation and temperature at the field locations were compared to the swainsonine concentration and the UD. Data were downloaded from public records at the National Oceanic and Atmospheric Administration (NOAA, 2006a, b, 2007a, b, 2008a, b, c, d), SNOTEL-Natural Resources Conservation Service (NRCS) and the New Mexico Climate Center (NMCC) (Table 2).

Statistical Analysis Undifilum DNA concentration (ng/μl) and swainsonine concentration (μg/ml) were both calculated in the qPCR and LC-MS instruments using line equations derived from linear regression. These equations were calculated automatically by the software of each instrument based on the readings of serial dilutions of a known standard concentration. The experiment was set up following a three-by-four factorial design. Results were analyzed by two-way ANOVA, using the GLM procedure in SAS 11. Data from the *in vitro* study were subjected to Non-Parametric Pearson correlation analysis to analyze the relationship between *Undifilum* DNA and swainsonine contained in the samples. Significant differences were established at α=0.05. All swainsonine and endophyte DNA measurements from field samples are presented as mean±standard error of the mean (SEM).

Results

Undifilum DNA Quantification Endophyte DNA quantification using the primer set ITS5/OR1 produced a melting curve with a main peak at 85.5°C and a secondary peak at 81.5°C (Fig. 1A). The secondary peak could be caused by the length of the PCR product and the presence of a high G/C rich region within it. The regression curve calculated from the standards followed a linear trend (r>0.99 in most runs) across the concentration range used to construct it (Fig. 1D). The lower quantification limit for this primer set was 61 fg/μl. Amplification of lower template concentrations can be achieved, but it was not consistent enough for quantification. Replacing OR1 with EqR4 improved amplification sensitivity about a hundred fold, detecting *Undifilum* DNA down to 100 ag/μl (Fig. 1E). However, the linearity of the relationship between template concentration and $C_{(t)}$ is not maintained over the lower concentrations. Therefore, we established a quantification limit of 1 fg/μl with the latter primer set. The melting curve produced by this amplicon has a single peak at 85.5°C (Fig. 1B). The *sr* amplicon produced a melting curve with a single peak at 85°C (Fig. 1C) but the fluorescence signal was lost around 10 pg/μl (Fig. 1F), leaving too narrow a range for quantification.

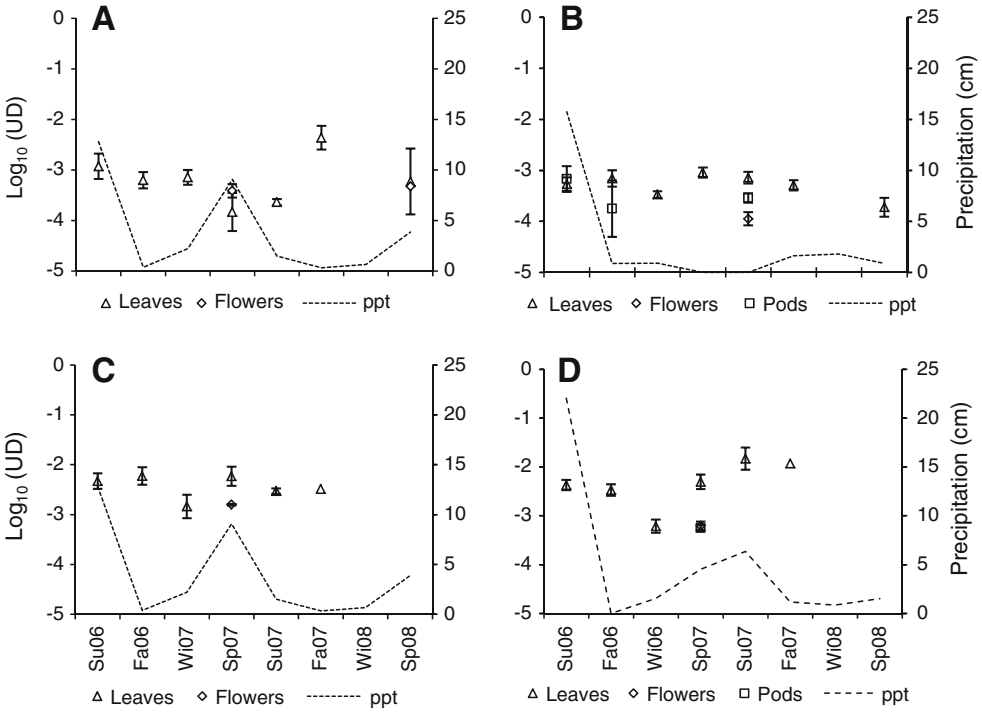

Fig. 4 *Undifilum* colonization in locoweeds and precipitation at the sampling sites: *Oxytropis sericea* in Capulin, NM (**a**), *O. lambertii* var. *bigelovii* (**b**), *Astragalus mollissimus* var. *mollissimus* (**c**) and *A. mollissimus* var. *bigelovii* in City of Rocks (**d**). *Oxytropis sericea* in Virginia Dale and *A. mollissimus* var. *bigelovii* in Jornada del Muerto were similar to their counterparts and thus their graphs were omitted. Sampling seasons are abbreviated by first two letters and year

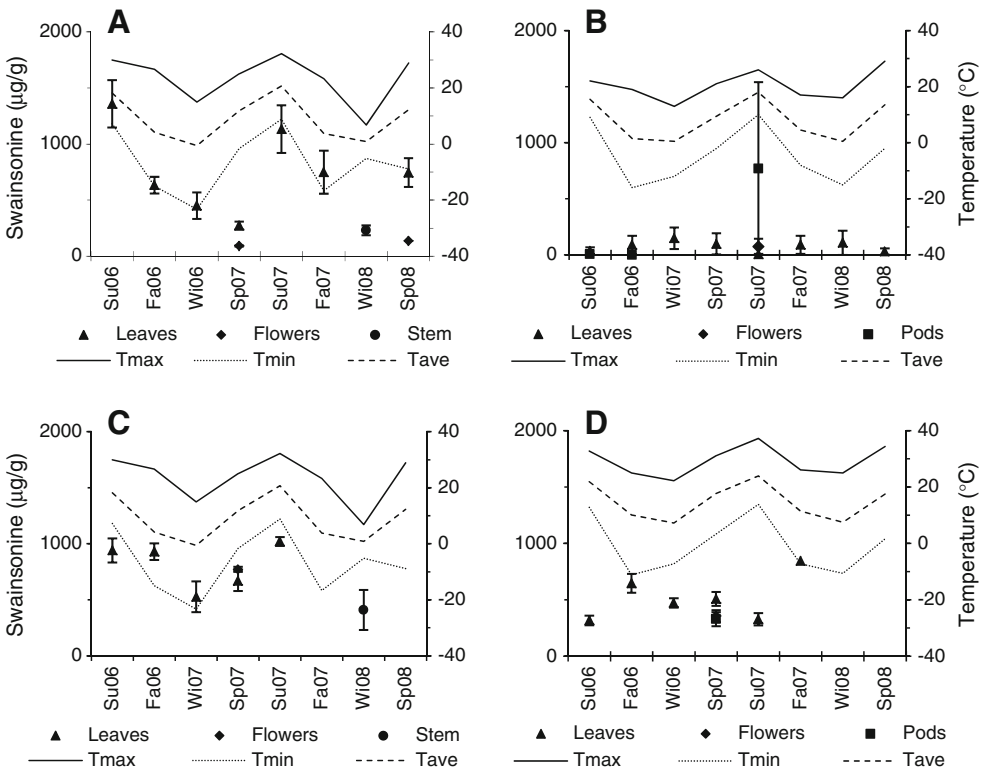

Fig. 5 Swainsonine content in locoweeds and environmental temperature at the sampling sites: *Oxytropis sericea* in Capulin, NM (**a**), *O. lambertii* var. *bigelovii* (**b**), *Astragalus mollissimus* var. *mollissimus* (**c**) and *A. mollissimus* var. *bigelovii* in City of Rocks (**d**). *Oxytropis sericea* in Virginia Dale and *A. mollissimus* var. *bigelovii* in Jornada del Muerto were similar to their varietal counterparts and thus their graphs omitted. Average daily temperature during the sampling month (*dashed line*) is shown as well as the highest and lowest temperatures recorded for the same month (*solid line* and *dotted line* respectively). Sampling seasons are abbreviated by first two letters and year

In vitro O. sericea. The *Undifilum* colonization increased over time, as indicated by the UD, reaching levels around 1 pg Undifilum DNA/ng of total DNA on all three media at the end of the fourth week (Fig. 2A). Plants grown in acidified and PEG ULT-0 showed colonization levels as high on the second week as plants grown in regular ULT-0 did on the fourth week. In general, UD was quite variable during the first three weeks, but it became more stable by the fourth week. Swainsonine concentration also increased over time, and it was even more influenced by the different media than endophyte colonization (Fig. 2B). Interestingly, the acidification of the media resulted in extremely low swainsonine content during the four weeks. Correlation between *Undifilum* DNA and swainsonine content shows that in general, higher levels of colonization translate into higher swainsonine content (Fig. 3). However, this correlation was high and significant only in plants grown on ULT-0 (ρ=0.972, P<0.001).

Field Plants Locoweeds were found in the vegetative state throughout most of the sampling seasons (Table 3); however, most individuals did produce seed as indicated by empty pods found still attached to the plants during summer and fall. The sample size decreased over time as some plants senesced and died, which occurred earlier and more extensively with *Astragalus* when compared to *Oxytropis*. The extent of endophyte colonization in leaves ranged from approximately 0.1 to 10 pg *Undifilum* DNA/ng of total DNA (Fig. 4). Colonization can be stable and be maintained within the same order of magnitude as in *O. lambertii* and *A. mollissimus* var. *mollissimus* (Fig. 4B and C). It can also change as much as a hundred fold within two seasons as in *O. sericea* and *A. mollissimus* var. *bigelovii* (Fig. 4 A and D). The seasonal changes in swainsonine content hinted at more of an annual cycle, with ranges more properly defined by the specific locoweed-endophyte association (Fig. 5). *Astragalus mollissimus* var. *bigelovii* from Jornada del Muerto exhibited a highly significant strong correlation between colonization and swainsonine content (ρ= 0.5439; P<0.001), where as *O. lambertii* and *O. sericea* from Virginia Dale exhibited significant, however moderate correlations (Table 4). The coefficients indicate that colonization is far from fully explaining the content of swainsonine in a plant. The average daily temperature was found to be strongly correlated with the average swainsonine content in leaves of

Table 4 Non-parametric correlation analysis between *Undifilum* colonization and swainsonine content in field plants

Population	n	ρ[1]	P value
Oxytropis lambertii var. *bigelovii* (BR)	65	0.3008	0.0149
O. sericea (C)	45	0.0586	0.7020
O. sericea (VD)	40	0.3989	0.0108
Astragalus mollissimus var. *bigelovii* (JM)	41	0.5439	0.0002
A. mollissimus var. *bigelovii* (CR)	60	0.0923	0.4830
A. mollissimus var. *mollissimus* (C)	27	0.2076	0.2989

[1] Spearman's correlation coefficient

Table 5 Non-parametric correlation between the average daily temperature at the sampling sites and the average swainsonine content in leaves

Population	n	ρ^1	*P* value
Oxytropis lambertii var. *bigelovii* (BR)	8	−0.8743	0.0045
O. sericea (C)	7	0.6071	0.1482
O. sericea (VD)	7	0.3214	0.4821
Astragalus mollissimus var. *bigelovii* (JM)	5	−0.7000	0.1881
A. mollissimus var. *bigelovii* (CR)	6	−0.5429	0.2657
A. mollissimus var. *mollissimus* (C)	5	0.9000	0.0378

[1] Spearman's correlation coefficient

O. lambertii (ρ=−0.8743; *P*=0.004) and *A. mollissimus* var. *mollissimus* (ρ=0.9; *P*=0.038), outlining seasonal swainsonine content cycles for these two populations (Table 5 and Fig. 6).

Discussion

Using *sr* to quantify *Undifilum* DNA results in a qPCR assay sensitive down to picograms per microliter, which is similar to what is used to detect *Alternaria* or *Neotyphodium* using low or single-copy genes (Gachon and Saindrenan, 2004; Andersen et al. 2006; Rasmussen et al., 2007). In the case of *Alternaria*, such a detection limit is roughly equivalent to eighty genome copies per reaction (Andersen et al. 2006). Unlike *sr*, the ITS region occurs in hundreds of copies per genome in fungi (Kobayashi, 2011), which allowed us to design qPCR assays orders of magnitude more sensitive, and just as accurate. The two assays we developed allow accurate quantification down to the order of femtograms of *Undifilum* DNA per reaction. Considering the data on *Alternaria* (Andersen et al. 2006), we speculate that this quantification limit could be equivalent to just a fraction of a genome per reaction. If correct, that would explain the successful but inconsistent amplification of endophyte DNA between 100 attograms and 10 femtograms per reaction.

This is not the first time the ITS region has been used to infer fungal biomass in plant tissues. Glynn et al. (2007) designed competitive PCR assays to quantify *Fusarium* and *Microdochium* infection in wheat seeds. The assay they designed is just as sensitive as that presented here; however, it requires two primer sets per reaction as well as visualization of the PCR products to interpret the outcome. Recently, Cook et al. (2009) reported a qPCR assay to quantify *Undifilum* DNA in locoweeds, also based on ITS amplification. They pointed out the need for an assay more sensitive than 10 pg *Undifilum* DNA per microliter, which we provide here by three orders of magnitude.

The concentration of endophyte DNA in the plant tissues appears to be a good indicator of the extent of the endophyte colonization. In the *in vitro* assays, colonization in *O. sericea* increased in the first four weeks after germination, independent of the stress that was simulated. Similarly, swainsonine increased over time, and it was detected at levels above baseline between the second and fourth week of development. In plants grown on baseline medium, the synthesis of swainsonine can be directly attributed to increasing fungal biomass. However, when plants were grown in modified media, the correlation was lost, and swainsonine production seemed to be delayed. Besides this lag, the relationship between colonization and the swainsonine content in the plant tissue was overall positive. At week four, the swainsonine concentration reached the maximum of 67.71±15.51 μg/g dry weight, similar to the 73 μg/g reported in eight-week-old plants grown under similar conditions (Oldrup et al., 2010).

The swainsonine content range in the populations sampled is similar to previous reports (Braun et al., 2003; Valloton and Sterling, 2007; Parker, 2008; Ralphs et al., 2008). The populations that showed the highest swainsonine content were from varieties known to be highly toxic. In

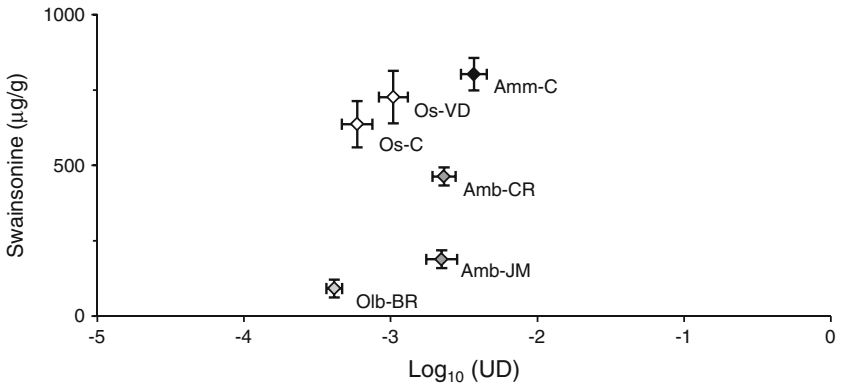

Fig. 6 *Undifilum* DNA and swainsonine contents from six locoweed populations (Mean±SEM). Abbreviations correspond to locoweed species and location. *Astragalus mollissimus var. bigelovii* (Amb), *Astragalus mollissimus var mollissimus* (Amm), *Oxytropis lambertii var. bigelovii* (Olb), *Oxytropis sericea* (Os); Black Range (BR), Capulin (C), Virginia Dale (VD), City of Rocks (CR), Jornada del Muerto (JM)

addition, during this sampling period, they were subjected to the most extreme environmental temperature differentials within the group. Water deficiency has been considered a likely elicitor of the swainsonine production pathway, but although rainfall was low in both years, the swainsonine content in *A. mollissimus* var. *mollissimus* was below the concentration Valloton and Sterling (2007) associate with drought stress.

Here, an annual cycle for toxicity in the field is proposed based on the data on leaf tissue of *A. mollissimus* var. *mollissimus* and *O. sericea* from Capulin, NM. Overwintering leaves have the lowest swainsonine concentrations; swainsonine content then increases during the spring as snow melts and the plants awaken from dormancy. Plants develop new foliage and start blooming early in the spring, then develop fruits that ripen before mid-summer. Meanwhile, swainsonine content increases in the leaves, without a major change in the extent of endophyte colonization, reaching a maximum swainsonine concentration around mid-summer, after seed dispersal. Frosts and cold wind sweep the pasture and leaves start senescing during the first half of fall, but they stay attached to the plant. Dead dry leaves and petioles protect the few leaves produced in the middle of the plant at the end of summer. The swainsonine content in the last leaves of summer is lower compared to the fully developed leaves around them, but the endophyte colonization is not different. During winter snow covers, the ground and the plants stay dormant, with few primordial leaves alive. Those overwintering leaves have the lowest swainsonine content throughout the year, but they are the same leaves that were produced at the end of summer, surviving through the fall. Since swainsonine was there at the beginning, it is potentially being degraded, transformed, or translocated to the underground tissues. The cycle is restarted as winter progresses onto spring and plants awaken from dormancy, and they either produce more swainsonine, translocate it from underground tissues or both.

It was not a surprise to find that locoweed toxicity changed over time, in part because toxicity data exist for those varieties and because research has been done over the last two decades on some of the populations we analyzed. Not only is the extent of endophyte colonization variable, but it also appears to be responsive to environmental cues. Using the amount of *Undifilum* DNA as a fair representation of the endophytic biomass, we are reporting a range of ten to a hundred-fold the amount of the symbiont biomass inside hosts belonging to the same population.

Swainsonine synthesis is influenced by the extent of endophyte colonization in the weeks following germination. The extent of plant colonization found at the end of the fourth week is similar to that found in mature plants from the field. This suggests that geographic location and/or weather conditions influence swainsonine production by locoweeds very early in plant development. Additional work will be necessary to elucidate the role of weather parameters such as temperature extremes on swainsonine production.

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References

ANDERSEN, B., SMEDSGAARD, J., JØRRING, I., SKOUBOE, P., and HAGSHOLM PEDERSON, L. 2006. Real-time PCR quantification of the AM-toxin gene and HPLC qualification of toxigenic metabolites from Alternaria species from apples. International J of Food Microbiology 111:105–111.

BRAUN, K., ROMERO, J., LIDDELL, C., and CREAMER, R. 2003. Production of swainsonine by fungal endophytes of locoweed. *Mycol. Res.* 107:980–988.

COOK, D., GARDNER, D. R., WELCH, K. D., ROPER, J. M., RALPHS, M. H., and GREEN, B. T. 2009. Quantitative PCR method to measure the fungal endophyte in locoweeds. *J. Agric. Food Chem.* 57:6050–6054.

DAVIS, D., SCHWARZ, P., HERNANDEZ, T., MITCHELL, M., WARNOCK, B., and ELBEIN, A. D. 1984. Isolation and characterization of swainsonine from Texas locoweed (*Astragalus emoryanus*). *Plant Physiol.* 76:972–975.

FOX, W.E., ALLRED, K.W., and ROALSON, E.H. 1998. A guide to the common locoweeds and milkvetches of New Mexico. NM Agric. Exp. Sta. Circ. 557.

GACHON, C. and SAINDRENAN, P. 2004. Real-time PCR monitoring of fungal development in Arabidopsis thaliana infected by *Alternaria brassicola* and *Botrytis cinerea*. *Plant Physiol. Biochem.* 42:367–371.

GARDNER, D. R., MOLYNEUX, R. J., and RALPHS, M. H. 2001. Analysis of swainsonine: Extraction methods, detection, and measurement in populations of locoweeds (*Oxytropis* spp.). *J. Agric Food Chem* 49:4573–4580.

GLYNN, N. C., RAY, R., EDWARDS, S. G., HARE, M. C., PARRY, D. W., BARNETT, C. J., and BECK, J. J. 2007. Quantitative *Fusarium* spp. and *Microdochium* spp. PCR assays to evaluate seed treatment for the control of *Fusarium* seedling blight of wheat. *J. Appl. Microbiol.* 102:1645–1653.

JAMES, L. F. and PANTER, K. E. 1989. Locoweed poisoning in livestock. pp 23-28 *in*: Swainsonine and related glycosidase inhibitors. Edited by L. F. James, A. D. Elbein, R. J. Molyneux and C. D. Warren, Iowa State University Press, Ames.

JAMES, L. F., GARDNER, D. R., LEE, S. T., PANTER, K. E., PFISTER, J. A., RALPHS, M. H., and STEGELMEIER, B. L. 2005. Important poisonous plants on rangelands. Management strategies based on toxin level in the plant, animal susceptibility, and grazing behavior can reduce the risk of poisoning. *Rangelands* 27:3–9.

KOBAYASHI, T. 2011. Regulation of ribosomal RNA gene copy number and its role in modulating genome integrity and evolutionary adaptability. *Cell. Mol. Life Sci.* 68:1395–1403.

LI, Y. Z. and NAN, Z. B. 2007a. Symptomology and etiology of a new disease, yellow stunt, and root rot of standing milkvetch caused by *Embellisia* sp. in Northern China. *Mycopathologia* 163:327–334.

LI, Y. Z. and NAN, Z. B. 2007b. A new species, *Embellisia astragali* sp. nov., causing standing milk-vetch disease in China. *Mycologia* 99:406–411.

MARSH, C.D. 1909. The locoweed disease of the plains. USDA Bureau of Animal Industries Bull. 112.

MCLAIN-ROMERO, J., CREAMER, R., ZEPEDA, H., STRICKLAND, J., and BELL, G. 2004. The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. *J. Anim. Sci.* 82:2169–2174.

MOLYNEUX, R. J. and JAMES, L. F. 1982. Loco intoxication: Indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). *Science* 216:190–191.

NOAA 2006a. Climatological Data. Annual Summary. Colorado 2006. 111(13)

NOAA 2006b. Climatological Data. Annual Summary. New Mexico 2006. 110(13)

NOAA 2007a. Climatological Data. Annual Summary. Colorado 2006. 112(13)

NOAA 2007b. Climatological Data. Annual Summary. New Mexico 2007. 111(13)

NOAA 2008a. Climatological Data. Colorado. February 2008. 113(02)

NOAA 2008b. Climatological Data. Colorado. May 2008. 113(05)

NOAA 2008c. Climatological Data. New Mexico. February 2008. 112(02)

NOAA 2008d. Climatological Data. New Mexico. May 2008. 112(05)

OLDRUP, E., MCLAIN-ROMERO, J., PADILLA, A., MOYA, A., GARDNER, D., and CREAMER, R. 2010. Localization of endophytic fungi in locoweed seed and influence of environmental parameters on a locoweed *in vitro* culture system. *Botany* 88:512–521.

PARKER, J. 2008. Effects of insect herbivory by the four-lined locoweed weevil *Cleonidius trivitattatus* (Say) (Coleoptera: Curculionidae), on the alkaloid swainsonine in locoweeds *Astagalus mollissimus* and *Oxytropis sericea*. MS thesis. New Mexico State University, Las Cruces.

PATTERSON, P. E. 1982. Loco, la yerba mala. *Rangelands* 4:147–148.

PFISTER, J. A., STEGELMEIER, B. L., GARDNER, D. R., and JAMES, L. F. 2003. Grazing of spotted locoweed (*Astragalus lentiginosus*) by cattle and horses in Arizona. *J. Anim. Sci.* 2003:2285–2293.

PRYOR, B. M., CREAMER, R., SHOEMAKER, R. A., MCLAIN-ROMERO, J., and HAMBLETON, S. 2009. *Undifilum*, a new genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuelleri* on legumes. *Botany* 87:178–194.

RALPHS, M. H., JAMES, L. F., NIELSEN, D. B., and PANTER, K. E. 1984. Management practices reduce cattle loss due to locoweed on high mountain range. *Rangelands* 6:175–177.

RALPHS, M. H., GRAHAM, D., MOLYNEUX, R. J., and JAMES, L. F. 1993. Seasonal grazing of locoweeds by cattle in northeastern New Mexico. *J. Rangeland Manage.* 46:416–420.

RALPHS, M. H., PFISTER, J. A., WELSH, S. L., GRAHAM, J. D., PURVINES, J., JENSEN, D. T., and JAMES, L. F. 2003. Locoweed population cycles. *Rangelands* 25:14–18.

RALPHS, M. H., CREAMER, R., BAUCOM, D., GARDNER, D. R., WELSH, S. L., GRAHAM, J. D., HART, C., COOK, D., and STEGELMEIER, B. L. 2008. Relationship between the endophyte *Embellisia* spp. and the toxic alkaloid swainsonine in major locoweed species (*Astragalus* and *Oxytropis*). *J. Chem. Ecol.* 34:32–38.

RASMUSSEN, S., PARSONS, A. J., BASSETT, S., CHRISTENSEN, M. J., HUME, D. E., JOHNSON, L. J., JOHNSON, R. D., SIMPSON, W. R., STACKE, C., VOISEY, C. R., XUE, H., and NEWMAN, J. A. 2007. High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. *New Phytol.* 173:787–797.

STEGELMEIER, B. L., JAMES, L. F., GARDNER, D. R., PANTER, K. E., LEE, S. T., RALPHS, M. H., PFISTER, J. A., and SPRAKER, T. R. 2005. Locoweed (*Oxytropis* sericea)-induced lesions in mule deer (*Odocoileius hemionus*). *Vet. Pathol.* 42:566–578.

VALLOTTON, A. D. and STERLING, T. M. STERLING. 2007. Physiology, growth and swainsonine content response to water deficit stress in *Astragalus mollissimus* and *Oxytropis sericea*. Paper presented at the 47th Annual Meeting of the Weed Science Society of America in Houston, TX.

WHITE, T.J., BRUNS, T., LEE, S., and TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315–352 *in*: PCR protocols: a guide to methods and applications. Edited by M.A. Innis, D.H. Gelfand, J. Sninsky and T.J. White, Academic Press, San Diego, CA.

WILLIAMS, M. C., JAMES, L. F., and BOND, B. O. 1979. Emory milkvetch (*Astragalus emoryanus* var *emoryanus*) poisoning in chicks, sheep, and cattle. *Amer. J. Vet. Res.* 40:403–406.

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RAPID COMMUNICATION

Hormaphis hamamelidis Fundatrices Benefit by Manipulating Phenolic Metabolism of Their Host

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Abstract We investigated the pattern and potential adaptive value of phenolic concentrations in galls induced by the aphid *Hormaphis hamamelidis* on leaves of *Hamamelis virginiana*. By the time that founding females began reproduction, galls had higher concentrations of condensed tannins and lower concentrations of hydrolyzable tannins than leaves. Galled and ungalled leaf laminas never differed significantly in any phenolic measure. Condensed tannin concentrations also were positively related to the number of offspring per gall when gall dry weight, another important correlate of fecundity, was accounted for. This could indicate the prior sink strength of the gall. Polyphenols may act as a repository for excess carbon drawn to the gall by increased sink strength, or be an indication of the fundatrix' ability to manipulate host physiology. This study is the first to demonstrate a tangible, quantitative association between phenolic accumulation in galls and gall-former reproductive performance, and illustrates that condensed tannins may play roles other than plant defense.

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Keywords Gall-former · Herbivory · Host manipulation · Polyphenols · Source-sink metabolism · Condensed tannin

Introduction

Gall-formers manipulate their hosts (Flaherty and Quiring, 2008; Detoni et al., 2010) and many galls have altered levels of phenolic metabolites (Ikai and Hijii, 2007), but the adaptive value of these chemical alterations is uncertain (Janzen, 1977; Inbar et al., 2009). Speculation regarding the role of altered phenolics in galls has included a potential defensive role against natural enemies (Schultz, 1992) or as a correlate of gall-former success at manipulating host quality (Nyman and Julkunen-Tiitto, 2000).

We tested two hypotheses in this study. H_1: Galls formed by the aphid *Hormaphis hamamelidis* have altered levels of phenolic metabolites. Our findings of elevated condensed tannins in galls led to the second hypothesis. H_2: Aphid fecundity is positively associated with concentrations of condensed tannins in galls.

Methods and Materials

Research System The witch hazel cone gall aphid, *Hormaphis hamamelidis* (Fitch), forms 7–10 mm tall conical galls on witch hazel leaves. The fundatrix bears the second generation parthenogenetically within the gall; all the aphids feed from phloem inside the gall. The number of second-generation aphids provides a fecundity measure for each fundatrix.

Study Site Witch hazel trees from a single site in Pennsylvania, USA, (centered at 40° 45′ 22″ N latitude and 77° 44′ 10″ W longitude) were used.

Chemistry Time Course: Material Collection Leaves were collected on 17 May, 28 May, 6 June, and 8 July 1997. Respectively, these dates corresponded to fundatrices being 1st, 2nd, and 3rd instar nymphs, then adults just beginning reproduction in fully grown galls. Six to twelve single-galled and ungalled leaves each were taken from each of 3 trees on the first three collection dates and from 6 trees on the last collection date. Our sample size was relatively small because we sought only a tissue level measure of phenolic concentrations. Each gall was opened with a sterile scalpel, and the fundatrix was removed by suction with a Pasteur pipette. Leaves and galls were flash frozen in liquid nitrogen and kept on dry ice until storage at −80 °C.

Chemistry Time Course: Phenolic Assays Tissues were lyophilized, ground in a mortar and pestle under liquid N_2, then extracted as in Adams et al. (2009). Methods used were Folin-Denis, potassium iodate, and n-butanol-HCl for total phenolics, hydrolyzable tannins, and condensed tannins, respectively, and quantification was based on standard curves generated with purified witch hazel tannins (Adams et al., 2009).

Gall Dry Weight, Fecundity and Phenolic Content On 25 July 1999, 120 galls were collected (30/tree × 4 trees). The mean number of offspring per gall was at its peak on that date (Rehill and Schultz, 2001). Galls were collected, stored, and handled as above. Only galls without signs of attack from natural enemies were used (N=70).

Statistical Analyses All statistical analyses were performed with SAS Version 8 (SAS Institute, Cary, NC, USA).

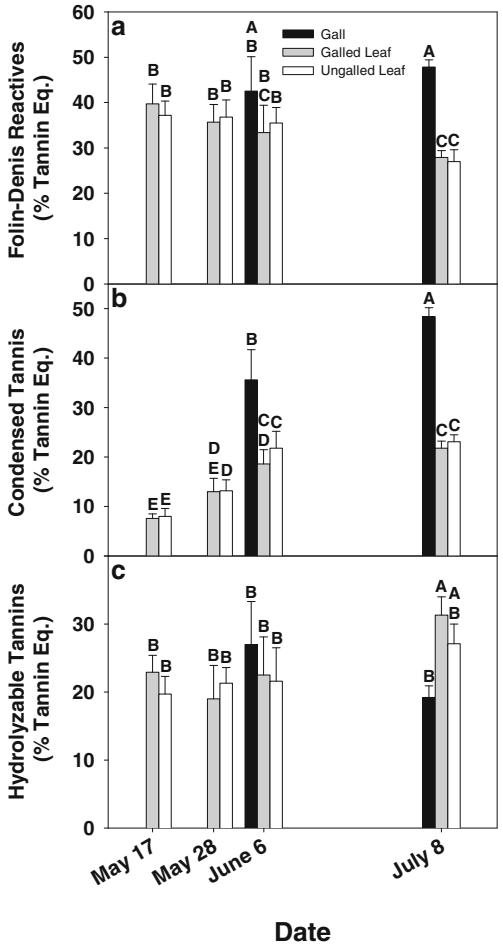

Fig. 1 Phenolic concentrations in the chemistry time course study: **a** Folin-reactive phenolics, **b** condensed tannins, and **c** hydrolyzable tannins

Results

Chemistry Time Course Folin-reactive phenolics decreased in leaves between 6/6/97 and 7/8/97, and concentrations in galls exceeded those in leaves on 7/8/97 (Fig. 1a; date: $F_{3,6}$=9.76; P=0.010; tissue: $F_{2,4}$=6.71; P=0.053; date by tissue: $F_{4,8}$=1.67; P=0.248).

Galls had higher concentrations of condensed tannins than leaves, and condensed tannins increased over time in all tissues (date: $F_{3,6}$=133.5; P<0.001; tissue: $F_{2,4}$=79.79; P<0.001; date by tissue: $F_{4,8}$=5.47; P=0.020). The concentrations in galls increased between the last two dates whereas those in leaves did not (Fig. 1b).

Hydrolyzable tannin concentrations started the same in all tissues (Fig. 1c), but diverged between 6/6/97 and 7/8/97 when galls contained lower concentrations than galled leaves due to an increase in the galled leaves (date: $F_{3,6}$= 8.81; P=0.013; tissue: $F_{2,4}$=1.68; P=.295; date by tissue: $F_{4,8}$=4.61; P=0.032). For all three phenolic measures, there were never significant differences between galled and ungalled leaves.

Gall Dry Weight, Fecundity and Phenolic Content Phenolic concentrations in individual galls were largely independent of one another and gall dry weight. Condensed tannin concentrations were not significantly correlated with Folin-reactive phenolic or hydrolyzable tannin concentrations (both: P>0.2), but Folin-reactive phenolics and hydrolyzable tannins were correlated (r_p=0.56; P<0.001).

Considered together with gall dry weight, condensed tannin concentrations positively affected reproductive output. In a stepwise regression, the effects of dry weight (P<0.001) and condensed tannin concentration on fecundity were significant (P=0.005), but Folin-reactive phenolics were not (P=0.230). In a regression that included only the significant factors, condensed tannin concentration explained 7.4 % of the variation of and was positively related to offspring per gall [#offspring=−14.6+3.00(dry weight (mg)]+0.52 [condensed tannin concentration

(% tannin equivalents)]; R^2=0.380; $F_{2,\ 67}$=20.52; P<0.001; coefficients for dry weight and condensed tannins; P<0.01). Similar to prior experiments, dry weight explained 30.6 % of the variation in offspring per gall. Thus, for a gall of a given dry weight, fecundity increased with increasing condensed tannin concentrations.

Discussion

Galls induced by *Hormaphis hamamelidis* fundatrices have markedly different phenolic concentrations from the adjacent leaf laminas. When galls reach full size and the fundatrices begin to reproduce parthenogenetically, galls have nearly twice the concentration of condensed tannins, but two thirds the hydrolyzable tannins of leaves. Furthermore, galled and ungalled leaves never differ in any of the measured phenolic metabolites, and thus, the changes seen in gall tissue are consistent with manipulation by the aphid, not the choice of leaf. Furthermore, these drastic alterations in phenolic metabolism occur due to the interaction of the fundatrix with the host tissue, as opposed to her offspring, since the changes begin long before they appear. Thus, the evidence suggests that *Hormaphis hamamelidis* fundatrices manipulate phenolic metabolism of their galls.

Hormaphis hamamelidis galls accumulated substantial concentrations of condensed tannins, like many other arthropod galls. Interestingly, we found a positive relationship between condensed tannin concentration and the reproductive performance of fundatrices. Condensed tannins, typically thought of as defenses, could in fact reflect nutritional enhancement. Galls are physiological sinks, and *Hormaphis hamamelidis* galls have upregulated sink metabolism (Rehill and Schultz, 2003). Increased sink strength draws additional photosynthate to a site, thus, condensed tannins might accumulate in these galls as enhanced sink strength—providing increased nutrient flow to the aphids—draws additional substrates for phenolic metabolism to the gall. Alternatively, by first inducing greater condensed tannin synthesis, fundatrices could induce sink enzyme activity to meet this demand. Enhanced nutrition could, thus, be a secondary effect of tannin induction.

Other explanations for phenolic accumulation in galls include defense for the gall inhabitants. Caterpillars rarely eat *Hormaphis* galls; folivory often extends up to or even surrounds the uneaten gall (Schultz, 1992, B. Rehill, pers.obs.). Since a gall dies once the fundatrix perishes, we only measured phenolic concentrations of inhabited galls, thus we cannot determine if gall-former survival was related to gall phenolic content.

Regardless of mechanism(s), the positive relationship between condensed tannins and gall-former performance demonstrates adaptive value other than plant defense. To our knowledge, this study is the first to demonstrate a relationship between condensed tannin concentrations in insect gall tissues (vs. galled leaves) and gall-former reproductive performance. Thus, these data suggest that regarding condensed tannins only as defenses in plant–animal interactions may be misleading in some cases.

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References

ADAMS, J. M., REHILL, B., ZHANG, Y. J., and GOWER, J. 2009. A test of the latitudinal defense hypothesis: herbivory, tannins and total phenolics in four North American tree species. *Ecol. Res.* 24:697–704.

DETONI, M. D., VASCONCELOS, E. G., SCIO, E., DE AGUIAR, J. A. K., ISAIAS, R. M. D., and SOARES, G. L. G. 2010. Differential biochemical responses of *Calliandra brevipes* (Fabaceae, Mimosoidae) to galling behaviour by *Tanaostigmodes ringueleti* and *T. mecanga* (Hymenoptera, Tanaostigmatidae). *Aust. J. Bot.* 58:280–285.

FLAHERTY, L. and QUIRING, D. 2008. Plant module size and dose of gall induction stimulus influence gall induction and galler performance. *Oikos* 117:1601–1608.

IKAI, N. and HIJII, N. 2007. Manipulation of tannins in oaks by galling cynipids. *J. For. Res.* 12:316–319.

INBAR, M., IZHAKI, I., KOPLOVICH, A., LUPO, I., SILANIKOVE, N., GLASSER, T., GERCHMAN, Y., PEREVOLOTSKY, A., and LEV-YADUN, S. 2009. Why do many galls have conspicuous colours? A new hypothesis. *Arthropod-Plant Interactions* 4:1–6.

JANZEN, D. H. 1977. Why fruits rot, seeds mold, and meat spoils. *Am. Nat.* 111:691–713.

NYMAN, T. and JULKUNEN-TIITTO, R. 2000. Manipulation of the phenolic chemistry of willows by gall-inducing sawflies. *Proc. Natl. Acad. Sci. USA* 97:13184–13187.

REHILL, B. J. and SCHULTZ, J. C. 2001. *Hormaphis hamamelidis* and gall size: a test of the plant vigor hypothesis. *Oikos* 95:94–104.

REHILL, B. J. and SCHULTZ, J. C. 2003. Enhanced invertase activities in the galls of *Hormaphis hamamelidis*. *J. Chem. Ecol.* 29:2703–2720.

SCHULTZ, B. B. 1992. Insect herbivores as potential causes of mortality and adaptation in gallforming insects. *Oecologia* 90:297–299.

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Interspecific Comparison of Constitutive Ash Phloem Phenolic Chemistry Reveals Compounds Unique to Manchurian Ash, a Species Resistant to Emerald Ash Borer

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Abstract The emerald ash borer (*Agrilus planipennis*, EAB) is an invasive wood-borer indigenous to Asia and is responsible for widespread ash (*Fraxinus* spp.) mortality in the U.S. and Canada. Resistance and susceptibility to EAB varies among *Fraxinus* spp., which is a result of their co-evolutionary history with the pest. We characterized constitutive phenolic profiles and lignin levels in the phloem of green, white, black, blue, European, and Manchurian ash. Phloem was sampled twice during the growing season, coinciding with phenology of early and late instar EAB. We identified 66 metabolites that displayed a pattern of variation, which corresponded strongly with phylogeny. Previously identified lignans and lignan derivatives were confirmed to be unique to Manchurian ash, and may contribute to its high level of resistance to EAB. Other compounds that had been considered unique to Manchurian ash, including hydroxycoumarins and the phenylethanoids calceolarioside A and B, were detected in closely related, but susceptible species, and thus are unlikely to contribute to EAB resistance of Manchurian ash. The distinct phenolic profile of blue ash may contribute to its relatively high resistance to EAB.

Keywords *Agrilus planipennis* · *Fraxinus* · Wood-borer · HPLC · Host plant resistance · Plant–insect interactions · Invasive species · Emerald ash borer · Coleoptera · Buprestidae

Introduction

Emerald ash borer [(EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)] is an invasive wood-boring beetle indigenous to Asia that has caused widespread mortality

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of ash (*Fraxinus* spp.) trees in the U.S., Canada, and Russia (Poland and McCullough, 2006; Baranchikov et al., 2008; Smith et al., 2009). Larvae feed on the phloem and outer xylem, disrupting translocation of nutrients and water and ultimately causing tree death (Herms et al., 2004; Cappaert et al., 2005; Poland and McCullough, 2006). Black (*F. nigra* Marshall), white (*F. americana* L.), green (*F. pennsylvanica* Marshall), and European (*F. excelsior* L.) ash have all experienced extensive mortality, while in one study blue ash (*F. quadrangulata* Michaux.) was less extensively colonized than white ash, suggesting that it may be more resistant to EAB (Cappaert et al., 2005; Poland and McCullough, 2006; Anulewicz et al., 2007; Rebek et al., 2008). Observations in Asia suggest that Manchurian ash (*F. mandshurica* Ruprecht), which has coevolved with EAB, is not aggressively attacked unless stressed (Wei et al., 2004; Liu et al., 2007). These observations were confirmed experimentally in a common garden experiment where Manchurian ash was found to be much more resistant to EAB than green and white ash, supporting the hypothesis that Asian species possess defenses by virtue of their evolutionary history with EAB (Rebek et al., 2008).

Resistance of deciduous trees to wood-boring, phloem feeding insects is hypothesized to result from a combination of constitutive and induced physical and chemical defenses (Dunn et al., 1990; Eyles et al., 2007). The secondary chemistry of the genus *Fraxinus* is highly diverse (Kostova and Iossifova, 2007). Recently, it was noted that constitutive phenolic compounds, including hydroxycoumarins and phenylethanoid glycosides (calceolariosides A and B), were found in phloem tissue of Manchurian ash but not in green and white ash. These compounds were hypothesized to contribute to the high resistance of Manchurian ash to EAB (Eyles et al., 2007; Cipollini et al., 2011).

Identification of resistance mechanisms inferred from interspecific comparisons of resistant and susceptible species can be confounded by phylogenetic variation in traits that do not contribute to variation in host quality (Agrawal, 2011). Within the genus *Fraxinus*, green and white ash are phylogenetically distant from Manchurian ash, and some of the previously documented variation in their phenolic profiles (Eyles et al., 2007; Cipollini et al., 2011) may be the result of evolutionary divergence that is not related to their differences in resistance to EAB. Black and European ash are phylogenetically closely related to the resistant Manchurian ash, with all three species belonging to the section Fraxinus, but both of the former species are highly susceptible to EAB (Wallander, 2008). Variation in the phloem chemistry of these closely related resistant and suceptible species may have resulted from differential selection pressure imposed by EAB. Hence, we conducted an extensive metabolite profile characterization of interspecific variation in the constitutive phenolic chemistry of the phloem of ash species, with the prediction that closely related Manchurian, black, and European ash would be more similar to each other than to green, white, and blue ash, and that compounds unique to Manchurian ash might be related to its resistance against EAB. Trees were sampled twice during the growing season to coincide with the phenology of early and late instar EAB. We also quantified interspecific variation in lignin concentrations, a phenolic polymer that can also contribute to insect resistance (Coley, 1986; Wainhouse et al., 1990; Kurokawa et al., 2004; Borg-Karlson et al., 2006), but that showed little variation among a number of *Fraxinus* species in an earlier study (Cipollini et al., 2011).

Methods and Materials

Experimental Design and Sampling Ash trees were grown in an experimental plantation that included Manchurian ash cv. 'Mancana', green ash cv. 'Patmore', and white ash cv. 'Autumn Purple', all of which are clonally propagated cultivars. The plantation also included blue, green, and European ashes that were propagated as open-pollinated seedlings. The three cultivars and three open-pollinated species were planted in a common garden in November 2007 in Bowling Green, OH, USA in a randomized complete block design with eight blocks. Each of the six taxa was replicated four times within each block (4 individual tree replicates × 6 taxa = 24 trees per block). Thirty-two black ash cv. 'Fallgold' trees were planted in a plot adjacent to the original common garden plantation on 1 May 2008. On 2 June 2008, the mean stem diameter (measured 15 cm above ground) of Manchurian ash cv. 'Mancana', black ash cv. 'Fallgold', green ash cv. 'Patmore', white ash cv. 'Autumn Purple', and seedling-propagated green ash, blue ash, and European ash were 3.1± 0.06 cm (SEM), 2.6±0.06, 3.0±0.05, 3.0±0.1, 2.5±0.1, 1.7± 0.09, and 1.8±0.08, respectively. A previous study has shown that ash trees of this size, including some of the same cultivars, are readily colonized by EAB under field conditions in a common garden in Michigan, while exhibiting differences in resistance to EAB attack (Rebek et al., 2008), with Manchurian ash cv. 'Mancana' clearly being resistant. The relative resistance of Manchurian ash cv. 'Mancana' to EAB attack was confirmed in a follow-up to this study in which our common garden was exposed to augmented populations of the insect in the year following this investigation (Whitehill, 2011).

Trees were sampled at each of two time points, 2 June and 6 August 2008, with one tree for each taxon per block randomly selected for the sampling. All samples were from unique trees except for one case in which the same white ash tree was sampled and analyzed in both June and August. Phloem tissue was sampled for phenolic analyses on those dates to correspond phenologically with the beginning of egg hatch and the presence of mature larvae (3 rd and 4th

instars), respectively (Cappaert et al., 2005). Two-yr-old branches were pruned from trees, placed on ice and transported to the lab where phloem tissue was excised immediately from the branch, frozen in liquid nitrogen, and stored at −80 °C until sample extraction.

Phenolic and Lignin Extraction Soluble phenolics were extracted according to Eyles et al. (2007) and Cipollini et al. (2011). Extracts were stored at −20° C and used in subsequent HPLC analyses within 3 wk following extraction. Lignin was extracted and measured according to Bonello and Blodgett (2003).

Analysis of Phenolic Compounds HPLC-UV analyses of phenolic extracts were performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler and a 996 Photodiode Array Detector (PDA). The autosampler and column temperatures were set to 4 and 30 °C, respectively. Chromatographic separations were accomplished using a Waters Xterra™ RP18, 5 μm, 4.6×150 mm column and a Waters Xterra™ RP18, 3.9 μm, 3.0×20 mm guard column. The binary mobile phase consisted of water/acetic acid (A) (98:2, *v*/*v*) and methanol/acetic acid (B) (98:2, *v*/*v*), with a flow rate of 1 ml/min. The elution program followed that of Eyles et al. (2007). The injection volume for all samples was 10 μl. Samples were passed through the PDA (scanning range, 200–400 nm) with individual peaks quantified at 280 nm. The absorbance limit for detection was set to a minimum peak area of 9,000 in the processing parameters prior to data export. Individual peaks were included in subsequent statistical analyses if they met the following criteria: 1) a peak height greater than 0.02 AU, 2) a clearly discernible UV spectrum that could be matched to known phenolic compounds and/or could be interpreted in conjunction with MS data, and 3) consistent detection in at least three individual trees within a taxon.

For each sampling date, phenolic extracts from each taxon also were analyzed using an HPLC-ESI-MS (Varian 500 MS; Palo Alto, CA, USA) in parallel with a PDA detector. For detailed methods used in the qualitative identification of phenolic compounds see Supplemental Materials and Methods.

Individual compounds were quantified following the methods of Eyles et al. (2007). Matching standards were used for compound quantification when available. In cases where no matching standard was available, standard curves generated by using related compounds were used for relative quantification. For detailed methods used in compound quantification see Supplemental Materials and Methods.

Statistical Analyses Principal component analysis (PCA) was used to investigate the overall relationships between phenolic metabolites and species through dimensionality reduction and feature extraction (Johnson and Wichern, 2002). The PCA was conducted using R software (R Development Core Team, 2011) for both species and metabolites. PCA was run twice in order to better visualize relationships between species. The second PCA was based on data obtained from a cluster analysis of species and metabolites following the first PCA. Cluster analysis was run to support the grouping of variables obtained in the PCA. The cluster analyses were carried out using the "pvclust" routine in the pvclust package in R (Suzuki and Shimodaira, 2006). The pvclust package generates approximately unbiased (AU) confidence values for the clusters using the bootstrap resampling technique to assess their reliability (Efron et al., 1996; Suzuki and Shimodaira, 2006). A total of 1,000 bootstrap replications were generated for each cluster, and the AU confidence values were used to assess the uncertainty. The significance level of the clusters was set to 95. A larger confidence value is indicative of a "true" cluster (Efron et al., 1996). In this study, we also used biplots constructed with R to identify the relationship between species and metabolites.

Individual peak areas and mg g^{-1} FW concentrations for lignin were compared among species using univariate analysis of variance (ANOVA). Exploratory analyses of data and Levene's test were used to evaluate variance equality and normality requirements of residuals. Square-root and logarithmic transformations were used to meet normality requirements of residuals and homogeneity of variance. Following significant *F*-tests, means were separated using the LSD test (α=0.05). All data were analyzed with IBM SPSS Statistics v. 19 (SPSS Inc., 2010).

Results

A total of 66 individual phenolic compounds (Figs. 1, 2 and 3) were selected for analysis from all seven taxa, including the clonally and seedling-propagated green ash, from the combined June (Table S1) and August (Table 1) datasets. Only August data are presented in the paper (Tables 1 and 2), while the June data are presented in supplemental materials (Tables S1 and S2). Classes of phenolic compounds that we found in ash phloem include simple phenolics, phenolic acids, hydroxycoumarins, lignans, secoiridoids, phenylethanoids, flavonoids, and coumarin-secoiridoids. These compounds are similar to the phenolic metabolites previously described for the genus *Fraxinus* (Eyles et al., 2007; Kostova and Iossifova, 2007; Cipollini et al., 2011). Qualitative patterns of phloem chemistry among the taxa reflected their phylogenetic relationships, and quantities of phenolic compounds tended to increase from June to August (Tables 1 and 2; Figs. 1, 2, 3 and 4; Tables S1 and S2; and Fig. S1, S2, S3, S4 and S5). Detailed information regarding fragmentation patterns of phenolic metabolites identified in this study and data analysis,

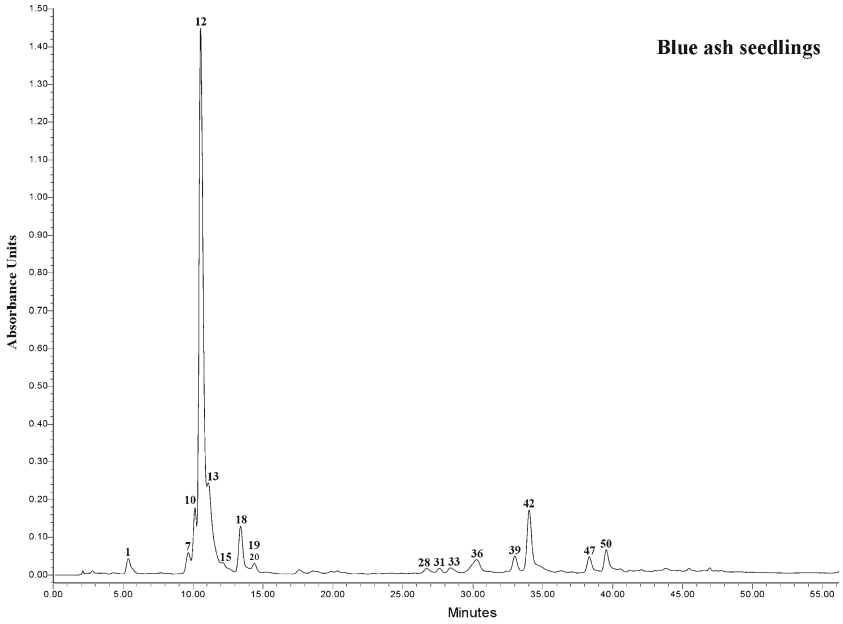

Fig. 1 Representative HPLC chromatogram at 280 nm of phloem tissue extracts from blue ash seedlings—a member of the section Dipetalae (Wallander, 2008). The chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

interpretation, and presentation of qualitative and quantitative differences in phenolic profiles can be found in Supplemental Results.

Lignin Lignin concentrations tended to be higher in August than in June and were consistently higher in phloem of blue ash and black ash cv. 'Fallgold' than in the other species (Tables 2 and S2). European ash had the lowest concentration of lignin in both June and August (Tables 2 and S2). White ash cv. 'Autumn Purple' and Manchurian ash cv. 'Mancana' had consistently higher lignin concentrations than green ash cv. 'Patmore' and seedling-propagated green ash.

Discussion

Manchurian ash cv. 'Mancana' is much more resistant to EAB than North American green ash cv. 'Patmore' or white ash cv. Autumn Purple', perhaps due to targeted selection imposed by its co-evolutionary history with EAB (Liu et al., 2007; Rebek et al., 2008; Whitehill, 2011). Earlier characterization of the constitutive phenolics in the phloem of these three cultivars revealed several compounds unique to Manchurian ash, including several hydroxycoumarins and two phenylethanoid compounds, calceolariosides A and B. These compounds were hypothesized to contribute to Manchurian ash resistance to EAB (Eyles et al., 2007; Cipollini et al., 2011). However, we detected hydroxycoumarins and calceolariosides A and B in the constitutive phloem of susceptible black and European ash in quantities comparable to or greater than those found in Manchurian ash, contradicting previous studies that were focused on differences between Manchurian, green, and white ash (Eyles et al., 2007; Cipollini et al., 2011). Of the ash species and cultivars we compared in this study, the qualitative phenolic profile of Manchurian ash cv. 'Mancana' differed most from green ash cv. 'Patmore' and white ash cv. 'Autumn Purple', which makes the comparison of these species the least informative when attempting to identify compounds potentially involved in resistance. This pattern is consistent with their distant phylogenetic relationship (Figs. 1, 2, 3 and 4) (Wallander, 2008). While some of the variation in the phenolic chemistry of Manchurian, white, and green ash may contribute to their differential resistance to EAB, much of their phytochemical variation may be the result of evolutionary divergence unrelated to selection imposed by EAB.

Black and European ash, which are highly susceptible to EAB, belong to the same section (Fraxinus) as Manchurian ash, and thus provide a phylogenetically-controlled comparison for identifying compounds that might be involved in EAB resistance . We found that phloem of black ash cv. 'Fallgold' and European ash contained a diverse array of hydroxycoumarins. Given their presence in the highly susceptible black and European ash, it is unlikely that these compounds play an important role in resistance of Manchurian ash to EAB. A role for hydroxycoumarins in resistance to EAB (Eyles et al., 2007) is not supported by our data.

Eyles et al. (2007) also hypothesized that calceolarioside A and B contributed to Manchurian ash resistance to EAB. However, we found ca. three-fold higher concentrations of calceolarioside A in black ash cv. 'Fallgold', and two-fold higher concentrations of calceolarioside B in European ash, than in Manchurian

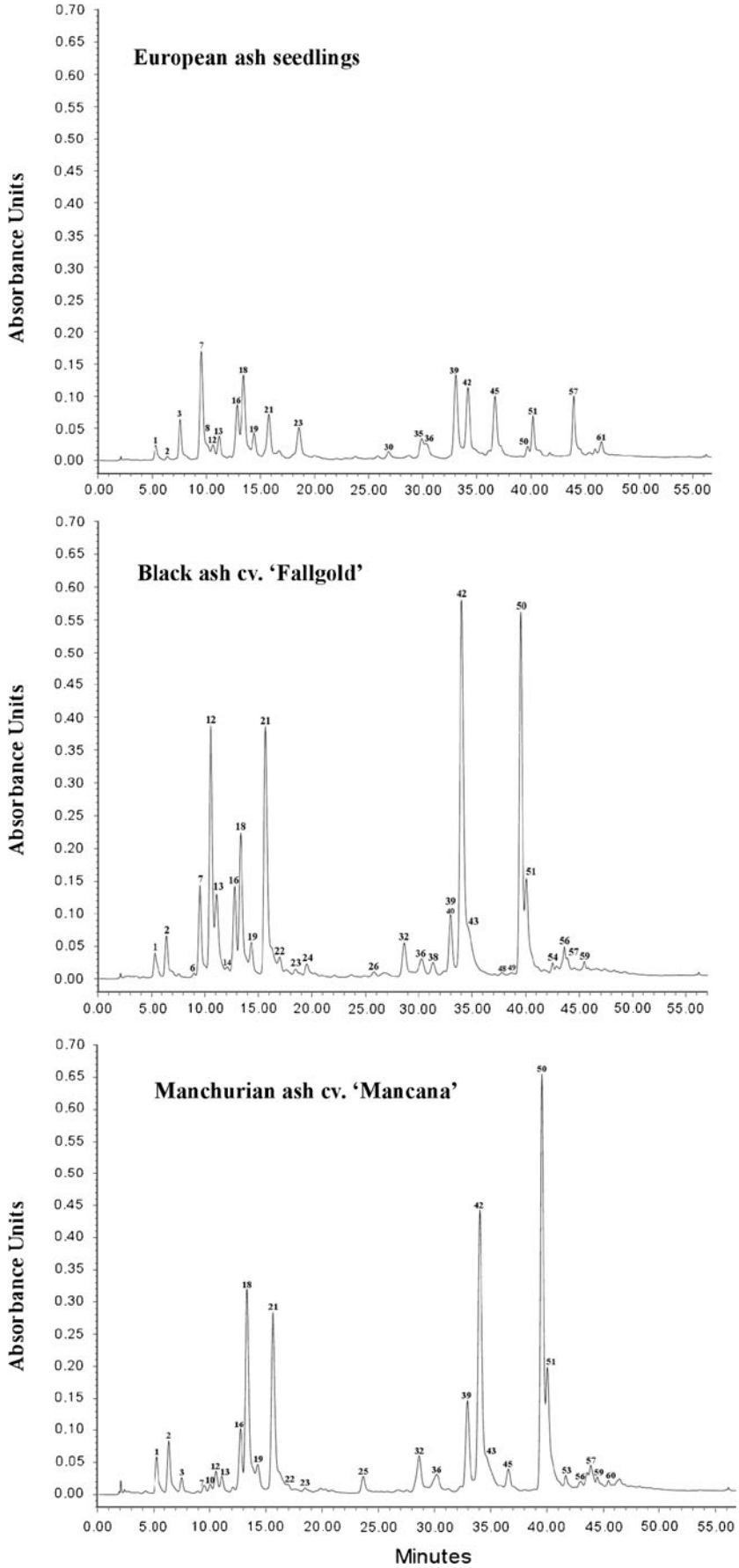

Fig. 2 Representative HPLC chromatograms at 280 nm of phloem tissue extracts from European ash seedlings, black ash cv. 'Fallgold', and Manchurian ash cv. 'Mancana'—all members of the section Fraxinus (Wallander, 2008). Each chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

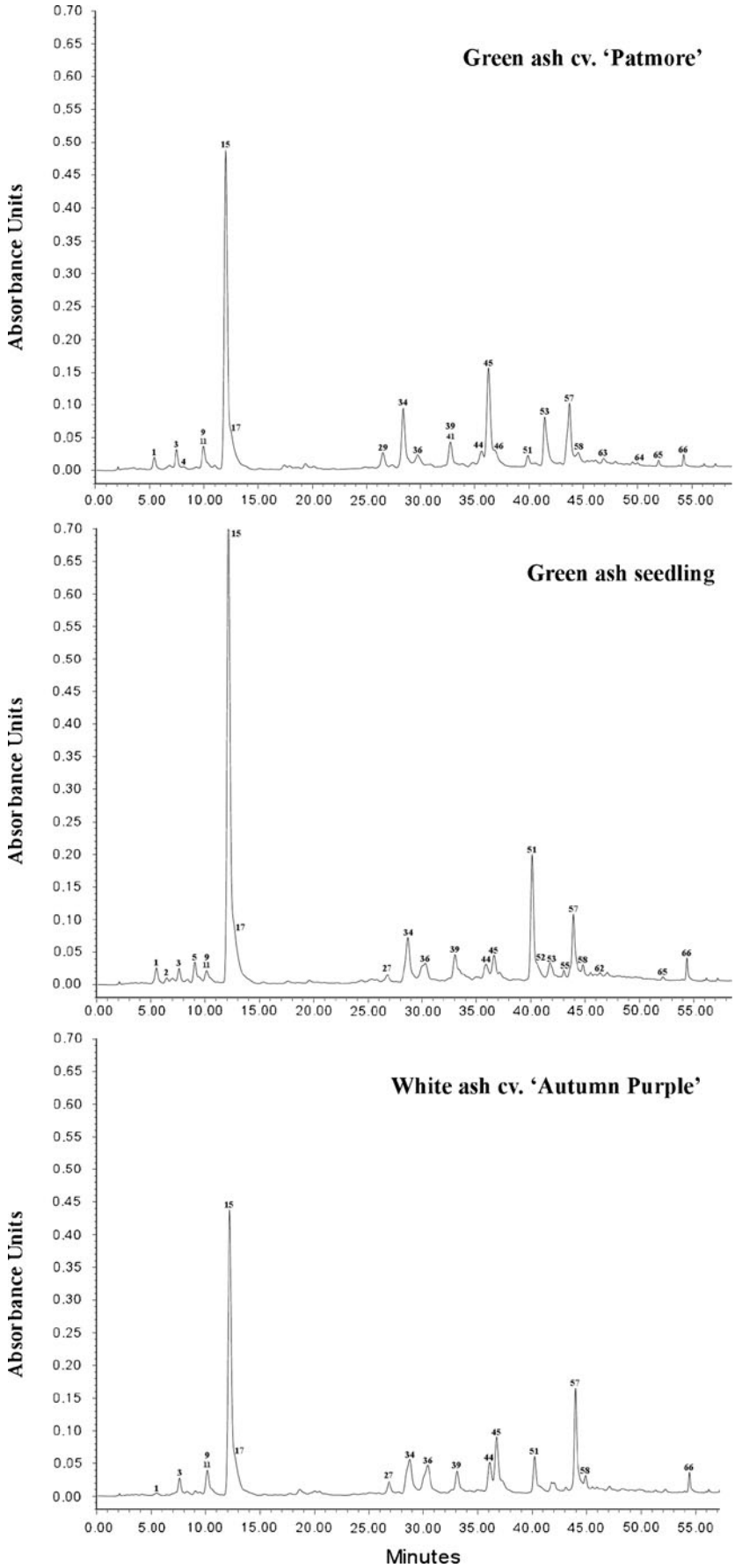

Fig. 3 Representative HPLC chromatograms at 280 nm of phloem tissue extracts from green ash cv. 'Patmore', green ash seedlings, and white ash cv. 'Autumn Purple'—all members of the section Melioides (Wallander, 2008). Each chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

Table 1 Phenolic compounds identified by HPLC-UV and LC-MS-PDA from samples collected on August 8th, 2008 from susceptible white ash cv. 'Autumn Purple', green ash cv. 'Patmore', green ash seedling, blue ash seedling, European ash seedling, black ash cv. 'Fallgold', and the resistant Manchurian ash cv. 'Mancana' grown in a common garden in Bowling Green, OH

Master Peak Number[a]	Species in which peak detected[b]	RT-HPLC-UV[c]	[M-H]- or [M-H]+[d*]	Fragments m/z (in order of decreasing abundance)[e]	λ max (nm)[f]	Putative ID[g]	Reference[h]
1	B, Q, E, GS, M, W	5.22	315	MS2: 135, 179; MS3: 107, 91, 117, 93, 79	281.2	Hydroxytyrosol hexoside	Cardoso et al., 2005; Eyles et al., 2007
2	B, GS, E, M	6.2	389	MS2: 329, 167, 161	276.5	Vanillic acid hexoside acetate adduct A	Eyles et al., 2007; Jimenez et al., 2010
3	GS, E, W, G, M	7.37	299	MS2: 179, 119, 143, 113, 161, 131	275.3	Tyrosol hexoside	Kammerer et al., 2005; Eyles et al., 2007
5	GS	8.59	389	MS2: 329, 167, 161	280.1	Vanillic acid hexoside acetate adduct B	Eyles et al., 2007; Jimenez et al., 2010
7	B, Q, E, M	9.24	177	MS2: 133; MS3: 89	289.5, 340.7	Esculetin A	Eyles et al., 2007
9	G, GS, W	9.76	601	MS2: 403, 223; MS3: 223, 371, 179; MS4: 121	264.7, sh[i] 300	Elenolic acid derivative 1 (m/z 431; 275 UV max)	Eyles et al., 2007
10	M, Q	10.02	177	MS2: 133; MS3: 89	258.8, sh 300, 340	Esculetin B	Eyles et al., 2007
12	B, Q, E, M	10.22	339	MS2: 177; MS3: 133, 105, 89, 149	334.7, sh 295	Esculin	Parejo et al., 2004; Eyles et al., 2007
13	B, Q, E, M	10.77	177	MS2: 133; MS3: 89	339.5, 289.5, 258	Esculetin C	Eyles et al., 2007
14	B	11.38	ND-LC-MS	ND-LC-MS	334.7, sh 293, 257.6	Unknown Coumarin 1	–
15	GS, W, Q, G	11.8	395*	MS2: 233, 185; MS3: 217, 203	264.7	Syringin	Eyles et al., 2007; Kostova and Iossifova, 2007
16	B, E, M	12.44	223*	MS2: 208, 163, 190, 107; MS3: 190, 180; MS4: 162, 134	289.5, 341.9	Fraxidin A	Yasuda et al., 2006
18	B, Q, E, M	12.99	369	MS2: 207; MS3: 192; MS4: 108, 164, 120, 175	341.9, sh 300	Fraxin	Godecke et al., 2005; Eyles et al., 2007
19	B, E, M	13.94	223*	MS2: 208, 163, 190, 107; MS3: 190, 180; MS4: 162, 134	293.1, 339.5	Fraxidin B	Yasuda et al., 2006
20	Q	13.94	177	MS2: 133; MS3: 89	345.4, 298, sh 260	Esculetin	Eyles et al., 2007
21	B, E, M	15.25	385*	MS2: 223; MS3: 208, 190; MS4: 190	328.7	Mandshurin	Terazawa and Sasaya, 1970; Eyles et al., 2007
22	B	16.17	209*	MS2: 149, 163, 181, 194; MS3: 121; MS4: 93, 65	339.5	Fraxetin	Eyles et al., 2007
23	B, E	17.64	353	MS2: 191, 179, 161	326.4, sh 298	3-Caffeoyl-quinic acid (Chlorogenic acid)	Cardoso et al., 2005
24	B	18.71	573	MS2: 537, 375; MS3: 375; MS4: 179, 327	277.7	Unknown Lignoid 1	–
25	M	23.64	717	MS2: 519, 357; MS3: 357; MS4: 151, 136	276.5	Pinoresinol dihexoside + $2H_2O$	Terazawa and Sasaya, 1970; Eyles et al., 2007
27	G, GS	26.66	523	MS2: 361; MS3: 165, 179, 313; MS4: 147	278.9	Ligustroside A	De la Torre-Carbot et al., 2005
28	Q	26.31	571	MS2: 373, 535, 343; MS3: 343; MS4: 285, 313, 207	281.2	(+)-1-Hydroxypinoresinol-4′-*O*-glucoside + $2H_2O$	Guo et al., 2007
29	W	26.44	525	MS2: 363, 301, 345; MS3: 301, 181, 199, 283; MS4: 283	278.9	Oleuropein Related Compound 1	Cardoso et al., 2005; Eyles et al., 2007
31	Q	27.24	ND by LC-MS PDA	ND by LC-MS PDA	278.9	Unknown Secoiridoid 1	–
32	B, M	27.69	477	MS2: 315, 203, 179, 341, 397; MS3: 135; MS4: 107	326.4, sh 290	Calceolarioside C	Eyles et al., 2007
33	Q	28.02	523	MS2: 361; MS3: 165, 179, 313	278.9	Ligustroside B	Ryan et al., 2002
34	G, GS, W	28.08	785	MS2: 623; MS3: 477, 461, 315; MS4: 315	331.1, sh 290	Forsythoside A *O*-glucoside	Guo et al., 2007
35	E	28.9	335	MS2: 179, 135 MS3: 135 MS4: 107, 117, 91, 79	327.6, 295	Caffeoylshikimic acid	Fang et al., 2002; Lin and Hamly, 2008
36	B, Q, E, G, GS, M, W	29.73	555		sh 280	10-Hydroxyoleuropein	

Table 1 (continued)

Master Peak Number[a]	Species in which peak detected[b]	RT-HPLC-UV[c]	[M-H]- or [M-H]+[d*]	Fragments m/z (in order of decreasing abundance)[e]	λ max (nm)[f]	Putative ID[g]	Reference[h]
				MS2: 393, 273, 307, 361, 375, 357, 419; MS3: 273, 307, 357, 343, 361; MS4: 137			Hosny, 1998; Kostova and Iossifova, 2007
38	B	30.43	525	MS2: 481, 195, 389, 319, 345; MS3: 345; MS4: 165	281.2	Dimethyoleuropein	Savarese et al., 2007
39	B, Q, E, G, GS, M, W	32.5	357	MS2: 151, 136, 311; MS3: 136; MS4: 92, 108	278.9	Pinoresinol	Eyles et al., 2007
42	B, Q, E, M	33.5	477	MS2: 315, 323, 179, 203, 341; MS3: 135 MS4: 107	328.7, sh 290	Calceolarioside A	Eyles et al., 2007
44	G, W	35.55	539	MS2: 377, 275, 291, 359; MS3: 275, 291, 359, 179; MS4: 111, 155	sh 280	Oleuropein A	Eyles et al., 2007
45	E, G, GS, M, W	36.04	623	MS2: 461; MS3: 315, 297; MS4: 135	331.1, sh 290	Verbascoside	Eyles et al., 2007
46	W	36.72	623	MS2: 461; MS3: 315, 297; MS4: 136	281.2, 331.1	Verbascoside A	Eyles et al., 2007
47	Q	37.89	725	MS2: 339; MS3: 177; MS4: 133	335.9, sh 295	Escuside	Iossifova et al., 2002;
50	B, E, Q, M	39.11	477	MS2: 315, 281, 251, 179, 221; MS3: 135; MS4: 107	328.7, sh 290	Calceolarioside B	Eyles et al., 2007
51	B, E, G, GS, M, W	39.68	539	MS2: 377, 307, 275; MS3: 307, 275, 345; MS4: 275, 139	281.2	Oleuropein	Cardoso et al., 2005; Eyles et al., 2007
52	GS	41.27	793.9	MS2: 403, 589, 743, 748, 595; MS3: 223, 371	265	Elenolic acid derivative 2	Eyles et al., 2007
53	GS, M, W	41.75	623	MS2: 461; MS3: 315, 297; MS4: 135	327.6, sh 288	Verbascoside B	Eyles et al., 2007
54	B	42.02	545	MS2: 369; MS3: 207, 192; MS4: 192	329.9, sh 295	Fraxin Related Compound	Godecke et al., 2005; Eyles et al., 2007
56	B, M	43.09	553	MS2: 391, 321, 289; MS3: 321, 289, 223; MS4: 139, 171, 167, 143, 261	280.1	Oleuropein Related Compound 2	Tanahashi et al., 1996; Hosny, 1998
57	B, E, G, GS, M, W	43.6	523	MS2: 361; MS3: 291, 259; MS4: 111, 139, 171, 143, 259	sh 280	Ligustroside	Tanahashi et al., 1996; Eyles et al., 2007
58	G, GS, W	44.36	431	MS2: 269; MS3: 225, 197, 117, 151	339.5, 268.2	Apigenin glucoside	Ryan et al., 2002
59	B, M	44.77	501	No fragmentation data available	328.7, sh 295	Unknown Coumarin 2	–
60	M	45.48	ND by LC-MS PDA	ND by LC-MS PDA	327.6, sh 298	Unknown Coumarin 3	–
61	E	46.01	447	MS2: 285, 255, 327, 227; MS3: 255, 227; MS4: 227, 211	350, sh 295, 264	Kaempherol galactoside	Ye et al., 2005
62	GS	46.03	ND LC-MS PDA	ND LC-MS PDA	sh 280	Unknown Secoiridoid 2	–
63	W	46.86	ND LC-MS	ND LC-MS	322.8, sh 282	Unknown Phenylethanoid 1	–
65	GS, W	51.82	285	MS2: 175, 199, 217, 241, 243, 151; MS3: 147, 131, 133, 119, 146; MS4: 119	254, sh 267, sh 290, 349.0	Luteolin	Ryan et al., 1999
66	G, GS, W	54.19	269	MS2: 225, 149, 151, 201; MS3: 181, 183, 197; MS4: 155, 141, 154	339.5, sh 290, 268.2	Apigenin	Eyles et al., 2007

