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COMMENTARY

New Synthesis – Visual and Chemical Ornaments: What Researchers of Different Signal Modalities Can Learn from Each Other

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Interestingly, the field of animal communication appears to suffer from a lack of communication between scientists studying different sensory channels. Communication theory has been developed primarily on the basis of visual and acoustic signals; chemical signals have been neglected, despite a long history of outstanding studies and a plethora of available literature. Conversely, chemical ecologists tend to focus on proximate mechanisms, often ignoring the concept structure developed by behavioral and evolutionary ecologists. Clearly, researchers of chemical and non-chemical communication would benefit from each other's ideas, and a joint effort could generate new insight into why and how signals evolve. A recent article by Hill (2011) proposes a useful framework to this end.

Condition and condition-dependent traits are well-known terms in the community working on communication and sexual selection, but as Hill (2011) points out they are poorly defined. He defines condition as the capacity to maintain system functionality and to withstand environmental challenges, and condition-dependent traits as "conspicuous features of an organism that enhance perception of condition". Moreover, he proposes four distinct if non-exclusive ways that ornaments (although he means visual ornaments, we can expand it to include chemical ornaments) can be linked to system functionality: 1) Resource Tradeoff Hypothesis: resources needed for signal production also are needed for the operation of essential physiological processes; 2) Mediator Hypothesis: a regulatory agent (e.g., hormone) promotes signal production, but suppresses a vital physiological process; 3) Pathway Functionality Hypothesis: signal production requires a product of a vital physiological process; and 4) Shared Pathway Hypothesis: signal production and vital systems share pathways. These hypotheses are not entirely new to pheromone researchers, as they invoke the biosynthesis of such chemical signals. However, the different possibilities of linkage between condition and the signal have never been stated as explicitly as Hill has done.

Most available evidence seems to support the first hypothesis, as pheromone synthesis often depends on limited nutritional resources. Yet, few studies provide a clear demonstration of a linkage between the specific resource and essential physiological processes. Exceptions are the well known examples of male moths and butterflies that sequester alkaloids derived from food plants. The alkaloids are essential for their own defense and the defense of their offspring, but also are the resource for sex pheromone production. A more recent exciting example is the study of Blaul and Ruther (2011), which demonstrates that dietary linoleic acid is not only a precursor of the male sex pheromone in *Nasonia vitripennis* wasps, but also affects sperm production and therefore male condition.

Evidence supporting Hill's second hypothesis comes from work that shows that juvenile hormone (JH) promotes pheromone production but suppresses immunity. However, the exact mechanism of this linkage is unknown, and as Hill (2011) pointed out for testosterone, JH might only regulate energy allocation, shunting either the energy to general homeostasis or to the production of pheromones. In this case, the Mediator Hypothesis would not differ from the Resource Tradeoff Hypothesis.

Hill's third and fourth hypotheses both predict that the pathway of ornament production is intimately linked to some essential physiological pathways, and thus are likely relevant to chemical signals. Evidence comes from studies on lizards (Lopez et al. 2009). Here, sex pheromone and Vitamin D production share the same biosynthetic pathway; more precisely, the pheromone is a precursor of Vitamin D. In mammals, Vitamin D positively influences immunity, and the same might be true for lizards (see references in Lopez et al. 2009). Another example comes from insects, in which many pheromones share their pathways with fatty acid metabolism, a critical physiological process responsible for supplying energy to the body. A high rate of pheromone production is possible only if lipid metabolism is functioning well. However, here we can also see the limitation of Hill's approach. Nearly each insect pheromone synthesized *de novo* is tightly linked to a pathway that is important for maintenance of system functionality. Does that mean that all these pheromones are condition dependent signals and used in mate choice? Presumably not. They only reflect condition if (1) pheromone production reaches a critical level, a level that reflects metabolic efficiency, otherwise there would not be much variation in chemical signaling between individuals. (2) The shared essential physiological pathway has to be prone to disturbance or somehow depend on individual differences in phenotype, otherwise there would be not much variability in condition and hence signal quantity or quality between individuals. I encourage chemical ecologists interested in sexual selection to focus on these last two mentioned aspects and investigate whether or not the metabolic machinery places any strain on signal production. In recent decades, the deciphering of metabolic pathways that connect visual signal production to condition has been a neglected research area. In contrast, the specific pathways of chemical signal production have been investigated extensively and, therefore, chemical ecologists are well positioned to provide new insights into the proximate mechanisms of sexually selected signals, from which researchers of all signal modalities would benefit.

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REVIEW ARTICLE

The Chemical Ecology of Cecidomyiid Midges (Diptera: Cecidomyiidae)

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Abstract The family of cecidomyiid midges (Diptera: Cecidomyiidae) exhibits diversified patterns of life history, behavior, host range, population dynamics and other ecological traits. Those that feed on plants include many important agricultural pests; most cultivated plants are attacked by at least one midge species. Several features of the reproductive biology of cecidomyiid midges point to an important role for chemical communication, with this topic last reviewed comprehensively 12 years ago. Here, we review progress on identification of sex pheromones, chemicals involved in location of host plants, the neurophysiology of reception of volatile chemicals, and application of semiochemicals to management of pest species of cecidomyiid midges that has occurred during the last decade. We hope this review will stimulate and sustain further research in these fields.

Key Words Cecidomyiidae · Diptera · Pheromone · Semiochemical · Synthesis · Biosynthesis · Olfaction · Monitoring · Traps · Control

Dedicated to the late Professor C.-H. Zhao.

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Introduction

The family of cecidomyiid midges (Diptera: Cecidomyiidae) exhibits diverse patterns in life history, behavior, host range, population dynamics and other ecological traits (Yukawa and Rohfritsch, 2005). There are more than 5,000 species described, although this is probably a "small fraction of the number that must exist" (Gagné, 1989). Their diverse feeding habits include zoophagy, mycetophagy, as well as phytophagy (Mamaev and Krivosheina, 1965). Those that feed on plants include many important agricultural pests, with most cultivated plants attacked by at least one midge species. As a result, most research has focused on phytophagous species, often referred to as gall midges, e.g., in the review of Harris and Foster (1999). However, in that not all cecidomyiid midges are phytophagous, and not all phytophagous midges form galls, this review will use the taxonomic definition. It is anticipated that the generalized conclusions will apply to the taxonomic family rather than to the behavioral groupings.

The Role of Chemical Communication in the Biology of Cecidomyiid Midges

The chemical ecology of cecidomyiid midges was last reviewed comprehensively by Harris and Foster (1999), although Wicker-Thomas (2007) included a summary of more recent work on the sex pheromones of Cecidomyiidae in her review of pheromonal communication in courtship behavior of Diptera. As noted by these authors, several features of the reproductive biology of these midges point

to an important role for chemical communication, especially as all exhibit sexual reproduction (Gagné, 1994). In general, adults are very small, with body and wing lengths <3 mm and body weight <2 mg (e.g., Bergh et al., 1990), and are weak fliers. The life span of adults is very short, sometimes only 1–2 days. Adult males emerge, take flight, find, and copulate with females. Females emerge with a full complement of mature eggs, generally mate once, and then search for host plants on which to oviposit (Gagné, 1989, 1994).

These features require efficient mechanisms for finding mates, such as the use of sex pheromones. Similarly, effective means of locating host plants are required. Cecidomyiid midges are generally monophagous or oligophagous and, even in the latter, larval development is restricted to two or more plant species within a single genus or to species in different genera within a family. Polyphagous midges with a host range extending beyond one plant family are relatively rare, and several of these species in fact alternate between specific hosts at different times of the year (Harris et al., 2003; Yukawa and Rohfritsch, 2005). Host plant ecology is crucial for the growth and survival of larvae. Yukawa (2000) outlined the importance of synchronization of the emergence of adults and the availability and suitability of hosts. Furthermore, neonate larvae are small and generally unable to move from one plant to another (Gagné, 1989). As a result of all these factors, the propagation of a species depends upon adult female midges finding a suitable host at the correct stage of development in a short space of time (Åhman, 1985; Harris and Rose, 1989).

A further intriguing feature of the biology of cecidomyiid midges is the structure of the antenna, the site of receptors for volatile chemicals. In addition to sensilla typically found on antennae of other insects, both males and females have curious, looped sense organs known as circumfila (Slifer and Sekhon, 1971; Lee and Lee, 1985; Solinas and Nuzzaci, 1987).

At the time of the review by Harris and Foster (1999), the structure of only one component of the pheromone of one species of cecidomyiid midge had been identified, that of the major component of the female-produced sex pheromone of the Hessian fly, *Mayetiola destructor* (Foster et al., 1991a). This discovery heralded a surge in identification of pheromone components of other midges and their use in pest management. Here, we review progress on identification of sex pheromones, chemicals involved in location of host plants, the neurophysiology of reception of volatile chemicals, and application of semiochemicals to management of pest species of cecidomyiid midges that has occurred during the last decade. We hope this review will stimulate and sustain further research in these fields.

Sex Pheromones of Cecidomyiidae

Demonstration of Presence of Sex Pheromones

The presence of female-produced sex pheromones, using laboratory bioassays and/or field experiments with live virgin females or female extracts as baits, has been demonstrated in 10 species of cecidomyiid midge: Douglas-fir cone gall midge, *Contarinia oregonensis* (Miller and Borden, 1984), Hessian fly, *M. destructor* (McKay and Hatchett, 1984), pea midge, *C. pisi* (Wall et al., 1985), pine gall midge, *Thecodiplosis japonensis* (Lee and Lee, 1985), rice gall midge, *Orseolia oryzae* (C.-H. Zhao, pers. comm., 1982; Sain and Kalode, 1985), blackcurrant leaf midge, *Dasineura tetensi* (Garthwaite et al., 1986), brassica pod midge, *D. brassicae* (Williams and Martin, 1986), sorghum midge, *Stenodiplosis (Contarinia) sorghicola* (Sharma and Vidyasagar, 1992), orange blossom wheat midge, *Sitodiplosis mosellana* (Pivnick, 1993), and apple leaf midge, *D. mali* (Harris et al., 1996; Heath et al., 1998, 2005).

The family Cecidomyiidae usually is divided into three sub-families: the Lestremiinae, Porricondylinae, and Cecidomyiinae (Gagné, 1989, 1994). Only species in the Cecidomyiinae have so far been shown to produce sex pheromones. The Cecidomyiinae is divided into two supertribes: the Lasiopteridi and the Cecidomyiidi. The genera *Dasineura* and *Mayetiola* are in the former, while *Contarinia, Thecodiplosis, Orseolia,* and *Sitodiplosis* are in the latter.

Identification of Sex Pheromones

Components of the female sex pheromones of 16 species of cecidomyiid midge have been identified and shown to attract conspecific males in laboratory and/or field bioassays (Table 1). Pheromones have been isolated by solvent extraction of whole insects or excised glands (Table 1, entries 1, 2, 6, 8, 9, 10, 11, 12), or by air entrainment onto Porapak (Table 1, entries 3, 4, 5, 6, 7, 13, 14, 15, 16). The latter procedure allows for easier processing of large numbers of insects and concentration of the collection prior to gas chromatographic (GC) analysis without contaminating the column with involatile material, although in some cases it may be difficult to get females to exhibit normal calling behavior in the laboratory (Hillbur, unpublished).

Cecidomyiid midges generally produce pheromone in very small amounts. For example, in *C. oregonensis* (Gries et al., 2002) and the aphidophagous gall midge, *Aphidoletes aphidimyza* (Choi et al., 2004), pheromone in extracts was undetectable by a mass spectrometer. Gland extracts of *C. pisi* contained a few picograms per female (Hillbur et al., 1999). Similarly, air entrainment of pheromone of *D. mali* gave approximately 20 pg per female (Cross and Hall,

Table 1 Pheromone components of cecidomyiid midges

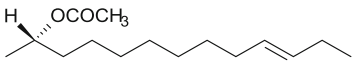
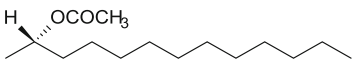
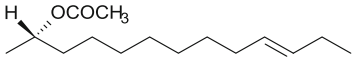
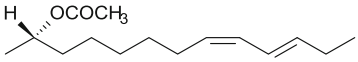
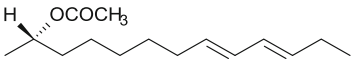
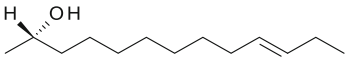
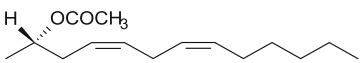
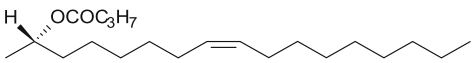
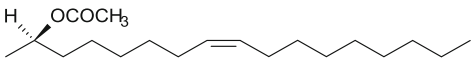
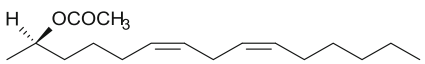
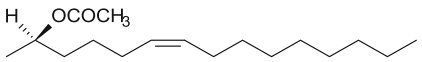
	Species	Pheromone components	Reference
	Monoester unsaturated		
1	Hessian fly *Mayetiola destructor*	 (2*S*,10*E*)-2-Acetoxy-10-tridecene	Foster et al. (1991a)
		 (2*S*)-2-Acetoxytridecane	Andersson et al., (2009a)
		 (2*S*,10*E*)-2-Acetoxy-10-tridecene	
		 (2*S*,8*Z*,10*E*)-2-Acetoxy-8,10-tridecadiene	
		 (2*S*,8*E*,10*E*)-2-Acetoxy-8,10-tridecadiene	
		 (2*S*,10*E*)-10-Tridecen-2-ol	
2	Douglas fir-cone gall midge *Contarinia oregonensis*	 (2*S*,4*Z*,7*Z*)-2-Acetoxy-4,7-tridecadiene	Gries et al. (2002)
3	Chinese chrysanthemum midge *Rhopalomyia longicauda*	 (2*S*,8*Z*)-2-Butyroxy-8-heptadecene	Liu et al. (2009)
4	Honey locust midge *Dasineura gleditchiae*	 (2*R*,8*Z*)-2-Acetoxy-8-heptadecene	Molnar et al., (2009)
5	Blackberry leaf midge *Dasineura plicatrix*	 (2*R*,6*Z*,9*Z*)-2-Acetoxy-6,9-pentadecadiene	Hall et al. (unpublished)
		 (2*R*,6*Z*)-2-Acetoxy-6-pentadecene	

Table 1 (continued)

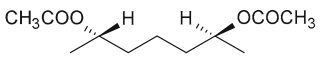
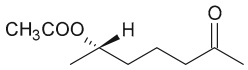
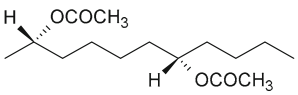
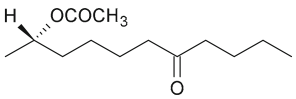
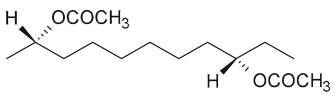
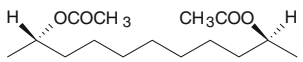
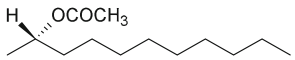
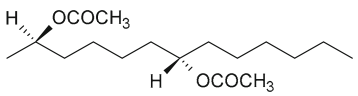
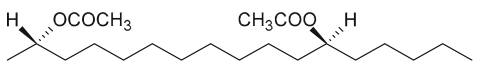
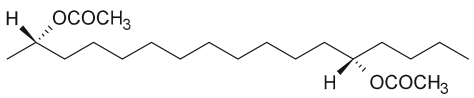
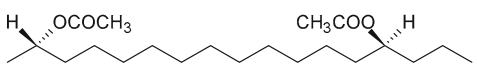
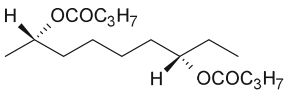
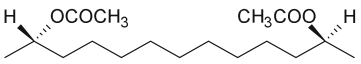
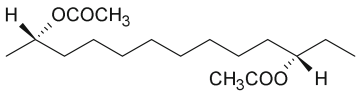
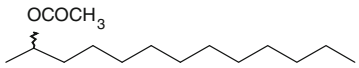
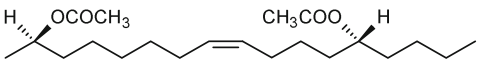
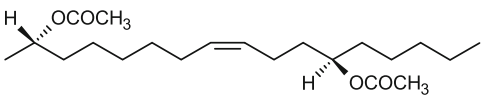
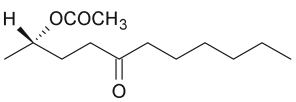
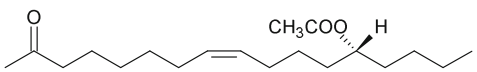
	Species	Pheromone components	Reference
	Diester		
6	Rice gall midge *Orseolia oryzae*	 (2*S*,6*S*)-2,6-Diacetoxyheptane  (6*S*)-6-Acetoxyheptan-2-one	Zhao (1982, unpublished) Zhao and Francke (unpublished) Hall et al. (unpublished)
7	Pear midge *Contarinia pyrivora*	 (2*S*,7*S*)-2,7-Diacetoxyundecane  (2*S*)-2-Acetoxyundecan-7-one	Amarawardana (2009)
8	Swede midge *Contarinia nasturtii*	 (2*S*,9*S*)-2,9-Diacetoxyundecane  (2*S*,10*S*)-2,10-Diacetoxyundecane  (2*S*)-2-Acetoxyundecane	Hillbur et al. (2005) Boddum et al. (2009)
9	Aphidophagous gall midge *Aphidoletes aphidimyza*	 (2*R*,7*S*)-2,7-Diacetoxytridecane	Choi et al. (2004)
10	Red cedar cone midge *Mayetiola thujae*	 (2*S*,12*S*)-2,12-Diacetoxyheptadecane  (2*S*,13*S*)-2,13-Diacetoxyheptadecane  (2*S*,14*S*)-2,14-Diacetoxyheptadecane	Gries et al. (2005)

Table 1 (continued)

	Species	Pheromone components	Reference
11	Orange wheat blossom midge *Sitodiplosis mosellana*	 (2*S*,7*S*)-2,7-Dibutyroxynonane	Gries et al. (2000)
12	Pea midge *Contarinia pisi*	 (2*S*,12*S*)-2,10-Diacetoxytridecane  (2*S*,11*S*)-2,10-Diacetoxytridecane  2-Acetoxytridecane	Hillbur et al. (1999, 2000)
	Diester unsaturated		
13	Pear leaf midge *Dasineura pyri*	 (2*R*,13*R*,8*Z*)-2,13-Diacetoxy-8-heptadecene	Amarawardana (2009)
14	Blackcurrant leaf midge *Dasineura tetensi*	 (2*S*,12*R*,8*Z*)-2,12-Diacetoxy-8-heptadecene	Amarawardana (2009); Hall et al., (unpublished)
	Keto-ester		
15	Raspberry cane midge *Resseliella theobaldi*	 (2*S*)-2-Acetoxyundecan-5-one	Hall et al. (2009)
	Keto-ester unsaturated		
16	Apple leaf midge *Dasineura mali*	 (13*R*,8*Z*)-13-Acetoxy-8-heptadecen-2-one	Cross and Hall (2007, 2009, unpublished)

2007). However, in the case of the raspberry cane midge, *Resseliella theobaldi*, air entrainment from 200 females yielded 1.5 μg of pheromone (i.e., 7.5 ng per female; Hall et al., 2009).

Analysis by coupled gas chromatographic-electroantennographic detection (GC-EAD) has proved effective at detecting pheromone components in collections from female Cecidomyiidae, with the male antenna capable of detecting components below a GC threshold of detection (Hillbur et al., 1999; Gries et al., 2002; Choi et al., 2004). Specially designed Plexiglas holders have been used for the antennal preparation (Hillbur et al., 2001, 2005; Andersson et al., 2009a), while Gries et al. (2002) used a severed head and antenna suspended between two glass electrodes. Male midges generally have long antennae relative to their bodies, and EAD preparations can be made by inserting a body into the glass reference electrode, after first removing wings and head, and then inserting one or both antennae into the recording electrode (Birkett et al., 2004; Amarawardana, 2009; Hall et al., 2009; Molnár et al., 2009).

Pheromone components of Cecidomyiidae generally are chiral (Table 1). In the original work on the major component of the pheromone of *M. destructor*, the absolute configuration of the pheromone, (10*E*)-2-acetoxy-10-tridecene, was determined by hydrolyzing extract, conversion to the acetyl (2*S*)-(+)-lactyl ester, and comparison of GC retention time with those of the diastereoisomers on a standard, non-polar GC column (Foster et al., 1991a). Subsequently, chiral cyclodextrin GC phases have been used, either commercially-available or custom-made versions (e.g., Hillbur et al., 1999). In our experience, these do not work well with chiral alcohols, but give much better resolution of esters. The resolution is particularly good when the ester functionality is at C-2 (e.g., Hall et al., 2009) and poorer when the functionality is further into the aliphatic chain.

Chemical Structures of Pheromone Components

The first published report of elucidation of the chemical structure of a component of the female sex pheromone of a cecidomyiid midge was that by Foster et al. (1991a). These authors identified the major component of the pheromone of *M. destructor* as (10*E*)-2-acetoxy-10-tridecene and showed that it was the *S*-enantiomer. Some 10 years earlier, Zhao (1982, unpublished personal communication) characterized the major component of the female sex pheromone of *O. oryzae* as 2,6-diacetoxyheptane. Knowledge of these two structures facilitated identification of the pheromone components of *C. pisi* by Swedish and German workers. Hillbur et al. (1999) reported the pheromone of this species to consist of a blend of (2*S*,11*S*)-2,11-diacetoxytridecane, (2*S*,12*S*)-2,12-diacetoxytridecane, and 2-acetoxytridecane.

These reports have been followed by identification of pheromone components in 13 more midge species (Table 1). Many are mono- or diacetates, related to the structures identified previously. Analogous mono- (Table 1, entry 3) and di-butyrates (Table 1, entry 11) have been reported, and acetoxyketones are a further structural type in four species (Table 1, entries 6, 7, 15, and 16). A preliminary report (Riolo et al. 2006) of di-esters with two different alkanoyloxy groups, as pheromone components of *C. sorghicola* has yet to be further confirmed.

The structures identified to date show several patterns. All are unbranched, indicating a biogenetic origin from the acetate pool. All have an odd number of carbons, with representatives of from 7–17 (Table 1), and all have an oxygenated functionality at C-2. These features suggest biosynthesis by decarboxylation of β-ketoacyl precursors. In the structures reported, the functionality is most commonly an acetoxy-, butyroxy-, or keto- group, although two alcohols have been reported: (2*S*,10*E*)-10-tridecen-2-ol is an essential component of the pheromone of *M. destructor* (Andersson et al., 2009a), and (2*S*,8*Z*)-8-heptadecen-2-ol was detected in volatiles released by females of the Chinese chrysanthemum midge, *Rhopalomyia longicauda.* It is possible that the latter is a precursor to, or artifact from, the major component, (2*S*,8*Z*)-2-butyroxy-8-heptadecene, as addition of the alcohol to the butyrate in a range of relative amounts reduced the attractiveness of the latter to male midges (Liu et al., 2009).

With the exception of minor components in the pheromones of *M. destructor*, *C. nasturtii* and *C. pisi* (Table 1, entries 1, 8, 12), all relevant components contain at least one additional functional group. These can be one or two double bonds (Table 1, entries 1–5), another oxygenated moiety (Table 1, entries 6–12), or one double bond and an additional oxygenated functionality (Table 1, entries 13–16). Double bonds have the *Z*-configuration, except those in components of *M. destructor* (Table 1, entry 1).

All cecidomyiid pheromone components are chiral. Production of a single stereoisomer by females has been demonstrated in six species (Table 1, entries 1, 3, 6, 8, 12, 15); in the remainder, it is presumed that only one of the stereosisomers is attractive to males. There are no particular patterns in chirality, although the *S*-configuration predominates in compounds with one chiral center (Table 1, entries 1, 2, 3, 15 with *S*-configuration, entries 4, 5, and 16 with *R*), and the *S,S*- in compounds with two chiral centers (Table 1, entries 6, 7, 8, 10, 11, 12, compared to entries 9, 13, 14 with at least one center with *R*-configuration).

Biosynthesis of Pheromone Components

There has been only one study on the biosynthesis of pheromone in Cecidomyiidae to date. Foster et al. (1991b)

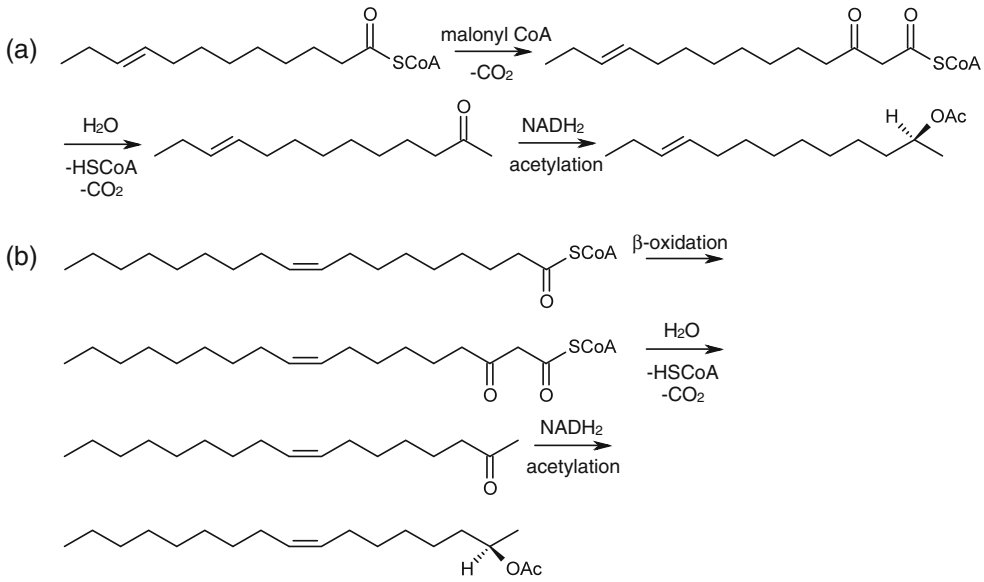


Fig. 1 Suggested biosynthetic routes to pheromone components of cecidomyiid midges, starting from fatty acid coenzyme A derivatives, by (**a**) chain elongation or (**b**) chain shortening

showed that decapitation stopped pheromone production by females of *M. destructor*, and this was partially restored by injecting them with either an extract of homogenized female head or the pheromone biosynthesis activating neuropeptide (PBAN) from *Heliothis zea.* This suggested biosynthesis may be under the control of a PBAN-like neuropeptide, as in many Lepidoptera (Raina, 1993; chapters in Cardé and Minks, 1997).

Foster et al. (1991b) also identified an unusual fatty acyl moiety, (9*E*)-9-dodecenoate, in the pheromone gland of female *M. destructor,* and proposed this was an intermediate in the biosynthesis of the major pheromone component, (2*S*,10*E*)-2-acetoxy-10-tridecene. As shown in Fig. 1a, two-carbon homologation of the dodecenyl precursor with malonyl coenzyme A (CoA) would give a 14-carbon, β-ketoacyl CoA. Hydrolysis, decarboxylation, and stereoselective reduction of the resultant methyl ketone, followed by acetylation, would yield the pheromone component.

The positions of double bonds in components of other cecidomyiid species suggest they may be generated by chain shortening of a fatty acid precursor, rather than chain elongation. The 2-acyl-8-heptadecenes (Table 1, entries 3, 4, 13, 14, 16) could be produced by β-oxidation of oleyl-CoA, followed by decarboxylation, reduction of the resultant methyl ketone, and acylation (Fig. 1b). The same process, preceded by β-oxidation and two-carbon chain shortening, could give (2*R*,6*Z*)-2-acetoxy-6-pentadecene (Table 1, entry 5). Homo-conjugated dienes (2*R*,6*Z*,9*Z*)-2-acetoxy-6,9-pentadecadiene (Table 1, entry 5) and (2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene (Table 1, entry 2) may be similarly derived from a linoleate precursor.

A second oxygenation along the chain can account for further structural variability of the pheromone components, and may generate a second stereogenic center. In unsaturated compounds, the second oxygen is always placed down the chain after the double bond. The mechanism is unknown, but it may involve hydroxylation of a former double bond or mid-chain oxygenation of a non-activated methylene group (Booth et al. 2009).

Synthesis of Pheromone Components

Synthesis of pheromone components of Cecidomyiidae has been important for chemical identification and for provision of material for bioassays and field tests. In the majority of species investigated so far, the amounts of material produced by female midges are very small, generally insufficient for obtaining full spectral data or microanalytical reactions. Identification thus has often relied on matching mass spectral data, GC retention data, and biological activity of synthetic standards with those of the natural compounds.

Synthesis of pheromone components generally has relied on a linear approach, assembling the aliphatic chain in a step-wise manner. An elegant exception was synthesis of (2*S*,7*S*)-2,7-dibutyroxynonane by Hooper et al. (2007), using metathesis of a mixed di-*t*-butyl-silaketal, prepared from (2*S*)-5-hexen-2-ol and prochiral 1,4-pentadien-3-ol, with Grubbs' catalyst.

As mentioned above, all the pheromone components of Cecidomyiidae identified to date have an oxygenated functionality at C-2, and production of this has been a key process in many syntheses. In the simplest approach, an aldehyde or acid is reacted with a methyl organometallic reagent to give the corresponding 2-alcohol or ketone. Thus, in the identification of the pheromone of *C. oregonensis*, Gries et al. (2002) observed an EAD response at certain GC retention times on different columns, consistent with those predicted for a 13-carbon compound with acetoxy function at C-2 and one or two non-conjugated double bonds. There was insufficient natural material to obtain a mass spectrum.

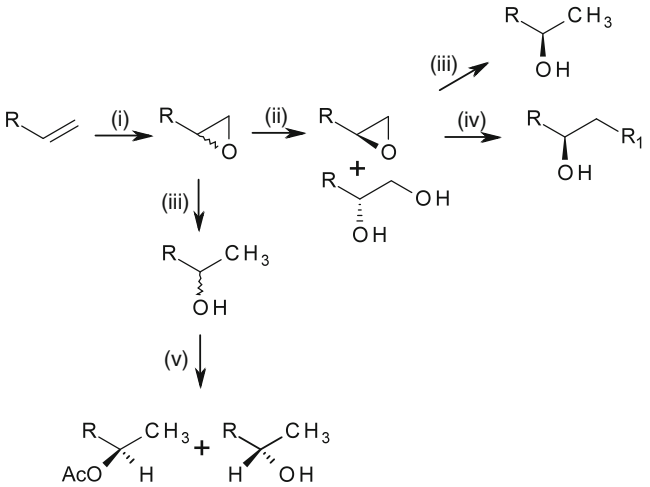


Fig. 2 Synthetic routes to chiral midge pheromone components using epoxides (Reagents: (i) 3-chloroperbenzoic acid; (ii) Jacobsen's reagent; (iii) $LiAlH_4$; (iv) R_1MgX + Cu(I); (v) lipase from *Candida antarctica*/vinyl acetate)

Therefore, all the positional and geometric isomers of 2-acetoxytridecene were synthesized by reaction of the corresponding dodecenals with a methyl Grignard reagent. The dodecenals were available from previous work on moth pheromones. It was then possible to calculate the shifts in GC retention times caused by the double bonds, and to predict two candidates for the EAD-active compound: (4*Z*,8*Z*)-2-acetoxy-4,8-tridecadiene and (4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene. Only the latter proved to have identical retention times to those of the EAD-active compound, and the 2*S*-enantiomer of this was confirmed to be the structure of the pheromone component. A similar approach was used to identify the two components of the pheromone of the blackberry midge, *D. plicatrix*, as (2*S*,6*Z*,9*Z*)-2-acetoxy-6,9-pentadecadiene and (2*S*,6*Z*)-2-acetoxy-6-pentadecene (Table 1, entry 5).

Three particularly effective approaches have been used to derive the enantiomers of pheromone components with a chiral oxygenated functionality at C-2 (Fig. 2). Both enantiomers of propylene oxide are commercially available, and reaction of these with an appropriate Grignard reagent under copper (I) catalysis gives stereospecific ring-opening (Table 1, entries 1, 8, 9, 10, 11, 12). Similarly, successive deprotonations of 1,3-dithiane and reaction with (*S*)-propylene oxide, were key steps in the synthesis of (2*S*,6*S*)-2,6-diacetoxyheptane, the major component of the pheromone of *O. oryzae* (Table 1, entry 6; Francke et al., unpublished). Enantiomers of several other terminal epoxides are also available, and these can be used to produce chiral oxygenated functionalities at other positions. Thus, for the synthesis of the pheromone components of the red cedar cone midge, *M. thujae*, Gries et al. (2005) reacted appropriate di-Grignard reagents with specific enantiomers of two terminal epoxides to give mixtures of three diols. For example, reaction of the di-Grignard reagent from 1,7-dibromoheptane with (*S*)-propylene oxide and (2*S*)-1,2-epoxyheptane gave a mixture of (6*S*,16*S*)-6,16-dihydroxyheneicosane, (2*S*,12*S*)-2,12-dihydroxyheptadecane, and (2*S*,12*S*)-dihydroxytridecane. Compounds with the 2-hydroxy functionality are much more polar than compounds with the hydroxyl group further into the chain, so column chromatography of the mixture on silica gel gave good separation of the three components, eluting in the above order. Acetylation of the desired (2*S*,12*S*)-2,12-dihydroxyheptadecane gave the corresponding diacetate as one of the pheromone components (Gries et al., 2005).

The commercially available terminal epoxides are produced by hydrolytic kinetic resolution of the racemic epoxides with a cobalt catalyst, developed by Jacobsen and coworkers (Tokunaga et al., 1997). This procedure can be used directly on appropriate terminal epoxides in the synthesis of pheromone components of Cecidomyiidae. For synthesis of (2*R*,7*S*)-2,7-diacetoxytridecane, the pheromone of the aphidophagous gall midge, *Aphidoletes aphidimyza,* the 2*R* functionality was generated by reaction of the Grignard reagent from 1-bromo-4-pentene with (*R*)-propylene oxide (Choi et al., 2004). The terminal alkene was epoxidized, and the epoxide reacted with (*R,R*)-N,N'-bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexane-diaminecobalt(II) (Jacobsen's reagent) in aqueous tetrahydrofuran, which selectively hydrolyzed the *S*-epoxide to the corresponding diol, leaving the *R*-epoxide. The latter was reacted with the Grignard reagent from 1-bromopentane, followed by acetylation to give the pheromone with 99% enantiomeric excess (ee). This approach was also used to synthesize several other Cecidomyiidae pheromone components (Table 1, entries 2, 8, 11, 15).