[a] Number corresponding to a single individual peak identified using HPLC-UV. Peak numbers were assigned according to their retention time

[b] Letters correspond to the species in which the compound was detected via HPLC-UV (*B* black; *E* European; *G* green; *GS* green seedling; *Q* blue; *M* Manchurian; *W* white)

[c] RT-HPLC-UV = the average retention time for all individual biological replicates for each compound from all species

[d] The dominant molecular ion $[M-H]^-$ detected via LC-MS. Compounds were run in parallel with a PDA detector and full scan and PDA chromatograms were overlaid in order to match mass data. In some instances a $[M-H]^-$ could not be detected, in which case the positive ion mode was utilized to detect and identify the compound under investigation. For compounds identified using the positive ion mode, the corresponding $[M-H]^+$ is denoted with an *

[e] Corresponding fragmentation of the dominant molecular ion. Mass spectra for MS2, MS3, and MS4 are shown where applicable. Fragments are ordered according to decreasing abundance, with bolded ions representing the main ions fragmented in subsequent fragmentations

[f] λ max associated with each compound

[g] Tentative compound identity assigned based on literature match

[h] Corresponding reference for which a compound has been previously described

[i] *sh* = shoulder

Table 2 Contents of individual phenolic compounds and lignin in susceptible white ash cv. 'Autumn Purple', green ash cv. 'Patmore', green ash seedling, blue ash seedling, European ash seedling, black ash cv. 'Fallgold', and the resistant Manchurian ash cv. 'Mancana' in samples collected on August 8th, 2008. Compounds are separated into groups by phenolic compound class, while species are separated into the sections to which they belong within the genus *Fraxinus* (Wallander, 2008). Contents are expressed in mg g^{-1} FW±SEM (n=8). Different letters within a row indicate significantly different means by the protected LSD test (α=0.05). Black ash data appear in bold and are separate from the other species because it was not part of the original experimental design, but can be visually compared to the other species

Peak #	Compound name	*Melioides*			*Dipetalae*	*Fraxinus*		
		White	Green 'Patmore'	Green Seedling	Blue	European	Manchurian	Black
Phenolic acids/simple phenolics								
1	Hydroxytyrosol hexoside	0.4±0.03 b	ND[a]	0.4±0.1 b	1.1±0.1 a	0.5±0.1 b	1.2±0.1 a	**1.0±0.04**
2	Vanillic acid hexoside acetate adduct A	ND	ND	0.1±0.02 b	ND	0.1±0.01 b	1.0±0.04 a	**0.8±0.1**
3	Tyrosol hexoside	0.6±0.1 b	0.5±0.04 bc	0.4±0.1 c	ND	1.2±0.2 a	0.5±0.05 bc	**ND**
5	Vanillic acid hexoside acetate adduct B	ND	ND	0.4±0.1	ND	ND	ND	**ND**
23	3-Caffeoyl-quinic acid (Chlorogenic acid)	ND	ND	ND	ND	1.2±0.4	ND	**0.2±0.03**
Coumarins								
7	Esculetin A	ND	ND	ND	0.4±0.1 b	1.2±0.3 a	$0.1±3e^{-3}$ c	**0.9±0.1**
10	Esculetin B	ND	ND	ND	0.4±0.2 a	ND	$0.1±3e^{-3}$ b	**ND**
12	Esculin	ND	ND	ND	63.0±5.0 a	1.0±0.2 c	1.3±0.1 b	**15.0±0.3**
13	Esculetin C	ND	ND	ND	1.5±0.3 a	0.2±0.1 b	0.2±0.01 b	**1.0±0.1**
14	Unknown Coumarin 1	ND	ND	ND	ND	ND	ND	**0.1±0.04**
16	Fraxidin A	ND	ND	ND	ND	1.6±0.3 a	1.3±0.2 a	**2.0±0.2**
18	Fraxin	ND	ND	ND	8.7±0.9 b	8.6±1.7 b	22.5±1.6 a	**16.0±0.6**
19	Fraxidin B	ND	ND	ND	ND	0.7±0.1 a	0.7±0.1 a	**0.9±0.1**
20	Esculetin	ND	ND	ND	0.2±ND	ND	ND	**ND**
21	Mandshurin	ND	ND	ND	ND	1.1±0.1 b	5.0±0.5 a	**6.9±0.8**
22	Fraxetin	ND	ND	ND	ND	ND	ND	**1.6±0.5**
54	Fraxin Related Compound	ND	ND	ND	ND	ND	ND	**0.7±0.1**
59	Unknown Coumarin 2	ND	ND	ND	ND	ND	1.1±0.1	**1.2±0.1**
60	Unknown Coumarin 3	ND	ND	ND	ND	ND	0.7±0.1	**ND**
Monolignol								
15	Syringin	7.3±1.2 b	7.6±0.6 a	11.4±1.4 a	0.6±0.4 c	ND	ND	**ND**
Lignans								
24	Unknown Lignoid 1	ND	ND	ND	ND	ND	ND	**1.6±0.2**
25	Pinoresinol dihexoside + $2H_2O$	ND	ND	ND	ND	ND	1.7±0.3	**ND**
28	(+)-1-Hydroxypinoresinol-4′-*O*-glucoside + $2H_2O$	ND	ND	ND	1.3±0.4	ND	ND	**ND**
39	Pinoresinol	3.5±0.7 b	2.7±0.3 b	2.5±0.4 b	3.4±0.6 b	9.5±1.7 a	10.1±0.7 a	**7.0±0.4**
Phenylethanoids								
32	Calceolarioside C	ND	ND	ND	ND	ND	3.7±0.4	**3.0±0.2**
34	Forsythoside A *O*-glucoside	7.2±2.6 a	3.9±0.2 a	3.8±0.6 a	ND	ND	ND	**ND**
42	Calceolarioside A	ND	ND	ND	10.1±1.4 b	6.5±1.6 b	26.3±1.8 a	**34.6±1.1**
45	Verbascoside	4.3±1.2 a	4.2±0.3 a	1.7±0.2 b	ND	5.0±1.4 a	1.7±0.2 b	**ND**
46	Verbascoside A	1.2±0.3	ND	ND	ND	ND	ND	**ND**
50	Calceolarioside B	ND	ND	ND	3.0±1.4 b	0.7±0.2 c	25.2±2.6 a	**21.2±1.3**
53	Verbascoside B	3.9±0.5 a	ND	0.3±0.1 c	ND	ND	1.1±0.1 b	**ND**
63	Unknown Phenylethanoid 1	0.4±0.1	ND	ND	ND	ND	ND	**ND**

Table 2 (continued)

Peak #	Compound name	*Melioides*			*Dipetalae*	*Fraxinus*		
		White	Green 'Patmore'	Green Seedling	Blue	European	Manchurian	Black
Secoiridoids								
27	Ligustroside A	ND	2.5±0.2 a	1.6±0.3 b	ND	ND	ND	**ND**
29	Oleuropein Related Compound 1	3.0±0.4	ND	ND	ND	ND	ND	**ND**
31	Unknown Secoiridoid 1	ND	ND	ND	1.7±0.2	ND	ND	**ND**
33	Ligustroside B	ND	ND	ND	2.6±0.3	ND	ND	**ND**
36	10-Hydroxyoleuropein	3.4±0.3 d	9.4±0.8 a	5.2±0.5 c	7.5±0.9 b	4.1±0.6 cd	5.4±0.3 c	**4.5±0.2**
38	Dimethyoleuropein	ND	ND	ND	ND	ND	ND	**3.0±0.5**
44	Oleuropein A	3.2±0.2 b	5.4±0.7 a	ND	ND	ND	ND	**ND**
51	Oleuropein	1.8±0.1 d	5.1±0.5 c	19.2±2.7 b	ND	6.9±0.8 c	26.2±2.1 a	**20.7±2.0**
56	Oleuropein Related Compound 2	ND	ND	ND	ND	ND	1.3±0.1	**4.0±0.5**
57	Ligustroside	10.4±1.7 b	14.8±1.2 a	9.8±1.1 b	ND	8.1±1.2 b	3.9±0.4 c	**1.4±0.5**
62	Unknown Secoiridoid 2	ND	ND	0.8±0.3	ND	ND	ND	**ND**
Coumarin-Secoiridoid								
47	Escuside	ND	ND	ND	2.1±0.2	ND	ND	**ND**
Flavonoids								
58	Apigenin glucoside	0.2±0.01 ab	0.2±0.02 a	0.1±0.02 b	ND	ND	ND	**ND**
61	Kaempherol galactoside	ND	ND	ND	ND	0.3±0.1	ND	**ND**
65	Luteolin	$0.1\pm8e^{-3}$ a	ND	$0.04\pm6e^{-3}$ b	ND	ND	ND	**ND**
66	Apigenin	$0.1\pm6e^{-3}$ b	0.1±0.01 a	0.1±0.01 a	ND	ND	ND	**ND**
Phenolic polymer								
	Lignin	18.5±1.0 b	14.6±1.2 c	13.6±1.0 c	21.6±0.8 a	5.8±0.8 d	18.1±1.2 b	**20.5±1.0**

[a] *ND* = not detected

ash cv. 'Mancana'. The high concentrations of these compounds in black and European ash make it unlikely that calceolariosides are involved in Manchurian ash resistance to EAB.

Of the 27 phenolic compounds that we identified in the phloem tissue of Manchurian ash cv. 'Mancana', only pinoresinol dihexoside **(25)**, and a compound tentatively identified as a coumarin derivative **(60)**, were unique to this cultivar. Because the other 25 compounds were also detected in susceptible ash species, they are unlikely to contribute to EAB resistance, unless they act synergistically with compounds unique to Manchurian ash.

Eyles et al. (2007) detected high concentrations of pinoresinol dihexoside in dormant tissue of Manchurian ash relative to green and white ash and suggested that it may play a role in EAB resistance. We confirmed that concentrations of this compound remained high during the growing season on dates highly relevant to the phenology of EAB larvae, as did Cipollini et al. (2011). Pinoresinol dihexoside is a lignan (phenylpropane dimer) (Strack, 1997), and its aglycone (pinoresinol) has antifeedant and growth/molt inhibiting activities against several insect species (Miyazawa et al., 1994; Cabral et al., 2000; Garcia et al., 2000). A recent proteomic analysis of ash phloem showed that a phenylcoumaran benzylic ether reductase (PCBER) is expressed constitutively at >25-fold higher levels in phloem of Manchurian ash cv. 'Mancana' than in black ash cv. 'Fallgold', green ash cv. 'Patmore', and white ash cv. 'Autumn Purple' (Whitehill et al., 2011). PCBER is an enzyme involved in the biosynthesis of lignans, providing further support for a potential role of lignans in resistance of Manchurian ash cv. 'Mancana' to EAB.

Our inability to identify conclusively the coumarin derivative **(60)** precludes informed speculation about its putative role in EAB resistance. The functions of this compound could be tested by bioassays with EAB larvae in a phloem-free artificial diet (Keena et al., 2010), with compounds tested at biologically relevant concentrations (Tables 2 and S2).

Lignin can act as an indirect chemical defense (Borg-Karlson et al., 2006), or as a dose-dependent physical defense against wood-boring insects (Wainhouse et al., 1990). However, we found little evidence that variation in lignin concentration of phloem contributes to interspecific variation in EAB resistance, confirming earlier observations by

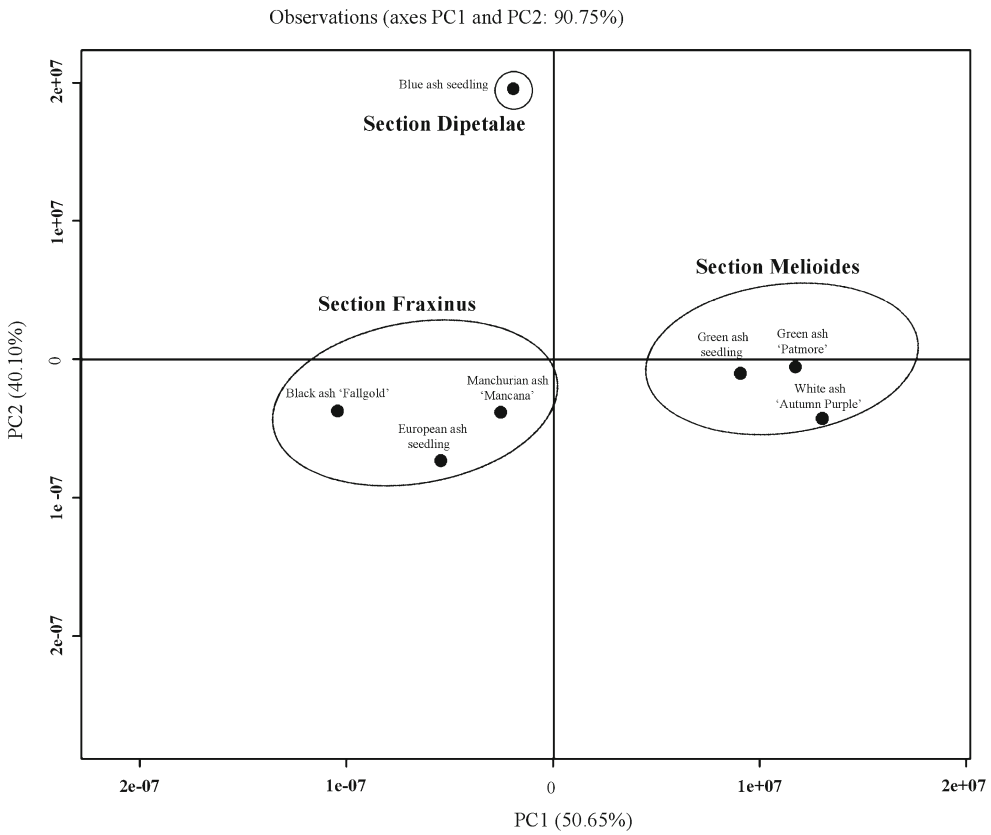

Fig. 4 Relationship between PC1 and PC2 scores for individual peak areas for 59 compounds, but excluding the most characteristic compounds (**12**, **15**, **18**, **21**, **42**, **50**, and **51**) that were associated with individual ash species (see Figs. 1, 2 and 3). Black dots represent the mean of eight biological replicates within a given taxon. The taxa cluster according to phylogenetic placement into the three groups Meliodes (green and white ash), Dipetalae (blue ash), and Fraxinus (Manchurian, European, and black ash)

Cipollini et al. (2011). Lignin concentrations in phloem of Manchurian ash cv. 'Mancana' were similar to those in white ash cv. 'Autumn Purple' and lower than in black ash cv. 'Fallgold', which are both highly susceptible. The high levels of lignin in blue ash were similar to the levels found in the highly susceptible black ash cv. 'Fallgold.'

Four of the taxa (black ash cv. 'Fallgold', green ash cv. 'Patmore', white ash cv. 'Autumn Purple', and Manchurian ash cv. 'Mancana') that we compared were clonally propagated cultivars, and thus their chemistry may not be representative of their respective species as a whole. However, their relative resistance to EAB is well characterized, so they provide relevant samples of these taxa to compare in the search for EAB resistance mechanisms. The other three taxa that we examined (blue, European, and green ash) were propagated as open-pollinated seedlings. We observed extremely high phytochemical similarity between the green ash clone and the seedling-propagated green ash population, as did Cipollini et al. (2011). We also observed high convergence in the phenolic profiles between species in the same taxonomic section (Fig. 4).

Blue ash, which is endemic to North America, has been colonized by EAB at lower levels than other North American species in the field (Anulewicz et al., 2007) and in a common garden study (Herms et al., unpublished). The very distinct phenolic chemistry of blue ash that we observed relative to that of green, white, and black ash cultivars may contribute to this resistance. Hence, blue ash may be a source of allopatric resistance (e.g., Harris and Frederiksen, 1984) that could be introgressed into the more susceptible North American congeners. However, the highly divergent phylogenetic relationships among these taxa (Jeandroz et al., 1997; Wallander, 2008) could complicate efforts to hybridize blue ash with more susceptible species.

The identification of resistant green and white ash genotypes could be important for host plant resistance breeding programs. A small proportion of 'lingering' green and white ash continue to survive in areas of high EAB-induced ash mortality, but further work is needed to establish their level of genetic resistance and to identify markers that could be used in programs to screen or breed for resistance (Koch et al., 2010). Pinoresinol dihexoside or unknown coumarin **(60)** may represent two of such biomarkers. Indeed, pinoresinol, the aglycone of pinoresinol dihexoside, was detected in all species we investigated. If a resistance role for pinoresinol is confirmed, the requisite genetic machinery for its production exists in susceptible North American ash species.

In summary, our broad-based interspecific survey of the phenolic profiles of ash phloem has confirmed that previously identified lignans and lignan derivatives may contribute to the high level of resistance of Manchurian ash cv. 'Mancana' to EAB. However, functional studies and/or bioassays are required to confirm the role of these compounds as deterrents or toxins for EAB larvae.

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References

AGRAWAL, A. A. 2011. Current trends in the evolutionary ecology of plant defence. *Funct. Ecol.* 25:420–432.

ANULEWICZ, A. C., MCCULLOUGH, D. G., and CAPPAERT, D. L. 2007. Emerald ash borer (*Agrilus planipennis*) density and canopy dieback in three North American ash species. *Arboricult. Urban For.* 33:338–349.

BARANCHIKOV, Y., MOZOLEVSKAYA, E., YURCHENKO, G., and KENIS, M. 2008. Occurrence of the emerald ash borer, *Agrilus planipennis* in Russia and its potential impact on European forestry. *EPPO Bull.* 38:233–238.

BONELLO, P. and BLODGETT, J. T. 2003. *Pinus nigra-Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Pathol.* 63:249–261.

BORG-KARLSON, A. K., NORDLANDER, G., MUDALIGE, A., NORDENHEM, H., AND UNELIUS, C. R. 2006. Antifeedants in the feces of the pine weevil *Hylobius abietis*: Identification and biological activity. *J. Chem. Ecol.* 32:943–957.

CABRAL, M. M. O., AZAMBUJA, P., GOTTLIEB, O. R., and GARCIA, E. S. 2000. Effects of some lignans and neolignans on the development and excretion of *Rhodnius prolixus*. *Fitoterapia* 71:1–9.

CAPPAERT, D., MCCULLOUGH, D. G., POLAND, T. M., and SIEGERT, N. 2005. Emerald ash borer in North America: a research and regulatory challenge. *Am. Entomol.* 51:152–165.

CARDOSO, S. M., GUYOT, S., MARNET, N., LOPES-DA-SILVA, J. A., RENARD, C. M. G. C., and COIMBRA, M. A. 2005. Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J. Sci. Food Agric.* 85:21–32.

CIPOLLINI, D., WANG, Q., WHITEHILL, J. G. A., POWELL, J., BONELLO, P., and HERMS, D. 2011. Distinguishing defensive characteristics in the phloem of ash species resistant and susceptible to emerald ash borer. *J. Chem. Ecol.* 37:450–459.

COLEY, P. D. 1986. Costs and benefits of defense by tannins in a neotropical tree. *Oecologia* 70:238–241.

DE LA TORRE-CARBOT, K., JAUREGUI, O., GIMENO, E., CASTELLOTE, A. I., LAMUELA-RAVENTOS, R. M., and LOPEZ-SABATER, M. C. 2005. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. *J. Agric. Food Chem.* 53:4331–4340.

DUNN, J. P., POTTER, D. A., and KIMMERER, T. W. 1990. Carbohydrate reserves, radial growth, and mechanisms of resistance of oak trees to phloem-boring insects. *Oecologia* 83:458–468.

EFRON, B., HALLORAN, E., and HOLMES, S. 1996. Bootstrap confidence levels for phylogenetic trees (vol 93, p. 7085, 1996). *Proc. Natl. Acad. Sci. USA.* 93:13429–13434.

EYLES, A., JONES, W., RIEDL, K., HERMS, D. A., CIPOLLINI, D., SCHWARTZ, S., CHAN, K., and BONELLO, P. 2007. Comparative phloem chemistry of Manchurian (*F. mandshurica*) and two North American ash species (*F. americana* and *F. pennsylvanica*). *J. Chem. Ecol.* 33:1430–1448.

FANG, N. B., YU, S. G., and PRIOR, R. L. 2002. LC/MS/MS characterization of phenolic constituents in dried plums. *J. Agric. Food Chem.* 50:3579–3585.

GARCIA, E. S., CABRAL, M. M. O., SCHAUB, G. A., GOTTLIEB, O. R., and AZAMBUJA, P. 2000. Effects of lignoids on a hematophagous bug, *Rhodnius prolixus*: feeding, ecdysis, and diuresis. *Phytochemistry* 55:611–616.

GODECKE, T., KALOGA, M., and KOLODZIEJ, H. 2005. A phenol glucoside, uncommon coumarins and flavonoids from *Pelargonium sidoides*. *Naturforsch B* 60:677–682.

GUO, H., LIU, A. H., YE, M., YANG, M., and GUO, D. A. 2007. Characterization of phenolic compounds in the fruits of *Forsythia suspensa* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Sp.* 21:715–729.

HARRIS, M. K. and FREDERIKSEN, R. A. 1984. Concepts and methods regarding host plant resistance to arthropods and pathogens. *Annu. Rev. Phytophathol.* 22:247–272.

HERMS, D. A., MCCULLOUGH, D. G., and SMITLEY, D. R. 2004. Under attack: the current status of the emerald ash borer infestation and the program to eradicate it. *Am. Nurseryman* 7:20–27.

HOSNY, M. 1998. Secoiridoid glucosides from *Fraxinus oxycarpa*. *Phytochemistry* 47:1569–1576.

IOSSIFOVA, T., VOGLER, B., and KOSTOVA, I. 2002. Escuside, a new coumarin-secoiridoid from *Fraxinus ornus* bark. *Fitoterapia* 73:386–389.

JEANDROZ, S., ROY, A., and BOUSQUET, J. 1997. Phylogeny and phylogeography of the circumpolar genus *Fraxinus* (Oleaceae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. *Mol. Phylogenet. Evol.* 7:241–251.

JIMENEZ, P., MASSON, L., BARRIGA, A., CHAVEZ, J., and ROBERT, P. 2010. Oxidative stability of oils containing olive leaf extracts obtained by pressure, supercritical and solvent-extraction. *Eur. J. Lipid Sci. Technol.* 113:497–505.

JOHNSON, R. and WICHERN, D. A. 2002. Applied Multivariate Statistical Analysis, 5th ed. Prentice-Hall, Upper Saddle River.

KAMMERER, B., KAHLICH, R., BIEGERT, C., GLEITER, C. H., and HEIDE, L. 2005. HPLC-MS/MS analysis of willow bark extracts contained in pharmaceutical preparations. *Phytochem. Anal.* 16:470–478.

KEENA, M. A., GOULD, J., and BAUER, L. S. 2010. Developing an effective and efficient rearing method for the emerald ash borer, *in*, Proceedings of the Emerald Ash Borer Research and Technology Development Meeting, October 20–21, 2009, Pittsburgh, Pennsylvania. Lance D, Buck J, Binion D, Reardon R, and Mastro V, compilers. USDA Forest Health Technology Enterprise Team, FHTET-2010-01. p. 136.

KOCH J. L., MASON M. E., CAREY D. W., KNIGHT K. S., POLAND T. M., and HERMS D. A. 2010. Survey for tolerance to emerald ash borer within North American ash species, pp. 64, *in* C. H. Michler, M.D. Ginzel (eds.), Proceedings of Symposium on Ash in North America, Gen. Tech. Rep. NRS-P-72. Newtown Square, PA: USDA Forest Service, Northern Research Station.

KOSTOVA, I. and IOSSIFOVA, T. 2007. Chemical components of *Fraxinus* species. *Fitoterapia* 78(2):85–106.

KUROKAWA, H., KITAHASHI, Y., KOIKE, T., LAI, J., and NAKASHIZUKA, T. 2004. Allocation to defense or growth in dipterocarp forest seedlings in Borneo. *Oecologia* 140(2):261–270.

LIN, L. Z. and HARNLY, J. N. 2008. Phenolic compounds and chromatographic profiles of pear skins (*Pyrus* spp.). *J. Agric. Food Chem.* 56:9094–9101.

LIU, H. P., BAUER, L. S., MILLER, D. L., ZHAO, T. H., GAO, R. T., SONG, L. W., LUAN, Q. S., JIN, R. Z., and GAO, C. Q. 2007. Seasonal abundance of *Agrilus planipennis* (Coleoptera: Buprestidae) and its natural enemies *Oobius agrili* (Hymenoptera: Encyrtidae) and *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) in China. *Biol. Control.* 42:61–71.

MIYAZAWA, M., ISHIKAWA, Y., KASAHARA, H., YAMANAKA, J., and KAMEOKA, H. 1994. An insect growth-inhibitory lignan from flower buds of *Magnolia-fargesii*. *Phytochemistry* 35:611–613.

PAREJO, I., JAUREGUI, O., SANCHEZ-RABANEDA, F., VILADOMAT, F., BASTIDA, J., and CODINA, C. 2004. Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* 52:3679–3687.

POLAND, T. M. and MCCULLOUGH, D. G. 2006. Emerald ash borer: invasion of the urban forest and the threat to North America's ash resource. *J. Forest.* 104:118–124.

R DEVELOPMENT CORE TEAM 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

REBEK, E. J., HERMS, D. A., and SMITLEY, D. R. 2008. Interspecific variation in resistance to emerald ash borer (Coleoptera: Buprestidae) among North American and Asian Ash (*Fraxinus* spp.). *Environ. Entomol.* 37:242–246.

RYAN, D., ROBARDS, K., and LAVEE, S. 1999. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J. Chromatogr. A* 832:87–96.

RYAN, D., ANTOLOVICH, M., HERLT, T., PRENZLER, P. D., LAVEE, S., and ROBARDS, K. 2002. Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's mammoth. *J. Agric. Food Chem.* 50:6716–6724.

SAVARESE, M., DE MARCO, E., and SACCHI, R. 2007. Characterization of phenolic extracts from olives (*Olea europaea cv.* Pisciottana) by electrospray ionization mass spectrometry. *Food Chem.* 105:761–770.

SMITH E. L., STORER A. J., and ROOSIEN B. K. Emerald ash borer infestation rates in Michigan, Ohio, and Indiana. McManus K, Gottschalk KW, eds. p. 82 *in:* Proceedings. 20th USDA Interagency Research Forum on Invasive Species 2009. Jan. 13–16, 2009, Annapolis, MD. USDA FS GTR NRC-P-51, Newtown Square, PA: USDA, Forest Service, Northern Research Station. 114 pp.

SPSS STATISTICS FOR MAC. Version 19. 2010. IBM-SPSS Inc., Chicago, IL. USA.

STRACK, D. 1997. Phenolic metabolism, pp. 387–416, *in* P. M. Dey and J. B. Harborne (eds.), Plant Biochemistry. Academic Press, New York.

SUZUKI, R. and SHIMODAIRA, H. 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 22:1540–1542.

TANAHASHI, T., TAKENAKA, Y., and NAGAKURA, N. 1996. Two dimeric secoiridoid glucosides from *Jasminum polyanthum*. *Phytochemistry* 41:1341–1345.

TERAZAWA, M. and SASAYA, T. 1970. Studies on the extractives of Yachidamo, Fraxinus mandshurica Ruprecht var. japonica Maxim. II. Glucosides in bark. *J. Jpn. Wood Res. Soc.* 16:192–199.

WAINHOUSE, D., CROSS, D. J., and HOWELL, R. S. 1990. The role of lignin as a defense against the spruce bark beetle *Dendroctonus micans*—effect on larvae and adults. *Oecologia* 85:257–265.

WALLANDER, E. 2008. Systematic of *Fraxinus* (Oleaceae) and evolution of dioecy. *Plant Syst. Evol.* 273:25–49.

WEI, X., REARDON, D., WU, Y., and SUN, J. H. 2004. Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), in China: a review and distribution survey. *Acta Entomol. Sin.* 47:679–685.

WHITEHILL, J. G. A. 2011. Investigations into Mechanisms of Ash Resistance to the Emerald Ash Borer. The Ohio State University, Columbus. 250 p.

WHITEHILL, J. G. A., POPOVA-BUTLER, A., GREEN-CHURCH, K. B., KOCH, J. L., HERMS, D. A., and BONELLO, P. 2011. Interspecific proteomic comparisons reveal ash phloem genes potentially involved in constitutive resistance to the emerald ash borer. *PLoS One* 6:e24863.

YASUDA, T., FUKUI, M., NAKAZAWA, T., HOSHIKAWA, A., and OHSAWA, K. 2006. Metabolic fate of fraxin administered orally to rats. *J. Nat. Prod.* 69:755–757.

YE, M., YAN, Y. N., and GUO, D. A. 2005. Characterization of phenolic compounds in the Chinese herbal drug Tu-Si-Zi by liquid chromatography coupled to electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 19:1469–1484.

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Electrophysiological and Behavioral Responses of the Bark Beetle *Dendroctonus rhizophagus* to Volatiles from Host Pines and Conspecifics

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Abstract The bark beetle *Dendroctonus rhizophagus* is endemic to northwestern Mexico where it kills immature pines<3 m tall. We report the first investigation of the chemical ecology of this pest of forest regeneration. We used GC-EAD to assess olfactory sensitivity of this species to volatile compounds from: resin of a major host, *Pinus arizonica*; mid/hindguts of single, gallery-initiating females; and mate-paired males within galleries of attacked host trees in the field. Antennae of both sexes responded to monoterpenes α-pinene, β-pinene and 3-carene as well as to the beetle-derived oxygenated monoterpenes fenchyl alcohol, myrtenal, *cis*-verbenol, *trans*-verbenol, verbenone, and myrtenol. These monoterpenes were quantified from pre-emerged *D. rhizophagus* adults forced to attack host tissue in the laboratory, and from individuals dissected from naturally-attacked hosts at different stages of colonization. In both bioassays, myrtenol and *trans*-verbenol were the most abundant volatiles, and *trans*-verbenol was the only one produced in significantly greater quantities by females than males in a naturally-colonized host. Two field experiments were performed to evaluate behavioral responses of *D. rhizophagus* to antennally-active monoterpenes. Results show that 3-carene was significantly attractive either alone or in a ternary (1:1:1) combination with α-pinene and β-pinene, whereas neither α-pinene nor β-pinene alone were attractive. None of the beetle-associated oxygenated monoterpenes enhanced the attractiveness of the ternary mixture of monoterpenes, while verbenone either alone or combined with the other five oxygenated terpenes reduced *D. rhizophagus* attraction to the ternary mixture. The results suggest that attraction of *D. rhizophagus* to the host tree *P. arizonica* is mediated especially by 3-carene. There was no conclusive evidence for an aggregation or sex attractant pheromone.

Keywords Coleoptera · Curculionidae · Scolytinae · Monoterpenes *Pinus arizonica* · Semiochemical · Gas chromatography-electroantennographic detection (GC-EAD) · Pest management · Host location · Mate finding · *trans*-verbenol

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Introduction

Bark beetles are important mortality agents of conifers in North and Central America (Wood S. L. 1982). Populations can remain at endemic levels for many years; however, changes in abiotic or biotic variables can lead to outbreaks that result in substantial host plant mortality (Bentz et al., 2010) and can consequently result in serious economic losses in the forest industry and disturbances to forest ecosystems.

Dendroctonus rhizophagus Thomas & Bright is a bark beetle species endemic to the Sierra Madre Occidental in northwestern Mexico that colonizes 11 different species of pine, particularly Arizona pine (*Pinus arizonica* Engelm.), Apache pine (*Pinus engelmannii* Carr.), Chihuahua pine (*Pinus leiophylla* Schlecht and Cham.), and Durango pine (*Pinus durangensis* Martínez) (Salinas-Moreno et al., 2004, 2010; Mendoza et al., 2011). This bark beetle has had significant impact on natural and commercial regeneration of pine in the region. For example, from 1977 to 1983, this beetle killed 2 million young trees in Mesa del Huracán, Chihuahua (29 ° 38′ N, 108 °14′ W) across an infested area of approximately 12,000 ha (Estrada-Murrieta 1983). The total land area susceptible to infestation by this beetle is estimated to be 100,000 ha (SEMARNAT, 2005).

Dendroctonus rhizophagus is a typical primary bark beetle species in that it can successfully attack apparently healthy pine trees and kill them (Cibrián-Tovar et al., 1995). It is morphologically similar to the red turpentine beetle, *D. valens* LeConte (Wood S. L. 1982), however, these sibling species differ substantially in their behavior. *Dendroctonus rhizophagus* infests only young hosts, ranging in size from seedlings (≥1.5 cm diam. base) to saplings (< 8 cm diam. at 1.4 m height and<3 m tall), whereas *D. valens* resembles most other members of the genus in colonizing mature hosts (> 20 cm diam. at 1.4 m height and >20 m tall), including >40 species of pine present in its extensive native range within North America (Wood S. L. 1982). Furthermore, *D. rhizophagus* is atypical of its genus in that generally just one or two pairs (rather than tens to thousands of pairs) colonize and kill a single host (Estrada-Murrieta 1983; Cibrián-Tovar et al., 1995). Thus, this species does not conspicuously aggregate or rely on mass attacks of conspecifics for neutralizing constitutive tree defenses (Sánchez-Martínez and Wagner, 2009), whereas many other tree-killing bark beetles extensively use mass attack as a host colonization strategy (Raffa et al., 1993). Each pair excavates a gallery under the bark of the tree's root collar where eggs are laid; as the larvae develop, they mine downward and feed in the roots where they subsequently pupate within ovoid chambers that penetrate the root wood. The species is univoltine, with brood adults emerging from the roots to attack new hosts during the onset of the rainy season in summer (Estrada-Murrieta 1983).

While numerous studies have examined the chemical ecology of sibling species *D. valens* and *D. terebrans* Olivier (Phillips et al., 1989; Hobson et al., 1993; Sun et al., 2004; Zhang and Sun, 2006; Erbilgin et al., 2007; Shi and Sun, 2010), the semiochemistry of *D. rhizophagus* has not previously been investigated. We, therefore, performed studies on *D. rhizophagus* to identify compounds of the host tree and the beetle that might mediate host and mate location for this species and possibly provide chemical baits for its management. Since both attractive and inhibitory semiochemicals play a key role in mediating host location and colonization by aggressive *Dendroctonus* and other bark beetles, we hypothesized that the host colonization behavior of *D. rhizophagus* would be associated with an atypical semiochemical system. In particular, we predicted the absence of an aggregation pheromone in this bark beetle.

To identify volatile host and beetle-associated compounds that are olfactorily perceived by *D. rhizophagus*, we first used coupled gas chromatography-electroantennographic detection (GC-EAD) analysis of extracts of host resin and beetle guts. Further, we investigated whether release of volatiles by *D. rhizophagus* is dependent on the beetle's sex and life history stage, since such dependencies often signify pheromone components. We quantified individual volatiles released by female and male insects before and after host attack and pairing, and under both laboratory and natural field conditions. Finally, we evaluated behavioral responses by this species to devices that release electrophysiologically-active host and insect-produced volatiles in the field.

Methods and Materials

Insects Adult bark beetles used in bioassays were collected from roots and root collars of naturally-infested Arizona pine, *Pinus arizonica* Engelm., in San Juanito, Bocoyna Municipality, Chihuahua State (27°55′54.9″N, 107°35′ 54.6″W; 2452 m elevation), Mexico. Two categories of insects were tested: "pre-emerged" beetles were fully-melanized brood adults present beneath the cortex of roots of dead trees colonized the previous year; "emerged" beetles were those found within new galleries under the bark of live hosts. Beetles were collected from the colonized pine by carefully shaving the bark away with a knife and removing each insect individually with soft forceps. Beetles were housed in vented plastic containers with moistened paper at 4°C for up to 3 d before use. Insects were sexed according to the shape of the seventh abdominal tergite (Lyon, 1958).

Electrophysiological Assays Olfactory sensitivity of *D. rhizophagus* to volatile compounds in the resin of *P. arizonica* and guts of emerged adults was studied with GC-EAD. Resin from young *P. arizonica* (< 2 m height) was collected by removing a 7 cm^2 section of bark with a hatchet and installing aluminum, "V"-shaped collector beneath the wound to funnel exuded resin into a 5 ml glass vial (Smith 2000). After 24 hr, 100 μl of the resin were subsampled with a Drummond capillary pipette (Scientific Co., Broomall, Pa., USA) and placed into a vial with 900 μl hexane (95 % n-hexane, HPLC purity, Baker, Phillipsburg, N.J., USA) and stored at 4°C. Gut volatiles were sampled from 38 solitary females before oviposition and 38 paired

males; the beetles were excised from naturally-attacked *P. arizonica* trees in August 2009. Immediately after excision of the beetles, the hindguts of males and females were pulled and extracted separately in 1 ml hexane (Sullivan, 2005). The extracts of each sex then were concentrated to 0.3 ml under nitrogen. Dilute resin and gut extracts were stored at −20°C.

The GC-EAD apparatus consisted of a model 8610 gas chromatograph (SRI instruments, Torrence, CA, USA) fitted with a helium ionization detector (HID). A high flow rate of makeup helium (~80 ml/min) carried the HID effluent through a PTFE transfer line (0.8 mm i.d., 50 cm long) and into a stainless-steel pipe (8 mm diam., 14 cm long) that delivered a constant flow (400 ml/min) of purified, humidified air over the antennal preparation. This arrangement (i.e., with the HID and EAD in series) resulted in a 1–2 s lag time in response of the EAD relative to the HID. Voltages from antennal electrodes were pre-amplified on a Syntech PRS-1 probe (Hilversum, North Holland, The Netherlands); the output was fed directly into an input on the multi-channel detector amplifier circuitry of the GC. HID and EAD traces were collected simultaneously and examined subsequently with PeakSimple software (SRI Instruments). The antennal preparation procedure was largely identical to that used in previous studies of *D. frontalis* Zimmermann (Sullivan, 2005). For each assay, a glass pipette Ag/AgCl reference electrode (containing Beadle-Ephrussi saline and 0.5 % polyvinylpyrrolidone) was inserted into the foramen of a beetle's excised head. The tip of a similar recording electrode was cut to match the diameter of the antennal club; the club then was laid flat against the electrode opening so that one entire side made contact with the saline. One microliter of dilute resin or 2 µl of hindgut extract were injected (split 1/10) into the GC-EAD equipped with a polyethylene glycol phase microcapillary column (HP-INNOWax 60 m x 0.25 mm x 0.25 µm film, Agilent Technologies, Wilmington, DE, USA). The oven program was 50°C for 1 min, 16°C/min to 80°C, then 7°C/min to 218°C, and then hold for a final 5 min. Antennae of 8–10 emerged individuals of each sex were assayed with each of the three volatiles samples (i.e., resin, male gut extract, and female gut extract). The HID and EAD data streams were converted to ASCII format and subjected to baseline filtering and summing in Microsoft Excel (Slone and Sullivan, 2007). Olfactory stimulants (i.e., HID peaks associated consistently with an EAD response) were subsequently identified by matching GC retention times to those of commercially obtained standards [fenchyl alcohol (96 % purity, Sigma-Aldrich Corp. Milwaukee, WI, USA), myrtenal (98 % purity, Sigma-Aldrich Corp.), myrtenol (95 % purity, Sigma-Aldrich Corp.), *cis*-verbenol (95 % purity, Sigma-Aldrich Corp.), *trans*-verbenol (95 % purity, PheroTech {now ConTech Inc.}, Delta, BC, Canada), and verbenone (99 % purity, Sigma-Aldrich Corp.)] and by analyzing the samples on a coupled gas chromatograph-mass spectrometer (GC-MS; see details below) operating under the same conditions as the GC of the GC-EAD. Olfactory activity of the identified compounds was confirmed through GC-EAD analyses of commercially obtained standards with antennae of 10 female and 10 male pre-emerged adults of *D. rhizophagus.* Pre-emerged beetles were used for these analyses due to their greater availability.

Quantification of Volatiles from Individual Beetles at Different Colonization Phases Volatiles were quantified from individual male and female *D. rhizophagus* in two different bioassay settings.

Bioassay 1 Pre-emerged adult males and females were sampled in three different treatments in the laboratory: 1) denied contact with fresh host bark, 2) solitary and actively mining after being confined on host bark for 24 hr, and 3) paired 24 hr following introduction of the male to a treatment 2 female gallery. Treatment 2 insects were confined within gelatin capsule halves secured over ~6 mm diam. holes drilled through the outer bark of bolts of healthy, immature *P. arizonica* (30 cm long, 6–10 cm diam.) following Sullivan (2005). These treatments were intended to simulate the normal colonization sequence involving female establishment of a nuptial chamber in the phloem followed by pairing with a subsequently arriving male; they also represent the life history periods of greatest pheromone production in bark beetles (Wood D. L. 1982). Females are the sex that initiates the construction of the gallery in *D. rhizophagus* (authors' pers. obs.) as in other species of *Dendroctonus*. Ten to fifteen replicates were collected for each sex and treatment.

Bioassay 2 Beetles of *D. rhizophagus* were sampled directly from naturally-colonized, 0.5 to 1.5 m-tall *P. arizonica* in July 2009 in San Juanito, Bocoyna Municipality, Chihuahua State, Mexico. The trees had single attacks and were identified by the presence of a pitch tube and boring dust on the soil surface near the root collar. Colonization stages sampled were: 1) solitary female boring a nuptial chamber into the bark, 2) paired male and female in a new gallery prior to beginning oviposition, 3) paired male and female during oviposition, and 4) solitary female inside a completed gallery system containing eggs (i.e., after the male had presumably left). Four to sixteen replicates were collected for each sex and treatment.

In both bioassays, volatiles were sampled by confining insects individually inside 100 µl-capacity, conical-interior glass vials containing≈0.3 mg of clean Super Q adsorbent (80–100 mesh; Alltech, Deerfield, IL, USA) during a 24 hr period at room temperature (Sullivan, 2005). Following the

aeration interval, each insect was removed from its vial, and the hindgut of each was dissected and placed immediately back into the original conical vial together with 50 μl hexane [spiked with 3.5 ng/μl of cycloheptanone (99 purity, Sigma-Aldrich Corp.) as an internal quantitative standard]. The hindgut was macerated against the inside vial wall with the tip of the forceps, and then the vial was capped, and the contents was extracted passively for 15 min at room temperature. The hindgut/adsorbent extract was removed, and then the vial contents were further rinsed with 50 μl non-spiked hexane that was subsequently combined with the original extract. Negative control extracts also were collected from conical vials to which no beetles had been introduced.

Three microliters of each extract were analyzed by GC-MS (Hewlett-Packard model G1800C, Palo Alto, CA, USA) in splitless mode with the same column used for GC-EAD assays; the oven temperature was 40°C for 1 min, 16°C/min to 80°C, then 5°C/min to 240°C for a final 8 min. Compounds were quantified relatively against the internal standard and then converted to nanogram amounts based on response curves calculated from analyses of sequential dilutions of known quantities of synthetic standards (mentioned in electrophysiological assays section) plus the sample internal standard (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).

Field Trials Two trapping experiments were performed in the field to evaluate behavioral responses of *D. rhizophagus* to olfactory stimulants identified in the GC-EAD studies. Experiments were conducted from 26 June to 31 August, 2010, in a stand of natural *P. arizonica* regeneration at San Juanito, Bocoyna Municipality, Chihuahua. Both experiments had a randomized complete block design, in which each experimental block consisted of a line of traps equal in number to the treatments, with a unique bait treatment randomly assigned to each trap in the line. The first experiment examined beetle responses just to the EAD-stimulating host monoterpenes and compared five treatments within six blocks: 1) unbaited control, 2) α-pinene, 3) β-pinene, 4) 3-carene, and 5) a 1:1:1 mixture of all three compounds. The second experiment tested the effect of beetle-identified, EAD-stimulating oxygenated monoterpenes on beetle responses to an attractive blend of host-produced hydrocarbon monoterpenes (α-pinene, β-pinene, and 3-carene). Ten treatments were assayed within ten blocks: 1) unbaited control, 2) 1:1:1 mixture of α-pinene, β-pinene, and 3-carene, 3) 2 plus myrtenal, 4) 2 plus *trans*-verbenol, 5) 2 plus verbenone, 6) 2 plus myrtenol, 7) 2 plus *cis*-verbenol, 8) 2 plus fenchyl alcohol, 9) 2 plus all six oxygenated monoterpenes, and 10) 9 without verbenone. Baits were supplied by Chemtica International (S.A. San José, Costa Rica) with purity, enantiomeric ratios, bait construction, and release rates given in Table 1.

The traps used in this study consisted of a series of identical black plastic funnels aligned vertically over a collection cup (Lindgren, 1983) as commonly used for bark beetles; however, they were constructed with smaller than typical funnels and deployed at ground level to better imitate the appearance of stems of the preferred host ages for *D. rhizophagus*.

Each trap was composed of six conical funnels (each 13.5 cm diam. at top, 6 cm diam. at bottom, and 7.0 cm height) with an overlap of 2 cm between them, a collection cup (9 cm diam. x 18 cm height), and a plastic cover (30 cm diam.) placed 20 cm above of the first funnel. The trap total length was≈80 cm. The last funnel was attached directly to the mouth of the collection cup, which was buried in the ground. Traps were suspended from a wooden post and positioned >1 m from the closest suitable host tree. Traps within a single block were spaced>5 m apart, whereas traps in adjacent blocks were >10 m apart. Trap collection cups contained the insecticide 2-isopropoxy phenyl methylcarbamate (Baygon, Chemagro Corporation, Kansas City, Mo., USA) to kill trapped insects. Baits were hung within the upper funnel to protect them from rain. Trapped *D. rhizophagus* were collected weekly, counted, sexed, and preserved in 70 % ethanol.

Statistical Analysis

Bioassay 1 Five of the analyzed compounds (fenchyl alcohol, myrtenal, *cis*-verbenol, *trans*-verbenol, and verbenone) did

Table 1 Baits used in trapping tests of electrophysiologically active compounds identified from host resin and mid/hindguts of host-attacking *Dendroctonus rhizophagus* males and females

Chemical name	Purity (%)	Chirality (%)	Device	Release rate (mg/d±SE)[a]
α-Pinene	97.4	75 (+)	polysleeve bag	672.6±97.5
β-Pinene	97.5	95 (−)	polysleeve bag	673.6±97.0
3-Carene	>92	92.5 (+)	polysleeve bag	670.3±97.9
Fenchyl Alcohol	>95	99 (+)	bubble cup	8.00±1.91
Myrtenal	>95	99 (−)	bubble cup	3.75±0.92
cis-Verbenol	99.5	92 (*S*)	bubble cup	3.33±1.35
trans-Verbenol	97.3	92 (*S*)	bubble cup	2.83±1.24
Verbenone	>93	92 (−)	bubble cup	6.33±0.76
Myrtenol	>95	99 (−)	bubble cup	3.66±1.27

[a] Measured gravimetrically in a fume hood at room temperature (mean≈26°C).

not exceed the GC-MS threshold of detection in more than half of the sampled insects. This invalidated conventional methods (e.g., parametric and nonparametric rank-based ANOVAs) for comparing mean quantities of compounds produced by the insects. Therefore, within each treatment and sex, the number of insects in which a compound was/was not detected was calculated as a binomial frequency, and these frequencies were subjected to contingency table tests with computation of exact *P*- values (SAS PROC FREQ). These *P*-values were adjusted by Bonferroni method in cases of multiple pairwise comparisons. A single compound (myrtenol) was detected in the great majority of samples, and in this instance the quantities detected in each insect were log transformed and subjected to a mixed-model ANOVA (PROC MIXED) with treatment and sex as fixed factors and experimental units as the random source of replication. In treatment categories 1 and 2, each sampled insect was an experimental unit, but in treatment 3, due to pairing of males and females, the insect pair was considered the experimental unit. All pairwise tests were performed on significant fixed factors by using LSD with Bonferroni adjustment (α=0.05).

Bioassay 2 Log transformed quantities of each compound were subjected separately to a mixed-model ANOVA (PROC MIXED) with insect sample category (i.e., each sex and colonization stage) included as a fixed factor and gallery system nested within colonization stage included as a random factor. Nine pairwise contrasts were performed (between females in all four stages, between males of stages 2 and 3, and between males and females of stages 2 and 3) using LSD with Bonferroni correction (α=0.05).

Total catches of male and female *D. rhizophagus* in each trap (all weeks summed) were transformed [log10(X+1)] to allow the data to meet the assumptions of parametric tests, and were then subjected to a mixed-model ANOVA (PROC MIXED) with fixed effects treatment, sex, and treatment/sex, and random effects block and block/treatment. If a significant interaction was detected between sex and treatment (α=0.05), then a 2-way ANOVA (PROC GLM) was performed on each sex individually with treatment and block as factors; otherwise, catches of males and females were summed, transformed, and subjected to this same type of 2-way ANOVA. For trapping experiment 1, treatment means were subjected to all pairwise comparisons with Tukey's test (α=0.05). For trapping

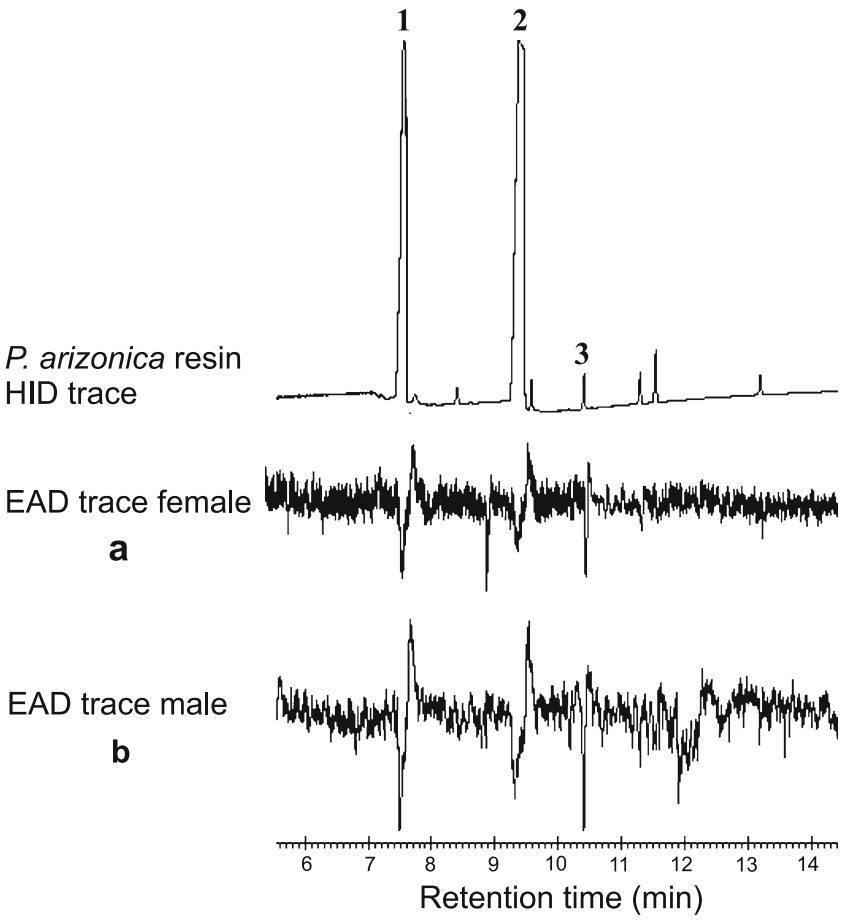

Fig. 1 Electrophysiological responses (GC-EAD) of antennae from emerged female (**a**) and male (**b**) *Dendroctonus rhizophagus* adults to compounds from *Pinus arizonica* resin. EAD = electroantennographic detection; HID = Helium ionization detection. Antennal traces represent the combined responses from 8 individual insects (single EAD traces were digitized in a spreadsheet to produce a composite race). Compounds eliciting consistent antennal voltage spikes were α-pinene (1), β-pinene (2), and 3-carene (3)

experiment 2, mean catches of all other bait treatments were contrasted against mean catches by the blank (the negative control) and the hydrocarbon monoterpene mix (the positive control) using LSD with a Bonferroni correction for 17 contrasts. All analyses were performed with SAS (SAS Institute 1989).

Results

Electrophysiological Assays The antennae of emerged female and male *D. rhizophagus* responded consistently to α-pinene, β-pinene, and 3-carene (Fig. 1) present in the constitutive resin of *P. arizonica*, and to fenchyl alcohol, myrtenal, *cis*-verbenol, *trans*-verbenol, verbenone, and myrtenol (Fig. 2) present in guts of host-attacking *D. rhizophagus* females and males. GC-EAD tests with commercially obtained standards of these compounds confirmed their electrophysiological activity in both sexes.

Quantification of Volatiles from Individual Beetles at Different Colonization Phases Bioassay 1 In root-collected adults that had not been allowed to feed on fresh host tissue, all six EAD-active oxygenated monoterpenes were either undetectable or present in sub-nanogram quantities, whereas myrtenal, *cis*-verbenol, *trans*-verbenol, and verbenone were detected in nanogram or greater levels from such beetles after mining in a pine log (Table 2, Fig. 3). Fenchyl alcohol was detected in merely a few unfed females. The quantity of myrtenol detected varied significantly by treatment category (F=54.54, df=2, 47.2, P<0.001) but not by sex (F=1.59, df=1, 49.1, P=0.214), although there was a strong sex by treatment interaction (F=6.33, df=2, 37.8, P=0.004). In solitary mining males, we detected significantly more myrtenol

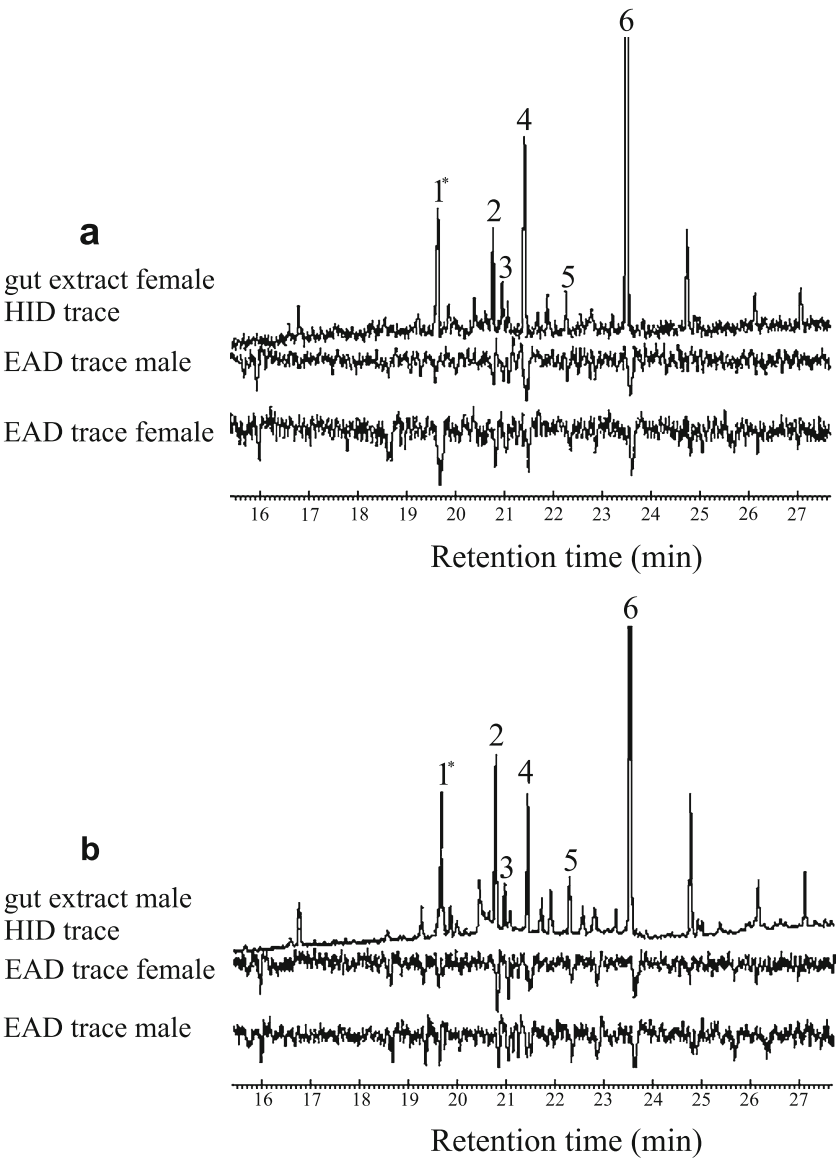

Fig. 2 Electrophysiological responses (GC-EAD) of antennae from emerged female (**a**) and male (**b**) *Dendroctonus rhizophagus* adults to compounds in mid and hindgut extracts of 38 conspecifics. EAD = electroantennographic detection; HID = Helium ionization detection. Antennal traces represent the combined responses from 8–10 individual insects (single EAD traces were digitized in a spreadsheet to produce a composite race). Compounds eliciting consistent antennal voltage spikes were fenchyl alcohol (1), myrtenal (2), *cis*-verbenol (3), *trans*-verbenol (4), verbenone (5), and myrtenol (6). *Fenchyl alcohol was identified tentatively on the basis of a retention time match to a standard and presence of major diagnostic ions

than in males from mining pairs, and both groups had more than unfed males; furthermore, paired and solitary mining females contained more myrtenol than unfed females (Fig. 3). The remaining five compounds were detected in an insufficient proportion of the sampled insects; no statistical analysis of quantities was possible.

An analysis of the frequencies of beetles containing these five compounds revealed no differences between the sexes (Table 2). The frequency of solitary mining beetles that contained detectable levels of myrtenal, *cis*-verbenol, *trans*-verbenol, or verbenone was significantly greater than the frequency of unfed beetles that contained these compounds. Furthermore, *cis*-verbenol, *trans*-verbenol, and verbenone were detected from a greater proportion of solitary mining beetles than of paired mining beetles (Table 2).

Bioassay 2 Five of the olfactory stimulants that were isolated from laboratory-sampled beetles and identified in the EAD studies also were detected and quantified from adult beetles sampled in different stages of colonization of naturally-infested pines in the field: *cis*-verbenol, *trans*-verbenol, verbenone, myrtenal, and myrtenol. Quantities of *trans*-verbenol (F=8.84, df=5, 41.4, P<0.001) and verbenone (F=3.51, df=5, 43.4, P=0.009) in these beetles varied significantly according to the sex and infestation stage classification, but not quantities of *cis*-verbenol (F=2.38, df=5, 42.9, P=0.055), myrtenol (F=1.48, df=5, 41, P=0.218), or myrtenal (F=0.83, df=5,43, P=0.534). The fenchyl alcohol was detected in both sexes but in insufficient quantities for its analysis. In pairwise comparisons, females in the four attack stages did not significantly differ in quantities of any one of the five compounds, nor did quantities differ significantly in males from preoviposition or ovipositing pairs (Fig. 4). However, females from either pre-oviposition (t=3.53, df=20.2, adjusted P=0.019) or ovipositing (t=5.02, df=20.2, adjusted P=0.001) pairs contained significantly greater quantities of *trans*-verbenol than their male counterparts.

Field Trials In trapping experiment 1, there was no significant interaction detected between sex and treatment (F=2.63, df=4, 25, P=0.058). Therefore, the sexes were pooled and reanalyzed. The presence of single (α-pinene, β-pinene, 3-carene) monoterpenes or their mixture in baits significantly affected catches of *D. rhizophagus* (F=12.2, df=4, 20, P<0.001). Baits composed of either 3-carene alone or the mix of all three hydrocarbon monoterpenes were attractive to *D. rhizophagus*; baits composed of α-pinene or β-pinene alone trapped insects but not in numbers significantly greater than the blank (Fig. 5). The 3-carene bait was superior to the α- and β-pinene baits, and the addition of these two monoterpenes did not significantly alter responses to 3-carene alone. In trapping experiment

Table 2 Proportion of *Dendroctonus rhizophagus* adults containing detectable quantities of EAD-active compounds; adults were collected from roots and sampled in the laboratory[a]

	Unfed			Mining, solitary			Mining, paired		
	Females	Males		Females	Males		Females	Males	
Fenchyl Alcohol*	5/15[b]	0/15	a[d]	0/14	0/14	a	0/9	0/9	a
	(13.5-25.5)[c]	–		–	–		–	–	
Myrtenal	0/15	0/15	a	11/14	8/14	b	5/9	2/9	b
	–	–		(11.5-83.5)	(13–68.5)		(23–48.5)	(9–17.5)	
cis-Verbenol	0/15	0/15	a	10/14	8/14	b	1/9	1/9	a
	–	–		(29–363)	(32.5-357)		(45)	(38)	
trans-Verbenol	4/15	1/15	a	12/14	12/14	b	4/9	1/9	a
	(24.5-45)	(30.5)		(15.5-496)	(1–1364.5)		(26.5-114.5)	(84)	
Verbenone	0/15	0/15	a	4/14	4/14	b	0/9	0/9	a
	–	–		(17–107)	(26.5-53.5)		–	–	

[a] Beetles were either sampled directly ("unfed") or first confined on the bark of a pine log to force them to initiate galleries. "Mining, solitary" beetles had mined singly in the log for ~24 hr; "mining, paired" beetles were paired in a gallery for 24 hr, with the male introduced to the log 24 hr after the female. Table includes only those compounds that were detected in fewer than half of the sampled insects.

[b] Numerator is the number of insects from which the compound was detected; denominator is the total number of insects sampled.

[c] Quantity range (ng) detected from individuals producing the compound.

[d] Treatment categories associated with the same letter were not significantly different in the proportion of individuals producing detectable quantities of the compound (sexes pooled; all-pairwise Fisher exact tests with Bonferroni adjustment, α=0.05).

*Fenchyl alcohol was identified tentatively on the basis of a retention time match to a standard and presence of major diagnostic ions.

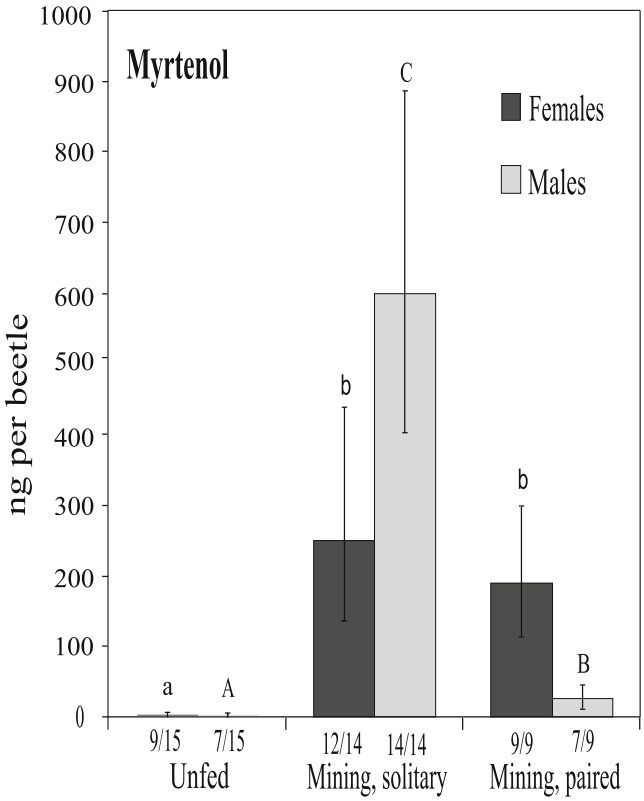

Fig. 3 Quantities (mean±SEM) of myrtenol isolated from *Dendroctonus rhizophagus* adults excised from roots in the laboratory and either sampled directly ("unfed") or first confined on the bark of a pine log to force them to initiate galleries. "Mining, solitary" beetles had mined singly in the log for ~24 hr whereas "mining, paired" beetles had been paired in a gallery with a mate for 24 hr, with the male introduced to the log 24 hr after the female. For females (respectively, males), bars labeled with the same lower case (respectively, upper case) letter indicate no significant difference (LSD with Bonferroni adjustment, α=0.05). ANOVA and contrasts were performed on log transformed data; the data shown here are the back-transformed means and back-transformed standard error bars. The fractions indicate detections of myrtenol/number of insects sampled

2, a significant interaction was detected between sex and treatment (F=2.94, df=9, 90, P=0.004); therefore, the sexes were reanalyzed separately. No effect of bait treatment on catches of female *D. rhizophagus* (F=0.62, df=9, 81, P=0.78) was detected, but there was a difference for males (F=4.18, df=9, 81, P<0.001). The hydrocarbon monoterpene mix alone or combined with either myrtenal or *trans*-verbenol was significantly attractive to male *D. rhizophagus* (Fig. 6). Addition of either verbenone or the full blend of the six oxygenated monoterpenes significantly reduced male responses to the hydrocarbon monoterpene mix, and none of the individual oxygenated monoterpenes significantly enhanced attraction to the hydrocarbon monoterpene mix. No species of *Dendroctonus* other than *D. rhizophagus* were trapped in either experiment.

Discussion

Our field investigations revealed that 3-carene produced by host trees is attractive to both sexes. These results suggest that this compound may mediate both host location and contact between the sexes for mate finding. The bicyclic monoterpene 3-carene also has been identified as a primary attractant for the closely related *D. valens* (Hobson et al., 1993; Erbilgin et al., 2007), and as an aggregation pheromone synergist in *D. ponderosae* Hopkins (Conn et al., 1983; Miller and Borden, 2000). In addition to 3-carene, two other monoterpenes in the resin of *D. rhizophagus* host trees, α- and β-pinene, stimulated olfactory receptors of both sexes, but it is unclear whether either has a behavioral function since they failed to significantly alter beetle responses to traps (Fig. 5). These findings for α- and β-pinene must be viewed cautiously because the statistical power of the trapping test was low due to low catches.

Our results did not provide evidence of a secondary attractant in *D. rhizophagus,* although we did identify several compounds in the gut of beetles with qualities suggestive of a possible role as pheromone components. Antennal receptors of both sexes were sensitive to six oxygenated monoterpenes in gut extracts of *D. rhizophagus* that had been initiating galleries (Fig. 2), the stage in the life history of bark beetles that is associated with production of attractant pheromones (Borden, 1982; Wood D. L. 1982). Two of these compounds, *cis*- and *trans*-verbenol, are aggregation or synergistic attractants for species of *Dendroctonus* (Borden, 1982; Skillen et al., 1997). However, these compounds did not enhance *D. rhizophagus* responses to an attractive combination of host monoterpenes (Fig. 6). Despite this, our evidence that *trans*-verbenol is detected disproportionately by females during preoviposition and oviposition stages suggests that this compound should be investigated further as a possible secondary attractant.

Our studies of the question whether the presence of olfactory stimulating compounds in the gut of *D. rhizophagus* adults depends on the life history stage or sex of the beetles did not reveal clear patterns. The major components of bark beetle aggregation or sex pheromones generally are detectable in only one sex or are produced in different quantities by the sexes (Borden, 1982; Blomquist et al., 2010). However, all six beetle-derived olfactory stimulants were detected in both sexes of *D. rhizophagus*, and there were no significant differences between the sexes, except for *trans*-verbenol, which was produced by beetle pairs infesting pines in nature (Fig. 4). The quantity of *trans*-verbenol detected in females was approximately 3–5 times greater than males occupying the same galleries in the trees. This sexual dimorphism was not detected in beetles that were collected from roots prior to emergence and forced to infest the host tissue in the laboratory (Table 2, Fig. 3). In addition, it is known that

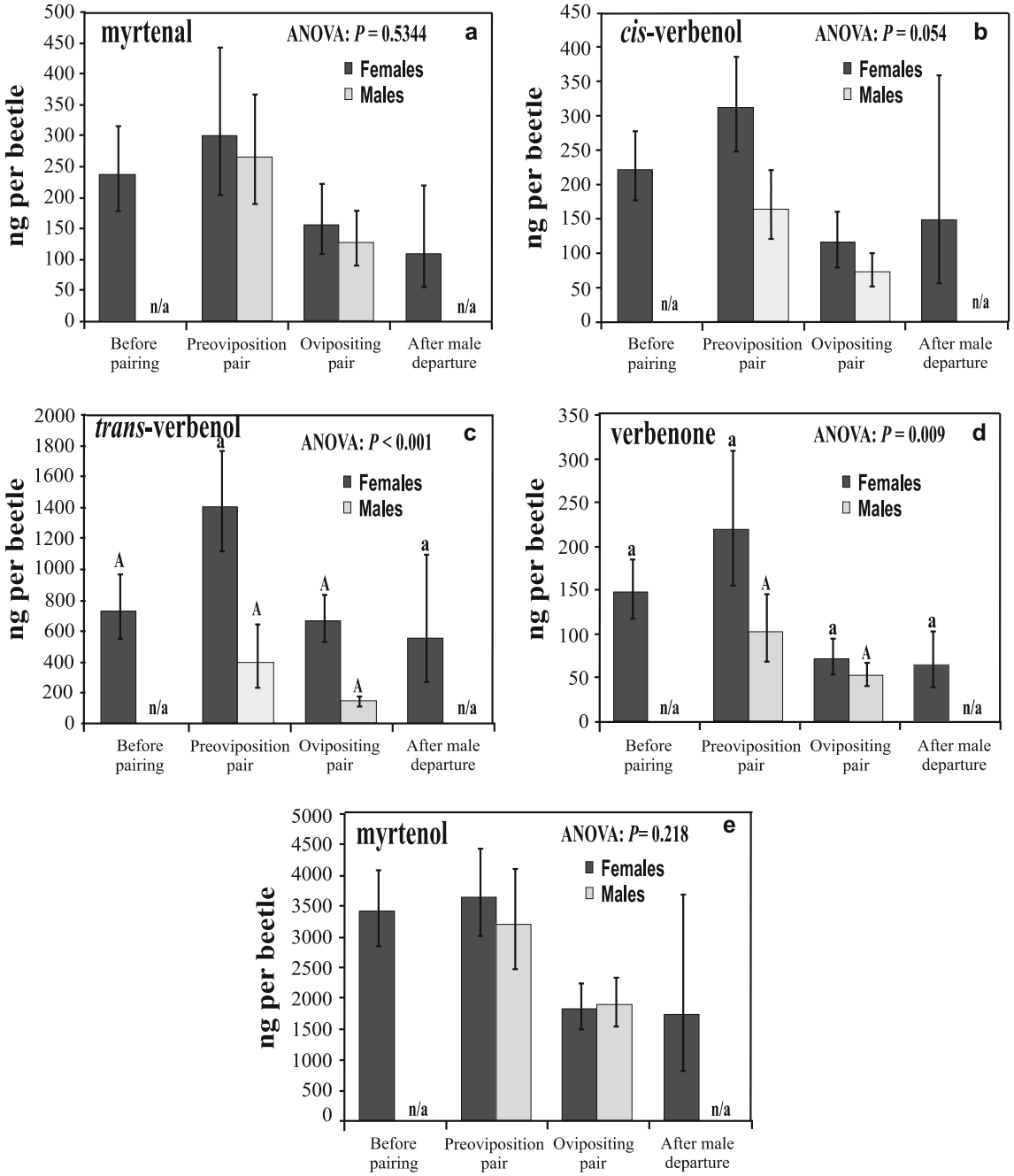

Fig. 4 Quantities (mean±SEM) of olfactory stimulants isolated from *Dendroctonus rhizophagus* individuals found in successive colonization stages of pines in the field: myrtenal (**a**), *cis*-verbenol (**b**), *trans*-verbenol (**c**), verbenone (**d**), and myrtenol (**e**). For females (respectively, males), bars associated with the same lower case (respectively, upper case) letter indicate no significant difference. An asterisk indicates a significant difference between males and females within a pair type (i.e., preoviposition or ovipositing) with respect to the quantities produced (α=0.05). ANOVA and contrasts were performed on log transformed data; the data shown here are the back-transformed means and back-transformed standard error bars. The olfactory stimulant, fenchyl alcohol, that had been detected in laboratory-collected beetles (Table 2) was not detected in this analysis

over the course of host colonization, bark beetles exhibit large quantitative variation in the production of their major pheromone components, i.e., increasing during gallery initiation (coinciding with aggregation and pairing) and then terminating or declining rapidly afterwards (Borden, 1982; Wood D. L. 1982). However, quantities of the six olfactory stimulants detected in *D. rhizophagus* males and females attacking pines in nature did not differ significantly before or after gallery construction (Fig. 4).

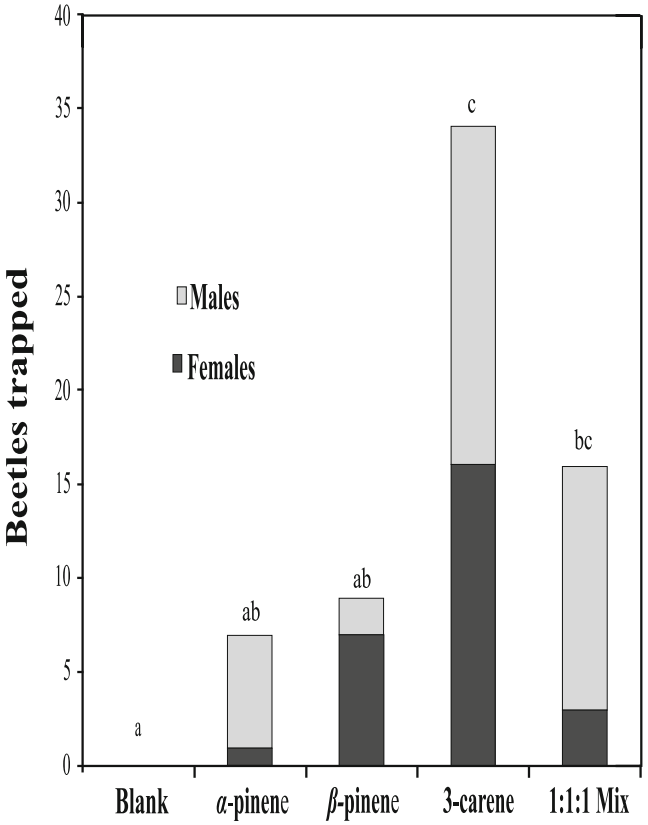

Fig. 5 Total numbers of *Dendroctonus rhizophagus* caught in funnel traps baited with any of three electrophysiologically-active host monoterpenes (α-pinene, β-pinene, and 3-carene) or a 1:1:1 mixture of all three. No interaction between sex and treatment was detected (ANOVA on transformed data), and bars associated with the same letter indicate no significant difference for total beetles trapped (i.e., sexes summed; α=0.05; Tukey's test on log transformed data). The test was replicated six times

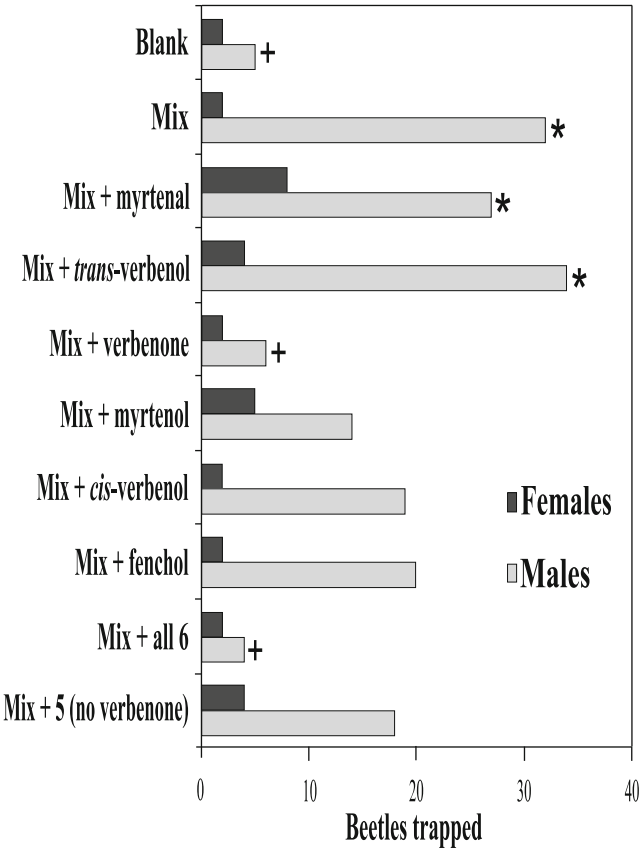

Fig. 6 Total number of *Dendroctonus rhizophagus* caught in funnel traps baited with a tertiary mixture of three hydrocarbon monoterpenes ("Mix"; i.e., α-pinene, β-pinene, and 3-carene in a 1:1:1 ratio) either alone or in combination with one or more beetle-derived, electrophysiologically-active oxygenated monoterpenes [myrtenal, *trans*-verbenol, verbenone, myrtenol, *cis*-verbenol, and fenchyl alcohol (fenchol)]. A significant interaction between sex and bait treatment was detected (ANOVA on log transformed data) but there were no treatment effects on female catches. For males, data associated with (*) were significantly different from the blank (an unbaited trap); those with (+) were significantly different from the hydrocarbon monoterpene mix alone (LSD with Bonferroni correction for 17 contrasts; i.e., α=0.003). The test was replicated ten times

All six of the olfactory stimulants detected in the beetles have been isolated from and demonstrated behavioral activity as attractants, arrestants, or inhibitors with one or more species of *Dendroctonus* bark beetles (Skillen et al., 1997; Sullivan, 2005; Blomquist et al., 2010). Five of these compounds (myrtenol, myrtenal, verbenone, and *cis*- and *trans*-verbenol) were found previously in association with bark beetle attacks on conifers. They are generated through the oxidation of α-pinene either spontaneously by the exposure of tree resin to the air or by oxidases from beetles or their associated microorganisms (see Seybold et al., 2006 and citations therein).

The beetle's enzymatic oxidation of host-produced α-pinene to these more water-soluble forms is a detoxification mechanism which helps protect the beetles from this defensive compound (Pierce et al., 1987; Tittiger et al., 2005). Exposure to α-pinene vapors stimulates the appearance of these oxidation products in the adult midgut and hindgut of several species of *Dendroctonus* including *D. rhizophagus* and its sibling species, *D. valens* (Hughes, 1973, 1975; Renwick et al., 1973; Shi and Sun, 2010; López et al., 2011). Given that the quantities of myrtenal, myrtenol, verbenone, and verbenol did not differ significantly in *D. rhizophagus* during colonization of a natural host (Fig. 4), and that their production was stimulated in both sexes by contact with the host (Table 2, Fig. 3), their presence in *D. rhizophagus* most likely represents the detoxification of α-pinene encountered during invasion of the host tissues (Pierce et al., 1987; Byers, 1995). This hypothesis is consistent with the overexpression that some *CYP* genes shown in the *D. rhizophagus* pre-emerged beetles gut exposed to α-pinene vapors and with our observation that juvenile hormone III, a stimulant of pheromone production in bark beetles (Blomquist et al., 2010), did not elicit the synthesis of these or any other

volatile compounds in hindguts of this species (authors' unpublished data).

One of the olfactory stimulants isolated from the beetle's gut, verbenone, was found to elicit behavioral activity of the beetles. It significantly reduced responses by male *D. rhizophagus* to attractive baits (Fig. 6). Verbenone inhibits responses by many bark and ambrosia beetles to attractants, and has been classified an antiaggregation pheromone (e.g., Borden, 1996; Skillen et al., 1997; Lindgren and Miller, 2002a,b; Dodds and Miller, 2010). This compound likely has received more effort at practical development as a tree and stand protector than any other identified bark beetle "repellent". *Dendroctonus rhizophagus* does not aggregate (Sánchez-Martínez and Wagner, 2009), and therefore, verbenone cannot be considered an 'antiaggregation pheromone' in this species. However, it could potentially perform a parallel function, serving as an indicator that a host has been fully utilized and is no longer suitable for (further) colonization, as has been suggested for many bark and woodboring beetles (Byers et al., 1989; Lindgren et al., 1996; Lindgren and Miller, 2002a). Verbenone concentrations associated with conifer host tissue tend to increase following bark beetle infestation, likely due to the greater exposure of α-pinene to the air and the action of oxidative enzymes from beetles and associated microorganisms that invade host tissues attacked by the beetles (Leufven and Birgersson, 1987; Birgersson and Bergstrom, 1989; Hunt et al., 1989; Hunt and Borden, 1990).

Our data indicate that the semiochemical system of *D. rhizophagus* is very similar to that of its sibling species, *D. valens*. As with *D. rhizophagus*, in *D. valens*: (1) both sexes produce the oxygenated monoterpenes myrtenal, myrtenol, verbenone, and *cis*- and *trans*-verbenol during infestation of a host (Zhang and Sun, 2006; Shi and Sun, 2010); (2) myrtenol and *trans*-verbenol are the two most abundant compounds produced by host-infesting beetles (Shi and Sun, 2010); (3) the bicyclic acetals frontalin and brevicomin, which are major components of *Dendroctonus* aggregation pheromones, are undetectable in beetles; (4) the host monoterpene 3-carene is an effective trap bait either alone or in combination with α- and β-pinene (Hobson et al., 1993; Sun et al., 2004; Erbilgin et al., 2007); and (5) verbenone inhibits beetle response to attractive host odors (Rappaport et al., 2001; Gillette et al., 2006; Sun et al., 2006). Noteworthy differences include: (1) greater production of *trans*-verbenol by female than male beetles that we observed in *D. rhizophagus* but which was not detected in *D. valens* (Shi and Sun, 2010), and (2) a modest enhancement of attractiveness of a host monoterpene lure by *trans*-verbenol and myrtenol was observed in *D. valens* (Zhang and Sun, 2006), but not in *D. rhizophagus* in the present study. Thus, the host/mate location semiochemistry of both species is characterized by a host-produced primary attractant but apparently not by a potent pheromonal compound.

This evidence for the overall similarity of the semiochemical systems of *D. rhizophagus* and *D. valens* contrasts sharply with the conspicuous differences in their respective host utilization and colonization behaviors. In *D. rhizophagus,* only one or a few pairs of beetles kill and colonize sapling-sized tree (Sánchez-Martínez and Wagner, 2009), whereas *D. valens* colonizes mature trees; tree death is not needed for successful reproduction (Wood S. L. 1982).

Despite their apparently differing requirements regarding host tree death, *D. valens* and *D. rhizophagus* resemble one another insofar as they can attack and reproduce in apparently vigorous hosts without aggregating. Consequently, selective pressures that have presumably favored the evolution and retention of aggregation pheromones in other aggressive bark beetles species are similarly lacking in both *D. valens* and *D. rhizophagus.* In *D. rhizophagus* as in other species of its genus, females are the sex that locates and establishes attacks on new hosts, whereas males must locate established female galleries to reproduce. While females presumably are attracted to 3-carene when locating a suitable host, the attraction of males by sex pheromone must be investigated.

In summary, our results support the following preliminary outline for the semiochemical system of *D. rhizophagus*. Host seeking females as well as mate seeking males are guided to hosts by 3-carene and possibly additional host-produced hydrocarbon monoterpenes. Secondary attractants released by the beetles probably do not play an important role in guiding beetles to hosts. However, the detection of greater quantities of *trans*-verbenol in females suggests that this compound must be investigated further as a possible secondary attractant for males. Additionally, an attraction inhibitor produced by both sexes, verbenone, may function in signaling unsuitability of colonized hosts to host- or mate-seeking *D. rhizophagus*. Confirmation of these results will require *in situ* field tests of attractive responses *by D. rhizophagus* to infested and uninfested trees. Regarding practical applications of our findings, 3-carene baits in conjunction with the trap design and deployment methods described here may prove valuable for the detection and monitoring of *D. rhizophagus* populations. Furthermore, the attraction inhibitor verbenone shows potential for possible use in protecting trees and stands from *D. rhizophagus* infestation.

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References

Bentz, B. J., Régnière, J., Fetting, C. J., Hansen, M., Hayes, J. L., Hicke, J. A., Kelsey, R. G., Negrón, J. F., and Seybold, S. J. 2010. Climate change and bark beetle of the Western United States and Canada: Direct and indirect effects. *BioScience.* 60:602–613.

Birgersson, G. and Bergstrom, G. 1989. Volatiles released from individual spruce bark beetle entrance holes - Quantitative variations during the first week of attack. *J. Chem. Ecol.* 15:2465–2483.

Blomquist, G. J., Figueroa-Teran, R., Aw, M., Song, M. M., Gorzalski, A., Abbott, N. L., Chang, E., and Tittiger, C. 2010. Pheromone production in bark beetles. *Insect Biochem. Mol* 40:699–712.

Borden, J. H. 1982. Aggregation pheromones, pp. 74–139, *in* J. B. Mitton and K. B. Sturgeon (eds.), Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press, Austin.

Borden, J. H. 1996. Disruption of semiochemical-mediated aggregation in bark beetles, pp. 421–438, *in* R. T. Cardé and A. K. Minks (eds.), Pheromone Research: New Directions. Chapman and Hall, New York.

Byers, J. A., Lanne, B. S., and Lofqvist, J. 1989. Host tree unsuitability recognized by pine shoot beetles in flight. *Experientia* 45:489–492.

Byers, J. A. 1995. Host tree chemistry affecting colonization in bark beetles, pp. 154–213, *in* R. T. Cardé and W. J. Bell (eds.), Chemical Ecology of Insects 2. Chapman and Hall, New York.

Cibrián-Tovar, D., Méndez Montiel, J. T., Campos Bolaños, R., Yates III, H. O., and Flores Lara, J. 1995. Insectos Forestales de México/Forests Insects of México COFAN/NAFC. Universidad Autónoma Chapingo, México.

Conn, J. E., Borden, J. H., Scott, B. E., Frieske, L. M., Pierce, H. D., and Oehlschlager, A. C. 1983. Semiochemicals for the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae) in British Columbia: field trapping studies. *Can. J. For. Res.* 13:320–324.

Dodds, K. J. and Miller, D. R. 2010. Test of nonhost angiosperm volatiles and verbenone to protect trap trees for *Sirex noctilio* (Hymenoptera: Siricidae) from attacks by bark beetles (Coleoptera: Scolytidae) in the Northeastern United States. *J. Econ. Entomol.* 103:2094–2099.

Estrada-Murrieta, O. 1983. Biología del descortezador del renuevo de pino *Dendroctonus rhizophagus* Thomas y Bright (Col.: Scolytidae) en la región Mesa del Huracán, Chih. Bachelor thesis. Universidad Autónoma de Chapingo, México.

Erbilgin, N., Mori, S. R., Sun, J. H., Stein, J. D., Owen, D. R., Merrill, L. D., Campos Bolaños, R., Raffa, K. F., Méndez Montiel, T., Wood, D. L., and Gillette, N. E. 2007. Response to host volatiles by native and introduced populations of *Dendroctonus valens* (Coleoptera: Curculionidae, Scolytinae) in North America and China. *J. Chem. Ecol.* 33:131–146.

Gillette, N. E., Stein, J. D., Owen, D. R., Webster, J. N., Fiddler, G. O., Mori, S. R., and Wood, D. L. 2006. Verbenone-releasing flakes protect individual *Pinus contorta* trees from attack by *Dendroctonus ponderosae* and *Dendroctonus valens* (Coleoptera: Curculionidae, Scolytinae). *Agr. For. Entomol.* 8:243–251.

Hobson, K. R., Wood, D. L., Cool, L. G., White, P. R., Ohtsuka, T., Kubo, I., and Zavarin, E. 1993. Chiral specificity in responses by the bark beetle *Dendroctonus valens* to host Kairomones. *J. Chem. Ecol.* 19:1837–1846.

Hughes, P. R. 1973. *Dendroctonus*: production of pheromones and related compounds in response to host monoterpenes. *Z. Angew. Entomol.* 73:294–312.

Hughes, P. R. 1975. Pheromones of *Dendroctonus*: origin of α-pinene oxidation products present in emergent adults. *J. Insect Physiol.* 21:687–691.

Hunt, D. W. A., Borden, J. H., Lindgren, B. S., and Gries, G. 1989. The role of autoxidation of α-pinene in the production of pheromones of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Can. J. For. Res.* 19:1275–1282.

Hunt, D. W. A. and Borden, J. H. 1990. Conversion of verbenols to verbenone by yeasts isolated from *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16:1385–1397.

Leufven, A. and Birgersson, G. 1987. Quantitative variation of different monoterpenes around galleries of *Ips typographus* (Coleoptera, Scolytidae) attacking Norway spruce. *Can. J. Bot.* 65:1038–1044.

Lindgren, B. S. 1983. A multiple funnel trap for scolytid beetles. *Can. Entomol.* 115:299–302.

Lindgren, B. S., Nordlander, G., and Birgersson, G. 1996. Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L) (Col, Curculionidae). *J. Appl. Entomol.* 120:397–403.

Lindgren, B. S. and Miller, D. R. 2002a. Effect of verbenone on attraction of predatory and woodboring beetles (Coleoptera) to kairomones in lodgepole pine forests. *Environ. Entomol.* 31:766–773.

Lindgren, B. S. and Miller, D. R. 2002b. Effect of verbenone on five species of bark beetles (Coleoptera: Scolytidae) in lodgepole pine forests. *Environ. Entomol.* 31:759–765.

López, M. F., Cano-Ramírez, C., Shibayama, M., and Zúñiga, G. 2011. α-Pinene and myrcene induce ultrastructural changes in the midgut of *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae). *Ann. Entomol. Soc. Am.* 104:553–561.

Lyon, R. L. 1958. Useful secondary sex character in *Dendroctonus* Bark Beetles. *Can. Entomol.* 90:552–558.

Mendoza, M. G., Salinas-Moreno, Y., Olivo-Martínez, A., and Zúñiga, G. 2011. Factors influencing the geographical distribution of *Dendroctonus rhizophagus* (Coleoptera: Curculionidae: Scolytinae) in the Sierra Madre Occidental. *México. Environ. Entomol* 40:549–559.

Miller, D. R. and Borden, J. H. 2000. Dose-dependent and species-specific responses of pine bark beetles (Coleoptera: Scolytidae) to monoterpenes in association with pheromones. *Can. Entomol.* 132:183–195.

Phillips, T. W., Nation, J. L., Wilkinson, R. C., and Foltz, J. L. 1989. Secondary attraction and field activity of beetle-produced volatiles in *Dendroctonus terebrans*. *J. Chem. Ecol.* 5:1513–1533.

Pierce, H. D., Conn, J. E., Oehlschlager, A. C., and Borden, J. H. 1987. Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, attacking ponderosa pine. *J. Chem. Ecol.* 13:1455–1480.

Raffa, K. F., Phillips, T. W., and Salom, S. M. 1993. Strategies and mechanisms of host colonization by bark beetles, pp. 103–128, *in* R. D. Schowalter and G. M. Filip (eds.), Beetle-Pathogen Interactions in Conifer forests. Academic, London.

Rappaport, N. G., Owen, D. R., and Stein, J. D. 2001. Interruption of semiochemical-mediated attraction of *Dendroctonus valens* (Coleoptera: Scolytidae) and selected nontarget insects by verbenone. *Environ. Entomol.* 30:837–841.

Renwick, J. A. A., Hughes, P. R., and Ty, T. D. 1973. Oxidation products of pinene in the bark beetle *Dendroctonus frontalis*. *J. Insect Physiol.* 19:1735–1740.

SALINAS-MORENO, Y., MENDOZA, M. G., BARRIOS, M. A., CISNEROS, R., MACÍAS-SÁMANO, J., and ZÚÑIGA, G. 2004. Areography of the genus *Dendroctonus* (Coleoptera: Curculionidae: Scolytinae) in Mexico. *J. Biogeogr.* 31:1163–1177.

SALINAS-MORENO, Y., AGER, A., VARGAS, C. F., HAYES, J. L., and ZÚÑIGA, G. 2010. Determining the vulnerability of Mexican pine forests to bark beetles on the genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae). *Forest Ecol. Manag.* 260:52–61.

SÁNCHEZ-MARTÍNEZ, G. and WAGNER, M. R. 2009. Host preference and attack pattern of *Dendroctonus rhizophagus* (Coleoptera: Curculionidae: Scolytinae): A bark beetle specialist on pine regeneration. *Environ. Entomol.* 38:1197–1204.

SAS INSTITUTE. 1989. SAS Users Guide. Release 6.09 edition. SAS. Institute, Cary, North Carolina.

SEMARNAT. 2005. Aprovechamiento de los recursos forestales, pesqueros y de la vida silvestre. México. http://app1.semarnat.gob.mx/dgeia/informe_resumen/pdf/5_info_resumen.pdf.

SEYBOLD, S. J., HUBER, D. P. W., LEE, J. C., GRAVES, A. D., and BOHLMANN, J. 2006. Pine monoterpenes and pine bark beetles: A marriage of convenience for defense and chemical communication. *Phytochem. Rev.* 5:143–178.

SHI, Z. H. and SUN, J. H. 2010. Quantitative variation and biosynthesis of hindgut volatiles associated with the red turpentine beetle, *Dendroctonus valens* LeConte, at different attack phases. *Bull. Entomol. Res.* 100:273–277.

SKILLEN, E. L., BERISFORD, C. W., CAMAAN, M. A., and REARDON, R. C. 1997. Semiochemicals of Forest and Shade Tree Insects in North America and Management Applications. USDA Forest Service Forest Health Technology Enterprise Team Publication FHTET-96-15. 182 p.

SLONE, D. H. and SULLIVAN, B. T. 2007. An automated approach to detecting signals in electroantennogram data. *J. Chem. Ecol.* 33:748–1762.

SMITH, R. H. 2000. Xylem monoterpenes of pines: Distribution, variation, genetics, function. Gen. Tech. Rep. PSW-GTR-177. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture.

SULLIVAN, B. T. 2005. Electrophysiological and behavioral responses of *Dendroctonus frontalis* (Coleoptera: Curculionidae) to volatiles isolated from conspecifics. *J. Econ. Entomol.* 98:2067–2078.

SUN, J., MIAO, Z. W., ZHANG, Z., ZHANG, Z. N., and GILLETTE, N. E. 2004. Red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), response to host semiochemicals in China. *Environ. Entomol.* 33:206–212.

SUN, J. H., ZHANG, L. W., and CLARKE, S. R. 2006. Effects of verbenone dose and enantiomer on the interruption of response of the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), to its kariomones. *Environ. Entomol.* 35:655–660.

TITTIGER, C., KEELING, C. I., and BLOMQUIST, G. J. 2005. Some insights into the remarkable metabolism of the bark beetle midgut. *Recent Adv. Phytochem.* 39:57–78.

WOOD, D. L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behaviour of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

WOOD, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Nat. Mem.* 6:1–1359.

ZHANG, L. and SUN, J. 2006. Electrophysiological and behavioral responses of *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae) to candidate pheromone components identified in hindgut extracts. *Environ. Entomol.* 35:1232–1237.

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Occurrence of Sarmentosin and Other Hydroxynitrile Glucosides in Parnassius (Papilionidae) Butterflies and Their Food Plants

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Abstract Sequestration of plant secondary metabolites is a widespread phenomenon among aposematic insects. Sarmentosin is an unsaturated γ-hydroxynitrile glucoside known from plants and some Lepidoptera. It is structurally and biosynthetically closely related to cyanogenic glucosides, which are commonly sequestered from food plants and/or *de novo* synthesized by lepidopteran species. Sarmentosin was found previously in *Parnassius* (Papilionidae) butterflies, but it was not known how the occurrence was related to food plants or whether *Parnassius* species could biosynthesize the compound. Here, we report on the occurrence of sarmentosin and related compounds in four different *Parnassius* species belonging to two different clades, as well as their known and suspected food plants. There were dramatic differences between the two clades, with *P. apollo* and *P. smintheus* from the Apollo group containing high amounts of sarmentosin, and *P. clodius* and *P. mnemosyne* from the Mnemosyne group containing low or no detectable amounts. This was reflected in the larval food plants; *P. apollo* and *P. smintheus* larvae feed on *Sedum* species (Crassulaceae), which all contained considerable amounts of sarmentosin, while the known food plants of the two other species, *Dicentra* and *Corydalis* (Fumariaceae), had no detectable levels of sarmentosin. All insects and plants containing sarmentosin also contained other biosynthetically related hydroxynitrile glucosides in patterns previously reported for plants, but not for insects. Not all findings could be explained by sequestration alone and we therefore hypothesize that *Parnassius* species are able to *de novo* synthesize sarmentosin.

Keywords *Parnassius* (Papilionidae) · Sarmentosin · Hydroxynitrile glucosides · Cyanogenic glucosides · Sequestration · Biosynthesis

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Introduction

Almost all plants and insects contain characteristic allelochemicals with defensive properties. The chemical interactions of insects and their food plants are variable and hard to predict, and are dependent on types of chemicals, and the insects' ability to sense and avoid, sequester, degrade/transform, or excrete chemicals as well as cost-benefit involved for the plants and the insects. Sarmentosin is an unsaturated γ-

hydroxynitrile glucoside (γ-HNG) (Fig. 1) known from plants as well as butterflies and moths (Nishida, 1994; Nishida et al., 1994; Nishida and Rothschild, 1995; Bjarnholt et al., 2008). The importance and biological function of the compound is not understood, although it is found in significant amounts in both types of organisms, i.e., 500–650 μg/insect (Nishida et al., 1994; Nishida and Rothschild, 1995) and up to 75 % of total HNG content in a given plant (Bjarnholt et al., 2008). In plants, sarmentosin is derived from the amino acid isoleucine (Ile), and most often co-occurs with other Ile-derived HNGs (Nishida, 1994; Bjarnholt et al., 2008) (Fig. 1). Commonly known are the α-HNGs, also denoted cyanogenic glucosides (CNglcs) because they release hydrogen cyanide (HCN) upon hydrolysis of the glucosidic bond. The HNGs are mainly β-glucosides, and most plants that harbor CNglcs also contain a β-glucosidase (BGD) committed to hydrolyzing the compounds upon herbivore attack causing HCN release. The CNglcs are thus considered to be part of the plants' chemical defense (Morant et al., 2008). Many CNglc-containing insects have been reported to be cyanogenic, and some have been shown to contain a corresponding β-glucosidase (Franzl et al., 1989), which may be released along with the CNglcs as a defense mechanism. Insects that contain either sarmentosin or the leucine (Leu)-derived analog sutherlandin are generally known to be deterrent to many predators, and sutherlandin itself has been reported to be deterrent to predating ants (Braekman et al., 1982; Aldrich et al., 1990; Nishida et al., 1994).

In *Lotus japonicus,* the six co-occurring Ile-derived α-, β-, and γ-HNGs (Fig. 1) share the first step of the biosynthetic pathway where Ile is converted to its corresponding oxime by a cytochrome P450, CYP79D3 (Takos et al., 2011) (Fig. 2). Furthermore, the presence or absence of the five non-cyanogenic HNGs in this plant is determined by a single recessive genetic trait, demonstrating a tight biosynthetic connection between these compounds further downstream in the pathway as well (Bjarnholt et al., 2008). Subsequent investigation of the pathway in *L. japonicus* has led to the proposed pathway seen in Fig. 2 (Saito et al., 2012), however, some key enzymes remain unknown. Sarmentosin is not present in *L. japonicus*, but the typical co-occurrence with the remaining Ile-derived HNGs in other plants renders it a likely product of rhodiocyanoside A hydroxylation. Sarmentosin is, therefore, presumably also biosynthetically linked to CNglcs and other HNGs in plants (Fig. 2). The CNglcs are widespread in the plant kingdom (Bak et al., 2006) and are also present in some insects, especially butterflies and moths (Zagrobelny et al., 2008). Insects either sequester the compounds from their food plants or biosynthesize the compounds themselves, and some are even able to do both (Zagrobelny et al., 2008). It is possible that the situation is the same for sarmentosin (Nishida, 1994). It has recently been established that the individual steps in CNglc biosynthesis are the same in plants and insects, although catalyzed by members of different subfamilies of the involved cytochrome P450s and glucosyl transferases (Jensen et al., 2011). Furthermore, the pathway of sequestration from plant to insect appears to be the same for cyanogenic and non-cyanogenic HNGs, as demonstrated for the larvae of the burnet moth *Zygaena filipendulae*. These larvae naturally sequester linamarin and lotaustralin from the food plant *Lotus corniculatus,* which does not contain non-cyanogenic HNGs. When reared instead on *L. japonicus, Z. filipendulae* also sequestered the remaining HNGs found in this plant (Zagrobelny et al., 2007a). In addition, the zygaenid moth *Pryeria sinica* was reported to contain sarmentosin as well as linamarin and lotaustralin, all of which also were found in its larval food plant *Euonymus japonicus* (Nishida, 1994). This indicates that it sequesters all HNGs from the food plant. On the other hand, the Magpie moth *Abraxas grossulariata* (Geometridae) appears to be able to *de novo* synthesize sarmentosin. The compound was found in pupae as well as adults regardless of whether the food plant was a *Prunus* species (only known to contain phenylalanine-derived HNGs) or *E. japonicus* (Nishida et al., 1994; Nishida and Rothschild, 1995). Several other *Abraxas* moths have also been found to contain sarmentosin, most of them after being reared on *E. japonicus*, but interestingly none were reported to contain other HNGs (Nishida, 1994).

Sarmentosin was additionally found in two species of *Parnassius* (Papilionidae) butterflies, *P. apollo* and *P. smintheus*[1] (Nishida, 1994; Shepard and Manley, 1998). These species are reported to feed on *Sedum* plants, which are

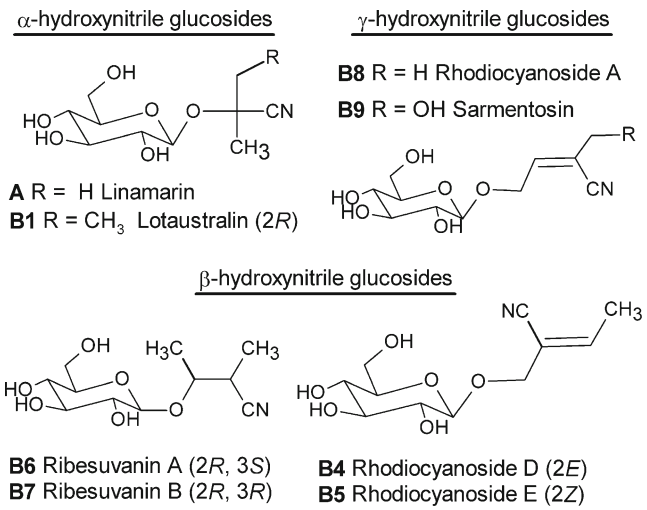

Fig. 1 Structure of sarmentosin and related cyanogenic and non-cyanogenic glucosides described in this study

[1] At the time of sampling *P. smintheus* was considered to be *P. phoebus* and the results reported at such (Nishida, 1994). Later, the two species were separated. Given the reported host plant and because *P. phoebus* does not exist in the state of Washington, USA, where the butterflies were sampled, the species investigated must have been *P. smintheus* (Shepard and Manley, 1998).

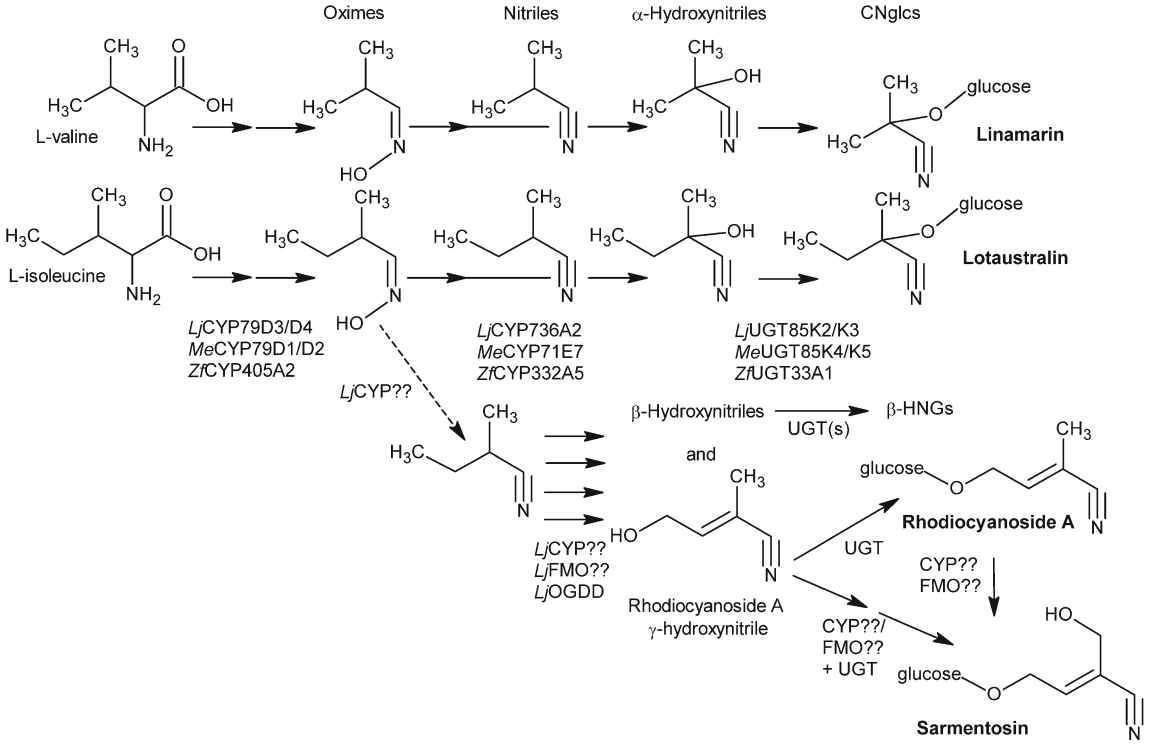

Fig. 2 Biosynthesis of Ile-derived HNGs and linamarin as known from plants and insects. *Lj=Lotus japonicus, Me=Manihot esculenta*, *Zf=Zygaena filipendulae*, CYP=cytochrome P450, FMO=flavin monooxygenase, OGDD=2-oxoglutarate-dependent dioxygenase. CNglc pathway in plants and insects: The first cytochrome P450 catalyzes the conversion of amino acids to oximes, and the second converts oximes to α-hydroxynitriles, most likely via a nitrile intermediate. The α-hydroxynitriles are glucosylated to CNglcs by family 1 UDP-glycosyl transferases (Takos et al., 2011). In *L. japonicus,* the pathway for non-cyanogenic HNGs share the first step with the CNglc pathway (Bjarnholt et al., 2008), the second step is catalyzed by an unknown cytochrome P450 converting the Ile-derived oxime to the corresponding nitrile (Saito et al., 2012). The following steps towards the β- and γ-hydroxynitriles are unknown but hypothesized to be catalyzed by any of the mentioned oxygenases or combinations of them (Saito et al., 2012), and the putative UGT(s) finally producing the β- and γ-HNGs has not been identified yet either. In sarmentosin producing plants, the extra hydroxylation can take place before or after glucosylation and may be catalyzed by CYPs or FMOs

likely to contain sarmentosin, as the compound was first isolated from *S. sarmentosum* (Lechtenberg and Nahrstedt, 1999) and subsequently found in *S. stenopetalum* (Nishida, 1994). There are no reports on other HNGs occurring in the butterflies or the plants. In order to shed some light on the possible co-occurrence and sequestration or biosynthesis of various cyanogenic and non-cyanogenic HNGs in these insects, we here report on the content of HNGs in four different species of *Parnassius* butterflies and larvae, and in their known and suspected food plants. Around 50 species of Parnassius butterflies are known, mostly occurring in areas in Central Asia, the Himalayas, and western China at high altitudes. Based on phylogenetic analyses, they are divided into eight clades (Michel et al., 2008; Omoto et al., 2009). The species examined here belong to two clades (Fig. 5): European *P. apollo* and North American *P. smintheus* represent the Apollo group (subgenus *Parnassius* s.str.) feeding on Crassulaceae, while European *P. mnemosyne* and North American *P. clodius* represent the Mnemosyne group (subgenus *Driopa*) feeding on Fumariaceae.

Methods and Materials

Samples and Sampling Procedure To elucidate the presence of sarmentosin and related compounds in Parnassius butterflies and their respective food plants, four butterfly species (*P. smintheus*, *P. mnemosyne*, *P. apollo*, *P. clodius*) and four plant species representing Crassulaceae (*S. telephium ssp. maximum*, *S. lanceolatum*) and Fumariaceae (*Dicentra uniflora*, *Corydalis solida*) were sampled as summarized in Table 1. All samples were collected alive in the field, where they were frozen quickly and subsequently transported on dry ice to Copenhagen University. Here, the samples were kept at −80°C until they were extracted and analyzed according to the procedures below.

Extraction Procedure Metabolites were extracted from frozen samples with 55 % MeOH containing 0.1 % formic acid and 0.044 mM amygdalin (phenylalanine derived CNglc) as internal standard. Butterfly samples were crushed with a mortar and pestle (in some cases with wings and body as separate

Table 1 Summary of insect and plant sampling

Parnassius sp.	Food plant	Year	No of specimens		Locality
			Insects[a]	Plants[b]	
P. apollo ssp. frankenbergeri[c]	*Sedum telephium ssp. maximum*	2010	7(L), 8(A,M), 5(A,F)	16 (l)[d]	Poland: Pieniny National Park, semi-natural colony
P. clodius	*Dicentra uniflora* (*Du*)	2009	3(A,M), 3(A,F)	2(*Sl*)(l+f)	USA:Moran, WY
	S. lanceolatum (*Sl*)	2010	5(A,M)), 3(A,F) 1(L)	3(*Sl*)(l), 3(*Du*)(l)	
		2011	8(A,M)		
P. mnemosyne[e]	*Corydalis solida*	2010	-	10 (l)	Poland: Pieniny National Park
		2011	2(A,M)	8 (l)	
P. smintheus	*S. lanceolatum*	2011	8(A)	10 (l)	Canada: Jumpingpound Ridge, Alberta, (50.95 °N, 114.91 °W)

[a] Insects = larvae (L), adults (A), males (M), females (F)

[b] Plants = leaves (l), flowers (f)

[c] According to permission of the Ministry of Environment (DOP/pn–4102–285–13235/10/RS) and General Director for Environmental Protection (DOPozgiz-4200/I-28/1261/10/km)

[d] Half of the samples were undamaged by insects and half had grazing marks

[e] According to permission of the Ministry of Environment (DOP/pn–4102–243–14230/11/RS) and General Director for Environmental Protection (DOP.OZGIZ-6401.01.81.2011.JRO)

samples), while plant samples were crushed in an eppendorf tube with a plastic pestle. Both sample types were subsequently passed through an anopore 0.45 mm filter (Whatman).

LC-MS Analyses The extracts were analyzed by LC-MS in their year of collection and subsequently stored at −20°C. All extracts were re-analyzed together in 2011 to be able to compare samples from different years, and the resulting chromatograms had not changed during storage of extracts (data not shown). All samples were diluted four times with water before analysis by LC-MS. Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A Zorbax SB-C18 column (Agilent; 1.8 μM, 2.1 × 50 mm) was used at a flow rate of 0.2 ml/min. The oven temperature was maintained at 35°C. The mobile phases were: A, water with 0.1 % (v/v) HCOOH and 50μM NaCl; B, acetonitrile with 0.1 % (v/v) HCOOH. The gradient program was: 0 to 0.5 min, isocratic 2 % B; 0.5 to 7.5 min, linear gradient 2 to 40 % B; 7.5 to 8.5 min, linear gradient 40 % to 90 % B; 8.5 to 11.5 isocratic 90 % B; 11.60 to 17 min, isocratic 2 % B. The flow rate was increased to 0.3 ml/min in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode. Mass spectral data were analysed with the native DataAnalysis software. Sodium adducts of sarmentosin (*m/z* 298, retention time (rt) 1.8 min), linamarin (*m/z* 270, rt 2.6 min), rhodiocyanoside A (*m/z* 282, rt 4.6 min), lotaustralin (*m/z* 284, rt 5.5 min), β-HNGs (ribesuvanins and rhodiocyanosides D+E)(*m/z* 282+284, rt 4.0-4.5 min) and amygdalin (*m/z* 480, rt 6.6 min) (Fig. 3) were detected and compared to standards of linamarin, lotaustralin, and sarmentosin. Total amounts of HNGs were estimated based on peak areas and quantified by injection of known amounts of linamarin, lotaustralin, and sarmentosin. As the ionization efficiency of linamarin, lotaustralin, and rhodiocyanoside A under these analytical conditions is the same (Bjarnholt et al., 2008), all rhodiocyanosides and ribesuvanins were quantified using the standard curve for lotaustralin.

Estimation of P. apollo Larval Leaf Consumption The food and energy budget of *P. apollo* reared in the semi-natural colony was experimentally estimated in 1996, 1997, and 2003 as described in detail for 1996 (Kędziorski et al., 1997). These numbers were used to calculate the estimated average amount of sarmentosin ingested by larvae (Table 2). Only the two last instars were considered, as the amount of food consumed at earlier instars is relatively small.

Results

Four different species of *Parnassius* butterflies were analyzed for their content of linamarin, lotaustralin, and the suite of known Ile-derived HNGs, including sarmentosin. The specimens of *P. apollo* had been reared in a semi-natural open-air colony and fed with food plants freshly collected in their natural biotopes, whereas the remaining three species originated from three different native habitats. In the cases of *P.*

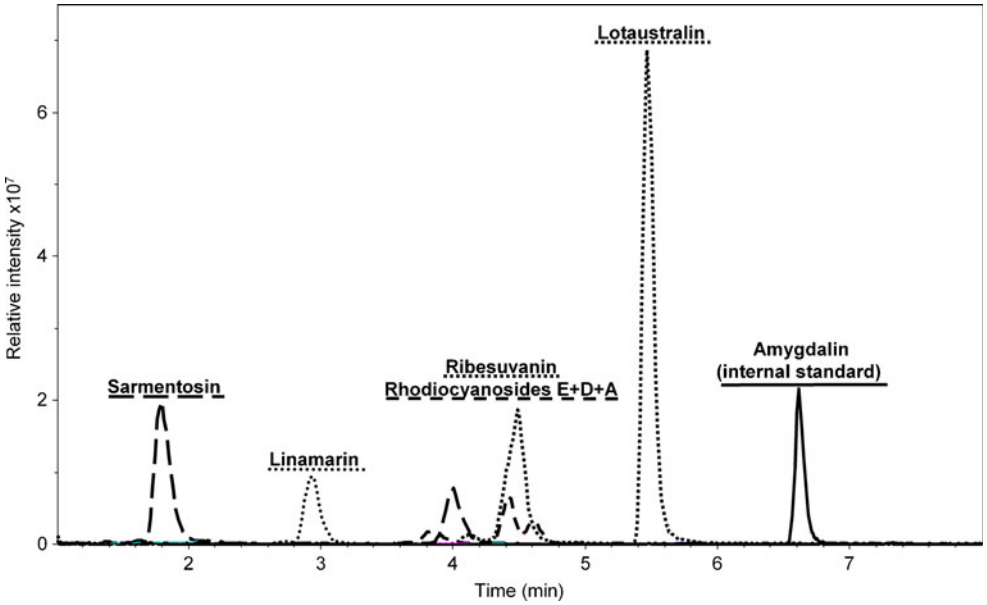

Fig. 3 Extracted ion chromatograms from LC-MS analyses of *Sedum lanceolatum* from 2009 USA illustrating the peaks of HNGs. The peak with a retention time of approximately 4 min and the same *m/z* value as sarmentosin is an unidentified glucoside (as determined by MS/MS-analysis, data not shown) which was not found in the insects

apollo and *P. clodius,* it was possible to analyze both larvae and adults, while only adults were available from the other species. Samples of plants known to or suspected to have served as the food plants of the larvae were also analyzed. The results on the overall occurrence of the compounds are shown in Table 3 (for details on number of replicates etc., see Table 1). *Corydalis solida* did not contain any of our compounds of interest, and in *P. clodius* from 2009 and 2010 we found only linamarin and lotaustralin. In the remaining plant and insect species, we found lotaustralin co-occurring with sarmentosin and/or the other Ile-derived HNGs, and/or sometimes with linamarin. Although it is not surprising that these compounds co-occur, this is the first report of rhodiocyanosides and ribesuvanins found in insects reared on a natural diet. Some of these compounds were found previously in *Z. filipendulae*, but only when larvae were reared on a species of *Lotus,* which was not their natural food plant (Zagrobelny et al., 2007a). To our knowledge, this is also the first report of CNglcs in the Fumariaceae (*D. uniflora*). The analyzed *Sedum* plants (Crassulaceae) had similar amounts of sarmentosin, lotaustralin, rhodiocyanoside D, and ribesuvanins across years and continents, but at least in the USA *S. lanceolatum* has the ability to produce linamarin and rhodiocyanoside A as well. Also, to the best of our knowledge, presence of linamarin has not been reported previously in the Crassulaceae, although several members of this order contain Ile-derived HNGs (Bjarnholt and Møller, 2008).

Sarmentosin was found in three of the four analyzed Parnassius species (Table 3). In *P. smintheus* and *P. apollo,* the levels were respectively 602±74 and 845±151 μg per individual adult. It is noteworthy that the *P. smintheus* imagines were on average approximately one third the size of the *P. apollo* imagines (130 vs. 420 mg). This means that the concentrations found in *P. smintheus* were almost three times higher than those found in *P. apollo*, as can be seen from Fig. 4. The total amounts are in accordance with the previously reported levels of 460 μg per individual for *P. smintheus* [Clade I (Omoto et al., 2009)], whereas the amount of sarmentosin found in *P. apollo* has to our knowledge not been published (Nishida, 1994; Nishida and Rothschild, 1995). Furthermore, the reported levels in various *Abraxas* moths are 270–670 μg per individual, and in one species of *Yponomeuta* moth 120 μg per individual (Nishida, 1994; Nishida et al., 1994). On the other hand, we found no sarmentosin in any individuals of *P. mnemosyne* from 2011, and in *P. clodius* the occurrence varied among the 3 years they were collected; furthermore, when sarmentosin was present, the level was very low (~0.5 μg/individual).

Table 2 Estimated average larval leaf consumption for late instars of *Parnassius apollo* (Kędziorski et al., 1997)

Series	Sex	Energy budget (kJ)		*Sedum telephium* (kJ/g FW)	Consumption (g FW)		
		L4	L5		L4	L5	L4+L5
I (early)[a]	F	5.8	47.7	1.392	4.2	34.3	38.4
	M	4.6	29.5	1.392	3.3	21.2	24.5
II (late)[a]	F	7.2	25.3	1.392	5.2	18.2	23.3
	M	8.8	25.6	1.392	6.3	18.4	24.7

[a]Early = larvae hatched in February; Late = larvae hatched mid March

Table 3 Occurrence of HNGs in butterflies and their known and suspected food plants

Butterfly/plant	Year	Sarmentosin	Rhodio A[a]	β-HNGs[b]	Lotaustralin	Linamarin
USA						
Parnassius clodius adults	2009	-[c]	-	-	+	(+)
adults/larva	2010	–/–	–/–	–/–	(+)/+	(+)/-
adults	2011	+	+	-	+	+
Dicentra uniflora leaves	2010	-	+	+	+	+
Sedum lanceolatum flowers/leaves	2009	+	+	+	+	+
leaves	2010	+	(+)	+	+	-
Poland						
P. apollo adults/larvae	2010	+/+	+/+	+/+	(+)/(+)	-
S. telephium leaves	2010	+	-	+	+	(+)
P. mnemosyne adults	2011	-	+	-	+	(+)
Corydalis solida leaves	2010	-	-	-	-	-
leaves	2011	-	-	-	-	-
Canada						
P. smintheus adults	2011	+	+	+	+	-
S. lanceolatum	2011	+	-	+	+	-

[a] Rhodio A = rhodiocyanoside A

[b] β-HNGs include rhodiocyanoside D and E and the ribesuvanins

[c] + = present; (+) = present in a few of the examined specimens; - = not detected

The larvae that gave rise to the examined *P. apollo* adults were from a batch of early hatched larvae, and their average leaf consumption can be estimated to be approximately 25 g for males and 38 g for females (Table 2). The difference is mainly due to sex differences in duration of the last instar (Kędziorski et al., 1997). The sarmentosin content of 25 g of *S. telephium* is approximately 1000 μg [40 μg/g FW (Fig. 4)], which is within the confidence interval ($P<0.05$) for the amount found in *P. apollo* adults (roughly 850 μg/individual ±150 μg). The "concentration factor" from larval food plant to imago was 55 (data from Fig. 4: $C_{P.\ apollo} : C_{S.\ telephium}$=2.2 μg/g : 0.04 μg/g). Interestingly, this factor for the other species containing high sarmentosin amounts was essentially the same ($C_{P.\ smintheus} : C_{S.\ lanceolatum}$=5.9 μg/g : 0.1 μg/g=59). As seen from Fig. 4, the remaining HNGs were not concentrated from food plant to butterflies.

For *S. telephium* and *C. solida,* intact leaves as well as leaves that had been damaged by larval feeding were analyzed. There were no differences in the amount of HNGs due to grazing stress (data not shown), and this had also previously been observed in *S. lanceolatum* in Canada for sarmentosin only (our unpublished results). This is consistent with the traditional perception that CNglcs – and possibly also the other HNGs – are phytoanticipins, and that the plants' inducible defense involves different compounds. There were no differences in the type or amounts of compounds between males and females in *P. apollo* and *P. clodius* (data not shown), although differences between the sexes were previously shown for linamarin and lotaustralin in *Z. filipendulae* adults (Zagrobelny et al., 2007b). Adult specimens of *P. apollo* and *P. clodius* from 2010 were divided into wings and body, which were extracted separately, and it was evident that 30–40 % of the sarmentosin content of the entire *P. apollo* butterflies was present in the wings (Table 4). Since the body is much heavier than the wings, wings actually contain up to 10 times as much sarmentosin per mg fresh weight. This was shown previously to also be the case for *P. phoebus* from the US (Nishida and Rothschild, 1995).

Discussion

Many Lepidoptera are strongly associated with poisonous plants and sequester toxic compounds instead of or in addition to manufacturing their own (Nishida, 1994, 2002; Opitz and Muller, 2009). The potential of insects to take up, transport, and sequester plant derived defense compounds to their own benefit probably derives from their general nutrient uptake system, which has a broad selectivity for transport of plant glucosides. If food plants provide compounds that match the substrate profile of the larval transport systems, the larvae have a high probability of adopting compounds into their defense system (Kunert et al., 2008; Zagrobelny and Møller, 2011). At first sight, the results of our study present a case of sequestration, in that the patterns of compounds found in larvae and butterflies roughly reflect those found in the food plants. However, some specimens contained "extra compounds" (Table 3). Larvae of the *P. apollo* ssp. *frankenbergeri* feed almost exclusively on *S.*

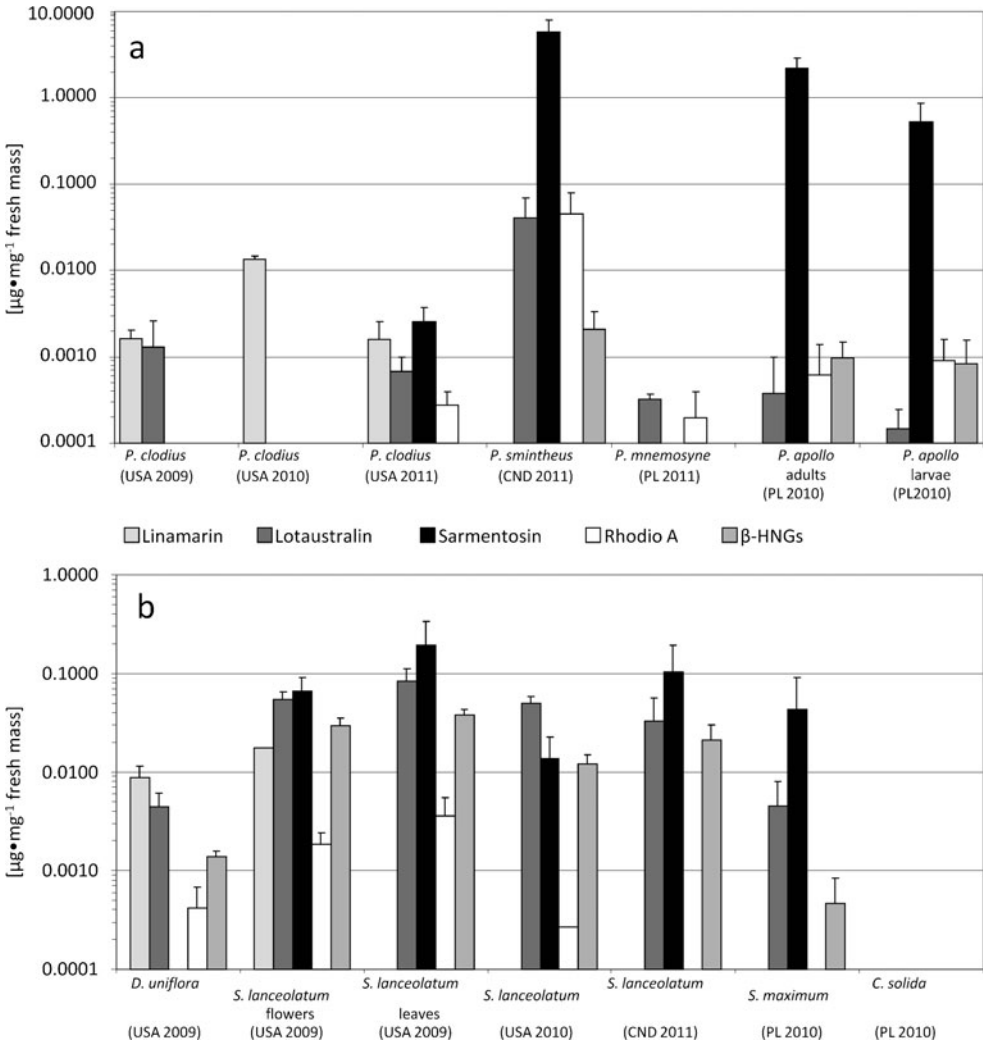

Fig. 4 Quantitative CNglc and β- and γ-HNG contents of the analyzed butterflies (*Parnassius*) (panel a) and their known and suspected food plants *(Dicentra, Sedum, Corydalis)* (panel b)

telephium spp. *maximum,* so this was the only food plant made available to the specimens in the semi-natural colony (Nakonieczny and Kedziorski, 2005). The pattern of compounds found in the insects and the food plant was the same, except for the presence of rhodiocyanoside A in larvae and butterflies. As this compound is likely to be the biosynthetic precursor of sarmentosin it could have been present in the food plant at other time points or in different tissues from the ones sampled. The same applies for the rhodiocyanoside A found in the sampled *P. smintheus*; the Canadian samples of its food plant *S. lanceolatum* (Matter et al., 2003) did not contain this compound, although *S. lanceolatum* from USA did. Furthermore, larvae of *P. clodius* are known to feed on *D. uniflora* (Scott, 1986), which did not contain sarmentosin,

Table 4 Distribution of HNGs between body and wings in *Parnassius apollo*[a]

	Average weight (mg±*s.d.*[b])	Lotaustralin (µg±*s.d.*[b])	Sarmentosin (µg±*s.d.*[b])	Rhodio A (µg±*s.d.*[b])	β-HNGs (µg±*s.d.*[b])
P. apollo body	361±120	0.26±0.21	542±155	0.13±0.14	0.38±0.33
% of total[c]	*88*		*66*		
P. apollo wings	48±12	0.06±0.05	279±80	0.05±0.05	0.06±0.04
% of total[c]	*12*		*34*		

[a] Results for *P. clodius* were not included in the table since the only detectable compound was linamarin, in very low amounts and not in all samples

[b] $P<0.05$

[c] Percentages were not included when amounts of compounds were very small and standard deviations were high

whereas *P. clodius* butterflies from 2011 did. It is also possible that these plants contain sarmentosin in different tissues from those sampled here, but other plants are known which produce rhodiocyanoside A without producing sarmentosin (Bjarnholt et al., 2008). Because of the limited total biomass available from the food plant *D. uniflora* and the co-occurence of *S. lanceolatum* at the population site, we wondered whether *P. clodius* specimens collected at this location may have supplemented their diet with *S. lanceolatum*. The presence of sarmentosin in *S. lanceolatum* supports this hypothesis, but it is also possible that the *P. clodius* specimens from 2011 have synthesized the compound, either *de novo* or by hydroxylation of rhodiocyanoside A sequestered from *D. uniflora*. The *P. mnemosyne* butterflies contained little or none of our compounds of interest, and in the food plant *C. solida* there were no detectable levels at all. This *Parnassius* species feeds exclusively on *Corydalis ssp.* (Turlin and Malin, 2005), and to our knowledge there are no other reports of HNGs in *Corydalis*. The present results, therefore, indicate that *P. mnemosyne* can biosynthesize HNGs, unless the compounds found in the butterflies originate from tissues or *Corydalis* species not analyzed. In all cases, it is theoretically possible that the compounds found in insects, but not in food plants, were bio-concentrated from undetectable levels in plants to detectable levels in insects. On the other hand, the remaining results show that sarmentosin is the only compound that is concentrated from plant to insect.

Interestingly, in larvae and butterflies of *P. apollo* and *P. smintheus* where the sarmentosin content was high, the content of the remaining HNGs were several orders of magnitude lower (Fig. 4). This is in stark contrast to the pattern found in the food plants where the levels of the different HNGs were of comparable magnitude. Similar HNG levels were previously also observed in plants from the genera *Rhodiola, Ribes,* and *Lotus* (Bjarnholt et al., 2008). From the report of co-occurring sarmentosin, lotaustralin, and linamarin in *Pryeria sinica*, it seems that the levels of the compounds were more equal in this species, but there are no exact measurements stated (Nishida, 1994). The dramatic increase in the ratio of sarmentosin to the remaining HNGs in *P. apollo* and *P. smintheus* demonstrates clearly that there is preferential accumulation in these species. This could be achieved either by preferential uptake of sarmentosin, preferential degradation of the remaining HNGs, or by *de novo* synthesis of sarmentosin. As previously mentioned, the uptake system in *Z. filipendulae* larvae does not appear to discriminate between CNglcs and other HNGs found in the food plant, and the ratio between the different CNglcs linamarin and lotaustralin is influenced by the ratio in the food plant at the larval stage (Zagrobelny et al., 2007a). During pupation, this changes to almost identical ratios of compounds in the imagines, irrespective of food plant composition (Zagrobelny et al., 2007a). Simultaneously, the total amount of CNglcs decreases (Zagrobelny et al., 2007a), indicating that the changed ratio is obtained by preferential degradation, but in *P. apollo* the preferential accumulation of sarmentosin appears already at the larval stage (Fig. 4). *De novo* synthesis of CNglcs is a widespread phenomenon in Papilionoidea, where imagines of butterflies from the Heliconiinae, Acraeinae, Nymphalinae, and Polyommatinae groups have been shown to accumulate linamarin and often lotaustralin, which is probably achieved by biosynthesis of the compounds (Engler et al., 2000; Turlin and Malin 2005; Rebourg et al., 2006). The ability to produce CNglcs has also been proposed to be present in all species of Zygaenidae (Zagrobelny et al., 2008). When the biosynthetic pathway was recently resolved for *Z. filipendulae* (Jensen et al. 2011), it was found to be highly similar to the pathways known from plants (Takos et al., 2011), although clearly convergent. In *L. japonicus,* it appears likely that only two more enzymes are necessary to produce all the non-cyanogenic HNGs, and they could very well be closely related to the two cytochromes P450 already known to be involved in the pathway (Bjarnholt et al., 2008; Takos et al., 2011; Saito et al., 2012). As previously mentioned, biosynthesis of sarmentosin presumably only requires an additional hydroxylation of rhodiocyanoside A, a reaction frequently used by insects to handle sequestered toxins (Langel and Ober, 2011; Pinto et al., 2011) and possibly present in *P. clodius* if it does not feed on *S. lanceolatum*. Given the similar structure of the CNglc biosynthetic pathway in insects and plants, it is likely that some insects could also possess the few additional enzymes needed to produce the remaining compounds as suspected for the *Abraxas* species (Nishida, 1994; Nishida et al., 1994).

The calculations of larval sarmentosin intake for the two *Parnassius* species from the Apollo group are based on estimates of larval leaf intake and variable sarmentosin concentrations in plants. However, they show that sequestration efficiency must be quite high if all the sarmentosin found in the male insects originate from sequestration during the larval stage. The literature contains few attempts to quantify sequestration of plant secondary metabolites by insects, but in general significantly less than 100 % of ingested compounds can be recovered in the insects. Furthermore, the concentration factors from larval food plant to imagines are much lower than the values of 55–60 found for *P. apollo* and *P. smintheus* in the present study. Larvae of *Heliconius* butterflies sequester CNglcs derived from the non-protein amino acid cyclopentenyl glycine from their Passifloraceae food plants, and in some cases possibly also minor amounts of linamarin and lotaustralin. Some or all of them are also able to biosynthesize linamarin and lotaustralin. In an extensive study of different *Heliconius* species reared on different *Passiflora* plants, the concentrations of CNglcs were measured in the butterflies and the food plants (Engler-Chaouat and Gilbert, 2007). No attempts were made to quantify sequestration, but the concentrations found in butterflies were generally equal to or much less than concentrations in the food plants, with few examples

of a 6–10 fold increase.[2] Our unpublished results indicate that *Z. filipendulae* larvae sequester around 80–85 % of CNglcs present in the leaves they eat, with the rest being lost due to degradation and HCN release during chewing. Before reaching maturity, *Zygeana* species lose additional CNglcs by at least two routes: degradation during pupation and degradation during the larval stage, where they have been shown to be continuously surrounded by a HCN "cloud" (Witthohn and Naumann, 1987; Zagrobelny et al., 2007a, b). In the case of *Z. filipendulae,* the concentration factor from larval food plant to adult moth was around 3 when the larvae were reared on natural food plants (Zagrobelny et al., 2007a). Larvae of *Thessalia leanira fulvia* (Nymphalidae) sequester iridoid glycosides (IGs) from *Castilleja integra*. Cultured larvae reared on this food plant contained IGs to the same order of magnitude as the plant tissue, and so did the larval frass (Mead et al., 1993), clearly demonstrating less than 100 % sequestration. Among Papilionidae sequestering non-glucosidic compounds such as aristolochic acids, quinolizidine alkaloids, or pyrrolizidine alkaloids, the recovery of compounds in larvae can be estimated to correspond to either 0.5-50 % of ingested compounds, or up to 1 g of plant material (Montllor et al., 1990; Vonnickischrosenegk and Wink, 1993; Pinto et al., 2011).

All in all, it appears somewhat unlikely that the *P. apollo* and *P. smintheus* larvae should have sequestered *and* retained all the sarmentosin ingested with their food plant. At the same time, not all HNGs found in *P. clodius* and *P. mnemosyne* can be unequivocally accounted for by sequestration, and there is also the question of the presence of rhodiocyanoside A in *P. apollo* and *P. smintheus*. Based on this, we hypothesize that *Parnassius* species are capable of biosynthesizing HNGs. We propose that a pathway for production of all HNGs evolved in the common ancestor of Zygaenoidea and Papilionoidea (Fig. 5) or even earlier. This made that ancestor able to produce all of the different Ile-derived HNGs and also valine-derived linamarin, which is biosynthesized by the same enzyme system as lotaustralin in insects and plants (Jensen et al. 2011; Takos et al., 2011). If the *Abraxas* moths (Geometridae) are indeed capable of biosynthesizing sarmentosin, the pathway must have evolved even before this branch was split from Zygaenoidea and Papilionoidea (Fig. 5). During evolution, some branches must have specialized to produce only one or two compounds, and some branches lost the ability altogether. However, most species in all branches probably retained the ability to handle and detoxify the compounds, since HNGs are widespread in plants (Bjarnholt and Møller, 2008), making it easy for species to sequester the compounds from newly colonized food plants during evolution.

Parnassius butterflies are divided into eight clades (Omoto et al., 2009) (Fig. 5). Clade I which is the most basal split on the *Parnassius* species tree, comprise species feeding on Crassulaceae, mainly *Sedum* spp., and in one instance Saxifragaceae (Michel et al., 2008), with several species from both plant families having been shown to contain sarmentosin (Nishida and Rothschild, 1995; Bjarnholt et al., 2008). The other seven *Parnassius* clades feed on Fumariaceae, mostly on *Corydalis* species, and in one instance on Scrophulariaceae (Michel et al., 2008), so far not shown to contain sarmentosin. According to molecular data *Parnassius* underwent a major speciation event some 24.3±4.1 MY BP (Omoto et al., 2009) which probably resulted in a shift from ancestral food-plants (possibly Aristolochiaceae) onto Crassulaceae and Fumariaceae (Rebourg et al., 2006; Michel et al., 2008). We hypothesize that *Parnassius* butterflies were able to biosynthesize HNGs even before they colonized Crassulaceae since the ability came from a proposed Ditrysian ancestor (Fig. 5). They were then immediately able to sequester the encountered compounds, when colonizing Crassulaceae, and this would have enabled them to conserve energy from the production of defense compounds. Their own biosynthetic activity may then have become lower over time, because it was not necessary to maintain a high activity anymore. The later shift to Fumariaceae as food plant probably entailed a shift in defense compounds as well, as *D. uniflora* contains poisonous alkaloids that *P. clodius* is suspected of sequestering, and *C. solida* apparently does not contain HNGs at all, rendering it unfavorable for *P. mnemosyne* to base its chemical defense on these compounds. This could be the reason why we found only trace amounts of HNGs in the Mnemosyne clade and why *P. mnemosyne* has apparently lost the ability to perform the last step of sarmentosin biosynthesis and accumulates rhodiocyanoside A instead.

Whether the sarmentosin found in *P. apollo*, *P. smintheus,* and *P. clodius* was a result of sequestering and/or biosynthesis, the high amounts and the comparatively low amounts of the remaining HNGs found in the two first species clearly show that sarmentosin is of importance to these larvae and butterflies. The observation that the sarmentosin concentrations were much higher in wings than in the bodies of all analyzed sarmentosin-containing specimens is consistent with sarmentosin being a defense compound, since wings and their scales are the first thing a predator comes in contact with when attacking a butterfly. In addition, *P. apollo* is avoided by naive chicks, either initially or after a few attacks (Bohlin et al., 2008). Likewise, *Abraxas* moths are known to be deterrent to a long list of predators (Nishida et al., 1994). The young black Apollo larvae are hard to detect by predators, while the colored spots appearing later significantly increase larval conspicuousness (Bohlin et al., 2008). This could be linked to the

[2] The concentrations stated are based on dry weight of insects and plants. Our calculations of concentration factors are based on the assumption that butterfly and plant dry matter constitute approximately 20 % and 10 % respectively.

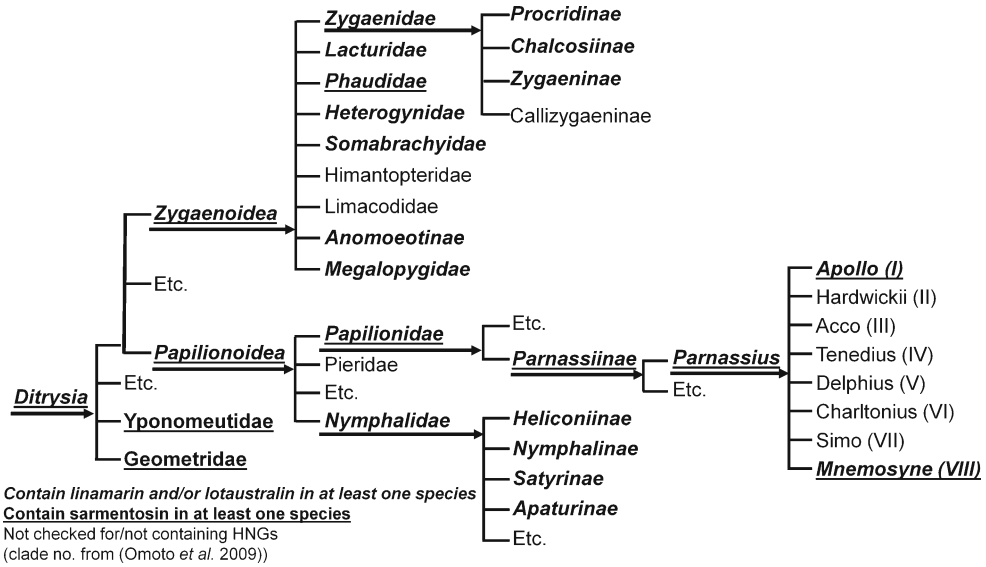

Fig. 5 Overview of sarmentosin, linamarin and lotaustralin occurrence in Ditrysian insects

lower amounts of sarmentosin presumably present during the first few instars where the larvae have not yet had the opportunity to sequester or biosynthesize as much sarmentosin as compared to later instars. Larvae of *P. smintheus* show a similar pattern with conspicuous yellow markings generally appearing after the second instar, whereas *P. clodius* larvae are more cryptic in coloration patterns, brown in background color with small dark brown and cream colored spots. Likewise, *P. apollo* and *P. smintheus* butterflies both have large and distinct red spots on their wings, suggesting aposematism, whereas the spots on adult *P. clodius* are smaller and more variable in color, and adult *P. mnemosyne* have no spots at all.