A third approach to esters of chiral secondary alcohols is by kinetic hydrolytic resolution with lipase. This is especially effective for 2-acyloxy compounds using the lipase from *Candida antarctica*, which can hydrolyze the *R*-ester or acetylate the *R*-alcohol selectively, according to conditions. Reaction of racemic (8*Z*)-2-butyroxy-8-heptadecene with this lipase in phosphate buffer gave the unreacted *S*-ester and the corresponding *R*-alcohol. These were separated by silica gel chromatography to give the *S*-butyrate with 99.8% ee and the alcohol, which, after acylation, gave the *R*-butyrate, with 99.1% ee. The latter is the sex pheromone of the honey locust gall midge, *D. gleditchiae* (Molnár et al., 2009). Successive hydrolysis and acetylation with this enzyme also were used to produce all three diastereomers of 2,6-diacetoxyheptane, the major pheromone component of *O. oryzae* (Hall et al., unpublished). In our experience, other enzymes and secondary acyloxy functionalities at other positions in the carbon chain give much lower ee values. This approach was used to synthesize the pheromone components of other cecidomyiid midges (Table 1,

entries 3, 5, 15), and for synthesis of (2*S*,12*Z*)-2-acetoxy-12-heptadecene, the female sex pheromone of the pistachio twig borer moth, *Kermania pistaciella* (Lepidoptera: Oinophilidae) (Gries et al., 2006).

High performance liquid chromatography (HPLC) has proved useful for isolating stereoisomers of pheromone components of Cecidomyiidae. Even with a standard analytical column, tens of micrograms can be obtained, which is usually sufficient for bioassay and field testing. For cecidomyiid pheromone components, the most widely used system has been a normal phase Chiralpak AD column (Diacel Chemical Industry Co. Ltd., Tokyo, Japan), eluted with hexane modified with a small amount of 2-propanol, as developed by Qin et al. (1997) for chiral moth pheromones. This was used to separate enantiomers of pheromones with one chiral center for field testing (Table 1, entries 15 and 16), and has proved particularly useful for separation of all stereoisomers of compounds with two chiral centers, which are much more difficult to synthesize (Table 1, entries 7, 11, 13, 14).

Alternatively, enantiomers have been separated by the method of Slessor et al. (1985), involving hydrolysis of racemic esters to alcohols, conversion to acetyl (2*S*)-lactate esters, and separation of the diastereomers by GC/HPLC. These esters were hydrolyzed and re-acylated to give pheromone enantiomers (e.g., Table 1, entry 2).

Pheromone Chemistry in Relation to Biological Activity

In 10 of the 16 species of cecidomyiid midges investigated to date, a single component is attractive to males (Table 1, entries 2, 3, 4, 7, 9, 11, 13, 14, 15, 16). In most of these, no other potential components have been reported. In the pear midge, *C. pyrivora*, the presence of an acetoxyketone, as well as the more abundant diacetate, was detected in volatiles collected from virgin females (Table 1, entry 7), but addition of the former to the latter did not affect attractiveness (Amarawardana, 2009). In *R. longicauda*, the corresponding alcohol was detected, as well as the major mono-butyrate component (Table 1, entry 3), but addition of the alcohol to the butyrate over a range of ratios only reduced catches of males (Liu et al., 2009).

In *M. thujae*, three 17-carbon diacetates were detected in extracts of pheromone glands of virgin females (Table 1, entry 10). All three elicited strong electroantennogram (EAG) responses from males, and a blend of these compounds was attractive to males in field tests. However, it was subsequently found that the individual components were each as attractive as the three-component blend, indicating an unusual redundancy (Gries et al., 2005).

In the other five species of cecidomyiid midges for which the pheromone has been identified, the pheromone consists of two or more components. For *O. oryzae* (Table 1, entry 6), preliminary results of field trials indicated that the major component, (2*S*,6*S*)-2,6-diacetoxyheptane, was attractive to males, and that addition of a minor component, (2*S*)-2-acetoxy-heptan-6-one, increased catches further (Liu et al., unpublished). Similarly, (2*R*,6*Z*,9*Z*)-2-acetoxy-6,9-pentadecadiene is the most abundant component in collections of volatiles from female blackberry midges, *D. plicatrix,* and is weakly attractive to males in field tests. However, attractiveness is greatly increased by addition of the minor component, (2*R*,6*Z*)-2-acetoxy-6-pentadecene (Hall et al., unpublished).

For the other three species, single components are unattractive to males. All three components are important for attraction of the swede midge, *C. nasturtii* (Table 1, entry 8) (Hillbur et al., 2005, Boddum et al., 2009) and *C. pisi* (Table 1, entry 12) (Hillbur et al., 2000, 2001). Although the major pheromone component of *M. destructor* (Table 1, entry 1) showed some activity in a wind tunnel, it was unattractive in field tests (Foster et al., 1991a). Subsequently, Andersson et al. (2009a) reported seven components in extracts of pheromone glands of females that elicited EAG responses from males. Six of these were identified and are required for attraction in the field.

In most cases, only the naturally occurring ratio of components has been evaluated with multicomponent pheromones. However, the importance of blend ratios was shown for *C. nasturtii* by Hillbur et al. (2005). In wind tunnel tests, varying the amount of (2*S*)-2-tridecyl acetate in the blend showed a maximum attractiveness at 1% of the major component, (2*S*,10*S*)-2,10-diacetoxyundecane, with a loss of attractiveness at higher or lower ratios.

As mentioned above, the pheromone components of cecidomyiid midges have all been demonstrated or deduced to be single enantiomers. In several cases, further studies have investigated the effects of other enantiomers on attractiveness, and these studies show some patterns.

Among pheromones with compounds containing one chiral center, for five of the cases the racemate is as attractive as the natural enantiomer (Table 1, entries 2, 3, 4, 15, 16), with the opposite enantiomer behaviorally inactive. However, for *D. plicatrix* (Table 1, entry 5), preliminary field tests indicated that only *R*-enantiomers of the pheromone components were attractive. The *S*-enantiomers and racemates were unattractive, indicating that the *S*-enantiomers inhibited attractiveness of the *R*-enantiomers (Hall et al., unpublished). With *M. destructor* (Table 1, entry 1), only the naturally occurring *S*-enantiomers of a six-component blend were tested in laboratory and field trials by Andersson et al. (2009a), although in the original work by Foster et al. (1991a) and Harris and Foster (1991), (2*S*,10*E*)-2-acetoxy-10-tridecene elicited 63% source contacts in a wind-tunnel bioassay, while the racemate gave reduced (38%) and the *R*-enantiomer little (7%) source contact.

Chirality seems to be more critical in pheromone components with two chiral centers. Only in *S. mosellana* (Table 1, entry 11) is the racemic mixture of all four diastereomers of 2,7-dibutyroxynonane as attractive to males as the 2*S*,7*S*-isomer, with the other diastereoisomers not affecting attractiveness (Gries et al., 2000). Although preliminary field studies suggest a similar situation may exist in *O. oryzae* (Table 1, entry 6) (Liu et al., unpublished), for all other examples one stereoisomer is attractive to males and the other stereoisomers and racemate are not attractive or only weakly so (Table 1, entries 7, 8, 9, 10, 12, 13, 14). Interestingly, in *C. pisi* (Table 1, entry 12), only blends containing the *S,S*-isomers of the two components with two chiral centers, 2,11-diacetoxytridecane and 2,12-diacetoxytridecane, are attractive to males. However, for the third component, 2-acetoxytridecane, containing one chiral center, blends with the racemate were as attractive to males as a gland extract in a windtunnel bioassay (Hillbur et al., 2000, 2001).

In several examples it has been shown that just one of the other stereoisomers is responsible for a reduction in attractiveness, although there are no obvious patterns in these interactions. In the pear leaf midge, *D. pyri* (Table 1, entry 13), Amarawardana (2009) separated the four stereoisomers of the pheromone, (8*Z*)-2,13-diacetoxy-8-heptadecene, by HPLC, and determined their configurations by correlation with the elution order of synthesized saturated analogs. Only the first-eluting isomer was attractive to males, and this had the 2*R*,13*R*-configuration. Addition of the 2*S*,13*R*- or 2*S*,13*S*-isomers to the attractive enantiomer had no effect on attractiveness, but addition of the 2*R*,13*S*-isomer completely negated attractiveness, explaining the inactivity of the racemate. Thus, while configurations at both chiral centers are important for attractiveness, synthesis fixing the stereochemistry at C-13 only (i.e., producing a mixture of the 2*R*,13*R*- and 2*S*,13*R*-isomers) should produce a mixture as attractive as the pure 2*R*,13*R*-isomer. A similar situation was found with *D. tetensi* (Table 1, entry 14), in which separation of stereoisomers by HPLC showed the third eluting isomer of (8*Z*)-2,12-diacetoxy-8-heptadecene to be the only attractive one (Amarawardana, 2009). Using the elution order correlations used for the pheromone of *D. pyri*, the active stereoisomer was predicted to be the 2*S*,12*R*-isomer. Addition of the 2*R*,12*S*- or 2*S*,12*S*-isomers to the active stereoisomer had no effect on attractiveness, but the mixture containing the 2*R*,12*R*-isomer was much less attractive to males. In *C. pyrivora* (Table 1, entry 7), field tests suggest that the first-eluting isomer of 2,7-diacetoxyundecane from the chiral HPLC column is attractive to males, while the second-eluting isomer is responsible for the lack of attractiveness of the racemate (Amarawardana, 2009). Choi et al. (2004) reported that (2*R*,7*S*)-2,7-diacetoxytridecane was attractive to male *A. aphidimyza* (Table 1, entry 9). Addition of the 2*R*,7*R*- or 2*S*,7*R*-isomers reduced attractiveness, but addition of the 2*S*,7*S*-isomer had little effect.

In two species with pheromones containing two components with two chiral centers, the chirality of only one of these seems to be important for attractiveness. Hillbur et al. (2001) found that addition of 5% or 20% of the 2*S*,11*R*- or 2*R*,11*S*-isomers of 2,11-diacetoxytridecane, to the blend containing the attractive 2*S*,11*S*-isomer, reduced attractiveness to male *C. pisi* (Table 1, entry 12) in field tests; addition of the 2*R*,11*R*-stereoisomer at 20% or 100% had no effect. For the other pheromone component, (2*S*,12*S*)-2,12-diacetoxytridecane, addition of 20% or 100% of the 2*R*,12*R*- or meso *R*,*S*-stereoisomers to the attractive blend did not reduce attractiveness.

For the swede midge, *C. nasturtii*, both wind tunnel and field tests showed that replacement of (2*S*,10*S*)-2,10-diacetoxyundecane in an attractive blend by an equivalent amount of its racemate completely stopped attractiveness. However, replacement of either (2*S*,9*S*)-2,9-diacetoxyundecane or (2S)-2-acetoxytridecane by their racemates did not affect attractiveness (Boddum et al., 2009).

Responses of Cecidomyiid Midges to Host Plant Volatiles

Successful host-plant finding by adult females is crucial to the subsequent survival of gall midge larvae. Attraction of cecidomyiid midges to volatiles from their host plants has been demonstrated in several species, although progress in identifying the chemicals involved has been slower than that with the sex pheromones.

The brassica pod midge, *D. brassicae*, is a pest of brassicas such as oilseed rape, *Brassica napus* L. *D. brassicae* flew passively with the wind until they came near a host-plant, whereby they started moving upwind (Sylvén, 1970), typical of when olfactory cues are involved. Åhman (1985) observed wild insects in the field in the presence of two *Brassica* species classified as "preferred" and "non-preferred". The preferred species generally was infested with more and bigger larvae of *D. brassicae* than the non-preferred one (Åhman, 1981). The number of females landing on the preferred plant was greater than on the non-preferred, indicating differences between the plants in olfactory and/or visual stimuli. Oviposition rate and egg batch size were similar between the two species, but females stayed longer on the preferred species, and hence deposited more eggs. In laboratory bioassays, Pettersson (1976) showed that females were attracted to *B. napus* plants, and the response was lost when the antennae were removed. Brassicas are characterized by production of isothiocyanates (ITCs) when damaged, due to interaction of glucosinolates and the enzyme myrosinase, which are stored in separate cells in the parenchymous tissues. *Dasineura brassicae* responded to 2-

allyl glucosinolate in an olfactometer (Pettersson, 1976), even though glucosinolates are involatile. It is possible that this response was actually to allyl ITC produced by hydrolysis. Males, but not females, of *D. brassicae* subsequently were found to be attracted to traps baited with allyl ITC (Lerin, 1984; Evans, 1991). Murchie et al. (1997) confirmed that large numbers of males of *D. brassicae* were caught in traps baited with allyl ITC, while females were also attracted, but in much lower numbers. Traps baited with 2-phenylethyl ITC were not attractive to *D. brassicae*, but were to both males and females of its parasitoid, *Platygaster subuliformis*. The parasitoid was not caught in traps baited with allyl ITC (Murchie et al., 1997).

In *R. theobaldi*, mated females oviposit within 24 hr of mating. Females exhibit a strong preference for splits in new-growth "primocanes" (Gordon and Williamson, 1991), and fresh splits are preferred over old ones or ones already occupied by larvae (Pitcher, 1952). Nijveldt et al. (1963) observed, in the laboratory, that odor from wounds and splits is an important stimulus for oviposition. They showed that spraying willow twigs with sap from young raspberry canes resulted in immediate egg laying by gravid female *R. theobaldi*; females did not lay on unsprayed twigs. This difference could be due to volatile and/or involatile chemicals in the sap, but the fact that older splits are less favored suggests that ephemeral, volatile chemicals may be responsible, at least in part. Recent work has shown that, on splitting, raspberry canes produce a characteristic burst of chemicals. The 18 main compounds have been identified, seven of which are produced by intact canes. Preliminary field trapping experiments have shown that both male and female *R. theobaldi* can be attracted to blends of these chemicals under certain conditions, but further work is required to understand this fully (Hall et al., 2010).

In windtunnel studies, Galanihe and Harris (1997) showed that females of *D. mali* distinguish apple from pear foliage, by flying upwind and landing on apple foliage. Hexanol and (3*Z*)-3-hexen-1-ol, identified from immature apple leaves, elicited EAG responses from female antennae (Anfora et al., 2005).

Crook and Mordue (1999) studied responses of *D. tetensi* in a four-way olfactometer. Newly emerged females or males showed no responses to volatiles from blackcurrant shoots. However, 2 hr after mating, females responded positively to these shoots. These authors suggested leaf volatiles may play a role in location of the host plant and carried out a detailed study of the morphology of the antennae, as described below. In further work, Crook et al. (2001) showed leaves of a susceptible and a resistant variety were equally attractive to mated females in an olfactometer. In the field, there was no difference in the incidence of oviposition on the two varieties, but larvae reared on the resistant variety were smaller than those reared on the susceptible variety, suggesting larval antibiosis rather than host selection is important as the main mechanism of resistance. Griffiths et al. (1999) also showed no obvious differences in the volatile profiles of resistant and susceptible varieties of blackcurrant.

Sitodiplosis mosellana is a cereal specialist, and field observations have shown that females fly upwind to wheat crops, often over considerable distances (Oakley et al., 1998). Using a four-way olfactometer, Birkett et al. (2004) found that females were attracted to freshly cut wheat panicles, and also to a collection of panicle volatiles on Porapak Q. GC-EAD analyses, using a female midge, of the volatile collection recorded six active compounds, identified as acetophenone, (*Z*)-3-hexenyl acetate, 3-carene, 2-tridecanone, 2-ethyl-1-hexanol, and 1-octen-3-ol. A blend of the six compounds, or a three-component mixture of acetophenone, (Z)-3-hexenyl acetate, and 3-carene, presented at the same ratios and concentrations as those found in the volatile collection, were as attractive to female midges as volatiles from a wheat panicle, when each was compared to no odor. No direct comparison was made of responses to synthetic blends and the natural stimulus (Birkett et al., 2004).

Sharma and Franzmann (2001a) showed that *C. sorghicola* responded to color and odor of host plants, and also demonstrated differential oviposition on various wild relatives of sorghum (Sharma and Franzmann, 2001b).

Whereas the above studies focused on the role of volatile chemicals in attraction of adult midges to host plants, selection of oviposition sites by *M. destructor* was influenced by visual and tactile cues, and by epicuticular leaf waxes (Harris and Rose, 1990; Foster and Harris, 1992). Fractionation of epicuticular wax extracts from wheat suggested at least two different compounds were present that stimulated oviposition (Foster and Harris, 1992), and these were identified as octacosanal and 6-methoxy-2-benzoxazolinone (Morris et al. 2000).

Odor Reception in Cecidomyiid Midges

Morphological and electrophysiological studies have been reported on the antennae of *C. sorghicola* (Slifer and Sekhon, 1971), *Thecodiplosis japonensis* (Lee and Lee, 1985), *Mycodiplosis erysiphes* (Solinas and Nuzzaci, 1987), *D. tetensi* (Crook and Mordue, 1999), *C. pisi* (Hillbur et al., 2001), *M. destructor*, and *C. nasturtii* (Boddum et al., 2010).

Morphology of Olfactory Sensilla

Four types of sensilla have been found on midge antennae: sensillum chaetica, s. trichodea, s. coeloconica, and s. circumfila, the last type being unique to cecidomyiid midges

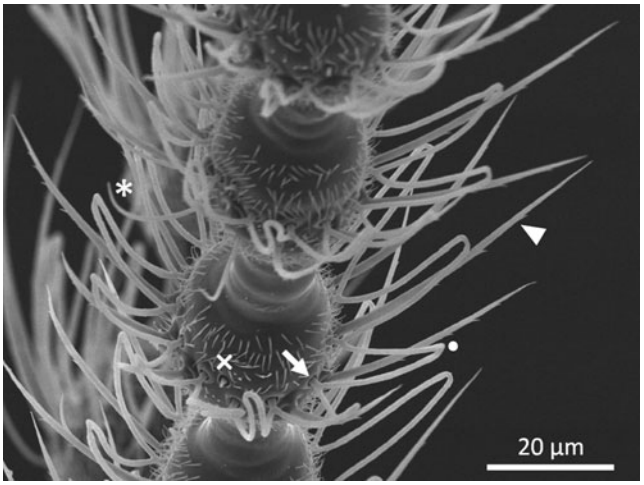


Fig. 3 Scanning electron micrograph of antenna of male *Contarinia nasturtii*. Four sensillum types are visible: s. trichodea (*), s. chaetica (Δ), s. coeloconica (×) and s. circumfila. Sensilla circumfila bifurcate (↓) and extend from the antennal surface where branches of two neighboring sensilla fuse (●)

(Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987; Crook and Mordue, 1999; Hillbur et al., 2001; Boddum et al. 2010). All types of sensilla, except s. chaetica, have been suggested to be involved in olfaction (Slifer and Sekhon, 1971; Gagné, 1989; Harris and Foster, 1999).

A sensillum circumfila is a compound structure, evolved from independent sensilla that have merged into one structure encircling the nodes of the dumbbell-shaped antennal segments (Hallberg and Hansson, 1999). There are two morphological types of s. circumfila. In female midges and in males of *D. tetensi* and *M. destructor*, they are located close to the antennal surface (Crook and Mordue, 1999; Boddum et al., 2010). In other males, they form elongated loops protruding from the antennal surface (Fig. 3) (Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987; Hillbur et al., 2001; Boddum et al., 2010). Sensilla circumfila have thin, single layer walls with multiple pores and are innervated by branching neurons (Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987; Crook and Mordue, 1999; Hillbur et al., 2001; Boddum et al., 2010). Their structure resembles that of s. basiconica, found in most insects (Hansson, 1995, Ochieng et al., 1998; Keil, 1999). However, s. basiconica have not been reported in midges; Boddum et al. (2010) suggested that s. circumfila might have evolved by fusion of a basiconic type of sensillum.

In *C. sorghicola,* s. coeloconica have been described as small peg-shaped structures with clearly visible grooves that run in parallel to the peg axis (Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987). They are innervated by five unbranched sensory neurons (Solinas and Nuzzaci, 1987).

The long and thin hair-like s. trichodea have wall pores, and are innervated by a few unbranched to moderately branched dendrites in *M. destructor*, *C. nasturtii*, *C. pisi,* and *D. tetensi* (Solinas and Nuzzaci, 1987; Crook and Mordue, 1999; Hillbur et al., 2001; Boddum et al., 2010), and by several highly branched dendrites in *C. sorghicola* (Slifer and Sekhon, 1971).

Function of Olfactory Sensilla

In many midge species, the s. circumfila are highly enlarged in males, suggesting that this structure is involved in detection of the female sex pheromone (Slifer and Sekhon, 1971; Hillbur et al., 2001). However, in *M. destructor* and *D. tetensi,* in which the s. circumfila in males are similar to those in females, the numerous s. trichodea on male antennae have been proposed to house neurons responding to pheromones (Crook and Mordue, 1999; Boddum et al., 2010). In species in which the s. circumfila of males are enlarged, s. trichodea occur in larger number in females than in males (Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987; Boddum et al., 2010), which might indicate that s. trichodea are involved in localization of host plants.

The function of the different sensillum types was studied by single sensillum recording (SSR) in *M. destructor*, in which there is no pronounced sexual dimorphism in s. circumfila, and in *C. nasturtii,* in which s. circumfila are enlarged in males (Boddum et al., 2010). Olfactory sensory neurons (OSNs) in male *C. nasturtii* s. circumfila, and in male *M. destructor* s. trichodea, responded to female sex pheromone components, indicating that the most abundant male sensillum type in each species house pheromone-sensitive OSNs. In the same study, a small number of receptors were found in the antennae of *C. nasturtii* males that responded only to stereoisomers of the pheromone which are not produced by females and which have an inhibitory effect on the behavioral response (Boddum et al., 2009).

The function of the complex structure of s. circumfila is still unknown. In both Lepidoptera and bark beetles, the ability to differentiate between behaviorally important odors is achieved by co-localization within the same sensillum of OSNs tuned to these odors (Baker et al., 1998, Andersson et al., 2009b). The fused s. circumfila might enhance detection of spatial and temporal coincidence over an extended area, as the circumfila cover the complete antennal circumference.

Exploitation of Pheromones for Management of Midge Pests

As noted by Harris and Foster (1999), many plant-feeding midges such as *M. destructor*, *D. brassicae*, *C. sorghicola*, and *O. oryzae*, are important as pests. A major outbreak of *S. mosellana* was recorded during 1983 in Saskatchewan, Canada, and caused yield losses estimated at $30 million

Table 2 Availability and use of pheromone lures for cecidomyiid midge pests as of 2011

Species	Suppliers[a]	Dispenser[b]	Loading	Field Life	Trap design	Trap height	Use[c]	Trap threshold[d]	Countries[e]
Dasineura mali	(6)	RS	3 μg racemate	1 yr	White delta	0.5 m	P, S	30/trap/wk	UK, FR, NL, N, CA
Resseliella theobaldi	(2)	RS	10 μg racemate	1 mo	White delta	0.5 m	P, S	30/trap/wk	EU
Dasineura tetensi	(6)	RS	5 μg active isomer	1 yr	Red delta	3 cm	P, S	10/trap/wk	UK
Sitodiplosis mosellana	(1), (8)	PV	1 mg racemate	1 yr	White delta	0.5 m	P, S	30–120/trap/d	UK, CA
Contarinia nasturtii	(3), (5)	PC	10 μg active enantiomers	1 mo	Delta	0.2 m	D, P, S	None	EU, CA, US
Contarina pisi	(3), (4)	PC	1 and 10 μg active enantiomers	1 mo	Delta	0.2 m	S	None	UK, FR
Mayetiola destructor	(3)	PC	10 and 100 μg of active enantiomers	2 wk	Delta	0.2 m	D, P	None	US
Contarinia oregonensis	(7)	RS							US

[a] (1) Agrisense BCS Ltd, Treforest Industrial Estate, Pontypridd, South Wales, CF37 5SU, UK (www.agrisense.co.uk); (2) Agralan Ltd, The Old Brickyard, Ashton Keynes, Swindon, Wilt SN6 6QR UK (www.agralan.co.uk); (3) Andermatt Biocontrol AG, Stahlermatten 6, 6146 Grossdietwil, Switzerland; (4) Oecos Ltd, 11a High Street, Kimpton, Herts, SG4 8RA UK; (5) PheroNet AB, PO Box 19, 230 53 Alnarp, Sweden ((niclas.ostlund@slu.se); (6) East Malling Research, New Road, East Malling, Kent ME19 6BJ UK (www.emr.ac.uk); (7) Alpha Scents Inc. 1089 Willamette Falls Drive, West Linn, OR 97068 (www.alphascents.com); (8) Contech Enterprises Inc., Unit 115 - 19 Dallas Road, Victoria, BC V8V 5A6, Canada (www.contech-inc.com).

[b] RS rubber septum; PV polyethylene vial; PC polyethylene cap

[c] D detection; P population monitoring; S spray timing

[d] Nominal threshold for spray timing

[e] UK United Kingdom; FR France, NL The Netherlands; N Norway, EU European Union; CA Canada; US United States

(Olfert et al., 1985). A similar outbreak of *S. mosellana* in the UK during 1993 caused crop losses estimated to be worth millions of pounds (Oakley, 1994). Since the 1920's, Australia has banned imports of apples from New Zealand because of the fear of introducing fireblight, European canker, and *D. mali*. This ban recently was overturned by the WTO, but strict monitoring for the presence of pests and diseases is required. *Contarinia nasturtii* is a quarantine pest in Canada and the USA, and many other species of midge are important pests in forestry, field crops (Barnes, 1956) and particularly in horticultural and fruit crops (Barnes 1948).

The pheromones of several species of cecidomyiid midge now are commercially available (Table 2). Practical use of these has, so far, been restricted to pest monitoring, but attempts to use these for direct control have been reported.

Use of Pheromones for Pest Monitoring

Populations of pest species of midge previously have been monitored by egg counts (Harris et al., 1996), water, or sticky traps (Williams et al., 1987; Williams, 1990), or destructive sampling of plant material for larvae and pupae (Barnes, 1956). In recent years, a temperature-based forecasting system for *R. theobaldi* has been available to raspberry growers in the UK (Gordon et al., 1989). Traps baited with synthetic pheromone can provide simpler, more accurate, and more local approaches to pest monitoring. Midges generally pupate in the soil under plants they infested previously. Monitoring with pheromone traps is, thus, likely to be particularly valuable in perennial crops, such as forests and many horticultural crops, in crops planted at the same site in successive seasons, and in places where more than one generation of the pest occur during a season.

Lures

Filter papers, cotton wicks, and dental rolls were used as pheromone dispensers in some initial field studies (e.g., Hillbur et al., 2000, 2005; Choi et al., 2004; Gries et al., 2005). However, rubber septa and polyethylene vials or caps are used as dispensers in field applications, as they give longer persistence and more uniform release rates (Table 2). The wide variation in molecular weight and polarity of midge sex pheromone components (Table 1), means that release rates of components vary widely. At one extreme, the pheromone of *D. mali* with a 17-carbon chain (Table 1, entry 16) is released at only 6–7 $ng.h^{-1}$ from polyethylene vials, and 0.5 $ng.h^{-1}$ from rubber septa loaded with 100 μg at 27°C (Cross and Hall, 2009), which is still much more than the 20 pg produced by a single female over several hours (Cross and Hall, 2007). The more volatile pheromone component of *R. theobaldi*, with an 11-carbon chain (Table 1, entry 15), is released at 20–80 $ng.h^{-1}$ from vials and septa under similar conditions (Hall et al., 2009). Release of the major component of the

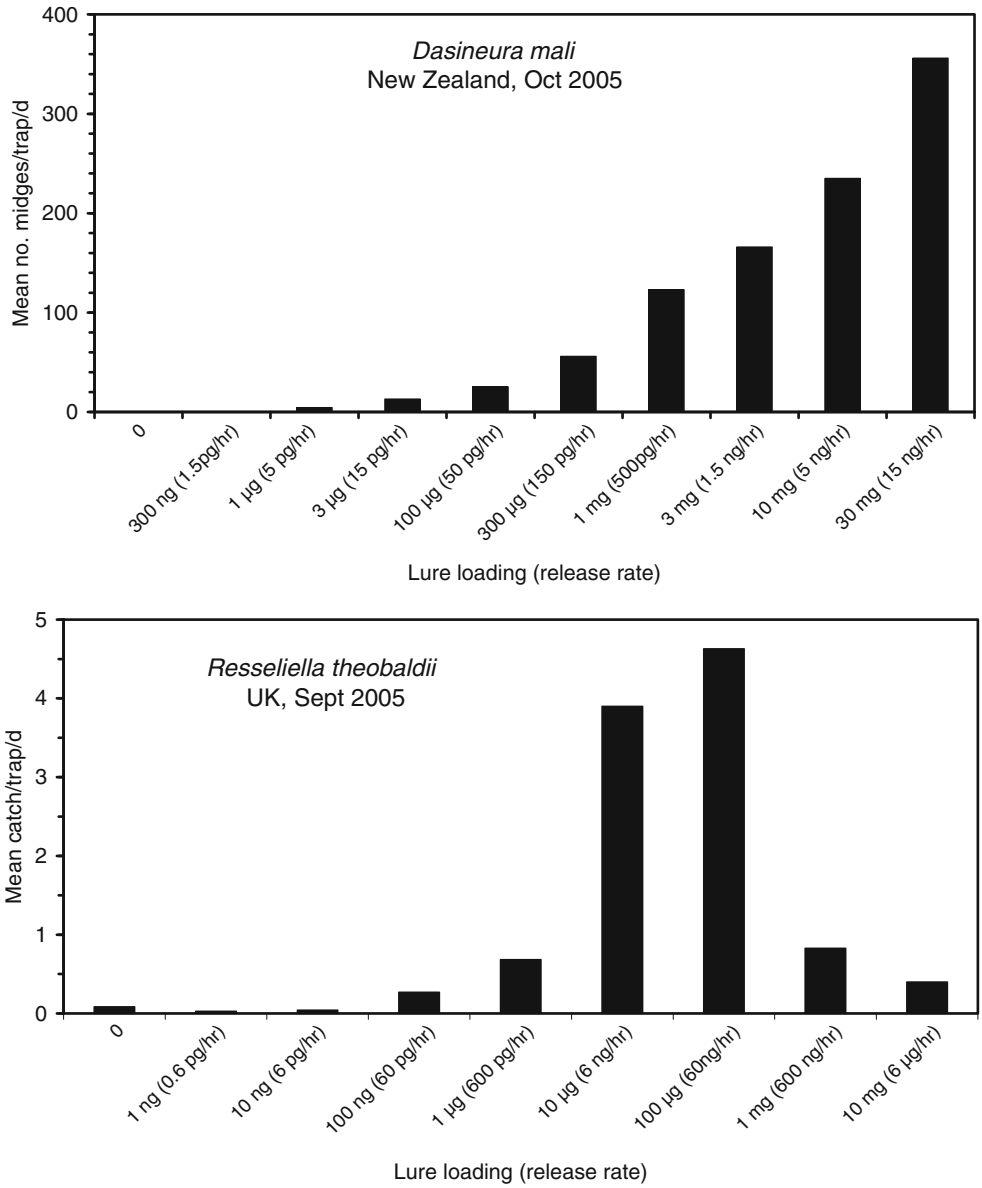


Fig. 4 Effect of lure loading and release rate of pheromone from rubber septa lures on catches of *Dasineura mali* in New Zealand in 2005 (Suckling et al., 2007) (*upper*) and *Resseliella theobaldi* males in Kent, UK in 2006 (Cross, 2010) (*lower*). Release rates estimated from measurements made in a laboratory wind tunnel at 27°C and 8 km.hr^{-1} airspeed

pheromone of *C. nasturtii* (Table 1, entry 8) was variable under field conditions, with up to 924 ng.h^{-1} released from polyethylene caps loaded with 100 µg; the ratio of the three components remained relatively constant during this period (Boddum et al., 2009). Release rates of the 9-carbon dibutyrate pheromone of *S. mosellana* (Table 1, entry 11), from a range of dispensers, were measured by Bruce et al. (2007). Rates from polyethylene vials and rubber septa with 5 mg loading were 0.73 µg.d^{-1} and 1.3 µg.d^{-1}, respectively, equivalent to 0.6 ng.h^{-1} and 1.1 ng.h^{-1} from lures loaded with 100 µg.

The effect of lure loading, and hence release rate, on the attractiveness of lures in the field varies with species. With *D. gleditchiae* (Table 1, entry 4), there was no difference in catches of male midges with lures loaded with 1–30 µg (Molnár et al., 2009). With several species, increasing pheromone loading increased catches of males over the range tested, as with *D. mali* over the range 300 ng–30 mg (Suckling et al., 2007) (Fig. 4) and 1–100 µg (Cross and Hall, 2009) in rubber septa, and *S. mosellana* (Table 1, entry 11) using polyethylene vials loaded with 10 µg and polyethylene sachets loaded with 24 mg (Bruce et al., 2007). In other species, attractiveness has a maximum at certain pheromone loadings. For *C. pisi,* catches of male midges, using dental rolls impregnated with 1 µg pheromone, were higher than catches to 10 or 100 µg (Hillbur et al., 2001). With *R. theobaldi*, increasing the loading of pheromone in rubber septa by decadic steps, from 1 ng to 10 mg, showed maximum attractiveness at 100 µg and 1 mg; further increasing this to 10 mg resulted in decreased catches (Fig. 4). However, it should be noted that trap catch maxima for certain species may not have been reached because of differences in

relative release rates of the different pheromones. For instance, as shown in Fig. 4, while the reduction in trap catch of *R. theobaldi* was observed at release rates above 600 ng.h^{-1}, the maximum release rate tested for *D. mali* was only 15 ng.h^{-1}.

In practice, lure type and loading for pest monitoring should be selected so that traps are sensitive for capturing males at low population densities, but not so attractive that they attract too many males with traps becoming saturated or the numbers caught so large that they cannot be readily counted (Cross, 2010).

Traps

Water traps and sticky traps of a wide range of design and size are suitable for capturing *D. mali*. The larger the trap used, the more midges that were caught (Cross and Hall, 2009). Sticky delta traps are used for most species, mostly for practical reasons including, ready availability, low cost, protection of lure and sticky base from rain, and ease of counting midges on sticky bases. Surprisingly for day-flying midges, trap color does not seem to affect catches, but can be manipulated to reduce catches of non-target insects (Amarawardana, 2009). Thus, red delta traps are recommended for monitoring *D. tetensii* in the UK, and are fitted with excluder grids at the entrances to prevent birds entering to feed on the trapped midges.

Most cecidomyiid species that are pests in temperate climates pupate in or on the surface of soil or leaf litter, with males emerging a short time before females (Gagné, 1989). Males probably wait on, or close to, the soil when females emerge and start emitting pheromone. Because midges are weak flyers, the height of trap deployment often has a strong influence on the number of midges captured and the sensitivity of the trap for pest detection and monitoring. Cross and Hall (2009) showed that traps deployed on the ground caught three-fold more *D. mali* than traps deployed at 0.5 m which, in turn, captured nine-fold more than those at 2 m. Almost no midges were captured at 2.5 m (Fig. 5). Similar results were found for *C. nasturtii* (Hillbur et al., 2005), *M. destructor* (Andersson et al., 2009a; Harris and Anderson, *pers. comm.*), and *D. tetensii* (Amarawardana, 2009).