The mechanism by which sarmentosin might work as a defense compound is not known. In plants, the action of the non-cyanogenic HNGs is most likely mediated by BGD-hydrolysis, as *L. japonicus* contains BGDs committed to rhodiocyanoside hydrolysis (Takos et al., 2010), and our unpublished results for eight cultivars of gooseberry show that sarmentosin is hydrolyzed upon tissue disruption. Insects may also possess such BGDs, but the γ-hydroxynitrile resulting from sarmentosin hydrolysis (Fig. 6) will not release HCN as do the α-hydroxynitriles derived from CNglcs hydrolysis. The hypothetical defensive effect of sarmentosin could simply be related to the reported bitterness of the intact compound (Nishida et al., 1994), which could also be the cause of the repellency towards predating ants reported for the Leu-derived analog, sutherlandin (Braekman et al., 1982). On the other hand, a number of reaction schemes may also be envisioned to transform sarmentosin into more potent compounds as described in Fig. 6 and the following. First, a related compound

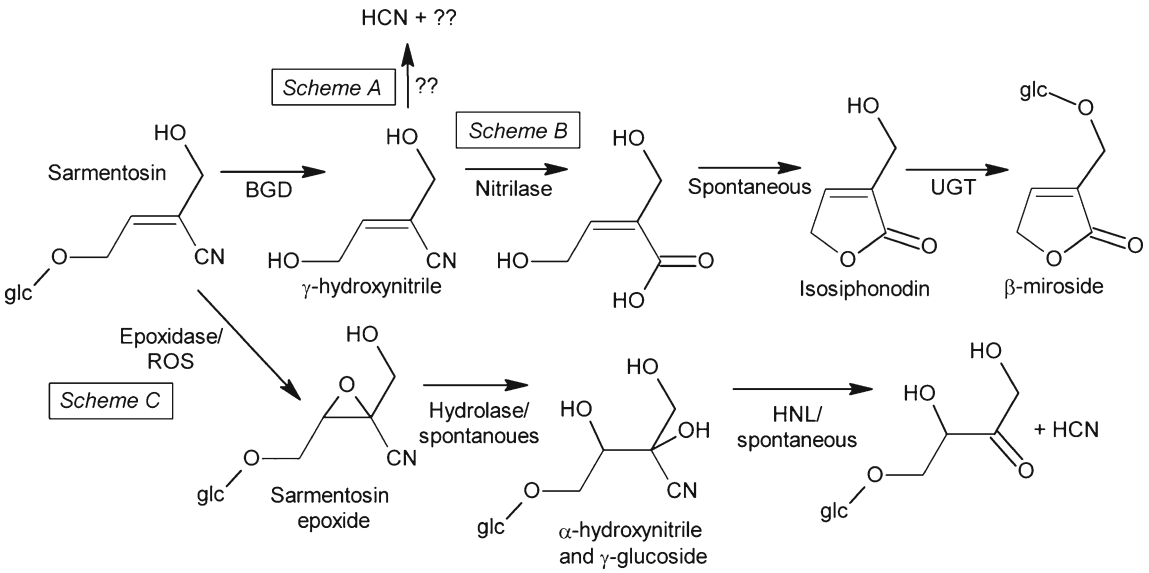

Fig. 6 Proposed schemes for production of toxic or deterrent compounds from sarmentosin. BGD = β-glucosidase, ROS = reactive oxygen species, HNL = hydroxynitrile lyase. All enzymes are putative

has been identified in *S. cepaea*, namely sarmentosin epoxide (Fig. 6) (Nahrstedt et al., 1982). The epoxide group can be hydrolyzed, causing the formation of a free α-hydroxynitrile moiety (Scheme C, Fig. 6). The reaction has been demonstrated to be accelerated in the presence of rat liver microsomes containing epoxide hydrolases (Nahrstedt et al., 1982). We investigated the possibility that the presently analyzed plants or insects also contained sarmentosin epoxide, but did not find clear evidence of the compound or its possible degradation products (scheme C, Fig. 6) being present. However, the compound may represent an activated form of sarmentosin, the production of which could depend on an enzyme released upon cell disruption, as is the case for the CNglc-BGD system, or upon epoxidation by reactive oxygen species. Alternatively, the action of sarmentosin may depend on BGD-mediated hydrolysis, which could lead to defense in several hypothetical ways: 1. HCN release from γ-hydroxynitriles by an unknown mechanism, probably enzyme-mediated (scheme A, Fig. 6); 2. Toxicity or deterrent effects of the γ-hydroxynitrile itself; 3. Nitrilase-mediated formation of a lactone, isosiphonodin (scheme B, Fig. 6) as we have previously suggested (Bjarnholt and Møller, 2008). So far, there is no experimental evidence to support possibilities 1 and 2, but isosiphondin has been found in several *Yponomeuta* larvae (Yponomeutidae) and in small amounts in *E. europaeus* on which some of them feed (Fung et al., 1988). As previously mentioned, one *Yponomeuta* moth and *E. japonica* contain sarmentosin (Nishida, 1994). Isosophonodin was subsequently also found in roots of *S. telephium* (Fung et al., 1990), and its glucoside, β-miroside, has been isolated from a gymnosperm, *Prymnopitys ferruginea* (Lorimer et al., 1995). In addition, analogous lactones and corresponding glucosides suspected to be derived from Leu-derived hydroxynitrile compounds in plants have been found in larvae and adults of true bugs (Braekman et al., 1982; Aldrich et al., 1990), and similar lactones and corresponding glucosides have been identified in various plants (Fung et al., 1988; Lorimer et al., 1995). Antifungal, antibacterial, and/or cytotoxic activities have been reported for β-miroside and several of the related compounds (Fung et al., 1988; Lorimer et al., 1995). We did not find any evidence of lactones in the analyzed specimens in this study either, but the analytical method was not optimized for such compounds. More importantly, if isosophonodin has a biological activity, sarmentosin could also in this case be the storage form that is enzymatically activated upon attack by predators and, therefore, not found in the intact insect.

This study has demonstrated that sarmentosin and related HNGs can be found in Crassulaceae-feeding *Parnassius* species as well as species feeding on Fumariaceae, albeit in highly variable amounts. Since the members of the remaining clades also feed on Fumariaceae species, it is likely that HNGs are widespread in this genus, just as is the case for sarmentosin in the *Abraxas* genus (Nishida, 1994) and for CNglcs in the *Zygeana* and *Heliconius* genera (Engler-Chaouat and Gilbert, 2007; Zagrobelny et al., 2008). Based on the observed discrepancies between occurrence of specific compounds in butterflies and known food plants, and on the apparently high accumulation of sarmentosin in *P. apollo* and *P. smintheus,* we have hypothesized that *Parnassius* species possess a biosynthetic pathway for production of HNGs. This has also been suggested for *Abraxas,* and is known to be the case for *Zygeana* and *Heliconius* (Nishida, 1994; Engler-Chaouat and Gilbert, 2007; Zagrobelny et al., 2008). The presence of such a biosynthetic pathway remains to be experimentally verified, e.g., by controlled feeding of the larvae or biochemical investigations of enzymatic activities. If the larvae are not able to *de novo* synthesize the compounds, the present study demonstrates a highly efficient selection for sarmentosin accumulation in species of the Apollo clade. Lack of such a biosynthetic pathway would also indicate that at least the populations of species from the Mnemosyne clade sampled in this study are or can be polyphagous since they apparently can not sequester all the observed compounds from their known food plants. It is of great interest to researchers from the insect as well as the plant field to uncover the biological role of sarmentosin and other non-cyanogenic HNGs, but this is more complicated. As a first step, it should be determined if any of the schemes in Fig. 6 can be verified, in order to identify compounds that may be tested for effects on predators, mates etc.

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References

ALDRICH, J. R., CARROLL, S. P., LUSBY, W. R., THOMPSON, M. J., KOCHANSKY, J. P., and WATERS, R. M. 1990. Sapindaceae, cyanolipids, and bugs. *J. Chem. Ecol.* 16:199–210.

BAK, S., PAQUETTE, S. M., MORANT, M., RASMUSSEN, A. B., SAITO, S., BJARNHOLT, N., ZAGROBELNY, M., JØRGENSEN, K., HAMANN, T., OSMANI, S., SIMONSEN, H. T., PEREZ, R. S., VAN HESSWIJCK, T. B., JØRGENSEN, B., and MØLLER, B. L. 2006. Cyanogenic glycosides: a case study for evolution and application of cytochromes P450. *Phytochem. Rev.* 5:309–329.

BJARNHOLT, N. and MØLLER, B. L. 2008. Hydroxynitrile glucosides. *Phytochemistry* 69:1947–1961.

BJARNHOLT, N., ROOK, F., MOTAWIA, M. S., JØRGENSEN, C., OLSEN, C. E., JAROSZEWSKI, J. W., BAK, S., and MØLLER, B. L. 2008. Diversification of an ancient theme: hydroxynitrile glucosides. *Phytochemistry* 69:1507–1516.

BOHLIN, T., TULLBERG, B. S., and MERILAITA, S. 2008. The effect of signal appearance and distance on detection risk in an aposematic butterfly larva (*Parnassius apollo*). *Anim. behav.* 76:577–584.

BRAEKMAN, J. C., DALOZE, D., and PASTEELS, J. M. 1982. Cyanogenic and other glucosides in a neo-guinean bug *Leptocoris isolata* - possible precursors in its host plant. *Biochem. Syst. Ecol.* 10:355–364.

ENGLER-CHAOUAT, H. S. and GILBERT, L. E. 2007. *De novo* synthesis vs. sequestration: Negatively correlated metabolic traits and the evolution of host plant specialization in cyanogenic butterflies. *J. Chem. Ecol.* 33:25–42.

ENGLER, H. S., SPENCER, K. C., and GILBERT, L. E. 2000. Insect metabolism - Preventing cyanide release from leaves. *Nature* 406:144–145.

FRANZL, S., ACKERMANN, I., and NAHRSTEDT, A. 1989. Purification and characterization of beta-glucosidase (linamarase) from the hemolymph of *Zygaena trifolii esper*, 1783 (Insecta, Lepidoptera). *Experientia* 45:712–718.

FUNG, S. Y., HERREBOUT, W. M., VERPOORTE, R., and FISCHER, F. C. 1988. Butenolides in small ermine moths, *Yponomeuta ssp.* (Lepidoptere, Yponomeutidae), and spindle tree, *Euonymus europaeus* (Celastraceae). *J. Chem. Ecol.* 14:1099–1111.

FUNG, S. Y., SCHRIPSEMA, J., and VERPOORTE, R. 1990. Alpha, beta-unsaturated gamma-lactones from *Sedum telephium* roots. *Phytochemistry* 29:517–519.

JENSEN, N. B., ZAGROBELNY, M., HJERNO, K., OLSEN, C. E., HOUGHTON-LARSEN, J., BORCH, J., MØLLER, B. L., and BAK, S. 2011. Convergent evolution in biosynthesis of cyanogenic defense compounds in plants and insects. *Nat. Comm.* 2: doi: 10.1038/ncomms1271.

KĘDZIORSKI, A., NAKONIECZNY, M., PYRAK, K., BEMBENEK, J., and ROSIŃSKI, G. 1997. Energy Metabolism in the Apollo Butterfly (*Parnassius apollo* L., Lepidoptera, Papilionidae). pp 114–120 *in*: D. Konopińska, G. Goldsworthy, R. J. Nachman, J. Nawrot, I. Orchard, and G. Rosiński, (eds.). Insects-Chemical, Physiological and Environmental Aspects. University of Wroclaw.

KUNERT, M., SOE, A., BARTRAM, S., DISCHER, S., TOLZIN-BANASCH, K., NIE, L., DAVID, A., PASTEELS, J., and BOLAND, W. 2008. De novo biosynthesis versus sequestration: A network of transport systems supports in iridoid producing leaf beetle larvae both modes of defense. *Insect Biochem. Mol. Biol.* 38:895–904.

LANGEL, D. and OBER, D. 2011. Evolutionary recruitment of a flavin-dependent monooxygenase for stabilization of sequestered pyrrolizidine alkaloids in arctiids. *Phytochemistry* 72:1576–1584.

LECHTENBERG, M. and NAHRSTEDT, A. 1999. Cyanogenic glycosides, pp. 147–191, *in* R. Ikan (ed.), Naturally Occurring Glycosides. John Wiley and Sons, Chichester.

LORIMER, S. D., MAWSON, S. D., PERRY, N. B., and WEAVERS, R. T. 1995. Isoltaion and synthesis of beta-miroside - an antifungal furanone glucosides from *Prumnopitys ferruginea*. *Tetrahedron* 51:7287–7300.

MATTER, S. F., ROLand, J., KEYGHOBADI, N., and SABOURIN, K. 2003. The effects of isolation, habitat area and resources on the abundance, density and movement of the butterfly *Parnassius smintheus*. *Am. Midl. Nat.* 150:26–36.

MEAD, E. W., FODERARO, T. A., GARDNER, D. R., and STERMITZ, F. R. 1993. Iridoid glycosides sequestration by *Thessalia leanira* (Lepidoptera, Nymphalidae) feeding on *Castilleja integra* (Scrophulariaceae). *J. Chem. Ecol.* 19:1155–1166.

MICHEL, F., REBOURG, C., COSSON, E., and DESCIMON, H. 2008. Molecular phylogeny of Parnassiinae butterflies (Lepidoptera: Papilionidae) based on the sequences of four mitochondrial DNA segments. *Ann. Soc. Entomol. Fr. (n. s.)* 44:1–36.

MONTLLOR, C. B., BERNAYS, E. A., and BARBEHENN, R. V. 1990. Importance of quinolizidine alkaloids in the relationship between larvae of *Uresiphita reversalis* (Lepidoptera, Pyralidae) and a host plant, *Genista monspessulana*. *J. Chem. Ecol.* 16:1853–1865.

MORANT, A. V., JØRGENSEN, K., JØRGENSEN, C., PAQUETTE, S. M., SÁNCHEZ-PEREZ, R., MØLLER, B. L., and BAK, S. 2008. β-glucosidases as detonators of plant chemical defense. *Phytochemistry* 69:1795–1813.

NAHRSTEDT, A., WALTHER, A., and WRAY, V. 1982. Sarmentosin epoxide, a new cyanogenic compound from *Sedum cepaea*. *Phytochemistry* 21:107–110.

NAKONIECZNY, M. and KEDZIORSKI, K. 2005. Feeding preferences of the Apollo butterfly (*Parnassius apollo ssp frankenbergeri*) larvae inhabiting the Pieniny Mts (southern Poland). *C. R. Biol.* 328:235–242.

NISHIDA, R. 1994. Sequestration of plant secondary compounds by butterflies and moths. *Chemoecology* 5:127–138.

NISHIDA, R. 2002. Sequestration of defensive substances from plants by Lepidoptera. *Annu. Rev. Entomol.* 47:57–92.

NISHIDA, R. and ROTHSCHILD, M. 1995. A cyanoglucoside stored by a *Sedum*-feeding apollo butterfly, *Parnassius phoebus*. *Experientia* 51:267–269.

NISHIDA, R., ROTHSCHILD, M., and MUMMERY, R. 1994. A cyanoglucoside, sarmentosin, from the magpie moth, *Abraxas grossulariata*, Geometridae: Lepidoptera. *Phytochemistry* 36:37–38.

OMOTO, K., YONEZAWA, T., and SHINKAWA, T. 2009. Molecular systematics and evolution of the recently discovered "Parnassian" butterfly (*Parnassius davydovi* Churkin, 2006) and its allied species (Lepidoptera, Papilionidae). *Gene* 441:80–88.

OPITZ, S. E. W. and MULLER, C. 2009. Plant chemistry and insect sequestration. *Chemoecology* 19:117–154.

PINTO, C. F., URZUA, A., and NIEMEYER, H. M. 2011. Sequestration of aristolochic acids from meridic diets by larvae of *Battus polydamas archidamas* (Papilionidae: Troidini). *Eur. J. Entomol.* 108:41–45.

REBOURG, C., PETENIAN, F., COSSON, E., and FAURE, E. 2006. Patterns of speciation and adaptive radiation in *Parnassius* butterflies. *J. Entomol.* 3:204–215.

SAITO, S., MOTAWIA, M. S., OLSEN, C. E., MØLLER, B. L., and BAK, S. 2012. Biosynthesis of rhodiocyanosides in *Lotus japonicus*: Rhodiocyanoside A is synthesized from (Z)-2-methylbutanaloxime via 2-methyl-2-butenenitrile. *Phytochemistry.* doi: dx.doi.org/10.1016/j.phytochem.2012.01.020

SCOTT, J. A. 1986. The Butterflies of North America: A Natural History and Field Guide. Stanford University Press, Stanford, California.

SHEPARD, J. H. and MANLEY, T. R. 1998. A species revision of the *Parnassius phoebus* complex in North America (Lepidoptera: Papilionidae), pp. 731–736, *in* T. C. Emmel (ed.), Systematics of Western North American Butterflies. Mariposa Press, Gainsville, FL.

TAKOS, A. M., KNUDSEN, C., LAI, D., KANNANGARA, R., MIKKELSEN, L., MOTAWIA, M. S., OLSEN, C. E., SAITO, S., TABATA, S., JØRGENSEN, K., MØLLER, B. L., and ROOK, F. 2011. Genomic clustering of cyanogenic glucoside biosynthetic genes aids their identification in *Lotus japonicus* and suggests the repeated evolution of this chemical defense pathway. *Plant J.* 68:273–86.

TAKOS, A., LAI, D., MIKKELSEN, L., ABOU HACHEM, M., SHELTON, D., MOTAWIA, M. S., OLSEN, C. E., WANG, T. L., MARTIN, C., and ROOK, F. 2010. Genetic screening identifies cyanogenesis-deficient mutants of *Lotus japonicus* and reveals enzymatic specificity in hydroxynitrile glucoside metabolism. *Plant Cell* 22:1605–1619.

TURLIN, B. and MALIN, L. 2005. Etude synoptique et Répartition mondiale des Espèces du Genre *Parnassius* Latreille 1804 (Lepidoptera Papilionidae). L. Manil, (ed.). Paris.

VONNICKISCHROSENEGK, E. and WINK, M. 1993. Sequestration of pyrrolizidine alkaloids in several arctiid moths (Lepidoptera, Arctiidae). *J. Chem. Ecol.* 19:1889–1903.

WITTHOHN, K. and NAUMANN, C. M. 1987. Genus *Zygaena F* and related taxa (Insecta, Lepidoptera) 53. Active cyanogenesis - in Zygaenids and other Lepidoptera. *Z. Naturforsch. C* 42c:1319–1322.

ZAGROBELNY, M., BAK, S., EKSTROM, C. T., OLSEN, C. E., and MØLLER, B. L. 2007a. The cyanogenic glucoside composition of *Zygaena filipendulae* (Lepidoptera: Zygaenidae) as effected by feeding on wild-type and transgenic lotus populations with variable cyanogenic glucoside profiles. *Insect Biochem. Mol. Biol.* 37:10–18.

ZAGROBELNY, M., BAK, S., and MØLLER, B. L. 2008. Cyanogenesis in plants and arthropods. *Phytochemistry* 69:1457–1468.

ZAGROBELNY, M., BAK, S., OLSEN, C. E., and MØLLER, B. L. 2007b. Intimate roles for cyanogenic glucosides in the life cycle of *Zygaena filipendulae* (Lepidoptera, Zygaenidae). *Insect Biochem. Mol. Biol.* 37:1189–1197.

ZAGROBELNY, M. and MØLLER, B. L. 2011. Cyanogenic glucosides in the biological warfare between plants and insects: the Burnet moth-Birdsfoot trefoil model system. *Phytochemistry* 72:1585–1592.

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Flavonoid Metabolites in the Hemolymph of European Pine Sawfly (*Neodiprion sertifer*) Larvae

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Abstract Flavonoids in the hemolymph of European pine sawfly (*Neodiprion sertifer*) larvae that were feeding on *Pinus sylvestris* needles were identified. HPLC–ESI–MS analysis revealed that the main components in the hemolymph were flavonol di- and triglucosides and a catechin monoglucoside. These compounds were isolated from the larval hemolymph and their structures were established by HPLC–MS, GC–MS, and NMR spectroscopy. The isolated flavonoids were identified as (+)-catechin 7-*O*-*β*-glucoside, isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside, kaempferol 3,7,4′-tri-*O*-*β*-glucoside, and quercetin 3,7,4′-tri-*O*-*β*-glucoside. The combined concentration of these four compounds in the hemolymph was 3.7 mg/ml. None of these compounds was present in the needles of *P. sylvestris*. Therefore, we propose that the flavonoid glucosides were produced by the larvae from flavonoid monoglucosides and (+)-catechin obtained from the pine needles.

Keywords European pine sawfly · Scots pine · Hemolymph · Flavonoid glycosides · Hymenoptera · Diprionidae

Introduction

Plants have evolved several strategies that protect them against insect herbivores. One option is to produce secondary metabolites such as alkaloids, phenolic compounds, or terpenoids, which may reduce the palatability of plant material or make it deterrent to herbivores (Langheim, 1994; Wink, 2003; Bernards, 2010). Scots pine (*Pinus sylvestris*) is a conifer species native to large areas of Europe and Asia. The secondary metabolites of *P. sylvestris* include oleoresin that protects the tree from needle-chewing insects (Larsson et al., 1986; Mumm and Hilker, 2006; Semiz et al., 2007). Other examples of secondary compounds in pine needles are phenolics such as proanthocyanidins (Lavola et al., 2003; Kanerva et al., 2008) and flavonoid glycosides (Oleszek et al., 2002; Lavola et al., 2003). Despite these chemicals, many insect herbivores consume *P. sylvestris*. One of these is the European pine sawfly, *Neodiprion sertifer* (Hymenoptera: Diprionidae). Its larvae are known for outbreaks when tens of thousands of larvae defoliate large areas of pine forests. It is unknown how the larvae of *N. sertifer* can have such dramatic outbreaks, but the needle chemistry and predation pressure on the insect may be important factors that control the *N. sertifer* populations (Larsson et al., 2000). An insect outbreak also can occur when the insect population escapes from the regulating influence of its natural enemies, such as parasitoids (Maron et al., 2001).

Previous studies have reported several plant-derived chemicals in the hemolymph of different sawfly larvae: e.g., steroid alkaloids (Schaffner et al., 1994), glucosinolates (Müller et al., 2001) and furostanol saponins (Prieto et al., 2007). Additionally, Bowers et al. (1993) found sequestered iridoid glycosides in the extracted larvae of a sawfly *Tenthredo grandis*. It has been shown that the sawfly larvae can use different plant chemicals that protect them against predators (Schaffner et al., 1994; Müller et al., 2002; Opitz et al., 2010). In our recent study, we found high concentrations of flavonol oligoglycosides in the hemolymph of birch-feeding sawfly larvae (Vihakas et al., 2010). These flavonoids were not present in the larval diet, but were produced by the insects from the precursor flavonoids in birch leaves. The

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flavonol oligoglycosides were only tentatively identified based on their mass and UV spectra because we were not able to isolate the compounds in a sufficient amount for further analyses. In the present study, we were interested to see if similar kinds of flavonoid metabolites also were present in the hemolymph of pine sawflies. We collected the larvae of *N. sertifer* from an outbreak area and analyzed flavonoids from the hemolymph and feces of the insect. The large number of larvae available for the study allowed us to isolate and purify four flavonoid glycosides and to characterize their structures.

Methods and Materials

Plant Material Needles of *P. sylvestris* were collected from an outbreak area of *N. sertifer* in Taivassalo, southern Finland, on 29 June 2009. Only second year or older needles were sampled because the larvae prefer them as food (Larsson and Tenow, 1984). A needle sample was pooled from 6 trees from the collection site of the larvae. The needle sample was frozen, lyophilized, and ground. The needle powder (three replicates of 10 mg) was suspended in 1 ml of 70 % aqueous acetone and shaken for 1 hr at room temperature. The extract was centrifuged, and the resulting pellet was re-extracted three times. Finally, the acetone was evaporated from the combined extracts, and the residual water solution was lyophilized. The dry crude extract was dissolved in 2 ml water, and the water-insoluble compounds were removed by filtration. The residual water-insoluble lipophilic compounds were dissolved in ethanol and filtered. Polar and lipophilic fractions of pine needle extracts were stored at −25°C for analysis with high performance liquid chromatography–mass spectrometry (HPLC–MS).

Insect Material On 29 June 2009, several hundred individuals of *N. sertifer* larvae were collected from the same trees as the needle samples. On the collection date, the larvae were in their 4th to 5th instar. Larvae were taken into the laboratory where they were reared for 5 d on branches of *P. sylvestris* that were collected from the same site as the larvae. Then, the larvae were dipped into liquid nitrogen and stored at −82°C until extraction and analysis of the hemolymph. Feces samples were pooled from several larvae at the end of the laboratory feeding period. Feces samples (three replicates) were extracted and stored the same way as the needle samples. Only the polar phenolic compounds were measured in the feces.

Hemolymph samples were collected from the insects by piercing a small hole into the integument of a larva and then gently pushing out a droplet of hemolymph. The hemolymph from 6 randomly chosen larvae were pooled (80 μl in total), and three replicates, each comprising 10 μl of this sample, were applied to a solid phase extraction cartridge (SPE, Sep-Pak® Plus Short tC18 Cartridge, Waters) that was preconditioned with methanol and equilibrated with water. The hemolymph metabolites were fractioned by their elution from a SPE cartridge with H_2O, 40 % aqueous MeOH, and MeOH. Only the 40 % aqueous MeOH fraction contained any flavonoids, and methanol was evaporated from this fraction and the remaining water solution was lyophilized. The metabolites were re-dissolved in H_2O and analyzed with HPLC–MS.

Isolation of Flavonoid Glycosides from Larvae From the preliminary HPLC analyses of the hemolymph, we tentatively identified the flavonoid peaks of the hemolymph. In order to accumulate these compounds for their structure elucidation we decided not to collect hemolymph from individual larvae, as this would have been difficult given the small volume of the hemolymph (10–20 μl) that could be isolated from each larva. Instead, the flavonoid glycosides were isolated from larval extracts. We established that the extract contained the same flavonoid glycosides as the hemolymph samples by comparing the HPLC profiles and mass spectra of the two sample types. Frozen larvae (109 g, over 1000 larvae) were thawed and gently broken in a mortar. The larvae were extracted with 70 % aqueous acetone four times, and the combined extract was filtered and rotary evaporated to remove the acetone, lyophilized, and re-dissolved in water. The solution was extracted several times with hexane to remove lipophilic compounds, and the water phase was lyophilized.

The lyophilized larval extract was re-dissolved in water and applied onto a Sephadex LH-20 column that was equilibrated with water. Flavonoid triglycosides (compounds **B**–**D**) eluted from the column with water, and the catechin monoglycoside (compound **A**) with 50 % aqueous MeOH. Sephadex fractions then were further purified with a semi-preparative HPLC system (Waters 600 Controller and Waters Delta 600, Waters Corporation, MA, USA) that was combined with a photodiode array detector (Waters 2998 Photodiode Array Detector, Waters Corporation, MA, USA) and a fraction collector (Waters Fraction Collector III, Waters Corporation, Japan). A column (25×320 mm) was filled with RP-18 material (LiChroprep® RP-18, 40–63 μm, Merck KGaA, Darmstadt, Germany). The mobile phases consisted of MeOH and H_2O (compounds **B**–**D**) and acetonitrile and 1 % aqueous formic acid (compound **A**; the eluents were changed between samples to improve chromatographic separation and shapes of the peaks). The elution was started with 1 % aqueous formic acid/H_2O, and the proportion of the organic solvent was gradually increased until the desired compounds eluted from the column. The detection wavelength was 280 nm. Four flavonoid fractions containing compounds **A**–**D** were collected, the organic solvent was evaporated and the fractions were

lyophilized and weighed: compound **A** (8.4 mg), **B** (17.7 mg), **C** (22.1 mg), and **D** (10.7 mg).

HPLC–MS Analyses Qualitative analyses of phenolic compounds together with their mass spectra and accurate masses were acquired with an HPLC system combined with a time–of–flight mass spectrometer (Bruker micrOTOF-Q, Bruker Daltonics GmbH, Bremen, Germany). The HPLC system (Agilent Tecnologies 1200 series, Agilent Technologies, Walbronn, Germany) consisted of a control module, a binary pump, a degasser, an autosampler, and a diode array detector. The column was a LiChroCART Purospher STAR (2×55 mm, 3 μm, Merck KGaA, Darmstadt, Germany) and the flow rate was 0.3 ml/min. Two solvents were used: 0.1 % formic acid (A) and acetonitrile (B). The elution profile was: 0–2 min, 100 % A; 2–33 min, 0–30 % B in A (linear gradient); 33–35 min, 30–70 % B in A (linear gradient); 35–55 min, column wash and stabilization. The detection wavelength was 280 nm. ESI (electrospray ionization) conditions were: capillary voltage +4500 V (positive ionization mode), end plate offset −500 V, nebulizer gas (N_2) pressure 1.6 bar and drying gas (N_2) flow 12.0 l/min and temperature 200°C. The mass spectra were acquired between *m/z* 50 and 2000.

Quantitative analysis of the samples, UV spectra of the flavonoids and HPLC-chromatograms were acquired with an ultra high performance liquid chromatography system (UHPLC, Acquity UPLC®, Waters Corporation, Milford, MA, USA) combined with a triple quadrupole mass spectrometer (Xevo® TQ, Waters Corporation, Milford, MA, USA). The UHPLC system consisted of a sample manager, a binary solvent manager, and a diode array detector. An Acquity UPLC® BEH Phenyl (2.1×100 mm, 1.7 μm, Waters Corporation, Wexford, Ireland) column was used. The flow rate of the eluent was 0.5 ml/min. The elution profile used two solvents, 0.1 % formic acid (A) and acetonitrile (B): 0–0.5 min, 100 % A; 0.5–5.0 min, 0–30 % B in A (linear gradient); 5.0–5.1 min, 30–90 % B in A (linear gradient); 5.1–8.5 min, column wash and stabilization. The detection wavelength was 280 nm and the negative ionization mode was used. ESI conditions were: Capillary voltage 2,4 kV, desolvation temperature 500°C, source temperature 150°C, desolvation and cone gas (N_2) 1000 and 100 l/h, respectively, and collision gas was argon. The unknown compounds were identified by comparing their UV and mass spectra and retention times with data from the literature and with the results obtained from authentic standards: (2*R*,3*S*)-(+)-catechin (Sigma Chemical Co., USA), isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, and quercetin-3-*O*-glucoside (Extrasynthèse Genay, France). The same standards were used to quantitate the corresponding flavonoids from the needle and feces samples. The purified flavonoids **A**–**D** from the larvae were used to quantitate the corresponding flavonoids from the hemolymph samples. For quantitation, external standard solutions were prepared, and compounds were quantitated from the samples with multiple reaction monitoring (MRM). For further information about quantitation, see the Supplemental Information.

GC–MS Analysis Acid hydrolysis was used to break the glycosidic bonds in the flavonoid glycosides **A**–**D**, after which the hydrolysis products were analyzed with gas chromatography–mass spectrometry (GC–MS). Two mg of compounds **A**–**D** were dissolved in 50 % aqueous methanol and hydrolyzed for 1 h at 90°C with 4 M HCl. After hydrolysis, the eluent was evaporated, and the carbonyl moieties were protected by using methoxylamine hydrochloride in pyridine (20 mg/ml). TMS-derivatives were prepared by adding N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 5 % of trimethylchlorosilane (TMCS).

The TMS derivatives were analyzed with a Perkin-Elmer GC-MS system (GC Autosystem XL with TurboMass Gold mass spectrometer, Norwalk, CT, USA). The mass spectrometer was used in the electron impact mode (EI^+); the data acquisition time was 0.3 scan/sec, and the scan range was from 50 to 620 *m/z*. A Perkin Elmer capillary column (PE-5MS, 30 m, 0.25 mm i.d., film 0.25 μm) was utilized, and helium was used as a carrier gas with a flow rate of 1.0 ml/min. The injection volume was 1 μl and the split ratio 20:1. The injector temperature was 280°C and the inlet line and the MS source were held at 290°C and 200°C, correspondingly. The oven temperature program was as follows: initial temperature 70°C (2.0 min) followed by temperature increase of 7°C/min up to 290°C, which was held for 4 min, and then 10°C/min to final temperature 300°C, which was held for 11.57 min. Total run time of the analysis was 50 min. After automatic deconvolution of the mass spectra of co-eluting peaks with AMDIS software (Automated Mass Spectral Deconvolution and Identification System; Halket et al., 1999), the hydrolysis products were identified by using reference compounds (glucose and galactose), retention indices (RI), and mass spectra of GC–MS databases (NIST-2008 and database of plant metabolites of Max-Plank Institute of Molecular Plant Physiology, Germany, http://csbdb.mpimp-golm.mpg.de).

NMR Spectroscopy The nuclear magnetic resonance (NMR) spectra of the isolated compounds were recorded without sample spinning at 25°C with a Bruker Avance 500 spectrometer (equipped with BBI and BBO-5-mm-Zgrad-ATM probes) operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. Compounds **A**, **B**, and **D** were measured in D_2O and compound **C** in CD_3OD. Additional NMR spectra of **B** and **D** were recorded in a mixture of D_2O and acetone-d_6 (1:1). The chemical shifts of the external standard TSP-d_4 (the sodium salt of 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate in

D_2O; 0.00 ppm for both ^1H and ^{13}C) were used as reference values for the D_2O samples. For the CD_3OD sample, the solvent residual peaks (^1H: 3.31 ppm; ^{13}C: 49.00 ppm) were used to calibrate the spectra. Standard ^1H and ^{13}C $\{^1H\}$ spectra were measured and analyzed for the isolated compounds. To facilitate a full assignment of the chemical shifts and complete analysis of the structures, a set of gradient selected multipulse NMR experiments were recorded for each isolated compound including 1D-NOESY (Nuclear Overhauser Effect Spectroscopy), 1D-ROESY (Rotating Frame Overhauser Effect Spectroscopy), 1D-TOCSY (Total Correlation Spectroscopy), and various 2D measurements: DQF-COSY (Double-Quantum Filtered Correlation Spectroscopy), multiplicity edited *f2*-undecoupled and fully decoupled HSQC (Heteronuclear Single Quantum Correlation), and HMBC (Heteronuclear Multiple Bond Correlation) and CIGAR-HMBC (constant time inverse-detected gradient accordion rescaled HMBC, compounds **C** and **D**). 1D-TOCSY detected glucosyl subspectra of compounds **B** and **C** were further analyzed with the aid of PERCH NMR software (Laatikainen et al., 1996). Finally, the measured NMR data (chemical shifts and coupling constants) was compared with literature data (Fossen et al., 1997; Fossen and Andersen, 2006; Raab et al., 2010). Further information about NMR measurements and the ^1H and ^{13}C NMR spectral data of the compounds **A–D** is presented in the Supplemental Information and Supplemental Table 1.

ECD Spectroscopy The electronic circular dichroism (ECD) spectra of (2*R*,3*S*)-(+)-catechin (Sigma Chemical Co., USA, 172.0 μM) and the isolated catechin 7-*O*-glucoside (164 μM, compound **A**) in water were measured over the range of 180–350 nm at 298 K using a Chirascan™ circular dichroism spectrometer (Applied PhotoPhysics, Leatherhead, UK) equipped with a 1 mm path length cell.

Results

This study focused on the secondary metabolites of *P. sylvestris* and one of its severe defoliators, *N. sertifer*. We analyzed flavonoids from the pine needles and from the hemolymph and feces of the insect. Flavonoids were detected only in the polar fraction of the pine needles. Some of these compounds were tentatively identified by comparing their retention times, molecular masses, and UV spectra with authentic standards and data in literature (Roitto et al., 2003; Karonen et al., 2004; Kang and Howard, 2010). The main types of flavonoids in needles were flavan-3-ols (catechin and gallocatechin), di- and trimeric proanthocyanidins, and flavonols (3-*O*-glucosides of isorhamnetin, kaempferol, and quercetin). The feces of the insect contained the same flavonol 3-*O*-glucosides as the pine needles. The feces also contained smaller amounts of other flavonoid-type compounds and compounds whose structures were not elucidated in this study.

Flavonoids in Hemolymph In the UHPLC–DAD chromatogram of the hemolymph, most of the peaks eluted between 2.2–3.7 min (Fig. 1). The relatively short retention times of compounds **A–H**, together with their UV spectral characteristics, indicated that the hemolymph might contain flavonoid oligoglycosides (Vihakas et al., 2010). The mass spectra of **A–H** showed a distinctive pattern where a molecular ion was preceded by ions that differed by 162 Da from each other (Table 1). This indicated that several hexopyranosyl units were attached to the aglycone, and the aglycones were revealed by their characteristic *m/z* values. Compounds **E–H** were tentatively identified from the hemolymph as flavonol di- and triglycosides (Table 1). Compounds **A–D** were isolated from the whole larval extracts for more detailed characterization (see below).

Acid hydrolysis of the isolated flavonoid glycosides **A–D** and GC–MS analysis of the hydrolysis products were performed to determine which sugar units were attached to the flavonoids. The results of the RI and MS-database searches and comparisons to the MS data of reference carbohydrates indicated that the sugar units in the flavonoid glycosides were glucosyls. This was further confirmed by the NMR data. The signals of the protons in sugar positions 2″, 3″, 4″, and 5″ showed large coupling constants (up to ^3J=9.5 Hz) in their 1D–spectra and distinctive coupling patterns, which indicated that these protons were diaxially oriented relative to their neighboring protons (Bubb, 2003; Roslund et al.,

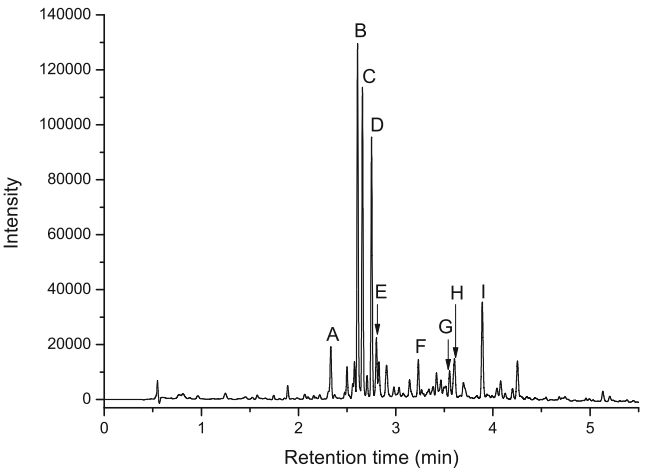

Fig. 1 An UHPLC–chromatogram of the hemolymph of *Neodiprion sertifer* at 280 nm. Interpretation of the symbols: *A*: (+)-catechin 7-*O*-*β*-glucoside, *B*: Quercetin 3,7,4′-tri-*O*-*β*-glucoside, *C*: Kaempferol 3,7,4′-tri-*O*-*β*-glucoside, *D*: Isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside, *E*: quercetin triglycoside, *F*: isorhamnetin diglycoside, *G*: quercetin diglycoside, *H*: kaempferol diglycosides, *I*: an unknown non-flavonoid compound

Table 1 Mass and UV spectral data of the flavonoid glycosides from the hemolymph of *N. sertifer*

Compound	Peak	λ max (nm)	Measured accurate mass (Da)	Theoretical accurate mass (Da)	Error (ppm)	*m/z* values in mass spectra				
						$[M+H]^+$	$[2M+H]^+$	$[M–glyc+H]^+$	$[M-2glyc+H]^+$	$[M–3\ glyc+H]^+$
(+)-Catechin 7-*O*-*β*-glucoside	A	203, 227, 278	452.1332	452.1319	2.9	453	905	291	–	–
Quercetin 3,7,4′-tri-*O*-*β*-glucoside	B	202, 253, 264, 344	788.2001	788.2011	– 1.3	789	1577	627	465	303
Kaempferol 3,7,4′-tri-*O*-*β*-glucoside	C	195, 265, 319, 338	772.2091	772.2062	3.8	773	1545	611	449	287
Isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside	D	202, 251, 265, 344	802.2187	802.2168	2.4	803	1605	641	479	317
Quercetin triglycoside	E	265, 347	788.1973	788.2011	– 4.8	789	1577	627	465	303
Isorhamnetin diglycoside	F	254, 351	640.1628	640.164	– 1.9	641	–	479	317	–
Quercetin diglycoside	G	265, 344	626.147	626.1483	– 2.1	627	–	465	303	–
Kaempferol diglycoside	H	265, 323	610.1522	610.1534	– 2.0	611	–	449	287	–

Letters a–h refer to peaks in Fig. 1

glyc a glycosyl group

2008). Therefore, each proton in the sugar positions 2″, 3″, 4″, and 5″ remained axial, which consequently confirmed that the sugar units must be glucosyls.

The UV spectrum of **A** resembled that of a flavan-3-ol (Table 1; Karonen, 2007), and its mass spectrum showed an ion at *m/z* 291, which was interpreted as a protonated catechin or epicatechin structure. The molecular ion at *m/z* 453 differed by 162 Da from the catechin ion, which indicated that compound **A** was a catechin monoglucoside. This was further proved by the NMR results (for NMR spectral data, see Supplemental Table 1). First, the ^{1}H-NMR spectrum of **A** was compared with literature data (Raab et al., 2010). It was concluded that the aglycone was catechin since the H–2 signal of **A** was a doublet with a large coupling constant (8.2 Hz) and H–3 represented a multiplet indicating a relative *trans*-configuration of the substituents at positions 2 and 3. In epicatechin, with a relative *cis*-configuration of the substituents, both H-2 and H-3 would have showed only small couplings (Hatano and Hemingway, 1997; Abd El-Razek, 2007). The 1D-ROESY and HMBC experiments indicated that the hydroxyl group in C–7 was glucosylated. The anomeric proton signal of **A** was a doublet with a rather large coupling constant ($^3J_{H1,H2}\approx$7.4 Hz), which indicated a relative diaxial orientation of the protons in sugar positions 1 and 2, and consequently a *β*-linkage to the aglycone. Finally, the absolute configuration of the aglycone in **A** was determined by comparing its ECD spectrum to the spectrum of (2*R*,3*S*)-(+)-catechin-7-*O*-β-D-glucopyranoside found in the literature (Friedrich and Galensa, 2002) and to the spectrum of (2*R*,3*S*)-(+)-catechin (Supplemental Fig. 1). The similarity of the spectra, and especially, the broad negative Cotton effect at ca. 280 nm, which is known to be indicative of (2*R*)-configuration (Korver and Wilkins, 1971; Slade et al., 2005), confirmed that compound **A** was (2*R*,3*S*)-(+)-catechin 7-*O*-*β*-glucoside (Fig. 2).

The mass spectrum of **B** showed an ion at *m/z* 303 as expected for the flavonol quercetin, and the fragmentation pattern indicated that **B** was a triglycoside (Table 1). Also, the GC–MS data of the hydrolysis products of **B** indicated that the aglycone was quercetin. The ^{1}H chemical shifts of

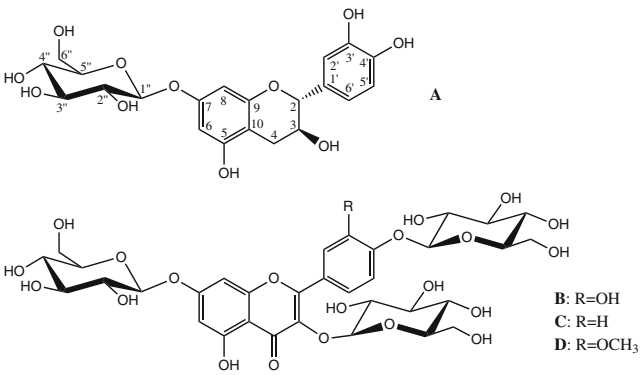

Fig. 2 Structures of (+)-catechin 7-*O*-*β*-glucoside (**A**), quercetin 3,7,4′-tri-*O*-*β*-glucoside (**B**), kaempferol 3,7,4′-tri-*O*-*β*-glucoside (**C**), and isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside (**D**). The glucosyls are depicted with D-configuration, as this is most often found for natural flavonoid glucosides. However, the absolute configurations of the glucosyls in the isolated compounds were not determined due to limited sample availability

the aglycone were compared with literature data (Fossen and Andersen, 2006), which confirmed the interpretation of the aglycone (Supplemental Table 1). The 1D-ROESY and HMBC experiments indicated that hydroxyl groups C–3, C–7, and C–4′ were glucosylated (Fig. 2). Two of the glucosyls in **B** were found to be β-linked to the aglycone ($^3J_{H1,H2}\approx$7.5 Hz) in D_2O. An exception was observed for the 4′-*O*-glucosyl for which $^3J_{H1,H2}$=5.7 Hz in D_2O. Typically, the $^3J_{H1,H2}$ values are in the range of 2–4 Hz for equatorial anomeric protons of α-linked glycopyranosyls and 7–9 Hz for axial anomeric protons of β-linked glycopyranosyls (Bubb, 2003; Roslund et al., 2008). However, the redetection of the ^{1}H-NMR spectrum in a mixture of D_2O and acetone-d_6 (1:1) revealed a large $^3J_{H1,H2}$ value (7.0 Hz), and thus, the observed intermediate value in D_2O could be due to the influence of the solvent. Finally, the $^1J_{C1,H1}$ was determined for each glucosyl of **B** in D_2O. It is well-known for D-glucopyranosides that for an α-linked anomer the $^1J_{C1,H1}\approx$170 Hz and for a β-linked anomer the $^1J_{C1,H1}\approx$160 Hz (Tvaroska and Taravel, 1995; Grindley, 2008). The observed values for 3-*O*- (161 Hz), 7-*O*- (162 Hz), and 4′-*O*-glucosyls (161 Hz) in **B** confirmed that they all are β-linked to the aglycone. Compound **B** was thus characterized as quercetin 3,7,4′-tri-*O*-β-glucoside (Fig. 2).

The mass spectrum of **C** showed an aglycone ion at *m/z* 287 (kaempferol) and a fragmentation pattern that indicated that **C** was a kaempferol triglycoside (Table 1). Also, the GC–MS results of the hydrolysis products of **C** and the comparison of the ^{1}H NMR data with the literature values (Fossen and Andersen, 2006) confirmed that the aglycone was kaempferol (Supplemental Table 1). Similar to **A** and **B**, the 1D-ROESY and HMBC results showed that glucosyls were attached to hydroxyl groups C–3, C–7, and C–4′. As described above, we concluded that all the glucosyls in **C** were β-linked to the aglycone ($^3J_{H1,H2}\approx$7.5 Hz). The structure of compound **C** was thus identified as kaempferol 3,7,4′-tri-*O*-β-glucoside (Fig. 2).

Compound **D** had a similar fragmentation pattern in the mass spectrum to **B** and **C**, and it was tentatively identified as a triglycoside (Table 1). The mass spectral data, GC–MS results of the hydrolysis products of **D** and comparison of NMR literature data indicated that the aglycone part was an isorhamnetin (Fossen and Andersen, 2006; Supplemental Table 1). Again, 1D-ROESY and HMBC spectra revealed that glucosyls were located in positions C–3, C–7, and C–4′. Intermediate sugar $^3J_{H1,H2}$ values (6.0–6.9 Hz) were observed for each glucosyl of **D** in D_2O. However, this was at least partially due to the poor solubility of **D** in D_2O and a resulting poor resolution in the spectrum. Similarly, as with **B**, the redetection of the ^{1}H-NMR spectrum in a mixture of D_2O and acetone-d_6 (1:1) revealed large $^3J_{H1,H2}$ values (7.0–7.6 Hz) for each glucosyl, which confirmed that they were β-linked to the aglycone in the used solvent mixture. Compound **D** was, therefore, identified as isorhamnetin 3,7,4′-tri-*O*-β-glucoside (Fig. 2).

Quantitative Data The results from the quantitative analysis of the needle, fecal, and hemolymph samples are displayed in Table 2. Flavonoid triglucosides were the most abundant compounds in the hemolymph, but these compounds were not detected in the needles or feces. The combined amount of flavonoid glucosides **A–D** in the hemolymph was 3.7 mg/ml. The needle and fecal samples contained (+)-catechin and flavonol monoglucosides, but only trace amounts of these compounds were detected in the hemolymph. Interestingly, the amount of (+)-catechin was rather high in pine needles but its concentration was lower in fecal samples.

Discussion

In this study, we examined flavonoids extracted from the larvae of *N. sertifer*. The most predominant compounds in the hemolymph of the larvae were 3,7,4′-tri-*O*-β-glucosides of quercetin, kaempferol, and isorhamnetin (compounds **B–D** in Fig. 2, respectively). In addition to those, the hemolymph contained (+)-catechin 7-*O*-β-glucoside (compound **A** in Fig. 2) and several compounds that were tentatively identified as flavonol di- and triglycosides (compounds **E–H** in Table 1). Catechin 7-*O*-β-glucoside has been reported previously from several plants (e.g., Foo and Karchesy, 1989; Benavides et al., 2006; Watanabe and Ayugase, 2009). Kaempferol 3,7,4′-tri-*O*-β-glucoside has been detected in saffron (Carmona et al., 2007), and quercetin 3,7,4′-tri-*O*-β-glucoside in red onions

Table 2 Concentrations of flavonoids in the needles of *P. sylvestris* and in the hemolymph and feces of *N. sertifer*

	Needles[a]	Hemolymph[b]	Feces[a]
(+)-Catechin	2.30	tr	0.04
(+)-Catechin 7-*O*-β-glucoside (compound **A**)	n.d.	0.28	n.d.
Isorhamnetin 3-*O*-β-glucoside	0.25	tr	0.20
Kaempferol 3-*O*-β-glucoside	0.23	tr	0.18
Quercetin 3-*O*-β-glucoside	2.23	tr	1.57
Isorhamnetin 3,7,4′-tri-*O*-β-glucoside (compound **D**)	n.d.	1.11	n.d.
Kaempferol 3,7,4′-tri-*O*-β-glucoside (compound **C**)	n.d.	0.81	n.d.
Quercetin 3,7,4′-tri-*O*-β-glucoside (compound **B**)	n.d.	1.48	n.d.

Values are means of three replicate analyses of pooled needle, hemolymph and feces samples

tr trace amounts; *n.d.* not detected

[a] Values are expressed as mg/g dry weight of needle or feces material

[b] Values are expressed as mg/ml in hemolymph

(Fossen et al., 1997). Isorhamnetin-, kaempferol-, and quercetin 3,7,4′-tri-*O*-*β*-glucosides have been detected in kale leaves (Schmidt et al., 2010). However, our study reports these compounds for the first time in insects. In our earlier study, we found similar flavonol oligoglycosides, including tri-, tetra-, penta-, and hexaglycosides of kaempferol and quercetin, in the hemolymph of larvae of several birch-feeding sawfly species (Vihakas et al., 2010).

None of these flavonoid glycosides was present in the pine needle diet of the larvae, which indicated that they were produced by the larvae from simpler flavonoid precursors of the needle diet. In our experiment, the larvae were fed in laboratory for five days with pine needles that were collected from the collection site of the larvae. However, it is possible that the larvae had consumed needles with different flavonoid contents before they were collected. The five-day feeding period might have been too short to eliminate the effects of earlier consumption on the flavonoid contents of the hemolymph. Thus, our experimental design allows us to make only tentative hypotheses about the possible starting materials of the flavonoid metabolites in the sawfly larvae. The analyses of the pine needles suggest that the flavonol 3-*O*-monoglucosides of isorhamnetin, kaempferol, and quercetin were probably used as starting material for flavonol di- and triglucosides, as flavonol aglycones were absent from the needles. We came to the same conclusion about the origin of the flavonol oligoglycosides from birch-feeding sawfly larvae (Vihakas et al., 2010). The needles of *P. sylvestris* contained (+)-catechin as one of the main compounds, and it is presumable that the larvae use this compound for the production of (+)-catechin 7-*O*-*β*-glucoside. Similarly, Schopf et al. (1982) found a catechin glycoside in the larval silk glands of spruce-feeding sawfly *Gilpinia hercyniae*, and this compound was probably formed from the catechin of spruce needles. A recent study (Crockett and Boevé, 2011) found several flavonoid glycosides in different body parts of sawfly *Tenthredo zonula* feeding on *Hypericum* plants, but it seems probable that the larvae had only sequestrated these compounds and not modified or glycosylated them. The sawfly species presented in the above examples belong to families Argidae, Cimbicidae, Diprionidae, and Tenthredinidae, which implies that the ability to glycosylate or sequester flavonoids may be a common feature among different sawfly species and families.

The amounts of flavonoids in both the plant and insect samples were determined by using a pooled sample, which prevented us from analyzing the interspecific variation of flavonoids in individual trees or larvae. The amounts of flavonol monoglucosides and (+)-catechin in pine needles were in accordance with earlier reports from *P. sylvestris* needles (Table 2, Lavola et al., 2003; Roitto et al., 2003, 2005). The concentrations of flavonol monoglucosides in fecal samples were at the same level as in birch-feeding sawfly larvae (Lahtinen et al., 2005). Catechin has been detected earlier in the feces of spruce-feeding sawfly *G. hercyniae* (Schopf et al., 1982). Interestingly, fecal concentrations of catechin showed a similar trend in both *G. hercyniae* and *N. sertifer*, as the amount of catechin was lower in feces than in spruce or pine needles (Table 2). Crockett and Boevé (2011) found flavonoid glycosides in sawfly *T. zonula*, but no flavonoids were detected in the feces of the larvae.

We estimated that the combined amount of the four main flavonoid glucosides in the hemolymph of *N. sertifer* was 3.7 mg/ml hemolymph. The hemolymph also contained smaller amounts of flavonol di- and triglycosides (compounds **E–H** in Fig. 1 and Table 1), which means that the total flavonoid contents of the hemolymph of *N. sertifer* may be as high as 4–5 mg/ml. In our study with birch sawflies, we estimated that the larval hemolymph contained 0.6–12.3 mg/ml of flavonoids, which is consistent with the present results (Vihakas et al., 2010). Several other plant-derived secondary compounds (e.g., glucosinolates, steroid alkaloids, or furastanol saponins) have been quantitated from the hemolymph of sawfly larvae that use different host plants (Schaffner et al., 1994; Müller et al., 2001; Prieto et al., 2007; Opitz and Müller, 2009). In general, the hemolymph concentrations of these compounds seem to be of the same order of magnitude as in *N. sertifer*. The amounts of flavonoid glycosides in the hemolymph of sawfly *T. zonula* (larvae and prepupae) were lower than in our study (Crockett and Boevé, 2011).

Many functions have been attributed to flavonoids in plants, such as protection against UV-B radiation and acting as oviposition stimulants for insects (Shirley, 1996; Harborne, 2001). These multiple roles make it difficult to interpret the precise function of flavonoids metabolites in sawfly larvae. Some flavonoids (e.g., taxifolin and its glycoside) have either negative or no effects on the performance of pine-feeding sawflies (Larsson et al., 1992; Auger et al., 1994). The high concentrations of flavonoid metabolites in the hemolymph of *N. sertifer* imply that these compounds may not be particularly harmful for the larvae.

It seems improbable that flavonoid glycosides would have defensive functions against *N. sertifer* predators, as flavonoids generally appear to have only limited defensive roles (Harborne, 2001). Defense mechanisms of *N. sertifer* have been well-reported. For example, when approached, the larvae regurgitate a droplet of resin-containing fluid from diverticular pouches of the foregut (Eisner et al., 1974) that deters ants, birds, and spiders (Prop, 1960; Eisner et al., 1974; Sillén-Tullberg, 1990; Björkman and Larsson, 1991). Some other sawfly species secrete a droplet of hemolymph containing plant secondary compounds through the integument of the larvae, and this secretion deters predators of the larvae (Boevé and Schaffner, 2003; Boevé and Müller, 2005). However, this hemolymph secretion has not

been reported for *N. sertifer* larvae, and was not observed in our study, which suggests that this species does not use hemolymph and flavonoid glycosides for defense.

The formation and accumulation of flavonoid glucosides could be a biochemical mechanism that reserves flavonoids or glucose for their later use in larval metabolism. Schopf et al. (1982) speculated that the sawfly *G. hercyniae* could use the phenolic compounds (e.g., catechin) of spruce needles in metabolic processes of the larvae. They proposed that the consumed catechin is first glycosylated by the larvae and then stored in silk glands of the insect. The catechin glycoside could subsequently be hydrolyzed, and the released catechin could take part in the sclerotization (tanning) of the cocoon. We have found flavonoid-type compounds in the cocoon shells of *N. sertifer* (unpublished data), which suggests that the glycosylation of flavonoids could have a metabolic function in *N. sertifer*. Further studies are needed to define how flavonoid glycosides function in *N. sertifer* and to identify the metabolic sources of these compounds.

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References

ABD EL-RAZEK, M. H. 2007. NMR assignments of four catechin epimers. *Asian J. Chem.* 19:4867–4872.

AUGER, M. A., JAY-ALLEMAND, C., BASTIEN, C., AND GERI, C. 1994. Quantitative variations of taxifolin and its glucoside in *Pinus sylvestris* needles consumed by *Diprion pini* larvae. *Ann. Sci. For.* 51:135–146.

BENAVIDES, A., MONTORO, P., BASSARELLO, C., PIACENTE, S., and PIZZA, C. 2006. Catechin derivatives in *Jatropha macrantha* stems: Characterisation and LC/ESI/MS/MS quali–quantitative analysis. *J. Pharm. Biomed. Anal.* 40:639–647.

BERNARDS, M. A. 2010. Plant natural products: A primer. *Can. J. Zool.* 88:601–614.

BJÖRKMAN, C. and LARSSON, S. 1991. Pine sawfly defence and variation in host plant resin acids: A trade-off with growth. *Ecol. Entomol.* 16:283–289.

BOEVÉ, J.-L. and MÜLLER, C. 2005. Defence effectiveness of easy bleeding sawfly larvae towards invertebrate and avian predators. *Chemoecology* 15:51–58.

BOEVÉ, J.-L. and SCHAFFNER, U. 2003. Why does the larval integument of some sawfly species disrupt so easily? The Harmful Hemolymph Hypothesis. *Oecologia* 134:104–111.

BOWERS, M. D., BOOCKVAR, K., and COLLINGE, S. K. 1993. Iridoid glycosides of *Chelone glabra* (Scrophulariaceae) and their sequestration by larvae of a sawfly, *Tenthredo grandis* (Tenthredinidae). *J. Chem. Ecol.* 19:815–823.

BUBB, W. A. 2003. NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Conc. Magn. Reson.* 19A:1–19.

CARMONA, M., SÁNCHEZ, A. M., FERRERES, F., ZALACAIN, A., TOMÁS-BARBERÁN, F. A., and ALONSO, G. L. 2007. Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins. *Food Chem.* 100:445–450.

CROCKETT, S. L. and BOEVÉ, J.-L. 2011. Flavonoid glycosides and naphthodianthrones in the sawfly *Tenthredo zonula* and its host-plants, *Hypericum perforatum* and *H. hirsutum*. *J. Chem. Ecol.* 37:943–952.

EISNER, T., JOHNESSEE, J. S., CARREL, J., HENDRY, L. B., and MEINWALD, J. 1974. Defensive use by an insect of a plant resin. *Science* 184:996–999.

FOO, L. Y. and KARCHESY, J. J. 1989. Polyphenolic glycosides from douglas fir inner bark. *Phytochemistry* 28:1237–1240.

FOSSEN, T. and ANDERSEN, Ø. M. 2006. Spectroscopic techniques applied to flavonoids, pp. 37–142, *in* Ø. M. Andersen and K. R. Markham (eds.), Flavonoids: Chemistry, Biochemistry, and Applications. CRC Press, Taylor & Francis Group, Boca Raton, USA.

FOSSEN, T., PEDERSEN, A. T., and ANDERSEN, Ø. M. 1997. Flavonoids from red onion (*Allium Cepa*). *Phytochemistry* 47:281–285.

FRIEDRICH, W. and GALENSA, R. 2002. Identification of a new flavanol glucoside from barley (*Hordeum vulgare* L.) and malt. *Eur. Food Res. Technol.* 214:388–393.

GRINDLEY, T. B. 2008. Structure and conformation of carbohydrates, pp. 3–55, *in* B. O. Fraser-Reid, K. Tatsuta, and J. Thiem (eds.), Glycoscience: Chemistry and Chemical Biology, 2nd ed. Springer, Berlin, Germany.

HALKET, J., PRZYBOROWSKA, A., STEIN, S., DOWN, S., and CHALMERS, R. 1999. Deconvolution gas chromatography/mass spectrometry of urinary organic acid—potential for pattern recognition and automated identification of metabolite disorders. *Rapid Commun. Mass Spectr.* 13:279–284.

HARBORNE, J. B. 2001. Twenty-five years of chemical ecology. *Nat. Prod. Rep.* 18:361–379.

HATANO, T. and HEMINGWAY, R. W. 1997. Conformational isomerism of phenolic procyanidins: Preferred conformations in organic solvents and water. *J. Chem. Soc. Perkin Trans.* 2:1035–1043.

KANERVA, S., KITUNEN, V., LOPONEN, J., and SMOLANDER, A. 2008. Phenolic compounds and terpenes in soil organic horizon layers under silver birch, Norway spruce and Scots Pine. *Biol. Fertil. Soils* 44:547–556.

KANG, Y.-J. and HOWARD, L. R. 2010. Phenolic composition and antioxidant activities of different solvent extracts from pine needles in *Pinus* species. *J. Food Sci. Nutr.* 15:36–43.

KARONEN, M., LOPONEN, J., OSSIPOV, V., and PIHLAJA, K. 2004. Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal. Chim. Acta* 522:105–112.

KORVER, O. and WILKINS, C. K. 1971. Circular dichroism spectra of flavanols. *Tetrahedron* 27:5459–5465.

LAATIKAINEN, R., NIEMITZ, M., WEBER, U., SUNDELIN, J., HASSINEN, T., and VEPSÄLÄINEN, J. 1996. General strategies for total-line-shape type spectral analysis of NMR spectra using integral transform iterator. *J. Magn. Reson.* A120:1–10.

LAHTINEN, M., KAPARI, L., OSSIPOV, V., SALMINEN, J.-P., HAUKIOJA, E., and PIHLAJA, K. 2005. Biochemical transformation of birch leaf phenolics in larvae of six species of sawflies. *Chemoecology* 15:153–159.

LANGHEIM, J. H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* 20:1223–1280.

LARSSON, S. and TENOW, O. 1984. Areal distribution of a *Neodiprion sertifer* (Hym., Diprionidae) outbreak on scots pine as related to stand condition. *Holarctic Ecol.* 7:81–90.

LARSSON, S., BJÖRKMAN, C., and GREF, R. 1986. Responses of *Neodiprion sertifer* (Hym., Diprionidae) larvae to variation in needle resin acid concentration in Scots Pine. *Oecologia* 70:77–84.

LARSSON, S., LUNDGREN, L., OHMART, C. P., and GREF, R. 1992. Weak responses of pine sawfly larvae to high needle flavonoid concentrations in scots pine. *J. Chem. Ecol.* 18:271–282.

LARSSON, S., EKBOM, B., and BJÖRKMAN, C. 2000. Influence of plant quality on pine sawfly population dynamics. *Oikos* 89:440–450.

LAVOLA, A., APHALO, P. J., LAHTI, M., and JULKUNEN-TIITTO, R. 2003. Nutrient availability and the effect of increasing UV-B radiation on secondary plant compounds in Scots Pine. *Environ. Exp. Bot.* 49:49–60.

MARON, J. L., HARRISON, S., and GREAVES, M. 2001. Origin of an insect outbreak: Escape in space or time from natural enemies? *Oecologia* 126:595–602.

MÜLLER, C., AGERBIRK, N., OLSEN, C. E., BOEVÉ, J.-L., SCHAFFNER, U., and BRAKEFIELD, P. M. 2001. Sequestration of Host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. *J. Chem. Ecol.* 27:2505–2516.

MÜLLER, C., BOEVÉ, J.-L., and BRAKEFIELD, P. M. 2002. Host plant derived feeding deterrence towards ants in the turnip sawfly *Athalia rosae*. *Entomol. Exp. Appl.* 104:153–157.

MUMM, R. and HILKER, M. 2006. Direct and indirect chemical defence of pine against folivorous insects. *Trends Plant Sci.* 11:351–358.

KARONEN, M. 2007. Plant proanthocyanidins. characterization and quantification by degradation methods, HPLC-DAD/ESI-MS and NMR. Annales Universitatis Turkuensis AI 374, 53 p. PhD dissertation. University of Turku, Turku, Finland.

OLESZEK, W., STOCHMAL, A., KAROLEWSKI, P., SIMONET, A. M., MACIAS, F. A., and TAVA, A. 2002. Flavonoids from *Pinus sylvestris* needles and their variation in trees of different origin grown for nearly a century at the same Area. *Biochem. Syst. Ecol.* 30:1011–1022.

OPITZ, S. E. W. and MÜLLER, C. 2009. Plant chemistry and insect sequestration. *Chemoecology* 19:117–154.

OPITZ, S. E. W., JENSEN, S. R., and MÜLLER, C. 2010. Sequestration of glucosinolates and iridoid glucsides in sawfly species of the genus *Athalia* and their role in defense against ants. *J. Chem. Ecol.* 36:148–157.

PRIETO, J. M., SCHAFFNER, U., BARKER, A., BRACA, A., SICILIANO, T., and BOEVÉ, J.-L. 2007. Sequestration of furostanol saponins by *Monophadnus* sawfly larvae. *J. Chem. Ecol.* 33:513–524.

PROP, N. 1960. Protection against birds and parasites in some species of Tenthredinid larvae. *Arch. Neerl. Zool.* 13:380–447.

RAAB, T., BARRON, D., ARCE VERA, F., CRESPY, V., OLIVEIRA, M., and WILLIAMSON, G. 2010. Catechin glucosides: Occurrence, synthesis and stability. *J. Agric. Food Chem.* 58:2138–2149.

ROITTO, M., MARKKOLA, A., JULKUNEN-TIITTO, R., SARJALA, T., RAUTIO, P., KUIKKA, K., and TUOMI, J. 2003. Defoliation-induced responses in peroxidases, phenolics, and polyamines in Scots pine (*Pinus sylvestris* L.) needles. *J. Chem. Ecol.* 29:1905–1918.

ROITTO, M., RAUTIO, P., JULKUNEN-TIITTO, R., KUKKOLA, E., and HUTTUNEN, S. 2005. Changes in the concentrations of phenolics and photosynthates in Scots pine (*Pinus sylvestris* L.) seedlings exposed to nickel and copper. *Environ. Pollut.* 137:603–609.

ROSLUND, M. U., TÄHTINEN, P., NIEMITZ, M., and SJÖHOLM, R. 2008. Complete assignments of the ^{1}H and ^{13}C chemical shifts and $J_{H,\,H}$ coupling constants in NMR spectra of D-glucopyranose and all D-glucopyranosyl- D-glucopyranosides. *Carbohydr. Res.* 343:101–112.

SCHAFFNER, U., BOEVÉ, J.-L., GFELLER, H., and SCHLUNEGGER, U. P. 1994. Sequestration of *Veratrum* alkaloids by specialist *Rhadinoceraea nodicornis* Konow (Hymenoptera, Tenthredinidae) and its ecoethological implications. *J. Chem. Ecol.* 20:3233–3250.

SCHMIDT, S., ZIETZ, M., SCHREINER, M., ROHN, S., KROH, L. W., and KRUMBEIN, A. 2010. Identification of complex, naturally occurring flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*) by high-performance liquid chromatography diode-array detection/electrospray ionization multi-stage mass spectrometry. *Rapid Commun. Mass Spectrom.* 24:2009–2022.

SCHOPF, R., MIGNAT, C., and HEDDEN, P. 1982. As to the food quality of spruce needles for forest damaging insects. 18. Resorption of secondary plant metabolites by the sawfly *Gilpinia hercyniae* Htg (Hym., Diprionidae). *Z. Angew. Entomol.* 93:244–257.

SEMIZ, G., HEIJARI, J., ISIK, K., and HOLOPAINEN, J. K. 2007. Variation in needle terpenoids among *Pinus sylvestris* L. (Pinaceae) provenances from Turkey. *Biochem. Syst. Ecol.* 35:652–661.

SHIRLEY, B. W. 1996. Flavonoid biosynthesis: 'New' functions for an 'old' pathway. *Trends Plant Sci.* 1:377–382.

SILLÉN-TULLBERG, B. 1990. Do predators avoid groups of aposematic prey? An experimental test. *Anim. Behav.* 40:856–860.

SLADE, D., FERREIRA, D., and MARAIS, J. P. J. 2005. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavanoids. *Phytochemistry* 66:2177–2215.

TVAROSKA, I. and TARAVEL, F. R. 1995. Carbon-proton coupling constants in the conformational analysis of sugar molecules. *Adv. Carbohydr. Chem. Biochem.* 51:15–62.

VIHAKAS, M., KAPARI, L., and SALMINEN, J.-P. 2010. New types of flavonol oligoglycosides accumulate in the hemolymph of birch-feeding sawfly larvae. *J. Chem. Ecol.* 36:864–872.

WATANABE, M. and AYUGASE, J. 2009. Chiral separation of catechins in buckwheat groats and the effects of phenolic compounds in mice subjected to restraint stress. *J. Agric. Food Chem.* 57:6438–6442.

WINK, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64:3–19.