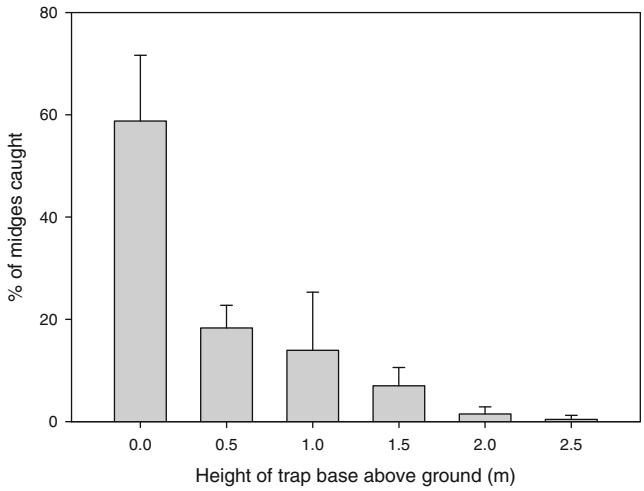


Fig. 5 Effect of trap height on catch of *Dasineura mali* (error bars are one standard deviation) (from Cross and Hall, 2009)

Orange Wheat Blossom Midge

Sitodiplosis mosellana is an increasingly important pest of wheat in the Northern Hemisphere, causing severe yield losses in years of high infestation; e.g., in the UK (Oakley, 1994) and China (Wu et al., 2008). Bruce et al. (2007) conducted field-trapping experiments with the synthetic sex pheromone in wheat crops in the UK. They demonstrated that pheromone traps gave a reliable indication of peak midge emergence, onset of flight, and abundance of midges throughout the season. A strong correlation between catch and crop infestation levels was obtained (Bruce et al., 2007). A simple decision-support model then was developed (Bruce and Smart, 2009), with monitoring intensified if catches of >30 males.d^{-1} are recorded, and treatment recommended when catches reach 120 males.d^{-1}. Use of sex pheromone-monitoring traps is an important component in integrated management strategies for the midge in the UK (Ellis et al., 2009).

Swede Midge

Contarinia nasturtii is a pest of most cultivated Brassicaceae, such as broccoli, canola, cauliflower, cabbage, swedes, and Brussels sprouts, with a high potential for economic impact (Hallett et al., 2009). It is a new invasive species in Canada and the USA (Hallett et al., 2009). Boddum et al. (2009) described optimization of pheromone traps for *C. nasturtii*, and the traps are currently used for pest monitoring in Switzerland, Canada (Hallett et al., 2007), the United States, Germany, and Norway. Linear relationships between trap catches and larval infestations were found, and 3 years of trap catch and crop damage data were used to establish a preliminary damage threshold of 10 males/trap/week for kohlrabi and broccoli crops (Baur, 2005; Sauer, 2008).

Pea Midge

Sex pheromone traps for *C. pisi* are used for monitoring emergence of males in fields where peas were grown the previous year, as males do not leave their overwintering site (Anthony Biddle, pers. comm.). Catches provide advance warning of infestation in nearby pea crops, but numbers of

females migrating to the current year's pea crop is dependant on weather conditions, prevailing wind direction, and the closeness of the current year's pea crop to the previous year's site. Consequently, catches are not clearly related to subsequent infestation levels. When a peak catch of more than 500 midges/trap/week is recorded, it is recommended that pea crops in the near vicinity should be examined for midge adults, and a spray of a suitable synthetic pyrethroid insecticide applied to those crops at the susceptible early green bud stage as soon as female midges are found (Anon, 2008).

Hessian Fly

Pheromone traps are used in North Dakota to establish the geographic distribution of *M. destructor*, and to monitor this pest's phenology and seasonal abundance. Adult emergence generally begins in early May, and ends in late September to early October, with as many as three broods of adult flies a season. Peak fly activity occurs in late July to early August, which is unusual, as this seems to be too early to attack winter-sown cereals and too late to utilize spring-sown cereals. Traps are also being used in the US winter wheat belt to validate fly-free planting dates (Harris and Anderson, pers. comm.).

Pheromone traps are used also as a convenient method for collecting *M. destructor* adults to determine the frequency of avirulence genes in populations of the fly in the southeastern US (Harris et al., 2003; Harris, pers. comm.).

Apple Leaf Midge

Dasineura mali is a pest of apples, particularly during the nursery stage, in the UK and other European countries, the Western US, and New Zealand. Cross and Hall (2009) described optimization of pheromone traps for *D. mali.* Cross et al. (2009) carried out trapping experiments in England, Italy, and New Zealand, and demonstrated a strong linear relationship between trap catch and number of galls observed subsequently, particularly during the first two generations (Fig. 6). There was also a good correlation between trap catch and number of larvae found in shoots (Cross et al., 2009).

Raspberry Cane Midge

Sex pheromone traps for *R. theobaldi* are used increasingly for monitoring and timing insecticide sprays in the UK and continental Europe (e.g., Bedard et al., 2010; Sipos et al.,

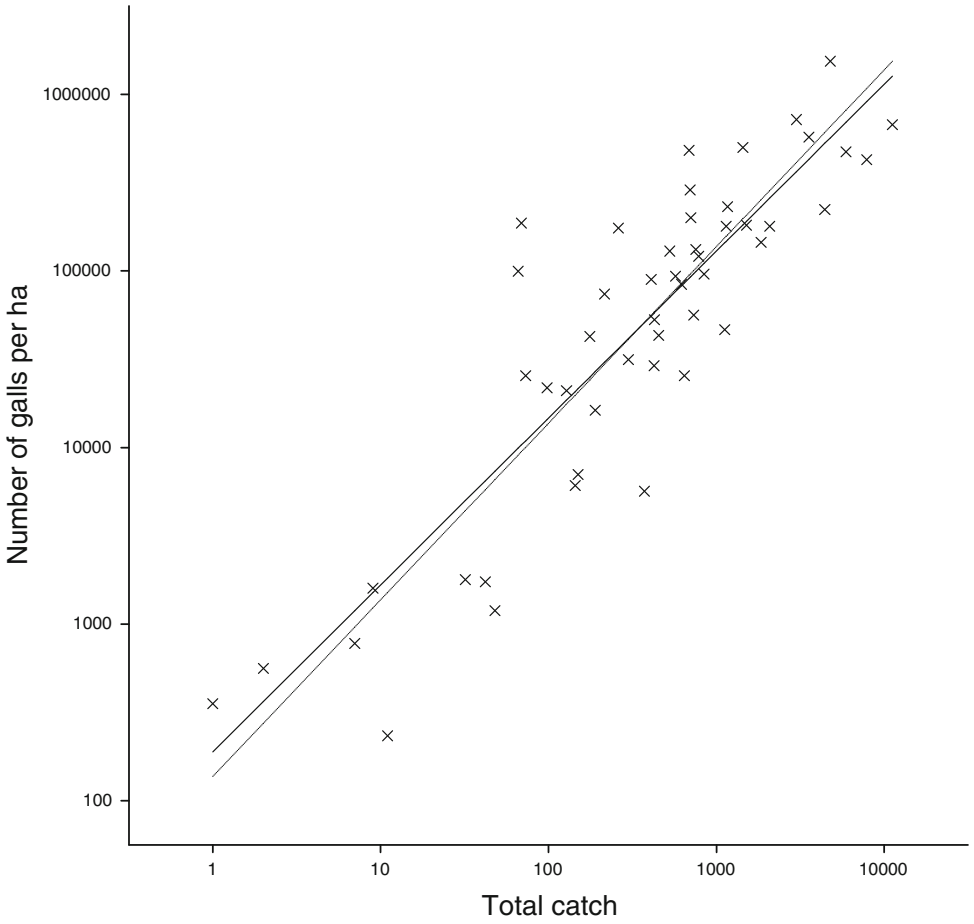


Fig. 6 Relationship between total catch per generation of first or second generation *Dasineura mali* males in standard sex pheromone traps and the number of galls formed by that generation subsequently. Best fit linear regressions on a log-log scale through the origin (*dashed line*) or unconstrained (*solid line*) are included (from Cross et al., 2009)

2010; Tanasković and Milenković, 2010). A ring test was conducted by fruit entomologists in nine EU countries and Russia in 2006, and a strong linear relationship established between trap catch of adult males and number of larvae found subsequently in splits in raspberry canes (Cross et al., 2008). This relationship has not been used directly for setting trap thresholds, because the relationship between larval infestation and crop damage has not been established. A low nominal threshold of 30 midges/trap/week was set as a threshold for spraying of insecticide.

Blackcurrant Leaf Midge

Use of air-temperature data to predict emergence of *D. tetensi* was reported by Cross and Crook (1999), but pheromone traps currently are being evaluated by UK blackcurrant growers for monitoring this pest. A low nominal trap threshold has been set to time sprays, which are best applied against the first generation in spring (Mitchell et al., 2011). Recent insecticide trials have shown that sprays of the insecticides bifenthrin or lambda cyhalothrin, applied a few days after a threshold catch, give a high degree of control, whereas sprays applied a week later have a markedly lower efficacy, demonstrating the importance of the traps for optimizing control by insecticides (Cross et al., unpublished).

Use of Pheromones for Pest Control

Because of their potency for modifying the behavior of male midges, synthetic sex pheromones of cecidomyiid midges have good potential for controlling target pests through mating disruption (MD), attract and kill (A&K), or mass trapping (MT), potentially at much lower doses than those used for moth pheromones, in which pheromone-based control approaches are most developed (Foster and Harris, 1997; Witzgall et al., 2010). In the Cecidomyiidae, unmated females do not lay viable eggs (Gagné, 1994, 1989). Females emerge with a full complement of eggs, and need mate only once to fertilize all their eggs (Harris and Foster, 1999). In contrast, males can mate several times (e.g., Suckling et al., 2007). Thus, a high degree of suppression of mating activity is likely required for successful control. Furthermore, some midge species, such as *M. destructor* (Sosa, 1981) or *O. oryzae* (Pasalu et al., 2004), have evolved biotypes that can survive on previously resistant varieties of their host plant. Thus, evolution of biotypes with modified responses to their pheromones might be possible under strong selection pressure from control with pheromones. However, several species of midge have been shown to produce unisexual progenies (Barnes 1931; Murchie and Hume, 2003 and refs therein), probably to reduce inbreeding within a population (Stuart and Hatchett, 1991). This feature would, presumably, act to augment the effect of disruption of mating by synthetic pheromone.

Apple Leaf Midge

Cross and Hall (2007) carried out trials of MD and A&K in the UK during 2004 and 2005 against *D. mali* on replicated 1 ha plots, in both established orchards with heavy infestation and newly-planted orchards with relatively light infestation. The application rate was restricted to 1 g.ha^{-1} pheromone per season, under the terms of the experimental permit. Racemic pheromone was used (Table 1, entry 16), as this was shown to be as attractive to males as the pure stereoisomer (Cross and Hall, 2007). MD dispensers were polyethylene caps loaded with 500 μg of pheromone (Table 1, entry 16). A&K devices were squares of plastic laminated card that had been surface-treated with microencapsulated lambda cyhalothrin and baited with a polythene cap lure containing 100 μg of pheromone. The MD and A&K devices were deployed at both 500 sources.ha^{-1} and 2000 sources.ha^{-1} in a regularly spaced lattice, fixed to tree stakes so that the lure was approximately 15 cm above the ground. For the 2000 sources.ha^{-1} treatments, release rates for the MD and A&K treatments were approximately 20 and 4 μg/ha/h, respectively. All treatments strongly suppressed catches of males in monitoring traps in the centers of the plots, compared to those in the untreated control plots, for at least 3 months. However, there was no evidence that either the MD or A&K treatments suppressed numbers of galls in shoots in either the heavily infested or the lightly infested orchards. Although the polyethylene cap dispensers showed relatively uniform release of pheromone for at least 270 d under laboratory conditions, little pheromone remained in those in the field for 4–5 months, possibly due to degradation of the pheromone in the lures by direct sunlight.

A trial of MT against *D. mali* was conducted in New Zealand in 2005 on replicated 1 ha plots by Suckling et al. (2007). Traps were Lynfield fruit fly traps, with oil as the catching medium, baited with rubber septa loaded with 3 mg of racemic pheromone. These were deployed at 500 traps. ha^{-1}. High levels of suppression (94%–98%) of trap catch in monitoring traps were observed, but numbers of galls that developed were too low to assess the effectiveness of the MT treatment for control of the midge. Further work is ongoing in New Zealand, but has not yet met with success (P. Shaw, pers. comm.).

Raspberry Cane Midge

Large-scale field trials have been conducted in the UK during the last 5 years, testing a wide range of formulations of the pheromone of *R. theobaldi* (Table 1, entry 15) for

control (Cross, 2010). As with *D. mali*, the racemic pheromone was used (Hall et al., 2009), with the maximum dose permitted under the experimental permit being 10 g/ha/season. Mating disruption formulations included polyethylene sachets, ethylene-vinyl acetate granules and SPLAT, a proprietary wax emulsion (ISCA Technologies, CA) applied with caulking guns in small dollops to the polythene mulch or irrigation pipe. Attack and kill devices were plastic laminated cards treated with microencapsulated lambda cyhalothrin, baited with a polythene cap or rubber septum dispenser, as for *D. mali* above. Mass trapping devices were Lynfield-type traps, baited with a rubber septum lure and containing a 50:50 water:glycol mixture to kill midges. Mixed results were obtained: some treatments worked well in some trials, but failed in others. Competitive attraction MD or A&K, using large numbers of sources with low release rates, appeared to be the most promising approach, but many of the formulations evaluated were impractical for use by growers. SPLAT, applied in $5{,}000 \times 0.5$ g dollops.ha^{-1}, gave good results in crops in polythene tunnels in which the initial populations of midge were low, although adjustment of the formulation is needed to sustain an adequate release rate.

Conclusions

Since the last review of the chemical ecology of cecidomyiid midges by Harris and Foster (1999), great progress has been made in identification of the female-produced sex pheromones of these insects. Pheromone components have been identified in at least 16 species (Table 1), despite the difficulties in working with these small, delicate and short-lived insects that generally produce pheromone in very small quantities. Synthetic compounds have been shown to be attractive to conspecific males, although rigorous comparisons of the attractiveness of the synthetic compounds and females have been made only in a few species (Table 1, entries 1, 2, 8, 9, 12). In others, further work to identify additional components may be required before identification of the complete pheromone can be claimed. The structures of the pheromone components share several features, most probably as a result of common biosynthetic pathways, although more detailed research on this is required. Nevertheless, the structural information gained to date should greatly facilitate identification of the pheromones of other species, as will the experience gained in synthesis of these molecules. The pheromone components generally are chiral, and several general and practical approaches to synthesis of single stereoisomers have been developed.

There is good evidence in several species that mated female midges use volatile semiochemicals to locate host plants. However, progress in identifying these chemicals has been slower than that for identifying pheromones. It is hoped that this will be an active area in future.

Similarly, research on the mechanisms of olfaction in cecidomyiid midges has only just begun. This area is particularly interesting in relation to responses to different stereoisomers of pheromone components. In several species, non-natural stereoisomers inhibit attraction to the natural stereoisomer, but it is not known whether this is due to unfavorable interactions at the receptor level or to inhibition involved in ensuring species-specificity. Furthermore, the s. circumfila is a unique and interesting sensory structure of the Cecidomyiidae.

Pheromone lures and traps have been developed for several midge species, and already are commercially available and in use for monitoring these pests. This approach is particularly valuable in forestry, perennial horticultural crops, and arable crops planted in the same or neighboring fields in successive years, because midges generally pupate and overwinter in the soil below the previous year's crop and are poor fliers.

Progress in use of pheromones for direct control of midge pests has been slower, at least in part because of the high cost of producing midge pheromones. Use of pheromones for control of some midge species is complicated by the sporadic nature of outbreaks, the ability to aestivate or diapause for long periods (Passlow, 1965), and the very high population levels that can occur during outbreaks (Harris and Foster, 1999). On the other hand, midges generally pupate in soil under plants they infested previously and have limited flight capacity. These latter factors favor approaches such as MD and A&K directed at adults, and may be enhanced by using pheromone traps for timing appearance of the adults. Pheromones may prove most viable for control of midge pests of crops planted at the same site in successive years and for perennial crops such as forests and high-value horticultural crops.

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n-Hexyl Laurate and Fourteen Related Fatty Acid Esters: New Secretory Compounds from the Julid Millipede, *Anaulaciulus* sp.

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Abstract A total of fifteen saturated fatty acid esters were newly identified from the secretions of an unidentified *Anaulaciulus* sp. (Julida: Julidae). The fatty acid components of the esters were composed of normal chain acids (from C_{10} to C_{14}) and of branched chain acids (from iso-C_{12} to iso-C_{15} and anteiso-C_{15}). The alcohol moieties were all composed of normal chain alcohols varying from *n*-butanol to *n*-octanol. The most abundant component found in the total esters was *n*-hexyl laurate (64.7%). Novel compounds identified from the millipede secretion extracts include six branched iso- and anteiso-fatty esters, an odd-numbered C_{11}-fatty acid ester, a C_{13}-fatty acid ester, and a C_7-alcohol ester, all of which were previously undescribed natural products. In addition, a characteristic mixture of benzoquinones, such as 2-methyl-1,4-benzoquinone, 2-methoxy-3-methyl-1,4-benzoquinone, 2,3-dimethoxy-1,4-benzoquinone, 2-methoxy-6-methyl-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-1,4-benzoquinone were identified from the secretions, together with trace amounts of 1,4-benzoquinone.

Keywords *n*-Hexyl laurate · Fatty acid ester · Branched fatty acid · *Anaulaciulus* sp. · Julid millipede · Benzoquinones · Defensive secretion

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Introduction

Most millipedes (Diplopoda) possess defensive glands in the form of integumental sacs arranged segmentally along the length of the body, from which they discharge secretory fluids when disturbed. The fluids have been studied in a wide variety of millipedes, and the order specificity for the distribution of the major components has been elucidated (Eisner et al., 1978; Kuwahara, 1999). The major components identified include cyanogenic compounds (order Polydesmida) (Blum and Woodring, 1962; Ômura et al., 2002a), phenolic compounds (some polydesmid species) (Blum et al., 1973; Duffey et al., 1977; Ômura et al., 2002b), 1,4-benzoquinones (orders Julida, Spirobolida, and Spirostreptida) (Monro et al., 1962; Röper and Heyns, 1977; Kuwahara et al., 2002), and alkaloids (orders Glomerida and Polyzoniida) (Meinwald et al., 1966; Meinwald et al., 1975; Wood et al., 2000; Kuwahara et al., 2007). These compounds are adhesive, irritating, or toxic, thus contributing to the millipede's chemical defense against various predators (Eisner and Meinwald, 1966; Pasteels et al., 1983).

Millipedes in genus *Anaulaciulus* are distributed in East Asia (Attems, 1909), and more than twenty species are known in Japan (Shinohara, 1990). In the present paper, we focused our research on a species known as "*fuji yasude*" in Japanese, *Anaulaciulus* sp. (Julida: Julidae). There is little published information on secretions of East Asian julid species. Here, we describe detailed characterization of a set of fatty esters by GC-MS, NMR and syntheses for *Anaulaciulus* sp., including new natural products comprised of branched or odd-numbered fatty acids and an odd-numbered alcohol. We also detected several 1,4-benzoquinone derivatives in the defensive volatile secretions. Excluding common defensive benzoquinones, secondary components such as aliphatic esters may be useful as

chemotaxonomic characters for the classification of East Asian Julida.

Materials and Methods

General Procedures The gas chromatography-mass spectroscopic (GC-MS) analyses were performed on a Network GC System (6890N; Agilent Technologies Inc.) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies Inc.) operated at 70 eV, using an HP-5MS capillary column (Agilent Technologies Inc, 0.25 mm I.D. × 30 m, 0.25 μm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.00 ml/min. Samples were analyzed in the splitless mode with temperature programmed to change from 60°C to 290°C at 10°C/min after an initial 2 min hold, and a final hold at 290°C for 5 min.

GC and GC-MS data were recorded using Chemstation (Agilent Technologies Inc.) with reference to an MS database (Agilent NIST05 mass spectral library, Agilent Technologies Inc.). ^{1}H NMR spectra (400 MHz, TMS at δ=0.00 as internal standard) and ^{13}C NMR spectra (100 MHz, $CDCl_3$ at δ=77.0 as internal standard) were recorded on a Bruker Biospin AC400M spectrometer. All chemicals and solvents used for analyses, extraction and syntheses, except $CDCl_3$, were of reagent grade (Sigma-Aldrich Co., Ltd., Tokyo, Japan and Wako Pure Chemical Industries, Ltd., Osaka, Japan). Column chromatography was performed on Wakosil silica gel C-200 with the specified solvents.

Millipedes Anaulaciulus sp. millipedes were collected as nymphs and adults from leaf litter on the campus of Kyoto Gakuen University in the winter of 2008 (voucher specimens (6 males, 4 females and a juvenile) have been deposited as D5124 in Faculty of Education, Kumamoto University, Japan). The millipedes were fed and kept alive in the laboratory on decaying plant material.

Extraction of Secretions and Body Surface Components The sex of each millipede was determined, and then they were individually treated as described below. Millipedes were squeezed with forceps causing emission of defensive secretion, which was collected in glass capillary tubes (0.5 mm id, hand-made), and extracted with hexane (0.1 ml) for 3 min. Body surface components of 1) frozen dead millipedes (-80°C for 1 h) and, 2) freshly decapitated millipedes were each extracted with 0.5 ml of hexane for 3 min. One μl portions of the resulting hexane extracts were analyzed by GC-MS.

Conventional Extraction and Collection of Ester Fraction In order to isolate the ester fraction, twelve adults were dipped in 1 ml of hexane for 3 min, the extract was loaded onto a SiO_2 (1.0 g) column, and eluted stepwise with the addition of 5 ml diethyl ether (1, 5, 10, 20, and 50%) in hexane. The resulting esters (2 mg) were recovered from the 5% ether-hexane fraction.

Syntheses of Standard Esters 1-Hexanol (1.0 g, 10.0 mmol) and lauric acid (100 mg, 0.5 mmol) were heated at 80°C for 1 h with a drop of sulfuric acid as a catalyst. After cooling to room temperature, the residual oil was chromatographed over SiO_2 (10 g, hexane:ethyl acetate, 10:1) to give *n*-hexyl laurate (compound **7**) (114 mg, 80%) as a colorless oil. GC-MS t_R: 18.52 min. ^{1}H NMR ($CDCl_3$, δppm): 0.88 (t, 3H, *J*= 6.6 Hz, CH_3), 0.90 (t, 3H, *J*=6.8 Hz, CH_3),1.26-1.36 (m, 22H, CH_2), 1.58-1.63 (m, 4H, CH_2), 2.29 (t, *J*=7.6 Hz, 2H, CH_2CO), 4.06 (t, *J*=6.6 Hz, 2H, CH_2O); ^{13}C NMR ($CDCl_3$, δppm): 174.0, 64.4, 34.4, 31.9, 31.4, 29.6 (x 2), 29.5, 29.3 (x 2), 29.2, 28.6, 25,6, 25.0, 22.7, 22.5, 14.1, 14.0.

Syntheses of the remaining 14 esters were achieved using the same procedure employed in the synthesis of *n*-hexyl laurate (compound **7**) via the reaction between the corresponding carboxylic acids and alcohols (see Table 2). The esters synthesized using this method included *n*-hexyl caprate (compound **1**), *n*-butyl laurate (compound **2**), *n*-hexyl *n*-undecanoate (compound **3**), *n*-pentyl laurate (compound **4**), *n*-pentyl 11-methyldodecanoate (compound **6**), *n*-hexyl laurate (compound **7**), *n*-hexyl 11-methyldodecanoate (compound **8**), *n*-heptyl laurate (compound **9**), *n*-hexyl *n*-tridecanoate (compound **10**), *n*-hexyl 12-methyltridecanoate (compound **11**), *n*-octyl laurate (compound **12**), *n*-hexyl myristate (compound **13**), *n*-hexyl 13-methyltetradecanoate (compound **14**), and *n*-butyl 12-methyltetradecanoate (compound **15**). The preparation of *n*-hexyl 10-methylundecanoate (compound **5**) by the same synthetic procedure was not possible as 10-methylundecanoic acid was not commercially available. The ester products were subsequently identified by GC-MS analysis without silica gel column purification.

Results

The prominent feature from the GC analyses of the millipede extract was the presence of fifteen compounds (compounds **1**–**15**), with multiple minor compounds recorded in the less-volatile range (t_R>16 min by GC-MS, 55.4% of total compound responses), together with seven compounds (compounds A–G) recorded in the volatile range (t_R< 13 min by GC-MS, 44.6% of total compound responses) (Fig. 1). No differences were observed in profiles of components between sexes, nor between the whole body extracts and the defensive secretions. The extracts from

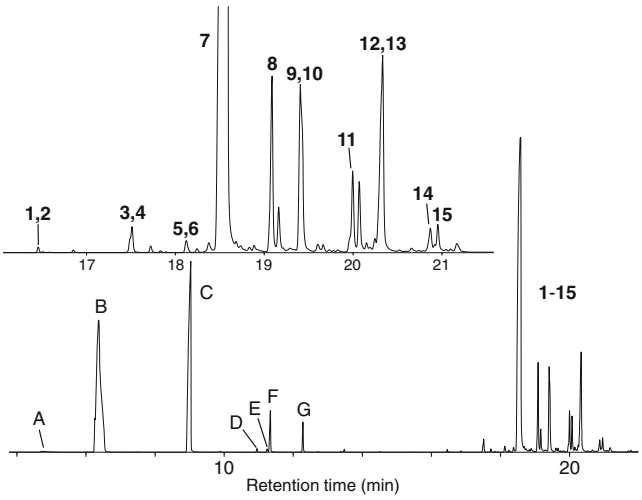


Fig. 1 Typical gas chromatogram of a hexane extract from *Anaulaciulus* sp.: A, benzoquinone; B, 2-methyl-1,4-benzoquinone; C, 2-methoxy-3-methyl-1,4-benzoquinone; D, 2,3-dimethoxy-1,4-benzoquinone; E, unknown; F, 2-methoxy-6-methyl-1,4-benzoquinone; G, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (see the text for details of compounds **1**–**15**)

frozen and decapitated millipedes did not contain any compounds (data not shown), indicating negligible hexane extractable components are present on the body surface. All components from the conventional hexane extract, therefore, represented the secretory compounds derived from the repugnatorial gland.

Compounds **1**–**15** eluting by GC in the less-volatile range were recovered from the 5% ether-hexane fraction using a SiO_2 column, which is indicative of esters. Compound **7** (t_R 18.52 min) was the most abundant product (64.7% of the ester fraction), was identified by NMR analysis with a mixture of minor esters also present. ^{1}H NMR spectrum of the compound **7** indicated two ω-methyls at δ_H 0.88 ppm (t, 6.6 Hz) and 0.90 ppm (t, 6.8 Hz), an *O*-substituted methylene at δ 4.06 ppm (t, 6.6 Hz), and a carbonyl-substituted methylene at δ 2.29 ppm (t, 7.6 Hz), together with multiplet methylenes recorded around δ 1.3 and 1.6 ppm. ^{13}C NMR spectrum gave a carbonyl carbon at δ_C 174.0 ppm and *O*-substituted carbon at δ 64.4 ppm, with a total of 14 methylene carbons recorded within δ 20–40 ppm. Two ω-methyls appeared at δ 14.0 and 14.1 ppm. Mass spectral analysis of compound **7** showed ions at *m/z* 284 (M^+) with the base ion at *m/z* 201 and a diagnostic ion at *m/z* 84. The base ion at *m/z* 201 was indicative of a fragment derived from a C_{12}-fatty acid moiety. The diagnostic ion recorded at *m/z* 84 was the second most intense ion, and was concluded to be derived from a C_6-alcohol moiety. The NMR spectra and GC-MS data of compound **7** were identical to those of synthetic *n*-hexyl laurate.

The GC-MS spectral data recorded for fifteen esters (compounds **1**–**15**) are listed in Table 1. Esters containing a C_6 alcohol moiety showed a fragment ion of *m/z* 84 as the base ion or as the second largest ion (compounds **1**, **3**, **5**, **7**, **8**, **10**, **11**, **13**, **14**, and **15**, underlined in Table 1). Similarly, *m/z* 56 (compound **2**), *m/z* 70 (compounds **4** and **6**), *m/z* 98 (compound **9**) and *m/z* 112 (compound **12**) corresponded to C_4-, C_5-, C_7- and C_8-alcohol moieties, respectively, as underlined in Table 1. The base ion of compound **7** recorded at *m/z* 201 was assigned as a C_{12} fatty acid, as previously mentioned (compounds **2**, **4**, **5**, **9**, and **12**, dashed-underlined in Table 1). Ions recorded from the mass spectra (Table 1; dashed-underlined) at *m/z* 173 (compound **1**), *m/z* 187 (compound **3**), *m/z* 215 (compounds **6**, **8**, and **10**), *m/z* 229 (compounds **11** and **13**), and *m/z* 243 (compounds **14** and **15**) suggest the presence of C_{10}-, C_{11}-, C_{13}-, C_{14}- and C_{15}-fatty acid moieties, respectively. The results from the MS analyses suggest that all recorded compounds (**1**–**15**) are esters derived from C_{10}-C_{15} saturated fatty acids and C_4-C_8 saturated alcohols, as summarized in Table 2. Consequently, compound **1** (t_R 16.46 min) was assigned as *n*-hexyl caprate, **2** (t_R 16.51 min) as *n*-butyl laurate, **3** (t_R 17.49 min) as *n*-hexyl *n*-undecanoate, **4** (t_R 17.52 min) as *n*-pentyl laurate, **7** (t_R 18.52 min) as *n*-hexyl laurate, **9** (t_R 19.40 min) as *n*-heptyl laurate, **10** (t_R 19.43 min) as *n*-hexyl *n*-tridecanoate, **12** (t_R 20.28 min) as *n*-octyl laurate, and **13** (t_R 20.33 min) as *n*-hexyl myristate.

Compound **5** (t_R 18.13 min) and compound **7** (t_R 18.52 min) showed the same M^+ ion at *m/z* 284, with a common alcohol moiety fragment recorded at *m/z* 84; these findings are suggestive of the isomeric relationship between the two compounds, indicating that compound **5** is *n*-hexyl isododecanoate on the basis of shorter GC t_R. Similarly, compound **8** (t_R 19.09 min, *m/z* 298 (M^+), with the base ion at *m/z* 215, and the alcohol moiety at *m/z* 84) is elucidated as *n*-hexyl isotridecanoate, an isomer to *n*-hexyl *n*-tridecanoate (compound **10**, t_R 19.43 min). Compound **11** (t_R 20.00 min, *m/z* 312 (M^+), with the base ion at *m/z* 229, and the alcohol moiety at *m/z* 84) is *n*-hexyl isotetradecanoate, an isomer to *n*-hexyl myristate (compound **13**, t_R 20.33 min). Mass fragmentation patterns recorded for compound **14** (t_R 20.87 min, *m/z* 326 (M^+), with the second largest ion at *m/z* 243, and the base ion and the alcohol moiety at *m/z* 84); and for compound **15** (t_R 20.95 min, *m/z* 326 (M^+), with the second largest ion at *m/z* 243, and the base ion and the alcohol moiety at *m/z* 84) were similar to that of *n*-hexyl *n*-pentadecanoate. However, the GC t_R for compound **14** and **15** were less than the calculated value (t_R ca. 21.2 min), based on comparison of the t_R observed from compound **3** (t_R 17.49 min, *n*-hexyl *n*-undecanoate), compound **7** (t_R 18.52 min, *n*-hexyl laurate), compound **10** (t_R 19.43 min, *n*-hexyl *n*-tridecanoate), and compound **13** (t_R 20.33 min, *n*-hexyl myristate). Therefore, compound **14** (t_R 20.87 min) was assigned as *n*-hexyl isopentadecanoate, and compound **15** (t_R 20.95 min) as *n*-hexyl anteisopentadecanoate. Compound **6** was assigned as *n*-pentyl isotridecanoate, based on

Table 1 GC-MS spectrum of ester from *Anaulaciulus* sp.

Peak	Retention time (min)	Diagnostic ions *m/z* (relative intensity %)
1	16.46	43(59), 56(43), 61(31), 69(27), 84(84), 115(6), 129(19), 155(41), 173(100), 213(3), 256(M^+,2)
2	16.51	41(36), 56(100), 73(30), 101(13), 116(12), 129(21), 157(11), 183(50), 201(87), 227(1), 256(M^+,6)
3	17.49	43(64), 56(43), 61(36), 69(30), 84(91), 115(6), 129(14), 169(39), 187(100), 227(1), 270(M^+,3)
4	17.52	43(44), 55(27), 70(100), 115(13), 129(13), 157(9), 183(36), 201(88), 227(1), 270(M^+,4)
5	18.13	43(64), 56(54), 61(17), 69(28), 84(100), 129(10), 157(9), 183(23), 201(81), 241(4), 284(M^+,4)
6	18.14	43(54), 55(31), 70(100), 97(14), 115(13), 129(14), 171(16), 197(21), 215(85), 241(4), 284(M^+,6)
7	18.52	43(33), 56(23), 61(18), 69(17), 84(58), 129(12), 157(11), 183(34), 201(100), 241(1), 284(M^+,6)
8	19.09	43(63), 56(41), 61(34), 69(31), 84(89), 97(16), 129(20), 157(7), 171(19), 197(20), 215(100), 255(3), 298(M^+,7)
9	19.40	43(34), 57(62), 61(31), 70(39), 83(16), 98(79), 129(11), 157(9), 183(30), 201(100), 227(1), 255(1), 298(M^+,4)
10	19.43	43(49), 56(34), 61(32), 69(25), 84(79), 98(14), 129(17), 171(11), 197(33), 215(100), 255(1), 298(M^+,7)
11	20.00	43(63), 56(36), 61(36), 69(29), 84(89), 129(18), 185(26), 211(16), 229(100), 269(4), 312(M^+,6)
12	20.28	43(60), 57(67), 61(26), 69(45), 83(44), 112(69), 129(11), 157(14), 183(43), 201(100), 281(3), 312(M^+,6)
13	20.33	43(51), 55(33), 61(31), 69(31), 84(80), 110(14), 129(18), 157(7), 185(11), 211(30), 229(100), 269(2), 312(M^+,9)
14	20.87	43(84), 57(47), 61(44), 69(43), 84(100), 110(16), 129(19), 157(10), 199(13), 225(13), 243(94), 283(2), 326(M^+,10)
15	20.95	43(65), 57(57), 61(41), 69(36), 84(100), 97(19), 129(18), 157(10), 185(24), 225(14), 243(93), 269(5), 283(3), 326(M^+,9)

Bold are the numbers of "peak"; underline; a fragment ion from alcohol moiety; dashed-underline; a fragment ion from fatty acid moiety.

the observation of the GC t_R of compound **6** (t_R 18.14 min, ion at *m/z* 284 (M^+), with the second largest ion at *m/z* 215, and the base ion and the alcohol moiety at *m/z* 70) being less than the calculated value (t_R ca. 18.55 min), which was determined by comparing the value expected for the corresponding ester between *n*-tridecanoic acid and *n*-pentyl alcohol.

The elucidated structures for compound **1** to **15**, excluding compound **5**, were verified by comparisons to synthetic standards prepared via acid-catalyzed esterifications between the corresponding fatty acids and alcohols. The compounds synthesized by this method exhibited identical GC t_R and mass spectra values to those of the corresponding natural products.

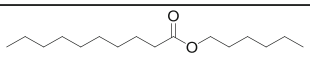
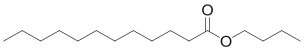
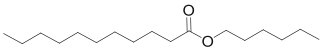
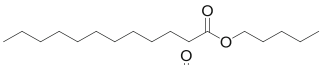
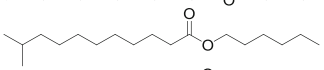
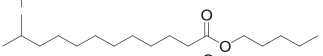
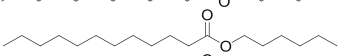
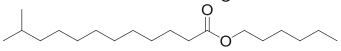
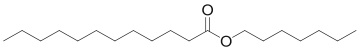
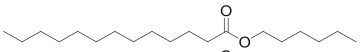
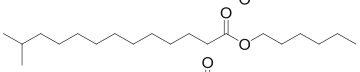
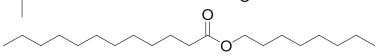
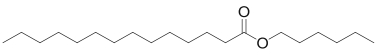
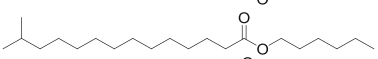
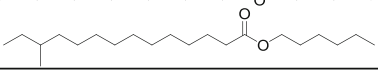
Compounds A-G were identified as benzoquinone derivatives. The GC-MS analyses of these compounds by co-injection with authentic samples (Sigma-Aldrich Co., Ltd., Tokyo, Japan) revealed that compound A (t_R 4.89 min, *m/z* 108 (M^+, base ion)), B (t_R 6.35 min, *m/z* 122 (M^+, base ion)), and G (t_R 12.28 min, *m/z* 182 (M^+, 66%) and the base ion at *m/z* 137) corresponded to 1,4-benzoquinone, 2-methyl-1,4-benzoquinone, and 2,3-dimthoxy-5-methyl-1,4-benzoquinone, respectively. Compound C (t_R 9.00 min, *m/z* 152 (M^+), base ion) was identified as 2-methoxy-3-methyl-1,4-benzoquinone. Compound D (t_R 10.95 min, *m/z* 168 (M^+, 76%) and the base ion at *m/z* 123) was identified as 2,3-dimethoxy-1,4-benzoquinone (Kuwahara et al., 2002). Compound E (t_R 11.25 min, *m/z* 152 (M^+, 36%) and the base ion at *m/z* 124) is suggestive of a di- or tri-substituted 1,4-benzoquinone. Compound F (t_R 11.33 min) also gave the same M^+ ion as compound E, at *m/z* 152 (53%) with the base ion at *m/z* 69, and the fragmentation pattern was virtually identical to that of 2-methoxy-6-methyl-1,4-benzoquinone derived from SDBS mass spectrum (National Institute of Advanced Industrial Science and Technology, 2010).

Discussion

In the julid millipede, *Anaulaciulus* sp. that we analyzed, there were no differences recognized in the composition of the conventional hexane extract from whole body and of the defensive secretion directly sampled by a glass capillary tube, indicating no significant additional hexane extractable compounds are present on the body surface. This is the first report of $C_{16\text{-}21}$ aliphatic esters in the defensive secretion of a julid millipede.

n-Hexyl laurate (compound **7**) was the major component of the millipede secretions. In the present study ca. 95.5% of total esters were identified; of these, 76.7% were *n*-hexyl esters. The remaining 4.1% of unknown esters are probably saturated branched-chain and monoenoic fatty acid esters.

Table 2 Constituent carboxylic acid and alcohol, and structures of fifteen esters from *Anaulaciulus* sp.

peak	carboxylic acid	alcohol	structure
1	*n*-C_9COOH	*n*-C_6OH	
2	*n*-C_{11}COOH	*n*-C_4OH	
3	*n*-C_{10}COOH	*n*-C_6OH	
4	*n*-C_{11}COOH	*n*-C_5OH	
5*	iso-C_{11}COOH	*n*-C_6OH	
6	iso-C_{12}COOH	*n*-C_5OH	
7	*n*-C_{11}COOH	*n*-C_6OH	
8	iso-C_{12}COOH	*n*-C_6OH	
9	*n*-C_{11}COOH	*n*-C_7OH	
10	*n*-C_{12}COOH	*n*-C_6OH	
11	iso-C_{13}COOH	*n*-C_6OH	
12	*n*-C_{11}COOH	*n*-C_8OH	
13	*n*-C_{13}COOH	*n*-C_6OH	
14	iso-C_{14}COOH	*n*-C_6OH	
15	anteiso-C_{14}COOH	*n*-C_6OH	

* Putative structure from mass spectrum and GC retention time

The identified branched iso- and anteiso-fatty esters (compounds **5**, **6**, **8**, **11**, **14**, and **15**) are novel products, while the odd-numbered C_{11}-fatty acid ester (compound **3**), C_{13}-fatty acid ester (compound **10**), and C_7-fatty alcohol ester (compound **9**) had not been previously described as natural products. Other major components of the millipede secretions were 2-methyl-1,4-benzoquinone (compound B) and 2-methoxy-3-methyl-1,4-benzoquinone (compound C), which are known as the most common defensive compounds in quinone-producing millipedes. In addition, one hitherto undescribed compound, 2-methoxy-6-methyl-1,4-benzoquinone (compound F), was characterized from the defensive secretion.

Julid species reportedly produce various 1,4-benzoquinones (Behal and Phisalix, 1900; Weatherston and Percy, 1969; Röper and Heyns, 1977; Eisner et al., 1978). Among them, the defensive secretion of a millipede, *Uroblaniulus canadensis* (Say), was shown to consist of a mixture of benzoquinones and unidentified aliphatic compounds (Weatherston and Percy, 1969). In quinone-producing spirobolid species, an aliphatic compound from *Rhinocricus insulatus* has been characterized as *trans*-2-dodecenal (Wheeler et al., 1964), while uncharacterized components have been reported for two species, *Narceus annularis* and *Floridobolus penneri* (Monro et al., 1962). The spirobolid millipede species, *Rhinocricus padbergi*, produces long-chain hydrocarbons C_{21}-C_{30} together with benzoquinones and alkaloids (Arab et al., 2003), but the glandular origin of the aliphatic compounds are uncertain.

Most arthropod defensive secretions that employ benzoquinones as major components also contain liquid hydrocarbons such as pentadecane and (*Z*,*Z*)-7,9-heptadecadiene, which may serve as solvents for the irritating quinones (Eisner et al., 2000). These hydrocarbons are effective carriers of benzoquinones, and surfactants promoting spread of secretion over the body following glandular discharge. In tests with insects, both hydrocarbons and benzoquinones proved to be repellent and irritating (Peschke and Eisner, 1987). Thus, the combination of benzoquinones and esters could be a more effective millipede defense than either type of compound alone. Excluding the new esters described hitherto, the remaining esters identified have been generally reported as chemical components of many cosmetics. In bioactivity studies, the honeybee *Apis mellifera* has been reported as producing the compounds associated with compounds **1**, **7**, **12**, and **13**, which are utilized in the queen fecal pheromones involved in nestmate recognition (Breed et al., 1992). Recently, the two laurates described as compounds **7** and **9** were evaluated for *in vitro* antimicrobial activity against the panel of Gram positive and Gram negative bacterial and fungal strains in quantitative structure activity relationship (QSAR) studies (Sarova et al., 2011). *n*-Hexyl

myristate, described as compound **13**, has been found to function as a species recognition signal and is reported to possess low-to-moderate aphrodisiac activity in two sulfur butterflies: *Colias eurytheme* and *C. philodice* (Grula and Taylor, 1979; Grula et al., 1980).

In summary, we demonstrated the presence of aliphatic esters, mainly composed of *n*-hexyl laurate, in the defensive secretion of a julid millipede, *Anaulaciulus* sp. These esters are discharged as a mixture of benzoquinones in response to disturbance. The composition and amount of newly identified esters was not influenced by seasonal change (data not shown). Furthermore, *Anaulaciulus* sp. extracts collected in Kameoka-shi, Kyoto prefecture, were identical to those collected from the three other Kansai regions (i.e. Kyoto-shi, Osaka, and Wakayama prefectures; data not shown). Further studies are necessary in order to elucidate ester profile differences in other julid species that may have chemotaxonomic value.

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The Absolute Configuration of Chrysomelidial: A Widely Distributed Defensive Component Among Oribotririid Mites (Acari: Oribatida)

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Abstract The absolute configuration of the iridoid monoterpene chrysomelidial from the oribatid mite, *Austrotritia dentate* Aoki, was elucidated by the GC-MS and GC comparisons with four synthetic stereoisomers of this well-known natural product. This identification was made possible by asymmetric synthesis of the known alcohol, (5*S*,8*S*)-chrysomelidiol. The GC retention time of diol derived from the natural oribatid dial agreed with that of the synthetic (5*S*,8*S*)-chrysomelidiol, confirming that the absolute configurations at C5 and C8 positions of the natural chrysomelidial are both *S*. Chrysomelidial was detected as a single or a major component in nine oribatid mites examined; thus, this compound is considered to be commonly distributed in Oribotririidae where it serves a defensive role.

Keywords (5*S*,8*S*)-Chrysomelidial · Absolute configuration · Allomone · Oribotririidae · Oribatida · Acari

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Introduction

The iridoid monoterpene, 2-methyl-5-(1-formylethyl)-1-cyclopentene-1-carbaldehyde (**1**) was first isolated from a chrysomelid beetle, *Plagiodera versicolora* (Meinwald et al., 1977); phytophagous leaf beetle larvae of the subfamily Chrysomelinae utilize the compound defensively (Blum et al., 1978; Sugawara et al., 1979a, b; Pasteels et al., 1982). This compound has also been discovered in glandular secretions from the oribatid mite, *Oribotritia berlesei* (Raspotnig et al., 2008). Iridoid monoterpene **1** possesses four possible stereoisomers known as chrysomelidial [(5*S*,8*S*)-**1** and (5*S*,8*R*)-**1**)] and dehydroiridodial [(5*R*,8*R*)-**1** and (5*R*,8*S*)-**1**)] (Fig. 1). In 1978, Meinwald and Jones (1978) reported the synthesis of two diastereomeric mixtures of chrysomelidial [(5*S*,8*S*)-**1** and (5*S*,8*R*)-**1**)] starting from (*R*)-limonene. Comparing the spectral data from the natural product **1** extracted from *P. versicolora* with the synthetic equivalent they concluded that the structure of the natural product was either (5*S*,8*S*)-**1** or (5*R*,8*R*)-**1**. Two diastereomeric mixtures of plagiolactone (Fig. 1), isolated along with **1** from *P. versicolora*, were also prepared from the same (*R*)-limonene precursor. As the optical rotatory dispersion curve of the synthetic plagiolactone exhibits a negative Cotton effect similar to the natural plagiolactone, the two chiral centers (4 and 4a) of plagiolactone were both determined to be *S* configurations (Fig. 1). Based on its biosynthetic aspects, Meinwald and Jones (1978) speculated that the absolute configuration of the natural product **1** is the same as that of plagiolactone. According to the studies described above, the natural product **1**, found from chrysomelid beetle larvae, rove beetles (Pasteels et al., 1982; Weibel et al., 2001), and oribatid mites (*O. berlesei*) (Raspotnig et al., 2008), has been regarded as (5*S*,8*S*)-**1** or a mixture of (5*S*,8*S*)-**1** and

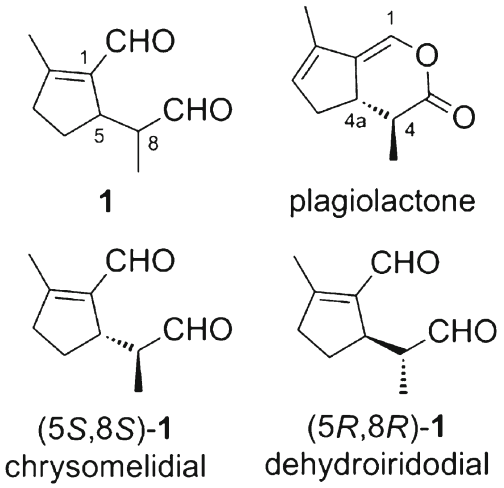


Fig. 1 Structures of **1** and plagiolactone

(5*S*,8*R*)-**1**. To the best of our knowledge, there are no previous reports available on the absolute stereochemistry.

Results from the study described herein reveal that the secretions extracted from *Austrotritia dentata* Aoki, of the Mixonomata, (one of the four glandulate cohorts of Oribatida) is composed mainly of **1**. The relative structure of natural **1** was determined by gas chromatographic (GC) retention time and mass spectral matches with those of one of the two peaks in a synthetic standard composed of an approximately equal mixture of all four stereoisomers of this compound. The absolute configuration of natural **1** was established via asymmetric synthesis of the known alcohol, (5*S*,8*S*)-chrysomelidiol (**9**), and subsequent GC retention time matches on a chiral column with diol **9** derived from the natural dial **1**. Further investigations were also conducted on the secretory components of the other eight Oribotririidae species present in Japan.

Materials and Methods

General Procedures All moisture-sensitive reactions were performed within a nitrogen atmosphere in oven-dried glassware. Reaction flasks were fitted with rubber septa for the introduction of substrates and reagents via standard syringe techniques. Solvents were dried and purified by conventional methods prior to use. All chemicals used were of reagent grade. Column chromatography was performed on Wakosil silica gel C-200 with the specified solvents. ^{1}H NMR spectra (400 MHz, TMS at δ=0.00 as internal standard) and ^{13}C NMR spectra (100 MHz, $CDCl_3$ at δ=77.0 as internal standard) were recorded on a Bruker Biospin AC400M spectrometer. NMR data are reported as follows: chemical shifts in parts per million, multiplicity, coupling constants, and number of protons. Optical rotations were taken on a SEPA-300 high-sensitive polarimeter (HORIBA). Gas chromatography–mass spectrometry (GC-MS) analysis was conducted using a Network GC System (6890N; Agilent Technologies Inc. or HP-5890 series II plus; Hewlett Packard Co.) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies Inc. or HP-5989B; Hewlett Packard Co.) operated at 70 eV using an HP-5MS capillary column (0.25 mm i.d. × 29 m, 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as the carrier gas at a flow rate of 1.00 ml/min, with a split-less mode at a temperature programmed to change from 60°C (2 min) to 290°C at a rate of 10°C/min. The temperature was maintained at 290°C for 5 min. GC was performed with an Agilent Technologies 6890N instrument equipped with a flame ionization detector using an InertCap CHIRAMIX capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness; GL Sciences Inc.). Helium was used as the carrier gas at a flow rate of 1.00 ml/min, with a split-less mode at a temperature programmed to change from 50°C to 180°C at a rate of 3°C/min. The temperature was then maintained at 180°C for 30 min.

Mites and Mite Extracts Soil samples were collected from various locations in Japan (Table 1). Representatives of nine species of Oribotririidae mites were extracted along with various other organisms using Berlese funnels. All mite species in Table 1 were identified by co-author Shimano, Oribatid taxonomist. Adult mites were analyzed within a week after their collection. The opisthonotal gland secretions were obtained by submerging individual mites in 3 μl of hexane for 3 min with the extraction solution subsequently analyzed by GC-MS. In order to determine the absolute configuration of the natural product **1**, an extract was prepared from five *A. dentata* adults by dipping in hexane (5 μl) for 3 min.

Chemical Identification Secretory components were identified by comparison of mass spectra, GC retention times, and co-injection with authentic samples. Authentic samples of saturated hydrocarbons, neral, and geranial were commercially available (Sigma-Aldrich Co., Ltd., Tokyo, Japan).

*2-Methyl-5-(1-formylethyl)-1-cyclopentene-1-carbaldehyde (**1**)* According to the procedure previously reported, citral (a mixture of geranial and neral) **2** (1.52 g, 10.0 mmol) was converted to dial **3** (747 mg) in 45% yield (Santangelo et al., 2001). A solution of 0.01 M NaOH (MeOH:H_2O= 98:2, 10 ml) was added to dial **3** (500 mg, 3.01 mmol) at room temperature. The reaction mixture was stirred for 10 h and poured into water. The solution was extracted with EtOAc, the organic layer was washed with 2N HCl and brine, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent *in vacuo* afforded four stereoisomers of **1** (390 mg, 78%), which were chromatographed using a silica

Table 1 Oribotririidae species collection sites and descriptions in Japan

Mite collection			
Taxon	Prefecture	Locality	Site
Austrotritia dentata	Kyoto	Kameoka-shi, Kyoto Gakuen University	soil and litter in Japanese ceder (*Cryptomeria japonica*) forest
Austrotritia ishigakiensis	Kyoto	Kyoto-shi, Kyoto University	soil, litter and woodchips under *Metasequoia glyptostroboides*
Austrotritia sp.	Kyoto	Kyoto-shi, Kyoto University	soil, litter and woodchips under *Metasequoia glyptostroboides*
Mesotritia okuyamai	Kagoshima	Soh-gun, Kihoku-tyo	soil and litter in unidentified forest
Oribotritia chichijimensis	Hiroshima	Mihara-shi, Minato-machi	soil and litter in secondary *Quercus* forest
Oribotritia sp.1	Hokkaido	Nakagawa-gun, Otoikoneppu-mura	soil and litter in *Quercus* and *Abies* forest with *Sasa sylvatica*
Oribotritia sp.2	Hokkaido	Sapporo-shi, Hokkaido University	soil and litter in deciduous *Ulmus japonica* forest
Oribotritia sp.3	Toyama	Toyama-shi, Hamakurosaki-kaigan	soil and litter in *Pinus thunbergii* forest at seashore
Sabacarus japonicus	Miyazaki	Higashimorokata-gun, Aya-tyo	soil and litter in *Castanopsis* forest

gel column [10:1-6:1 benzene/EtOAc] to separate two pairs of enantiomers, A [(5*S*,8*S*)-**1** and (5*R*,8*R*)-**1**] and B [(5*S*,8*R*)-**1** and (5*R*,8*S*)-**1**]. Racemic A was more polar than racemic B.

Racemic A: GC-MS t_R: 12.28 min. MS *m/z* (%): 166 (M^+, 5), 148 (32), 138 (14), 133 (7), 123 (9), 109 (52), 108 (30), 105 (32), 91 (23), 81 (100), 79 (55), 77 (27), 67 (14), 53 (18), 41 (16). ^{1}H NMR ($CDCl_3$, ppm): 0.90 (d, *J*=6.8 Hz, 3H), 1.53 (m, 1H), 1.96 (m, 1H), 2.19 (s, 3H), 2.54 (m, 2H), 3.12 (ddd, *J*=6.80, 3.20 and 0.4 Hz, 1H), 3.68 (m, 1H), 9.71 (d, *J*=0.4 Hz, 1H), 10.02 (s, 1H). ^{13}C NMR ($CDCl_3$, ppm): 7.7, 14.4, 23.0, 40.1, 42.8, 48.1, 137.4, 165.1, 187.9, 204.3.

Racemic B: GC-MS t_R: 12.26 min. MS *m/z* (%): 166 (M^+, 3), 148 (16), 138 (21), 133 (6), 123 (11), 109 (60), 108 (51), 105 (23), 91 (19), 81 (100), 79 (51), 77 (24), 67 (14), 53 (17), 41 (14). ^{1}H NMR ($CDCl_3$, ppm): 1.02 (d, *J*=7.2 Hz, 3H), 1.65 (m, 1H), 2.12 (m, 1H), 2.17 (s, 3H), 2.54 (m, 2H), 2.83 (ddd, *J*=7.20, 3.60 and 0.4 Hz, 1H), 3.43 (m, 1H), 9.68 (d, *J*=0.8 Hz, 1H), 10.02 (s, 1H). ^{13}C NMR ($CDCl_3$, ppm): 10.8, 14.6, 25.5, 39.4, 45.4, 48.2, 137.7, 165.3, 188.1, 204.7.

*Methyl (2S,2'S)-2-[1'-(tert-butoxy)-1'-oxopropan-2'-yl]-5-oxocyclopentane-carboxylate (***6***)* A solution of (*R*,*R*)-2,2'-isopropylidene-bis(4-phenyl-2-oxazoline) (27 mg, 0.08 mmol) in CH_2Cl_2 (1 ml) was added to a solution of $Cu(OTf)_2$ (29 mg, 0.08 mmol) in CH_2Cl_2 (2 ml). The reaction mixture was stirred at room temperature for 2 h and the solvent evaporated. Toluene (5 ml) was added to the bis(oxazoline)-Cu(II) ligand and the mixture cooled to −78°C. A solution of ketoester **4** (108 mg, 0.77 mmol) and silylketene acetal **5** (171 mg, 0.70 mmol) in toluene (2 ml) was added to the mixture and the resulting solution was stirred at −78°C for 2 h (Bernardi et al., 1996; Bernardi et al., 1997). Water was then added to the solution and extracted with EtOAc. The organic layer was washed with water and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent yielded a residual oil that upon purification by silica gel column chromatography [3:1 hexane/EtOAc] afforded **6** (85 mg, 45%). $[\alpha]_D^{20}$ +44.4 (*c* 1.0 $CHCl_3$); MS *m/z* (%): 197 ([M−O*t*-Bu]$^+$, 3), 156 (31), 139 (31), 111 (30), 99 (15), 97 (20), 83 (53), 57 (100), 41 (30); ^{1}H NMR ($CDCl_3$, ppm): 1.17 (d, *J*=6.8 Hz, 3H), 1.44 (s, 9H), 2.22 (m, 1H), 2.31-2.49 (m, 4H), 2.90 (ddt, *J*=11.2, 11.2 and 7.2 Hz, 1H), 3.12 (d, *J*=11.2 Hz, 1H), 3.75 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm): 14.6, 25.0, 28.0, 38.3, 43.2, 44.0, 52.5, 59.2, 80.9, 170.0, 174.0, 211.3.

*Methyl (2'S,5S)-5-[1'-(tert-butoxy)-1'-oxopropan-2'-yl]-2- [(diethoxyphosphoryl)oxy)]cyclopeant-1-enecarboxylate (***7***)* Sodium hydride (50 mg of a ca. 60% suspension in mineral oil, 1.25 mmol) was covered with Et_2O (1 ml), and a solution of keto diester **6** (48 mg, 0.18 mmol) in Et_2O (1 ml) was added to the suspension at 0°C. After stirring the mixture for 30 min, a solution of diethyl phosphorochloridate (215 mg, 1.25 mmol) in Et_2O (1 ml) was added at 0°C. The resulting mixture was stirred for 30 min, quenched with water, and extracted with EtOAc. The organic layer was successively washed with saturated NH_4Cl and brine. After drying over anhydrous Na_2SO_4, the solvent was removed *in vacuo*, leaving an oil that upon purification by silica gel column [1:1 hexane/EtOAc] afforded **7** (88 mg, 83%). $[\alpha]_D^{20}$ −12.0 (*c* 1.0 $CHCl_3$); MS *m/z* (%): 406 (M^+, <1), 350 (29), 333 (24), 318 (24), 272 (63), 245 (100), 217 (43), 189 (46), 109 (19); ^{1}H NMR ($CDCl_3$, ppm): 0.95 (d, *J*=7.2 Hz, 3H), 1.38 (t, *J*=7.2 Hz, 6H), 1.45 (s, 9H), 1.75 (m, 1H), 1.99 (m, 1H), 2.73 (br m, 2H), 3.03 (ddd, *J*=14.4, 7.2 and 3.6 Hz, 1H), 3.54 (br m, 1H), 3.74 (s, 3H), 4.23 (q, *J*=7.2 Hz, 4H); ^{13}C NMR ($CDCl_3$, ppm): 10.0, 16.1, 20.9, 28.1, 32.8, 41.4, 43.3, 51.2, 64.9, 80.2, 115.1, 159.2, 163.9, 174.6.

*Methyl (2'S,5S)-5-[1'-(tert-butoxy)-1'-oxopropan-2'-yl]-2-methylcyclopeant-1-enecarboxylate (***8***)* A solution of 1.0 M MeLi in Et_2O (1.54 ml, 1.54 mmol) was added dropwise to a suspension of CuI (147 mg, 0.77 mmol) in Et_2O (4 ml) at 0°C. After stirring the mixture for 15 min, the resulting clear solution was cooled to −78°C, and a solution

of enol phosphate **7** (57 mg, 0.14 mmol) in Et_2O (1 ml) was added. After stirring the mixture for 30 min at −78°C, followed by stirring for 1.5 h at −50°C, the reaction was quenched at −20°C using saturated NH_4Cl. The product was extracted with Et_2O, and the organic layer successively washed with saturated NH_4Cl and brine. The organic layer was dried over anhydrous Na_2SO_4, and the evaporation of the solvent *in vacuo* yielded the crude diester **8** (30 mg, 80%), which was submitted to the following reduction without purification. MS *m/z* (%): 212 ($[M-(CH_3)_2C=CH_2]^+$, 16), 195 (23), 180 (14), 166 (100), 152 (43), 139 (44), 107 (43), 79 (31), 57(24).

*(5S,8S)-Chrysomelidiol (***9***)* Diester **8** (30 mg, 0.11 mmol) dissolved in Et_2O (1 ml) was slowly added while stirring to an ice-cooled suspension of $LiAlH_4$ (17 mg, 0.44 mmol) in Et_2O (1 ml). The mixture was then stirred for an additional 30 min at room temperature, after which EtOAc and water were carefully added at 0°C. The organic layer was washed with water and brine and dried over anhydrous Na_2SO_4. After evaporation of the solvent *in vacuo*, the resulting oil was chromatographed in a silica gel column [1:10 hexane/EtOAc] to give **9** (10 mg, 52%). $[\alpha]_D^{20}$ +14.0 (*c* 1.0 $CHCl_3$); MS *m/z* (%): 170 (M^+, <1), 152 (23), 137 (15), 121 (37), 111 (41), 93 (100), 79 (29), 67 (16), 55(20); ^{1}H NMR ($CDCl_3$, ppm): 0.74 (d, *J*=6.8 Hz, 3H), 1.58 (m, 1H), 1.72 (d, *J*=0.8 Hz, 3H), 1.79 (m, 1H), 2.02 (m, 1H), 2.26 (m, 2H), 3.08 (br m, 1H), 3.55 (d, *J*=6.8 Hz, 2H), 4.07 (br d, *J*=12.0 Hz, 1H), 4.30 (d, *J*=12.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, ppm): 11.0, 14.0, 21.9, 36.6, 38.0, 46.0, 57.3, 67.1, 135.4, 137.4.

Preparation of diol **9** *from natural* **1** To the hexane extract prepared from five *A. dentata* adults after concentration of solvent, Et_2O (250 μl) was added. $LiAlH_4$ (1 mg) was added to the solution of natural **1** at 0°C, and the reaction mixture kept for 5 min. After quenching the mixture with water, the product was extracted with ether, and the organic layer was washed with brine. The resulting ether layer of the natural **9** was then subjected to the GC-MS (t_R 13.23 min) and the GC analyses.

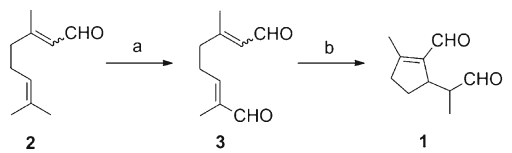
Determination of the stereochemistry of natural **1** The absolute configuration of the natural product **1** was determined after $LiAlH_4$ reduction, via analysis of the GC retention times and co-chromatography, using an InertCap CHIRAMIX capillary column. The racemic mixture of (5*S*,8*S*)-**9** and (5*R*,8*R*)-**9** was obtained from the $LiAlH_4$ reduction of racemic **1** prepared as above. The racemic **9** gave two peaks at t_R 63.23 min and 63.65 min using a chiral GC analysis. Enantiomer (5*S*,8*S*)-**9** was assigned to the peak observed at t_R 63.23 min.

Results and Discussion

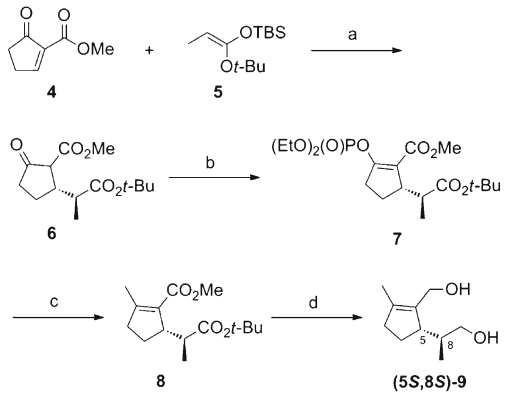
A single predominant compound **1** was obtained from the extraction of the opisthonotal gland secretions from an adult *A. dentata*. Analysis of the mass spectrum of **1**, revealed a base ion peak at *m/z* 81 (100%) with characteristic ions observed at *m/z* 166 (M^+, 5), 148 (32), 138 (14), 133 (7), 123 (9), 109 (52), 108 (30), 91 (23), 79 (55), 67 (14), 53 (18), and 41 (16). The fragmentation pattern of **1** was virtually identical to that of 2-methyl-5-(1-formylethyl)-1-cyclopentene-1-carbaldehyde referenced from the Agilent NIST05 Mass spectral library. In order to determine the relative configuration of **1**, all four stereoisomers of **1** were synthesized as shown in Scheme 1.

The oxidation of citral **2** to 8-oxocitral **3** was achieved using stoichiometric amounts of selenium dioxide (Santangelo et al., 2001). The subsequent cyclization performed under basic conditions afforded the four stereoisomers of **1** (Bellesia et al., 1986). The stereoisomers of **1** were separated into two pairs of enantiomers, A [(5*S*,8*S*)-**1** and (5*R*,8*R*)-**1**] and B [(5*S*,8*R*)-**1** and (5*R*,8*S*)-**1**], using silica gel column chromatography. The relative configurations of the two pairs of enantiomers was elucidated via NMR spectral comparison of the synthetic equivalents in conjunction with spectra reported by Yoshihara et al. (1978) and Enders and Kaiser (1997). Stereoisomers displayed two peaks (t_R) at 12.26 min and 12.28 min when analyzed using GC-MS with an achiral HP-5MS capillary column. The latter peak (t_R) at 12.28 min was assigned to the racemic mixture of A with the retention time and mass fragmentation pattern was consistent with those of the natural product **1**, derived from the *A. dentata* extract.

To elucidate the absolute configuration of **1**, optically active (5*S*,8*S*)-chrysomelidiol (**9**) was synthesized in four steps as summarized in Scheme 2. Successful stereo-control of the C5 and C8 positions of keto diester **6** was achieved via the enantioselective Mukaiyama-Michael reaction between 2-carbomethoxy cyclopentenone **4** and propionate silylketene acetal **5** (Bernardi et al., 1996). We employed (*R*,*R*)-2,2'-isopropylidene-bis(4-phenyl-2-oxazoline) and $Cu(OTf)_2$ to form a chiral bis(oxazoline)-Cu(II) ligand in toluene under several reaction conditions, as reported by Bernardi et al. (1996). Keto diester **6** was then treated with sodium hydride and diethyl phosphorochloridate followed by Me_2CuLi to afford the desired **8** (Kato et al., 1990; Bernardi et al., 1997). Finally, the formation of the corresponding diol **9** was achieved by the reduction of diester **8** with $LiAlH_4$; the spectral data of the resultant diol was identical to previous reports (Bernardi et al., 1997). The



Scheme 1 Reagents and conditions: (a) SeO_2, CH_2Cl_2, 45%; (b) NaOH, MeOH, H_2O, 78%



Scheme 2 Reagents and conditions: (a) (*R*,*R*)-2,2'-isopropylidene-bis(4-phenyl-2-oxazoline), $Cu(OTf)_2$, toluene, 45%; (b) NaH, $ClP(O)(OEt)_2$, Et_2O, 83% (c) MeLi, CuI, Et_2O, 80% (d) $LiAlH_4$, Et_2O, 52%

GC-MS analysis of the synthetic **9** on an achiral HP-5MS capillary column showed a single peak (t_R) at 13.23 min. Reduction of two pairs of enantiomers, A [(5*S*,8*S*)-**1** and (5*R*,8*R*)-**1**] and B [(5*S*,8*R*)-**1** and (5*R*,8*S*)-**1**] with $LiAlH_4$ afforded the corresponding diols, which showed two peaks (t_R) at 13.23 min and 13.09 min, respectively. The retention time of diols from A agreed with that of asymmetrically synthesized **9**.

The enantiomeric excess of the initial Mukaiyama-Michael addition was determined at the stage of diol **9** (ca. 60% ee) by GC analysis using a chiral InertCap CHIRAMIX column. Judging from the specific rotation of **9** ($[\alpha]_D^{20}$ +14.0 (*c* 1.0, $CHCl_3$)), the synthetic **9** was concluded to be (5*S*,8*S*)-chrysomelidiol (Sakai et al., 1980; Bernardi et al., 1997). Referencing the results of Bernardi et al. (1996), we expected the enantioselective Mukaiyama-Michael addition to yield **6** as an *R*-isomer. However, the product **6** yielded from the addition was an *S*-isomer, determined according to the sign of specific rotation of the diol **9**. Although we substituted CH_2Cl_2 for toluene as the solvent in accordance with Bernardi's reports, the adduct **6** was also concluded to be *S*-isomer, which led to (5*S*,8*S*)-chrysomelidiol **9** (33% ee, $[\alpha]_D^{20}$ +10.1 (*c* 1.0, $CHCl_3$)) via an identical synthetic route.

Bernardi et al. (1996) reported that the adduct **6** was transformed to the known mandelate to establish the absolute configuration, although we found no data and descriptions to prove the absolute configuration of the mandelate derivative in any related literature. According to the described reference (Bernardi et al., 1995), the absolute configuration of **6** prepared by enantioselective syntheses using chiral Ti complexes was only assumed by the analogy with that found for the similar compound prepared in the same synthetic method. We deduced that insufficient verification may result in incorrect assignment of the absolute configuration of the adduct **6** in Bernardi et al. (1996). In order to unambiguously establish the absolute configuration, Bernardi et al. (1997) transformed **6** to (5*S*,8*S*)-chrysomelidiol **9** ($[\alpha]_D$ +5.2 (*c* 0.76, $CHCl_3$)). Although the reported diastereo- and enantioselective Mukaiyama-Michael reaction was informative (Bernardi et al., 1996) for determining the stereochemistry of dial **1**, we concluded that the absolute configuration of our synthesized **6** to be *S*-isomer, and not *R*-isomer as stated in Bernardi et al. (1996), in light of more reliable and adequately verified assignments (Sakai et al., 1980; Bernardi et al., 1997).

The absolute configuration of (5*R*,8*S*)-dehydroiridodiol isolated from the cat- and lacewing-attracting plant *Actinidia polygama* Miq. was determined by ex-chiral pool synthesis starting from (*S*)-limonene (Sakai et al., 1980). The assignment of the relative and absolute configuration of (5*R*,8*S*)-diol validated not only based on the polarimetric data, but also the X-ray structure analysis of related compound prepared by MIRC reaction (Enders and Kaiser, 1997). Sakai et al. (1980) also synthesized another single (5*R*,8*R*)-diol ($[\alpha]_D^{27}$ −15.3 (*c* 0.78, $CHCl_3$)), which is the diastereomer of (5*R*,8*S*)-diol, by the same synthetic route. For these reasons, the specific rotation of resulting assigned (5*R*,8*R*)-diol is unambiguous and trustworthy as the decisive factor in determining the absolute configuration of our synthetic (5*S*,8*S*)-**9**.