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Millipedes That Smell Like Bugs: (*E*)-Alkenals in the Defensive Secretion of the Julid Diplopod *Allajulus Dicentrus*

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Abstract The secretions from serial defensive glands of the Austrian diplopod *Allajulus dicentrus* (Julidae, Cylindroiulini) were extracted and analyzed by means of gas chromatography – mass spectrometry. In adults, 13 components from two chemical classes were detected: 1) The common juliform benzoquinones were represented by four compounds (2-hydroxy-3-methyl-1,4-benzoquinone, 2-methoxy-3-methyl-1,4-benzoquinone, 2,3-dimethoxy-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-1,4-benzoquinone). From this series, 2-methoxy-3-methyl-1,4-benzoquinone was most abundant, comprising about 40 % of the whole secretion. 2) All remaining compounds were identified as aliphatic (*E*)-alkenals [(*E*)-2-heptenal, *(E)*-2-octenal, *(E)*-2-nonenal, *(E)*-2-decenal)] along with their corresponding alcohols. *(E)*-2-Octenal was most abundant, roughly accounting for another 35 % of the secretion. In juveniles, different stages in the ontogenetic development of the secretion were observed, with early instars (stadium III and IV) exclusively containing the benzoquinone fraction. Alkenols and alkenals were added in later instars (stadium V and VI), with secretions of stadium VI-juveniles being already similar to those of adults. Representatives of Spirostreptida, Spirobolida, and Julida traditionally have been considered to produce benzoquinonic secretions only ("quinone millipedes"), and information on secretion components from other chemical classes is still scarce. We here provide evidence for the participation of non-quinonic compounds in the defensive exudates of the Cylindroiulini. The occurrence of additional, non-quinonic compounds in certain species within a chemically homogenous, benzoquinone-producing taxon indicates the rapid adoption of novel exocrine compounds, possibly in order to meet the demands in a changed ecological environment.

Keywords Quinone diplopods · Chemical defense · *(E)*-2-octenal · 2-methoxy-3-methyl-1,4-benzoquinone · Juliformia · Julidae · Cylindroiulini.

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Introduction

Diplopods are well-known to produce various kinds of defensive secretions that include: 1) unusual alkaloidal compounds in the Glomerida and Polyzoniida; 2) mainly cyanogenic compounds and their breakdown products, including hydrogen cyanide, in the Polydesmida; 3) phenolic compounds in the Callipodida; and 4) particularly, benzoquinone-rich exudates in the Juliformia (Meinwald et al., 1975; Eisner et al., 1978; Ćurčić et al., 2009; Makarov et al., 2010; Vujisić et al., 2011; Shear et al., 2011; Shimizu et al., 2012 and references therein). To describe and characterize the secretion chemistry of the latter, Eisner et al. (1978) introduced the term "quinone millipedes", implying that these millipedes mainly or even exclusively produce quinonic secretions. Indeed, this situation was true for most species hitherto studied, including representatives of all three juliform orders, Spirostreptida, Spirobolida, and Julida. Most of these investigations have been conducted with the Spirobolida and Spirostreptida, which are represented by many large and conspicuous species in America,

Africa, and the Indo-Australian region, such as the giant African species *Archispirostreptus gigas* (e.g., Smolanoff et al., 1975) and others (e.g., Eisner et al., 1965, 1998; De Bernardi et al., 1982; Wood, 1974).

Consistently, there is evidence that quinonic secretions also are characteristic for the holarctic – mainly European – Julida. This order of Juliformia is chemically less studied than the Spirobolida and Spirostreptida. However, at least some early reports on the defensive chemistry of European Julida (e.g., Schildkecht and Weis, 1961; Röper and Heyns, 1977) as well as more recent publications (e.g., Huth, 2000) are available, referring to 16 thus far analyzed species of the Julidae (Vujisic et al., 2011) and at least one species of Blaniulidae (Weatherston et al., 1971). In addition, one report deals with julids from South East Asia (Shimizu et al., 2012).

All these investigations have underlined the picture of a widely homogenous, quinonic juliform chemistry (Attygalle et al., 1993; Williams et al., 1997; Deml and Huth, 2000; Weldon et al., 2003; Schmitt et al., 2004; Bedoussac et al., 2007; Wu et al., 2007; Vujisic et al., 2011). The widely-realized, uniform chemical structure of juliform defensive exudates appears to be an old, plesiomorphic trait, and seems to rely on a pool of about 15 different benzoquinones and related hydroquinones, with toluquinone (2-methyl-1,4-benzoquinone) and 2-methoxy-3-methyl-1,4-benzoquinone being the most common.

Only a few studies have indicated the presence of other – i.e., non-quinonic – components in some groups of the Juliformia. For example, Wheeler et al. (1964) reported on the secretion of the spirobolid *Rhinocricus insularis* that contained large amounts of the *(E)*-alkenal, (*E*)-2-dodecenal, together with the common juliform toluquinone. The secretion of this species is distinct by its anomalous smell. Additionally, from Julida, examples of chemically unusual secretions have been published. Weatherston et al. (1971) found long chain acetates in filter papers loaded with defensive exudates of the blaniulid *Blaniulus guttatus*, again together with a benzoquinonic compound. Recently, Shimizu et al. (2012) identified a series of fatty acid esters together with benzoquinones from a species of the East Asian julid *Anaulaciulus*. There also is evidence that such esters may have a wide distribution among certain Julidae, possibly together with n-alkanols (Huth, 2000), but conclusive and comprehensive data are thus far missing. Such novel groups of compounds in certain taxa, however, would strongly increase the chemotaxonomic potential of juliform secretions, and could represent important tools for integrative taxonomy.

We, here, report on the defensive chemistry in the European julid *Allajulus dicentrus* (Julidae, Cylindroiulini) that is able to discharge secretions possessing a distinct smell that is reminiscent of the alkenal-rich exudates of true bugs.

Materials and Methods

Collection of Species Specimens of *Allajulus dicentrus* (Latzel) were collected by hand and by means of a soil sifter from the leaf litter layer at different locations in Austria and Slovenia (Table 1). As an authentic reference for several quinones (see Vujisić et al., 2011), we used secretion-loaded extracts of *Cylindroiulus boleti* (C.L. Koch), found at the Gloriette Warte (near Eisenstadt, Austria), and specimens of *Leptoiulus trilineatus* (C.L. Koch) from Plana Mt. (near Pasarel Village, Bulgaria; kindly provided by Boyan Vagalinski, University of Sofia). *Pella cognata* (Märkel), a staphylinid beetle (and a reference for quinones as well, see below), was kindly provided by Michael Stoeffler (University of Hohenheim, Germany).

Extraction of Secretions Defensive secretions were either 1) obtained by dabbing secretions on small filter paper pieces (2×2 mm) from mechanically stimulated and visibly secretion-discharging species, followed by extraction of the secretion-loaded filter papers in 100 µl of hexane, or 2) by whole body extraction of single individuals in 50–500 µl of hexane (depending on size of specimens) for about 15 min. With the latter technique, the defensive secretions were directly discharged into the solvent, visibly turning it yellow after a few seconds of extraction. In total, individual extracts of the *A. dicentrus*-secretions of 120 specimens were prepared (34 males, 42 females, and 44 juveniles). Juvenile specimens were allocated to a specific instar or stadium by the number of podous segments and according to the classification scheme of Blower (1985) and Blower and Miller (1977). For *A. dicentrus*, in analogy to *A. nitidus* (see Blower and Miller, 1977), we expected adults to occur from stadium VIII (from 36–37 podous segments) on. Particularly, adult specimens of *A. dicentrus* that discharged secretions, as well as whole body extracts, exhibited a smell being redolent of the scent of many true bugs.

Analysis of Secretions An optimal concentration of compounds for routine analysis was achieved by dilution of extracts with hexane until only a faint yellow color remained. Aliquots of diluted extracts (1.5 µl) were subject to gas chromatography - mass spectrometry (GC-MS), using a Trace gas chromatograph coupled to a DSQ I mass spectrometer (MS), both from Thermo (Vienna, Austria). The GC was equipped with a ZB-5 fused silica capillary column (30 m× 0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Germany). Injection was splitless with helium (at 1.2 ml min^{-1}) as a carrier gas. The column temperature was programmed from 50 °C (held for 1 min) to 300 °C at 10 °C min^{-1}, and held at 300 °C for 5 min. The ion source of the MS and the transfer line were kept at 200 °C and 310 °C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Table 1 Collection of *Allajulus dicentrus*

Population	Location	Date	
1	Maria Rain (Carinthia, Austria) N 46.54870°; E 14.29704°; 474 m	Oct. 15, 2011	7 ♂, 4 ♀
		Oct. 22, 2011	9 ♂, 12 ♀
		Nov. 6, 2011	8 ♂, 13 ♀, 15 juv.
		Dec. 12, 2011	7 ♂, 10 ♀, 10 juv.
2	Rauth – Waidischbach (Carinthia, Austria) N 46.51329°; E 14.33405°; 546 m	Dec. 10, 2011	1 ♂, 2 ♀, 18 juv.
3	Rauth – near Dixer (Carinthia, Austria) N 46.51928°, E 14.33133°; 532 m	June 3, 2011	1 ♀
4	near Lovnik (Slovenia) N 46.27951°; E 15.56617°; 337 m	Aug. 15, 2011	1 ♂
5	near Podplat (Slovenia) N 46.24292°; E 15.57964°; 246 m	Aug. 15, 2011	1 ♂

Reference Compounds Authentic *(E)*-2-heptenal, *(E)*-2-octenal, *(E)*- 2-nonenal, *(E)*-2-decenal, and their corresponding alcohols for a comparison of GC-MS data were purchased from Sigma (Vienna, Austria). As references for some remaining compounds, we used natural sources from which these compounds had already been identified: Authentic 2-methoxy-3-methyl-1,4-benzoquinone was found in the extracts of the staphylinid beetle *Pella cognata* (Stoeffler et al., 2011) as well as in two julid species, *Cylindroiulus boleti* and *Leptoiulus trilineatus* (Vujisić et al., 2011). In detail, whole body extracts of specimens of the mentioned species were prepared, examined by GC-MS, and were found to contain this compound as a part of extract patterns as previously described (Stoeffler et al., 2011; Vujisić et al., 2011). The latter two species also served as references for authentic 2-hydroxy-3-methyl-1,4-benzoquinone, 2,3-dimethoxy-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-1,4-benzoquinone, which occurred in the extracts exactly in those patterns as reported (Vujisić et al., 2011). In contrast to *A. dicentrus*, these two julids exhibited the common and characteristic quinonic smell of juliform diplopods.

Quantification of Secretion Components and Statistics Absolute amounts of secretions were evaluated for 17 adult specimens. These specimens were extracted in a well-defined hexane volume (mostly 500 µl), and all further dilutions were recorded. Absolute amounts for *(E)*-2-octenal were determined using a calibration curve, showing a linear correlation between peak area and absolute amounts of synthetic *(E)*-2-octenal across a range from 1.5–500 ng. For the determination of the amounts of whole secretion per individual, the relative abundance of *(E)*-2-octenal per individual extract was extrapolated, giving a (half-quantitative) measure for the absolute amount of the whole secretion per individual. Secretion patterns, based on the integration of peak areas in the chromatograms and resulting in chemical profiles that express the relative abundance of each component in % of whole secretion, were calculated for all individuals. For a statistical comparison of profiles, we used 2D-non metric multidimensional scaling (2D-nMDS) and the Gower coefficient of dissimilarity, expressing the distances between individual profiles based on the weighted mean of the contributions of each variable in these profiles (Gower, 1967).

Results

Identification of Extract Components Hexane whole-body extracts of adult individuals of *Allajulus dicentrus* as well as extracts of secretion-loaded filter paper pieces exhibited a consistent GC pattern of 13 compounds (peaks A-M in Fig. 1, Table 2). Compounds were identified as follows: 9 by comparison of their mass spectra and retention times to authentic references (compounds A, C, E, H-M), 1 compound on the basis of comparison of mass spectral data and retention index (compound D), and the remaining 3 (B, F, G) tentatively on the basis of their mass spectra only (Table 2).

Four compounds E, J, L, and M appeared to be substituted benzoquinones, showing abundant molecular ions at *m/z* 138, *m/z* 152, *m/z* 168, and *m/z* 182, respectively. A comparison of their spectra and GC retention times confirmed full consistence with authentic 2-hydroxy-3-methyl-1,4-benzoquinone (compound E), 2-methoxy-3-methyl-1,4-benzoquinone (compound J), 2,3-dimethoxy-1,4-benzoquinone (compound L), and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (compound M).

Compounds A-D, F-I, and K had the spectra of 1) monounsaturated aliphatic aldehydes and 2) monounsaturated aliphatic alcohols. The aldehyde-series contained seven compounds (peaks A-C, F-H, K), exhibiting molecular ions of generally low intensity at *m/z* 112 (compound A), *m/z* 126 (compounds B, C), *m/z* 140 (compounds F, G, H), *m/z* 154 (compound K), and thus corresponding to a heptenal, two octenals, three nonenals, and a decenal. The mass spectra as well as the retention times of compounds A, C, H, and K fully matched authentic *(E)*-2-heptenal, *(E)*-2-octenal, *(E)*-2-nonenal, and *(E)*-2-decenal, respectively. The geometry of the remaining aldehydes in the extracts (all of which are minor compounds) remained uncertain. These were

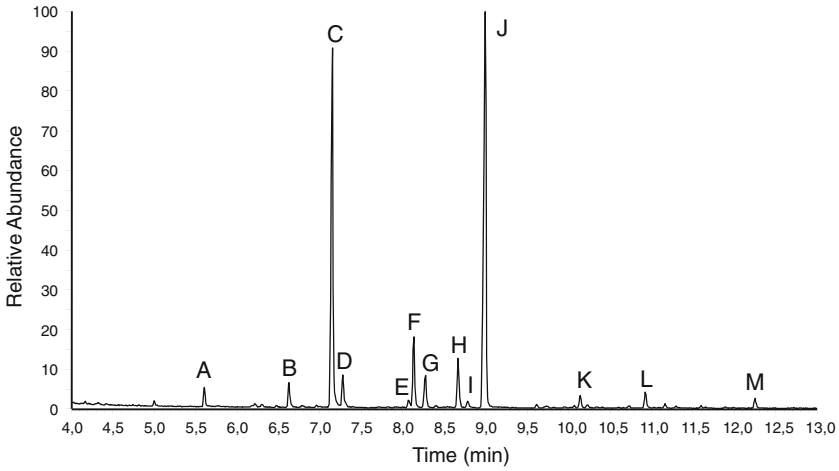

Fig. 1 Typical gas chromatographic profile of the defensive secretion of adult *Allajulus dicentrus*. Main compounds are *(E)*-2-octenal (peak C) and 2-methoxy-3-methyl-1,4-benzoquinone (peak J). For a complete list of compounds see Table 2

tentatively identified as a further (isomeric) octenal (= compound B) and two isomeric nonenals (= compounds F, G). With respect to their retention indices (see Table 2), none of these was *(Z)*-2-octenal nor *(Z)*-2-nonenal, respectively.

The alcohol-series included two components (D, I). The GC retention time as well as the MS fragmentation pattern of compound I were identical to authentic *(E)*-2-nonenol. This component eluted shortly after its corresponding aldehyde (compound H = *(E)*-2-nonenal). The remaining compound D appeared to be an octenol (M^+ at *m/z* 142), and comparably, it eluted shortly after its corresponding aldehyde, *(E)*-2-octenal (= compound C). Although no authentic

Table 2 Gas chromatographic and mass spectral data to components from extracts of adult *Allajulus dicentrus*

Peak no.	Retention index RI**	Mass spectrometric fragmentation (*m/z*)	Identified as
A	955	112 (M^+, 3), 111(3), 97 (19), 94 (8), 84 (26), 83 (100), 70 (41), 69 (44), 68 (36), 57 (40), 56 (31), 55 (39), 43 (14), 41 (34)	(*E*)-2-heptenal
B	1023	126 (M^+, 1), 111 (2), 108 (1,67), 97 (5), 95 (10), 93 (9), 83 (48), 82 (56), 70 (100), 69 (50), 57 (38), 56 (28), 55 (43), 41 (38)	isomeric octenal*
C	1058	126 (M^+, 1), 125 (1), 111 (4), 108 (5), 98 (18), 97 (27), 93 (20), 84 (25), 83 (93), 82 (51), 70 (100), 69 (53), 57 (43), 55 (63), 41 (41)	(*E*)-2-octenal
D	1067	128 (M^+, 1), 110 (9), 95 (19), 82 (40), 81 (41), 69 (22), 68 (42), 67 (36), 57 (100), 55 (33), 43 (24), 41 (27)	(*E*)-2-octenol
E	1120	138 (M^+, 100), 137 (1), 110 (10), 83 (12), 82 (40), 81 (15), 54 (10)	2-hydroxy-3-methyl-1,4-benzoquinone
F	1124	140 (M^+, 1), 125 (8), 122 (4), 109 (6), 107 (39), 98 (16), 97 (53), 96 (47), 95 (15), 84 (38), 83 (65), 81 (52), 70 (100), 69 (76), 57 (31), 56 (27), 55 (67), 43 (63), 41 (37)	isomeric nonenal (1)*
G	1133	140 (M^+, 1), 125 (2), 122 (2), 111 (3), 109 (6), 107 (12), 97 (19), 96 (33), 84 (52), 83 (88), 81 (29), 70 (100), 69 (41), 57 (28), 55 (59), 43 (32), 41 (34)	isomeric nonenal (2)*
H	1159	140 (M^+, 1), 125 (1), 122 (3), 111 (13), 107 (9), 98 (26), 97 (27), 96 (45), 93 (27), 84 (44), 83 (82), 81 (43), 70 (100), 69 (60), 57 (35), 55 (63), 43 (42), 41 (42)	(*E*)-2-nonenal
I	1168	142 (M^+, 3), 124 (2), 99 (7), 96 (24), 95 (48), 82 (51), 81 (40), 69 (22), 68 (39), 67 (44), 57 (100), 55 (23), 43 (31), 41 (21)	(*E*)-2-nonenol
J	1182	152 (M^+, 100), 151 (22), 137 (5), 122 (32), 109 (21), 94 (10), 83 (24), 82 (28), 81 (21), 67 (14), 66 (29), 55 (10), 54 (19), 53 (22)	2-methoxy-3-methyl-1,4-benzoquinone
K	1262	154 (M^+, 1), 153 (1), 136 (3), 121 (4), 111 (10), 110 (12), 107 (11), 98 (36), 97 (31), 98 (36), 97 (31), 84 (22), 83 (65), 81 (32), 70 (100), 69 (42), 57 (31), 56 (28), 55 (53), 43 (47), 41 (29)	(*E*)-2-decenal
L	1319	168 (M^+, 100), 153 (50), 139 (4), 125 (15), 123 (83), 122 (25), 97 (12), 95 (19), 94 (13), 82 (34), 69 (53), 54 (22)	2,3-dimethoxy-1,4-benzoquinone
M	1420	182 (M^+, 87), 167 (66), 153 (17), 139 (23), 137 (100), 136 (29), 121 (8), 111 (23), 96 (16), 83 (77), 69 (30), 68 (28)	2,3-dimethoxy-5-methyl-1,4-benzoquinone

*tentatively identified on the basis of mass spectral comparison and RI. **RI = Retention index, calculated as $RI_x = 100n_0 + (100t_x - -100tn_0)/(tn_1 - tn_0)$, with x: target compound; t_x: retention time of target compound; n_0: number of carbon atoms in the alkane directly eluting before x; tn_0: retention time of alkane directly eluting before x; tn_1: retention time of alkane directly eluting after x

sample was available for a direct comparison, we tentatively suggest compound D to be *(E)*-2-octenol. A comparison of its mass spectrum to that of *(E)*-2-octenol from the NIST-mass spectral library showed high correspondence (direct matching factor from NIST library search: SI 910="excellent match"). Furthermore, the compound had a retention index (RI) of 1067, matching the RI for *(E)*-2-octenol reported in the literature (e.g., Thakeow et al., 2008).

Secretion Profiles in Adults When evaluating chromatograms based on the relative abundance of components, most of the adult individuals showed a stable, homogenous profile, independent of sex and population: The two main components in all 76 samples were 2-methoxy-3-methyl-1,4-benzoquinone (44.3±14.8 % of secretion) and *(E)*-2-octenal (31.6±12.0 %). All other components occurred in minor amounts as summarized in Table 3. In order to assess the range of intraspecific variability of the secretion profile of adult *A. dicentrus*, a box plot showing the statistical distribution of the abundance of single components in the secretions of all adult individuals analyzed is given in Fig. 2. Individuals with extreme values in the abundance of certain compounds could neither be allocated with a specific gender nor with a specific population, and profiles from males and females as well as from individuals of different populations clustered together as shown in a scatter plot obtained by 2D-nMDS (Fig. 3a, b).

Amounts of Secretion Absolute amounts of secretion were quantified for 17 adult specimens. Using a calibration curve for *(E)*-2-octenal, an average of 219±122 μg of secretion per individual (span 54 up to 568 μg) was calculated. Based on this calculation, one pair of glands statistically contained about 5.7±3.1 μg of secretion.

Secretion Profiles in Juveniles In contrast to adults, the secretions of early-instar juveniles (10 up to 18 leg-bearing body segments, corresponding to stadium III and IV) showed only the benzoquinone-fraction, leading to profiles consisting to about 95 % of 2-methoxy-3-methyl-1,4-benzoquinone (Fig. 4a). Later instars (with 22–23 podous body segments, corresponding to stadium V) still exhibited profiles dominated by 2-methoxy-3-methyl-1,4-benzoquinone along with small amounts of *(E)*-2-octenol (Fig. 4b). Finally,

Table 3 Gas chromatographic profiles of defensive gland secretions in adults and juveniles of *Allajulus dicentrus*

Peak	Compound	Adults[1]	Early instars[2] (stadium III, IV)	Middle instars[3] (stadium V)	Late instars[4] (stadium VI)	Late instars[5] (stadium VI)	Late instars[6] (stadium VI)
A	*(E)*-2-heptenal	1.1±0.5	–	–	–	0.9	0.6
A'	*(E)*-2-heptenol	–	–	–	–	0.4	–
B	isomeric octenal	1.8±0.5	–	–	–	1.2	1.0
B'	isomeric octenol	–	–	–	0,2	1.3	–
C	*(E)*-2-octenal	**31.6±12.0**	–	–	0,2	**18.7**	**20.8**
D	(*E*)-2-octenol	0.8±1.2	–	4.0±2.8	3,0	**13.3**	2.5
E	2-hydroxy-3-methyl-1,4 benzoquinone	2.7±2.6	0.1±0.3	0.4±0.5	0,6	0.6	0.6
F	isomeric nonenal (1)	5.8±1.7	–	–	–	3.4	3.7
F'	isomeric nonenol	–	–	–	1,2	5.6	2.5
G	isomeric nonenal (2)	2.6±0.8	–	0.9±0.6	–	–	–
G'	(*Z*)-2-nonenal	–	–	–	0,6	1.5	–
H	(*E*)-2-nonenal	4.6±1.6	–	–	–	1.9	2.5
I	(*E*)-2-nonenol	0.2±0.3	–	0.6±0.7	0,3	2.6	0.4
J	2-methoxy-3-methyl-1,4-benzoquinone	**44.3±14.8**	**89.5±4.9**	**88.6±4.1**	**84,2**	**43.7**	**60.8**
K	(*E*)-2-decenal	1.7±0.9	–	–	–	0.6	1.1
K'	(*E*)-2-decenol	–	–	–	–	0.9	–
L	2,3-dimethoxy-1,4-benzoquinone	2.1±0.9	9.6±4.9	4.7±2.2	8,7	2.9	2.8
M	2,3-dimethoxy-5-methyl-1,4-benzoquinone	0.7±0.3	0.8±0.4	0.7±0.1	1,0	0.4	0.6

Compounds in % of whole secretion, calculated from peak areas in the chromatograms. For adults, early and middle instars, mean values ± SD are given. For late instars of stadium VI, three individual profiles are shown. Main components (>10 % of secretion) in bold

[1] Males and females (see Fig. 1), *N*=76. [2] Instars with 10 (stadium III) and 16–18 podous segments, showing benzoquinone-rich secretions (stadium IV; see Fig. 4a), *N*=37. [3] Middle instars with 22–23 podous segments, *N*=4, still showing benzoquinone-rich secretions (stadium V; see Fig. 4b). [4] One late instar specimen with 28 podous segments (stadium VI) showing a benzoquinonic secretion similar to middle instars. [5] One late instar specimen with 28 podous segments (stadium VI), showing an intermediate, alcohol-rich secretion along with first aldehydes (see Fig. 4c). [6] One late instar specimen with 29 podous segments (stadium VI), already showing an aldehyde-rich secretion similar to adults (see Fig. 4d)

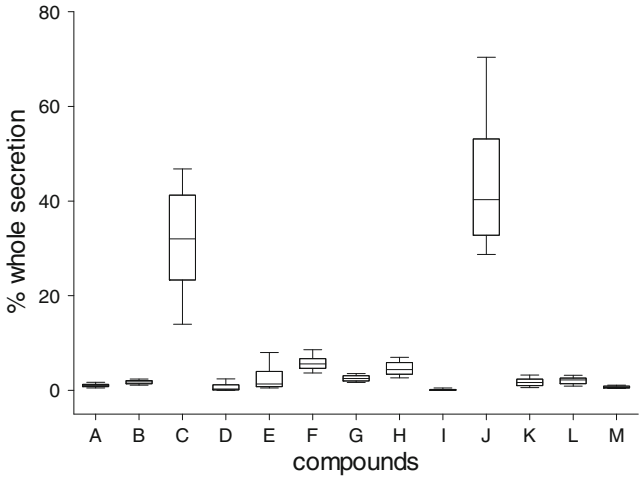

Fig. 2 Intraspecific variability of the secretion profile in adults of *Allajulus dicentrus*. Box plot, showing median, likely (25 %–75 %) and full range of variation (min–max) for each component in the secretion profiles, based on individual secretions of 76 adults of both sexes and from different populations

secretions of late instars (= 28 and 29 leg-bearing body segments, corresponding to stadium VI) were found to be either 1) benzoquinone-rich and similar to stadium V, or 2) to contain an abundant alkenol-fraction along with medium amounts of the corresponding alkenals (Fig. 4c). 3) The profile of one individual of stadium VI was already similar to the profiles of adults (Fig. 4d). In addition to *(E)*-2-octenol (peak D) and *(E)*-2-nonenol (peak I), the alkenol-fraction of late instars contained some compounds not found in adults, namely peak A' (M^+ at *m/z* 114; tentatively identified as heptenol), peak B' (M^+ at *m/z* 128; tentatively identified as an octenol-isomer), peak F' (M^+ at *m/z* 142; tentatively identified as a nonenol-isomer), and peak K' (M^+ at *m/z* 156; tentatively identified as decenol). For the heptenol and the decenol, we found RI values of 965 and 1268, corresponding to values given for *(E)*-2-heptenol and *(E)*-2-decenol in literature (http://webbook.nist.gov/chemistry/). In extracts of certain late instars, a further alkenal, most likely *(Z)*-2-nonenal, was detected (compound G': M^+ at *m/z* 140; RI 1142: corresponding to literature data of *(Z)*-2-nonenal as given in Georgiou et al., 2010). All gas chromatographic and mass spectrometric data for these additional compounds of late juvenile instars are summarized in Table 4. Secretions profiles of juveniles of different stadium are compared in Table 3. Profiles of some late-instar juveniles (as in Fig. 4d) were statistically indistinguishable from adults and clustered with adult profiles as shown by 2D non-metric multidimensional scaling (Fig. 5).

Discussion

Evidence for Non-quinonic Compounds in the "Quinone Millipedes"? Apart from the occurrence of certain esters and alcohols in a few species of Julidae (Huth, 2000; Shimizu et al., 2012), our finding of *(E)*-2-alkenals as a major class of components in the defensive secretion of *Allajulus dicentrus* represents a rare example for non-quinonic compounds in the "quinone millipedes" (= Juliformia *sensu* Eisner et al., 1978). Along with *(E)*-2-dodecenal from *Rhinocricus insulatus* (Wheeler et al., 1964), this is the second record of the occurrence of *(E)*-2-alkenals in the secretions of juliform

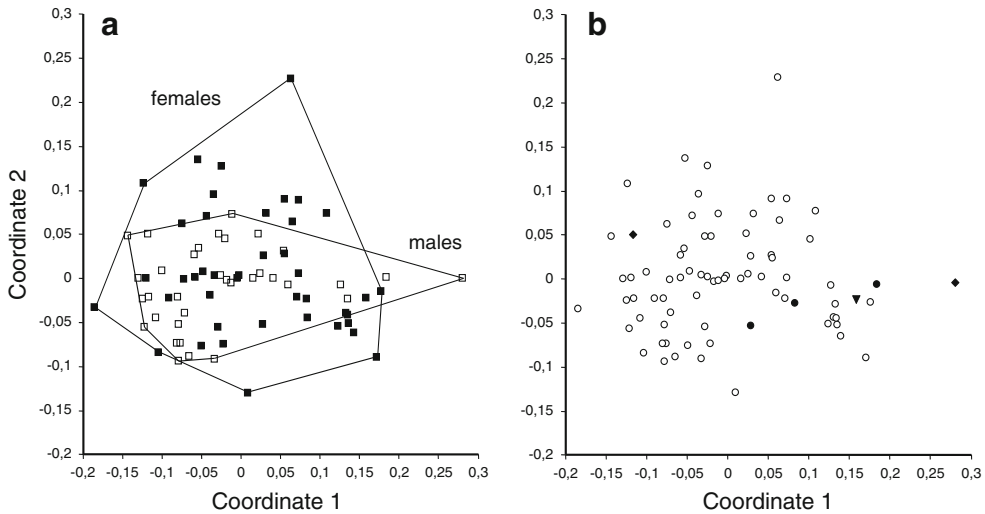

Fig. 3 Statistical comparison of profiles of both sexes and among individuals of different populations of *Allajulus dicentrus*. Multidimensional scaling (2D nMDS) of Gower dissimilarity calculated from individual adult profiles. **a** Profiles of males and females cluster together and cannot be distinguished. **b** Profiles of individuals of different populations appear to be merged within one large cluster. Hollow circles: population 1 (70 individuals); black dots: three individuals from population 2; black diamond: two individuals from Slovenia (population 4, 5), and black triangle: one individual from population 3

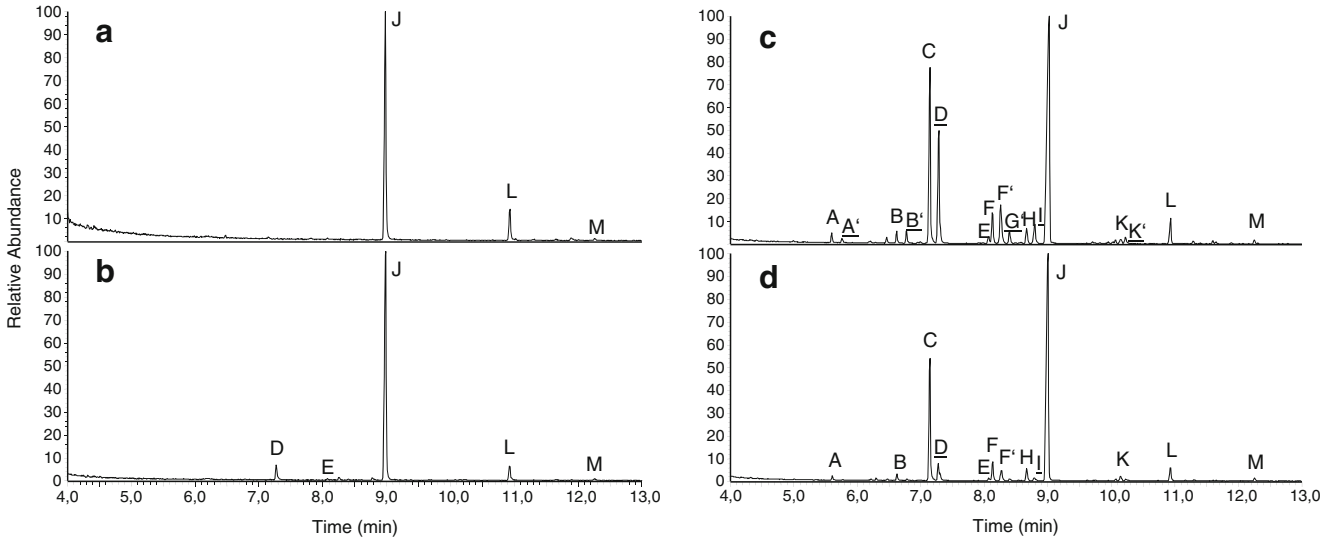

Fig. 4 Ontogenetic development of the defensive secretion in *Allajulus dicentrus*. **a** Profile 1, as found in early-instars (stadium III, IV), showing exclusively benzoquionones (peak J: 2-methoxy-3-methyl-1,4-benzoquinone; peak L: 2,3-dimethoxy-1,4-benzoquinone), but lacking both *(E)*-alkenals and *(E)*-alkenols. **b** Profile 2, as found in middle-instar juveniles (stadium V), showing small amounts of *(E)*-alkenols (peak D: *(E)*-2-octenol) in addition to benzoquinones (J, L), but still lacking *(E)*-alkenals. **c** Profile 3, as found in late-instar juveniles (stadium VI), showing benzoquinones (peaks J, L, M) together with large amounts of *(E)*-alkenols [peak A': *(E)*-2-heptenol; peak B': isomeric octenol; peak D: *(E)*-2-octenol; peak F': isomeric nonenol; peak I: *(E)*-2-nonenol); peak K': *(E)*-2-decenol] and increasing amounts of *(E)*-alkenals (peaks A, B, C, F, G, H, K: see Fig. 1). For better visibility, all *(E)*-alkenol-peaks are underlined. **d** Profile 4, as found in late-instar juveniles of stadium VI as well, is already indistinguishable from adults

Diplopoda, and the first incidence of alkenals in the secretions of Julida. For other Juliformia, sporadic reports on non-benzoquinone compounds are available, but these either deal with probably cuticle-derived components (e.g., Monro et al., 1962; Weatherston and Percy, 1969), or the identification of these components is only tentative and/or might need reconsideration. For example, analytical data for the identification of a putative alkaloid, 3,3a,4,5-tetrahydro-1H-pyrrolo-[2,3-b] pyridine-2,6-dione, from the secretion of *R. padbergi* are largely missing (Arab et al., 2003). However, both the molecular weight of this compound (M=152) as well as its chromatographic characteristics (as indicated in Fig. 1 in Arab et al., 2003) are reminiscent of the common juliform 2-methoxy-3-methyl-1,4-benzoquinone.

Regarding the secretion of *A. dicentrus,* it is interesting to note that all alkenals found were (*E*)-isomers. Only one (*Z*)-isomer, (*Z*)-2-nonenal, was present additionally in the extracts of some late instars (compound G'). Generally, the (*E*)-isomers of 2-alkenals must be regarded as more stable than (*Z*)-isomers, and the predominance of (*E*)-2-alkenals also is reflected in the exocrine secretions of other Arthropoda. The exudates of true bugs, for instance, represent the best known

Table 4 Additional compounds from the secretions of late-instar juveniles (stadium VI) in *Allajulus dicentrus*

Peak no.	Retention index RI	Mass spectrometric fragmentation (*m/z*)	Identified as*
A'	965	114 (M^+,2), 96 (13), 86 (14), 81 (64), 72 (11), 71 (24), 70 (9), 68 (31), 67 (18), 57 (100), 56 (14), 55 (34), 54 (19), 43 (11), 42 (16), 41 (22)	(*E*)-2-heptenol
B'	1034	128 (M^+, 1), 110 (7), 97 (6), 95 (44), 83 (11), 82 (94), 81 (21), 71 (14), 70 (19), 69 (75), 66 (29), 67 (43), 57 (100), 56 (72), 55 (43), 54 (19), 44 (11), 43 (55), 42 (19), 41 (61)	isomeric octenol
F'	1132	142 (M^+, 1), 124 (4), 111 (2), 109 (10), 97 (2), 96 (7), 95 (22), 85 (6), 83 (8), 82 (48), 81 (58), 71 (24), 70 (19), 69 (71), 68 (35), 67 (36), 66 (7), 57 (100), 56 (23), 55 (25), 54 (8), 43 (46), 42 (34)	isomeric nonenol
G'	1142	140 (M^+, 1), 109 (6), 97 (8), 96 (29), 95 (43), 83 (22), 82 (13), 81 (17), 71 (19), 70 (100), 69 (41), 66 (21), 67 (34), 57 (61), 56 (22), 55 (36), 43 (32), 42 (9), 41 (30)	(*Z*)-2-nonenal
K'	1268	156 (M^+, 1), 112 (12), 110 (4), 109 (9), 108 (3), 97 (6), 96 (27), 95 (17), 84 (9), 83 (21), 82 (56), 81 (47), 71 (33), 69 (36), 68 (41), 67 (57), 66 (6), 57 (100), 56 (20), 55 (44), 54 (24), 53 (7), 44 (14), 43 (37), 41 (30)	(*E*)-2-decenol

*all components were tentatively identified on the basis of mass spectral comparison; for compound A', G' and K', we additionally found RI values in literature (http://webbook.nist.gov/chemistry/) that correspond to (*E*)-2-heptenol, (*Z*)-2-nonenal, and (*E*)-2-decenol, respectively (see text)

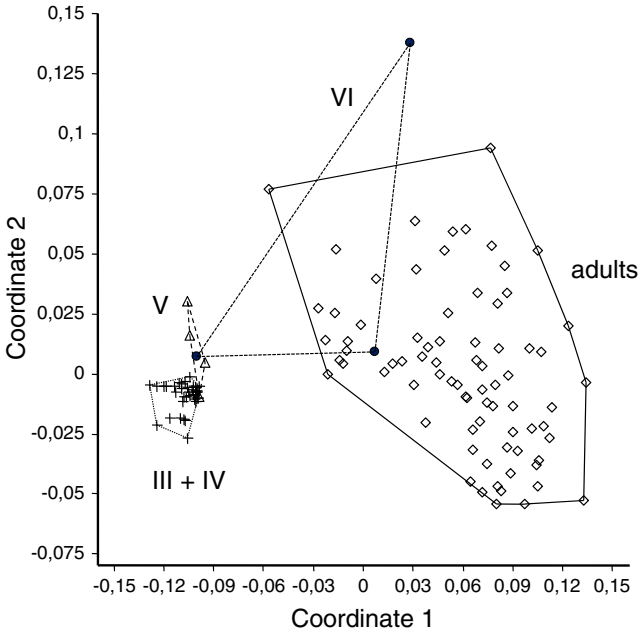

Fig. 5 Statistical discrimination of profiles of different developmental stages of *Allajulus dicentrus* by multidimensional scaling (2D-nMDS) of Gower dissimilarity calculated from individual profiles of juveniles and adults. Specimens with benzoquinonic profiles up to stadium IV cluster together but can be distinguished from individuals of stadium V. Individuals of stadium VI are chemically intermediate between the benzoquinone-rich profiles of early instars and the alkenal-rich secretions as found in adults

natural source for (*E*)-2-alkenals, and the major alkenal of *A. dicentrus*, (*E*)-2-octenal, is a common component in the secretions of representatives of different heteropteran families as well (Collins, 1968; Levinson et al., 1974; Farine et al., 1993; Aldrich et al., 1997; Siljander et al., 2008). (*E*)-2-Octenal contributes strongly to the offensive smell of some of these bug-species, and the characteristic bug-like smell of the *Allajulus*-secretion also relies on the presence of this compound.

In order to exclude the idea that the occurrence of *(E)*-alkenals in *A. dicentrus* was an incidental finding, being true only for one specific population, we compared individuals from different populations. Austrian populations 2 and 3 (see Table 1) were several kilometres from our main "population 1" and separated from it by the Drava-river, and other individuals were even further from populations in Slovenia (more than 100 km from main population 1, and separated by the Karavanke-mountains). Indeed, all specimens irrespective of their allocation to populations consistently exhibited an abundant *(E)*-alkenal-fraction in their secretions. In these terms, it seems justified to consider the *(E)*-alkenals (plus alkenols) as basic and consistent elements in the defensive chemistry of *A. dicentrus*. Since a systematic screening for these compounds throughout different taxonomic groups of the Julidae (or even Juliformia) has never been performed, we cannot conclude whether these *(E)*-alkenals represent an autapomorphy of *A. dicentrus*, or whether they may show a wider distribution in millipede secretions. When considering the rather distant relation to the only other *(E)*-alkenal producing diplopod species, the spirobolid *Rhinocricus insularis* (Wheeler et al., 1964), an independent evolution of these compounds in juliform secretions seems to be the most likely explanation at this time.

We also provide data for the ontogenetic development of the secretion chemistry in *A. dicentrus*, and to our knowledge, this topic is addressed for the first time for Diplopoda in general. Interestingly, early instars displayed nearly exclusively 2-methoxy-3-methyl-1,4-benzoquinone in their secretions, and *(E)*-alkenols (such as octenol) were added in later instars. These findings may indicate the uncoupled biosynthesis of benzoquinones and *(E)*-alkenals, with the latter formed during ontogenesis from their corresponding alcohols by oxidation. This process may take place during stadium VI, as evidenced by the three different profiles that we obtained from individuals with 28 and 29 podous segments. Additionally, the finding of adults with lesser amounts of alkenals in their secretions may be attributed to this phenomenon, and we speculate that these individuals are in a state of glandular refill, as may be possibly the case in a period shortly after molting. According to Blower (1985) and Blower and Miller (1977), 13 stadiums can be observed in the conspecific *A. nitidus*, and adults occur from stadium VIII on.

Chemosystematics in the Cylindroiulini? The genus *Allajulus* is classed with the julid tribe Cylindroiulini (Read, 1990) from which the secretions of some representatives of genera *Cylindroiulus* and *Enantiulus* have been analysed, all of them showing the typical toluquinone- and 2-methoxy-3-methyl-quinone-rich juliform secretion profiles (Schildknecht and Weis, 1961; Röper and Heyns, 1977; Röper, 1978; Huth, 2000; Vujisic et al., 2011). In a recent paper on the secretion chemistry of several cylindroiuline species, Vujisic et al. (2011) pointed out that the similarity of secretion profiles limits chemosystematic applications – but genus *Allajulus* may indeed be different. Röper and Heyns (1977) previously mentioned that the secretion of *A. nitidus* was exceptional in displaying only a single component, 2-methoxy-3-methyl-1,4-benzoquinone, and lacking toluquinone. This lack of toluquinone – also proved for *A. dicentrus* in the present study – appears to be characteristic for *Allajulus*. In a preliminary analysis of *A. groedensis* (data not shown), we found the secretion of this species to be devoid of toluquinone as well, and mainly made up of 2-methoxy-3-methyl-1,4-benzoquinone, hence resembling the secretion of *A. nitidus*. We have not yet checked the fourth Austrian *Allajulus*, *A. molybdinus*.

The genus *Allajulus* formerly consisted of about 100 species, most of which have now been transferred to other genera. According to Read (1990), seven species of "true" *Allajulus* are recognized. Interestingly, Read (1990) pointed

out that the taxonomy of *Allajulus* is in a state of confusion and that *Allajulus* is probably paraphyletic, with *A. dicentrus* possibly being sister to all other *Allajulus* species plus *Enantiulus* (see also Enghoff et al., 2011). The special position of *A. dicentrus* is also chemically reflected by its aberrant secretion. For cylindroiulines such as from genera *Styrioiulus* and *Kryphioiulus,* no chemical data are available at present.

Evolutionary Development of (E)-Alkenals In regard to the second *(E)*-alkenal-producing milliped, *Rhinocricus insularis*, several analogies to the situation in *Allajulus dicentrus* become obvious. For example, no sign of *(E)*-alkenals was found in the close relatives of *R. insularis* (*R. padbergi* and other rhinocricidid species such as *Acladocricus setigerus* and *Anadenobolus putealis*: Arab et al., 2003; Bedoussac et al., 2007; Wu et al., 2007). As in *A. dicentrus*, this possible sporadic occurrence of aberrantly-structured secretions within a chemically homogenous taxonomic group raises several questions. One of these refers to the selection of additional non-benzoquinone compounds in an already well-established and proven system for chemical defense in the course of evolution. One reason for this may be found in evolutionary adaptations to novel predators, i.e., although being defended by benzoquinones, predators from different taxonomic groups are known to specifically prey on millipedes, in many cases by circumventing the chemical protection devices of their prey. For instance, the spirobolid *Floridobolus penneri* is specifically preyed by a phengodid beetle larva (Eisner et al., 1998), and in some cases – such as documented for the spirobolid *Pelmatojulus tigrinus* – the originally defensive benzoquinones even act as kairomones for necrophagous beetles (Schmitt et al., 2004; Bedoussac et al., 2007) and for an array of other predators such as assassin bugs (Schmitt et al., 2004). Thus, the scattered occurrence of aberrant secretions within chemically homogenous, benzoquinone-producing taxa may indicate the rapid adoption of non-benzoquinonoid compounds in response to major changes in the ecological environment of these species.

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References

ALDRICH, J. R., LEAL, W. S., NISHIDA, R., KHRIMIAN, A. P., LEE, C.-J., and SAKURATANI, Y. 1997. Semiochemistry of aposematic seed bugs. *Entomol. Exp. Appl.* 84:127–135.

ARAB, A., ZACARIN, G. G., FONTANETTI, C. S., CAMARGO-MATHIAS, M. I., DOS SANTOS, M. G., and CABRERA, A. C. 2003. Composition of the defensive secretion of the Neotropical millipede *Rhinocricus padbergi* Verhoeff 1938 (Diplopoda: Spirobolida: Rhinocricidae). *Entomotropica* 18:79–82.

ATTYGALLE, A. B., XU, S. C., MEINWALD, J., and EISNER, T. 1993. Defensive secretion of the millepede *Floridobolus penneri*. *J. Nat. Prod.* 56:1700–1706.

BEDOUSSAC, L., FAVILA, M. E., and LÓPEZ, R. M. 2007. Defensive volatile secretions of two diplopod species attract the carrion ball roller scarab *Canthon morsei* (Coleoptera: Scarabaeidae). *Chemoecology.* 17:163–167.

BLOWER, J. G. 1985. Millipedes, *in* D. M. Kermack and R. S. K. Barnes (eds.), Synopses of the British Fauna (New series) No. 35. E.J. Brill/Dr. W. Backhuys, London. 242 p.

BLOWER, J. G. and MILLER, P. F. 1977. The life-history of the julid millipede *Cylindroiulus nitidus* in a Derbyshire wood. *J. Zool.* 183:339–351.

COLLINS, R. P. 1968. Carbonyl compounds produced by the bed bug, *Cimex lectularius*. *Ann. Entomol. Soc. Am.* 61:1338–1340.

ĆURČIĆ, B. P. M., MAKAROV, S. E., TEŠEVIĆ, V. V., JADRANIN, M. B., and VUJISIĆ, L. V. 2009. Identification of secretory compounds from the European callipodian species *Apfelbeckia insculpta*. *J. Chem. Ecol.* 35:893–895.

DE BERNARDI, M., MELLERIO, G., VIDARI, G., VITA-FINZI, P., DEMANGE, J. M., and PAVAN, M. 1982. Quinones in the defensive secretions of African millipedes. *Naturwissenschaften* 69:601–602.

DEML, R. and HUTH, A. 2000. Benzoquinones and hydroquinones in defensive secretions of tropical millipedes. *Naturwissenschaften* 87:8082.

EISNER, T., HURST, J. J., KEETON, W. T., and MEINWALD, Y. 1965. Defense mechanisms of arthropods. XVI. Para-benzoquinones in the secretion of spirostreptid millipedes. *Ann. Entomol. Soc. Am.* 58:247–248.

EISNER, T., ALSOP, D., HICKS, K., and MEINWALD, J. 1978. Defensive secretions of millipedes, pp. 41–72, *in* S. Bettini (ed.), Arthropod Venoms, Handbook of Experimental Pharmacology, Vol. 48. Springer, Berlin.

EISNER, T., EISNER, M., ATTYGALLE, A. B., DEYRUP, M., and MEINWALD, J. 1998. Rendering the inedible edible: Circumvention of a millipede's chemical defense by a predaceous beetle larva (Phengodidae). *Proc. Natl. Acad. Sci. USA* 95:1108–1113.

ENGHOFF, H., PETERSEN, G., and SEBERG, O. 2011. Phylogenetic relationships in the millipede family Julidae. *Cladistics* 27:606–616.

FARINE, J.-P., EVERAERTS, C., BROSSUT, R., and LE QUÉRE, J.-L. 1993. Defensive secretions of nymphs and adults of five species of Pyrrhocoridae (Insecta: Heteroptera). *Biochem. Syst. Ecol.* 21:363–371.

GEORGIOU, C., KOUTSAVITI, A., BAZOS, I., and TZAKOU, O. 2010. Chemical composition of *Echinophora tenuifolia* subsp. *sibthorpiana* essential oil from Greece. *Rec. Nat. Prod.* 4:167–170.

GOWER, J. C. 1967. A comparison of some methods of cluster analysis. *Biometrics* 23:623–637.

HUTH, A. 2000. Defensive secretions of millipedes: more than just a product of melting point decrease? *Frag. Faunistica* 43 (suppl):191–200.

LEVINSON, H. Z., LEVINSON, A. R., and MASCHWITZ, U. 1974. Action and composition of the alarm pheromone of the bedbug *Cimex lectularius* L. *Naturwissenschaften* 61:684–685.

MAKAROV, S. E., CURCIC, B. P. M., TEŠEVIC, V. V., JADRANIN, M. B., VUJISIC, L. V., CURCIC, S. B., MANDIC, B. M., SEKULIC, T. L., AND MITIC, B. M. 2010. Defensive secretions in three species of polydesmids (Diplopoda, Polydesmida, Polydesmidae). *J. Chem. Ecol.* 36:978–982.

MEINWALD, J., SMOLANOFF, J., MCPHAIL, A. T., and MILLER, R. W. 1975. Nitropolyzonamine: a spirocyclic nitro compound from the

defensive glands of a milliped (*Polyzonium rosalbum*). *Tetrahedon Lett.* 28:2367–2370.

MONRO, A., CHADHA, M., MEINWALD, J., and EISNER, T. 1962. Defense mechanisms of arthropods. IV. Para-benzoquinones in the secretion of five species of millipedes. *Ann. Entomol. Soc. Am.* 55:261–262.

READ, H. J. 1990. The generic composition and relationships of the Cylindriulini – a cladistic analysis (Diplopoda, Julida: Julidae). *Ent. Scand.* 21:97–112.

RÖPER, H. 1978. Ergebnisse chemisch-analytischer Untersuchungen der Wehrsekrete von Spirostreptiden, Spiroboliden und Juliden (Diplopoda), von *Peripatopsis* (Onychophora) und von *Polyzonium* (Diplopoda, Colobognatha). *Abh. Verh. Naturwiss. Ver. Hamburg* 21(22):353–363.

RÖPER, H. and HEYNS, K. 1977. Spurenanalytik von *p*-Benzochinon- und Hydrochinon-Derivaten mit Gaschromatographie und Gaschromatographie/Massenspektrometrie. Identifizierung von Wehrsekret-Komponenten europäischer Juliden. *Z. Naturforsch.* 32c:61–66.

SCHILDKNECHT, H. and WEIS, K. H. 1961. Chinones als aktives Prinzip der Abwehrstoffe von Diplopoden. *Z. Naturforsch.* 16b:810–816.

SCHMITT, T., KRELL, F. T., and LINSENMAIR, K. E. 2004. Quinones mixture as attractant for necrophagous dung beetles specialized on dead millipedes. *J. Chem. Ecol.* 30:731–740.

SHEAR, W. A., JONES, T. H., and WESENER, T. 2011. Glomerin and homoglomerin from the North American pill millipede *Onomeris sinuata* (Loomis, 1943) (Diplopoda, Pentazonia, Glomeridae). *Int. J. Myriapod.* 4:1–10.

SHIMIZU, N., KUWAHARA, Y., YAKUMARU, R., and TANABE, T. 2012. n-Hexyl laurate and fourteen related fatty acid esters: New secretory compounds from the julid millipede, *Anaulaciulus* sp. *J. Chem. Ecol.* 38:23–28.

SILJANDER, E., GRIES, R., KHASKIN, G., AND GRIES, G. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. *J. Chem. Ecol.* 34:708.

SMOLANOFF, J., DEMANGE, J. M., MEINWALD, J., and EISNER, T. 1975. 1,4-Benzoquinones in African millipeds. *Psyche* 82:78–80.

STOEFFLER, M., TOLASCH, T., and STEIDLE, J. L. M. 2011. Three beetles – three concepts. Different defensive strategies of congeneric myrmecophilous beetles. *Behav. Ecol. Sociobiol.* 65:1605–1613.

THAKEOW, P., ANGELI, S., WEIßBECKER, B., and SCHÜTZ, S. 2008. Antennal and behavioral responses of *Cis boleti* to fungal odor of *Trametes gibbosa*. *Chem. Senses.* 33:379–387.

VUJISIĆ, L. V., MAKAROV, S. E., ĆURČIĆ, B. P. M., ILIĆ, B. S., TEŠEVIĆ, V. V., GOĐEVAC, D. M., VUČKOVIĆ, I. M., ĆURČIĆ, S. B., and MITIĆ, B. M. 2011. Composition of the defensive secretion in three species of European millipedes. *J. Chem. Ecol.* 37:1358–1364.

WEATHERSTON, J. and PERCY, J. E. 1969. Studies of physiologically active arthropod secretions. III. Chemical, morphological, and histological studies of the defence mechanism of *Uroblaniulus candensis* (Say) (Diplopoda: Julida). *Can. Entomol.* 101:280–285.

WEATHERSTON, J., TYRRELL, D., and PERCY, J. E. 1971. Long chain alcohol acetates in the defensive secretion of the millipede *Blaniulus guttulatus*. *Chem. Phys. Lipids* 7:98–100.

WELDON, P. J., ALDRICH, J. R., KLUN, J. A., OLIVER, J. E., and DEBBOUN, M. 2003. Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus* spp.). *Naturwissenschaften* 90:301–304.

WHEELER, J. W., MEINWALD, J., HURST, J. J., and EISNER, T. 1964. Trans-2-Dodecenal and 2-methyl-1,4-quinone produced by a millipede. *Science* 144:540–541.

WILLIAMS, L. A. D., SINGH, P. D. A., and CALEB-WILLIAMS, L. S. 1997. Biology and biological action of the defensive secretion form a Jamaican millipede. *Naturwissenschaften* 84:143–144.

WOOD, W. F. 1974. Toluquinone and 2-methoxy-3-methylbenzoquinone from the defensive secretions of three African millipedes. *Ann. Entomol. Soc. Am.* 67:988–989.

WU, X., BUDEN, D. W., AND ATTYGALLE, A. B. 2007. Hydroquinones from defensive secretion of a giant Pacific millipede, *Acladocricus setigerus* (Diplopoda: Spirobolidae). *Chemoecology* 17:131–138.

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Chemistry and Anatomy of the Frontal Gland in Soldiers of the Sand Termite *Psammotermes hybostoma*

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Abstract A great diversity of defensive chemicals has been described in termite soldiers equipped with a unique defensive organ, the frontal gland. Along with the functional diversity of these compounds, reflecting the evolutionary history of particular lineages and their defensive strategies, a considerable degree of chemical variability often occurs among species and populations. Thus, the chemistry of termite defense may provide information on the phylogeny and geographic dispersal of species and populations. In this paper, we report on the anatomy of the frontal gland and on the diversity of soldier defensive chemicals in the sand termite, *Psammotermes hybostoma*, from nine colonies and five different localities in Egypt. Using gas chromatography–mass spectrometry, a total of 30 sesquiterpene hydrocarbons, or their oxygenated derivatives, were detected, and the chemical identity of most of them identified. In addition, a ketone, an ester, and a diterpene were identified in some colonies. Within colonies, the chemical composition was stable and did not differ among soldier size categories. However, there were pronounced quantitative and qualitative differences in frontal gland chemicals among colonies and geographic locations. The findings are discussed in a broader comparison with other termite taxa.

Keywords Termites · Chemical defense · Frontal gland of soldiers · Rhinotermitidae · *Psammotermes hybostoma* · Sesquiterpenes

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Introduction

Collective defense is a prominent characteristic of insect societies, manifested by defendable nests and defensive adaptations of inhabitants (Crespi, 1994). In termite ancestors, the caste of soldiers evolved as the first altruistic caste, completely sterile and fully devoted to defense (Roisin, 2000). Along with a plethora of anatomic and behavioral adaptations for mechanical defense, termite soldiers also possess efficient chemical weaponry, especially for the advanced families Rhinotermitidae, Serritermitidae, and Termitidae, which are equipped with a unique defensive organ, the frontal gland (Deligne et al., 1981; Prestwich, 1984; Quennedey, 1984; Šobotník et al., 2010).

A fascinating diversity of defensive chemicals produced by this gland has been discovered over the past four decades, with several hundred compounds from various chemical classes, including alcohols, mono-, sesqui-, di-terpenoid hydrocarbons, ketoaldehydes, fatty acids, macrocyclic lactones, heterocyclic and aromatic compounds, having being identified. The functional diversity of these compounds and their mixtures includes use as irritants, repellents, glues, antihealants, and contact poisons (Prestwich, 1984; Šobotník et

al., 2010). Besides their defensive function, volatiles from the frontal gland also may be involved in signaling alarm by fighting or irritated soldiers (Šobotník et al., 2010).

The functional chemistry of the frontal gland secretion co-evolved with structural aspects (anatomy of the glandular reservoir, the frontal pore, shape of the head and mandibles, and related behavior) used in association with defense, into a multitude of defensive strategies, ranging from contact discharge combined with mandibular biting, to non-contact delivery by spraying (Quennedey, 1984; Šobotník et al., 2010). Thus, the chemistry and anatomy of the frontal gland provide information on the evolutionary history of defensive strategies in particular lineages. At the same time, several studies have highlighted that defensive blends often are highly variable at interspecific and intercolonial scales, both in quality and quantity, thus making the frontal gland chemistry an interesting tool for studies on taxonomy and biogeography (see e.g., Goh et al., 1984; Bagnères et al., 1990; Quintana et al., 2003; Perdereau et al., 2010).

Psammotermes, Desneux, 1902, is the most arid-adapted isopteran genus, living in arid areas of the Old World, namely Arabia, Sahara, and Sahel (*P. hybostoma* Desneux, 1902; *P. assuanensis* Sjoestedt, 1912; *P. fuscofemoralis* Sjoestedt, 1904; *P. senegalensis* Sjoestedt, 1924), South Africa (*P. allocerus* Silvestri, 1908), Madagascar (*P. voeltzkowi* Wasmann, 1910), and South-West Asia (*P. rajasthanicus* Roonwal & Bose, 1960). The nesting and foraging habits, as described e.g., by Grassé, (1984) for *P. hybostoma*, are reminiscent of advanced rhinotermitids with populous colonies that inhabit a large polycalic system of hypogeal constructions with peripheral centers in the feeding substrate (i.e., various cellulose-containing material, from dead trees, through wind-blown vegetal debris to animal feces) (Harris, 1970; Grassé, 1984). Despite their wide distribution and economic importance as pests of timber and crops, many aspects of *Psammotermes* biology remain poorly understood, especially the caste system (only a pronounced size polymorphism of workers and soldiers has been noted; Clément, 1952; Roonwal, 1988) and chemical ecology, including the composition of the frontal gland. Therefore, we are investigating some of these aspects, namely chemical communication (Sillam-Dussès et al., 2011), the caste system (Bourguignon, unpublished data), and the chemistry of defensive compounds from the frontal gland.

In this study, we report on the anatomy of the frontal gland and on the chemistry of the frontal gland secretion in soldiers of the sand termite, *Psammotermes hybostoma*, collected from nine colonies in five localities in Egypt. We compare, among colonies and localities, data on soldiers of different body sizes, and discuss results in a phylogenetic context.

Methods and Materials

Insect Origin and Sampling Nine fragments of *P. hybostoma* colonies were collected at five different localities (A–E, see Fig. 1) in the Nile Valley and Egyptian Western Desert in March 2010. Eight colony fragments were extracted from tamarisk wood, and one from a palm tree, and brought live to Prague. The colonies were held under laboratory conditions in their original wood at 26°C and at a low relative humidity. The colonies are hereafter denominated with the locality code (A–E), followed by the colony number.

Given the pronounced size polymorphism of *Psammotermes* soldiers, we aimed to interpret our findings with respect to body size. The observed variability in soldier size was extreme, with head widths ranging from 0.9 to 2 mm. Therefore, we defined three categories based on the head width (HW): small soldiers (SS; HW<1.1 mm), medium soldiers (MS; HW=1.1–1.5 mm), and large soldiers (LS; HW> 1.5 mm) (Fig. 2).

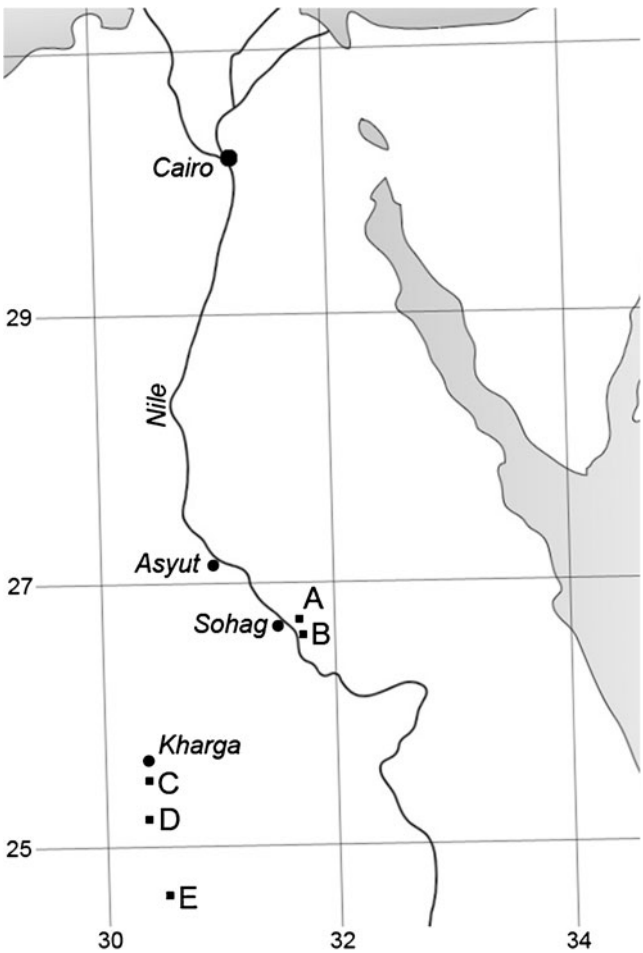

Fig. 1 Situation of the five localities, A–E, in the Nile Valley and Western Desert

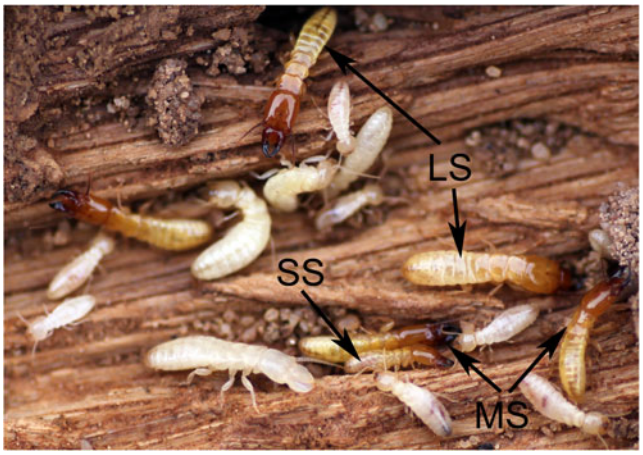

Fig. 2 A group of *Psammotermes hybostoma* termites (workers, soldiers, one presoldier) foraging in palm tree trunk. Soldiers of the three size categories considered in this paper are marked with arrows. Abbreviations: SS, small soldier; MS, medium soldiers; LS, large soldiers

Optical and Electron Microscopy Five soldiers of each size category were removed from colony B1, submerged in a drop of fixative (2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2), and dissected into head (mandibles carefully removed), thorax, and abdomen. After 24 h at 4°C, the samples were washed in 0.1 M phosphate buffer, post-fixed for 2 h in 1.5 % osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in an ethanol series, and embedded into standard Spurr resin. Subsequently, 1 μm-thick sections were cut with an Ultracut Reichert-Jung, stained with Azure II solution, and studied using a Carl Zeiss Amplival optical microscope equipped with a Canon EOS 500D camera. Ultrathin sections (50–80 nm) were stained using the standard protocol developed by Reynolds (1963) and observed with a JEOL 1011 transmission electron microscope. Entire soldier bodies were desiccated, gold-coated, and observed with a JEOL JSM-7401F scanning electron microscope.

Extracts Soldiers, freshly removed from colonies, were frozen at −20°C for 5 min. In order to open the frontal gland reservoir and extract its content, soldiers were dissected into head and rest of body, and extracted with distilled hexane (20 μl per soldier). The samples were shaken for 10 min. and stored at −20°C. Extracts of workers were prepared in the same way, but without the dissection.

The following extracts were prepared: i) extracts of 10 individual SS from colony A1 and 10 individual LS from colony E2 for evaluating intracolonial chemical diversity among soldiers of the same size category; ii) extracts of 5 soldiers of each size category from colony B1, for comparing chemical diversity among the three categories; iii) extracts of 10 soldiers from each colony, for identification and quantification, and intercolonial comparison; and iv) extracts of 10 workers from each of the colonies, to compare the profile of cuticular hydrocarbons among colonies and, thus, verify that all collections belonged to the same species; these extracts also allowed us to discriminate compounds specific to the frontal gland of soldiers from compounds present on the cuticle.

Gas Chromatography–Mass Spectrometry (GC-MS) Chemical identification and quantification were carried out by GC-MS (quadrupole DSQ II, Thermo Scientific) using a DB-5 column (30 m, id 0.25 mm, 0.25 μm film). The oven-temperature program was 50°C (1 min.) to 320°C (5 min.) at 7°C.min^{-1}. 1-Bromodecane (40 ng/μl) was added as an internal standard. Splitless injection was used, with helium as the carrier gas (1 ml.min^{-1}). Identification of compounds was based on comparison of retention indices and MS fragmentation patterns (70 eV, electron impact ionization) with published data and with synthetic and/or natural standards, as specified in Table 1. The retention indices of particular compounds were calculated using the retention times of *n*-alkanes (C10–C30).

Solid-Phase Microextraction (SPME) Several standard terpenes were extracted by SPME from natural sources: ar-curcumene and β-elemene were obtained, together with other sesquiterpenes, from ginger rhizome; valencene was obtained from Valencia orange peel. The samples (10 g) were heated at 50°C, and the volatile fraction adsorbed for 40 min. on a grey SUPELCO SPME fiber (50 μm DVB/CAR/PDMS coating) situated 5 cm above the heated sample. After this, the SPME fiber was injected into a GC and analysis performed under the conditions as for the hexane extracts.

Solvents and Standards β-Bisabolene and (*E*)-α-bisabolene were synthesized from (*Z*)-nerolidol (Sigma-Aldrich), using the same experimental conditions as described for synthesis from (*E*)-nerolidol by Svatoš and Attygale (1997). (*Z*)-γ-Bisabolene and (*E*)-γ-bisabolene were obtained from stored termite imagoes of *Prorhinotermes simplex* (Piskorski et al., 2009). Hexane was purchased from Merck and redistilled prior to use.

Statistics Differences in chemical composition of soldier extracts were evaluated using the formula of Nei (1972). The calculation was based on relative abundances of the 33 compounds listed in Table 1; the resulting matrix of Nei distances then was plotted as a cluster tree with the unweighted pair-group average clustering method (performed with Statistica 8).

Results

Anatomy of the Frontal Gland The frontal pore was located on the anterior frons, at the beginning of a shallow groove

Table 1 List of the frontal gland chemicals of *Psammotermes hybostoma* soldiers

No.	RI	Name	Ref.	Class	Colony										
					A1	A2	A4	B1 LS	B1 MS	B1 SS	C1	C2	D1	E1	E2
1	1188	*p*-methylacetophenone	1	ketone	-	-	-	-	-	-	tr	tr	+	+	tr
2	1394	iso-β-elemene	2	sesquiterpene	tr	tr	+	+	+	+	+	tr	tr	tr	+
3	1402	β-elemene	1,3	sesquiterpene	++	++	+++	++++	++++	++++	+++	+++	+++	+++	+++
4	1451	2-methylene-5-(1-methylvinyl)-8-methylbicyclo[5.3.0]decane	4	sesquiterpene	+	+	tr	tr	tr	tr	tr	tr	-	-	tr
5	1457	selina-5,11-diene	5	sesquiterpene	+	+	tr	tr	tr	tr	tr	tr	-	-	-
6	1464	α-helmiscapene	6	sesquiterpene	+++	+++	++	++	++	++	+	++	+	tr	tr
7	1473	unidentified		sesquiterpene	tr	tr	-	tr	tr	tr	-	-	-	-	-
8	1481	unidentified		sesquiterpene	tr	tr	tr	tr	tr	tr	tr	-	-	-	-
9	1488	*cis*-eudesma-6.11-diene	1	sesquiterpene	+	+	+	+	tr	tr	tr	tr	-	-	-
10	1489	ar-curcumene	1,3	sesquiterpene	-	-	-	-	-	-	-	tr	+	-	tr
11	1490	*cis*-β-guaiene	1	sesquiterpene	tr	tr	tr	tr	tr	tr	-	-	-	-	-
12	1495	selina-4.11-diene	5	sesquiterpene	-	-	-	-	-	-	++	+	++	+	++
13	1497	aristolochene	1	sesquiterpene	+	+	+	+	+	+	-	-	-	-	-
14	1500	eremophilene	5	sesquiterpene	tr	tr	tr	tr	tr	tr	tr	tr	-	-	-
15	1504	valencene	1,3	sesquiterpene	++++	++++	++++	+++	+++	+++	+++	+++	++	+	+
16	1515	β-bisabolene	1,7	sesquiterpene	-	-	-	-	-	-	+	++	+++	++	++
17	1518	germacrene A	1	sesquiterpene	tr	-	tr	tr	tr	+	-	-	-	-	-
18	1523	(*Z*)-γ-bisabolene	1,3	sesquiterpene	-	-	-	-	-	-	tr	tr	tr	tr	tr
19	1532	7-epi-α-selinene	1	sesquiterpene	tr	tr	+	tr	tr	tr	+	+	+	tr	+
20	1541	(*E*)-γ-bisabolene	1,3	sesquiterpene	-	-	-	-	-	-	+++	+++	+++	+++	+++
21	1549	(*E*)-α-bisabolene	1,7	sesquiterpene	-	-	-	-	-	-	tr	tr	+	+	+
22	1631	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	tr	tr	tr	tr	tr
23	1648	gossonorol	1	oxygenated sesquiterpene	-	-	-	-	-	-	tr	+	+	+	tr
24	1661	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	-	tr	tr	tr	tr
25	1668	unidentified		oxygenated sesquiterpene	-	tr	+	tr	tr	tr	tr	tr	tr	tr	tr
26	1679	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	-	tr	tr	+	-
27	1690	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	tr	+	+	+	tr
28	1713	nootkatol	1	oxygenated sesquiterpene	-	tr	tr	-	-	-	-	-	-	-	-
29	1756	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	tr	tr	tr	tr	tr
30	1796	unidentified		oxygenated sesquiterpene	-	-	-	-	-	-	tr	tr	+	+	tr
31	1824	nootkatone	1	oxygenated sesquiterpene	-	tr	tr	-	-	-	-	-	-	-	-
32	1986	hexadecenyl acetate	8	ester	+	-	tr	+	tr	tr	-	-	-	tr	-
33	2036	(*E*,*E*)-geranyllinalool	1	diterpene alcohol	tr	tr	tr	tr	+	tr	tr	tr	-	tr	-

Footnote: tr≤1 %; +≤5 %; ++<10 %; +++<50 %; ++++>50 %. RI – retention index, Ref. – the reference or chemical standard used for identification, 1 – Adams (2007), 2 – Kilic et al. (2004), 3 – natural standard, 4 – Cheng et al. (2005), 5 – Jouilan and König (1998), 6 – Adio et al. (2004), 7 – synthetic standard, 8 – Marques et al. (2000)

leading to the labrum (Fig. 3). The size of the frontal gland reservoir differed considerably among soldier categories; it reached as far as to the fourth abdominal segment in SS (Fig. 4B), while it was confined to the head in MS and LS (Fig. 4A).