Gas chromatographic analysis of the racemic diols, (5*S*,8*S*)-**9** and (5*R*,8*R*)-**9**, on a chiral column showed two peaks (t_R) observed to have adequate separation at 63.23 min and 63.65 min (Fig. 2A). The synthetic (5*S*,8*S*)-chrysomelidiol (**9**) (ca. 60% ee) was assigned to the former peak (t_R) at 63.23 min (Fig. 2B). The natural product **1** was extracted from five *A. dentata* adults and reduced to the corresponding diol **9** by $LiAlH_4$. The natural diol **9** was enantiomerically pure, consisting of a single peak that corresponded to the former peak (t_R) at 63.23 min (Fig. 2C). The retention time of **9** from the natural product **1** agreed with that of the former-eluting enantiomer (5*S*,8*S*)-**9** of the synthesized diol. Identity was also confirmed by GC co-chromatography. From the analyses, the absolute configuration of the mite-produced dial **1** was concluded to be *S* at both the C5 and C8 positions.

According to the study by Meinwald and Jones (1978), the natural product **1** extracted from leaf beetle larvae (Chrysomelinae) has been presumed to be (5*S*,8*S*)-chrysomelidial (Sugawara et al., 1979a; Pasteels et al., 1982), although no further evidence was procured that confirms the absolute configuration. Iridoid monoterpene **1** has been recently identified in the opisthonotal gland secretions of the oribatid mite, *O. berlesei* (Raspotnig et al., 2008). However, evidence in the literature is limited, describing only the relative stereochemistry of **1** without the absolute stereochemistry, despite this being the first identification of **1** outside of the Coleoptera. This may be the first study to report that the natural product **1** extracted from the oribatid

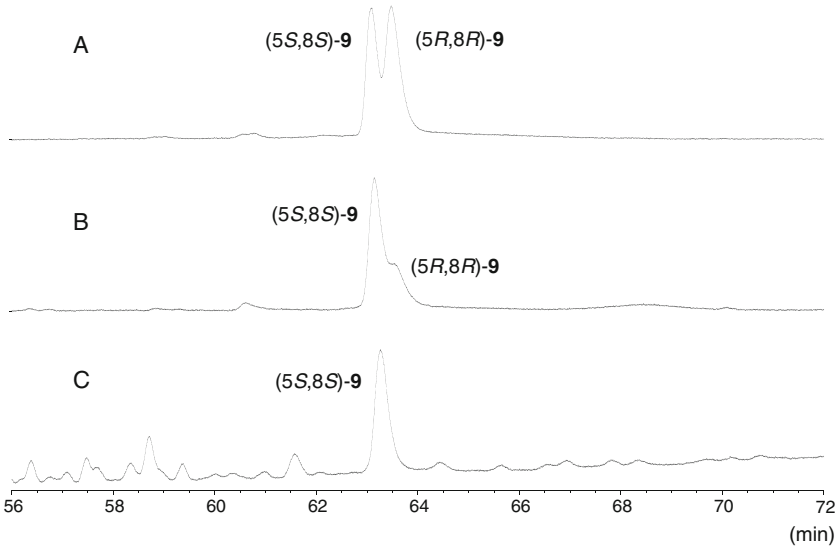


Fig. 2 Gas chromatograms of **a**, a racemic mixture of (5*S*,8*S*)-**9** and (5*R*,8*R*)-**9**, **b**, synthetic diol **9** via the asymmetric synthesis, and **c**, natural **9** derived from the mite-produced dial **1**

mite, *A. dentate,* is (5*S*,8*S*)-chrysomelidial and not (5*R*,8*R*)-dehydroiridodial.

We examined the secretions from nine related adult mite species, including *A. dentata*, all belonging to the family Oribotririidae of Oribatida (Table 2). Chrysomelidial **1** is a common and characteristic secretory component of all species in this family, but is not known to occur in Astigmata or the other family of Oribatida. Recently, the other *Oribotritia* species (*O. banksi*, *O. hermanni*, and *O. storkani*) of the family Oribotririidae was also shown to possess **1** as a single or a mixture of diastereomers (Raspotnig et al., 2011). Stereochemical studies on each of the examined species as well as comparisons between the stereochemistry of Oribotririidae mites and chrysomelid beetle larvae will be the subject of further research. The mass of natural product **1** (average±SD, n=4) extracted from each *A. dentata* adult was calculated at 3.68±1.0 μg. This is approximately 10 to 100-fold greater than the mass of the major secretory components found in species from Astigmata and Oribatida. Common secretory components from the glands of the Oribotririidae family are the M272 and C17 hydrocarbons, although they were not detected in *A. dentata* and *Sabacarus japonicus* (Table 2). The structure of M272 was suggested as the diterpene, (3*E*,6*E*,10*E*)-3,7,11,15-tetramethylhaxadeca-1,3,6,10,14-pentaene, which was evaluated from fragmentation patterns, with mass spectrum analysis showing a molecular ion at *m/z* 272. Interestingly, *A. ishigakiensis* secretes a certain amount of heptadecane instead of heptadecene, the latter a commonly occurring compound among most other species. All four species of the genus *Oribotritia* also secrete several minor compounds, such as some hydrocarbons, neral and geranial.

Table 2 Chemical profiles of Oribotririidae species

Taxon	**1**	M272	C17:0	C17:1	C13:0	C15:0	C19:1	neral	geranial
Austrotritia dentata	+++								
Austrotritia ishigakiensis	+++	+	++	(+)					
Austrotritia sp.	+++	+		(+)					
Mesotritia okuyamai	+++	++		+					
Oribotritia chichijimensis	+++			++	(+)		(+)	+	+
Oribotritia sp.1	+++	+		++		(+)	(+)		
Oribotritia sp.2	+++	+		++	(+)				
Oribotritia sp.3	+++	+		++	(+)		(+)		
Sabacarus japonicus	+++								

relative quantity of mite secretion +++>++>+>(+)

M272, (3*E*,6*E*,10*E*)-3,7,11,15-tetramethylhaxadeca-1,3,6,10,14-pentaene (not identified); C17:0, heptadecane; C17:1, heptadecene; C13:0, tridecane; C15:0, pentadecane; C19:1, nonadecene

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Chemical Ecology of Astigmatid Mites LXXXVII. *S*-(+)-Isopiperitenone: Re-identification of the Alarm Pheromone as the Female Sex Pheromone in *Tyrophagus similis* (Acari: Acaridae)

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Abstract Behavioral analysis revealed that *S*-(+)-isopiperitenone [(*S*)-3-methyl-6-isopropenyl-2-cyclohexen-1-one], previously identified as an alarm pheromone, is also the female sex pheromone of *Tyrophagus similis* (Astigmata: Acaridae), showing maximum male attraction at a dose of 0.1 female equivalent. Although the antipode, *R*-(−)-isopiperitenone, was not detectable in the mite extract, this synthetic optical isomer (80% e.e.) also induced activity at a dose of 100 ng, a response indicative of *S*-(+)-isopiperitenone being the active compound. The average content was determined to be 38.5 ng per female and 19.8 ng per male. This is the first example of an astigmatid mite species possessing a compound that functions as an alarm as well as a sex pheromone.

Keywords Sex pheromone · Alarm pheromone · *S*-(+)-isopiperitenone · *Tyrophagus similis* · (*S*)-3-methyl-6-isopropenyl-2-cyclohexen-1-one · Acaridae

Introduction

Alarm, aggregation, and sex pheromones are distributed among species of Astigmata. The active compounds, including monoterpenes, hydrocarbons, or aromatic compounds, are emitted from a pair of opisthonotal glands as mixtures of many related compounds, for most of which biological functions are unknown (Kuwahara, 2004). Recently, it has been discovered that some species of Astigmata display at least three kinds of pheromone combinations: alarm-aggregation, aggregation-sex, or alarm-sex. For two species of Astigmata, both functions are governed by the same compound, and in four species each function is due to different compounds emitted together in the opisthonotal gland mixture, as summarized in Kuwahara (2004).

Tyrophagus similis (hourensou kenaga-konadani in Japanese) is a pest species in horticulture, causing serious damage to spinach, melon, cucumber, pumpkin, and maize in green houses and fields (Nakao and Kurosa, 1988). This species reproduces via mating between males and females, and mating pairs are observable continually in the stock culture, strongly suggesting the presence of a female sex pheromone in this species. Here, we report a bioassay driven isolation of a female sex pheromone in *Tyrophagus similis*, identified as *S*-(+)-isopiperitenone [(*S*)-3-methyl-6-isopropenyl-2-cyclohexen-1-one], previously identified as the alarm pheromone of this mite (Kuwahara et al., 1987).

Methods and Materials

Mites *Tyrophagus similis* (Astigmata: Acaridae) is a species derived from a spinach field in Chiba Prefecture, Japan. The culture line was established in our laboratory, and has been maintained for several years on dried yeast at 70% relative humidity and 25°C.

Bioassay When encountering receptive females, males exhibit a characteristic foreleg tapping behavior before

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climbing upon the female's abdomen and, after a number of trials, successfully mating. Previously isolated and rested males initiated the same foreleg touches when exposed to a filter paper (1×2 mm) soaked in an extract of females. Based on these observations, an assay method was established where a group of 10 males together with a small amount of dry yeast as food was placed into a small culture dish (10×5 mm) whose bottom had been covered with moistened filter paper. The dishes were covered with a glass plate (18×18 mm) and maintained for 2 h at room temperature. A piece of filter paper (1×2 mm), impregnated with an isolated fraction, or a candidate compound, dissolved in hexane (1 μl) was introduced with minimal disturbance to the culture dish. The subsequent frequency of male foreleg tapping behavior (defined as more than two successive taps towards an individual) was counted for 3 min under a microscope. A new group of males was used for each observation, and each isolated compound was tested in a range of 0.003 to 3 female equivalents and replicated 6 times. Filter paper treated with just hexane was used as a control. Statistical significance was evaluated as indicated.

Instrumental Analyses Electron impact mass spectra (MS) were obtained at 70 eV (ion source temperature, 200°C) in the split-less mode on a Hewlett-Packard (HP) 5989B coupled with a gas chromatograph (GC) HP 5890 Plus, using an HP-5 MS capillary column (0.25 mm id×30 m, 0.25 μm in film thickness). The carrier gas was helium delivered at a constant flow of 1.2 ml/min, and the oven temperature was programmed from 60°C (2 min hold) to 290°C at a rate of 10°C/min. The GC analysis was conducted with a Hewlett Packard 5890 Series II Plus equipped with a flame ionization detector (FID), using identical column and running conditions. To separate *R*-(−)- and *S*-(+)-isopiperitenone, a chiral capillary column of CP-cyclodextrin-B-2,3,6-M-19 (0.25 mm id×25 m, 0.25 μm in film thickness, Chrompack) was used at 110°C with a split mode (100 : 1) of injection, and the optical purity of preparations was determined. Optical rotation was determined by a JASCO DIP-370 polarimeter, using 0.1 dm cell at c=0.2 in hexane. ^{1}H- and ^{13}C-NMR (nuclear magnetic resonance) spectra were obtained with a Bruker AC300 (^{1}H at 300 MHz, ^{13}C at 75 MHz) in a $CDCl_3$ solution with TMS (tetramethylsilane) as an internal standard.

Solvent Extraction and Isolation Two hundred female mites (8 mg) were transferred with a needle from the stock culture into a conical-bottomed glass tube (handmade, 8 mm od× 40 mm in height), and dipped into 200 μl of hexane for 3 min. The extracts, after separation from mite bodies using a microsyringe (100 μl, Hamilton Co. Ltd.), were applied without concentration to a SiO_2 column (Wako-gel C-200, 200 mg). The column was eluted successively with 2-ml aliquots of hexane, mixtures of ether in hexane (1, 3, 5, 10, and 20%), and ether. All fractions were bioassayed to locate the active fraction, and were also subjected to GC and GC/MS analyses.

Quantitative Determination of S-(+)-Isopiperitenone One female or 2 males were dipped into hexane (4 μl) containing dodecane as an internal standard (10 ppm), and extracted for 3 min. The extract, after mite bodies were removed by a micro-syringe (10 μl, Hamilton Co. Ltd), was subjected to GC using FID on an HP-5 capillary column under the conditions described above. Contents (ng/mite) of *S*-(+)-isopiperitenone were calculated by relative abundances, determined initially by using synthesized *S*-(+)-isopiperitenone and dodecane.

Synthesis of S-(+)-and R-(−)-Isopiperitenone To *R*-(+)-limonene [(*R*)-1-methyl-4-isopropenyl-1-cyclohexene](2.04 g, 15 mmol) dissolved in dry CH_2Cl_2 was added pyridium chlorochromate (PCC, 16.2 g, 75 mmol) with stirring, and the mixture was refluxed for 40 h. The mixture was filtered through a celite layer and applied to a florisil column before elution with ether. The eluate, after being concentrated, was applied to a SiO_2 column, and eluted with 5% ether in hexane to give *S*-(+)-isopiperitenone (200 mg, 8.9% yield) with $[\alpha]_D^{19}$=+59 (96% e.e.). ^{1}H- and ^{13}C-NMR were the same as those reported in Kuwahara et al. (1987). *S*-(−)-Limonene [(*S*)-1-methyl-4-isopropenyl-1-cyclehexene] (2.04 g, 15 mmol) was likewise oxidized as described above to give *R*-(−)-isopiperitenone [(*R*)-3-methyl-6-isopropenyl-2-cyclohexen-1-one] (130 mg, 5.7% yield) with $[\alpha]_{19}^{D}$=−40 (80% e.e.).

Results

GC and GC/MS Analyses The GC profile of mite hexane extracts contained two major components, peak C at t_R 9.96 min and peak F at t_R 10.28 min (Fig. 1) that were identified by mass spectrometry as the alarm pheromone isopiperitenone (Kuwahara et al., 1987) and tridecane (Howard et al., 1988), respectively, as well as several components previously described for this species as summarized in Kuwahara (2004). No qualitative sexual differences were identified in GC profiles.

Demonstration of Female Sex Pheromone Activity As shown in Fig. 2, the hexane extract at a dose of 0.1 female equivalent (1/10th of the female content) indicated sex pheromone activity. The frequency of tapping behavior in 3 min was 4.3±1.1 (N=6) among tested male mites, while with a control it was 1.1±0.4 (N=6), which is a statistically significant difference (Mann–Whitney U test, P<0.05). In

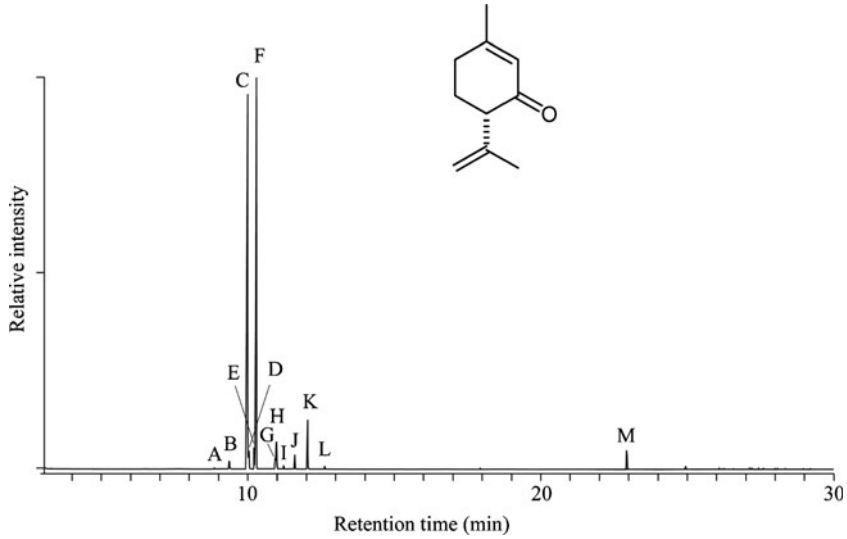

Fig. 1 Typical gas liquid chromatogram of *Tyrophagus similis* extracts and the chemical structure of *S*-(+)-isopiperitenone. See text for conditions. *A*, dodecane; *B*, neral; *C*, isopiperitenone; *D*, neryl formate; *E*, γ-acaridial; *F*, tridecane; *G*, unknown compound; *H*, isorobinal; *I*, 7-hydroxyphthalide; *J*, tetradecane; *K*, robinal; *L*, pentadecane; *M*, hexyl linolate

contrast, females exhibited no tapping behavior upon exposure to the male extract at a dose of 0.1 male equivalent (data not shown).

Purification and Determination of the Female Sex Pheromone The active hexane extracts from females were separated on a SiO_2 column into seven fractions as described above. In order to monitor pheromonal activity, all column fractions were grouped into four fractions: the hexane eluate as Fr. 1; a combined fraction (the 1, 3, and 5% ether-in-hexane eluates) as Fr. 2; the 10% ether-in-hexane fraction (containing the known alarm pheromone isopiperitenone) as Fr. 3; and the remaining combined fractions (20% ether-in-hexane and ether eluates) as Fr. 4. These fractions were assayed at a dose of 0.1 female equivalent. The results are summarized in Fig. 3. Only one fraction (Fr. 3) elicited tapping behavior (4.6±1.0). The other fractions and the control were inactive, as indicated by the following scores: Fr. 1 (1.7±0.5), Fr. 2 (2.0±0.6), Fr. 4 (1.5±0.5), and control (1.7±0.6). There was a significant difference between Fr. 3 and the other fractions (Tukey-Kramer test followed by Dunn's multiple-comparison test, $P<0.05$, $N=30$) (Adachi, 2000). As a result, Fr. 3 (the 10% ether-in-hexane eluate), in which isopiperitenone is the major component, was concluded to contain the female sex pheromone.

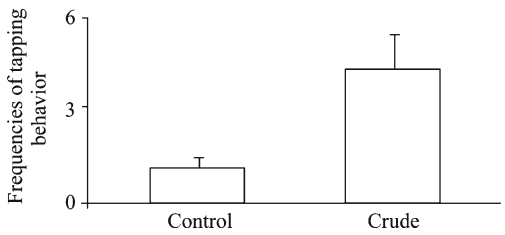

Fig. 2 The sex pheromone activities of hexane extracts at a dose of 0.1 female-equivalent against tested males. Significant difference (*, $P<0.05$) determined by the Mann–Whitney *U* test relative to the *control*

Sex Pheromone Activity of Isopiperitenone *S*-(+)-Isopiperitenone (96% e.e.) was subjected to a bioassay at doses of 0.1, 1, 10, and 100 ng (Fig. 4a). The 10 ng dose had maximum activity and produced a tapping behavior count of 5.4±0.8, while for the control and doses of 0.1, 1, and 100 ng, these counts decreased to 1.8±0.5, 2. 5±0.6, 3.1±0.6, and 2.3±0.4, respectively. This count difference was statistically significant (Tukey-Kramer test followed by Dunn's multiple-comparison test, $P<0.05$, $N=30$) (Adachi, 2000). *R*-(−)-Isopiperitenone (80% e.e.) exhibited maximum activity at 100 ng (4.8±0.9) with a similar convex dose–response relationship within the 1–1000 ng range (Fig. 4b). The control and doses of 1, 10, 1000 ng were inactive with the following scores: 2.1±0.6, 1.7±0.4, 2.8±0.7, 3.7±0.4, respectively. There was a significant difference between the count (4.8±0.9) at 100 ng and that in the control (Tukey-Kramer test followed by Dunn's multiple-comparison test, $P<0.05$, $N=30$) (Adachi, 2000). Considering that 10% of *R*-(−)-isopiperitenone (80% e.e.) corresponded to the *S*-(+)-isomer and that its activity was one-tenth that of the *S*-(+)-preparation (10 ng, 96% e.e.), the

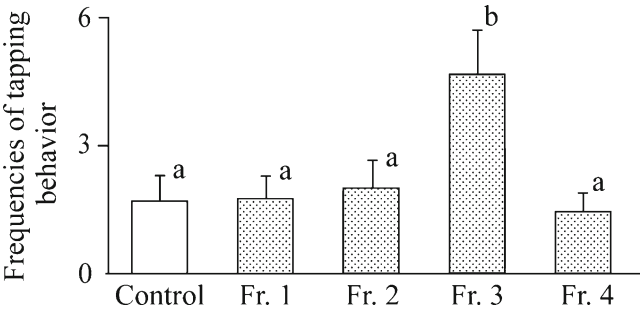

Fig. 3 Sex pheromone activities of the SiO_2 column's eluates, assayed at a dose of 0.1 female-equivalent. Fr. 1, the hexane eluate; Fr. 2, a mixture of the 1, 3 and 5% ether-in-hexane eluates; Fr. 3, the 10% ether-in-hexane eluate consisting only of isopiperitenone; Fr. 4, a mixture of the 20% ether-in-hexane and 100% ether eluates. Significant differences (*, $P<0.05$) determined by the Kruskal-Wallis test followed by Dunn's multiple-comparison test relative to the control

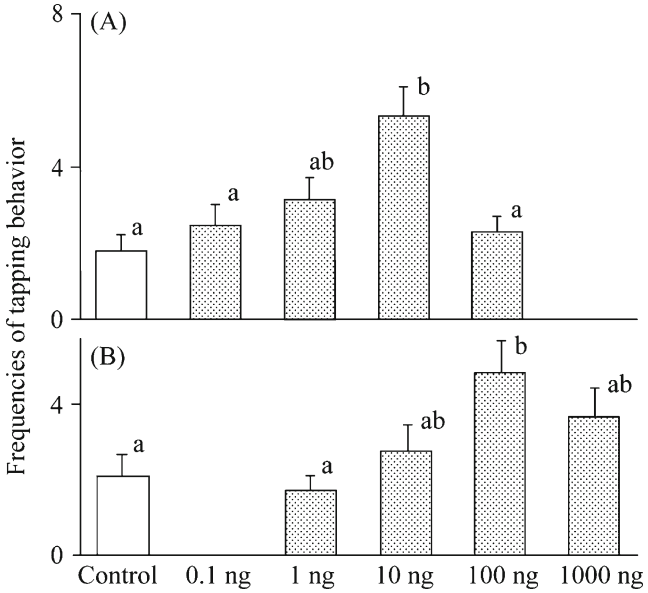

Fig. 4 Sex pheromone activities of *S*-(+)- and *R*-(−)-isopiperitenones. (**a**) *S*-(+)-isopiperitenone (98% e.e.); (**b**) *R*-(−)-isopiperitenone (80% e.e.). * Significant differences (*, $P<0.05$) determined by the Kruskal-Wallis test followed by Dunn's multiple-comparison test relative to the control

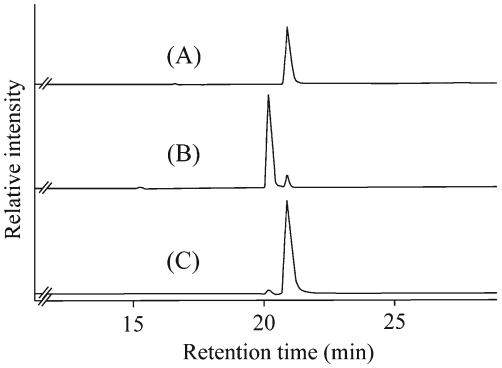

Fig. 5 Optical purity of natural isopiperitenone determined by GLC using a chiral capillary column. See text for conditions. (*A*) Preparation from mites, (*B*) synthetic *R*-(−)-isopiperitenone, and (*C*) synthetic *S*-(+)-isopiperitenone

active principle of the pheromone was concluded to be *S*-(+)-isopiperitenone.

All sex pheromones identified from female astigmatid mites are detected not only in females, but also in males. In fact, coupling behavior between males is sometimes observed in the stock culture; however, quantitative chemical differences between the sexes facilitate discriminationof females by males (Mori and Kuwahara, 2000). Furthermore, the dose–response curves for activity showed a convex shape for three species of astigmatid mites (Mori et al., 1996, 1998, 1995), and that one of the female sex pheromones (undecane) induces escape behavior at high dosages (Mori et al., 1995). The role(s) of the female sex pheromones in male opisthonatal glands remain unknown.

Stereochemistry of Isopiperitenone Both isomers, *R*-(−)- and *S*-(+)-isopiperitenone, exhibited well-separated peaks at 20.1 min and 20.5 min in a chiral GC analysis (Fig. 5). The isopiperitenone fraction from *T. similis* consisted only of the *S*-isomer, with no trace of the *R*-isomer (Fig. 5*A*).

Quantitative Determination of S-(+)-Isopiperitenone Female mites contained 38.5 ng of *S*-(+)-isopiperitenone on average, while males contained 19.8 ng on average.

Discussion

S-(+)-Isopiperitenone, previously identified as the alarm pheromone of the *Tyrophagus similis* (Kuwahara et al., 1987), was here concluded to be the female sex pheromone. Among 61 previously investigated species of Astigmata, a female sex pheromone has been demonstrated in 14 species, a male sex pheromone in one species, an alarm pheromone in 19 species, and an aggregation pheromone in four species (Kuwahara, 2004). All of these pheromones are emitted from a pair of opisthonotal glands, which occur only in Astigmata and some species of Oribatida (Sakata et al., 2003). It had been assumed that each species can possess only one kind of pheromone. However, at least seven species have been shown to possess more than one type, suggesting either a pheromone with a dose-dependent function, a pheromone response that depends on the mite's condition (disturbed or non-disturbed), or chemically different pheromones (Kuwahara, 2004).

Although the present study of *T. similis* corresponds to the 14th identification of a sex pheromone in astigmatid mites, it is the first example of a species where the same compound, *S*-(+)-isopiperitenone, functions as an alarm as well as a female sex pheromone. Our bioassay gave a maximum sex pheromone activity at a dose of 10 ng (or of 0.1 female equivalent of the mite hexane extract), while alarm pheromone activity has been demonstrated previously with 100 ppm in solution (Kuwahara et al., 1987), a dose corresponding to at least three female equivalents of the mite extract (at least 100 ng). It is interesting that sex pheromone activity developed at a lower dose range, whereas alarm pheromone activity developed at higher doses. The astigmatid mite *Schwiebea elongata* uses neral [(*Z*)-3,7-dimethyl-2,6-octadienal] as the alarm pheromone at a high dose range (Kuwahara et al., 2001), and as an aggregation pheromone at lower dose ranges (Nishimura et al., 2002). Furthermore, *Caloglyphus* (=Sankasania) *polyphyllae* employs β-acaridial [2(*E*)-(4-methyl-3-pentenylidene)butanedial] as the sex pheromone in a conditioned state (Leal et al., 1989), and as the aggregation pheromone in an unconditioned state (Shimizu et al., 2001). In total, three two-

pheromone combinations have been identified in separate astigmatid mite species; an alarm-aggregation combination in *S. elongata*, an aggregation-sex combination in *C. polyphyllae,* and a sex-alarm combination in the present species. The alternative scenario, in which two pheromonal functions are induced by separate compounds, has been found for the following four species, of which three utilize neryl formate as the alarm pheromone but as a sex pheromone; *R. robini* uses α-acaridial [2(*E*)-(4-methyl-3-pentenyl)butenedial] (Mizoguchi et al., 2003), *R. setosus* uses *S*-isorobinal (4-isopropenyl-3-oxo-1-cyclohexene-1-carboxyaldehyde) (Mizoguchi et al., 2005), and *T. putrescentiae* uses β-acaridial in (Maruno et al., 2006). In the fourth species, *Lardoglyphus konoi,* citral functions as the alarm pheromone (Kuwahara et al., 1980a, b) and lardlure (1*R*,3*R*,5*R*,7*R*-1,3,5,7-tetramethyldecyl formate) as the aggregation pheromone (Kuwahara et al., 1982; Mori and Kuwahara, 1986a, b). Among these two-pheromone-combinations for astigmatid mites, a sex-aggregation combination is still unknown.

Using GC with a chiral capillary column, the stereochemistry of the natural isopiperitenone was confirmed to be *S* with no trace of antipode contamination, although this configuration has been suggested from the alarm pheromone bioassay of *R*- and *S*-preparations (Kuwahara et al., 1987).

S-(+)-Isopiperitenone displayed maximum activity as the sex pheromone at a dose of 10 ng with a convex dose–response relationship, and the crude hexane extract indicated activity at a dose of 0.1 female equivalent. Results from the bioassay at a dose of 1 female equivalent did not indicate any sex pheromone activity (data not shown), whereas squashed mite bodies exhibited alarm pheromone activity (Kuwahara et al., 1987). Based on these results, it may be reasonable to assume that the compound manifests alarm pheromone activity at high doses, and female sex pheromone activity at low doses.

The *S*-(+)-isopiperitenone preparation (96% e.e.) used for the bioassay was composed of 98% *S*- isomer and 2% *R*-isomer, while the *R*-preparation (80% e.e.) was composed of 10% *S*-isomer and 90% *R*-isomer. Although both preparations exhibited sex pheromone activity, the *S*-preparation had 10 times higher activity than the *R*-preparation. This suggests that the active principle of the sex pheromone is *S*-(+)-isopiperitenone, and the *R*-(−)-isomer not only is inactive but also does not mask the activity of the *S*-(+)-isomer. The same phenomenon has been demonstrated in an alarm pheromone study of this species (Kuwahara et al., 1987), and a sex pheromone study of 2*R*,3*R*-epoxyneral in *Caloglyphus* (=*Sankasania*) sp. (Mori et al., 1996). In contrast, the aggregation pheromone lardolure of *L. konoi* functions differently: contamination with epimers and antipodes masks pheromone activity (Kuwahara et al., 1994).

Isopiperitenone is one of the rare monoterpenes, occurring in just five of the 61 species of astigmatid mites examined. The present species and *Tyrobolus lini* contain this compound as a major component, while three other species, *S. araujoae*, *Caloglyphus* spp. "amami", and "kouchidai-chiba", contain isopiperitenone as a minor component. With the exception of the alarm and sex pheromone functions in the present species, no such biological functions of this compound have been observed among the remaining four species investigated (Kuwahara, 2004).

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Hydrocarbon Signatures of Egg Maternity, Caste Membership and Reproductive Status in the Common Wasp

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Abstract In most ants, bees, and wasps, the workers are capable of challenging the reproductive monopoly of the queen by laying unfertilized, male eggs. An important mechanism that can resolve this conflict is policing, whereby the queen or workers prevent successful worker reproduction by selectively eating worker-laid eggs or by attacking egg-laying workers. Egg policing by workers has been shown to occur in several social wasp species, but the information used by worker wasps to discriminate between queen-laid and worker-laid eggs has never been investigated. Our aim, therefore, was to investigate if hydrocarbons might be used in egg policing by workers in the common wasp, *Vespula vulgaris*, where worker policing previously has been shown to be effective. Our results show that 51 different hydrocarbons are present on the surface of newly-laid eggs, and that there are pronounced quantitative differences in the hydrocarbon profiles of queen-laid and worker-laid eggs, with longer-chained alkenes and methylated alkanes (C_{28}–C_{31}) in particular being more abundant on the surface of queen-laid eggs. We further show that the hydrocarbon profiles on the surface of queen-laid and worker-laid eggs resemble those found on the mother queen's and workers' cuticles. Interestingly, longer-chained methylated alkanes also were more abundant on the cuticle of both mother queens and reproductive workers, suggesting that these compounds are linked to fertility, as has also been found to be the case in several ant species.

Key Words Hydrocarbons · Reproductive conflict · Worker policing · Social insects · Vespinae · *Vespula vulgaris*

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Introduction

Insect societies are among the most highly complex and well-organized societies known in the animal kingdom, to the extent that they are often referred to as "super-organisms" (Hölldobler and Wilson, 2009; Queller and Strassmann, 2009; Strassmann and Queller, 2010). This high level of integration is surprising, given the fact that social insect colonies are typically families, and not genetically identical clones and that, under such circumstances, social conflicts among colony members might be expected to be rife. Over the last decades,

however, it has been established that one of the main reasons for some of these conflicts to be less apparent than expected is the presence of various evolved mechanisms that reduce conflict (reviewed in Ratnieks et al., 2006; Ratnieks and Wenseleers, 2008). In most ants, bees and wasps, for example, the workers are capable of challenging the reproductive monopoly of the queen by laying unfertilized, male eggs. However, an important mechanism that can resolve this conflict is policing, which is the phenomenon whereby the queen or workers prevent successful worker reproduction by selectively eating worker-laid eggs or by attacking egg-laying workers (reviewed in Wenseleers and Ratnieks, 2006).

At a proximate level, the fact that the queen or workers are capable of discriminating eggs laid by queens from those laid by workers, and between laying and non-laying workers, implies the presence of accurate recognition mechanisms. Most researchers believe that these are based on the detection of differences in the surface chemistry of queen- and worker-laid eggs, and differences in the chemical profiles of laying and non-laying workers (reviewed in Monnin, 2006; Le Conte and Hefetz, 2008; Liebig, 2010). Worker policing by egg eating was first discovered in the Western honeybee (Ratnieks and Visscher, 1989), and since then, extensive studies on egg surface chemistry of queen-laid and worker-laid eggs in honeybees have been conducted (reviewed in Le Conte and Hefetz, 2008; Liebig, 2010). Nevertheless, it remains unclear exactly how honeybee workers discriminate between queen-laid and worker-laid eggs. Thus far, it has been established that differences in surface hydrocarbon profiles do not seem to play a role (Martin et al., 2004). Instead, discrimination is probably based on the presence of certain esters derived from the Dufour's gland, since queens produce higher levels of esters in their Dufour's gland than laying workers (Katzav-Gozansky et al., 1997). Treatment of worker-laid eggs with Dufour's gland extracts reduced policing rates (Ratnieks, 1995). Nevertheless, experiments to directly show the function of the esters in egg discrimination in the honeybees have failed; artificial applications of queen-like esters either did not protect the worker-laid eggs at all (Katzav-Gozansky et al., 2001) or only delayed egg-eating (Martin et al., 2002).

Aside from honeybees, attempts have been made to identify pheromones used in worker policing in several ant species (reviewed in Monnin, 2006; Le Conte and Hefetz, 2008; Liebig, 2010). In *Pachycondyla inversa*, early results based on gas chromatography (GC) and electroantennography showed that the hydrocarbon, 3,11-dimethylheptacosane, was more abundant on the surface of queen-laid eggs, and that this compound triggered a specific response in the antennae of workers (D'Ettorre et al., 2004b), suggesting that it was used as a discrimination pheromone enabling selective policing of worker-laid eggs (D'Ettorre et al., 2004a). Nevertheless, later bioassays have been unable to prove this assertion when field colonies were used (van Zweden et al., 2009). In the ant *Camponotus floridanus*, queen- and worker-laid eggs exhibit cuticular hydrocarbon profiles that resemble those of the mother queens and workers, respectively, and when hydrocarbons from the queen's cuticle were transferred onto worker-laid eggs they were policed at a reduced rate (Endler et al., 2004). In addition to chemical differences between queen- and worker-laid eggs, Meunier et al. (2011) showed that the social origin of queen-laid eggs can influence their acceptance by foreign workers. The most direct proof of the involvement of a cuticular hydrocarbon in worker policing is in the ant *Aphaenogaster cockerelli*. In this species, workers that activate their ovaries have more n-pentacosane on their cuticle, and the transfer of this compound onto the cuticle of non-reproductive workers induced biting by workers in queenright colonies (Smith et al., 2009). Thus, pentacosane is an indicator of fertility and is most likely used by workers to identify and police nestmates that activate their ovaries. Consistent quantitative differences in cuticular hydrocarbon profiles of laying and non-laying workers also occur in several other ant species (reviewed in Monnin, 2006; Le Conte and Hefetz, 2008; Liebig, 2010), but their use in worker policing has not been demonstrated.

The aim of the present study was to investigate if hydrocarbons might be used in egg policing by workers in the common wasp, *Vespula vulgaris* (Vespinae), where worker policing has evolved independently from ants and bees (Foster and Ratnieks, 2001). Because workers in this species selectively eat worker-laid eggs (Foster and Ratnieks, 2001), our study aimed at quantifying the differences in the surface hydrocarbon profiles of queen- and worker-laid eggs by using GC-mass spectrometric (MS) analysis. In addition, we tested whether egg profiles are closest to either cuticular or Dufour's gland profiles of reproductive individuals, thereby enabling determination of the possible exocrine source of the hydrocarbons used in egg policing. In addition, we investigated whether cuticular hydrocarbons provide information on caste membership, colony membership, and the reproductive status of queens and workers.

Methods and Materials

Colony Collection In August and September 2007, five mature colonies of *V. vulgaris* were collected in the vicinity of Leuven, Belgium. Nests were found underground or in attics and contained ca. 400–600 workers, and a single reproductive mother queen. After collection, the nests were transferred to 30 cm (wide) × 32 cm (deep) × 40 cm (high) wooden observation boxes with a 5 cm diameter entrance hole, which allowed the workers to forage naturally. The colonies were used to collect queen- and worker-laid eggs

(*N*=44 and *N*=39, respectively), workers (*N*=101), virgin and reproductive queens (*N*=17 and *N*=4, respectively). Seven nest-searching *V. vulgaris* queens (referred to as spring-collected queens hereafter) also were collected in the surroundings of Kampenhout, Belgium, in April 2007, as well as nine reproductive queens from colonies which had been used in another experiment.

Queen-laid and Worker-laid Eggs Queen-laid eggs were collected from the five queenright nests by removing the eggs present in the lowest comb, which consists of large cells used to rear gynes and males. To allow this comb to be easily taken out and returned to the colony, the comb was first glued onto a thin iron wire attached to a piece of modeling clay, and then reinserted into the nest. After 24 hr, the comb was removed, queen-laid eggs were collected, and visually checked for damage on a glass plate. Only intact eggs were used for chemical analysis. Most or all of the eggs collected this way were queen-laid eggs since in queenright colonies of *V. vulgaris* only 1.0% of the workers have functional ovaries, and in a policing trial all 120 worker-laid eggs were removed within 16 hr (Foster and Ratnieks, 2001). Furthermore, the rate of ovary activation of *V. vulgaris* workers in queenright colonies does not differ significantly between Belgian and British populations (A. Van Oystaeyen and T. Wenseleers, unpublished results). Subsequently, the colonies were orphaned, and the reproductive mother queens were frozen at −20°C (except for one colony from which the mother queen could not be retrieved). Approximately 2 weeks after being orphaned, workers started laying eggs, and these eggs were collected in the same way as queen-laid eggs.

Surface hydrocarbons from queen- and worker-laid eggs were extracted by washing each individual egg in 40 μl of HPLC-grade pentane (Acros Organics, Belgium) for 1 min, of which the first and last 15 sec involved gentle mixing. The solvent then was allowed to evaporate at room temperature in a laminar flow hood. Each extract was resuspended in 10 μl pentane, of which 2 μl were injected splitless in an Agilent 6850 GC (Agilent Technologies, USA), equipped with an HP-1 capillary column (30 m × 320 μm × 0.25 μm), a split–splitless injector, a flame ionization detector (FID), and a helium carrier gas flow of 1.1 ml/min. After an initial hold of 1 min at 70°C, the temperature was raised to 150°C at 20°C/min, then to 320°C at 3.5°C/min, with a final hold at 320°C for 5 min.

Cuticular and Dufour's Gland Hydrocarbons After having collected worker-laid eggs, all workers inside the nests, as well as virgin queens from two colonies, were killed by freezing at −20°C. Wasps were dissected to measure ovary development and to obtain the Dufour's gland. Workers were classified in three categories based on the size of the biggest oocyt being smaller than 25%, 25–50%, and greater than 50% the size of a freshly laid egg, respectively: those with undeveloped ovaries (UND, *N*=49), partially developed ovaries (PD, *N*=8), or fully developed ovaries (FD, *N*=44). The cuticular hydrocarbons of adult wasps were extracted by immersing head and thorax for 10 min, of which the first and the last 15 sec involved gentle mixing in 600 μl or 1 ml of pentane for adult workers and queens, respectively. We used only the head and thorax since the abdomen was used to obtain the Dufour's gland. Solvent then was allowed to evaporate at room temperature in a laminar flow hood. Each extract was resuspended in 50 μl of pentane for workers and 100 μl pentane for queens, of which 2 μl were analyzed by GC in the splitless mode. The Dufour's gland was dissected and placed into a micro-insert to which 100 μl of pentane were added, and the contents were crushed with forceps and vortexed for 10 sec. Solvents were evaporated as above, and each extract was resuspended in 10, 100, and 200 μl of pentane for workers and reproductive queens, virgin queens and spring-collected queens, respectively, of which 2 μl were analyzed by GC as above. Different amounts of pentane were necessary to obtain comparable chromatograms (total concentration of compounds) for the different classes of queens.

Identification of Compounds Compound identities were determined on the basis of their mass spectra from GC-MS analysis performed using an Agilent 7890A GC, equipped with a ZB-5HT capillary column (30 m × 320 μm × 25 μm) coupled to a 5975C Inert XL EI/CI MSD with electron ionization (70 eV), and a helium carrier gas flow of 1.5 ml/min, using the same temperature program as above, and by subsequently comparing diagnostic ions of the mass spectra with published data (e.g., Bonavita-Cougourdan et al., 1987; Howard et al., 2001).

Analysis of Hydrocarbon Profiles Peak areas of 51 identified hydrocarbons (or mixtures of several co-eluting hydrocarbons) found on the surface of queen- and worker-laid eggs, and the cuticle of workers and queens (Fig. 1, Table S1), as well as those found in the Dufour's gland of workers (UND, *N*=23; PD, *N*=3; FD, *N*=28) and queens (reproductive queens Q, *N*=13; spring-collected queens, SQ, *N*=7; virgin queens, VQ, *N*=17) were quantified using the software Agilent ChemStation (Rev. A.09.01, Agilent Technologies) normalized to relative concentrations with a Z-transformation (Aitchison, 1986). These normalized concentrations then were used as variables in a principal component analysis (PCA), after which the scores for all principal components with an eigenvalue greater than 1 were used as independent variables in further discriminant analyses to determine if variation in hydrocarbons allowed us to differentiate among worker- and queen-laid eggs, as

well as among workers from different colonies, and to determine if hydrocarbon profiles contained information regarding caste and fertility. We divided the hydrocarbons into two groups (shorter-chained and longer-chained) based on their loadings on the PC explaining the most variation. Differences in the total relative abundances of these particular classes of compounds (i.e., shorter-chained and longer-chained alkanes, alkenes, and methylated alkanes) were tested using independent *t*-tests. Lastly, the mean City-block (Manhattan) distances between the surface hydrocarbon profiles of queen-laid eggs and the cuticular and Dufour's gland compounds of reproductive queens on the one hand, and between worker-laid eggs and the cuticular and Dufour's gland hydrocarbon profiles of reproductive workers on the other hand, were calculated. These values were used in Wilcoxon matched pairs tests to determine if the surface hydrocarbon profiles of eggs were closer to the cuticular than to the Dufour's gland hydrocarbon profiles.

To investigate in more detail which compounds could be used to identify colony identity, we preformed a separate principal component analysis using only the cuticular hydrocarbon profiles of workers with undeveloped ovaries, after which the scores for all principal components with an eigenvalue greater than 1 were used as independent variables in a discriminant analysis. We used this approach because colony membership information might be masked by fertility, and because recognizing sterile workers returning from foraging is essential in the context of nestmate recognition. The program STATISTICA 9.1 (StatSoft Inc., USA) was used for all statistical analyses.

Results

Identity of Compounds A total of 51 hydrocarbons were identified from the surface of queen-laid and worker-laid eggs, and on the cuticle and in the Dufour's gland of queens and workers. The hydrocarbon profiles consisted of linear alkanes and alkenes, and branched alkanes with a chain length ranging from C_{23} to C_{31} (Fig. 1, Table S1). A PCA analysis produced six principal components with eigenvalues higher than 1, which together explained 84.6% of the variance in the data (Table S2). Based on the loadings of the hydrocarbons on the PC explaining the most variation (PC 1) we could divide the hydrocarbons into two groups, shorter-chained (C_{23}–C_{27}) and longer-chained (C_{28}–C_{31}) hydrocarbons.

Egg Maternity PCA analysis shows that the surface hydrocarbons of worker-laid eggs were significantly different from those of queen-laid eggs, and that it was possible to correctly assign 89.2% of all eggs to the right egg-type (Table S3; *Wilks'* λ=0.53, $F_{6,76}$=7.89, P<0.001), with three PCs being significant for the discrimination according to egg-type (PC 1, *Partial Wilks'* λ=0.80, P<0.001; PC 2, *Partial Wilks'* λ=0.91, P=0.006; PC 5, *Partial Wilks'* λ=0.93, P=0.02; Fig. 2). Furthermore, all the variation could be explained by a single significant discriminant function (canonical correlation=0.72; *Wilks'* λ=0.48, $\chi 2$=57.55, *df*=6, P<0.001), which was determined principally by PC 1 (standardized coefficient −0.76, correlation −0.65), and hence by the compounds 3-, 5-, 7-, 9-, 11-MeC_{23}, 5,x-diMeC_{23}, 3,x-diMeC_{23}, 10-,12-,14-MeC_{24}, 6-MeC_{24}, 4-MeC_{24}, 11-, 13-, 15-MeC_{25}, 5,x-diMeC_{25}, 3,x-diMeC_{25}, and 3-MeC_{29} (absolute value of factor loadings >0.9, Table S2). Univariate tests on the total relative abundance of particular classes of compounds (Table S1) show that queen-laid eggs had a higher proportion of longer-chained alkenes and methylated alkanes (C_{28}–C_{31}) as well as shorter-chained *n*-alkanes (C_{23}–C_{27}) on the surface (three independent *t*-test, t_{93}=5.24, P<0.001; t_{93}=3.67, P<0.001; t_{93}=4.03, P<0.001, respectively), while shorter-chained methylated alkanes (C_{23}–C_{27}) were more abundant on worker-laid eggs (independent *t*-test, t_{93}=−5.14, P<0.001). The relative abundance of shorter-chained alkenes (C_{23}–C_{27}) and longer-chained *n*-alkanes (C_{28}–C_{31}), however, did not differ between the two types of eggs (two independent *t*-test, t_{93}=0.50, P=0.61; t_{93}=−1.17, P=0.24, respectively).

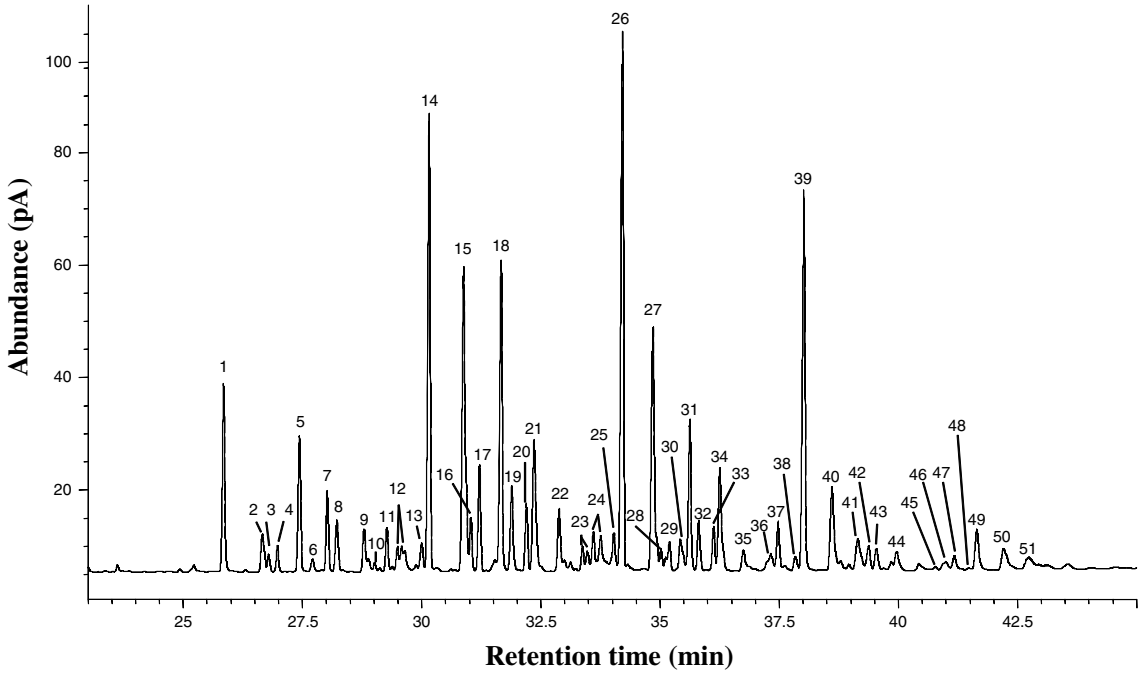
The surface hydrocarbon profiles of queen-laid eggs were closer to the cuticular hydrocarbon profiles of queens than to the queen's Dufour's gland hydrocarbon profiles, as measured by the average City-block (Manhattan) distances (Wilcoxon matched pairs test, P<0.001). The same was true for worker-laid eggs i.e., the hydrocarbon profile of a worker-laid egg resembled the cuticular hydrocarbon profile of a reproductive worker more than the Dufour's gland hydrocarbon profile of a reproductive worker (Wilcoxon matched pairs test, P<0.001).

Caste Membership and Fertility Reproductive queens, spring-collected queens, virgin queens, and workers with undeveloped, partially developed, or fully developed ovaries could be discriminated based on their cuticular hydrocarbon profile (*Wilks'* λ=0.0059, $F_{30,510}$=44.47, P<0.001). Of all individuals, 81.2% were assigned correctly, and the majority of misclassifications occurred between workers with partially and fully developed ovaries (Table S4). When workers with partially developed ovaries were excluded from the analysis, 83.8% of all individuals were correctly classified (Table S4; *Wilks'* λ=0.0053, $F_{24,419}$=61.27, P<0.001) with all PCs being significant but PC 1 and PC 5 having the lowest partial Wilks' λ (PC 1, *Partial Wilks'* λ=0.15; PC 5, *Partial Wilks'* λ=0.26; other PCs *Partial Wilks'* λ>0.64). Furthermore, all variation could be explained by four significant discriminant functions (function 1 explaining 61.3%, canonical correlation=0.95; *Wilks'* λ=0.0053, $\chi 2$=648.2, *df*=24, P<0.001; function 2 explaining

a

Peak #	Identification	Peak #	Identification
1	*n*-C_{23}	14	*n*-C_{25}
2	9-, 11-MeC_{23}	15	11-, 13-, 15-MeC_{25}
3	7-MeC_{23}	16	7-MeC_{25}
4	5-MeC_{23}	17	5-MeC_{25}
5	3-MeC_{23}	18	3-MeC_{25}
6	5,x-diMeC_{23}	19	5,x-diMeC_{25}
7	*n*-C_{24}	20	*n*-C_{26}
8	3,x-diMeC_{23}	21	3,x-diMeC_{25}
9	10-, 12-, 14-MeC_{24}	22	10-, 12-, 14-MeC_{26}
10	6-MeC_{24}	23	4-MeC_{26}
11	4-MeC_{24}	24	$C_{27:1}$ (+isomer)
12	$C_{25:1}$ (+ isomer)	25	4,x-diMeC_{26}
13	4,x-diMeC_{24}	26	*n*-C_{27}

Peak #	Identification	Peak #	Identification
27	11-, 13-MeC_{27}	40	11-, 13-, 15-MeC_{29}
28	7-MeC_{27}	41	7-MeC_{29}
29	5-MeC_{27}	42	3-MeC_{29}
30	11,15-diMeC_{27}	43	5,9-diMeC_{29}
31	3-MeC_{27}	44	*n*-C_{30}
32	5,x-diMeC_{27}	45	x_1-MeC_{30}
33	*n*-C_{28}	46	x,y-diMeC_{30}
34	3,x-diMeC_{27}	47	$C_{31:1}$
35	10-, 12-, 14-, 16-MeC_{28}	48	x_2-MeC_{30}
36	4-MeC_{28}	49	*n*-C_{31}
37	$C_{29:1}$	50	11-, 13-, 15-MeC_{31}
38	4, x-diMeC_{28}	51	11,17-, 13,17-, 15,19-diMeC_{31}
39	*n*-C_{29}		



b

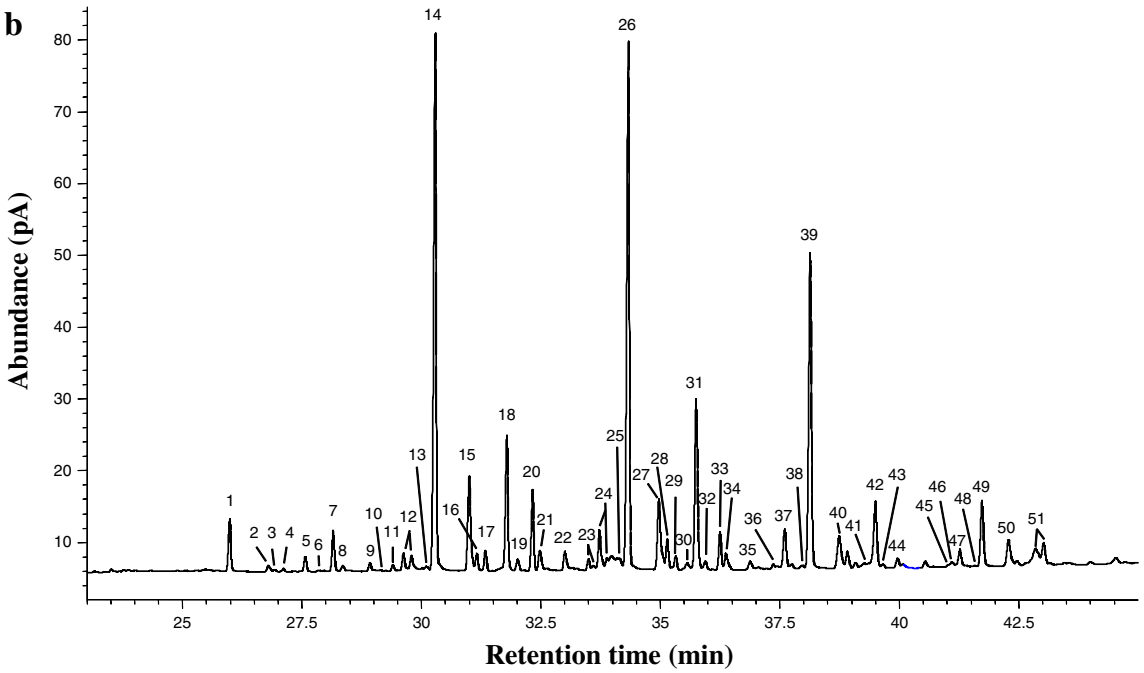


Fig. 1 Typical gas chromatogram of the surface hydrocarbon profile of **a** a *Vespula vulgaris* worker-laid egg and **b** a *V. vulgaris* queen-laid egg. The identity of the peaks, as established by GC-MS analysis, is given in the inset table. For dimethyl alkanes, x represents the position of the second methyl group (see Table S1)

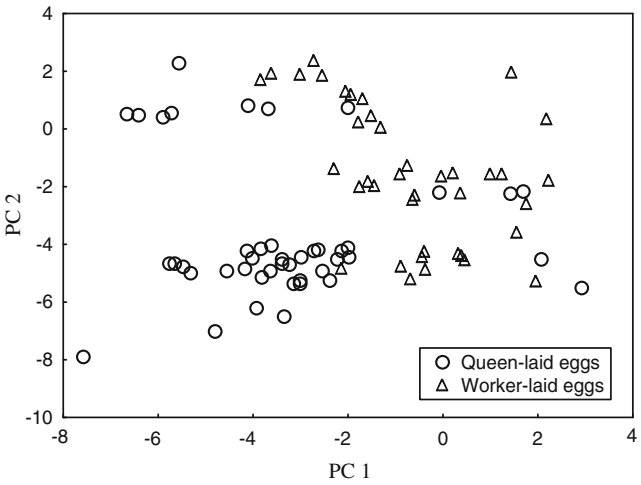


Fig. 2 Plot of the first two principal components showing the chemical difference between queen-laid and worker-laid eggs

26.0%, canonical correlation=0.90; *Wilks' λ*=0.058, $\chi 2$=352.4, *df*=15, *P*<0.001; function 3 explaining 11.9%, canonical correlation=0.81; *Wilks' λ*=0.30, $\chi 2$=147.7, *df*=8, *P*<0.001; function 4 explaining 0.8%, canonical correlation=0.34; *Wilks' λ*=0.89, $\chi 2$=14.89, *df*=3, *P*=0.002). The first discrimination function separated the queens from the workers, the second separated the different classes of queens, the third separated the reproductive queen from virgin and spring-collected queens, and the fourth moderately separated the workers with fully developed and undeveloped ovaries (Fig. 3).

As for egg maternity, the discrimination function separating the cuticular profiles of queens and workers (Fig. 3a) was determined mostly by PC 1 (standardized coefficient −1.20, correlation −0.79), and hence by the compounds 3-, 5-, 7-, 9-, 11-MeC_{23}, 5,x-diMeC_{23}, 3,x-diMeC_{23}, 10-,12-,14-MeC_{24}, 6-MeC_{24}, 4-MeC_{24}, 11-, 13-, 15-MeC_{25}, 5,x-diMeC_{25}, 3,x-diMeC_{25}, and 3-MeC_{29} (absolute value of factor loadings >0.9, Table S2). As with the egg discrimination results, univariate tests showed that the cuticle of reproductive queens contained more longer-chained *n*-alkanes, alkenes, and methylated alkanes (C_{28}–C_{31}), as well as shorter-chained *n*-alkanes (C_{23}–C_{27}) (four independent *t*-tests, t_{55}=17.97, *P*<0.001; t_{55}=2.04, *P*=0.046; t_{55}=2.96, *P*=0.005; t_{55}=15.37, *P*<0.001, respectively), while the cuticle of reproductive workers contained a higher proportion of shorter-chained alkenes and methylated alkanes (C_{23}–C_{27}) (two independent *t*-test, t_{55}=−7.33, *P*<0.001; t_{55}=−18.80, *P*<0.001). The discrimination function separating the three classes of queens (Fig. 3a) was determined primarily by PC 5 (standardized coefficient −1.00, correlation −0.97), with 4,x-diMeC_{28} being the compound with the highest factor loading on PC 5 (absolute value of factor loading >0.5, Table S2). The discrimination function separating the reproductive queen from virgin and spring-collected queens (Fig. 3b) was determined mainly by PC 3 (standardized coefficient −0.85, correlation −0.54), and hence by the compounds 10-, 12-, 14-MeC_{26}, 11-, 13-MeC_{27}, 5,x-diMeC_{27}, 10-, 12-, 14-, 16-MeC_{28}, 4,x-diMeC_{28}, and 5,9-diMeC_{29} (absolute value of factor loadings >0.5, Table S2). The discrimination function separating workers with fully developed ovaries from workers with undeveloped ovaries (Fig. 3c), was determined principally by PC 4 (standardized coefficient 0.63, correlation 0.95), and hence by the compounds $C_{25:1}$, x_1-MeC_{30} and x,y-diMeC_{30} (absolute value of factor loadings >0.5, Table S2).

Since the hydrocarbons found in the Dufour's gland were qualitatively identical to the ones found on the cuticle, and since previous studies have shown the importance of Dufour's gland hydrocarbons in signaling fertility in the paper wasp *Ropalidia marginata* (Mitra and Gadagkar, 2011; Mitra et al., 2011), the previous discriminant analysis (excluding workers with partially developed ovaries) could also be performed using the Dufour's gland hydrocarbons. However, the accuracy of the classification using cuticular hydrocarbon profiles was significantly better (Wilcoxon matched pairs test on the percentages of correctly classified classes of individuals, one-sided *P*=0.04).

Colony Membership Cuticular hydrocarbon profiles of workers from the five colonies studied were significantly different, and it was possible to correctly assign 64.4% of all individuals to their colony of origin (Table S5; Wilks' λ=0.23, $F_{24,318}$=7.01, *P*<0.001), with three PCs being significant for the discrimination (PC 1, *Wilks' λ=0.45*, *Partial Wilks' λ*=0.50, $F_{4,91}$=22.41, *P*<0.001; PC 3, *Wilks' λ*=0.26, *Partial Wilks' λ*=0.89, $F_{4,91}$=2.86, *P*=0.03; PC 5, *Wilks' λ*=0.28, *Partial Wilks' λ*=0.83, $F_{4,91}$=4.74, *P*=0.002). Two functions, explaining 94% of the variation, were significant (function 1 explaining 82%, canonical correlation=0.81; *Wilks' λ*=0.23, $\chi 2$=139.68, *df*=24, *P*<0.001; function 2 explaining 12%, canonical correlation=0.47; *Wilks' λ*=0.68, $\chi 2$=36.70, *df*=15, *P*=0.001) with PC 1 and PC 3 having the most weight on the first and second discrimination functions, respectively (PC 1, standardized coefficient −1.09, correlation −0.54; PC 3, standardized coefficient −0.43, correlation −0.73).

When the analysis of cuticular hydrocarbon profiles was restricted to that of undeveloped workers, a PCA analysis produced seven principal components with eigenvalues higher than 1, which together explained 92.1% of the variance in the data (Table S6). In a subsequent discriminant analysis, it was possible to correctly assign 85.7% of all individuals to their colony of origin (Table S7; Wilks' λ=0.017, $F_{28,138}$=10.31, *P*<0.001), with all PCs but one (PC 6) being significant. All variation could be explained by four significant functions of which the first two functions explained 87% of the variation (function 1 explained 62%,

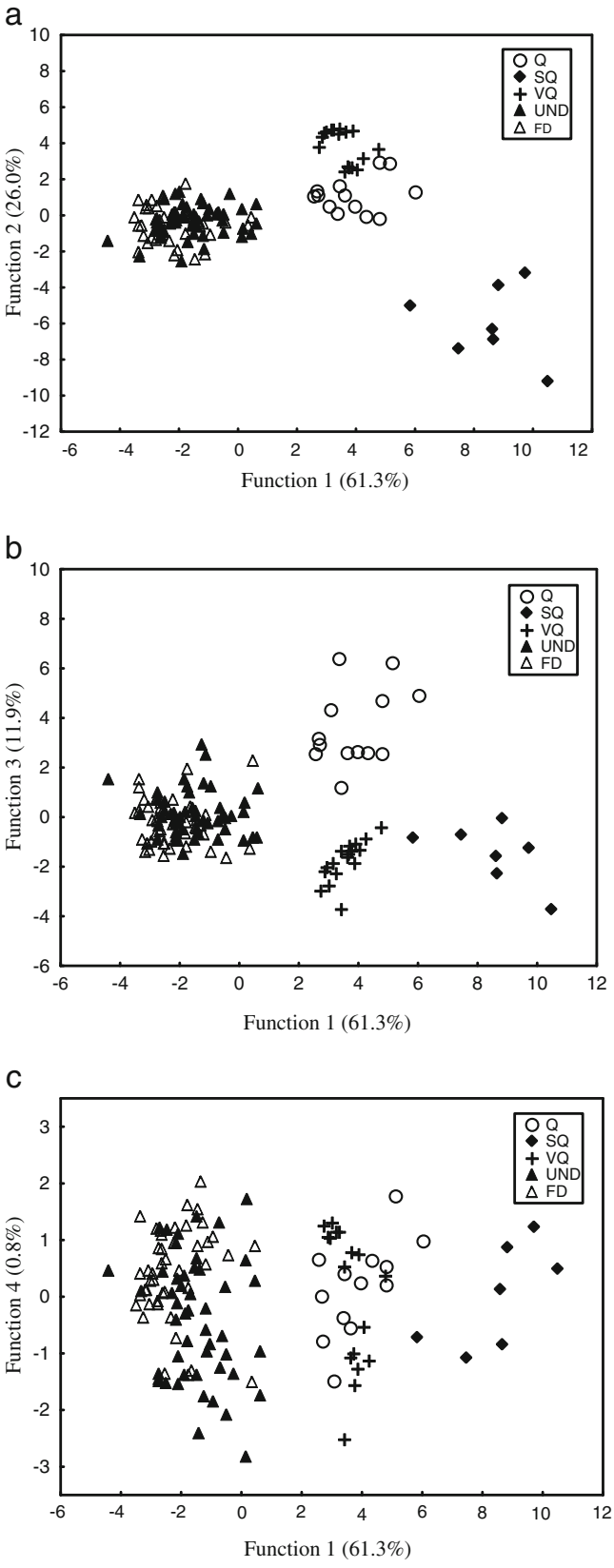


Fig. 3 Discriminant analysis based on the relative proportions of 51 cuticular hydrocarbons from reproductive queens (Q), spring-collected queens (SQ), virgin queens (VQ), workers with fully developed ovaries (FD), and workers with undeveloped ovaries (UND). The percentage of variance explained by function 1 and 2 (**a**), function 1 and 3 (**b**), function 1 and 4 (**c**) is given in parentheses

canonical correlation=0.93; *Wilks'* λ=0.017, $\chi 2$=170.64, *df*=28, *P*<0.001; function 2 explained 25%, canonical correlation=0.84; *Wilks'* λ=0.12, $\chi 2$=89.30, *df*=18, *P*<.001). The first and second discrimination function were determined chiefly by PC 2 (standardized coefficient −1.32, correlation −0.31) and PC 1 (standardized coefficient 0.88, correlation 0.63), respectively. Furthermore, PC 2 and PC 1 were mostly determined by shorter-chained and longer-chained hydrocarbons, respectively. This means that practically all hydrocarbons are needed to correctly classify the workers according to their colony of origin. Since the hydrocarbons found in the Dufour's gland were qualitatively identical to the ones found on the cuticle, and since previous studies on paper wasps have shown that individuals can be correctly classified according to their colony of origin based on Dufour's gland hydrocarbons (Dani et al., 1996; Mitra et al., 2011), the previous discrimination analysis could also be done using the Dufour's gland hydrocarbon profiles of workers with undeveloped ovaries. This led to a correct classification of 90.5% of all individuals to their colony of origin (Wilks' λ=0.069, $F_{21,32}$=2.35, *P*<0.014), which was not significantly different from the correct assignment rate based on the cuticular hydrocarbon profiles, 85.7% (Wilcoxon matched pairs test on the percentages of correctly classified classes of individuals, two-sided *P*=0.42). However, only one function, explaining 54.7% of the variation, was actually significant (canonical correlation=0.85; *Wilks'* λ=0.069, $\chi 2$=38.76, *df*=21, *P*=0.01).

Discussion

Our results show that queen-laid and worker-laid eggs can be discriminated based on their surface hydrocarbon profiles, with longer-chained alkenes and methylated alkanes (C_{28}–C_{31}) being more abundant on the surface of queen-laid eggs. This means that the hydrocarbons on the surface of queen- and worker-laid eggs, in principle, contain all the necessary information for workers to effectively recognize and police worker-laid eggs. Moreover, the hydrocarbon profiles on the surface of queen- and worker-laid eggs resemble those on queens' and workers' cuticles, respectively. This is in concordance with previous results found in other social Hymenoptera (reviewed in Monnin, 2006; Le Conte and Hefetz, 2008; Liebig, 2010), which suggests that the hydrocarbons on the cuticle and on the egg surface have the same exocrine source. In insects, cuticular hydrocarbons are produced in oenocytes associated with epidermal tissue or are present in the peripheral fat body, and are transported

for deposition on the cuticle by lipophorin (reviewed in Bagnères and Blomquist, 2010). In addition, in the German cockroach *Blattella germanica* and the house fly *Musca domestica*, it has been shown that lipophorin transports hydrocarbons to the ovaries (Schal et al., 2001; Fan et al., 2002). Despite the fact that this pathway has never been confirmed for social Hymenoptera, several studies provide indirect evidence that hydrocarbons on the surface of eggs originate from the same source as cuticular hydrocarbons (Monnin and Peeters, 1997; D'Ettorre et al., 2004a; Endler et al., 2004, 2006; Dapporto et al., 2007; Lommelen et al., 2008). Nevertheless, it should be noted that in this study all hydrocarbons found on the surface of the eggs were also found in the wasps' Dufour's glands, so it is possible that this gland also contributes to the hydrocarbon composition found on the surface of the eggs. Indeed, in contrast to the situation in ants, the posterior part of the Dufour's gland duct in wasps opens in the dorsal vaginal wall, making it potentially suitable for egg marking (Billen, 1987, 2006). In addition, Dani et al. (1996) demonstrated that in the paper wasp, *Polistes dominulus*, Dufour's gland of foundresses contained the same hydrocarbons as those found on the cuticle, perhaps as a result of the spreading of the secretion of the Dufour's gland over the body surface during self-grooming. Therefore, our results suggest that in *Vespula vulgaris* both the oenocytes and Dufour's gland might contribute to the formation of the specific hydrocarbon profiles present on the surface of the eggs and cuticle.