The secretory epithelium consisted entirely of class 1 secretory cells, whereas there was an abundance of class 3 secretory cells around the fontanelle (Figs. 3 and 4A). This epithelium usually was between 5–20 μm thick (Fig. 4) and lined with a highly modified cuticle made of fibrous material, occurring in electron lucent and electron dense patches (see Fig. 4D). A single continuous electron-dense layer (about 25 nm thick) occurred at the cuticle apex. The apical plasma membrane formed irregular projections (Fig. 4D) supported with numerous microtubules. The basement membrane was formed by a single lamina, 0.5–0.75 μm thick, connected to the secretory cells by numerous focal contacts. Intercellular junctions consisted of a single apical zonula adherens, followed by a septate junction; gap junctions were rare. The membranes of neighboring cells were free in their basal halves. Nuclei were centrally located, irregular, about 5 μm in the largest

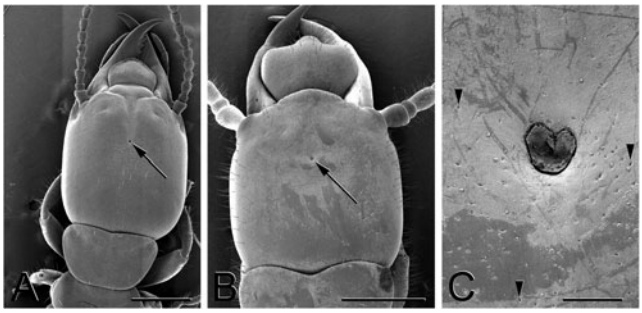

Fig. 3 Scanning electron microscopy of the head of *Psammotermes hybostoma* soldiers. **A.** Small soldier. The scale bar represents 0.5 mm. **B.** Large soldier. The scale bar represents 1 mm. **C**. Detail of the frontal pore in a large soldier. The scale bar represents 50 μm. The arrows mark the frontal pore; the arrowheads mark the openings of class 3 secretory cells

dimension. Secretory organelles included smooth and rough endoplasmic reticulum. Secretory vesicles were of two types, more abundant lipid-like droplets and less frequent electron-lucent vesicles; both originated from a conversion of large electron-dense granules (up to 3 μm diam.) and were released at the cell apex. Mitochondria were moderately abundant and often located next to the secretory vesicles.

Comparison of Cuticular Profiles, Specific Identity of the Collected Colonies Cuticular hydrocarbons profiles of workers from all colonies did not differ qualitatively in chemical composition, and only minor quantitative variations were observed (Fig. 5A). These results suggested that all collected colonies belonged to one species, *P. hybostoma*, widely distributed and abundant in the Sahara.

Chemical Composition of the Frontal Gland Secretion Soldier extracts were rich in volatiles that were lacking in the extracts of workers (Fig. 5B). Altogether, 33 compounds specific to soldiers were detected (Table 1). Most of these were sesquiterpenes (20) or oxygenated sesquiterpenes (10). Sesquiterpenes quantitatively dominated (over 95 % of) the extracts. In addition, a few other compounds, such as *p*-methylacetophenone, hexadecenyl acetate, and (*E*,*E*)-geranyllinalool, were identified as minor or trace compounds in some colonies. The method of identification of particular compounds is specified in Table 1. For most compounds, characteristic fragmentation patterns of mass spectra and retention indices allowed unambiguous identification based on published data. However, due to the high similarity of the mass spectra and retention indices of some sesquiterpenes, the identity of several compounds was confirmed using synthetic or natural standards. Nevertheless, the

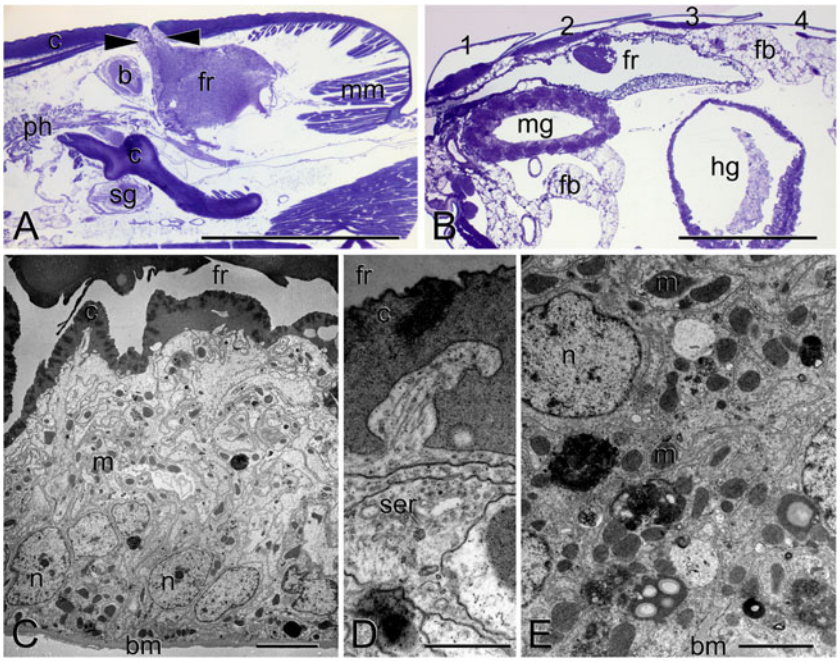

Fig. 4 Structure of the frontal gland in *Psammotermes hybostoma* soldiers. **A.** Sagittal section of a large soldier head. The arrowheads mark groups of class 3 secretory cells. The scale bar represents 1 mm. **B.** Sagittal section of the anterior abdomen of a small soldier. The scale bar represents 200 μm. **C.** Transmission electron microscopy of the frontal gland secretory epithelium in a small soldier. The scale bar represents 5 μm. **D.** Detail of the secretory cell apex. Note the electron lucent and electron dense patches in the cuticle as well as the irregular projection of the apical plasma membrane supported with microtubules. The scale bar represents 1 μm. **E.** The basal part of the secretory cell showing various interstages of secretory vesicle development. The scale bar represents 2 μm. Abbreviations: 1–4, abdominal segment 1–4; b, brain; bm, basement membrane; c, cuticle; fb, fat body; fr, frontal gland reservoir; hg, hindgut; m, mitochondria; mg, midgut; mm, mandibular muscles; n, nucleus; ser, smooth endoplasmic reticulum; sg, suboesophageal ganglium

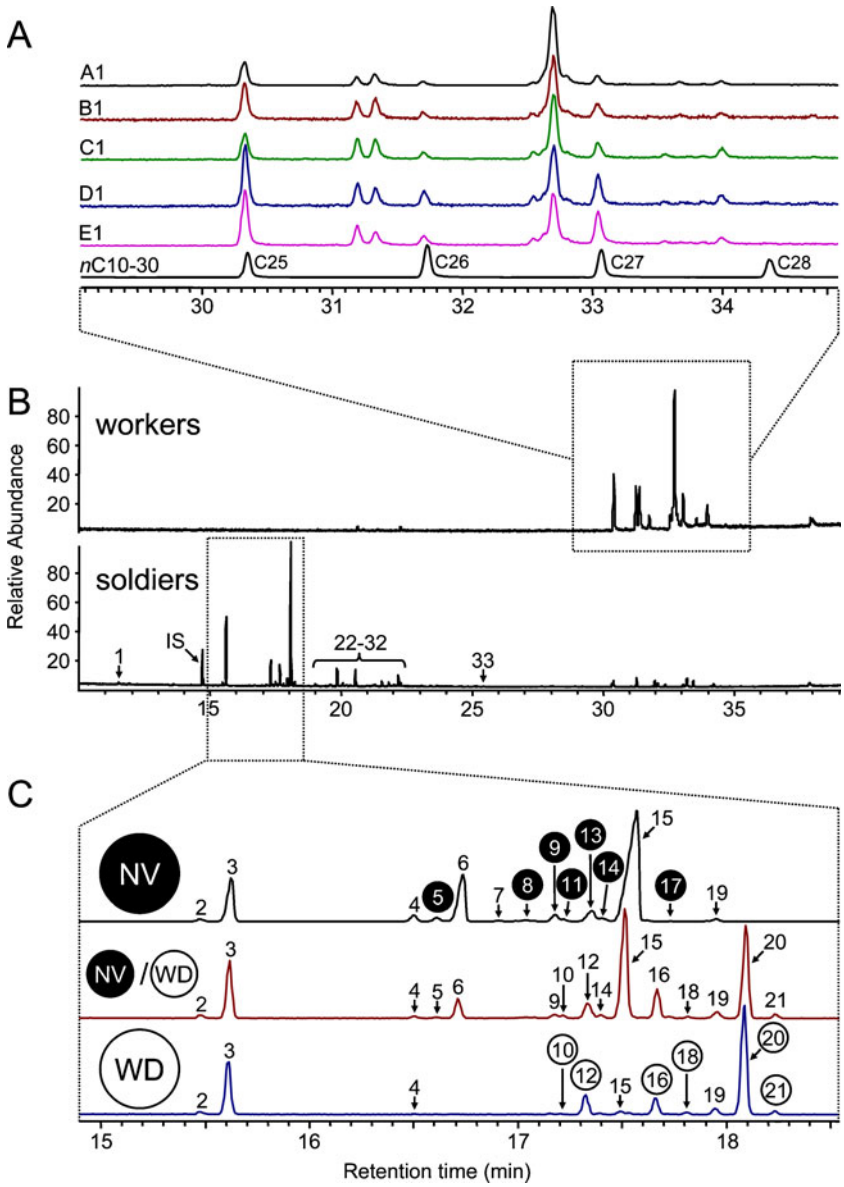

Fig. 5 Typical gas chromatograms of *Psammotermes hybostoma* workers and soldiers. **A.** Cuticular hydrocarbons of workers from one colony at each locality (A–E); for reference, a chromatogram of *n*-alkanes is shown. **B.** A characteristic gas chromatogram of a worker (colony B1) and of a soldier (colony E2). **C.** Gas chromatograms of soldier-specific sesquiterpenes, characteristic of the two distinct chemotypes (top: NV, colony B1, bottom: WD, colony E2), and the transitional situation observed in colony C2 (middle). Abbreviations: A1–E1, colony codes; IS, internal standard; NV, Nile Valley chemotype; WD, Western Desert chemotype. Peak numbers correspond to compounds listed in Table 1. The circles indicate sesquiterpenes exclusive to particular chemotypes

identification of β-elemene should be treated with caution, because of the risk of the possible Cope rearrangement of germacrene structures to elemenes under GC conditions (Takeda, 1974).

Total quantities of volatiles in individual soldiers were relatively high, ranging from tens to hundreds of micrograms per soldier, reflecting the relatively large size of *Psammotermes* soldiers. However, quantities were highly variable among individual soldiers, likely because of the differences in body size and in fullness of the gland reservoir. The maximum total quantities roughly corresponded to size differences among soldier categories, large (520 μg), medium (230 μg), and small soldiers (170 μg), and to the estimated sizes of their respective gland reservoirs. Hence, the quantities of particular compounds allowed a reliable relative quantification and comparison among individual soldiers.

Intracolonial Chemical Variability Chromatographic patterns of ten individual small soldiers from colony A1 and ten individual large soldiers from colony E2 were similar within each colony; the chemical dissimilarity between each pair of soldiers depicted in Fig. 6 was lower than 0.015 for both colonies. The same applies for the differences in relative abundances among large, medium and small soldiers, evaluated in colony B1. Thus, intracolonial chemical variability within particular size categories, and among the three size categories, can be considered as low.

Chemical Variability Between Colonies and Localities While the chromatographic patterns were alike within individual colonies, a higher level of variability was recorded among colonies from the same locality, as shown in Fig. 6 and Table 1. Colonies A1 and A2 were similar, with their

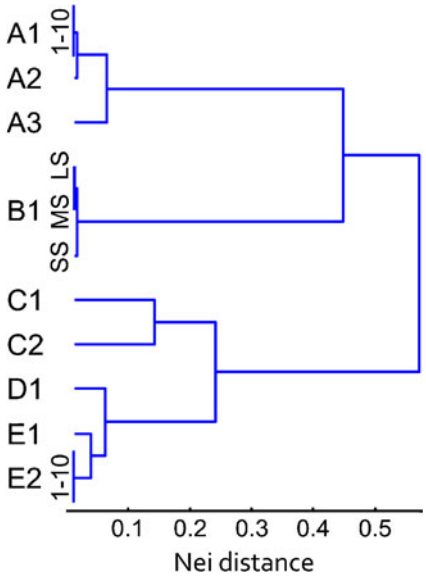

Fig. 6 Cluster tree depicting variability in relative abundances of 33 compounds listed in Table 1 among soldiers from nine colonies and five localities. In colonies A1 and E2, ten individuals were evaluated; in colony B1, three soldier categories were compared. The tree is constructed using unweighted pair-group average clustering method from the matrix of Nei distances

chemical difference being comparable to intracolonial variability. All other colonies were different from each other within the same locality (A1 + A2 vs. A3, C1 vs. C2, and E1 vs. E2), mostly due to quantitative differences in major compounds or qualitative differences in minor or trace components. The highest intercolonial chemical difference was observed between the two colonies from locality C, owing to the quantitative shift in the two major sesquiterpenes, β-elemene and valencene (34 and 19 % in C1 vs. 15 and 31 % in C2). Colonies E1 and E2, extracted from the tamarisk and palm trees, respectively, did not differ more than other pairs of colonies from one locality.

Fundamental differences were observed among localities. Quantitative patterns of relative abundances account for the differences between localities A and B from the Nile Valley, mostly due to the shift between valencene and β-elemene, the former being dominant in locality A (62± 3 %) and the latter being the major compound in locality B (59±5 %). Chemical differences between localities D and E from the Western Desert were low, a result of slight quantitative variations. In contrast, locality C can be distinguished easily from the two other localities, based on relative abundances of four major sesquiterpenes, β-elemene, valencene, β-bisabolene, and (*E*)-γ-bisabolene; i.e., 24±5, 25±6, 5±2, and 24±1 %, respectively, in colonies C1+C2, contrasting with 18±4, 4±1, 10±3, and 43±7 % in colonies D1+E1+E2.

A clear distinction can be made between colonies from the Nile Valley and those from the Western Desert, representing two dramatically different chemotypes, hereafter called NV and WD chemotypes. Each of the chemotypes is characterized by the presence of exclusive compounds, such as aristolochene and germacrene A in the NV phenotype, and ar-curcumene, selina-4,11-diene, four bisabolene isomers, and several oxygenated sesquiterpenes in the WD chemotype. The differences between the two chemotypes are apparent from the representative chromatograms depicted in Fig. 5C and peaks listed in Table 1.

Discussion

The frontal gland of *Psammotermes hybostoma* is among the smallest of all Rhinotermitidae (Šobotník et al., 2010). Along the continuum of soldier body sizes, the gland reservoir is developed in two modal forms. In small soldiers, it reaches deep into the abdomen, as in numerous other Rhinotermitidae, while in medium and large soldiers it resembles that of *Termitogeton*, the only rhinotermitid with the gland situated only in the head (Quennedey, 1984). The ultrastructure of the gland corresponds to the description by Quennedey (1984), and is similar to that in *Prorhinotermes simplex* (Šobotník et al., 2004), especially in the following traits: the structure of the glandular cuticle, irregular projections of the apical plasma membrane, scarce secretory organelles, and the process of secretion formation.

The total amount of defensive volatiles increases with body size; small soldiers may contain up to 170 μg, while the largest soldiers may contain as much as 500 μg. However, the relative quantity of defensive chemicals was much smaller than in most other rhinotermitids, e.g., *Coptotermes* or *Prorhinotermes* (Waller and La fage, 1987; Hanus et al., 2006). Despite these dramatic size differences among soldiers, we did not observe any chemical polymorphism. Therefore, the size variability is unlikely to be linked with a differential use of the frontal gland.

We observed a trend in chemical variability among colonies and localities. Intercolonial variability at one locality was usually low. Very similar chemical composition was recorded in colonies A1 and A2; these two samples likely represented two fragments of one large colony, given the distance of only a few tens of meters between the two sites. Other colonies had slightly different quantitative patterns of volatiles from each other at the same locality. However, the five localities showed two fundamentally different chemotypes. All colonies from the Nile Valley were of the NV chemotype, while all colonies from the Western Desert (25 km from the Nile Valley) were the WD chemotype. By our own sense of smell (unpublished results) we could distinguish samples from each colony (5 soldiers in a Petri dish) with respect to chemotype.

Aside from the two chemotypes, more subtle trends can be seen. In colonies C1 and C2, even though they belonged to the WD chemotype, characteristics of the NV chemotype are apparent, such as the presence of selina-5,11-diene and *cis*-eudesma-6,11-diene, or a higher proportion of valencene. This introgression of the NV chemotype into the WD chemotype corresponds well with the central position of locality C. Moreover, this locality was situated in the suburbs of a large Kharga oasis with a great probability of a passive transport of colony fragments or alates with human traffic. Colony D1, collected close to C locality but outside the urban area, corresponded much better with colonies E1 and E2.

Interspecific differences in frontal gland chemistry are well known (Šobotník et al., 2010) and, along with cuticular hydrocarbons, can be used as chemotaxonomic markers (e.g., Bagnères et al., 1990). Conspicuously different chemotypes also have been noted within species (e.g., Valterová et al., 1989) or even within a single chemotype of cuticular hydrocarbons (Bagnères et al., 1990; Perdereau et al., 2010), thus allowing the evaluation of another level of chemodiversity when disentangling the relatedness and dispersal history of populations or sibling species. Our results represent another example of intraspecific polymorphism in biosynthetic pathways of defensive compounds, independent of cuticular hydrocarbon chemotypes and feeding substrate, and subject to genetic mixing among populations.

The richness and variability of the sesquiterpenoid secretion suggest that its defensive potential results from the complex blend, rather than from the individual compounds. Among the biological functions attributed to terpenoids, their use as deterrents, repellents and irritants is widespread (e.g., Gershenzon and Dudareva, 2007). Multiple explanations have been proposed for the occurrence of terpenoids in mixtures, including the optimization of physicochemical properties, enlargement of the spectrum of target organisms, decreased likelihood of development of resistance to them, and functional constraints of biosynthetic pathways (Fischbach and Clardy, 2007; Gershenzon and Dudareva, 2007). It is interesting to note that the sesquiterpenes characteristic for each of the two chemotypes typically have a common biogenesis, differing only in the final steps; e.g., the bisabolene isomers and their oxygenated derivatives in WD, or valencene, nootkatol and nootkatone in NV.

Terpenoid mixtures are common in termite frontal glands (see Šobotník et al., 2010), and their repellent and toxic properties have been confirmed experimentally, especially sesquiterpene hydrocarbons (Scheffrahn et al., 1983) and oxygenated sesquiterpenes (Wadhams et al., 1974; Zhu et al., 2003). A strong toxicity has been shown for the diterpene geranyllinalool, frequently found in *Reticulitermes* sp. (Lemaire et al., 1990) and occurring in trace quantities in *Psammmotermes*. Hence, we hypothesize that the sesquiterpene mixtures of *Psammotermes* have a repellent and/or irritant function, or participate in unpalatability. In addition, sesquiterpenes can act as alarm pheromones, such as in *Prorhinotermes* (Šobotník et al., 2008) or *Reticulitermes* (Reinhard et al., 2003). Due to the limited survival of *Psammotermes* in captivity, we were not able to test their function.

In Rhinotermitidae, sesquiterpenes have been recorded in soldiers of several *Reticulitermes* species, as well as in soldiers and imagoes of *Prorhinotermes*, always as a mixture with other classes of compounds (Quintana et al., 2003; Hanus et al., 2006; Piskorski et al., 2007, 2009). Several recent phylogenetic hypotheses situate *Psammotermes* as a sister or closely related taxon to *Prorhinotermes* (Austin et al., 2004; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007). Despite the dramatic differences in ecology, the close relationship of the two genera is supported by similarities in anatomy of the frontal gland (Šobotník et al., 2004), by the unique shape of the nymphal wing buds (Štys and Šobotník, 1999, Bourguignon, unpublished data), and by the primitive type of caste development, which lacks an early divergence of a permanent worker caste (Roisin, 1988; Bourguignon, unpublished data).

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References

ADAMS, R. P. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. Allured Publishing Corporation, USA.

ADIO, A. M., PAUL, C., TESSO, H., KLOTH, P., and KÖNIG, W. A. 2004. Absolute configuration of helminthogermacrene. *Tetrahedron: Asymmetry* 15:1631–1635.

AUSTIN, J. W., SZALANSKI, A. L., and CABRERA, B. J. 2004. Phylogenetic Analysis of the Subterranean Termite Family Rhinotermitidae (Isoptera) by Using the Mitochondrial Cytochrome Oxidase II Gene. *Ann. Entomol. Soc. Am.* 97:548–555.

BAGNÈRES, A.-G., CLÉMENT, J.-L. A., BLUM, M. S., SEVERSON, R. F., JOULIE, C., and LANGE, C. 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Feytaud): polymorphism and chemotaxonomy. *J. Chem. Ecol.* 16:3213–3244.

CHENG, S. S., LIN, H. Y., and CHANG, S. T. 2005. Chemical composition and antifungal activity of essential oils from different tissues of Japanese Cedar (*Cryptomeria japonica*). *J. Agric. Food Chem.* 53:614–9.

CLÉMENT, G. 1952. Recherches sur le polymorphisme de *Psammotermes hybostoma* Desneux. *Ann. Sci. Nat. Zool.* 14:95–116.

CRESPI, B. J. 1994. Three conditions for the evolution of eusociality: Are they sufficient? *Insectes Soc.* 41:395–400.

DELIGNE, J., QUENNEDEY, A., and BLUM, M. S. 1981. The enemies and defense mechanisms of termites, pp. 1–76, *in* H. R. Hermann (ed.), Social insects, Vol. 2. Academic, New York, London etc.

FISCHBACH, M. A. and CLARDY, F. 2007. One pathway, many products. *Nat. Chem. Biol.* 3:353–355.

GERSHENZON, J. and DUDAREVA, N. 2007. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3:408–14.

GOH, S. H., CHUAH, C. H., THO, Y. P., and PRESTWICH, G. D. 1984. Extreme intraspecific chemical variability in soldier defense secretions of allopatric and sympatric colonies of *Longipeditermes longipes*. *J. Chem. Ecol.* 10:929–944.

GRASSÉ, P. P. 1984. Termitologia: anatomie, physiologie, biologie, systématique des termites. Tome 2. Fondation des sociétés - construction. Masson, Paris, New York etc.

HANUS, R., ŠOBOTNÍK, J., VALTEROVÁ, I., and LUKÁŠ, J. 2006. The ontogeny of soldiers in *Prorhinotermes simplex* (Isoptera, Rhinotermitidae). *Insectes Soc.* 53:249–257.

HARRIS, W. V. 1970. Termites of palearctic region, pp. 295–313, *in* K. Krishna and F. M. Weesner (eds.), Biology of Termites, Vol. 2. Academic, London & New York.

INWARD, D. J. G., VOGLER, A. P., and EGGLETON, P. 2007. A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Mol. Phylogenet. Evol.* 44:953–967.

JOUILAN, D. and KÖNIG, W. A. 1998. The Atlas of Spectral Data of Sesquiterpene Hydrocarbons. E. B.-Verlag, Hamburg.

KILIC, A., HAFIZOGLU, H., KOLLMANNSBERGER, H., and NITZ, S. 2004. Volatile constituents and key odorants in leaves, buds, flowers, and fruits of *Laurus nobilis* L. *J. Agric. Food Chem.* 52:1601–1606.

LEMAIRE, M., NAGNAN, P., CLÉMENT, J.-L. A., LANGE, C., PERU, L., and BASSELIER, J. J. 1990. Geranyllinalool (diterpene alcohol). An insecticidal component of pine wood and termites (Isoptera: Rhinotermitidae) in four European ecosystems. *J. Chem. Ecol.* 16:2067–2079.

LO, N., KITADE, O., MIURA, T., CONSTANTINO, R., and MATSUMOTO, T. 2004. Molecular phylogeny of the Rhinotermitidae. *Insectes Soc.* 51:365–371.

MARQUES, F. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Kováts retention indexes of monounsaturated C12, C14, and C16 alcohols, acetates and aldehydes commonly found in lepidopteran pheromone blends. *J. Braz. Chem. Soc.* 11:592–599.

NEI, M. 1972. Genetic distance between populations. *Am. Nat.* 106:283–292.

OHKUMA, M., YUZAWA, H., AMORNSAK, W., SORNNUWAT, Y., TAKEMATSU, Y., YAMADA, A., VONGKALUANG, C., SARNTHOY, O., KIRTIBUTR, N., NOPARATNARAPORN, N., KUDO, T., and INOUE, T. 2004. Molecular phylogeny of Asian termites (Isoptera) of the families Termitidae and Rhinotermitidae based on mitochondrial COII sequences. *Mol. Phylogenet. Evol.* 31:701–710.

PERDEREAU, E., DEDEINE, F., CHRISTIDES, J. P., and BAGNÈRES, A.-G. 2010. Variations in Worker Cuticular Hydrocarbons and Soldier Isoprenoid Defensive Secretions Within and Among Introduced and Native Populations of the Subterranean Termite, *Reticulitermes flavipes*. *J. Chem. Ecol.* 36:1189–1198.

PISKORSKI, R., HANUS, R., KALINOVÁ, B., VALTEROVÁ, I., KŘEČEK, J., and ŠOBOTNÍK, J. 2009. Chemical composition of the frontal gland secretion in imagoes of the termite genus *Prorhinotermes* (Isoptera: Rhinotermitidae): geographic and temporal variations. *Biol. J. Linnean Soc.* 98:384–392.

PISKORSKI, R., HANUS, R., VAŠÍČKOVÁ, S., CVAČKA, J., ŠOBOTNÍK, J., SVATOŠ, A., and VALTEROVÁ, I. 2007. Nitroalkenes and sesquiterpene hydrocarbons from the frontal gland of three *Prorhinotermes* termite species. *J. Chem. Ecol.* 33:1787–1794.

PRESTWICH, G. D. 1984. Defense mechanisms of termites. *Annu. Rev. Entomol.* 29:201–232.

QUENNEDEY, A. 1984. Morphology and ultrastructure of termite defense glands, pp. 151–200, *in* H. R. Hermann (ed.), Defensive Mechanisms in Social Insects. Praeger, New York, Philadelphia etc.

QUINTANA, A., REINHARD, J., FAURE, R., UVA, P., BAGNÈRES, A.-G., MASSIOT, G., and CLÉMENT, J.-L. A. 2003. Interspecific variation in terpenoid composition of defensive secretions of European *Reticulitermes* termites. *J. Chem. Ecol.* 29:639–652.

REINHARD, J., QUINTANA, A., SRENG, L., and CLÉMENT, J.-L. A. 2003. Chemical signals inducing attraction and alarm in European *Reticulitermes* termites (Isoptera, Rhinotermitidae). *Sociobiology* 42:675–691.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. *J. Cell Biol.* 17:208–12.

ROISIN, Y. 1988. Morphology, development and evolutionary significance of the working stages in the caste system of *Prorhinotermes* (Insecta, Isoptera). *Zoomorphology* 107:339–347.

ROISIN, Y. 2000. Diversity and Evolution of Caste Patterns, pp. 95–119, *in* T. Abe, D. E. Bignell, and M. Higashi (eds.), Termites: Evolution, Sociality, Symbioses. Ecology. Kluwer Academic Publishers, London.

ROONWAL, M. L. 1988. Field ecology and soldier polymorphism in the Indian sand termite, *Psammotermes rajasthanicus* (Rhinotermitidae, Psammotermitinae). *Indian J. Ent.* 47:455–460.

SCHEFFRAHN, R. H., GASTON, L. K., SIMS, J. J., and RUST, M. K. 1983. Identification of the defensive secretion from soldiers of the North American termite, *Amitermes wheeleri* (Desneux) (Isoptera: Termitidae). *J. Chem. Ecol.* 9:1293–1305.

SILLAM-DUSSÈS, D., HANUS, R., ABD EL-LATIF, A. O., JIROŠ, P., KRASULOVÁ, J., KALINOVÁ, B., VALTEROVÁ, I., and ŠOBOTNÍK, J. 2011. Sex pheromone and trail pheromone of the sand termite *Psammotermes hybostoma* (Rhinotermitidae: Psammotermitinae). *J. Chem. Ecol.* 37:179–188.

SVATOŠ, A. and ATTYGALE, A. B. 1997. Characterization of Vinyl-Substituted, Carbon-Carbon Double Bonds by GC/FT-IR Analysis. *Anal. Chem.* 69:1827–1836.

ŠOBOTNÍK, J., WEYDA, F., HANUS, R., KYJAKOVÁ, P., and DOUBSKÝ, J. 2004. Ultrastructure of the frontal gland in *Prorhinotermes simplex* (Isoptera: Rhinotermitidae) and quantity of the defensive substance. *Eur. J. Entomol.* 101:153–163.

ŠOBOTNÍK, J., HANUS, R., KALINOVÁ, B., PISKORSKI, R., CVAČKA, J., BOURGUIGNON, T., and ROISIN, Y. 2008. (*E, E*)-α-Farnesene, an Alarm Pheromone of the Termite *Prorhinotermes canalifrons*. *J. Chem. Ecol.* 34:478–486.

ŠOBOTNÍK, J., HANUS, R., and JIROŠOVÁ, A. 2010. Chemical warfare in termites. *J. Insect Physiol.* 56:1012–1021.

ŠTYS, P. and ŠOBOTNÍK, J. 1999. Comments on classifications of insect ontogenies, and occurrence of proneometabolous wing development in termite genus *Prorhinotermes* (Hexapoda: Isoptera). *Acta Soc. Zool. Bohem.* 63:483–492.

TAKEDA, K. 1974. Stereospecific cope rearrangement of the germacrene-type sesquiterpenes. *Tetrahedron*. 30:1525–1534.

VALTEROVÁ, I., KŘEČEK, J., and VRKOČ, J. 1989. Intraspecific variation in the defence secretions of *Nasutitermes ephratae* soldiers and the biological activity of some of their components. *Biochem. Syst. Ecol.* 17:327–332.

WADHAMS, L. J., BAKER, R., and HOWSE, P. E. 1974. 4,11-Epoxy-*cis*-eudesmane: A novel oxygenated sesquiterpene in the frontal gland secretion of the termite *Amitermes evuncifer* Silvestri. *Tetrahedron Lett.* 18:1697–1800.

WALLER, D. A. and LA FAGE, J. P. 1987. Unpalatability as a defense of *Coptotermes formosanus* Shiraki soldiers against ant predation. *J. Appl. Entomol.* 103:148–153.

ZHU, B. C., HENDERSON, G., SAUER, A. M., YU, Y., CROWE, W., and LAINE, R. A. 2003. Structure-activity of valencenoid derivatives and their repellence to the Formosan subterranean termite. *J. Chem. Ecol.* 29:2695–2701.

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Sex-Pairing Pheromone in the Asian Termite Pest Species *Odontotermes formosanus*

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Abstract The sex-pairing pheromone of the black winged subterranean termite, *Odontotermes formosanus* (Shiraki) (Isoptera, Termitidae), was investigated using headspace-SPME, GC-MS, GC-EAD, and attraction bioassays. Females secrete the pheromone from their sternal gland to attract males. The sex-pairing pheromone is composed of (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol, estimated at 9 to 16.64 ng and 0.2 to 0.54 ng, respectively. Both short- and long-distance sex attraction bioassays were employed to show that these compounds act in synergy at long distance, but only (*Z,Z*)-dodeca-3,6-dien-1-ol is active at short distance. The pheromone may be useful in efforts to control this pest, which is considered one of the most harmful termite species in Southeast Asia.

Keywords Sternal gland · (*Z*)-dodec-3-en-1-ol · (*Z,Z*)-dodeca-3,6-dien-1-ol · GC-EAD · Olfactometer · SPME · Isoptera · Termitidae · Macrotermitinae · Pest

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Introduction

The establishment of a new colony is a fundamental step in the life history of termites. In most species, it consists of a mass exodus of winged reproductive forms during swarming. This dispersal flight of alates usually is followed by dealation, and courtship consisting of calling and tandem behaviors with some important variations among species (Nutting, 1969; Myles, 1988). Courtship is facilitated by sex-pairing pheromones that play a key role in both aspects.

Because of the seasonal occurrence of the swarming, there have been few studies of termite sex-pairing pheromones. However, (*Z,Z,E*)-dodeca-3,6,8-trien-1-ol, (*Z,Z*)-dodeca-3,6-dien-1-ol, and neocembrene have been identified as sex-pairing pheromones in some termites species (reviewed in Bordereau and Pasteels, 2011). Recently, female sex-pairing pheromones of *Zootermopsis angusticollis* and *Z. nevadensis* have been identified as (5*E*)-2,6,10-trimethylundeca-5,9-dienal, and the male sex-pairing pheromones as *syn*-4,6-dimethyldodecanal (Bordereau et al., 2010). Even more recently, the major sex-specific compound of *Hodotermopsis sjostedti* females was shown to be (5*E*)-2,6,10-trimethylundeca-5,9-dienal, while *syn*-4,6-dimethylundecanal, a homolog of *syn*-4,6-dimethyldodecanal, is the male sex-pairing pheromone (Lacey et al., 2011). Finally, in *Cornitermes* spp. (*Z,Z,E*)-dodeca-3,6,8-trien-1-ol alone is the sex-pairing pheromone in *C. bequaerti*, whereas this compound is associated with (*E*)-nerolidol in *C. cumulans*, and with (*E*)-nerolidol and (*Z*)-dodec-3-en-1-ol in *C. silvestrii* (Bordereau et al., 2011).

Interestingly, termite sex-pairing pheromone compounds often are not species-specific. The parsimonious use of sex-pairing pheromone components also is evident for worker trail-following pheromones since many compounds used as sex-pairing pheromones also are used as trail-following

Fig. 1 A dealated *Odontotermes formosanus* calling female (*arrow* indicates the exposed sternal gland)

pheromones in termites. Moreover, only three exocrine glands are known to release sex-pairing pheromones: the sternal gland, the tergal glands, and the posterior sternal glands (Bordereau and Pasteels, 2011).

Although the mechanical activity of termites has a beneficial effect on renewal of organic matter and the soil environment (Holt and Lepage, 2000; Evans et al., 2011), about two hundred termite species are economically important due to their damage and subsequent repair costs. Among them, *Odontotermes formosanus* is considered one of the most important termite pests of dykes, dams, plantations, and forests in China, costing hundreds of millions of dollars annually. For example, many flood disasters in South China have been caused by dyke and dam breaks due to this termite (Huang et al., 2000). Recently, a field study was performed in order to test the effects of toxic baits on *O. formosanus* colonies with promising results (Huang et al., 2006). An alternative termite pest management technique could be the use of sex-pairing pheromones in mass trapping and mating disruption. Therefore, we investigated the chemical nature of the sex-pairing pheromone of *O. formosanus* using headspace solid phase microextraction (SPME), gas chromatography (GC), GC-mass spectrometry (MS), GC-electroantennographic detection (EAD), and short- and long-distance attraction bioassays.

Methods and Materials

Insects *Odontotermes formosanus* belongs to the most advanced termite subfamily, Macrotermitinae, the fungus-growing termites; it occurs in Southeast Asia, mainly South China (Huang et al., 2000; Zhong and Liu, 2002; Cheng et al., 2007). *Odontotermes formosanus* builds large subterranean nests with cavities linked by a network of galleries. Swarms occur every 2 or 3 years (Hu et al., 2007), usually during a rainy evening from late spring to early summer when the daily minimum temperature reaches 21 °C (Liu et al., 1985). After the flight, the female raises her abdomen and exposes her sternal gland to release sex-pairing pheromone during the characteristic calling behavior (Fig. 1). Once the male has found the female, the two reproductives exhibit a tandem behavior in which the male follows the female (Fig. 2). Dispersing alates were collected in the arboretum and at the dykes at the Nanjing Forestry University (Nanjing, China) using ultra-violet light traps (360 nm) or nylon sieve traps. Wings were removed within 1 hr after collection. Unwounded females and males were kept in separate Petri dishes with clean damp filter paper at 23 °C in darkness.

Extracts of Females Females were frozen at −40 °C, and the abdominal integument was cut into three parts using a sterile scalpel; the 8th to the last abdominal segments (AE), the 2nd to the 7th sternal segments (S), and the 1st to the 7th tergal segments (T). The fresh dissections were used directly in bioassays either on dry or damp (15 μl of bi-distilled water) filter papers, or were extracted in hexane for 3 hr prior to bioassays.

Gland Extracts The sternal gland is present in females and males; they were dissected from cold anesthetized imagoes under a stereomicroscope with microscissors by gently pressing the abdomen to expose the glands. The sternites with the attached gland were removed, and extracted in hexane for 3 hr prior to bioassays or chemical analyses.

Standards (*Z*)-Dodec-3-en-1-ol was synthesized by IU-CHEM chemical company (Shanghai, China), and (*Z*,*Z*)-dodeca-3,6-dien-1-ol was synthesized as described in Robert et al. (2004).

Solid Phase Microextraction (SPME) Living imagoes were held under a clean airflow with forceps under a stereomicroscope to expose their sternites. A 65-μm PDMS/DVB fiber (Supelco, USA) was used to rub the inter-sternal space between 4th and 5th sternites. The fiber then was desorbed in the injection port of a gas chromatograph (GC) or GC-mass spectrometer (MS). The same method was used to rub the inter-tergal space of the abdomen of other imagoes as a control. A range of synthetic (*Z*,*Z*)-dodeca-3,6-dien-1-ol (1 μl of 0.1, 1, 10, 20, 50, 100, 1000 ng/μl) was used for quantification of termite (*Z*,*Z*)-dodeca-3,6-dien-1-ol. Since (*Z*,*Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol have similar elemental compositions, the (*Z*,*Z*)-dodeca-3,6-dien-

Fig. 2 An *Odontotermes formosanus* female followed by a male in tandem

1-ol/(*Z*)-dodec-3-en-1-ol ratios in extracts were calculated, and the quantities of (*Z*)-dodec-3-en-1-ol were determined based on these ratios.

Headspace-SPME One female was placed in a 10-ml vial with a humidified airflow (1.5 L/min). A 65-μm PDMS/DVB SPME fiber was inserted into the vial 1 cm from the female allowing the adsorption of volatile compounds until calling behavior ceased. Subsequently, the fiber was removed and inserted into the GC or GC-MS. Controls were performed with non-calling females, and with empty clean vials following the above procedure. A range (5, 10, 15, 20, 50, 100 ng) of synthetic (*Z*,*Z*)-dodeca-3,6-dien-1-ol was applied to a round piece of filter paper (1.0 cm, od) impregnated with 15 μl of bi-distilled water, and extracted in a time series (1, 5, 10, 15, 20, 25 min) for quantification. (*Z*)-Dodec-3-en-1-ol was not detectable in the headspace extraction of females; therefore, an estimate was made from the ratio analyzed by SPME.

Gas Chromatography (GC) GC analyses were carried out on an HP6890 GC system (Agilent Technologies, US). The injection port (250 °C) was in the splitless mode, and the flame ionization detector (FID) was heated to 250 °C. Two capillary columns were used with N_2 (37 cm/s) as carrier gas; a (5 %-phenyl)-methylpolysiloxane HP-5 column (30 m×0.32 mm, 0.25 μm, Agilent J&W; 100 °C (1 min) to 275 °C at 5 °C/min), and a polyethylene glycol DB-WAX column (30 m×0.32 mm, 0.25 μm, Agilent J&W; 40 °C (2 min) to 240 °C at 15 °C/min with a 10-min final hold).

Gas Chromatography–Mass Spectrometry (GC-MS) GC-MS analyses were performed on an HP7890A-5975C system (Agilent Technologies, US) with the injector at 250 °C in the splitless mode. Analyses were performed using an HP-5ms column (30 m×0.25 mm, 0.25 μm, Agilent Technologies; 37 cm/s He as carrier gas, temperature programmed from 40 °C (2 min) to 260 °C at 4 °C/min with a 10-min hold), and a DB-WAX column (30 m×0.32 mm, 0.25 μm, Agilent J&W; 42 cm/s He as carrier gas, temperature programmed from 40 °C (2 min), to 240 °C at 15 °C/min with a 10-min hold). Temperatures of the transfer line, ion source and quadrupole were 250, 230, and 150 °C, respectively. Electron ionization (EI) mass spectra were recorded (with a 4-min solvent delay) at 70 eV over the range *m/z* 25 to 350 with the threshold abundance of 10.

Gas Chromatography Coupled with Electroantennographic Detector (GC-EAD) A quarter of the head with an antenna was cut from imagoes, and the terminal flagellum was cut open vertically with a razor blade. The dissected antenna was fixed between two Ag/AgCl electrodes. The glass pipettes of the electrodes were filled with saline solution (NaCl 9.0 g/L, KCl 0.71 g/L, $CaCl_2$ 0.46 g/L). Polyvinyl pyrrolidone (20 g/L) was added to the saline solution to prevent evaporation of water at the tip of the pipettes. The electrodes were connected to an IDAC-2 (Syntech, NL) data collector through an EAG Kombi-Probe (Syntech, NL). Samples were fractionated on an HP6890N GC system (Agilent Technologies, US). Elution was conducted to FID and EAD in a 1:1 split ratio. A DB-WAX capillary GC column (as described above) was used with splitless injection, programmed from 100 °C (1 min) to 250 °C at 15 °C/min with a 6-min final hold. FID and EAD transfer-lines were heated to 250 °C.

Short-Distance Attraction Bioassay Bioassays were conducted at 25 °C, RH>80 % and 130 lux white light illumination. They consisted of introducing a dealate in a 15-cm Petri dish containing a piece of round filter paper (1.0 cm, od). Before the introduction of the dealate, the piece of paper was impregnated with 15 μl of bi-distilled water (damp conditions) or not (dry conditions), and then with 5 μl of extract, standard, or hexane control. The paper disc was placed in the Petri dish after solvent evaporation. The duration of the first contact of the dealate with the paper was recorded. Only the first contact was considered so as to avoid contamination of the paper by the insect. After each test, the Petri dish was cleaned and a new piece of paper and new dealates were used for the next test. Results were analyzed using *Student's T-test*. The *Tukey's LSD* method was applied for multiple comparison analysis. Quantification of pheromone compounds was performed by comparing behavioral responses to the gland extracts with those to standards.

Long-Distance Attraction Bioassay These bioassays were preformed in a Y-shape olfactometer (Fig. 3). The core of the apparatus consisted of a glass Y-tube, and two odor source flasks. A silicone rubber tube was used to balance flow to the arms. The apparatus was placed on sponge cushions to reduce the vibrations. Teflon tubes were used to conduct the airflow. Damp rounded pieces of filter paper (1.0 cm, od) impregnated with 5 μl of an extract, a standard, or hexane control were placed in the flasks for comparisons. A dealate was introduced in the inlet chamber made of a nylon sieve and a glass tube (12 mm, id) to prevent the individual from moving before the beginning of the experiment. Once the airflow was turned on, the individual was allowed into the base of the Y-tube, and then to choose one arm or the other (Fig. 3). The distance traveled from the starting point to the odor source was 30 cm. Results were analyzed with a χ^2 *test*. The glass components were cleaned after each test (ultrasonic washing, 10 times bi-distilled water washing, and 210 °C heating for 30 min). Quantification of pheromone compounds was performed by comparing the

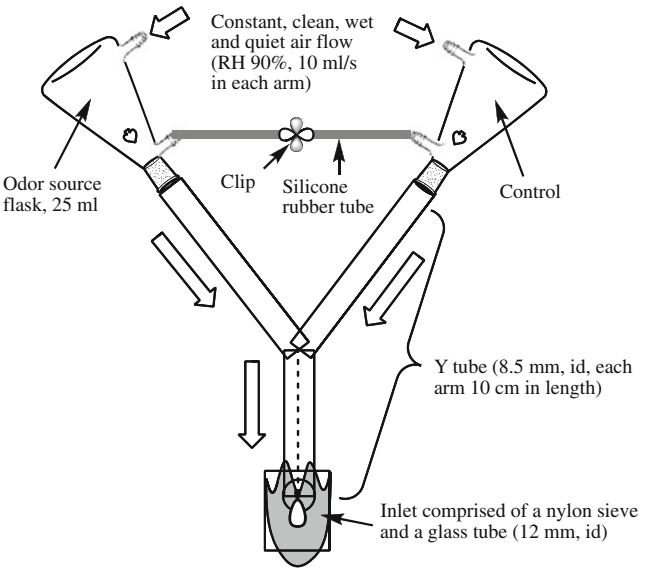

Fig. 3 Setup of Y-shape olfactometer test. A piece of round filter paper was impregnated with 5 μl of an extract, standard, or hexane (as a control), and placed in each flask at the end of the arms (*open arrows* indicate airflow). The dealate was free to go into the base of the olfactometer, and then to one of the two arms

behavioral responses to the standards and to the gland extracts.

Results

Origin of Sex-Pairing Pheromone The origin of the sex-pairing pheromone was determined by the short-distance attraction bioassay. Damp sternal extracts (S; 0.1 female-equivalents) showed a strong attraction toward males (30.9± 10.1 sec, N=16), while other extracts (AE and T) did not show any activity (Fig. 4). Interestingly, males were very excited, and ran quickly around the Petri dish with S extract at 10 times higher concentration. Bioassays performed with dry samples did not show any significant activity. In addition, sternal glands dissected from females were attractive to males, whereas abdomens without this gland elicited no attraction (10.4± 2.4 sec, N=32, P<0.001).

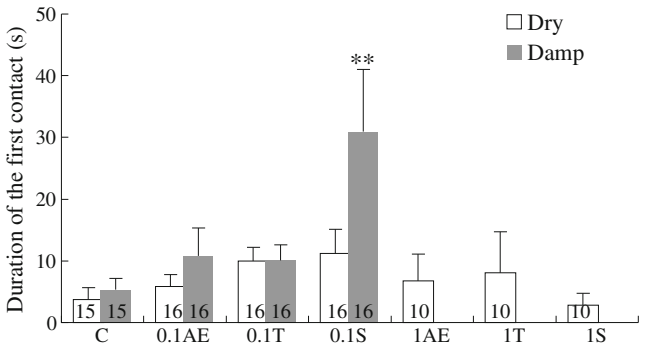

Fig. 4 Results of short-distance attraction bioassays of female *Odontotermes formosanus* sternal gland extracts on dry or damp filter paper discs. Extracts used were the 8th to the last abdominal segments (AE), integument from the 1st to the 7th tergites (T), and integument from the 2nd to the 7th sternites (S). Hexane was used as a control (C), as well as the solvent to produce 10 times dilutions of the dissected parts, e.g., 0.1AE, 0.1T and 0.1S. Results were analyzed with *Multiple Turkey LSD Comparison, P*<0.01. Number of termites used for each test is indicated at the base of the columns

Chemistry and Electrophysiology Four compounds were specific to the sternal glands of females (Fig. 5). Co-injection of the sternal gland extract with a series of n-alkanes revealed that the Linear Retention Indices (LRI) (van Den Dool and Kratz, 1963) of the putative active compounds were 1996, 2012, 2030, and 2047 on the DB-WAX column. The injection of the standards under the same condition showed that the compounds 1 (LRI 1996) and 2 (LRI 2047) were (*Z*)-dodec-3-en-1-ol and (*Z,Z*)-dodeca-3,6-dien-1-ol, respectively. The same procedure on a non-polar HP-5 or HP-5ms column showed that the LRIs of these two compounds corresponded to (*Z*)-dodec-3-en-1-ol (LRI 1457) and to (*Z,Z*)-dodeca-3,6-dien-1-ol (LRI 1449). Their EI mass spectra were identical to those of the standards (*Z*)-dodec-3-en-1-ol (Fig. 6a) and (*Z,Z*)-dodeca-3,6-dien-1-ol (Fig. 6b). The mass spectra of the compounds 3 (LRI 2012) and 4 (LRI 2030) had ion peaks with the same *m/z* value but not the same proportion as (*Z*)-dodec-3-en-1-ol and (*Z,Z*)-dodeca-3,6-dien-1-ol, respectively, indicating that they were isomers of these compounds (Fig. 6c,d). These four compounds were also identified in males (results not shown).

GC-EAD analyses of sternal gland extracts showed that only two compounds induced reproducible EAD responses in both females and males (Fig. 7). The compound with a retention time of 6.81 min (LRI 1996) matched that for (*Z*)-dodec-3-en-1-ol, and the compound with a retention time of 7.15 min (LRI 2047) matched that for (*Z,Z*)-dodeca-3,6-dien-1-ol. Moreover, the injection of the standards induced FID peaks and EAD responses at the same retention times as the two natural products. EAG experiments showed that imagoes were slightly more sensitive to (*Z*)-dodec-3-en-1-ol than to (*Z,Z*)-dodeca-3,6-dien-1-ol (results not shown). Responses to extracts were similar between both sexes.

Short-Distance Attraction Males were strongly attracted to damp papers impregnated with from 0.1 to 1 gland equivalents of extracts from females, (Fig. 8). Interestingly, the same extract did not show significant activity when applied on dry papers. The activity threshold of (*Z,Z*)-dodeca-3,6-dien-1-ol was 5 pg on damp papers and 50 ng on dry papers. Moreover, (*Z*)-dodec-3-en-1-ol was not attractive to males, and it did not significantly enhance the activity of (*Z,Z*)-dodeca-3,6-dien-1-ol.

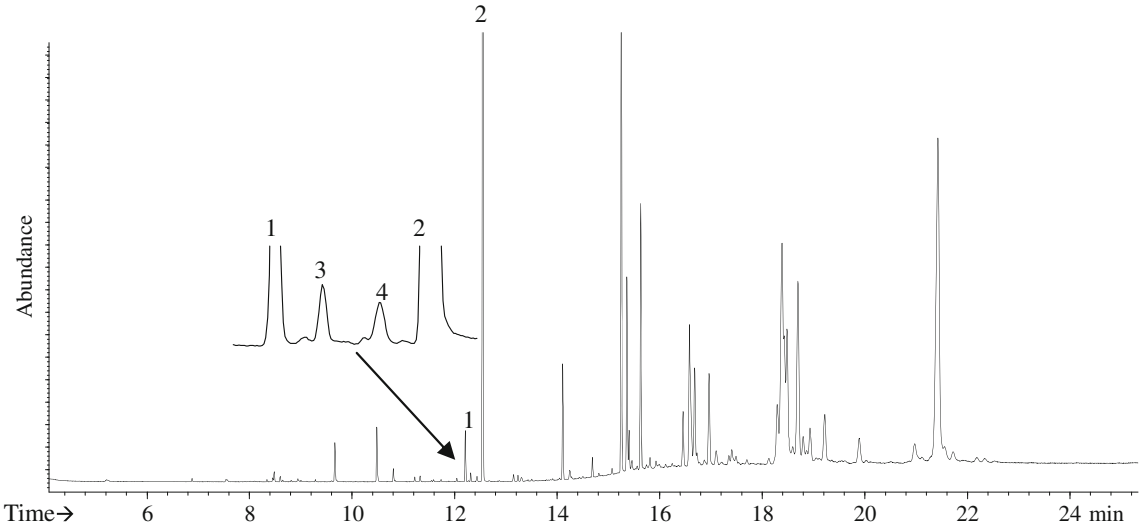

Fig. 5 Results of GC-MS analysis on a DB-WAX column after SPME of female *Odontotermes formosanus* sternal glands (compounds 1, 2, 3, and 4 were glandular-specific)

Females were not attracted by (*Z*)-dodec-3-en-1-ol or (*Z,Z*)-dodeca-3,6-dien-1-ol except at high concentration (50 ng). A mixture of (*Z*)-dodec-3-en-1-ol and (*Z,Z*)-dodeca-3,6-dien-1-ol did not significantly attract females (Fig. 9).

Long-Distance Attraction Males were highly attracted by females or sternal gland extracts from females, but females were not attracted to males (Table 1). Males were significantly attracted by (*Z,Z*)-dodeca-3,6-dien-1-ol and by (*Z*)-dodec-3-en-1-ol, with threshold concentrations of 0.5 and 0.05 ng, respectively. Some synergism was observed between (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol, since a mixture of 0.05 ng of (*Z,Z*)-dodeca-3,6-dien-1-ol and 0.005 ng of (*Z*)-dodec-3-en-1-ol was significantly more attractive to males than the separate compounds at the same quantity as in the mixture. In choice tests, males showed no preference between

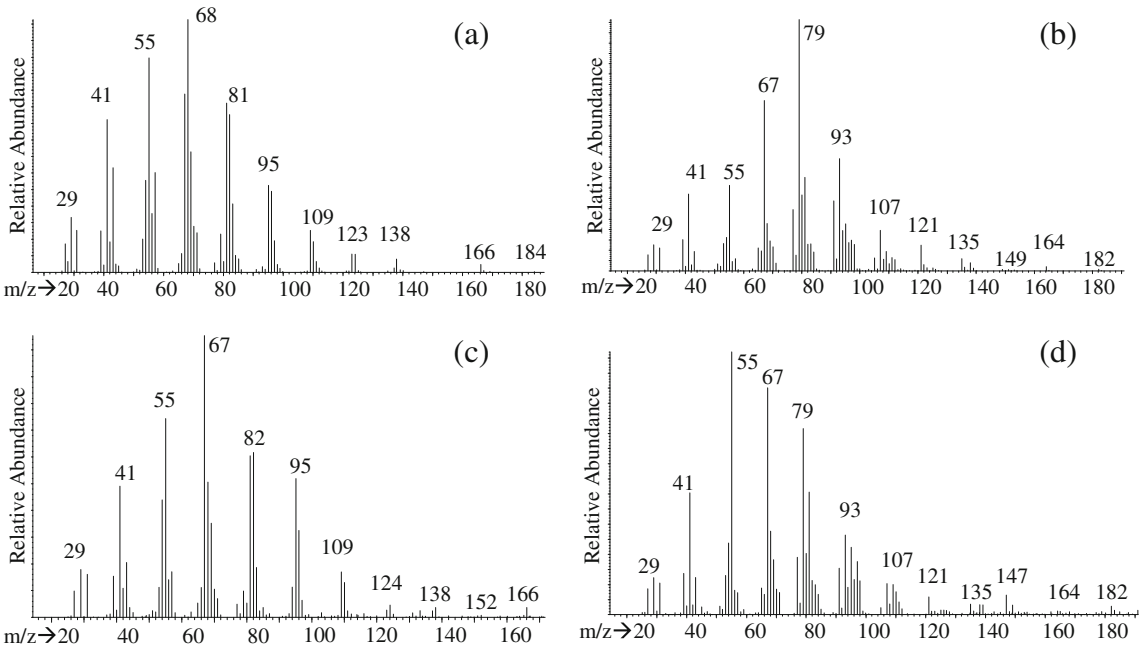

Fig. 6 EI mass spectral analysis of gland-specific compounds from sternal gland extracts of female *Odontotermes formosanus*. Mass spectra of the compounds LRI 1996 (**a**), LRI 2047 (**b**), LRI 2012 (**c**), and LRI 2030 (**d**) corresponding to (*Z*)-dodec-3-en-1-ol (**a**), (*Z,Z*)-dodeca-3,6-dien-1-ol (**b**), an isomer of dodecenol (**c**), and an isomer of dodecadienol (**d**), respectively

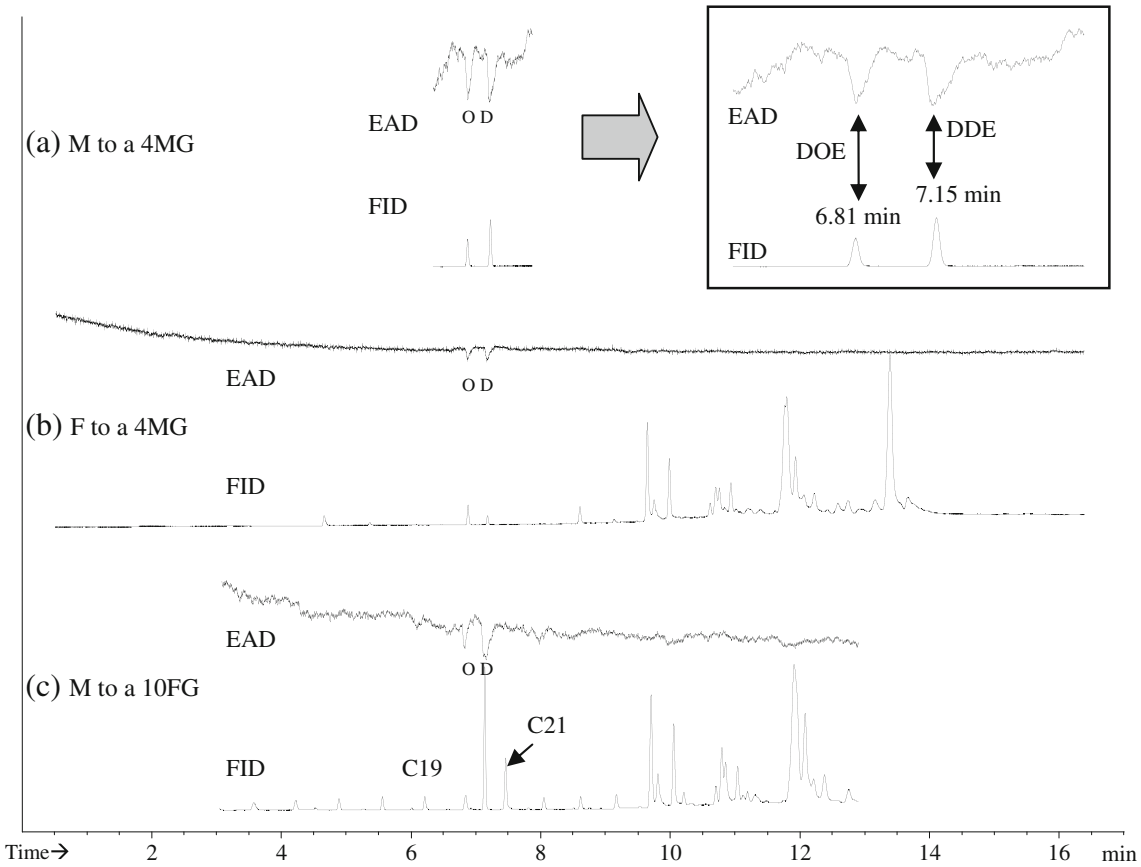

Fig. 7 GC-EAD responses of imagoes to sternal gland extracts from a female or a male *Odontotermes formosanus* on a DB-WAX column. Peaks corresponding to (*Z*)-dodec-3-en-1-ol (DOE) and (*Z,Z*)-dodeca-3,6-dien-1-ol (DDE) are indicated with the letters *O* and *D*, respectively. **a** Antennae of a male (M) to a gland extract from males (four males equivalent) (MG) (EAD 0.5 mV/div, FID 5 mV/div). **b** Antennae of a female (F) to a gland extract from males (4 male equivalent) (EAD 0.2 mV/div, FID 5 mV/div). **c** Antennae of a male to a gland extract from females (10 females equivalent) (FG) using a series of C8-40 alkanes (EAD 1 mV/div, FID 10 mV/div). Details of the active compounds are shown in the *box* at the top right corner of the figure

extracts of 0.1 female gland equivalent and standard samples of (*Z,Z*)-dodeca-3,6-dien-1-ol at 5 ng. Males always selected gland extracts of females rather than (*Z*)-dodec-3-en-1-ol at every concentration. Similarly, males always preferred (*Z,Z*)-dodeca-3,6-dien-1-ol to (*Z*)-dodec-3-en-1-ol. In choice tests between 0.1 female gland extracts and a mixture of 0.5 ng of (*Z,Z*)-dodeca-3,6-dien-1-ol and 0.05 ng of (*Z*)-dodec-3-en-1-ol, males showed no preference unless the mixture was 10 times higher in concentration (Table 1).

Quantification Analysis Quantification of (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol from the sternal gland secretion was performed using several techniques (Table 2). Based on the combination of these techniques, (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol in females were estimated at 9 to 16.64 ng and 0.2 to 0.54 ng, respectively. Moreover, less (*Z,Z*)-dodeca-3,6-dien-1-ol (0.25–8.15 ng) and more (*Z*)-dodec-3-en-1-ol (0.40–1.71 ng) were present in the sternal glands of males than in those of females (Table 2). Headspace-SPME showed that non-calling females released less (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol (DDE/DOE ratio 15.76±3.64, N=21) than calling females exposing their sternal gland (DDE/DOE ratio 40.48±19.8, N=6).

Discussion

Few compounds have been identified as sex-pairing pheromones in termites (Bordereau and Pasteels, 2011). Most of the trail-following pheromones and sex-pairing pheromones are apparently compound blends. (*Z,Z,E*)-Dodeca-3,6,8-trien-1-ol is the only compound of the sex-pairing pheromone in *C. bequaerti*, but the latter compound is mixed with (*E*)-nerolidol in *C. cumulans*, and with (*E*)-nerolidol and (*Z*)-dodec-3-en-1-ol in *C. silvestrii* (Bordereau et al., 2011). The sex-pairing pheromone of *Embiratermes festivellus* is a mixture of (*Z,Z,E*)-dodeca-3,6,8-trien-1-ol and (*Z*)-dodec-3-en-1-ol (Bordereau and Pasteels, 2011). In females of *Nasutitermes corniger* and *N. ephratae*,

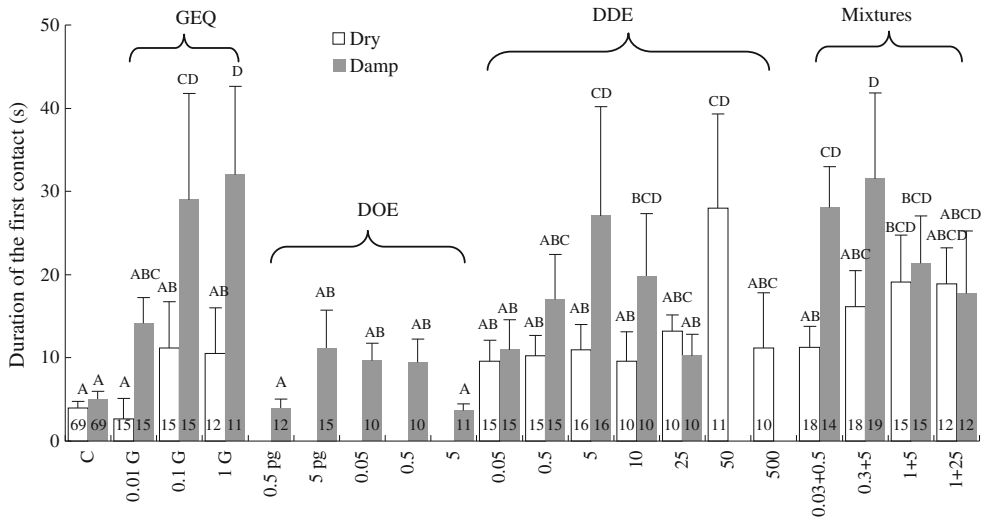

Fig. 8 Results of short-distance attraction bioassays with *Odontotermes formosanus* males in dry or damp (addition of 15 µl of bi-distilled water) conditions. Males had a choice between two pieces of filter paper impregnated with sternal gland extract from females, (*Z*)-dodec-3-en-1-ol (DOE), (*Z,Z*)-dodeca-3,6-dien-1-ol (DDE), or a mixture of both compounds (DOE + DDE). Quantities are indicated in ng, unless mentioned otherwise. *Columns with the same letter* are not significantly different (*Multiple Turkey LSD Comparison, P*<0.01). The number of termites used for each test is indicated at the base of the columns

neocembrene and (11*E*)-trinervita-1(14),2,11-triene are present in the tergal glands (Buděšínský et al., 2005; Bordereau and Pasteels, 2011). Herein, we demonstrated that the sex-pairing pheromone of *O. formosanus* is a blend of (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol, a combination not previously observed. However, (*Z,Z*)-dodeca-3,6-dien-1-ol is the sole compound found as the sex-pairing pheromone of *Ancistrotermes pakistanicus* (Robert et al., 2004) and *Armitermes euhamignathus* (Bordereau and Pasteels, 2011), while (*Z*)-dodec-3-en-1-ol is the only compound reported as the sex-pairing pheromone of *Syntermes praecellens* (Bordereau and Pasteels, 2011). Interestingly, although only (*Z,Z*)-dodeca-3,6-dien-1-ol was present at the surface of the sternal gland of *An. pakistanicus,* (*Z*)-dodec-3-en-1-ol, which was not attractive to males, also was found in the sternal gland extracts (Robert et al., 2004). The preference of males for pheromone released from damp filter papers could be related to the phenomenon of swarming in rainy evenings. Also, water could reduce the sorption capacity and/or surface binding ability of the filter paper, and make the release of the pheromone molecules from damp filter paper easier than from dry filter paper.

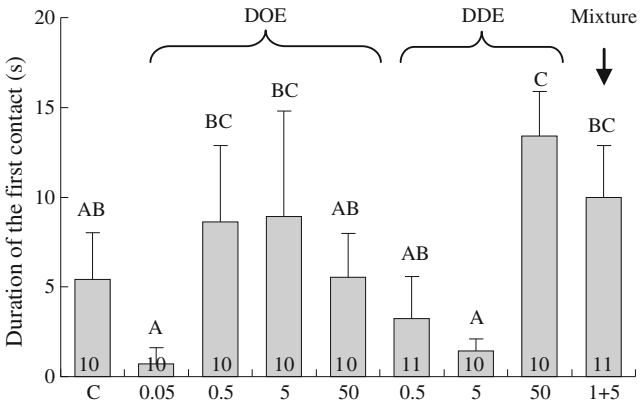

Fig. 9 Results of short-distance attraction bioassays with *Odontotermes formosanus* females in damp conditions using sternal gland extract from females, (*Z*)-dodec-3-en-1-ol (DOE), (*Z,Z*)-dodeca-3,6-dien-1-ol (DDE), or a mixture of both compounds (DOE + DDE). Quantities are indicated in ng, unless mentioned otherwise. *Columns with the same letter* are not significantly different (*Multiple Turkey LSD Comparison, P*<0.01). The number of termites used for each test is indicated at the base of the columns

Results reported here highlight once again the conservative nature of chemical communication in termites. (*Z*)-Dodec-3-en-1-ol is the trail-following pheromone of all Kalotermitidae species studied (Sillam-Dussès et al., 2009) and of some Macrotermitinae (Peppuy et al., 2001a,b), while (*Z,Z*)-dodeca-3,6-dien-1-ol is a component of the trail-following pheromone of *An. pakistanicus* (Robert et al., 2004), *O. latericius*, and *Ar. euhamignathus* (Bordereau and Pasteels, 2011). Some studies also showed that both (*Z,Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol are the components of the trail-following pheromone used by workers in *O. formosanus* (data not shown). These results underline the evolutionary parsimony between the trail-following pheromones of workers and the sex-pairing pheromones of imagoes (Bordereau et al., 1991, 2010; Robert et al., 2004; Sillam-Dussès et al., 2011).

In termites, sexual attraction usually occurs at distances less than 20 cm, (Leuthold, 1975, 1977); the only exception being *Hodotermes mossambicus* whose males can attract the females at a distance of 250 cm with their enlarged sternal gland (Leuthold and Bruinsma, 1977; Leuthold, 1977). The very short-distance attraction in termites may be explained

Table 1 Long-distance attraction bioassays using Y-shape olfactometer

Individual tested	Samples	Dosage	Stay at the base	Choose one branch			Control	*N*
				Sample	Control	*P*		
Male	DDE (ng)	0.005	22	5	3	>0.05	Hexane	30
		0.05	0	19	11	>0.05	Hexane	30
		0.5	0	23	7	<0.05	Hexane	30
		0.5	0	0	15	<0.01	0.1 FG	15
		5	4	26	0	<0.01	Hexane	30
		5	0	7	8	>0.05	0.1 FG	15
		50	2	28	0	<0.001	Hexane	30
	DOE (ng)	0.005	16	7	7	>0.05	Hexane	30
		0.05	0	30	0	<0.001	Hexane	30
		0.5	2	13	0	<0.01	Hexane	15
		0.05	0	0	15	<0.01	0.1 FG	15
		0.05	0	0	15	<0.01	0.5 ng DDE	15
		0.5	0	0	15	<0.01	0.1 FG	15
		0.5	0	2	13	<0.05	0.5 ng DDE	15
		1	0	0	15	<0.01	0.5 ng DDE	15
	DDE + DOE (ng)	0.005+ 0.005	9	16	5	>0.05	hexane	30
		0.05+ 0.005	3	27	0	<0.001	hexane	30
		0.5+0.05	0	6	9	>0.05	0.1 FG	15
		5+0.5	0	14	1	<0.01	0.1 FG	15
	FG	1	0	15	0	<0.01	hexane	15
	Female	1	5	23	2	<0.01	hexane	30
Female	0.1 MG	1	10	3	2	>0.05	hexane	15

Discs of filter paper placed in the flasks of the olfactometer were impregnated with (*Z*,*Z*)-dodeca-3,6-dien-1-ol (DDE), (*Z*)-dodec-3-en-1-ol (DOE), a mixture of both compounds, 1 female gland extract equivalent (FG), 0.1 female gland extract equivalent, and 0.1 male gland extract equivalent (MG), or 5 μl of hexane. A live female was also used as an attracting 'substance' in experiments. Values were analyzed by χ^2 test

by the relatively short distance they can fly (5–100 m on average) (Nutting, 1969; Jones et al., 1988), although some spectacular distances traversed in some species have been inferred or recorded (Harvey, 1934; Jones et al., 1988; García et al., 2002; Messenger and Mullins, 2005). Termite sex-pairing pheromones are considered to possess a dual role with the long-distance attraction of females and males, and the short-distance attraction maintaining tandem behavior (Nutting, 1969; Bordereau and Pasteels, 2011). As shown in this report, in *O. formosanus* long-distance attraction is achieved by (*Z*,*Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol acting in synergy, while (*Z*,*Z*)-dodeca-3,6-dien-1-ol alone is attractive at short-distance.