In addition to our data that suggested that hydrocarbons on the cuticle and on the egg surface have the same exocrine source, specific longer-chained methylated alkanes (C_{28}–C_{31}) were more abundant on the cuticle of queens relative to workers, as well as on reproductive workers relative to nonreproductive workers. This suggests that these compounds are linked to fertility, as has been found in several ant species (reviewed in Monnin, 2006; Le Conte and Hefetz, 2008; Liebig, 2010). For example, methylated alkanes C_{27}–C_{34} were significantly more abundant on the cuticle of *Linepithema humile* reproductive queens compared to the cuticle of virgin queens (de Biseau et al., 2004), and in *Lasius niger* specific long-chained hydrocarbons (3-MeC_{31}, $C_{31:1}$, and 3-MeC_{29}) were related to productivity and maturation in queens, and 3-MeC_{31} was identified as a sterility-regulating ant queen pheromone (Holman et al., 2010a,b). As with the queen pheromone in *L. niger*, the surface of queen-laid eggs and cuticle of *V. vulgaris* queens have a higher proportion of a similar compound (3-MeC_{29}) than do worker-laid eggs and the cuticle of workers. Therefore, it would be interesting to test if 3-MeC_{29} is used as a queen pheromone in *V. vulgaris*. However, it is clear that longer-chained methylated alkanes are not always linked to fertility in social insects, and that these compounds acquired a fertility signaling role independently several times in eusocial Hymenoptera. For example, in the bald-face hornet, *Dolichovespula maculata,* reproductive queens did not differ in their longer-chained methylated alkanes from workers (Butts et al., 1991), and in the ant *Ectatomma tuberculatum*, the alkane *n*-C_{27} strongly correlated with fertility (Hora et al., 2008). Nevertheless, our data adds credence to the idea that ovarian development in social insects is interwoven with the mechanism of hydrocarbon biosynthesis (e.g., Heinze et al., 2002; Smith et al., 2009). In contrast to queens, the relative abundance of $C_{25:1}$, x_1-MeC_{30}, and x,y-diMeC_{30} mainly determined the discrimination between the cuticular hydrocarbon profiles of workers with developed and undeveloped ovaries. The fact that the cuticular hydrocarbon profiles of reproductive workers differ from those of non-reproductive ones, could potentially be used to police reproductive workers via aggression. To reiterate, the most direct proof of the involvement of cuticular hydrocarbons in worker policing is for the ant *Aphaenogaster cockerelli* (Smith et al., 2009). Despite the fact that egg eating is most likely the main form of worker policing in *V. vulgaris*, the fact that some worker policing is done via aggression remains plausible.

In summary, our data show that the hydrocarbons correlated with fertility can be different for different castes in the same species. This is unique from the situation in the ants *Myrmecia gulosa*, *Aphaenogaster cockerelli*, and *Pachycondyla inversa*, where reproductive workers resemble the profiles of reproductive queens and, hence, the same hydrocarbons convey information on fertility in both castes (Heinze et al., 2002; Dietemann et al., 2003; Smith et al., 2008). Alternately, in the paper wasp, *Polistes dominulus,* dominance among foundresses determine the cuticular hydrocarbon profiles more than fertility (Dapporto et al., 2007, but see Bonavita-Cougourdan et al., 1991), suggesting that in these wasps cuticular hydrocarbon profiles represent social rather than reproductive status. Our results suggest that all hydrocarbons are necessary for nestmate recognition, whereas only a subset of specific hydrocarbons are used to signal caste and fertility in *V. vulgaris*.

Although we do not know the proximate mechanism driving the observed differences in cuticular hydrocarbon profiles in *V. vulgaris*, endocrine factors such as juvenile hormone might be important, as juvenile hormone plays an important role in determining the division of labor and influences cuticular hydrocarbon profiles in social Hymenoptera (e.g., Huang et al., 1994; Bloch et al., 2000; Lengyel et al., 2007).

Overall, our results show for the first time in social wasps that surface hydrocarbon profiles of queen-laid eggs and worker-laid eggs contain the necessary information for workers to effectively police worker-laid eggs. Bioassays are needed to determine if workers do indeed use these hydrocarbons for worker policing. Furthermore, our results indicate that it is possible that the Dufour's gland contributes, together with the oenocytes, to the formation of characteristic hydrocarbon profiles on the egg and body surfaces of vespines.

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Male-Produced Pheromone in the European Woodwasp, *Sirex noctilio*

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Abstract A male-produced pheromone that attracts both males and females was identified for the European woodwasp, *Sirex noctilio*, a serious pest of pine trees. Males displayed excitatory behaviors when placed in groups, and were attracted to the odors from males that were 2-5-d-old, but not to odors from males that were 0-1-d-old. An unsaturated short-chain alcohol, (*Z*)-3-decen-1-ol, was discovered in samples collected on SuperQ filters over groups of males and identified by using micro-derivatization reactions and gas chromatography coupled with mass spectrometry (GC-MS). The compound was not detected in volatile samples from females. Gas chromatography coupled electroantennographic detection (GC-EAD) of antennae from males exposed to male headspace odors produced strong antennal responses to the main peak of (Z)-3-decen-1-ol, as well as to an unknown minor component that had a similar retention time. Antennae from both males and females responded to synthetic (*Z*)-3-decen-1-ol. Several different synthetic candidates for the GC-EAD active minor components were selected based on GC-MS and GC-EAD responses to male headspace collections. These synthetic compounds were tested for antennal activity using GC-EAD, and those that produced strong responses were blended with the major component and tested for male attraction in the Y-tube olfactometer at different concentrations and ratios. Males tested in the Y-tube olfactometer were attracted to a synthetic blend of (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol at a ratio of 100:1. Whereas the addition of some suspected minor compounds reduced attraction, the addition of a third compound found in male emanations that produced strong male antennal responses, (*E,E*)-2,4-decadienal (at a ratio of 100:1:1), resulted in attraction of both males (Y-tube and wind tunnel) and females (wind tunnel).

Key Words Pheromone · Y-tube Olfactometer · Wind tunnel · Attractant · Wood wasp · Hymenoptera · Siricidae · Invasive insect

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Introduction

The European woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae) has become a major pest of pines outside of its native range of Eurasia and northern Africa due to human-assisted transport of infested wood into those areas where susceptible host species grow. The wasp, introduced to New Zealand around 1905 (Coutts, 1965), spread to Tasmania, Australia, Brazil, Uruguay, Argentina, South Africa, and Chile (Smith and Schiff, 2002; Ciesla, 2003; Collett and Elms, 2009). Most recently, it was discovered in the northeastern United States and subsequently in Canada (Hoebeke et al., 2005; Collett and Elms, 2009). Its introduction into the United States is of concern because, in pine plantations

in other parts of the world, it is a serious pest of pine species native to North America (Carnegie et al., 2006).

The adult female has a long serrate ovipositor used to drill into the sapwood of the host tree (Madden, 1974). During oviposition, she also deposits a symbiotic fungus, *Amylostereum areolatum* (reviewed by Slippers et al., 2003) and a phytotoxic mucus, which together can kill the tree. Larvae feed and develop in the wood, and rely on the fungus to aid in the digestion of cellulose (Rawlings, 1953; Gilmour, 1965). Typically, larvae overwinter inside the trees and emerge the following summer (Zondag, 1969). Adult wasps live up to 12 d (Zondag, 1969), and have haplo-diploid sex determination (Rawlings, 1953) in which unmated females produce unfertilized eggs that develop into haploid males. Mated females can deposit either unfertilized eggs that become males, or fertilized eggs that develop into diploid females.

Until recently, most chemical ecology research on *S. noctilio* has been directed towards the discovery of host tree volatiles attractive to gravid females (Simpson, 1976; Simpson and McQuilkin, 1976). A female contact sex pheromone was discovered recently that elicits copulation attempts from males upon contact (Böröczky et al., 2009). However, little is known about how *S. noctilio* males and females find each other in the wild to mate. There have been reports of male *S. noctilio* swarming near the tops of trees (Madden, 1982; 1988). Such behavior suggests the presence of a male aggregation pheromone. In this study, we report production by male *S. noctilio* of a major compound, *(Z)*-3-decen-1-ol, and when blended with two minor components, *(Z)*-4-decenol and (*E,E*)-2,4-decadienal, the blend is attractive to both males and females in laboratory bioassays.

Methods and Materials

Staggering Adults for Emergence In upstate New York, *S. noctilio* adults start to emerge in the field around the beginning of July, and emerge over a period of several weeks (Zylstra et al., 2010). Adults live only 6–12 d (Madden, 1982). At the USDA APHIS PPQ facility in Syracuse, NY, USA (hereafter referred to as 'Syracuse laboratory'), infested pine logs are routinely taken in from the field at the end of the fall and kept in plastic barrels (0.6 m tall× 0.45 m diam.) with screen covers until adult emergence. Artificially increasing the number of degree days in the laboratory results in earlier emergence. In order to increase the window of time when adult wasps were available for behavioral bioassays at both the Syracuse laboratory and the USDA APHIS PPQ Otis Quarantine Laboratory, in Buzzards Bay, MA, USA (hereafter referred to as 'Otis laboratory'), the overwintering period was manipulated by bringing infested wood into the laboratory at different times during the winter. In the fall in Oswego county (2008, 2009) and Oneida county (2010), NY, Scots pine trees (*Pinus sylvestris* L.) that had been girdled prior to *S. noctilio* flight the preceding June, were felled, cut into bolts of about 0.6 m in length, and sealed at the ends (Waxlor End Sealant, Willamette Valley Co., Eugene, OR, USA).

For volatile collection studies conducted at the Syracuse laboratory in 2009, live insects were provided in two batches. Barrels of logs that were placed indoors at the end of 2008 and stored at approximately 25ºC produced adult males and females from February to March 2009. Barrels of logs that were brought inside in April 2009 produced adults from May to June 2009.

For the behavioral experiments performed in the quarantine facility at the Otis laboratory, a group of barrels was brought indoors on a monthly basis from November to March, from 2008 to 2011. The average environmental conditions for barrel storage were approximately 23.5ºC and 16% R.H. The barrels were monitored daily for *S. noctilio* emergence. By staggering when barrels full of logs were brought in from the cold over a period of 5 mo, a small but steady supply of *S. noctilio* adults were available for study between the end of January and mid-June.

Insects for Electrophysiology and Behavior As wasps emerged at the Otis Laboratory, they were placed individually in labeled, 18 ml plastic vials (Bioquip, Rancho Dominguez, CA, USA) with a piece of filter paper and a single air hole in the snap cap. Insects were not given an opportunity to mate. Vials of wasps were held in a walk-in environmental chamber at 23–24ºC and 42–61% R.H., with a light regime of 16:8 L:D, which was also used for behavioral bioassays. Vials of males and females were kept in separate plastic shoeboxes. Adults of *S. noctilio* are not known to feed, so no food was provided (Morgan and Stewart, 1966; Zondag, 1969). Wasps tested in behavioral bioassays were 0-5-d-old; wasps used in volatile collections were 0-10-d-old; and wasps used in electrophysiology were 2–6 d old. Bioassays were performed in the same walk-in environmental chamber between 0830 and 1600 h.

Lighting Bright lighting was used for odor collections and bioassays. At the Syracuse laboratory, a 250 W light source was used in addition to the ceiling lights. At the Otis laboratory, bright light was provided by 4 40 W Gro-Lux wide-spectrum fluorescent bulbs (Sylvania, Danvers, MA, USA) for odor collections and Y-tube olfactometer experiments, and 2 full spectrum fluorescent bulbs illuminated the working section of the wind tunnel (Aqualine T5 Reef White, 10 K, 54 W, 115 cm×16 mm, Aqua Medic, Germany). At the Otis Laboratory, light intensity measured above the aeration chambers and the Y-tube olfactometer was about 2,600 lux, and light intensity measured inside the

wind tunnel was about 6,600 lux at the ceiling, 5,050 lux at mid-height, and about 2,390 lux on the wind tunnel floor.

Volatile Collections In 2009 at the Syracuse laboratory, the first volatile collections were performed. Different numbers of live *S. noctilio* males or females (3–30) were kept in a Plexiglas® cage (61×46×46 cm), and volatile compounds were collected on SuperQ filters (30 mg/filter) by flushing the cage with charcoal-filtered air. Volatile collections started with the insect-free cage in the morning, then 3 males (or females) were added, after which the number of males (or females) was increased gradually to 10, to 20, and finally to 25–30. Plastic screening (70×50 cm) and Scots pine branches inside the cage afforded walking surfaces for the insects. Volatiles were collected for 30 min after which the filter was changed. The ends of used filters were sealed with Teflon tape, and the filters were wrapped in aluminum foil. Filters were eluted at the Penn State laboratory with 100 μl of a hexane:dichloromethane 1:1 mix.

Volatile collection experiments continued at the Otis laboratory in 2010 and 2011, and provided samples for both electrophysiological and chromatographic experiments. Virgin males or females, individually or up to 20, were placed in 4-L glass chambers with filter paper for collection of headspace volatiles. The headspace volatiles were sent through Teflon tubing to the Y-tube bioassay apparatus (described below), or collected using either a 100-μm polydimethylsiloxane-coated solid phase micro-extraction (SPME) fiber (Supelco, Bellefonte, PA, USA), a 20 mg activated carbon trap, or a 20 mg Porapak-Q trap (Analytical Research Systems, Inc., Gainesville, FL, USA). SPME fibers were conditioned between uses by baking for 45 min at the injection port temperature of 250°C. Charcoal and Porapak-Q traps were rinsed with 0.2 ml of either hexane, acetone, dichloromethane, or ethyl acetate. Solvents of differing polarity were used in attempts to separate unknown active volatile components. Volatile collection times ranged from 1 to 3,480 min. Control headspace volatiles were sampled from a chamber containing only filter paper. SPME fibers also were exposed to the headspace of individual males or females in 5 dram plastic vials for 1–95 min.

Y-tube Olfactometer Choice Bioassays A glass Y-tube olfactometer was used for choice bioassays inside the walk-in environmental chamber in the quarantine at the Otis laboratory. The olfactometer consisted of the glass "Y" (34 mm I. D.×20 cm base and 12 cm arms) with the two upwind arms at a 90º angle from each other (Analytical Research Systems, Inc., Gainesville, FL, USA). A Y-shaped piece of fiberglass window screen was cut 1 cm wide to fit inside the length of the Y-tube to allow wasps to have traction inside the glass tube. This was replaced with a clean piece between treatments. Air pressure was supplied by an oil-less air compressor. Air passed through a carbon filter, a splitter, bubbled through distilled water, through two flow meters set to 0.2 L/min, then through Teflon tubing attached to ports directly on the Y-tube olfactometer. In aeration bioassays, air traveled into the two 4-L glass aeration chambers prior to entering the Y-tube olfactometer. Cardboard was placed between the aeration chambers and the Y-tube, and white sheets were suspended around the sides of the Y-tube to remove visual directional cues.

Adult *S. noctilio* males were individually placed in the downwind entry port of the Y-tube olfactometer and were observed for 3 min. If the wasp travelled to the upwind 5 cm of one of the arms on the Y-tube olfactometer, its choice was recorded and the wasp was removed. If no choice was made within 3 min, it was marked as non-responsive and removed. In preliminary tests, the number of times the following behaviors commenced was recorded: resting, walking, grooming, vibrating abdomen, antennating, splaying of genitalia, flying, and fanning wings.

Preliminary tests revealed that males performed well in the Y-tube olfactometer, whereas females had difficulty maneuvering and rarely responded to odors in the confined tube. Subsequently, only males were used in the Y-tube choice tests. The first set of Y-tube bioassays involved odors naturally emitted by groups of males in two 4-L glass chambers. Air was pushed through those chambers and into the upwind arms of the Y-tube olfactometer. In the second set of bioassays, synthetic compounds in varying concentrations and combinations were applied to grey rubber septa (West Pharmaceutical Services, Kearney, NE, USA). The olfactometer was flipped every 3 replicates to avoid directional bias due to lighting or visual cues, and all glassware was cleaned between tests with Citranox odorless detergent (Alconox, Jersey City, NJ, USA) and hot water, rinsed with acetone, and baked at 140ºC for at least 1 h.

Gas Chromatography and Electrophysiology Antennal responses to odors were analyzed at the Otis laboratory using gas chromatography (GC) coupled with electroantennographic detection (EAD). An Agilent 6890 GC in splitless mode, initially with an HP-5 column (30 m×0.320 mm I. D.×0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA) and helium carrier gas, was used with a starting temperature of 50ºC for 0.75 min, ramped 20ºC/min to 250ºC, and then held for 15 min. In attempts to improve peak separation, this temperature program was made slower by changing the start temperature to 40ºC and the ramp to 10ºC/min, 5ºC per min, or 2.5ºC per min. The GC injector temperature was 250ºC, and the FID temperature was 275ºC. At the Otis Laboratory, both HP-5 (Agilent) columns and polar Stabilwax (Restek) columns with the same dimensions were used on both the GC-MS and GC-EAD in attempts to improve peak separation. Near the end of the

GC column, a glass Y-connector split the effluent to the flame ionization detector (FID) and to the EAD. The EAD effluent traveled through a temperature controlled arm (Syntech Temperature Controller, Kirchzarten, Germany) at 200ºC, and into the side of a glass L-shaped stimulus delivery tube (7 mm i.d.), then to the antennae. Charcoal-filtered air passed through a humidifier, a flow meter, and into the stimulus delivery tube with flow of 0.5 L/min.

Antennae were removed at the basal flagellomere, and the tip of the terminal flagellomere was cut off using a razor blade against a piece of filter paper moistened with insect saline (see Cooperband et al., 2008). Four antennae were inserted between glass capillary saline electrodes, placed at the end of the stimulus delivery tube, beneath an aluminum foil cover that acted as a faraday cage. Electrodes were connected to 2 mm-diam. capillary electrode holders (World Precision Instruments, Sarasota, FL, USA) that were held in place by magnetic micropositioners (Signatone Corp., Gilroy, CA, USA) on a stainless steel platform (Syntech, Kirchzarten, Germany). The whole platform was placed on a lab jack that allowed for vertical positioning of the antennal preparation at the end of the stimulus delivery tube. All metal objects were grounded.

The signal from the antennae was first amplified and filtered (Grass Amplifier Model P55, Astro-Med, Inc., West Warwick, RI, USA) and then filtered again (HumBug, Quest Scientific, North Vancouver, BC, Canada). That signal and the GC signal were sent to a six-channel integrator (Model 302, SRI International, Menlo Park, CA, USA). PeakSimple v. 3.85 software (SRI International, Menlo Park, CA, USA) was used to capture the data.

Chemical Identification and Synthesis of the Main Component An Agilent 6890 N GC coupled with a 5973 mass-selective detector (GC-MS) equipped with an Equity-5 capillary column (30 m×0.2 mm×0.2 μm; Supelco, Bellefonte, PA, USA) was used to analyze odor samples at the Penn State laboratory. The oven program started at 45°C, held for 1 min, followed by a temperature ramp of 10°C/min to 300°C, held for 5 min. The temperature of the injector and the transfer line was held at 260°C and 300°C, respectively. Helium was used as a carrier at an average linear velocity of 30 cm/s, and samples were injected splitless after which the split valve was opened at 0.75 min. The MS was operated in the electron impact (EI) mode. Compounds were identified based on their mass spectra (NIST 05) and Kovats indices (Van Den Dool and Kratz, 1963; Kovats, 1965).

Micro-scale epoxidation reaction (Attygale 1998) was performed on the major male specific compound, decen-1-ol, that was detected in the Super Q aeration samples to determine the double bond position and geometry. To 100 μl of an aeration sample, 100 μl of a 0.2 mg/ml solution of *m*-chloroperbenzoic acid (*m*-CPBA) in dichloromethane were added, and the reaction mixture was held at room temperature for an hour in a closed 1-ml screw-cap vial. The mixture then was washed with 10% solution of sodium carbonate and the phases were separated. The solvent was evaporated under a gentle stream of nitrogen from the organic phase, and the residue was resuspended in 50 μl of hexane. The reaction was performed on two aeration samples and the position of the double bond was determined by examining the EI-MS fragmentation pattern of the epoxide.

To synthesize (*Z*)-3-decen-1-ol a sample of commercially available 3-decyn-1-ol (ALFA Aesar, Ward Hill, MA, USA) was hydrogenated over P-2 nickel (Brown and Ahuja, 1973) at atmospheric pressure (conducted at Virginia Military Institute by THJ). Analysis by GC-MS showed this product to be >99% pure with a small amount (<1%) of decanol present.

Identification of Minor Components Minor component peaks were difficult to distinguish due to their presence in minute quantities and their similarity in retention time to the main component. However, male antennal responses in GC-EAD revealed a second strong antennal response immediately after the response to the main component. Based on the GC-MS results using the NIST database at the Otis Laboratory, a list of 12 tentative compounds was compiled, and those compounds were acquired commercially (Sigma-Aldrich) or synthesized (THJ). Synthetic suspected compounds were serially diluted to 100 ng/μl in hexane, and tested for male antennal activity using 0.2 to 1.0 μl injections in the GC-EAD. After testing tentative minor compounds, the major component was tested on the same antennae to verify the antennal preparation was still functioning and responsive. Tentative minor compounds that produced strong male antennal responses were tested for male attraction at varying concentrations and blends in the Y-tube olfactometer against hexane controls, using gray rubber septa (West Pharmaceutical Services, Kearney, NE, USA).

Wind Tunnel Choice Bioassays The two blends that attracted males in the Y-tube olfactometer were tested in a wind tunnel to evaluate attraction in both males and females. A laminar flow, push-pull wind tunnel, with a working section of 120 cm long×91 cm tall×75 cm deep, was used, with an air speed of 35–45 cm/s. The wind tunnel was located inside a walk-in environmental chamber and operated at 23.5°C and 50% R.H. Air from the environmental chamber passed through an activated carbon honeycomb filter (50.8×63.5×5 cm, 4.9 kg activated carbon per m^2, Air Handler, Niles, IL, USA) before entering the wind tunnel. Air exiting the tunnel passed through an additional activated carbon filter for cleaning before returning to the environmental chamber (50.8×63.5×2.5 cm, 3.4 kg activated carbon per m^2, Air Handler, Niles, IL, USA).

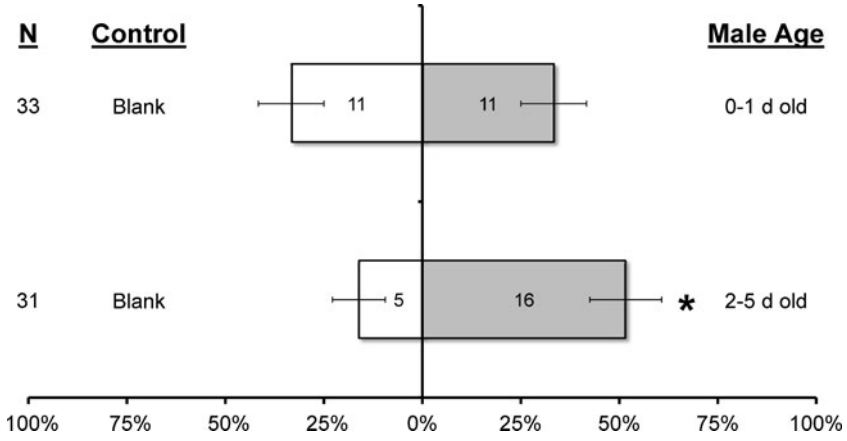

Fig. 1 Individual male *Sirex noctilio* tested in the Y-tube olfactometer were given a choice of odors emanating from either a blank jar or a jar with groups of males of different ages. *Asterisk* indicates significant difference between choices at α=0.05; *error bars* represent standard errors; *numbers in bars represent* number of individuals making each choice

Taking advantage of the phototactic nature of *S. noctilio* (Madden, 1974), two rubber septa lures in wind tunnel bioassays were hung 3.5 cm below the ceiling of the wind tunnel at the upwind end of the tunnel, and approximately 15 cm apart. A 5 cm disc cut from a black panel trap (AlphaScents, Bridgeport, NY, USA) was suspended directly below each lure. Male or female *S. noctilio* adults were released individually at the downwind end of the wind tunnel and observed for 3 min per trial. Each wasp was observed for upwind flight, whether they landed on or touched one of the two targets, and which target was chosen.

Statistical Analysis Dual choice bioassays conducted in the Y-tube olfactometer and in the wind tunnel were used to test the null hypothesis that both stimuli were chosen at the same frequency. Results were analyzed using the *Chi Square* Goodness-of-fit test. Significant differences between sides occurred when the test statistic $G \geq 3.841$ (df=1, α=0.05) (Sokal and Rohlf, 1995).

Results

Staggered Emergence Trees used in 2009 were naturally infested, whereas trees used in 2010 and 2011 were healthy trees that had been girdled prior to *S. noctilio* flight. Infested logs brought into the Otis Laboratory in five groups staggered over 5 months yielded 124, 388, and 735 *S. noctilio* wasps, in 2009, 2010, and 2011, respectively. These supplied the wasps used in aerations, electroantennograms, and behavioral bioassays at the Otis Laboratory. The sex ratios (males/total) for the respective years were 0.82, 0.73, and 0.70.

Male Response to Males When males were placed in groups of three or more in the 4-L aeration chamber, they were observed to engage in increased walking and flying while simultaneously everting their genitalia. These behaviors were also observed when placed in the Y-tube olfactometer. Additional behaviors observed during Y-tube experiments included vibrating their abdomen or antennae, and fanning their wings. Males were attracted to odors from groups of males that were 2-d-old or more, but not groups of males that were 1-d-old or less (Fig. 1).

Identification of Main Pheromone Component Headspace samples analyzed using GC-MS and GC-EAD revealed that groups of males 2-d-old or more released major amounts of a compound not detected in female emanations, or from males less than 2-d-old (Table 1). Of 12 individual males in vials that were sampled by SPME, two individuals produced the major component.

The main male specific compound found in the SuperQ samples has a Kovats index of 1,256 (on the Equity-5 column) and the EI-MS spectrum matched well (90%) with a few decen-1-ol isomers. The fragment *m/z* 138 indicated loss of water. The very low intensity of *m/z* 128 and the relatively low intensity of *m/z* 57 compared to that of *m/z* 55 suggested it was not decanal, which otherwise has the same molecular mass (156). There were no alkyl fragments or loss of alkyl fragments indicating branching of the chain. In order to determine the position of the double-bond, two samples containing the alcohol were subjected to epoxidation. Mass spectral analysis of the derivative showed

Table 1 Number of aerations at the Otis laboratory that contained (*Z*)-3-decen-1-ol out of total number of aerations conducted with males and females of different ages, either in groups or individually

Age	Grouping	No. of aerations	
		Male	Female
<2 d old			
	Single	0/10	0/1
	Multiple	–	0/2
2+ d old			
	Single	2/7	0/2
	Multiple	21/26	0/9

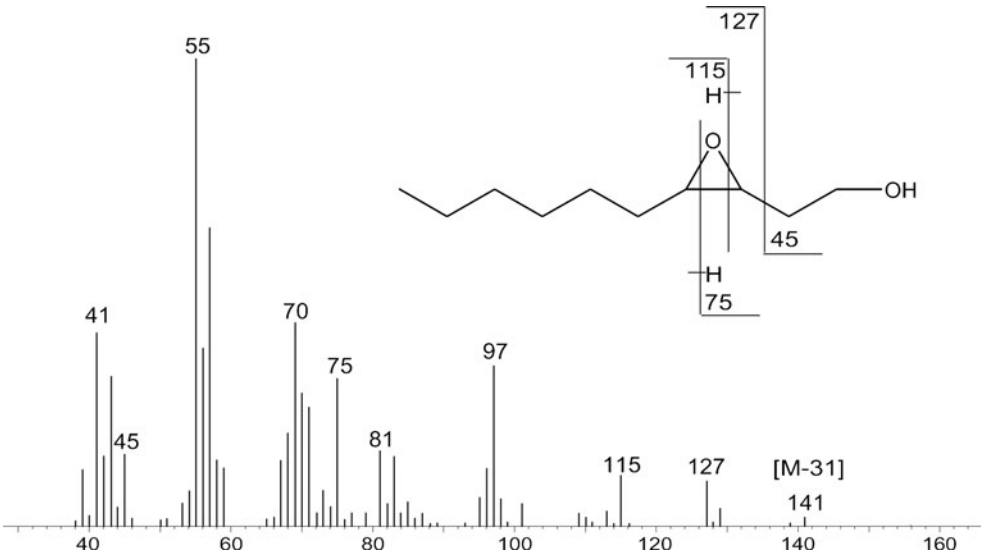

Fig. 2 EI-MS spectrum of the epoxide of (*Z*)-3-Decen-1-ol

diagnostic ions at *m/z* 127 and 45 corresponding to fragments formed by α-cleavage (adjacent to the epoxide group) on the alcohol-end of the molecule (Fig. 2). Transannular fragmentation resulted in the characteristic ions $C_7H_{15}CH{=}OH^+$ and $HOC_2H_4CH{=}OH^+$ at *m/z* 115 and 75, respectively. Thus, the double bond was identified to be at the 3rd position. The (*Z*)-isomer of 3-decen-1-ol was synthesized, as described above, and was used to determine the geometry of the double bond of the natural product. The retention times of the epoxide derivative of the synthetic (*Z*)-3-decen-1-ol and that of the natural product on the Equity-5 column matched perfectly, thus confirming the (*Z*)-geometry. No (*E*)-isomer of the synthetic standard was observed after derivatization.

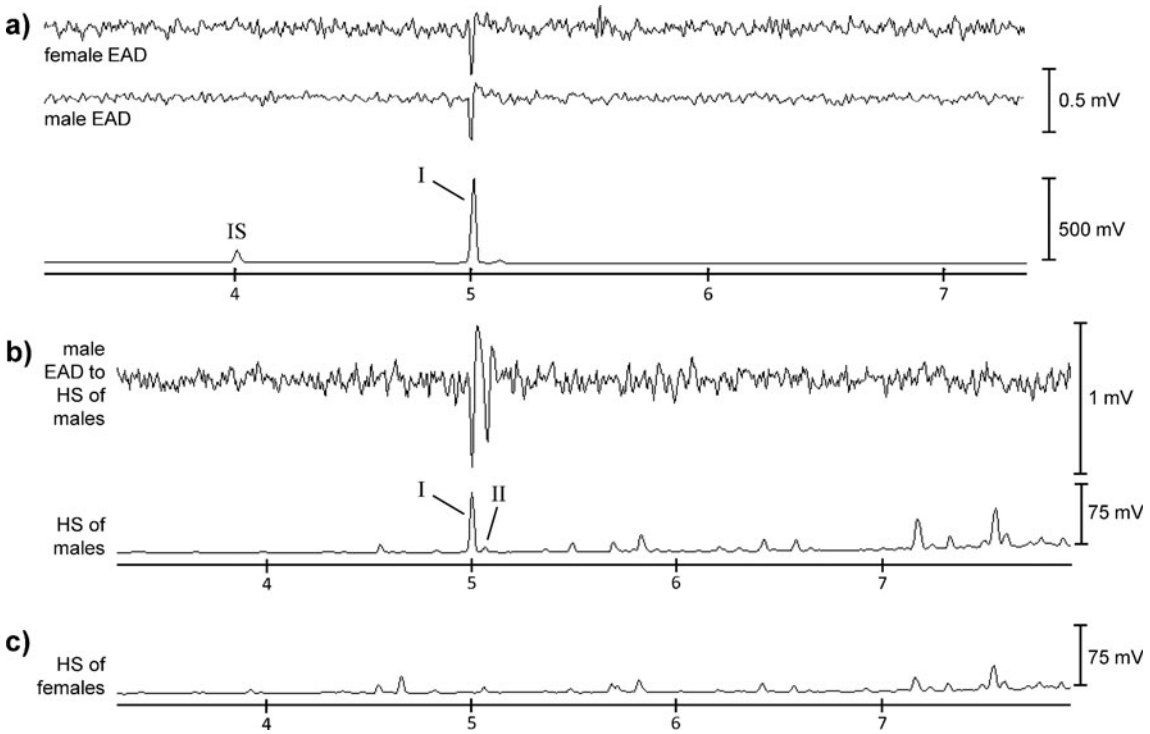

Fig. 3 Chromatographic traces of **a** GC-EAD showing no response to 5 ng of the internal standard (*E*)-cyclodecene (IS), and positive antennal responses by both male and female *Sirex noctilio* to 50 ng of synthetic (*Z*)-3-decen-1-ol (I); **b** GC-EAD of odors collected for 870 min on SPME fiber from the headspace of 4 *S. noctilio* males showing two typical male antennal responses to HS components (I) (*Z*)-3-decen-1-ol, and (II) unknown minor component; and **c** GC trace of odors collected for 870 min on SPME fiber from the headspace of 4 *S. noctilio* females, for comparison. *Tick marks* represent minutes of elution time on an HP-5 column with temperature program starting at 50°C and ramping 20°C/min to 250°C

Antennal Responses to Natural and Synthetic Compounds Strong antennal responses were elicited from both male and female antennae exposed to 10 to 100 ng of synthetic (*Z*)-3-decen-1-ol in the GC-EAD at the Otis Laboratory (Fig. 3a). When natural male headspace odor was tested against male antennae in the GC-EAD, two strong reproducible antennal responses were elicited: one response to (*Z*)-3-decen-1-ol, and another response immediately after the first, apparently in response to an unknown minor component that occurred in much smaller amounts (Fig. 3b). In comparison, the large peak of (*Z*)-3-decen-1-ol was absent from headspace odors collected from females (Fig. 3c).

Several smaller antennal responses corresponded to other small peaks from male odors. Using Kovats indices and peak shapes, male headspace peaks that elicited antennal responses in the GC-EAD were tentatively matched to male headspace peaks in the GC-MS, which was used to compile a list of possible identities for unknown minor compounds. The synthetic versions of those tentative compounds were obtained, and retention times of synthetic compounds were compared to the male headspace GC-MS results. The list of suspected minor components is given in Table 2, along with retention times and a summary of antennal responses.

The average ratio of the main active component and the peak suspected of being the minor component in SPME samples was 1000:8 (N=11). The minor component produced a very strong male antennal response despite its minute quantity.

Bioassays with Synthetic Blends The synthetic (*Z*)-3-decen-1-ol was serially diluted in hexane and applied in aliquots of 100 μl to rubber septa. Five concentrations were tested for

Table 2 Retention times (RT) of synthetic compounds using the HP-5 column and the polar Stabilwax column, compared to RT of odors of interest from headspace (HS) collections of male *Sirex noctilio*. For comparison, the major component is in *bold* and *shaded gray*. Kovats indices for synthetic compounds are also provided, as well as intensity of male antennal responses to synthetic compounds (strong, weak/inconsistent, or not detected)

	Kovats Index of Synthetic Compound		RT (min) of Synthetic Compound		Male EAD Response to Synthetic Compound[c]	RT (min) of Unknown Peak of Interest from HS		Synthetic RT Matches HS RT[d]
	Column		Column			Column		
Compound[a]	HP-5	Stabilwax[b]	HP-5	Stabilwax[b]		HP-5	Stabilwax[b]	
α-Pinene	939		2.64		*	2.63		Y
β-Pinene	980		2.95		*	2.96		Y
(+)-α-Longipinene		1376		4.97	n.d.		4.97	Y
Nonanal		1399		5.08	***		5.08	Y
Verbenol		1556		6.06	*		5.93	N
(1S)-(-)-Verbenone		1633		7.06	*		7.06	Y
Myrtenol		1686		6.83	n.d.		7.30	N
(*E*)-3-Decen-1-ol	1250		4.95		***	5.06[e]		N
(*Z*)-5-Decen-1-ol	1256		4.98		***	5.06[e]		N
(*Z*)-3-Decen-1-ol	**1255**	**1671**	**4.99**	**6.74**	***	**4.95-5.08[f]**	**6.71-6.83[f]**	**Y**
(*Z*)-4-Decen-1-ol	1259	1680	5.01	6.79	***	5.06[e]	6.80[e]	Y
9-Decen-1-ol	1266		5.06		n.d.	5.06[e]		Y
(*E,E*)-2,4-Decadienal[b]	1319	1823[b]	5.42	6.89[b]	***	5.43	6.90[b]	Y

[a] Compounds were matched between the GC-EAD and GC-MS equipped with a DB-5 or Stabilwax column using Kovats indices and shape, identified by GC-MS, and purchased from Sigma-Aldrich (St. Louis, MO, USA), except for (*E*)-3-Decen-1-ol, (*Z*)-5-Decen-1-ol, and (*Z*)-3-Decen-1-ol which were synthesized by THJ. All data in this table used a temperature program starting at 50°C for 0.75 min, ramping at 20°C/min to 250°C. Slower temperature programs used in attempts to improve peak separation are not shown

[b] Data shown for (*E,E*)-2,4-Decadienal on the Stabilwax column were collected on the instrument used for GC-MS. Data shown for the other compounds were collected on the instrument used for GC-EAD

[c] Three asterisks indicate a consistently strong antennal response, one asterisk indicates a weak or inconsistent response, n.d. indicates that an antennal response was not detected

[d] RT matches within 0.03 min

[e] Unknown peak of interest was hidden under large natural peak of (*Z*)-3-Decen-1-ol, but RT from antennal response was used as an estimate

[f] The peak of (*Z*)-3-Decen-1-ol was approximately 0.12 min wide in samples containing enough material to identify smaller peaks, thus concealing any smaller peaks that eluted during that time

male attraction in the Y-tube olfactometer. Responses by males to dilutions of (*Z*)-3-decen-1-ol alone did not differ significantly from the control, however, the least negative response was to 100 μg (*Z*)-3-decen-1-ol, so this concentration was used in other tests (Fig. 4a). All Y-tube tests conducted with synthetic compounds, and their statistical analyses, are summarized in Table 3. Only three blends tested were significantly different from the control.