Reproductive isolation in termites may be mediated by minor compounds. (*E*)-Nerolidol and (*Z*)-dodec-3-en-1-ol play a role in the species-specificity of the sympatric species of *Cornitermes* from South America (Bordereau et al., 2011). Another example is trinervitatriene secreted by the sternal gland of *Nasutitermes ephratae* females, but which is absent from the sternal gland of the sympatric species *N. corniger* in Central and South America (Buděšínský et al., 2005; Bordereau and Pasteels, 2011). As we have shown,

Table 2 Estimated quantities of (*Z*,*Z*)-dodeca-3,6-dien-1-ol (DDE) and (*Z*)-dodec-3-en-1-ol (DOE) in sternal glands of females and males (GEQ)

Techniques	GEQ	DDE (ng)	DOE (ng)
Bioassay	0.1 female	5	–
	1 female	5	–
SPME	1 female	2.15 to 16.64 (*N*=26)	0.07 to 0.54 (*N*=26)
	1 male	0.25 to 8.15 (*N*=14)	0.40 to1.71 (*N*=14)
Headspace-SPME	1 female	9 to 31 (*N*=5)	0.2 to 1.5* (*N*=6)

Values indicated by asterisk were not from detection, but were estimated from SPME ratio analyses of the tested females after headspace-SPME

(*Z*)-dodec-3-en-1-ol, in addition to its the orientation effect, may play a role in species-specific attraction for sympatric species from Southeast Asia. *Odontotermes formosanus* is sympatric in this area with other Macrotermitinae species (Huang et al., 2000; Zhong and Liu, 2002; Cheng et al., 2007). Among them, *An. pakistanicus* secretes a sex-pairing pheromone comprised of (*Z*,*Z*)-dodeca-3,6-dien-1-ol only (Robert et al., 2004). It is hypothesized that the absence of (*Z*)-dodec-3-en-1-ol in the secretion of *An. pakistanicus* females could eliminate attraction of *O. formosanus* males from a distance; bioassays are needed to confirm this hypothesis. Sex-pairing pheromones in the sympatric *Macrotermes annandalei* and *M. barneyi* also were shown to be species-specific, but they remain unidentified (Peppuy et al., 2004). At short distance, (*Z*)-dodec-3-en-1-ol does not play any other role in *O. formosanus*, but cuticular hydrocarbons may prevent hybridization (Howard and Blomquist, 2005).

The secretion of (*Z*,*Z*)-dodeca-3,6-dien-1-ol and (*Z*)-dodec-3-en-1-ol in females of *O. formosanus* involves the exposure of sternal gland while females are calling. Headspace-SPME showed that females secrete much more pheromone during the calling behavior than when they are not calling; bending and shaking of the abdomen appears to increase aeration of the pheromone. The sternal gland also is involved in the tandem behavior, and it is possible that the sex-pairing pheromone is essentially used as a trail-following pheromone to help the male to follow the female. It is usually observed that the female resumes the calling posture to restore contact if she is disconnected from her mate. The particular exposure of the sternal gland contributes to the tandem behavior in imagoes, which has been demonstrated in other species, such as *Pseudacanthotermes* spp. (Bordereau et al. 1991). Other glands are sometimes involved in termite sexual behavior, such as the tergal gland (Bordereau et al., 2002, 2011; Hanus et al., 2009) and the posterior sternal glands (Peppuy et al., 2004). In *O. formosanus*, only the sternal glands of the female are used for calling. Interestingly, a male also possesses this gland containing (*Z*,*Z*)-dodeca-3,6-dien-1-ol (estimated at 0.25 to 8.15 ng) and (*Z*)-dodec-3-en-1-ol (0.40 to 1.71 ng), but which is not involved in sexual behavior according to the bioassay data reported herein. Thus, the presence of these compounds may be a vestigial character, since males and females may attract reciprocally in some basal termites (Bordereau et al., 2010). A similar phenomenon has been previously observed in males of *An. pakistanicus* (Robert et al., 2004), in males of *Pseudacanthotermes spiniger* (Bordereau et al., 1991), and in males of *Trinervitermes bettonianus* (McDowell and Oloo, 1984). Possibly, males and females lay down trails on the ground to mark a territory to delineate the construction of their new nest.

In termites, pheromones are usually released by females, rarely by males (Leuthold, 1977; Leuthold and Bruinsma, 1977) or both sexes (Pasteels, 1972; Bordereau et al., 2010). The presence of a sternal gland in males could be explained as a primitive vestigial occurrence from the multiple glands in *Mastotermes darwiniensis* (Ampion and Quennedey, 1981; Quennedey et al., 2004). An analogous situation occurs in *Prorhinotermes simplex* since both females and males have tergal glands but only females are chemically attractive (Hanus et al., 2009). In *O. formosanus*, homosexual tandems between males was observed, but they did not last long (15.6±2.4 sec, N=30) in comparison to heterosexual tandems (47.1±12.1 sec, N=14, P<0.001). Males never were seen being followed by females. Homosexual tandems have been observed in many species (Buchli, 1960; Bordereau et al., 1991; Hanus et al., 2009). It could arise because the male secretion also contains identical components, but in different proportions and quantity, as the female secretion. Thus, homosexual tandems could be due to uncertain sexual recognition. It is also possible that these homosexual tandems are used as an adaptive anti-predatory strategy as described in *Reticulitermes speratus* (Matsuura et al., 2002).

Odontotermes formosanus is an important termite pest species in Southeast Asia. Understanding the chemical nature of its sex-pairing pheromone could enable application of this pheromone to help control this pest species, perhaps for mass trapping or mating disruption.

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References

AMPION, M. and QUENNEDEY, A. 1981. The abdominal epidermal glands of termites and their phylogenetic significance, pp. 249–261, *in* P. E. Howse and J. L. Clément (eds.), Biosystematics of Social Insects. Academic, London.

BORDEREAU, C. and PASTEELS, J. M. 2011. Pheromones and chemical ecology of dispersal and foraging in termites, pp. 279–320, *in* E. B. David, R. Yves, and L. Nathan (eds.), Biology of Termites: A Modern Synthesis. Springer, Netherlands.

BORDEREAU, C., ROBERT, A., BONNARD, O., and QUERE, J. L. 1991. (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol: Sex pheromone in a higher fungus-growing termite, *Pseudacanthotermes spiniger* (Isoptera, Macrotermitinae). *J. Chem. Ecol.* 17:2177–2191.

BORDEREAU, C., CANCELLO, E. M., SÉMON, E., COURRENT, A., and QUENNEDEY, B. 2002. Sex pheromone identified after Solid Phase Microextraction from tergal glands of female alates in *Cornitermes bequaerti* (Isoptera, Nasutitermitinae). *Insect Soc.* 49:209–215.

BORDEREAU, C., LACEY, M. J., SÉMON, E., BRAEKMAN, J. C., GHOSTIN, J., ROBERT, A., SHERMAN, J. S., and SILLAM-DUSSÈS, D. 2010. Sex pheromones and trail-following pheromone in the basal termites *Zootermopsis nevadensis* (Hagen) and *Z. angusticollis*

(Hagen) (Isoptera: Termopsidae: Termopsinae). *Biol. J. Linn. Soc.* 100:519–530.

BORDEREAU, C., CANCELLO, E. M., SILLAM-DUSSÈS, D., and SÉMON, E. 2011. Sex-pairing pheromones and reproductive isolation in three sympatric *Cornitermes* species (Isoptera, Termitidae, Syntermitinae). *J. Insect Physiol.* 57:469–474.

BUCHLI, H. 1960. Les tropismes lors de la pariade des imagos de *Reticulitermes lucifugus*. *R. Vie Milieu.* 11:308–315.

BUDĚŠÍNSKÝ, M., VALTEROVÁ, I., SÉMON, E., CANCELLO, E., and BORDEREAU, C. 2005. NMR structure determination of (11*E*)-trinervita-1(14),2,11-triene, a new diterpene from sexual glands of termites. *Tetrahedron* 61:10699–10704.

CHENG, M. L., MO, J. C., DENG, T. F., MAO, W. G., and LI, D. 2007. Biology and ecology of *Odontotermes formosanus* in China (Isoptera: Termitidae). *Sociobiology* 50:45–61.

EVANS, T. A., DAWES, T. Z., WARD, P. R., and LO, N. 2011. Ants and termites increase crop yield in a dry climate. *Nat. Commun.* 2:262.

GARCÍA, J., MAEKAWA, K., MIURA, T., and MATSUMOTO, T. 2002. Population structure and genetic diversity in insular populations of *Nasutitermes takasagoensis* (Isoptera: Termitidae) analyzed by AFLP markers. *Zool. Sci.* 19:1141–1146.

HANUS, R., LUXOVA, S., ŠOBOTNÍK, J., KALINOVÁ, B., JIROŚ, P., KŘEČEK, J., BOURGUIGNON, T., and BORDEREAU, C. 2009. Sexual communication in the termite *Prorhinotermes simplex* (Isoptera, Rhinotermitidae) mediated by a pheromone from female tergal glands. *Insect Soc.* 56:111–118.

HARVEY, P. A. 1934. Life history of *Kalotermes minor*, pp. 217–233, *in* C. A. Kofoid (ed.), Termites and Termite Control, 2nd ed. University of California Press, Berkeley.

HOLT, J. A. and LEPAGE, M. 2000. Termites and soil properties, pp. 389–407, *in* T. Abe, D. E. Bignell, and M. Higashi (eds.), Termites: Evolution, Sociality, Symbioses, Ecology. Kluwer, Dordrecht.

HOWARD, R. W. and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–393.

HU, J., ZHONG J. H., GUO, M. F. 2007. Alate dispersal distances of the black-winged subterranean termite *Odontotermes formosanus* (Isoptera: Termitidae) in southern China. *Sociobiology* 50:1–8.

HUANG, F. S., ZHU, S. M., PING, Z. M., HE, X. S., LI, G. X., and GAO, D. R. 2000. Fauna Sinica, Insecta, Vol. 17, Isoptera. Science Press, Beijing.

HUANG, Q., LEI, C., XUE, D. 2006. Field evaluation of a fipronil bait against subterranean termite *Odontotermes formosanus* (Isoptera: Termitidae). J. Econ. Entomol. 99:455–461.

JONES, S. C., LA-FAGE, J. P., and HOWARD, R. W. 1988. Isopteran sex ratios: Phylogenetic trends. *Sociobiology* 14:89–156.

LACEY, M. J., SÉMON, E., KRASULOVÁ, J., SILLAM-DUSSÈS, D., ROBERT, A., CORNETTE, R., HOSKOVEC, M., ŽÁČEK, P., VALTEROVÁ, I., and BORDEREAU, C. 2011. Chemical communication in termites: *syn*-4,6-dimethylundecan-1-ol as trail-following pheromone, *syn*-4,6-dimethylundecanal and (5*E*)-2,6,10-trimethylundeca-5,9-dienal as the respective male and female sex pheromones in *Hodotermopsis sjoestedti* (Isoptera, Archotermopsidae). *J. Insect Physiol.* 57:1585–1591.

LEUTHOLD, R. H. 1975. Orientation mediated by pheromones in social insects, pp. 197–211, *in* C. Noirot, P. E. Howse, and G. Le-Masne (eds.), Pheromone and Defensive Secretions in Social Insects. French Section of IUSSI, Dijon.

LEUTHOLD, R. H. and BRUINSMA, O. 1977. Pairing behavior in *Hodotermes mossambicus* (Isoptera). *Psyche* 84:109–119.

LIU, Y. Z., TANG, G. Q., PAN, Y. Z., CHENG, L. D., and HE, Y. Z. 1985. Observation on the reproductive caste of *Odontotermes formosanus* (Shiraki): Larval development and nuptial flight. *Acta Entomol. Sin.* 51:714–771.

MATSUURA, K., KUNO, E., and NISHIDA, T. 2002. Homosexual tandem running as selfish herd in *Reticulitermes speratus*: Novel antipredatory behavior in termites. *J. Theor. Biol.* 214:63–70.

MCDOWELL, P. G. and OLOO, G. W. 1984. Isolation, identification, and biological activity of trail-following pheromone of termite *Trinervitermes bettonianus* (Sjöstedt) (Termitidae: Nasutitermitinae). *J. Chem. Ecol.* 10:835–851.

MESSENGER, M. T. and MULLINS, A. J. 2005. New flight distance recorded for *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Fla Entomol.* 88:99–100.

MYLES, T. G. 1988. Dealation in termites (Isoptera). *Sociobiology* 14:61–88.

LEUTHOLD, R. H. 1977. Postflight communication in two termite species, *Trinervitermes bettonianus* and *Hodotermes mossambicus*, pp 62–64, *in* Proc VIII Congr IUSSI, Wageningen, The Netherlands.

NUTTING, W. L. 1969. Flight and colony foundation, pp. 233–282, *in* K. Krishna and F. M. Weesner (eds.), Biology of Termites (Vol. 1). Academic, New York and London.

PASTEELS, J. M. 1972. Sex-specific pheromones in a termite. *Cell. Mol. Life Sci*. 28:105–106.

PEPPUY, A., ROBERT, A., SÉMON, E., BONNARD, O., SON, N. T., and BORDEREAU, C. 2001a. Species specificity of trail pheromones of fungus-growing termites from northern Vietnam. *Insect Soc.* 48:245–250.

PEPPUY, A., ROBERT, A., SÉMON, E., GINIES, C., LETTERE, M., BONNARD, O., and BORDEREAU, C. 2001b. (*Z*)-dodec-3-en-1-ol, a novel termite trail pheromone identified after solid phase microextraction from *Macrotermes annandalei*. *J. Insect Physiol.* 47:445–453.

PEPPUY, A., ROBERT, A., and BORDEREAU, C. 2004. Species-specific sex pheromones secreted from new sexual glands in two sympatric fungus-growing termites from northern Vietnam, *Macrotermes annandalei* and *M. barneyi*. *Insect Soc.* 51:91–98.

QUENNEDEY, A., PEPPUY, A., COURRENT, A., ROBERT, A., EVERAERTS, C., and BORDEREAU, C. 2004. Ultrastructure of posterior sternal glands of Macrotermes annandalei (Silvestri): New members of the sexual glandular set found in termites (Insecta). *J. Morphol.* 262:683–691.

ROBERT, A., PEPPUY, A., SÉMON, E., BOYER, F. D., LACEY, M. J., and BORDEREAU, C. 2004. A NEW C12 ALCOHOL IDENTIFIED AS A SEX PHEROMONE AND A TRAIL-FOLLOWING PHEROMONE IN TERMITES: THE DIENE (Z, Z)-DODECA-3,6-DIEN-1-OL. *NATURWISSENSCHAFTEN* 91:34–39.

SILLAM-DUSSÈS, D., SÉMON, E., ROBERT, A., and BORDEREAU, C. 2009. (*Z*)-Dodec-3-en-1-ol, a common major component of the trail-following pheromone in the termites Kalotermitidae. *Chemoecology* 19:103–108.

SILLAM-DUSSÈS, D., HANUS, R., ABD EL-LATIF, A., JIROŠ, P., KRASULOVÁ, J., KALINOVÁ, B., VALTEROVÁ, I., and ŠOBOTNÍK, J. 2011. Sex pheromone and trail pheromone of the sand termite *Psammotermes hybostoma*. *J. Chem. Ecol.* 37:179.

VAN DEN DOOL, H. and KRATZ, D. P. 1963. A generalization of the retention index system including linear temperature programmed gas–liquid partition chromatography. *J. Chromatogr. A* 11:463–471.

ZHONG, J. H. AND LIU, L. L. 2002. Termite fauna in China and their economic importance. *Sociobiology* 40:25–32.

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The Sex Pheromones of Mealy Plum (*Hyalopterus pruni*) and Leaf-Curl Plum (*Brachycaudus helichrysi*) Aphids: Identification and Field Trapping of Male and Gynoparous Aphids in Prune Orchards

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Abstract Mealy plum, *Hyalopterus pruni*, and leaf-curl plum, *Brachycaudus helichrysi*, aphids are the primary arthropod pests in orchards that produce dried plums (i.e., prunes). The sexual stage of their respective lifecycles occurs on prune trees in the fall, during which time males respond to sex pheromones produced by oviparous females. Air-entrainment collections confirmed that oviparous *H. pruni* and *B. helichrysi* emitted combinations of (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol. The responses of *H. pruni* and *B. helichrysi* to these compounds in ratios of 1:0, 0:1, 1:1, 2.6:1, 3.4:1, 5:1, 7:1, and 0:0 (no-pheromone control) using water traps were determined in field experiments conducted in prune orchards during the fall. The greatest number of male *H. pruni* was caught in traps releasing a 1:1 ratio of (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol, while male *B. helichrysi* were caught in similar numbers in traps releasing any of the two-component ratios tested. There was no evidence that any of the pheromone treatments influenced trap catches of gynoparae of either species. Results suggest that addition of sex pheromone lures increases trap catches of male *H. pruni* and *B. helichrysi*, and that this approach may improve monitoring and management of these pests in prune orchards. Knowledge gained from this study contributes to the understanding of the ecology of insect pests in prune orchards.

Keywords (4a*S*, 7*S*, 7a*R*)-Nepetalactone · (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol · Monitoring · Management · Dried plum · Chemoreception · Hemiptera · Sternorrhyncha · Aphididae

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Introduction

The principal arthropod pests in orchards producing dried plums (i.e., prunes) are the holocyclic heteroecious mealy plum aphid, *Hyalopterus pruni* (Geoffroy), and the leaf-curl plum aphid, *Brachycaudus helichrysi* (Kaltenbach) (Hemiptera: Sternorrhyncha: Aphididae). Spring populations cause economic losses of prunes due to the effects of feeding and the production of honeydew that encourages fungal growth that may lead to fruit cracking and reduced sugar content. *Hyalopterus pruni* and *B. helichrysi* gynoparae, which give birth to egg-laying sexual females (oviparae), and males colonize prune trees, *Prunus domestica* L., their primary hosts in the fall. Mated oviparae lay eggs that overwinter and give rise to the following spring populations. This crop provides an ideal system to investigate the potential for exploiting the sexual stage of the aphid life cycle to improve pest management. Monitoring populations of fall migrants could provide information as to the damage potential in the subsequent season and influence treatment decisions.

Additionally, disruption of the lifecycle at this point could reduce the abundance of fertile overwintering eggs, leading to a decrease in damaging spring populations.

During the sexual generation, male aphids locate conspecific oviparae for mating by utilizing a sex pheromone released by oviparae (Pettersson 1970; Marsh 1972). To date, all aphid species investigated produce and release sex pheromones in which the active components comprise the iridoids nepetalactone and nepetalactol (Hardie et al. 1999; Pickett and Glinwood 2007). With the exception of a single species, the damson hop aphid *Phorodon humuli* (Schrank), the sex pheromones identified from members of the subfamily Aphidinae comprise a mixture of (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol (Hardie et al. 1999; Birkett and Pickett 2003; Pickett and Glinwood 2007; Stewart-Jones et al. 2007, Dewhirst et al. 2010). The sex pheromone of *P. humuli* comprises two alternate nepetalactol diastereoisomers, (1*S*, 4a*R*, 7*S*, 7a*S*)-nepetalactol and (1*R*, 4a*R*, 7*S*, 7a*S*)-nepetalactol (Campbell et al. 1990). (1*S*,2*R*,3*S*)-Dolichodial has been proposed as a third minor component of the sex pheromone for some Aphidinae species (Dewhirst et al. 2008). Headspace collection (air entrainment) of oviparae of a number of aphid species indicate that the ratio of the two pheromone components emitted by oviparae is relatively species-specific (Hardie et al. 1999; Boo et al. 2000; Goldansaz et al. 2004; Stewart-Jones et al. 2007 and references therein; Dewhirst et al. 2010 and references therein), and prior research indicates that while male aphids will respond to a range of ratios of the two components, they tend to exhibit a greater response to the ratio emitted by conspecific oviparae (Dawson et al. 1990; Hardie et al. 1990; Lilley and Hardie 1996; Boo et al. 2000). It also has been suggested that aphid sex pheromones may act as aggregation pheromones for gynoparae (Lilley and Hardie 1996; Powell and Hardie 2001; Zhu et al. 2006).

Several laboratory and field studies have shown the impacts of synthetic and natural sources of aphid sex pheromones on male aphid behavior and trap catches, many reporting significant responses to specific sex pheromone ratios (e.g., Pettersson 1970; Dawson et al. 1988; Campbell et al. 1990; Hardie et al. 1990, 1996; Guldemond et al. 1993; Lilley and Hardie 1996; Thieme and Dixon 1996; Boo et al. 2000; Goldansaz et al. 2004; Zhu et al. 2006; Dewhirst et al. 2008). The respective sex pheromones for *H. pruni* or *B. helichrysi* have not previously been identified, and identification would provide a foundation for developing pheromone-based approaches for monitoring and management of these aphid pests in damson and prune crops. The objectives of the current study were to collect and identify the sex pheromone components of *H. pruni* and *B. helichrysi*, and to investigate responses of these aphids to different ratios of the identified compounds, (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol, in prune orchards.

Methods and Materials

Insects *Hyalopterus pruni* (UK strain) and *Brachycaudus helichrysi* (UK strain) oviparae were collected from blackthorn (*Prunus spinosa* L.) in hedgerows surrounding plum (*Prunus domestica* L.) orchards (HRI-East Malling, New Road, East Malling, Kent, UK). Oviparous *H. pruni* (California strain) were collected from a prune (*P. domestica*, cv. 'Improved French') orchard (California, USA). Oviparae were maintained on blackthorn (*P. spinosa*) in a controlled environment room (12L: 12D photoperiod, 18 °C).

Isolation of Volatiles The base of an excised blackthorn (*P. spinosa*) branch, bearing leaves infested with mature *H. pruni* oviparae (UK strain, N=12, various adult ages), was placed in a glass vessel (50 ml) containing water. The vessel was then placed into a glass entrainment vessel (0.5 l) that contained a hole for the blackthorn branch. As a hole was present in the entrainment set-up (an open system), air that had been purified by passage through an activated-charcoal filter (BDH, 10–14 mesh, 50 g) was pushed into (850 ml.min^{-1}) and pulled out of (600 ml.min^{-1}) the vessel. As a control, volatiles also were collected from leaf-bearing, non-infested blackthorn branches. Volatiles were trapped for 72 h by drawing air from the vessel through Porapak Q 50/80 (50 mg; Supelco, Bellefonte, PA, USA) in glass tubing (5 mm o.d.) between two plugs of silanized glass wool. Porapak Q was conditioned by washing with redistilled diethyl ether (4 ml) and heating at 132 °C for 2 h under a stream of purified N_2. After air entrainment, volatiles were eluted from the Porapak Q with redistilled diethyl ether (750 µl), and then stored in a freezer (−22 °C) in tightly capped microvials until required for analysis. The same methods were used for volatile collections from blackthorn infested with mature *H. pruni* oviparae (California strain, N=20, various adult ages), mature *B. helichrysi* oviparae (UK strain, heavily infested, various adult ages), and non-infested blackthorn leaves.

Chemical Analysis Air-entrainment samples were analyzed by gas chromatography (GC), using both polar (DB-wax, 30 m×0.32 mm i.d.×0.5 µm film thickness) and non-polar (HP-1, 50 m×0.32 mm i.d. 0.5-µm film thickness) capillary columns, with an HP5890 GC (Agilent Technologies, UK). The columns were attached via deactivated retention gaps (1 m×0.53 mm i.d.) to a cool-on-column injector, and a flame ionization detector. The GC oven temperature was maintained at 30 °C for 1 min. after sample injection and then increased by 5 °C.min^{-1} to 150 °C, then 10 °C.min^{-1} to 240 °C. The carrier gas was hydrogen. Peak enhancement, by co-injection with authentic standards, was carried out to confirm tentative identifications. A multiple-point external standard method was used to quantify the compounds present in the air-entrainment samples. Coupled gas chromatography–

mass spectrometry (GC-MS) was performed on a Thermofinnigan Instrument MAT95 XP double-focusing magnetic sector mass spectrometer coupled to a TRACE GC fitted with an HP-1 column and integrated data system (Fisons Instruments, Manchester, UK). The GC oven temperature was maintained at 30 °C for 5 min. and then programmed at 5 °C min^{-1} to 250 °C. Ionization was electron impact at 70 eV (250 °C source temperature).

Chemical Standards (4a*S*, 7*S*, 7a*R*)-Nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol were synthesized as in Dawson et al. (1996).

Chemical Lures for Field Trials Polyvinyl chloride (PVC) polymer strips impregnated separately with (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol (Agrisense BCS Limited, Pontypridd, Wales), a formulation that provides slow and consistent release rates of the components, were used for the field experiments (Birkett and Pickett 2003). As release rates of each component are known for standard lengths of the strips (Graves 2003; Koczor et al. 2010), the pheromone ratios tested in the experiment were created by cutting individual PVC polymer strips to particular lengths to achieve the desired ratios while maintaining a constant total release rate of ~0.200 mg.day^{-1}.

Field Trials To determine the impacts of selected ratios of (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol on trap catches of *H. pruni* and *B. helichrysi*, 18 replicates (three or five replicates per orchard) of a randomized complete block design were established in four prune orchards (*P. domestica* L., cv. 'Improved French') in Yolo and Sutter Counties, California, USA. Orchard trials commenced 8 October 2008 and lasted until 10 December 2008. Water traps, consisting of clear 16 oz. polyethylene terephthalate containers (No. DM16-0090, Solo Cup Co., Urbana, IL, USA), were deployed in the southeast quadrant of the tree canopy at a height of 1.5–1.8 m and filled with approximately 0.38 l of a 1 % solution of detergent (Planet® Ultra Dishwashing Liquid, Planet Inc., Victoria, BC, Canada). There was a minimum of 25 m between treatment trees within replicate rows, and a minimum of 39 m between replicate rows. Lures were suspended approximately 2.5–5 cm (due to water evaporation between weekly trap changes) above the surface of the water using 20 gauge soft galvanized utility wire. The following (4a*S*, 7*S*, 7a*R*)-nepetalactone:(1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol ratios were tested: 1:0, 0:1, 1:1, 2.6:1, 3.4:1, 5:1, 7:1, and 0:0 (no-pheromone control). The ratios 3.4:1 and 2.6:1 were chosen to reflect those identified in the volatile collection from *H. pruni* (California strain) and *B. helichrysi* oviparae, respectively. Traps were changed and re-randomized within replicates weekly, and chemical lures replaced at week four of the experiment.

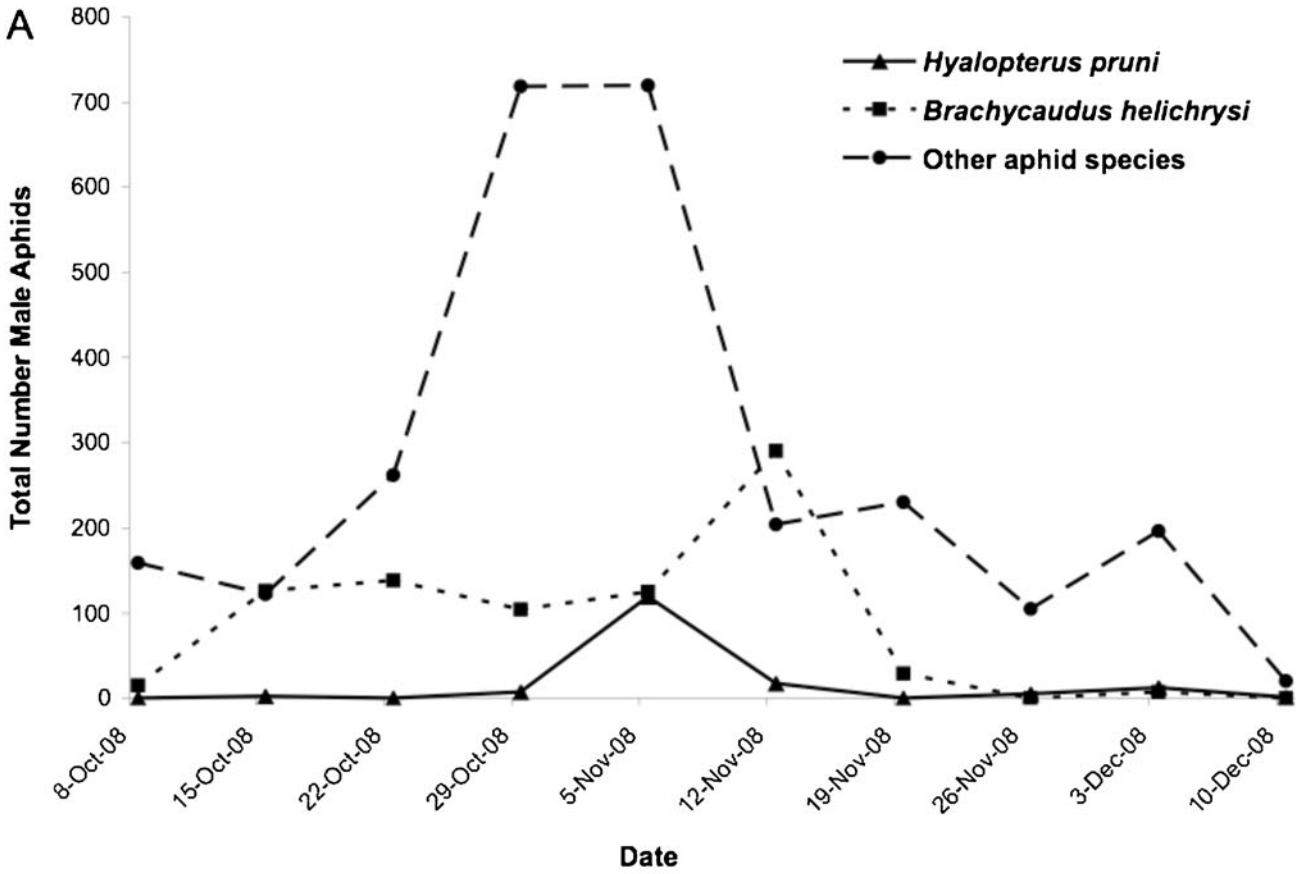
Statistical Analyses Numbers of *H. pruni*, *B. helichrysi*, and non-target aphid species trapped were summed over the trial for analyses. The following data were compared among the treatments: mean numbers of *H. pruni* males, *H. pruni* gynoparae, and *B. helichrysi* males, and mean percent of *H. pruni* and *B. helichrysi* males relative to the total number of male aphids trapped. Data from the field trials did not meet the criteria of normality and homogeneity of variances required for standard analyses of variance (ANOVA), and were, therefore, analyzed using Friedman's nonparametric ANOVA on ranked means (α=0.05), followed by least squares (LS) means multiple comparison tests with alpha adjusted to account for the number of comparisons (Bonferroni α=0.00179) (PROC RANK, PROC GLM, SAS Institute, 2008) (Ipe 1987; Conover 1999).

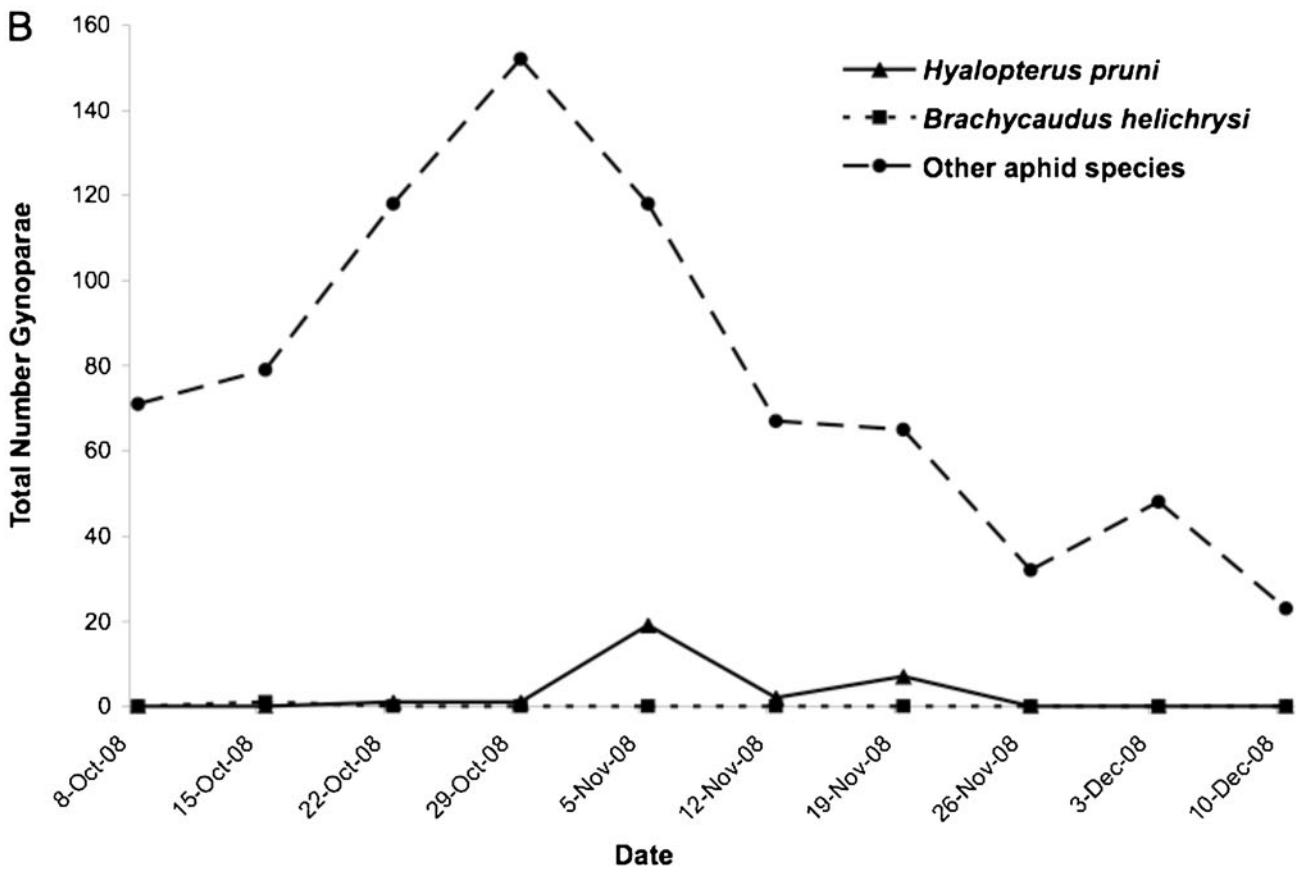
Results

Isolation of Volatiles GC-MS and peak enhancement by co-injection, using non-polar (HP-1) and polar (DB-Wax) columns, confirmed the presence of (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol in the volatiles collected from leaves infested with *H. pruni* oviparae (UK and Californian strains) and *B. helichrysi* oviparae (UK strain). The volatile samples from the UK strains of *H. pruni* and *B. helichrysi* contained a 2.5:1 and 2.6:1 ratio of (4a*S*, 7*S*, 7a*R*)-nepetalactone:(1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol, respectively. Volatile samples from the California strain of *H. pruni* had a ratio of 3.4:1 (4a*S*, 7*S*, 7a*R*)-nepetalactone:(1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol. These compounds were not detected in the air-entrainment samples of blackthorn leaves. (1*S*,2*R*,3*S*)-Dolichodial was not detected in the air-entrainment samples of *B. helichrysi* or *H. pruni* oviparae, or blackthorn leaves. Based on these results, 2.6:1 and 3.4:1 ratios of (4a*S*, 7*S*, 7a*R*)-nepetalactone:(1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol were tested in the field trials.

Field Trials Overall, fewer *H. pruni* males (163) were trapped than *B. helichrysi* males (834), whereas more *H. pruni* gynoparae (30) were trapped than *B. helichrysi* gynoparae (1), over the course of the field trials. Male aphids of all species were trapped in much higher numbers than gynoparae of all species, 3732 and 804, respectively.

Total trap catches (all treatments combined) of male and gynoparous aphids throughout the aphid migration and mating period (fall 2008) are shown in Fig. 1. Male *H. pruni* were trapped in low numbers throughout the season, with the exception of a single, distinct peak in early November, while male *B. helichrysi* were trapped consistently after early October, with peak trap catches in mid-November (Fig. 1a). Trap catches for males of both species declined


A
Total Number Male Aphids
800
700
600
500
400
300
200
100
0
Hyalopterus pruni
Brachycaudus helichrysi
Other aphid species
8-Oct-08
15-Oct-08
22-Oct-08
29-Oct-08
5-Nov-08
12-Nov-08
19-Nov-08
26-Nov-08
3-Dec-08
10-Dec-08
Date


B
Total Number Gynoparae
160
140
120
100
80
60
40
20
0
Hyalopterus pruni
Brachycaudus helichrysi
Other aphid species
8-Oct-08
15-Oct-08
22-Oct-08
29-Oct-08
5-Nov-08
12-Nov-08
19-Nov-08
26-Nov-08
3-Dec-08
10-Dec-08
Date

◀ **Fig. 1** Weekly totals of **a** male aphids and **b** gynoparae caught in water traps (control and all pheromone treatments combined) in prune orchards during fall 2008

considerably thereafter, and remained low after the third week of November. In addition, males of a number of non-target aphid species (total trap catch = 2735) were caught during the field trial, with highest numbers in late October through early November, but declining after the third week of November (Fig. 1a). Peak trap catches of *H. pruni* and non-target (total trap catch = 773) gynoparae (Fig. 1b) followed the same trends exhibited by males; the single *B. helichrysi* gynopara was trapped in mid-October. Non-target aphid species were not identified in the current study due to the complexity of aphid species identification when host-plant associations are unknown.

Statistical differences among mean numbers of male and gynoparous *H. pruni* and *B. helichrysi* caught in traps baited with different pheromone ratios are shown in Fig. 2. Traps with the 1:1 (4a*S*, 7*S*, 7a*R*)-nepetalactone:(1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol ratio caught more *H. pruni* males than any of the other treatments ($P<0.001$, Fig. 2a). Male *B. helichrysi* were caught in higher numbers in all of the two-component treatments compared to the no-pheromone control, the nepetalactone only, and nepetalactol only treatments ($P<0.001$, Fig. 2b). No differences among mean numbers of *H. pruni* gynoparae caught were found ($P=0.590$, Fig. 2c); data for *B. helichrysi* gynoparae were not analyzed, as only a single gynopara was trapped all season.

Discussion

Volatile samples collected from *Hyalopterus pruni* (UK and Californian strain) and *Brachycaudus helichrysi* oviparae (UK strain) contained (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol, the same compounds reported as major pheromone components of a number of other aphid species (Hardie et al., 1999; Boo et al., 2000; Goldansaz et al., 2004; Stewart-Jones et al., 2007 and references therein; Dewhirst et al., 2010 and references therein). As these chemicals are present in the hind tibae of oviparae of other aphid species (e.g., Dawson et al., 1987, 1989), and we recorded a behavioral response by male *H. pruni* and *B. helichrysi* to these compounds in the field, we conclude that they are components of the *H. pruni* and *B. helichrysi* sex pheromones. The results of this study increase our understanding of the ecology of insects in prune orchards, in particular the sexual phase semiochemical communication of aphid species that share a common primary host.

The specificity of sex pheromones is important for maintaining species integrity when different aphid species co-occur on the same primary host. In this case, the ratios of components identified from *H. pruni* and *B. helichrysi* oviparae (UK strain), which often co-occur on their primary hosts in agricultural environments, were slightly different, perhaps aiding species specificity. In addition, peak trap catch of male *H. pruni* occurred one week earlier than that of *B. helichrysi*, indicating that specificity of the sex pheromones may be increased by temporal division in migration, calling, or mating period of the two species. The ratio of components also differed between the UK and California strains of *H. pruni*, suggesting geographic differences in pheromone profiles of aphid species, an aspect of aphid chemical ecology that has not yet been specifically addressed for any aphid species. However, the difference in pheromone ratio between the UK and California *H. pruni* strains could also result from differences in the age structures of the populations of oviparae assayed, as a decrease in relative levels of nepetalactol in aged oviparae has been recorded previously for the vetch aphid, *Megoura viciae* (Hardie et al. 1990), the potato aphid *Macrosiphum euphorbiae* (Goldansaz et al., 2004), and the rosy apple aphid, *Dysaphis plantaginea* (Stewart-Jones et al. 2007). In the field, more male *H. pruni* were caught in traps baited with a 2.6:1 ratio than in the control traps, whereas there was no difference in catches between traps baited with the 3.4:1 ratio and the controls, indicating that the 2.5:1 ratio of the UK strain may be more biologically relevant.

Male *H. pruni* were caught in greatest numbers to traps baited with the 1:1 blend (nepetalactone: nepetalactol), whereas male *B. helichrysi* were caught in similar numbers in traps baited with any of the two-component treatments. In terms of practical application, these results suggest that the 1:1 pheromone ratio would be most useful for monitoring and management programs simultaneously targeting both species. This is important, as *H. pruni* and *B. helichrysi* populations tend to co-occur in infested orchards.

It has been suggested that aphid sex pheromones may act as aggregation pheromones for gynoparae (Lilley and Hardie 1996; Powell and Hardie 2001; Zhu et al. 2006). However, in our study neither *H. pruni* nor *B. helichrysi* gynoparae were caught in significant numbers (i.e., different from that in the control) in traps emitting (4a*S*, 7*S*, 7a*R*)-nepetalactone and (1*R*, 4a*S*, 7*S*, 7a*R*)-nepetalactol, or blends of the two compounds. Only a single *B. helichrysi* gynopara was trapped during the sampling period.

The current study examined the impacts of various pheromone ratios at a single total release rate, which was chosen based, in part, on known optimized release rates of the available pheromone lures. The total release rate of the ratios tested in this study was comparable to those employed by other researchers in field-trapping studies (Gabryś et al. 1997, Goldansaz et al. 2004). Although aphid responses to varying concentrations of sex pheromone components have been investigated in a number of laboratory experiments (Dawson et al. 1988, Campbell et al. 1990, Park et al.

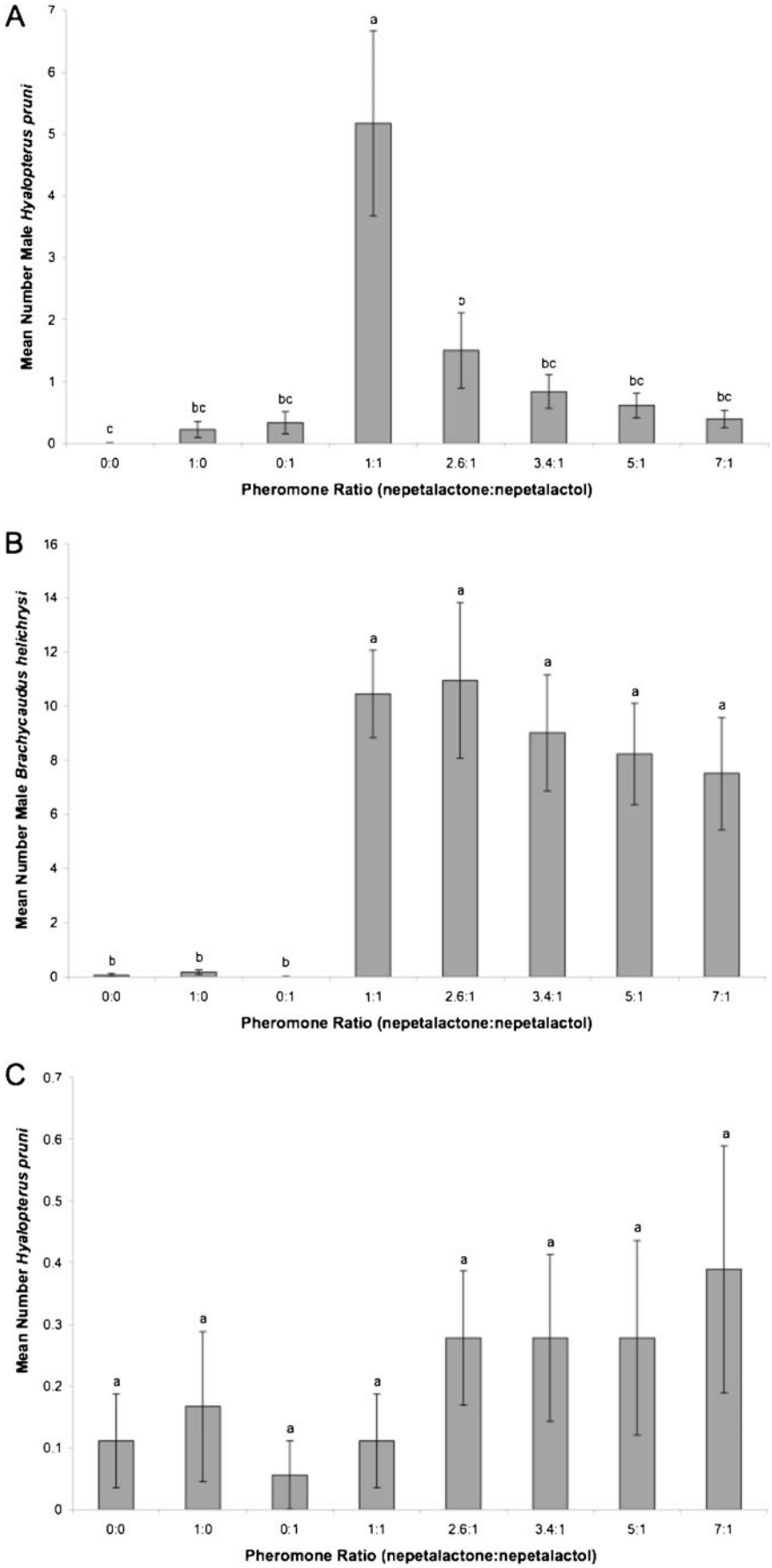


Fig. 2 Mean (± SE) numbers of **a** male *Hyalopterus pruni*, **b** male *Brachycaudus helichrysi*, and **c** *H. pruni* gynoparae caught in water traps baited with different ratios of aphid sex pheromone components, in prune orchards during fall 2008. Treatments with the same letters are not different (Friedman nonparametric ANOVA on ranked means, followed by least squares means multiple comparisons. Male *H. pruni*: $F=10.73$, $df=7, 119$, $P<0.001$; male *B. helichrysi*: $F=38.57$, $df=7, 119$, $P<0.001$; *H. pruni* gynoparae: $F=0.80$, $df=7, 119$, $P=0.590$)

2000), only one field-based study has examined the effect of different doses of a single, most attractive ratio on trap catches (Zhu et al. 2006). In that study, all pheromone concentrations caught male aphids, relative to control traps, with dose-dependent responses evident from 0 to 30 mg. Further research, investigating the responses of *H. pruni* and *B. helichrysi* to different release rates and ratios is warranted.

In addition to the two key aphid pests in prune orchards, a large number of non-target aphids, of numerous species, were trapped in the current study. The numbers of *H. pruni* and *B. helichrysi* caught were relatively low (4 and 19 % of the total, respectively) compared to the overall numbers of aphids caught in pheromone-baited traps. Tentative identification suggested that many of the species are not known to colonize prune trees or cause economic damage in prune orchards. Other research, examining the impact of different ratios of aphid sex pheromone components on captures of sexual aphids during the fall, has also reported this phenomenon (e.g., Boo et al. 2000). The process by which migratory sexual aphid forms locate their primary host plants and conspecific oviparae for reproduction and overwintering in the fall is not well understood. Research suggests that the termination of migratory translocation is based on visual cues, and that aphids are attracted to particular color wavelengths of landing targets. Further discrimination may occur after aphids have landed on and probed potential host plants, or may be mediated by plant chemical cues at very short (cm) distances (Irwin et al. 2007 and references therein). The large number of non-pest aphids caught in our study may have resulted from a large abundance of transient, non-colonizing aphid species passing though the orchards. Unable to find suitable host plants in this environment, they were attracted to pheromone lures deployed for the target aphid pests. Catches of a large number of non-target aphids may be an obstacle for developing monitoring applications, as identifying aphid species, particularly males, generally requires assistance by taxonomic specialists. Additionally, although water traps were utilized in the current study to facilitate aphid identification, sticky traps are the standard for aphid trapping. Use of sticky traps further complicates the identification of aphid species, due to the difficulty in removing aphids from trap glues while preserving their integrity for gender and species classification. There is evidence that interactions and/or synergism exist between aphid sex pheromone components, host-plant volatiles (Hardie et al. 1999; Powell and Hardie 2001; Pickett and Glinwood 2007), and visual cues (Hardie et al. 1996). Therefore, future research should investigate whether specificity toward target aphid pests could be increased by adding other semiochemicals, such as host-plant volatiles, to sex pheromone lures, as well as examining the effects of visual and other stimuli.

Pheromone-based monitoring and management targeting the sexual phase of the seasonal cycle of aphid pests that impact a primary host holds considerable promise. The demonstration of increased trap catches of males of specific pest species through use of sex pheromone lures shows promise for improving current approaches to monitoring and managing aphid pests of primary hosts. The development of management tactics, such as mating disruption, mass trapping, attract and kill, and dissemination of entomopathogens into aphid populations from pheromone traps, may also be possible (Hartfield et al. 2001; Pickett and Glinwood 2007). Establishment of significant and predictable correlations among data from pheromone-based fall trapping and overwintering egg and damaging spring aphid population densities, would provide useful measures by which more accurate determinations of the need for insecticide intervention to manage aphid pests can be made.

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References

BIRKETT, M. A. and PICKETT, J. A. 2003. Aphid sex pheromones: From discovery to commercial production. *Phytochemistry* 62:651–656.

BOO, K. S., CHOI, M. Y., CHUNG, I. B., EASTOP, V. F., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2000. Sex pheromone of the peach aphid, *Tuberocephalus momonis*, and optimal blends for trapping males and females in the field. *J. Chem. Ecol.* 26:601–609.

CAMPBELL, C. A. M., DAWSON, G. W., GRIFFITHS, D. C., PETTERSSON, J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Sex attractant of the damson-hop aphid *Phorodon humuli* (Homoptera: Aphididae). *J. Chem. Ecol.* 16:3455–3465.

CONOVER, W. J. 1999. pp. 584, Practical Nonparametric Statistics, 3rd ed. Wiley, New York.

DAWSON, G. W., GRIFFITHS, D. C., JANES, N. F., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1987. Identification of an aphid sex pheromone. *Nature*. 325:614-616.

DAWSON, G. W., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1988. The sex pheromone of the greenbug, *Schizaphis graminun*. *Entomol. Exp. Appl.* 48:91–93.

DAWSON, G. W., JANES, N. F., MUDD, A., PICKETT, J. A., SLAWIN, A. M. Z., WADHAMS, L. J., and WILLIAMS, D. J. 1989. The aphid sex pheromone. *Pure Appl. Chem*. 61:555–558.

DAWSON, G. W., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid semiochemicals—A review, and recent advances on the sex pheromone. *J. Chem. Ecol.* 16:3019–3030.

DAWSON, G. W., PICKETT, J. A., and SMILEY, D. W. M. 1996. The aphid sex pheromone cyclopentanoids: Synthesis in the elucidation of structure and biosynthetic pathways. *Bioorg. Med. Chem.* 4:351–361.

DEWHIRST, S. Y., BIRKETT, M. A., FITZGERALD, J. D., STEWART-JONES, A., WOODCOCK, C. M., WADHAMS, L. J., HARDIE, J., and PICKETT, J. A. 2008. Dolichodial: A new aphid sex pheromone component? *J. Chem. Ecol.* 34:1575–1583.

DEWHIRST, S. Y., PICKETT, J. A., and HARDIE, J. 2010. Aphid pheromones. *Vitam. Horm.* 83:547–570.

GABRYŚ, B. J., GADOMSKI, H. J., KLUKOWSKI, Z., PICKETT, J. A., SOBOTA, G. T., WADHAMS, L. J., and WOODCOCK, C. M. 1997. Sex pheromone of cabbage aphid *Brevicoryne brassicae*: identification and field trapping of male aphids and parasitoids. *J. Chem. Ecol.* 23:1881–1890.

GOLDANSAZ, S. H., DEWHIRST, S., BIRKETT, M. A., HOOPER, A. M., SMILEY, D. W. M., PICKETT, J. A., WADHAMS, L., and MCNEIL, J. N. 2004. Identification of two sex pheromone components of the potato aphid, *Macrosiphum euphorbiae* (Thomas). *J. Chem. Ecol.* 30:819–834.

GRAVES, S. 2003. The role of component ratio integrity in host plant selection: a chemical and biological approach. PhD dissertation. University College, London, UK.

GULDEMOND, J. A., DIXON, A. F. G., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1993. Specificity of sex pheromones, the role of host plant odour in the olfactory attraction of males, and mate recognition in the aphid *Cryptomyzus*. *Physiol. Entomol.* 18:137–143.

HARDIE, J., HOLYOAK, M., NICHOLAS, J., NOTTINGHAM, S. F., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid sex pheromone components: Age-dependent release by females and species specific male response. *Chemoecology* 1:63–68.

HARDIE, J., STORER, J. R., COOK, F. J., CAMPBELL, C. A. M., WADHAMS, L. J., LILLEY, R., and PEACE, L. 1996. Sex pheromone and visual trap interactions in mate location strategies and aggregation by host-alternating aphids in the field. *Physiol. Entomol.* 21:97–106.

HARDIE, J., PICKETT, J. A., POW, E. M., and SMILEY, D. W. M. 1999. Aphids, pp. 227–250, *in* J. Hardie and A. K. Minks (eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford.

HARTFIELD, C. M., CAMPBELL, C. A. M., HARDIE, J., PICKETT, J. A., and WADHAMS, L. J. 2001. Pheromone traps for the dissemination of an entomopathogen by the damson–hop aphid *Phorodon humuli*. *Biocontrol Sci. Tech.* 11:401–410.

IPE, D. 1987. Performing the Friedman test and the associated multiple comparison test using PROC GLM. *Proc SAS Users Group Int Conf* 12:1146–1148.

IRWIN, M. A., KAMPMEIER, G. E., and WEISSER, W. W. 2007. Aphid movement: process and consequences, pp. 153–186, *in* H. F. van Emden and R. Harrington (eds.), Aphids as Crop Pests. CAB International, Wallingford.

KOCZOR, S., SZENTKIRALYI, F., BIRKETT, M. A., PICKETT, J. A., VOIGHT, E., and TOTH, M. 2010. Attraction of *Chrysoperla carnea* complex and *Chrysopa* spp. lacewings (Neuroptera: Chrysopidae) to aphid sex pheromone components and a synthetic blend of floral compounds in Hungary. *Pest Manag. Sci.* 66:1374–1379.

LILLEY, R. and HARDIE, J. 1996. Cereal aphid responses to sex pheromones and host-plant odours in the laboratory. *Physiol. Entomol.* 21:304–308.

MARSH, D. 1972. Sex pheromone in the aphid *Megoura viciae*. *Nature* 238:31–32.

PARK, K. C., ELIAS, D., DONATO, B., and HARDIE, J. 2000. Electroantennogram and behavioral responses of different forms of the bird-cherry oat aphid, *Rhopalosiphum padi*, to sex pheromone and a plant volatile. *J. Insect Physiol.* 46:597–604.

PETTERSSON, J. 1970. An aphid sex attractant I. Biological studies. *Entomol. Scan.* 1:63–73.

PICKETT, J. A. and GLINWOOD, R. T. 2007. Chemical ecology, pp. 235–260, *in* H. F. van Emden and R. Harrington (eds.), Aphids as Crop Pests. CAB International, Wallingford.

POWELL, G. and HARDIE, J. 2001. The chemical ecology of aphid host alternation: How do return migrants find the primary host plant? *Appl. Entomol. Zoolog.* 36:259–267.

SAS INSTITUTE 2008. SAS OnlineDoc, Version 9.2. SAS Institute, Cary.

STEWART-JONES, A., DEWHIRST, S. Y., DURRANT, L., FITZGERALD, J. D., HARDIE, J., HOOPER, A. M., PICKETT, J. A., and POPPY, G. M. 2007. Structure, ratios and patterns of release in the sex pheromone of an aphid, *Dysaphis plantaginea*. *J. Exp. Biol.* 210:4335–4344.

THIEME, T. and DIXON, A. F. G. 1996. Mate recognition in the *Aphis fabae* complex: Daily rhythm of release and specificity of sex pheromones. *Entomol. Exp. Appl.* 79:85–89.

ZHU, J. W., ZHANG, A. J., PARK, K. C., BAKER, T. C., LANG, B., JURENKA, R., OBRYCKI, J. J., GRAVES, W. R., PICKETT, J. A., SMILEY, D., CHAUHAN, K. R., and KLUN, J. A. 2006. Sex pheromone of the soybean aphid, *Aphis glycines* Matsumura, and its potential use in semiochemical-based control. *Environ. Entomol.* 35:249–257.

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Timing of Male Sex Pheromone Biosynthesis in a Butterfly – Different Dynamics under Direct or Diapause Development

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Abstract The life history traits and behavior of the butterfly *Pieris napi* are well-known, as the species is often used as a model organism for evolutionary and ecological studies. The species has two or more generations per year in the major part of its temperate distribution, and as different selection pressures affect the different generations, both behavioral and physiological seasonal polyphenisms have been shown previously. Here, we explored the dynamics of male sex pheromone production. The two generations are shown to have significantly different scent compositions early in life; the direct developers—who have shorter time for pupal development—need the first 24 hr of adult life after eclosion to synthesize the sex pheromone citral (geranial and neral 1:1)—whereas the diapausing individuals who have spent several months in the pupal stage eclose with adult scent composition. Resource allocation and biosynthesis also were studied in greater detail by feeding butterflies ^{13}C labeled glucose either in the larval or adult stage, and recording incorporation into geranial, neral, and other volatiles produced. Results demonstrate that the pheromone synthesized by newly eclosed adult males is based on materials ingested in the larval stage, and that adult butterflies are able to synthesize the pheromone components geranial and neral and the related alcohols also from adult intake of glucose. In summary, our study shows that time-stress changes the timing in biosynthesis of the complete pheromone between generations, and underpins the importance of understanding resource allocation and the physiological basis of life history traits.

Keywords Aphrodisiac · Citral · Green-veined white butterfly · Polyphenism · Sex pheromone

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Introduction

Although Darwin (1871) already included chemical signals along with visual and auditory ones as important in sexual selection, these different modalities commonly assume different importance in different groups of animals. With respect to the diurnal pattern of activity, it seems logical to assume that chemical signals play a more important role for nocturnal than day-active animals, and indeed the sex pheromones released by a vast number of moths have now been described and are known to be the dominant modality by which sexual selection is exercised in these species (Wyatt, 2003, 2009). By comparison, pheromones traditionally have been assumed to play a less important role in butterflies, but this picture is now beginning to change. Another change of focus is related to which sex is actively dispensing the sex pheromone; in moths it is usually the female that attracts males from afar by dissemination of a pheromone, but in butterflies recent studies, alongside some traditional studies, have shown that pheromones emitted by males during courtship can be important for female acceptance (Brower et al., 1965; Andersson et al., 2007; Costanzo and Monteiro, 2007; Nieberding et al., 2008).

In butterflies, males customarily use both visual and olfactory stimuli during courtship, and both are needed for female acceptance of a mate (Vane-Wright and Boppre, 1993; Breuker and Brakefield, 2002; Wiklund, 2003; Robertson and Monteiro, 2005; Costanzo and Monteiro, 2007; Papke et

al., 2007; Kemp, 2007, 2008). Pheromones usually are employed at short-range during the courtship (Myers 1972; Vane-Wright and Boppre, 1993) and are emitted from scent-releasing organs, androconia, found on the legs, abdomen, thorax, or wings of the male (Birch et al., 1990).

Male scents or pheromones are a prevalent phenomenon and can be explained by several mutually non-exclusive functions; facilitating female acceptance, conveying information of male quality, species-specific recognition, sex-specific recognition, or intra-sexual signals between males (e.g., Andersson et al., 2007; Costanzo and Monteiro, 2007). As strong selection against interspecific matings (Andersson, 1994) can be expected, male sex pheromones that are both species and sex-specific would be beneficial.

Studying the relationship between diet, nutritional physiology, and life history can help us understand how holometabolous insects, such as butterflies, handle the temporal separation between nutrient intake and demand due to their shift in diet between life stages. The diet of most butterflies changes substantially with development, from herbivory in the larval stage to nectarivory in the adult stage. As these diets are nutritionally different, it is of interest to examine the use of dietary resources for manufacture of sex pheromones. If a sex pheromone, for example, only can be synthesized from larval derived nutrients and not from adult-derived nectar, the male will be constrained by his larval host plant. Consequently, how organisms use nutritional resources can be fundamental to predictions about life history and evolutionary ecology (e.g., Reznick, 1985; Stearns, 1992) and physiological complexity of resource allocation has received increasing interest (Rose and Bradley, 1998; Zera and Harshman, 2001; Stjernholm and Karlsson, 2006).

In the genus *Pieris,* the male sex pheromone is known for both *Pieris brassicae, P. rapae* (Yildizhan et al., 2009), and *P. napi* (Andersson et al., 2007). The green-veined white butterfly, *P. napi*, is a well-documented model organism for evolutionary studies, and is one of the few butterfly species (see Nieberding et al., 2008) where both behavioral (e.g., Larsdotter-Mellström et al., 2010) and chemical (Andersson et al., 2000, 2007) background information is available on male sex pheromones. Females control mating (Wiklund, 2003; Bergström and Wiklund, 2005) and usually mate 1–5 times during their lifetime in the wild, with an average of 2.7 times (Bergström et al., 2002). In *P. napi*, visual cues seem to be less important (Larsdotter-Mellström et al., 2010), whereas in other butterfly species such as *Bicyclus anynana*, visual and olfactory cues are equally important for mating success (Costanzo and Monteiro, 2007). *Pieris napi* males emit a strong scent, perceivable also by humans, of citral—a 1:1 mixture of the geometric isomers neral [(2*Z*)-3,7-dimethylocta-2,6-dienal] and geranial [(2*E*)-3,7-dimethylocta-2,6-dienal (Bergström and Lundgren, 1973)]. The amount of citral released by a male is correlated to male flight activity (Andersson et al., 2007), and it the pheromone seems to be passively emitted during flight. The adult male scent composition consists of around 95 % of the two isomers of citral (Andersson et al., 2007), and pure citral was sufficient to elicit a mate acceptance behavior as shown by behavioral assays, whereas a male model without citral never was accepted by a female (Andersson et al., 2007). In the same study, electroantennograms showed that both males and females react to citral, but the antennal response by females was approximately 10 times stronger.

The sex pheromone in *P. napi* can in addition to facilitating female acceptance also be used by males to assess male-male competition for matings and thereby allocation to the ejaculate (Larsdotter-Mellström and Wiklund, 2009). This shows the evolutionary potential of sex pheromones and highlights the importance of understanding physiology for both life history and ecology.

Species with two or more discrete generations per year, such as *P. napi*, may evolve to differ in life history traits, as selection pressures may vary with season and affect different generations of the same species in different ways. In *P. napi*, as well as in other butterflies, seasonal polyphenism influences both physiology and behavior (Larsdotter-Mellström and Wiklund, 2010; Larsdotter-Mellström et al., 2010). The bivoltine life cycle confers developmental time constraints for this species, consisting of a difference in time available for larval and pupal development of the two generations (see Friberg et al., In press). The diapause generation, eclosing in spring after having spent winter as pupae, suffers little from developmental time stress, as they spend several weeks in the pupal stage after the termination of diapause (Wiklund and Solbreck, 1982; Forsberg and Wiklund, 1988)—the more time-constrained, directly-developing generation generally has only about a week of pupal development before eclosion of the adult. As shorter time in the pupa yields shorter time for physiological processes during morphogenesis of the adult butterfly (Wiklund and Solbreck, 1982; Forsberg and Wiklund, 1988), we predict that males of the two generations will differ with respect to the composition of volatiles at eclosion and the timing of pheromone synthesis. This then will affect how soon after eclosion males can mate (Andersson et al., 2007, Larsdotter-Mellström et al., 2010).

To study the differences in physiology between generations that experience different levels of time stress, we used a bivoltine central Swedish population of *P. napi*. Our first objective was to assess pheromone biosynthesis in *P. napi*, and to compare sexual maturity at the time of adult eclosion, between the two generations. The time constrained directly-developing generation takes, on average, more than 6 hr longer to mate for the first time after eclosion than do diapausing males (23 vs. 29,5 hr) (Larsdotter-Mellström et al., 2010). Therefore, we hypothesized that the directly-developing males would take longer to reach adult scent composition than diapausing males. Second, we also more specifically studied

whether geranial and neral are biosynthesized from larval and/or adult-derived material by using stable carbon isotopes. With the *P. napi* butterfly system and stable carbon isotopes, we can describe citral production by studying time allowed for feeding on labeled glucose together with the appearance of labeled pheromone components. This then can be compared with known geranial and neral biosynthetic pathways in plants (Iijima et al., 2006; Schilmiller et al., 2009) to investigate whether *P. napi* uses similar or different biosynthetic pathways. Iijima et al. (2006) suggested, based on molecular methods, that the biosynthetic route to geranial and neral in plants to be formed from geranylpyrophosphate via geraniol-synthase, first to geraniol, then oxidized to geranial and isomerized to neral, followed by reduction of neral to nerol. If *P. napi* males use the same biosynthetic route, we would expect labeled compounds to show up in the order; geraniol – geranial – neral – nerol.

Methods and Materials

Study Species *Pieris napi* is a wide-spread, common temperate butterfly species. The larvae feed on a variety of species in the Brassicaceae family, such as *Cardamine pratensis (L.)*, *Barbarea vulgaris* (*R. Br.*), *Armoracia rusticana* (*G. M. S.*), and *Alliaria petiolata (Scop.)*. The founders of the laboratory population originates from the Stockholm area where *P. napi* has two generations per year, over-wintering in the pupal stage and flying during spring (diapausing individuals) and summer (directly-developing individuals).

The butterflies used were the offspring of wild-caught *P. napi*. Larvae were reared in climate cabinets (Termaks KB 8000L) and allowed to feed *ad libitum* throughout larval development until pupation on one of their natural crucifer host plants *A. rusticana* or *A. petiolata*. For comparison of the two generations (experiment 1), we synchronized adult eclosion from both pathways (see Larsdotter-Mellström et al., 2010). During the fall of 2007 and 2008, the light conditions in the climate cabinets were set to induce diapause (12:12 hr L:D at 23 °C), and pupae were then kept in a refrigerator at −1 °C. After 5 mo, half of the overwintering pupae were incubated at 23 °C, which led to adult eclosion approximately 1 wk later. These adults were mated and were allowed to lay eggs on *A. petiolata*, and eggs randomly chosen from >15 females founded the next generation. These larvae were reared under conditions that induce direct development (20:4 hr L:D, 23 °C). They eclosed as directly-developing adults simultaneously with the adults eclosing from the remaining pupae of the diapausing generation that had spent approximately 6.5 mo in diapause, and had thereafter been incubated at 20:4 hr L:D at 23 °C about a week before eclosion. For experiments 2 and 3, directly-developing males were used.

Experimental Setup The butterflies were kept indoors at the Department of Zoology, Stockholm University, Sweden. Experiments were carried out during May 2008 and April 2009 (experiment 1) and January 2010 (experiments 2 and 3). The flight cages (0.8×0.8×0.5 m) were located in a room with large windows and 400 W metal halide lamps over the cages to simulate daylight. The lamps were lit 8 hr/day between 9 am and 5 pm. The bottom of each cage was covered with paper soaked with water to maintain high humidity. In each cage, a *Kalanchoe sp.* plant, with drops of 20 % sucrose solution added to the flowers, was present for feeding.

Experiment 1 focused on the progression of the pheromone composition in males of the two developmental pathways. The volatile emission from the wings was compared between the two generations 4, 6, 8, and 24 hr after eclosion. Sixteen males from the directly-developing generation and 32 males from the diapausing generation, evenly distributed over the four treatments (4, 6, 8, or 24 hr), were released into flight cages upon eclosion. The males then were euthanized by freezing 4, 6, 8, or 24 hr after adult eclosion. For the directly-developing generation, an extra 5 males were allowed 1 wk of activity in the cages before freezing, to make sure that we had fully mature males for comparison of pheromone emission. The wings of all males were extracted separately for volatiles (see below). Five last instar male pupae from the diapausing pathway (approximately 1 hr before eclosion) and 10 directly-developing male pupae (5 approximately 24 hr before eclosion and 5 approximately 1 hr before eclosion) also were extracted.

In experiment 2, the effect of larval feeding on pheromone biosynthesis was studied. The light regime during larval development was 20:4 hr L:D, 23 °C securing direct development. Larvae were kept in climate cabinets and reared in plastic jars with leaves of *A. rusticana* in water culture present *ad libitum* throughout development. To examine if larval-derived nutrients contribute to the synthesis of sex pheromones emitted as adults, 6 male larvae during the last larval instar were fed leaves coated with fully ^{13}C labeled glucose. Ingested amounts were approximately 0.025 g of labeled glucose per larva. The males were, after eclosion, allowed 6 hr of activity in the flight cages, before they were transferred to a cold room (8 °C). The 6 males then were transferred from the cold room to be individually assayed for live emission of volatiles.

In experiment 3, the production of the pheromone components by adult males was studied by allowing them to feed on ^{13}C labeled glucose. The order of appearance of labeled pheromone components gives an indication of the biosynthetic pathway used in the species. Males were put in a cold room (8 °C) as they eclosed from direct development, for a maximum of 4 d, until sufficient numbers of individuals had emerged for an experiment to start. In total, 35

males were evenly assigned to one out of seven treatments (4, 6, 8, 16, 24, 32, or 48 hr of activity) and then released into flight cages. In each cage, a *Kalanchoe sp.* plant with drops of fully ^{13}C labeled glucose dissolved in 20 % sucrose solution on the flowers was added for feeding. The males were allowed 4, 6, 8, 16, 24, 32, or 48 hr of activity and foraging in the cages before they were euthanized by freezing. The wings of all males were extracted separately for volatiles (see below). Twenty five individuals were successfully analyzed.

Extraction and Chemical Analyses For extraction of volatiles from euthanized adults, the four wings of each individual were detached and placed in a 1.7-ml glass vial without crushing. For extraction from last stage pupae of directly-developing males, the pupae were crushed, and for diapause-generation males the abdomen was removed and the thorax with wings was placed individually in a 1.7-ml glass vial without crushing. Hexane (200 μl Lancaster pa grade spiked with 0.08 mg/ml n-pendadecane as internal standard) was added, and extraction of the wings/pupae was made during 12 hr at room temperature. Separation and identification of the wing scent components was made by using a Varian 3400 GC coupled to a Finnigan SSQ 7000 MS instrument. Injected amounts were 1 μl (splitless injection, 45 sec) using a A200S Finnigan MAT autosampler. A DB-WAX column (internal diameter 0.25 mm, film thickness 0.25 μm and length 30 m) was used, programmed at 40 °C (for 1 min), then increased 5 °C/min to 110 °C, followed by 10 °C/min to 200 °C (for 10 min), with an injector temperature at 225 °C and He carrier gas at 69 kPa. Identification of the compounds was made by comparison of retention times and mass spectra with authentic reference standards.

Emissions of the living adult males (experiment 2) were collected individually after the butterfly had been given 6 hr of activity in the flight cage. Collection of volatiles was made with solid-phase micro-extraction (SPME) with 65 μm polydimethylsiloxane/divinylbenzene fibers (Supelco) during 1 hr at room temperature. During collection of the emissions, the butterfly was kept in a glass cylinder (height 11 cm, diam 4.5 cm) that was sealed with aluminum foil. Before the experiments, the glass cylinder was cleaned by keeping it overnight at 150 °C. Chemical analysis was made as described above, with manual injection of the SPME fiber.

In experiment 1 the relative amounts of wing odors were calculated using the area of each compound divided by the sum of the areas of all four compounds (geranial, neral, nerol, and geraniol), multiplied by 100.

In order to show that the butterflies can use both larval and adult-derived materials for the production of pheromone components, we measured the incorporation of ^{13}C into pheromone components in experiments 2 and 3. After comparing the mass spectra of labeled and non labeled wing components, the fragments 71 and 69 were selected (see Fig. 1). Fragment 71 was chosen, as it did not appear in the mass spectra of the pheromone components geranial and neral obtained by non labeled male butterflies, and as such represented the level of labeled molecules. The relative abundance of the ion fragment 71 was divided with the relative abundance of the ion fragment 69, which is the equivalent ion of non labeled males. The level of incorporation using the molecular ion and corresponding isotopes was not used due to very low abundances in the four wing components. To avoid variation in labeled proportion due to individuals ingesting different amounts of labeled glucose, the incorporation of labeled carbon in the four odor components was calculated for each individual. As the amount of citral released by a male is strongly correlated to male flight activity (Andersson et al., 2007), the composition of volatiles emitted is reported as relative amounts.

Representative mass spectra of wing scents of one adult male who fed on labeled glucose for 48 hr that shows the level of incorporation in geraniol, nerol, geranial, and neral is seen in Fig. 1. For comparison, non-labeled mass spectra of the compounds are shown in the right hand panel.

Statistical Analyses Alpha was set at 0.05. All tests were performed in R (Version 2.10.1, 2009). Non-parametric tests were used, as data did not meet parametric test assumptions and could not be transformed to do so using *Box-Cox* transformations.

In experiment 1, the maturity of the two generations measured as proportion citral (consisting of neral + geranial) out of total scent composition was compared using *Mann–Whitney U-tests*.

In experiment 2, the level of incorporation in wing emissions was measured as the proportion of labeled (larval derived) volatiles compared to non labeled (ions 71/69) during live emission after 6 hr of activity after eclosion.

In experiment 3, differences in the abundance of the selected labeled and non-labeled fragments (71/69) were analyzed using *Spearman's rank correlation*.

Results

Experiment 1 The volatiles of the wing scent consisted of geraniol, nerol, geranial, neral in variable amounts depending on the age and generation of the butterflies (Fig. 2). At 4 hr of age, none of the directly-developing males, in contrast to the diapause-generation males, had any measurable amount of neral, and only one out of four directly-developing males had any geranial. The proportion of citral (geranial + neral) in the scent composition (alcohols + aldehydes) differed between generations during the first 6 hr of adult life (at 4 hr, *Mann–Whitney U test,* $U=1$, $P=0.02$; at 6 hr, *Mann–Whitney*

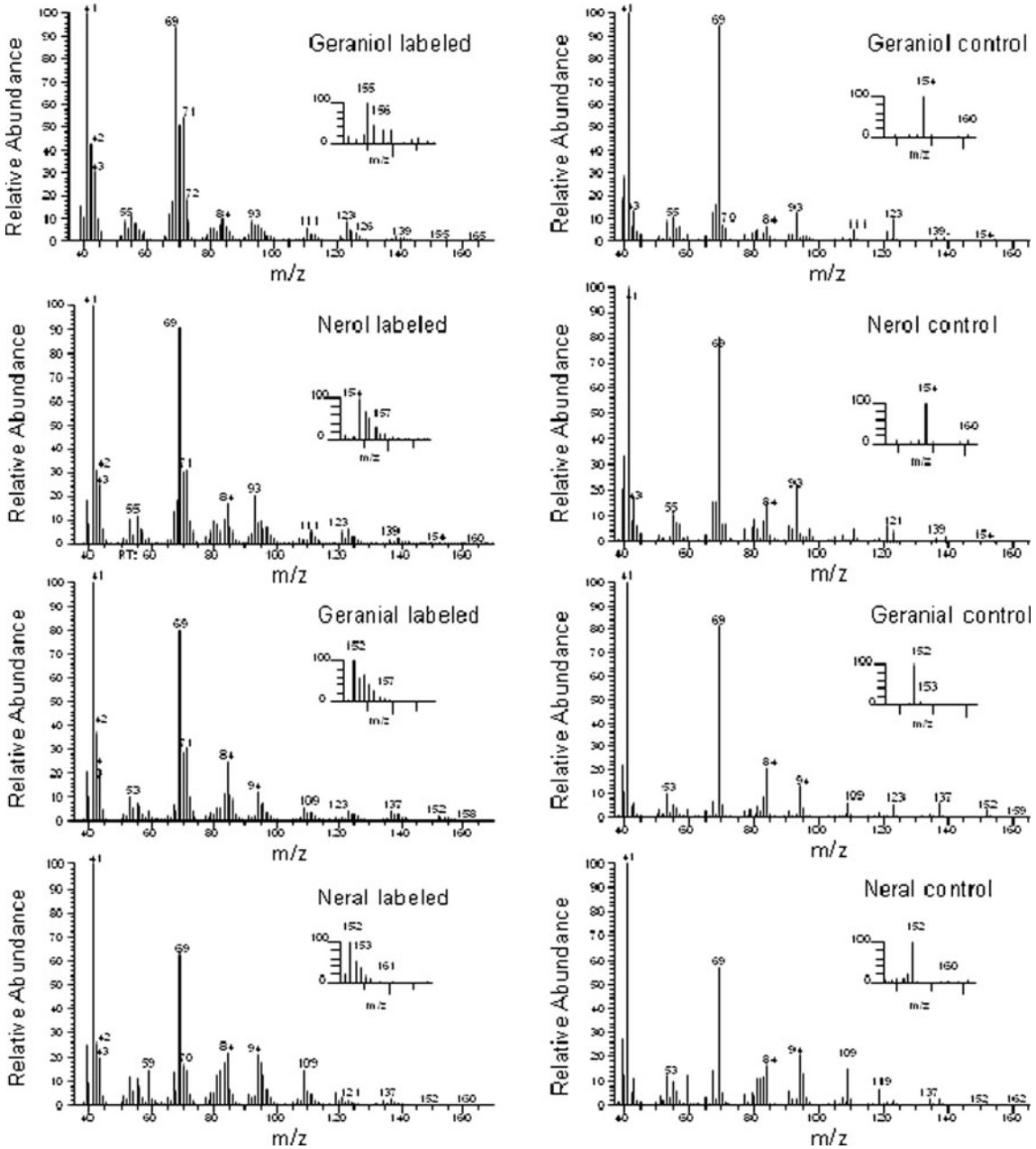

Fig. 1 Representative mass spectra for the four wing odor compounds from *Pieris napi* male wing after 48 hr of feeding on ^{13}C labeled glucose (*left column*). Non-labeled mass spectra of the compounds on the right

U test, U=4, *P*=0.03). After 8 and 24 hr, the difference in sex pheromone composition had disappeared, and the generations no longer differed in citral proportion (8 hr, *Mann–Whitney U test, U*=28, *P*=0.77; 24 hr, *Mann–Whitney U test, U*=27, *P*=0.20).

Directly-developing males could be considered mature as they reached 24 hr of age, since the scent composition no longer changed with age and citral proportion (neral + geranial out of total scent) was at 24 hr (median; 89 % citral) not significantly different from one-wk-old males (median; 93 % citral) (*Mann–Whitney U test, U*=8, *P*=0.4).

One out of the five diapausing pupae had geraniol present in small amounts approximately 1 hr before eclosion. None of the ten directly-developing pupae had traceable amounts of any of the four wing scent components.

Experiment 2 Larval feeding: Labeled ^{13}C of the fully labeled glucose, ingested in the larval stage, showed up in geraniol, nerol, geranial, and neral in the adult stage as measured by volatiles emitted. Around half of the emitted substances were at 6 hr of age derived from the labeled carbon. The average proportion ± SE labeled to non-

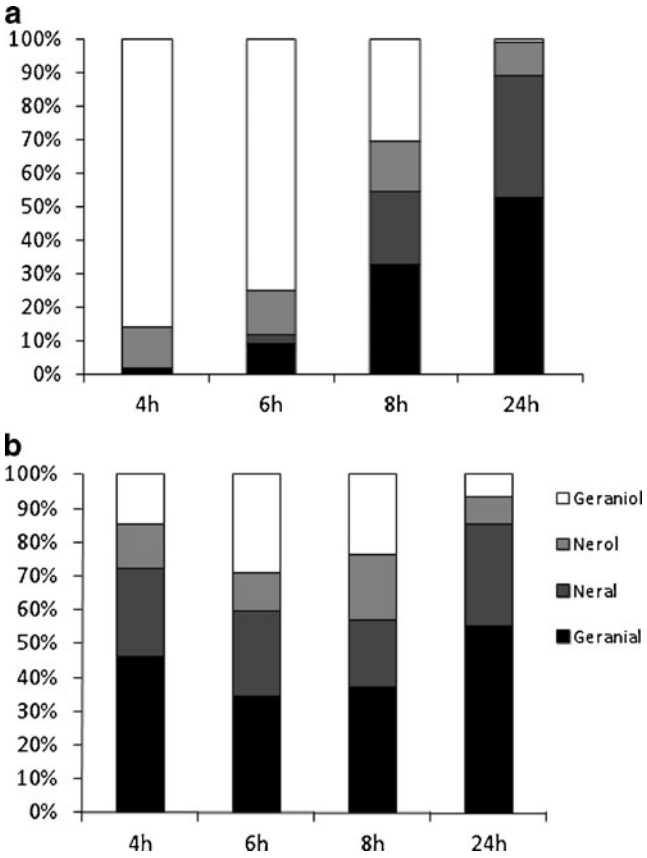

Fig. 2 Scent composition of the four major compounds by age. **a** Directly-developing *Pieris napi* males. **b** Diapausing *Pieris napi* males

labeled (ions 71/69) was; geraniol 0.9±0.2; nerol 0.8±0.2; geranial 1.0±0.3; neral 0.9±0.2.

Experiment 3 Adult feeding: As adult butterflies were allowed to feed on labeled glucose, the amount of labeled pheromone components increased significantly with the time allowed for foraging. For ions 71\69 (Fig. 3); Spearman's rank correlation, geranial $\rho=0.83$, $P<0.001$; geraniol $\rho=0.82$, $P<0.001$; neral $\rho=0.62$, $P<0.001$; nerol $\rho=0.87$, $P<0.001$.

Interestingly, when comparing the proportion of the ion fragments 71/69, a clear time difference in incorporation of ^{13}C was observed between the compounds. Incorporation of ^{13}C into geranial and neral appeared later than incorporation of ^{13}C into the corresponding alcohols. Already after 4 hr of feeding on labeled glucose, labeled geraniol and nerol were produced (Fig. 3). Labeled geranial appeared after 8 hr, and labeled neral was detected only after 32 hr of adult feeding (Fig. 3).

Discussion

From our experiments with ^{13}C-labeled glucose, we showed that male *P. napi* can synthesize geranial and neral from both larval (experiment 2) and adult-derived glucose (experiment 3, Fig. 3). The results of this study also show that males of the directly-developing generation do not emit adult scent composition at eclosion but have less geranial and neral and more of the corresponding alcohols early in life, indicating that males in the directly-developing generation eclose less sexually mature than diapausing individuals (Fig. 2). This could explain the observation that directly-developing males have a longer teneral period before mating (Larsdotter-Mellström et al., 2010); in the presence of a surplus of receptive females, diapause-generation males mated on average more than 6 hr sooner after eclosion than directly-developing males.

We contend that time constraints are responsible for the differences between generations (see Larsdotter-Mellström et

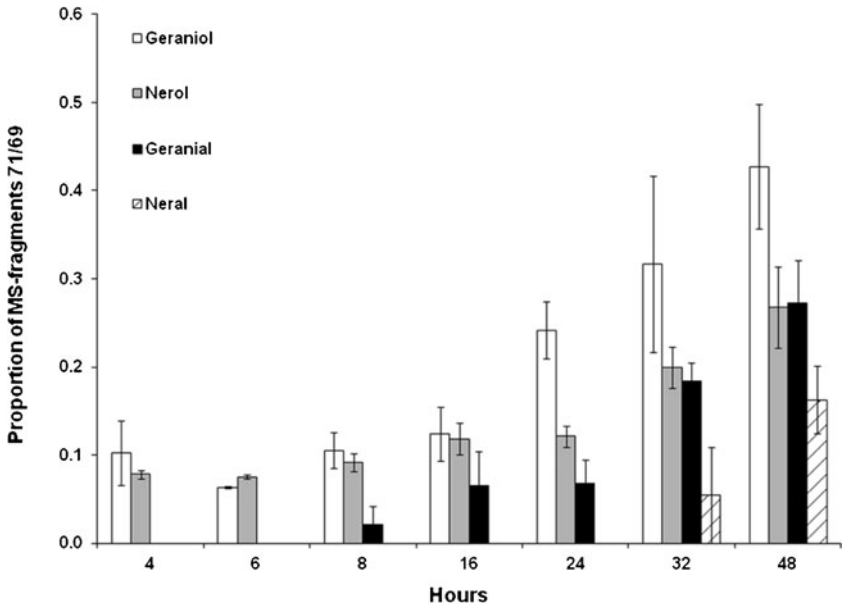

Fig. 3 Adult ^{13}C feeding. Proportion of labeled to non-labeled fragments (71/69) in the four components of *Pieris napi* wing scent. Average ± SE

al., 2010), as it is hard to envisage why males would evolve to postpone their maturity and first mating. In the butterfly *Bicyclus anynana*, the same pattern has been shown, with male sex pheromone synthesis being incomplete at eclosion (Nieberding et al., 2008, 2012), and older males having higher mating success than younger males (Fischer et al., 2008). Developing without strong time stress, may allow the adults of the diapausing spring generation to emerge in a sexually more mature state than adults of the time-constrained, directly-developing, summer generation. Why directly-developing *P. napi* eclose before they are sexually mature could have several, mutually non-exclusive explanations. The development of reproductive tissue might be faster in the adult stage than in the pupal stage, or survival might be higher in the adult stage. In the majority of insects, males emerge earlier than females, and protandry generally has been explained as a reproductive strategy that maximizes male mating success under the assumption that males are ready to mate already at the time of eclosion (Wiklund and Fagerström, 1977; Iwasa et al., 1983). The present results add a novel aspect to the nearly ubiquitous occurrence of protandry, as males not only benefit from emerging before females in terms of mating opportunities, but they also may need to emerge early to be sexually mature at the time when females start eclosing.

Wedell (2010) reported that males suffer a substantial cost, not only of mating, which is expected in a paternally investing species such as *P. napi*, but also from courtship behavior *per se*. A possible reason for this is that production of aphrodisiacs in the high amounts required for courtship could be nutritionally expensive. In this study, we have now shown that the male sex pheromones geranial and neral can be biosynthesized from glucose, a substance that is abundantly available, as a common nectar constituent, for feeding adult males. This means that male *P. napi* are able to keep up a steady production of the sex pheromone, to replenish what is emitted during flight (Andersson et al., 2007) and courtship. When comparing the results from experiment 1 and 3, we can see that directly-developing males have synthesized neral at the age of 8 hr (Fig. 2) but labeled neral only after 32 hr of adult feeding (Fig. 3). This may be due to the fact that neral is produced entirely from larval feeding in the first 32 hr after emergence, and gives an indication of the transition from larval to adult-derived sources of the pheromones. Additional experiments to formally test this would be interesting.

The results of the directly-developing males (Fig. 2) and labeling experiments (Fig. 3) suggest that the production of the male sex pheromone starts with the biosynthesis of geraniol via nerol, followed by geranial, and ending with neral. Iijima et al. (2006) suggest, based on molecular methods, the biosynthetic route to geranial and neral in plants to be formed from geranylpyrophosphate, first to geraniol, then oxidized to geranial, and isomerized to neral, followed by reduction of neral to nerol. By the feeding studies of *P. napi* using ^{13}C-labeled glucose and studies of pheromone progression in directly-developing males by GC-MS, we have shown that the fragments representing the labeled geranial and neral appear later in time, thus indicating a delayed production of neral and geranial compared to geraniol and nerol. We can only speculate whether there is a direct isomerization to nerol from geraniol, or whether there is the presence of two synthases—one producing geraniol and one producing nerol, followed by a subsequent oxidation step in the final formation of the pheromone. Monoterpenes in the glandular trichomes of tomato recently have shown to be synthesized from a neryl diphosphate precursor rather than geranyl diphosphate (Schilmiller et al., 2009), which supports our hypothesis of one *trans* and one *cis* synthase.

In summary, our study shows that time-stress can change the timing of pheromone production, between generations, in our model species *P. napi*. Studying the underlying resource allocation and the physiological basis of life history traits and mating success greatly contributes to the understanding of the ecology of the species.

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References

ANDERSSON, M. 1994. PP. 599, Sexual Selection. Princeton University Press, Princeton.

ANDERSSON, J., BORG-KARLSON, A. K., and WIKLUND, C. 2000. Sexual cooperation and conflict in butterflies: A male-transferred anti-aphrodisiac reduces harassment of recently mated females. *Proc. R. Soc. B.* 267:1271–1275.

ANDERSSON, J., BORG-KARLSON, A. K., VONGVANICH, N., and WIKLUND, C. 2007. Male sex pheromone release and female mate choice in a butterfly. *J. Exp. Biol.* 210:964–970.

BERGSTRÖM, G. and LUNDGREN, L. 1973. Androconial secretion of three species of butterflies of the genus *Pieris* (Lep., Pieridae). *Zoon Suppl.* 1:67–75.

BERGSTRÖM, J. and WIKLUND, C. 2005. No effect of male courtship intensity on female remating in the butterfly *Pieris napi*. *J. Insect Behav.* 18:479–489.

BERGSTRÖM, J., WIKLUND, C., and KAITALA, A. 2002. Natural variation in female mating frequency in a polyandrous butterfly: Effects of size and age. *Anim. Behav.* 64:49–54.

BIRCH, M. C., POPPY, G. M., and BAKER, T. C. 1990. Scents and eversible scent structures of male moths. *Annu. Rev. Entomol.* 35:25–58.

BREUKER, C. J. and BRAKEFIELD, P. M. 2002. Female choice depends on size but not symmetry of dorsal eyespots in the butterfly *Bicyclus anynana*. *Proc. R. Soc. B.* 269:1233–1239.

BROWER, L. P., BROWER, J. V. Z., and CRANSTON, F. P. 1965. Courtship behavior of the queen butterfly, *Danaus gilippus berenice*. *Zoologica* 50:1–39.

COSTANZO, K. and MONTEIRO, A. 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. *Proc. R. Soc. B.* 274:845–851.

DARWIN, C. 1871. The Descent of Man, and Selection in Relation to Sex. J. Murray, London.

FISCHER, K., PERLICK, J., and GALETZ, T. 2008. Residual reproductive value and male mating success: Older males do better. *Proc. R. Soc. B.* 275:1517–1524.

FORSBERG, J. and WIKLUND, C. 1988. Protandry in the green-veined white butterfly, *Pieris napi*. *Funct. Ecol.* 2:81–88.

FRIBERG M., DAHLERUS J., and WIKLUND C. In press. Strategic larval decision-making in a Bivoltine Butterfly. *Oecologia*. doi:10.1007/s00442-011-2238-z

IIJIMA, Y., WANG, G. D., FRIDMAN, E., and PICHERSKY, E. 2006. Analysis of the enzymatic formation of citral in the glands of sweet basil. *Arch. Biochem. Biophys.* 448:141–149.

IWASA, Y., ODENDAAL, F. J., MURPHY, D. D., EHRLICH, P. R., and LAUNER, A. E. 1983. Emergence patterns in male butterflies—A hypothesis and a test. *Theor. Popul. Biol.* 23:363–379.

KEMP, D. J. 2007. Female butterflies prefer males bearing bright iridescent ornamentation. *Proc. R. Soc. B.* 274:1043–1047.

KEMP, D. J. 2008. Female mating biases for bright ultraviolet iridescence in the butterfly *Eurema hecabe* (Pieridae). *Behav. Ecol.* 19:1–8.

LARSDOTTER-MELLSTRÖM, H. and WIKLUND, C. 2009. Males use sex pheromone assessment to tailor ejaculates to risk of sperm competition in a butterfly. *Behav. Ecol.* 20:1147–1151.

LARSDOTTER-MELLSTRÖM, H. and WIKLUND, C. 2010. What affects mating rate? Polyandry is higher in the directly developing generation of the butterfly *Pieris napi*. *Anim. Behav.* 80:413–418.

LARSDOTTER-MELLSTRÖM, H., FRIBERG, M., BORG-KARLSON, A. K., MURTAZINA, R., PALM, M., and WIKLUND, C. 2010. Seasonal polyphenism in life history traits: Time costs of direct development in a butterfly. *Behav. Ecol. Sociobiol.* 64:1377–1383.

MYERS, J. 1972. Pheromones and courtship behavior in butterflies. *Am. Zool.* 12:545–551.

NIEBERDING, C. M., DE VOS, H., SCHNEIDER, M. V., LASSANCE, J. M., ESTRAMIL, N., ANDERSSON, J., BANG, J., HEDENSTRÖM, E., LÖFSTEDT, C., and BRAKEFIELD, P. M. 2008. The male sex pheromone of the Butterfly *Bicyclus anynana*: Towards an evolutionary analysis. *PLoS One* 3(7).

NIEBERDING, C. M., FISCHER, K., SAASTAMOINEN, M., ALLEN, C. E., WALLIN, E. A., HEDENSTRÖM, E., and BRAKEFIELD, P. M. 2012. Cracking the olfactory code of a butterfly: The scent of ageing. *Ecol. Lett.* 15:415–424.

PAPKE, R. S., KEMP, D. J., and RUTOWSKI, R. L. 2007. Multimodal signalling: Structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). *Anim. Behav.* 73:1083–1083.

REZNICK, D. 1985. Costs of reproduction—an evaluation of the empirical evidence. *Oikos* 44:257–267.

ROBERTSON, K. A. and MONTEIRO, A. 2005. Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. *Proc. R. Soc. B.* 272:1541–1546.

ROSE, M. R. and BRADLEY, T. J. 1998. Evolutionary physiology of the cost of reproduction. *Oikos* 83:443–451.

SCHILMILLER, A. L., SHAUVINHOLD, I., LARSON, M., XU, R., CHARBONNEAU, A. L., SCHMIDT, A., WILKERSON, C., LAST, R. L., and PICHERSKY, E. 2009. Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. *Proc. Natl. Acad. Sci. U.S.A.* 106:10865–10870.

STEARNS, S. C. 1992. The Evolution of Life Histories. Oxford University Press, New York. p 249.

STJERNHOLM, F. and KARLSSON, B. 2006. Reproductive expenditure affects utilization of thoracic and abdominal resources in male *Pieris napi* butterflies. *Funct. Ecol.* 20:442–448.

VANE-WRIGHT, R. I. and BOPPRE, M. 1993. Visual and chemical signalling in butterflies—Functional and phylogenetic perspectives. *Philos. Trans. R. Soc. B.* 340:197–205.

WEDELL, N. 2010. Variation in male courtship costs in butterflies. *Behav. Ecol. Sociobiol.* 64:1385–1391.

WIKLUND, C. 2003. Sexual selection and the evolution of butterfly mating systems, pp. 67–90, *in* C. L. Boggs, W. Watt, and P. R. Erlich (eds.), Butterflies: Ecology and Evolution Taking Flight. The University of Chicago Press, Chicago.

WIKLUND, C. and FAGERSTRÖM, T. 1977. Why do males emerge before females—Hypothesis to explain incidence of protandry in butterflies. *Oecologia* 31:153–158.

WIKLUND, C. and SOLBRECK, C. 1982. Adaptive versus incidental explanations for the occurrence of protandry in a butterfly, *Leptidea Sinapis* L. *Evolution* 36:56–62.

WYATT, T. D. 2003. pp. 391, Pheromones and Animal Behaviour. Cambridge University Press, Cambridge.

WYATT, T. D. 2009. Fifty years of pheromones. *Nature* 457:262–263.

YILDIZHAN, S., VAN LOON, J., SRAMKOVA, A., AYASSE, M., ARSENE, C., TEN BROEKE, C., and SCHULZ, S. 2009. Aphrodisiac pheromones from the wings of the small cabbage white and large cabbage white butterflies, *Pieris rapae* and *Pieris brassicae*. *ChemBioChem* 10:1666–1677.

ZERA, A. J. and HARSHMAN, L. G. 2001. Physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* 32:95–106.

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Estimating Insect Flight Densities from Attractive Trap Catches and Flight Height Distributions

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Abstract Methods and equations have not been developed previously to estimate insect flight densities, a key factor in decisions regarding trap and lure deployment in programs of monitoring, mass trapping, and mating disruption with semiochemicals. An equation to estimate densities of flying insects per hectare is presented that uses the standard deviation (SD) of the vertical flight distribution, trapping time, the trap's spherical effective radius (ER), catch at the mean flight height (as estimated from a best-fitting normal distribution with SD), and an estimated average flight speed. Data from previous reports were used to estimate flight densities with the equations. The same equations can use traps with pheromone lures or attractive colors with a measured effective attraction radius (EAR) instead of the ER. In practice, EAR is more useful than ER for flight density calculations since attractive traps catch higher numbers of insects and thus can measure lower populations more readily. Computer simulations in three dimensions with varying numbers of insects (density) and varying EAR were used to validate the equations for density estimates of insects in the field. Few studies have provided data to obtain EAR, SD, speed, and trapping time to estimate flight densities per hectare. However, the necessary parameters can be measured more precisely in future studies.

Keywords Estimating flight density · Flight height · Computer simulation in three dimensions · Mass trapping · Monitoring · Mating disruption

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Introduction

Knowledge about densities of flying insects at particular times and places is of fundamental interest in ecology and chemical ecology, as well as in integrated pest management. Currently, there are no methods and equations that use trap catch data to estimate insect flight densities, a crucial factor in the success of mass trapping and mating disruption programs (Shorey, 1977; Cardé, 1990; Cardé and Minks, 1995; Miller et al., 2006a, b; El-Sayed et al., 2006, 2009; Byers, 2007, 2008). In order to develop methods and equations for determining flight densities from trap catch data, several parameters need to be estimated and understood. The first parameter is the *effective attraction radius* (EAR) that describes the strength of a semiochemical lure with regard to blend and release rate, which allows comparisons among species (Byers et al., 1989). The EAR is a spherical radius that would intercept the same number of insects as caught by a semiochemical-baited trap. The EAR is a spatial parameter based on catch, which conveniently substitutes in simulation models for the complex spatial dimensions of attractive odor plumes. The EAR can be determined by the catch ratio of attractive (baited) and non-attractive (blank) sticky traps, and the silhouette area of the trap (Fig. 1). In mass trapping for control of insects, the most effective lure is that with the largest EAR ascertained by field-testing the semiochemical at increasing release rates. The EAR has been used in simulation models of mass trapping and mating disruption (Byers, 2007) that are important tools of integrated pest management (Shorey, 1977; Cardé, 1990; Cardé and Minks, 1995; Miller et al., 2006a, b; El-Sayed et al., 2006, 2009; Byers, 2008). These and other studies, however, have relied upon subjectively testing densities of lures/traps by trial and error until some negative effect on mating success was observed. This approach would be more efficient with a better

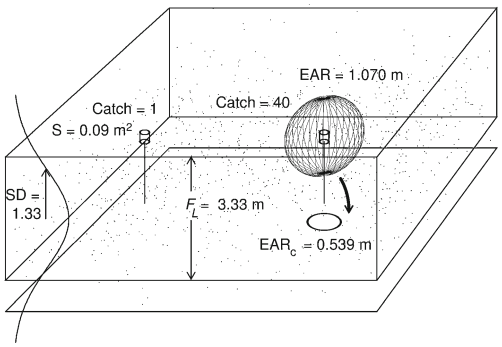

Fig. 1 Two cylindrical sticky traps, a blank catching one insect (Cb=1) and a pheromone trap catching 40 insects (Ca=40), are each 0.09 m^2 in silhouette area (S), giving a spherical EAR $= [(Ca \cdot S)/(\pi \cdot Cb)]^{0.5} = 1.070$ m that can be converted to a circular $EAR_c = \pi \cdot EAR^2/(2 \cdot F_L) = 0.539$ m (where $F_L = SD \cdot \sqrt{2 \cdot \pi} = 3.33$ m) (Byers, 2008). Based on the above EAR, SD, and catch of 40 given an 8 hr trap exposure with 2 m/sec flight speed, the 2D density estimate is 6.43 insects/hectare (from Eq. 6 in Results). Small dots represent 1000 insects distributed vertically in a normal distribution (SD=1.33 m)

knowledge of flight densities, EAR of attractants, and use of simulation models to determine the trap density most likely to succeed in control measures (Byers, 2007, 2008).

The *circular* EAR (termed EAR_c), used in encounter-rate simulations of mass trapping in two dimensions (2D), is not identical to the spherical EAR obtained from insect catches in the field (Byers, 2008, 2009). A transformation of EAR is necessary (Fig. 1) to obtain an accurate EAR_c for 2D simulations. This conversion equation requires an estimation of the effective flight layer (F_L), which reflects the vertical layer where the particular insect species flies in search of mates and host plants. Essentially, if the vertical flight density distribution described by the standard deviation (SD) is squeezed into a layer of uniform density equal to that at the mean flight height, then the thickness of this layer is the F_L (Byers, 2008, 2009). However, the F_L does not describe a real layer, so hereafter to avoid confusion the F_L will be replaced with its equivalent, which is $SD \cdot \sqrt{2 \cdot \pi}$. It is expected that the SD is a species characteristic that might vary somewhat due to habitat, but otherwise would often be quite different for each species, as indicated by SD-analyses of 116 species from previous studies (Byers, 2011).

Although more than 100 publications report trap catches of insects at different heights, the mean flight height or its SD have seldom been estimated (Byers, 2011), probably because iterative equations to calculate SD require inputting data for every insect caught (possibly thousands). By using an iterative algorithm, however, the SD can readily be determined (Byers, 2011). Knowledge about the mean flight height ± SD is not only useful in models, but can be applied in practice when deploying semiochemically baited traps for monitoring, mating disruption, and mass trapping. For example, Byers et al. (1989) estimated flight densities of spruce bark beetles, *Ips typographus* L., searching for host trees in Denmark. The densities were estimated in 2D (per hectare) by placing sticky-traps of known radius on metal poles throughout the flight volume (0.7 to 11.5 m high), and recording the number of beetles trapped over time. In these experiments during the spring swarming flight (May 17, 19, 20, 21, and 22, 1984), an estimated 38, 5, 55, 32, and 90 beetles per hectare were in continuous flight each day, respectively.

The first objective of the present investigation was to develop the equations necessary to obtain density estimates of flying insects from a combination of data including (1) catches on passive sticky traps at three or more heights to determine mean height and ± SD, and (2) catches on attractive and passive sticky traps to determine EAR and EAR_c. The equation for estimating densities in 2D also depends, in part, on the average flight speed, which is affected by both the insect's inherent flight speed in still air and the average wind speed.

The second objective addressed herein was to validate the density equation, and explore parameter effects by simulation of flying insects in three dimensions (3D). Simulated individuals can fly anywhere, but the population maintains a specified mean flight height, and a normal vertical distribution of flight heights (Byers, 2009, 2011). During these simulated population flights under constant parameters for number per area, flight speed, trap EAR, and exposure time, the stochastic catches on a spherical trap at the mean flight height were counted and compared to predicted results from the density equation. The simulation results should indicate the reliability and variability of the density estimates.

My third objective was to use the equations developed in the study to determine 2D insect densities from previous publications reporting passive and attractive trap data.

Methods and Materials

Equations for Estimating Densities of Insects per Hectare The trap catch data and a series of equations are presented that allow estimation of flight density in two dimensions, i.e., number per hectare. The equations are based on trap catch at several heights, and on data from a passive sticky trap placed at the mean flight height. The same equations, with little modification, can be used for chemically or visually attractive traps.

Given a passive cylindrical sticky trap, if its radius is 0.15 m and the height is 0.3 m, then the area S of the silhouette, as seen from a horizontal direction, is $S = 2 \cdot ra$

$dius \cdot height = 0.09\ m^2$. Using these values, the spherical effective radius, ER, of the trap is simply:

$$ER = \sqrt{S/\pi} = 0.169 \quad (1)$$

The mean flight height ($\overline{h}$) and SD of a particular insect species is found from catches on a series of passive traps placed at several heights (at least three) using the following formulas (Byers, 2011):

$$\overline{h} = sumx/n \quad (2)$$

$$SD = \sqrt{\left(n \cdot sumx^2 - (sumx)^2\right)/(n \cdot (n-1))} \quad (3)$$

where $sumx = \sum_{i=1}^{t} \sum_{j=1}^{c_i} h_i$, $sumx^2 = \sum_{i=1}^{t} \sum_{j=1}^{c_i} h_i^2$, and $n = \sum_{i=1}^{t} c_i$ with t = number of trap levels, h_i = height of trap level, c_i = catch at trap level, and i = trap level. In previous studies (Byers, 2008, 2009), $F_L = SD \cdot \sqrt{2 \cdot \pi}$ was used in an equation to convert spherical to circular radii. However, since F_L is a mathematical construct and not a real flight layer, while SD describes a real distribution, subsequent equations are in terms of SD.

The ER in three dimensions is converted to a circular effective radius ER_c in two dimensions (Byers, 2008, 2009) by the following formula:

$$ER_c = \pi \cdot ER^2/\left(2 \cdot SD \cdot \sqrt{2 \cdot \pi}\right) \quad (4)$$

The two-dimensional density D_2 of flying insects is then found from:

$$D_2 = C/(2 \cdot ER_c \cdot T \cdot V \cdot K) \quad (5)$$

where C is the catch on the trap (K=1) or traps (K>1) in the field, T is the seconds of trap exposure, and V is the average velocity (m/s) of the flying insects (K was added to equation 2 of Byers et al., 1989). Thus, if ER=0.169 m^2 and SD=1.67 m then ER_c=0.01075 m. Given that C=4, K=1, T=3600 sec, and V=2 m/sec, then D_2=0.0258 insects per m^2 or 258 insects per hectare. In 3D at the trap level, there are $D_2/\left(SD \cdot \sqrt{2 \cdot \pi}\right) = 0.0062$ insects per m^3 (Byers, 2009, 2011).

Density can more readily be estimated from a trap with a lure attractive to the particular insect species. First, calculate an EAR from an attractive trap placed at the mean flight height as shown in Byers (2008, 2009). For example, assuming the same catch occurred on a blank trap (Cb=4) of the same size as above, and Ca=80 were caught on the attractive trap, then $EAR = [(Ca \cdot S)/(\pi \cdot Cb)]^{0.5} = 0.757$ m. Substituting EAR for ER in Eq. (4), then EAR_c=0.215 m and this is substituted for ER_c in Eq. (5), where $C = Ca$, gives the same D_2=0.0258 per m^2 with the same above parameters. This means that EAR converted to EAR_c can be used to estimate 2D densities much more easily since catch is more readily obtained on an attractive trap than on a blank trap. The EAR and EAR_c need to be estimated previously from an attractive trap catch compared to the blank trap catch (Cb>0; Byers et al., 1989), as well as the SD from a series of trap heights and catches (Byers, 2008, 2009, 2011). Once the SD and EAR/EAR_c are estimated for a particular attractant release rate, subsequently only an attractive trap is needed to calculate the density of flying insects.

Equation (5) requires the average flight speed of the insects, which can be estimated by the flight speed in still air (for bark beetles this is about 1.6 m/sec, Byers et al., 1989). However, wind speed will affect flight speed either additively when the insect flies with the wind, or negatively when the insect flies upwind. Assuming most insects fly in all possible horizontal directions with respect to moderate wind speed as observed for moths, flies, and bark beetles (Elkinton and Cardé, 1983; Judd and Borden, 1988, 1989; Byers et al., 1989), then the affect of wind on average flight speed appears complex. Given that wind speed can range from 1 to 5 m/sec and the insect flies at 2 m/sec in still air, the average flight speed can be calculated by computer (from Eq. 7 in Results).

Simulation of Flight Densities and Catch on a Spherical Trap in 3D Insects were simulated in a volume with X-axis (xa), Y-axis (ya), and Z-axis (za) of 50×50×10 m, respectively. Various numbers of insects were placed initially at random according to a specified normal distribution of SD (Byers, 2001). Insects flew in a correlated random walk using spherical (x, y, z) coordinates (Hearn and Baker, 1994). The walk progressed as a series of steps, each of length $step$ (0.1 m), and 6° standard deviation of angular turns as in Byers (2001, 2009, 2011). Insects rebounded at random angles when striking the volume sides, and rarely when intercepting the ground and top boundaries. The coordinates of insects were transformed to 3D perspective coordinates when viewing the simulations (p. 57, Adams, 1987). Insects flew within the volume such that the population had a mean height of za/2 and was distributed normally with SD=1.67 m, as accomplished by an algorithm in Byers (2009, 2011). Insects flew at 2 m/sec for 1 hr except when the period of simulation was varied. The algorithm to determine whether insects entered or passed through the spherical EAR of a trap was as described in Byers (2009, 2011). Insects that were caught on the trap were replaced at random according to the population distribution within the volume to maintain a constant density. Simulations and calculations, for general demonstration on the Internet with a web browser (http://www.chemical-ecology.net/java2/den-2d.htm), were programmed in QuickBASIC 4.5 (Microsoft Corp., Redmond, WA, USA) as well as Java 6.0 (Oracle, Redwood City, CA, USA).

Estimating Densities from Insect Catch Studies in the Literature The scientific literature was explored (BIOSIS Previews) for articles that reported catch on traps at three or more heights in which catch diminished with height. These data were used to determine the mean height of flight and SD. These parameters were calculated previously (Tables 1–3 in Byers, 2011), but only the data on unattractive traps (his Table 3) were further analyzed to determine densities with Eq. (5). The catch at the mean flight height was estimated from the normal equations (Byers, 2011). In addition, some of these studies also reported catch on an attractive sticky trap as well as on a blank sticky trap that caught at least one insect in order to find an EAR. In studies where an attractive-colored sticky card was used (Gillespie and Vernon, 1990), the height by width area S is calculated for all possible angles that insects can intercept the trap giving $S = 0.637 \cdot height \cdot width$ (Byers et al., 1989). Attractive-colored cross-pane barrier traps (Zhang et al., 2011) have a mean interception area of $S = 0.9 \cdot height \cdot width$ (Byers, 2009). The formulas for cards and cross-pane traps also work for attractive odors. Using the attractive catch, EAR, and SD, it is possible to calculate densities per hectare (Eq. 6 in Results).

Results

Equations for Estimating Densities of Insects per Hectare The EAR_c does not need to be calculated explicitly to calculate 2D density because the EAR and SD can be used instead. Using EAR for ER in Eq. (4) gives the equation $EA R_c = \pi \cdot EAR^2 / \left(2 \cdot SD \cdot \sqrt{2 \cdot \pi}\right)$ that, when substituted into Eq. (5), gives the final portion of Eq. (6) in terms of EAR and SD:

$$D_2 = C/(2 \cdot EAR_c \cdot T \cdot V \cdot K) = C/\left(\pi \cdot EAR^2 \cdot T \cdot V \cdot K/\left(SD \cdot \sqrt{2 \cdot \pi}\right)\right) \quad (6)$$

The density in 3D at the trap would be $D_3 = C/(T \cdot V \cdot K \cdot \pi \cdot EAR^2)$, which yields values identical to $D_2/\left(SD \cdot \sqrt{2 \cdot \pi}\right)$.

Assuming insects fly in all horizontal directions with respect to the wind while searching (Elkinton and Cardé, 1983; Judd and Borden, 1988, 1989; Byers et al., 1989), then many possible ground speeds are possible depending on the direction of flight with respect to wind direction (Fig. 2, bottom right inset). The average ground speed ($\overline{s}$) that results from all possible directions of insect flight for a

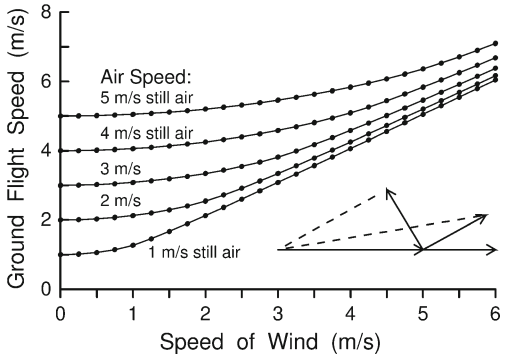

Fig. 2 Relationship between wind speed and ground flight speed when insect flies at 1 to 5 m/sec in still air (air speed); results generated by Eq. (7). Insert at lower right shows three possible ground flight speeds (3 m/sec straight line and dashed lines of 2.91 m/sec and 1.73 m/sec) based on vectors of air speed in still air (2 m/sec) and wind speed (1 m/sec)

speed in still air (s_i) of 2 m/sec and a wind speed (s_w) of 2 m/sec is found from the equation:

$$\overline{s} = \frac{1}{n} \sum_{a=0}^{n} \sqrt{s_i^2 + s_w^2 - 2 \cdot s_i \cdot s_w \cdot \cos(a \cdot \pi / n)} \quad (7)$$

where n=1000, giving 2.54 m/sec ground flight speed with the above values. Equation (7) was used with varying wind speed and flight speed to show graphically the effects on the average ground flight speed (Fig. 2).

Simulation of Flight Densities and Catch on a Spherical Trap in 3D The simulations attempted to fly a population of insects of known constant density in a volume (X=50, Y=50, Z=10 m). The population of individuals flies at a mean height of Z/2 and exhibits a normal distribution about the mean height with SD=1.67 m. The insects can be caught by a spherical trap of specified EAR placed at the mean flight height (5 m) in the center of the volume. The catch on the trap at the end of the simulation (T=3600 sec, with insects taking 72,000 steps of 0.1 m each) was used with the appropriate parameters in Eq. (6) to calculate the number of insects per hectare (means and 95 % CL, Fig. 3). The dashed line is the density of insects maintained throughout the simulations (10 insects in the volume gives 40 per hectare). As can be seen (Fig. 3), the means calculated from the catches are near the expected values of 40 or 160 per hectare. The 95 % CL is larger when the trap EAR is smaller since more variation in catch is expected on smaller traps.

When the time of exposure of the 0.5 m EAR trap was increased, there was no effect on the calculation of density, which was kept constant at 160 per hectare (Fig. 4). The 95 % CL of the means overlap the expected density, with generally more variation at the shorter exposures, an expectation when traps are not exposed long enough. Equation (6) predicted the densities of insects per hectare based on the

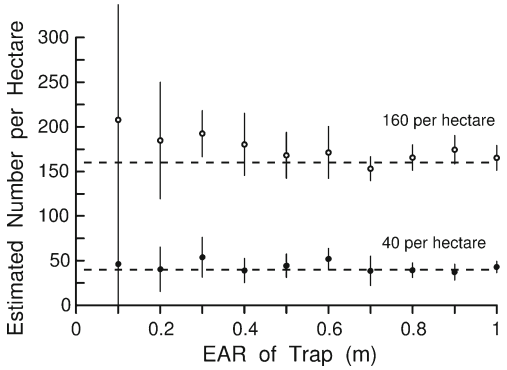

Fig. 3 Mean density of insects (number per hectare) flying at 2 m/sec calculated from catches on a trap of varying EAR exposed for 1 hr in simulations (N=8 each point) in three dimensions with constant densities of 40 or 160 insects per hectare (bars represent 95 % CL). The dashed lines indicate the expected values

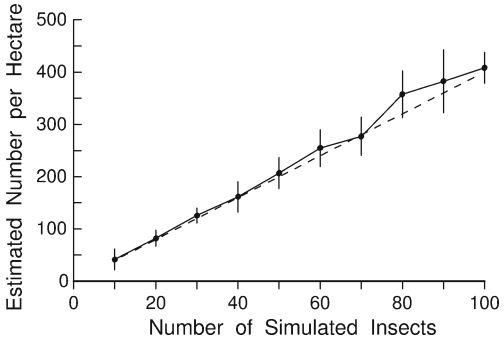

Fig. 5 Mean density of insects (number per hectare) flying at 2 m/sec calculated from catches on a trap of EAR=0.5 m exposed for 1 hr in simulations (N=8 each point) in three dimensions with varying densities of insects per simulation volume (one fourth hectare; bars represent 95 % CL). The dashed line indicates the expected values

trap catches of a spherical trap of 0.5 m EAR during an hour exposure when the simulated density of insects was increased from 10 to 100 in the volume (Fig. 5). The number per hectare is four times the number simulated since the volume represents one fourth of a hectare in surface area. The 95 % CL variation did not decrease with higher numbers of simulated insects as might be expected from the results on increasing EAR and exposure period. In fact, the larger numbers caught simply allowed more variation about the means, as seen when comparing the means for 40 and 160 densities in Fig. 3. These simulation results, which aim to represent stochastic processes in nature, show that the equations are valid assuming catch, mean ground speed, exposure period, and EAR were obtained with reasonable accuracy.

Estimating Densities from Insect Catch Studies in the Literature Over 100 articles were found on insect flight heights of which only about 60 were suitable for estimating SD (Tables 1–3 in Byers, 2011). Of these, about 30 species were caught on passive (blank) traps, and the catch at the mean flight height (estimated from normal equations in Byers, 2011) was used to calculate densities per hectare (Table 1) using Eqs. (4) and (5). These 30 species were matched with studies that had determined EAR from an attractive trap and a blank trap (preferably sticky traps) of reported dimensions, with the blank trap or traps catching >0 insects. Of these matches, only a few species were found that met these requirements to allow estimation of 2D densities (Table 2) from attractive traps using Eq. (6). These examples show how density can be estimated and serve as a model for future work.

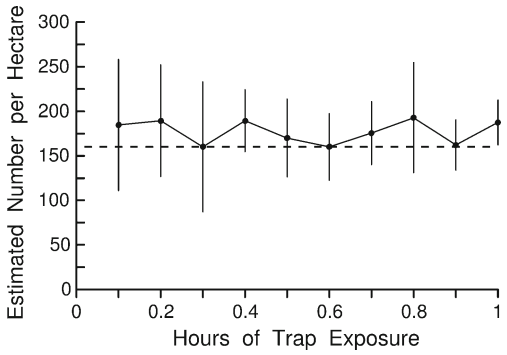

Fig. 4 Mean density of insects (number per hectare) flying at 2 m/sec calculated from catches on a trap of EAR=0.5 m exposed for varying periods of time in simulations (N=8 each point) in three dimensions with a constant density of 160 insects per hectare (bars represent 95 % CL). The dashed lines indicate the expected values

Discussion

Equations (5) and (6) were derived in part from the equation: Density = catch/(2 × radius × time × speed) presented in Byers et al. (1989) who were unaware of an earlier equation by Holling (1959) for calculating densities. Holling's type I functional response equation calculates how many prey would be eaten by a predator (or caught on sticky trap) as $H_a = aH$, where H_a is catch per time, H is prey density, and a is attack rate. In the case of traps, the attack rate, a, would be the diameter of the trap ($2R$) times average speed of the insects times the exposure time. Equations (5) and (6) simply solve for H (which is D_2 here). However, the trap's radius R that was implicit in Holling's equation is not appropriate in 3D with different vertical flight distributions. Thus, R is corrected by using SD (Eq. 3) to calculate ER (Eq. 1) and ER_c (Eq. 4) for passive traps to find insect densities by Eq. (5). If the vertical flight SD of a species and the EAR of an attractive trap are known, then densities are calculated via Eq. (6).

In order to estimate densities of flying insects over a certain length of time, one can use passive sticky traps.

Table 1 Estimation of mean density of insects (n/hectare) based on published data for non-attractive traps (window, sticky card, cross-barrier, or sticky cylinder) at various heights

Species	Mean height of catch ± *SD* (m)[a]	Catch at mean height[b]	Trap area S (m^2)[c]	ER_c (m)[d]	Hours of trapping (*N*)[e]	Density (number per hectare)[f]
Hemiptera:						
Alydus eurinus (Say)[g]	1.35±1.03	76	0.37	0.072	1400 (4)	0.26
Sinea diadema (F.)[h]	1.29±0.87	27	0.37	0.085	600 (4)	0.18
Sinea spinipes (Herrich-Schaeffer)[h]	4.06±2.06	6	0.37	0.036	900 (4)	0.06
Nabis americoferus Carayon[i]	2.48±1.83	13	0.37	0.040	600 (4)	0.19
Nabis roseipennis Reuter[i]	3.07±1.76	27	0.37	0.042	600 (4)	0.37
Orius insidiosus (Say)[i]	3.45±1.81	1109	0.37	0.041	600 (4)	15.72
Antillocoris pilosulus (Stål)[j]	4.27±1.80	66	0.37	0.041	2100 (4)	0.27
Crophius disconotus (Say)[j]	3.09±1.76	18	0.37	0.042	900 (4)	0.17
Geocoris punctipes (Say)[j]	2.37±1.74	19	0.37	0.042	1300 (4)	0.10
Corythucha ciliata (Say)[k]	4.27±1.75	15	0.37	0.042	300 (4)	0.42
Gargaphia solani Heidemann[k]	1.85±1.50	29	0.37	0.049	900 (4)	0.22
Amnestus basidentatus Froeschner[l]	4.63±1.43	168	0.37	0.051	600 (4)	1.89
Amnestus pallidus Zimmer[l]	3.51±1.85	21	0.37	0.040	600 (4)	0.31
Amnestus spinifrons (Say)[l]	3.33±2.02	15	0.37	0.036	450 (4)	0.31
Brochymena quadripustulata (F.)[l]	3.01±1.17	179	0.37	0.063	900 (4)	1.10
Euschistus servus (Say)[l]	2.12±1.54	41	0.37	0.048	1350 (4)	0.22
Diptera:						
Leptoconops noei Clastrier et Coluzzi[m]	2.11±0.50	1365	0.0382	0.015	35 (3)	1194.50
Leptoconops irritans (Noé)[m]	2.12±0.59	332	0.0382	0.013	35 (3)	340.72
Bibio johannis (L.) males[n]	0.71±0.42	2374	0.04	0.018	225 (72)	11.22
Bibio johannis (L.) females[n]	0.88±0.45	183	0.04	0.017	225 (72)	0.93
Sergentomyia bedfordi Newstead in open[o]	2.85±2.13	9	0.637	0.060	14 (5)	3.00
Above in wooded area[o]	4.80±2.69	38	0.637	0.047	14 (5)	16.11
Sergentomyia antennatus New. in open[o]	2.17±1.94	9	0.637	0.065	14 (5)	2.85
Above in wooded area[o]	2.85±2.72	23	0.637	0.047	14 (5)	9.58
Coleoptera:						
Hoplia spectabilis Medvedev[p]	0.87±0.76	2423	0.135	0.035	75 (3)	421.99
Hylurgops palliatus (Gryllenhal)[q]	4.98±2.63	16	0.09	0.007	6 (3)	177.50
Ips typographus (L.) males[q]	4.63±2.66	9	0.09	0.007	30 (3)	20.17
Ips typographus (L.) females[q]	4.63±2.85	9	0.09	0.006	30 (3)	21.83
Tomicus piniperda (L.)[q]	5.98±3.00	2	0.09	0.006	12 (3)	10.37
Trypodendron domensticum (L.)[q]	2.82±1.67	5	0.09	0.011	6 (3)	33.72
Cryphalus abietus (Ratz.)[q]	3.44±2.73	46	0.09	0.007	6 (3)	542.54
Pityogenes bidentatus (Herbst)[q]	3.10±1.62	7	0.09	0.011	6 (3)	48.08
Pityogenes chalcographus (L.)[q]	6.89±2.90	13	0.09	0.006	6 (3)	166.53
Pityogenes quadridens (Hartig)[q]	4.08±2.80	9	0.09	0.006	6 (3)	105.28
Balanogastris kolae (Desbr.)[r]	0.68±0.44	395	0.057	0.026	150 (3)	47.28

[a] Calculated from Eqs. (2) and (3)

[b] Estimated from normal equations (Table 3 in Byers, 2011)

[c] Based on equations to calculate *S* for either flat, cross-barrier, or cylindrical traps as presented in Methods

[d] Using Eq. (1)

[e] Rough estimate of trapping times based on trap days and 5 hr flight per day, *N* = number of trap stations

[f] Average density over trapping period as estimated from Eq. (6) with *V* assumed at 1 m/sec in all cases, *T* in seconds from trapping hours, and *N* was number of traps at each height level

[g] McPherson and Weber, 1981a; [h] McPherson and Weber, 1981b, [i] c, [j] d, [k] e, [l] 1980, [m] Carrieri et al., 2007, [n] D'Arcy-Burt and Blackshaw, 1987, [o] Basimike et al., 1989, [p] Zhang et al., 2011, [q] Byers et al., 1989, [r] Ivbijaro and Daramola, 1977

Table 2 Densities of flying insects calculated from Eq. (7), using EAR and EAR_c of semiochemicals or colors attractive to various species based on passive and active catches of sticky traps, SD ($SD = F_L/\sqrt{2 \cdot \pi}$ from Table 1 in Byers, 2011), and estimated average flight speed (V) of 2 m/sec for Scolytidae and Scarabaeidae, and 0.5 m/sec for Thripidae (trapping exposure times from literature unless specified)

Species and conditions	Insect catch		Trap area S (m^2)	EAR (m)	SD (m)	EAR_c (m)	Density (n per hectare)
	Blank	Active					
Scarabaeidae							
Hoplia spectabilis Medvedev[a]							
Yellow cross-panel	1867	12448	0.135	0.54	0.76	0.237	144.82
White cross-panel	1867	9274	0.135	0.46	0.76	0.176	144.82
Blue cross-panel	1867	5944	0.135	0.37	0.76	0.113	144.82
Host Plant	288	405	0.135	0.25	0.76	0.050	78.19
Host plant + 200 Hoplia	288	509	0.135	0.28	0.76	0.063	78.19
Curculionidae (Scolytinae)							
Tomicus piniperda L.							
Scots pine log I[b]	52	623	0.06	0.48	3.00	0.048	283.25
Log I + 30 males + 30 females[b]	52	774	0.06	0.53	3.00	0.059	283.25
(+)-3-Carene[b]	7	48	0.06	0.36	3.00	0.027	19.06
(+)-α-Pinene[b]	7	60	0.06	0.40	3.00	0.034	19.06
(−)-α-Pinene[b]	7	79	0.06	0.46	3.00	0.045	19.06
Terpinolene[b]	7	104	0.06	0.53	3.00	0.059	19.06
Scots pine Log II[b]	7	256	0.06	0.84	3.00	0.146	19.06
April 15, Monoterpenes[c]	2.33	27	0.09	0.58	3.00	0.069	67.69
April 21, Monoterpenes[c]	1	19	0.09	0.74	3.00	0.114	29.06
Ips typographus *L.*							
High release pheromone[d]	6	753	0.06	1.55	2.75	0.546	3.74
Medium release pheromone[e]	7	80	0.06	0.47	2.75	0.050	4.37
May 17, Pheromone[f]	5.67	194	0.09	0.99	2.75	0.223	241.5
May 20, Pheromone[f]	16.67	269	0.09	0.68	2.75	0.105	426.01
May 22, Pheromone[f]	8.5	215	0.09	0.85	2.75	0.165	402.26
Ips paraconfusus Lanier							
Log + 50 males[g]	1.27	339	0.1185	3.17	5.14[h]	1.228	2.13
Thripidae							
Frankliniella occidentalis (Pergande)[i]							
Blue card (greenhouse I)	29	639	0.0046	0.18	0.39	0.051	693.47
Yellow card (greenhouse I)	29	529	0.0046	0.16	0.39	0.042	693.47
Yellow card (greenhouse II)	-	71	-	0.16	0.39	-	97.11[j]

[a] Zhang et al. (2011): passive catch on black cross-panel trap, host plant was *Dasiphora fruticosa* (L.) Rydb. (Syn. *Potentilla fruticosa* L.) tested 4 d×6 h, N=3; colored traps in pastureland, 7 d×6 h, N=6

[b] Byers et al. (1985): released about 30 mg each compound/day; Scots pine (*Pinus sylvestris* L.) Log I tested 2 d×8 h, Log II and chemicals (30 mg each compound/day) tested 4 d×8 h, N=2 in all cases

[c] Byers et al. (1989): released about 10–20 mg/day of (+)-α-pinene, (−)-α-pinene, and (+)-3-carene, and 3–5 mg/d of terpinolene, 240 min trapping each date, N=1

[d] Schlyter et al. (1987): high release of 57 mg 2-methyl-3-buten-2-ol (MB)/day and 1 mg (4*S*)-*cis*-verbenol (cV)/day at 12 m trap separation, 16 d×8 h, N=2

[e] Schlyter et al. (1987): medium release: 5.8 mg MB/day and 1 mg cV/day, 16 d×8 h, N=2

[f] Byers et al. (1989): medium release MB and cV, trapping times as indicated in their Table 1, N=1

[g] Byers (1983): ponderosa pine log (*Pinus ponderosa* Laws.), passive catch average of 15 traps on row 3; 9 d×10 h, N=1

[h] Gara (1963)

[i] Gillespie and Vernon (1990): passive catch average of green and black sticky traps at 2.4 m height and average F_L, 1 d×10 h, N=5

[j] Density estimated in second greenhouse from catch and yellow card's EAR in first greenhouse, N=5

These should be nearly invisible to the insects of interest in order to obtain an accurate density estimate. The most appropriate insects are those with limited visual acuity with interommatidial angles >2°, including many herbivorous beetles, aphids, thrips, whiteflies, and true bugs (Land, 1997). Also, nocturnally flying insects would not avoid clear sticky traps under low light conditions. In addition, the SD must have been previously calculated from trap catches at several heights. The mean flight height also is obtained from these data so that the density-monitoring trap can be placed at this height. The habitat where densities are measured should be the same as where SD was obtained, since this value can sometimes vary depending on the habitat. For example, over open areas of grass, some species had mean flight heights that were lower than when flying in orchard/wooded areas (analyzed in Byers, 2011). In most cases, there were little or no differences in vertical flight distributions between the sexes of a species, but in species with sex-specific behaviors or territoriality there were significant differences (Byers, 2011). In the present study, only vertical distributions sampled with passive traps were used because a few insects appear to alter their vertical flight distribution when semiochemical lures are used at all trap heights (Byers et al., 1989; Byers, 2011). In most studies though, visible or olfactory attractants at all trap heights on the poles did not appear to affect the flight height distribution compared to passive traps (Byers, 2011).

The dimensions of the density-monitoring trap are easily measured, as well as the time of trap exposure. Average wind speed, but not direction, also need to be measured during the trapping period. Measurement of flight speed in still air would make estimates more accurate for a particular insect species, and then ground flight speed adjusted based on the observed average wind speed using Eq. (7). This procedure would allow more accurate estimates of density as compared to most previous studies where these parameters have been ill defined. Values for flight speed in still air and wind speed giving ground flight speeds (Fig. 2) can be reasonably assumed, based on the species and conditions tested. In any case, these two parameters can be measured.

A more efficient way of estimating densities is to use attractive traps since more insects are caught than with passive traps. Again, the SD must have been calculated previously, and the wind speed, time of trapping, and flight speed in still air is important to measure as precisely as possible. In addition, an EAR needs to be estimated prior to monitoring flight densities. The larger the passive trap catch, the more reliable and accurate the EAR estimate. Therefore, it is advisable to deploy several passive traps in order to obtain a sufficient catch for a reliable average to compare to the active trap catch. The magnitude of the EAR depends on the abilities of the species in question to find the attractant, and on the attractant's qualities (e.g., spectral reflectance; chemical components, and release rates). The EAR is a function of the ratio of catch on attractant and passive traps, and the effective radius of the passive trap. The EAR, being a ratio of trap catch, is expected to be robust and not affected by changes in population density. This has been shown previously in simulations (Byers, 2008, 2009), and is indicated from EAR calculations of trap data (Table 2). The EAR of attractive odors also is defined by a specified release rate, which would not be maintained by evaporative dispensers if temperature declines. Thus, flight density measurements are valid only under temperature conditions similar to those under which the EAR was obtained for a particular dispenser/release rate.

Other environmental factors may alter the EAR for chemical lures by altering the insect's flight height and orientation efficiency. The flight height and/or orientation efficiency is influenced by wind speed and wind turbulence in different habitats, which could affect EAR magnitude and reduce accuracy of density estimates. Further work is needed to test the variability of EAR estimates; however, some data are available for comparisons (Table 2). For example, bark beetles (*T. piniperda*) attracted to freshly cut host logs on different occasions had EAR of 0.48, 0.53, and 0.84 m; bark beetles (*I. typographus*) attracted to a specific release rate of aggregation pheromone had EAR of 0.68, 0.85, 0.99 m; scarab beetles (*H. spectabilis*) attracted to host plants had EAR of 0.25 and 0.28 m; and western flower thrips (*F. occidentalis*) attracted to yellow colored traps in two greenhouses had identical EAR of 0.16 m. In some species, the EAR for pheromone baits would be expected to be different for each sex, such as the sexes of *Ips* bark beetles that respond differentially to aggregation pheromone. The males avoided the highest pheromone concentrations indicating resident males, while females were attracted to high pheromone concentrations (Byers, 1983; Schlyter et al., 1987).

Different baits with different EAR calculated in the same experiment will report the same density (Table 2), since the catch on the attractive trap is used for both the density and EAR calculation. In practice, the measurement of density should be based on a previous EAR determination (which might involve several attractive and passive traps), and then only one attractive trap need be set up in any area in the future to estimate flight densities based on the catches. Multiple traps can be averaged to increase the accuracy, since flight densities vary locally and temporally. In one study, however, a "medium" and a "high" release rate of synthetic pheromone gave two different EAR for *I. typographus* in the same forest area, and the densities calculated were remarkably similar (4.37 and 3.74 beetles per hectare, Table 2), as should occur if the density methods are valid. These densities represent the average flight density over a 16-day period in 1982, but if most flight occurred over 4 days (not unusual for middle Sweden), then the densities

would be four times higher. The daily densities over a week period in May 1984 were certainly higher (241 to 426 beetles per hectare, Table 2) in Denmark as beetles were emerging from large piles of tree trunks after clear-cut operations.

When using passive traps to estimate densities, it is probably more important to place them at the mean flight height than when using traps with semiochemical lures in which insects can be attracted from different heights by following odor plumes or by visual attraction. Many previous studies were found in which proportions of catch were reported without total number. In these cases, SD can be calculated, but no calculation of trap catch at the mean flight height, or at any height is possible; thus, no densities can be estimated. The density estimates are for the flying population, and do not count resting insects. For moths, density measurements with EAR of pheromone are for flying males, including the entire population during the night in many species. Since the sex ratio is about 1:1 in moths, then the female population that is resting can also be estimated as equal to that found for the males. In any case, the flight density should correlate well with the total population density.

The equations presented here provide new methods to estimate flight densities for many insect species in the field. Additionally, estimates of flight densities of pest insects in agricultural systems obtained by other methods can be entered into computer models of mating disruption and mass trapping developed earlier (Byers, 2007). Even without such use in models, knowledge of flight population densities is critical to efficient deployment of lures and traps in monitoring, mating disruption and mass trapping (El-Sayed et al., 2006, 2009).

References

ADAMS, L. 1987. High Performance Interactive Graphics. Tab Books, Blue Ridge Summit, PA.

BASIMIKE, M., MUTINGA, M. J., and MUTERO, C. M. 1989. Vertical distribution of phlebotomine sandflies in two habitats in Marigat leishmaniases endemic focus, Baringo district, Kenya. *Insect Sci. Appl.* 10:645–650.

BYERS, J. A. 1983. Sex-specific responses to aggregation pheromone: Regulation of colonization density in the bark beetle *Ips paraconfusus*. *J. Chem. Ecol.* 9:129–142.

BYERS, J. A. 2001. Correlated random walk equations of animal dispersal resolved by simulation. *Ecology* 82:1680–1690.

BYERS, J. A. 2007. Simulation of mating disruption and mass trapping with competitive attraction and camouflage. *Environ. Entomol.* 36:1328–1338.

BYERS, J. A. 2008. Active space of pheromone plume and its relationship to effective attraction radius in applied models. *J. Chem. Ecol.* 34:1134–1145.

BYERS, J. A. 2009. Modeling distributions of flying insects: Effective attraction radius of pheromone in two and three dimensions. *J. Theor. Biol.* 256:81–89.

BYERS, J. A. 2011. Analysis of vertical distributions and effective flight layers of insects: Three-dimensional simulation of flying insects and catch at trap heights. *Environ. Entomol.* 40:1210–1222.

BYERS, J. A., LANNE, B. S., LÖFQVIST, J., SCHLYTER, F., and BERGSTRÖM, G. 1985. Olfactory recognition of host-tree susceptibility by pine shoot beetles. *Naturwissenschaften* 72:324–326.

BYERS, J. A., ANDERBRANT, O., AND LÖFQVIST, J. 1989. Effective attraction radius: A method for comparing species attractants and determining densities of flying insects. *J. Chem. Ecol.* 15:749–765.

CARDÉ, R. T. 1990. Principles of mating disruption, pp. 47–71, *in* R. L. Ridgway and R. M. Silverstein (eds.), Behavior-Modifying Chemicals for Pest Management: Applications of Pheromones and Other Attractants. Marcel Dekker, New York.

CARDÉ, R. T. and MINKS, A. K. 1995. Control of moth pests by mating disruption: Successes and constraints. *Annu. Rev. Entomol.* 40:559–585.

CARRIERI, M., MONTMURRO, E., VALENTINO, S. V., and BELLINI, R. 2007. Study on the flying height of *Leptoconops noei* and *Leptoconops irritans* in southern Italy. *Bull. Insectol.* 60:83–87.

D'ARCY-BURT, S. and BLACKSHAW, R. P. 1987. Effects of trap design on catches of grassland Bibionidae (Diptera: Nematocera). *Bull. Entomol. Res.* 77:309–315.

ELKINTON, J. S. and CARDÉ, R. T. 1983. Appetitive flight behavior of male gypsy moths (Lepidoptera: Lymantriidae). *Environ. Entomol.* 12:1702–1707.

EL-SAYED, A. M., SUCKLING, D. M., WEARING, C. H., and BYERS, J. A. 2006. Potential of mass trapping for long-term pest management and eradication of invasive species. *J. Econ. Entomol.* 99:1550–1564.

EL-SAYED, A. M., SUCKLING, D. M., BYERS, J. A., JANG, E. B., and WEARING, C. H. 2009. Potential of "lure and kill" in long-term pest management and eradication of invasive species. *J. Econ. Entomol.* 102:815–835.

GARA, R. I. 1963. Studies on the flight behavior of *Ips confuses* (Lec.) (Coleoptera: Scolytidae) in response to attractive material. *Contrib. Boyce Thompson Inst.* 22:51–66.

GILLESPIE, D. R. and VERNON, R. S. 1990. Trap catch of western flower thrips (Thysanoptera: Thripidae) as affected by color and height of sticky traps in mature greenhouse cucumber crops. *J. Econ. Entomol.* 83:971–975.

HEARN, D. and BAKER, M. P. 1994. Computer Graphics. Prentice Hall, Englewood Cliffs, N.J., USA.

HOLLING, C. S. 1959. Some characteristics of simple types of predation and parasitism. *Can. Entomol.* 91:385–398.

IVBIJARO, M. F. and DARAMOLA, A. M. 1977. Flight activity of the adult kola weevil, *Balanogastris kolae* (Coleoptera: Curculionidae) in relation to infestation. *Entomol. Exp. Appl.* 22:203–207.

JUDD, G. J. R. and BORDEN, J. H. 1988. Long-range host-finding behaviour of the onion fly *Delia antiqua* (Diptera: Anthomyiidae): ecological and physiological constraints. *J. Appl. Ecol.* 25:829–845.

JUDD, G. J. R. and BORDEN, J. H. 1989. Distant olfactory response of the onion fly, *Delia antiqua*, to host-plant odour in the field. *Physiol. Entomol.* 14:429–441.

Land, M. F. 1997. Visual acuity of insects. *Annu. Rev. Entomol.* 42:147–177.

MCPHERSON, J. E. and WEBER, B. C. 1980. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 1. Pentatomoidea. *Great Lakes Entomol.* 13:177–183.

MCPHERSON, J. E. and WEBER, B. C. 1981a. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 2. Coreoidea. *Great Lakes Entomol.* 14:11–13.

MCPHERSON, J. E. and WEBER, B. C. 1981b. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 3. Reduvioidea. *Great Lakes Entomol.* 14:15–17.

McPherson, J. E. and Weber, B. C. 1981c. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 4. Cimicoidea. *Great Lakes Entomol.* 14:19–22.

McPherson, J. E. and Weber, B. C. 1981d. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 5. Lygaeoidea. *Great Lakes Entomol.* 14:133–136.

McPherson, J. E. and Weber, B. C. 1981e. Seasonal flight patterns of Hemiptera in a North Carolina black walnut plantation. 6. Tingidae and Aradidae. *Great Lakes Entomol.* 14:137–140.

Miller, J. R., Gut, L. J., de Lame, F. M., and Stelinski, L. L. 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 1): Theory. *J. Chem. Ecol.* 32:2089–2114.

Miller, J. R., Gut, L. J., de Lame, F. M., and Stelinski, L. L. 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): Case studies. *J. Chem. Ecol.* 32:2115–2143.

Schlyter, F., Byers, J. A., and Löfqvist, J. 1987. Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol.* 13:1503–1523.

Shorey, H. H. 1977. Manipulation of insect pests of agricultural crops, pp. 353–367, *in* H. H. Shorey and J. J. McKelvey Jr. (eds.), Chemical Control of Insect Behaviour: Theory and Application. Wiley, New York.

Zhang, Q. H., Ma, J. H., Yang, Q. Q., Byers, J. A., Klein, M. G., Zhao, F. Y., and Luo, Y. Q. 2011. Olfactory and visual responses of the long-legged chafer *Hoplia spectabilis* Medvedev (Coleoptera: Scarabaeidae) in Qinghai province, China. *Pest Manag. Sci.* 67:162–169.