When offered two-component blends of (*Z*)-3-decen-1-ol in combination with 1 μg of (*E*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, (*Z*)-5-decen-1-ol, (*E,E*)-2,4-decadienal, or nonanal, males were attracted to the two-component blend of 100:1 μg of (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol, but to none of the other blends. When offered a two-component blend of 10:1 μg of (*Z*)-3-decen-1-ol and nonanal, males chose the hexane control septum significantly more than the blend (Fig. 4b). When (*Z*)-3-decen-1-ol was tested for male attraction with different concentrations of (*Z*)-4-decen-1-ol, the only ratio tested that was chosen significantly more than the hexane control was 100:1 μg (*Z*)-3-decen-1-ol: (*Z*)-4-decen-1-ol (Fig. 4c).

When 1 μg of a minor compound was added to the attractive 2-component blend of 100:1 μg (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol, the resulting three-component blend of 100:1:1 μg of (*Z*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, and (*E, E*)-2,4-decadienal produced significant attraction by males. However, the addition of 1 μg of (*E*)-3-decen-1-ol or nonanal to the attractive 2-component blend removed the attraction. The addition of 1 μg of (*Z*)-5-decen-1-ol or nonanal to the attractive 3-component blend also removed attraction (Fig. 4d).

Wind tunnel flights and choices when males and females were offered either the 2- or 3-component attractive blends are shown in Fig. 5. Both males and females had increased upwind flight, increased choices (by landing on or touching either target), and increased correct choices (blend vs. hexane control) when the three-component blend was presented rather than the two-component blend.

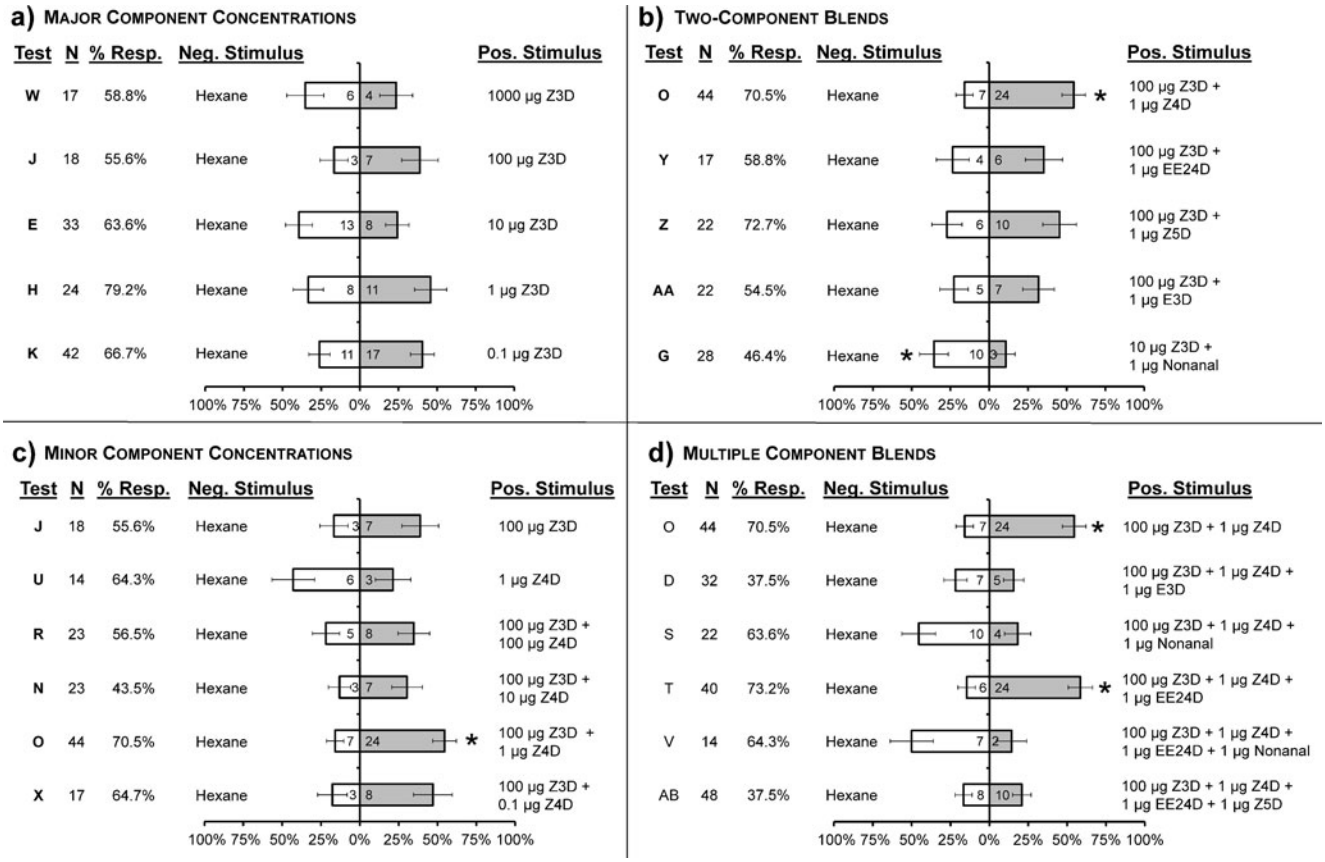

Fig. 4 Behavioral responses by male *Sirex noctilio* in the Y-tube olfactometer when presented with **a** different concentrations of (*Z*)-3-decen-1-ol (Z3D); **b** 2-component blends of (*Z*)-3-decen-1-ol in combination with 1 μg of either (*E*)-3-decen-1-ol (E3D), (*Z*)-4-decen-1-ol (Z4D), (*Z*)-5-decen-1-ol (Z5D), (*E,E*)-2,4-decadienal (EE24D), or nonanal; **c** (*Z*)-3-decen-1-ol and different concentrations of (*Z*)-4-decen-1-ol; **d** the attractive blends of 100 μg of (*Z*)-3-decen-1-ol and 1 μg of (*Z*)-4-decen-1-ol when combined with 1 μg of either (*E*)-3-decen-1-ol, nonanal, or (*E,E*)-2,4-decadienal. Percent responding (% Resp.) refers to the frequency that a choice was made. *Asterisk* indicates significant difference at α=0.05; *error bars* represent standard errors; *numbers in bars* represent number of individuals making each choice

Table 3 Tests conducted in the Y-tube olfactometer to evaluate attraction of male *Sirex noctilio* in response to different synthetic blends. *Percent* response refers to how many males travelled upwind in the Y-tube and made a choice. If the direction of the choices made were statistically significant, the direction towards the hexane control (Neg.) or the test blend (Pos.) is indicated. Compound amounts in blends tested are shown in μg per gray rubber septum

Test	Amounts of compounds (μg) in blend								*N*	% Resp.	*Chi-Sq.*	Choice
	Z3D	E3D	Z5D	Z4D	EE24D	Nonanal	α-pinene	β-pinene				
A	100	10		10					18	44.4%	0.0000	
B	10	1		1					28	50.0%	2.6566	
D	100	1		1					32	37.5%	0.3349	
E	10								33	63.6%	1.2020	
F	10					1	1	1	17	58.8%	1.6457	
G	10					1			28	46.4%	3.9765	Neg.
H	1								24	79.2%	0.4757	
I							1	1	32	62.5%	0.2003	
J	100								18	55.6%	1.6457	
K	0.1								42	66.7%	1.2957	
L	100							3	26	53.8%	0.2867	
M	100		10						20	50.0%	0.4027	
N	100			10					23	43.5%	1.6457	
O	100			1					44	70.5%	9.8572	Pos.
P	100			30					19	47.4%	0.1113	
R	100			100					23	56.5%	0.6986	
S	100			1		1			22	63.6%	2.6566	
T	100			1	1				41	73.2%	11.5647	Pos.
U				1					14	64.3%	1.0194	
V	100			1	1	1			14	64.3%	2.9419	
W	1,000								17	58.8%	0.4027	
X	100			0.1					17	64.7%	2.3583	
Y	100				1				17	58.8%	0.4027	
Z	100		1						22	72.7%	1.0107	
AA	100	1							22	54.5%	0.3349	
AB	100		1	1	1				48	37.5%	0.2227	

Z3D=(*Z*)-3-Decen-1-ol, E3D=(*E*)-3-Decen-1-ol, Z5D=(*Z*)-5-Decen-1-ol, Z4D=(*Z*)-4-Decen-1-ol, EE24D=(*E,E*)-2,4-Decadienal

Discussion

Since the first discovery of pheromones in the silk moth, *Bombyx mori* (Lepidoptera: Bombycidae) (Butenandt et al., 1959), most pheromone identifications have been in the Lepidoptera, Coleoptera, and social insects (Robacker and Hendry, 1977). In addition to the social Hymenoptera, volatile sex pheromones have been discovered in several families of the Apocrita: Eulophidae (Consoli et al., 2002), Eurytomidae (Leal et al., 1997), Ichneumonidae (Vinson, 1972), Pteromalidae (Yoshida, 1978), and Trichogrammatidae (Pompanon et al., 1997). In the Symphyta, however, few examples of volatile pheromones are available: Cephidae (Cossé et al., 2002), and Diprionidae (Jewett et al., 1978).

The pheromone described in this study likely functions as a lekking pheromone in *S. noctilio*. Such a pheromone would facilitate the formation of male swarms for females to fly through to mate. Similar behaviors have been described in other Hymenoptera (Marshall and Alcock, 1981). Although EAD of male headspace only reliably revealed two large antennal depolarizations, all three compounds (i.e., (*Z*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, and (*E,E*)-2,4-decadienal) are important and act together synergistically. Strong antennal responses were elicited from all three synthetic compounds. Some of the other compounds found in the male headspace could have originated from the host tree, since many of them are known plant odors. Although nonanal produced strong antennal responses, adding it to the attractive blend reversed attraction, and it appeared to have a repellent effect.

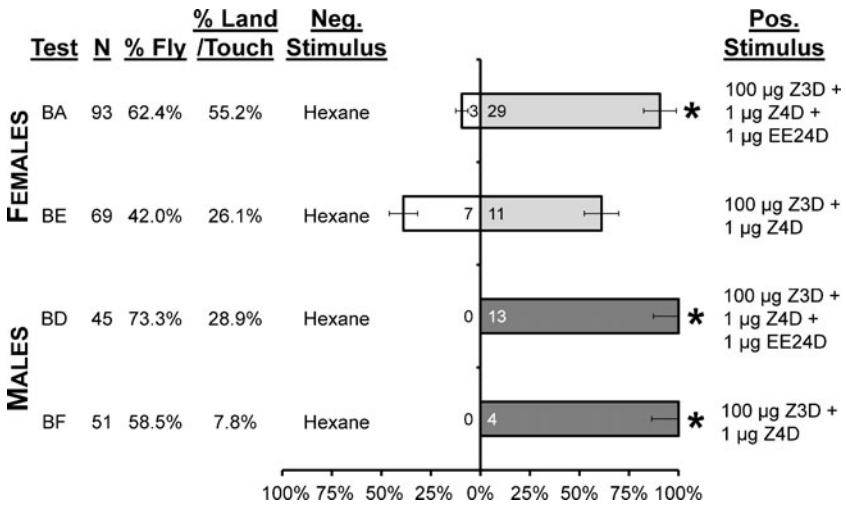

Fig. 5 Wind tunnel tests evaluated the attraction of female (*light gray bars*) and male (*dark gray bars*) *Sirex noctilio* to one of two 5-cm discs attached to lures with either the 2- or 3-component blends, or the hexane control (*white bars*). Displayed are the percent of males and females that flew upwind, the percent of those that flew upwind that made a choice by landing on or touching one of the two targets, and of those that made a choice, the percent that chose the hexane control vs. the lure with the synthetic blend. *Asterisk* indicates that choices were significant at α=0.05. Z3D=(*Z*)-3-decen-1-ol, Z4D=(*Z*)-4-decen-1-ol, and EE24D=(*E,E*)-2,4-decadienal. *Error bars* represent standard errors; *numbers* in bars represent number of individuals making each choice

The main component, (*Z*)-3-decen-1-ol, recently was described as a minor component of an aggregation pheromone of the banded alder borer, *Rosalia funebris* Mots. (Coleoptera: Cerambycidae) (Ray et al., 2009). The minor component (*E,E*)-2,4-decadienal was found in whole body washes of female *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), but its behavioral function has not been determined. It was also found as an insect semiochemical in two species of stink bugs (Ho et al., 2003; Aldrich et al., 2007). The components also are emitted by various plants, especially those in the Orchidaceae family (Kaiser, 1993).

This study used only virgin males and females, but preliminary tests that included potentially mated females gave less consistent results. For this reason, we think this pheromone may be attractive only to virgin females. Males appear to have a 2-d maturation period in which they do not yet produce the pheromone, but they are still attracted to it during that time. It is unknown whether males have a similar maturation period for mating. Regardless, if this pheromone proves useful in trapping programs, it could potentially capture wasps before they have an opportunity to mate or oviposit.

Behavioral differences were noted between males and females in the wind tunnel when offered a choice between two 5-cm discs suspended 15 cm apart, one with the hexane control, and the other with either the 2-component blend or the 3-component blend. The 3-component lure worked better than the 2-component lure for both males and females in terms of activating upwind flight, frequency of making a choice, and correct choice being made. Females showed a strong preference for only the 3-component lure, and not the 2-component lure. Some females chose the hexane control, whereas none of the males chose it. With both lures, upwind flight was activated more often in males than in females, whereas landing or touching the target occurred more often in females than in males.

The part of the male genitalia that is displayed during excitation, the latomeres, are known in siricids to be used for grasping the female during copulation (Schulmeister, 2001; 2003), and this was observed in the laboratory during mating. The males also have long, robust hind legs that hook forward to hold onto the female while mating (MFC, pers. obs.). The fact that the latomeres are everted when no females are present suggests that they could have other roles as well. Males splayed their latomeres when rapidly walking or flying, or immediately before taking flight, suggesting they may provide stabilization. However, genitalia were not always everted during flight (MFC, pers. obs.), and usually were everted when other males or their odors were present, suggesting a possible role in emitting pheromone.

One challenge for management of *S. noctilio* in the northeastern United States is the lack of efficient detection tools, particularly effective trap designs. The lack of a strong attractant has confounded trapping efforts in the delineation of the expanding range of this invasive species. An attractive pheromone could improve detection capabilities for this species by facilitating research to improve trap designs, and allowing control measures to be focused in problem areas. Field studies are underway to examine the utility of this pheromone for surveillance applications.

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Mating Disruption of Guatemalan Potato Moth *Tecia Solanivora* by Attractive and Non-Attractive Pheromone Blends

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Abstract The behavioral mechanisms of mating disruption in Guatemalan potato moth *Tecia solanivora* were studied using the sex pheromone components, (*E*)-3-dodecenyl acetate, (*Z*)-3-dodecenyl acetate, and dodecyl acetate, formulated in a 100:1:20-ratio mimicking the female-produced blend, and in a 100:56:100 off-blend ratio. The mode of action of these two blends was tested in mating disruption experiments in the field and in a greenhouse, as well as in a laboratory wind tunnel. Field treatments with both blends at 80 g pheromone per ha reduced male attraction to trap lures baited with 100 μg of female sex pheromone. In mesh-house treatments, these two blends were equally effective at reducing male attraction to traps baited with live females and mating of caged females. Subsequent flight tunnel tests corroborated that both blends reduced attraction of naive males to calling females, and pre-exposure of males with either dispenser blend for 24 hr resulted in a strongly reduced response to calling females. The pre-exposure effect was reversible, with males again responsive after 24 hr in clean air. The two dispenser formulations produced a similar effect on male behavior, despite the differences in blend composition. One mating disruption dispenser formulated with either the female-blend or off-blend elicited the same rate of male upwind attraction in a wind-tunnel bioassay. Sensory overload and camouflage, therefore, are contributing mechanisms to mating disruption using either blend. The off-blend, which is more economical to synthesize, is a valuable tool for further development of mating disruption against this major pest of potatoes in Latin America.

Key Words *Tecia solanivora* · Guatemalan potato moth · Mating disruption · Wind tunnel · Camouflage · Sensory imbalance · Sensory fatigue · Lepidoptera · Gelechiidae

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Introduction

Pheromone-mediated mating disruption has been developed against insect pests of labor-intensive horticultural crops, in part because consumers value reduced insecticide input in food crops (Witzgall et al., 2008; Jones et al., 2009; Ioriatti et al., 2011). Other examples of widespread use of pheromone use concern insects that cannot be controlled effectively with insecticides. A prominent example is gypsy moth *Lymantria*

dispar, a serious defoliator of deciduous forests in the Eastern USA. Mating disruption was adopted because it was neither effective nor sustainable to cover large forest areas with insecticide sprays (Sharov et al., 2002; Tobin et al., 2007).

Guatemalan potato moth *Tecia solanivora* (Povolny), (Lepidoptera: Gelechiidae), originates from Central America and has become an important invasive pest of potato in Central America and in the Andean region of South America. It causes great economic losses, both in potato fields and in storage facilities (Inoue et al., 1994; Pollet, 2001; Puillandre et al., 2008). The underground lifestyle of larvae renders control with insecticides difficult, and the resulting intensive use of highly toxic compounds from knapsack sprayers affects the health of the rural population and the environment (Feola and Binder, 2010).

In times of increasing food insecurity, more efficient methods must be developed to limit insect damage of major food crops. In addition, it remains a challenge to diminish and avoid the adverse environmental and health effects of insecticides. However, mating disruption has, with the exception of cotton, not been widely used in field crops (Baker et al., 1990; Witzgall et al., 2010), and the development of economic and efficient pheromone-based control methods against potato insects is a current research challenge.

The sex pheromone of *T. solanivora* is a blend of (*E*)-3-dodecenyl acetate (E3-12Ac), (*Z*)-3-dodecenyl acetate (Z3-12Ac), and dodecyl acetate (12Ac). Females release these three compounds in a 100:2:8 ratio. Lures, containing up to 10% of the *Z*-isomer, attracted as many males as calling females in wind-tunnel tests, but male attraction to an off-blend containing 50% of the *Z*-isomer was reduced (Nesbitt et al., 1985; Bosa et al., 2005, 2006). In comparison, the amount of 12:Ac in the blend had no effect on male attraction. An economical synthetic route produced a 100:56:100-blend, which was not attractive by itself, but resulted in trap shutdown in pheromone-treated potato fields (Bosa et al., 2005, 2006).

A crucial question for establishing mating disruption as a control method for Guatemalan potato moth is whether this off-blend is as effective as a blend that mimics the pheromone released by calling females. The goal of this study was to compare the mating disruption efficacy of two synthetic blends, and to investigate their behavioral modes of action in field and laboratory tests.

Methods and Materials

Insect Rearing Insects originating from potato fields near Bogota (Mosquera, Colombia) were reared in the laboratory on potato tubers (*Solanum tuberosum* cv. Maria) under a 12:12 hL:D photoperiod at 50% RH and 22°C. Pupae were separated by gender, according to the number of abdominal segments. Recently eclosed adults were collected daily and kept in 30×30×30 cm Plexiglass cages.

Pheromone Dispensers Sex pheromone, E3-12Ac, Z3-12Ac, and 12Ac, was formulated at 100:1:20 (female blend) to mimic the female-produced blend, and at 100:56:100 (off-blend), in polyethylene rope dispensers (Shin-Etsu Chemical Co., Tokyo, Japan) at 100 mg/dispenser. The estimated average pheromone release from these dispensers was 0.1 mg/day during the first 30 d after application. This estimate is based on the release of other dodecenyl acetates, (*E*)- and (*Z*)-8-dodecenyl acetate, from the same dispenser material (unpubl. res.).

Field Experiment A mating-disruption field experiment was carried out in a commercial potato crop of *S. tuberosum andigenum, cv.* Pastusa, near Siachoque (Boyacá, Colombia), comprising two adjacent blocks of 2 ha. Female-blend and off-blend dispensers were applied at a rate of 800 dispensers/ha. A third block, which was ca. 500 m apart, served as a control.

Male attraction was monitored with Tetra traps (Phero. Net AB, Alnarp, Sweden), baited with 3 different lures in the center of each block. The trap lures were red rubber septa containing 100 μg of the female blend (N=9), mating-disruption dispensers formulated with the female blend (N=6), and mating-disruption dispensers formulated with the off-blend (N=6). Trap lures were formulated with pheromone diluted in heptane (100 μl/lure). Captures were recorded weekly throughout the crop season, lasting up to 4 mo.

Mesh-house Experiment Behavioral observations and trap tests were done in a mesh house (metal screen, 3×3 mm mesh) at the Corpoica Biological Control Laboratory (Mosquera, Colombia), which was divided into five compartments of 8.3×8.2 m (2.4 m high), separated with solid plastic sheets. The walls toward the outside and the roof were made of metal screen. Each compartment contained 80 potato plants (*cv.* Pastusa) in the flowering stage. The two treatments (9 mating disruption dispensers with female blend, and the off-blend, respectively) and control (dispensers containing no pheromone) were separated by empty compartments. Treatments were replicated (N=4) in different chambers, after replanting potatoes.

The effect of the dispenser treatments was evaluated by using 3 water traps per treatment, each baited with live females, and three mating cages (1×0.5×0.5 m), each containing a pair of unmated 2-d-old moths. Trap captures were recorded over 1 wk, after 100 unmated males were released into each treatment and control (N=4). After 5 d of exposure, the mating status of 15 females in each treatment was determined by dissecting for the presence of a spermatophore (N=4).

Wind-tunnel Bioassays Behavioral observations were carried out in a wind tunnel made of Plexiglas, with a flight section of

180×60×60 cm, illuminated from above at 6 lux. The wind speed was 30 cm.sec^{-1}, with incoming air (21–24°C, 50–60% RH) filtered through active charcoal (Witzgall et al., 2001). Insects were kept individually, in 2.5×12.5-cm holding glass tubes closed with gauze, in the wind-tunnel room for 1 hr before testing. Insects were tested individually, in batches of 10 (N=5). Experiments were carried out 1–4 hr after onset of the scotophase.

Two-day-old unmated males were observed for 3 min to 5 treatments, in randomized order. Treatments were: five 2-d-old calling females (emission rate 0.02±0.01 ng/female; Bosa et al., 2005) in a 2.5×12.5-cm glass tube, either one female-blend or one off-blend mating disruption dispenser (see above), and five calling females placed 10 cm from either a female-blend or an off-blend mating-disruption dispenser in a side-by-side arrangement. Odor plumes from dispensers did not touch the wind-tunnel walls, according to visualization tests with titanium chloride. The wind tunnel was decontaminated with ethanol and ventilated for 1 day between tests with different dispenser blends.

For each treatment, the following parameters were recorded: the number of males that activated from rest (activation), left the glass tube by flight (take-off), flew upwind over 1 m, approached the source (within <25 cm), landed at the source, and the time spent in the glass tube before take-off.

In a subsequent pre-exposure experiment, males were kept with one mating-disruption dispenser, either the female- or off-blend, in a 30×30×30 cm mesh cage for 24 hr. The dispenser was enclosed in a mesh pouch, so as to exclude direct contact. Pre-exposed males were tested 1 and 24 hr after they had been removed from the dispenser cage. Two males were tested simultaneously, one pre-exposed male and one naïve male (N=50), to five calling females or to mating disruption dispensers.

Statistical Analyses All data were analyzed using R (version 2.8.0, R Development Core Team, 2008). In order to test the effect of week, treatment, and trap on the number of individuals captured per trap in the field, a general linear model (GLM) with a Poisson distribution was used. For the mesh-house experiment, the effect of dispenser treatments on the percentage of males captured per trap per wk, and on the percentage of males exhibiting different steps of the pheromone-induced behavioral sequence (activation, take-off, upwind flight, source approach and landing), were tested using a GLM with a binomial distribution (N=4), followed by pair-wise comparisons when a significant treatment effect was found. For wind-tunnel tests in which males were flown individually, a χ^2 test was calculated to test for differences in the frequencies of insects exhibiting each behavioral step (see above) between treatments. In addition, time to take-off was compared between treatments using a Wilcoxon test. In wind-tunnel tests with two males, the difference between control and pre-exposed males was tested for significance using a McNemar's χ^2 test. For multiple comparisons, P values were adjusted with a false discovery rate (Benjamini and Hochberg, 1995).

Results

Field Experiments The number of insects captured in mating-disruption field treatments differed among treatments (χ^2=24.7, df=2, P<0.001) and among trap lures within treatments (χ^2=105.8, df=6, P<0.001; Fig. 1). Fewer males were captured with 100-µg monitoring lures in pheromone treatments. Traps baited with mating-disruption dispensers containing pheromone in the female-blend ratio, captured more males in the off-blend than in the female-blend treatment (t=5.86, df=1, P=0.016) and in the control field (t=12.1, df=1, P=0.001) (Fig. 1).

Mesh-house Bioassays Behavioral observations showed that males in the off-blend treatment were less active, displaying less pheromone-induced orientation flights than in the female-blend treatment. However, male attraction to traps baited with females was reduced in both treatments, compared to in the control (Table 1). The difference between pheromone treatments and control was significant (F=22.6, df=2, P<0.001; GLM ANOVA), but not the difference between treatments. The number of matings in cages was reduced compared to control, for both pheromone treatments (F=23.6, df=2, P<0.001; GLM ANOVA), but there was no difference between the two treatments (Table 1).

Wind-tunnel Bioassays Few naive males flew upwind toward mating-disruption dispensers, with no apparent difference in attraction to female- and off-blend mating-disruption dispensers. Attraction to five calling females was reduced in the presence of these dispensers (χ^2=9.865 P=0.005 female blend; χ^2=13.310, P=0.002 off-blend), and did not differ from attraction to dispensers alone (Fig. 2). The time for male activation and take-off toward calling females increased with the presence of dispensers in the wind tunnel (Table 2).

In mating-disruption field treatments, males are continuously exposed to pheromone, but males may also move between potato fields and natural vegetation and thus leave and re-enter the pheromone treatment. The effect of pheromone pre-exposure was studied by keeping males close to one mating-disruption dispenser in a cage for 24 hr. Males were then placed in clean air for 1 or 24 hr before testing in the wind tunnel. Male attraction to calling females was reduced (landing at pheromone blend: χ^2=29.032, P< 0.001; off-blend: χ^2=22.781, P<0.001) 1 hr after males were removed from pheromone pre-exposure treatments

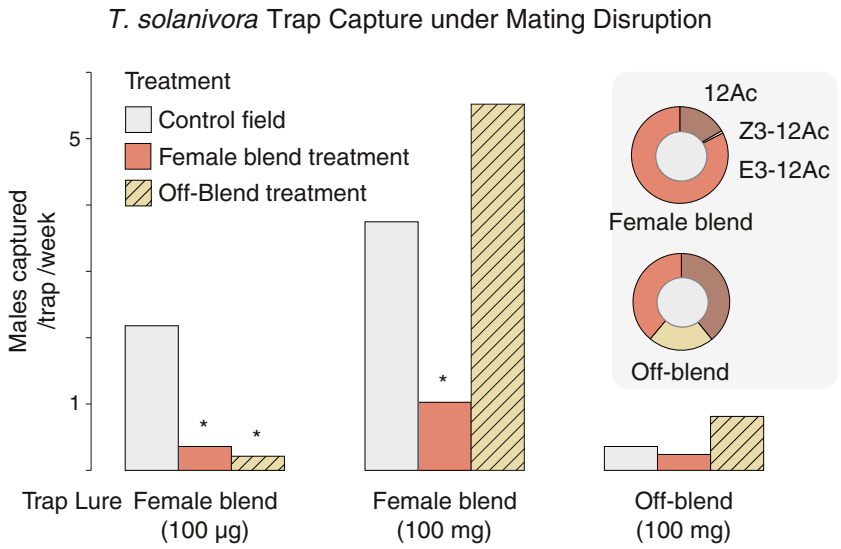

Fig. 1 Trap capture of Guatemalan potato moth, *Tecia solanivora*, males in two mating disruption treatments: a pheromone blend mimicking the female-produced pheromone blend, and an off-blend. Trap lures were the female blend formulated at 100 µg, and single mating disruption dispensers containing the female blend or the off-blend (100 mg/lure). Asterisks show differences ($P<0.05$) in trap captures between treatments and control

(Fig. 3). However, when males were kept in clean air for 24 hr after pre-exposure, there was no difference between control and treatment. Interestingly, there was a tendency for off-blend pre-exposed males to respond more strongly after 24 hr in clean air (Fig. 3). Pre-exposure, followed by a 24 hr period in clean air, did not have an effect on male attraction to mating-disruption dispensers (data not shown).

Presence of mating-disruption dispensers in the wind tunnel, or pheromone pre-exposure, increased the time males spent in the holding-glass tube before flying out (take-off; Table 2). The female blend and off-blend dispenser increased time to take-off, compared to calling females. Males took longer to fly toward the off-blend than to the female-blend dispenser, showing that the two blends did not produce identical behavioral effects. Pre-exposed males, 1 hr after removal from the pheromone atmosphere, took longer to respond than did naive males; however, 24 hr after removal from the pheromone atmosphere their time to take-off was similar to that of naive males (Table 2).

Discussion

Competitive and non-competitive mechanisms can contribute to pheromone-mediated communication disruption, and include false trail following, camouflage, sensory overload, and sensory imbalance (Cardé and Minks, 1995; Miller et al., 2006). These may act individually or in combination, and may vary according to pheromone blend and dispenser density, and in relation to the pheromone-mediated behavior of a target species and its population biology (Bengtsson et al., 1994; Knight, 2007; Jones et al., 2008).

The female sex pheromone of *T. solanivora* contains small amounts of the Z-isomer of the main component, E3-12Ac, while an economical large-scale synthesis of the main component produces an isomer blend with large amounts of Z3-12Ac. This off-blend produced shutdown of pheromone trap captures during the first pheromone air-permeation tests in potato fields (Bosa et al., 2005, 2006). The goal of this study was to compare the efficacy of the off-blend with a blend similar to the female pheromone for communication disruption, prior to organizing area-wide mating disruption campaigns against *T. solanivora*.

The conclusion from our trapping and behavioral studies in a pheromone-permeated atmosphere under field and semi-field conditions, and from male-attraction tests in a wind tunnel, is that both blends are equally effective for disruption. Attraction to traps baited with a female-pheromone blend, to traps baited with calling females in a mesh house, and to calling females in a wind tunnel, was reduced in the presence of both blends; captures or attraction rates, differed little between both blend treatments. Even pre-exposure to either of these two blends had similar effects on male attraction to

Table 1 Disruption of attraction and mating in Guatemalan potato moth, *Tecia solanivora*, in a mesh-house experiment, in which 68-m^2 compartments were treated with dispensers containing either a female blend or an off-blend dispensers. Treated compartments contained traps baited with calling females ($N=4$) and mating cages ($N=4$). Numbers in the same row followed by the same letter are not different ($P>0.05$), according to GLM ANOVA

	Control	Female blend	Off-blend
Males captured in traps baited with live females	24±9 a	3±1 b	6±3 b
Percent mating in mating cages	85±10 a	38±7 b	45±9 b

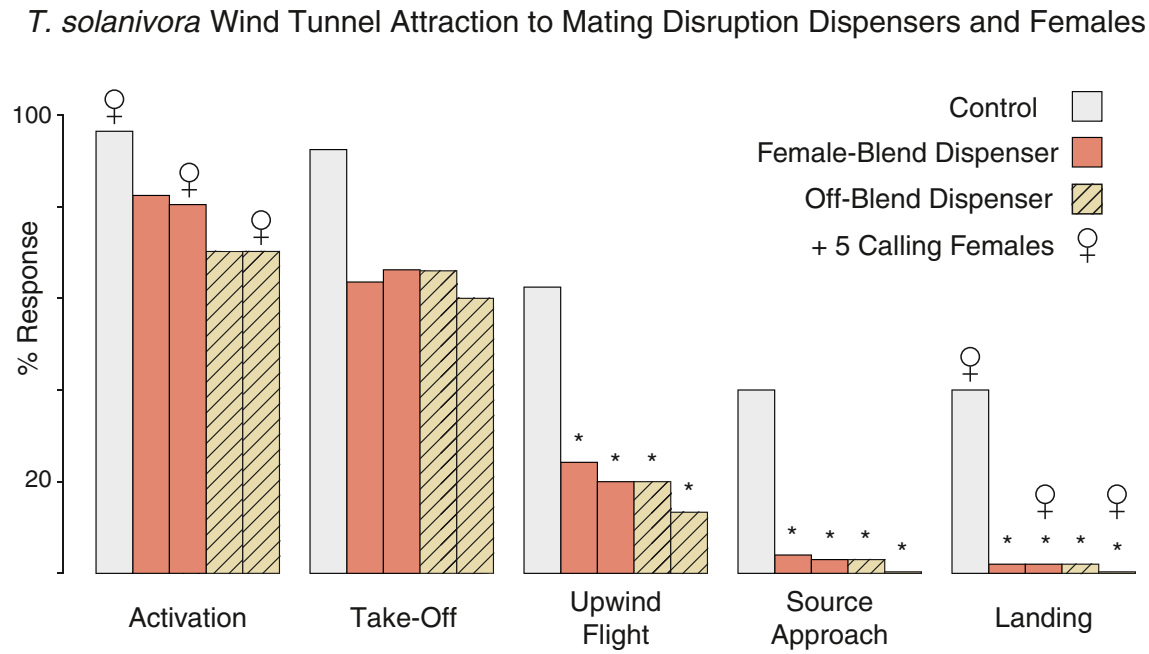

Fig. 2 Wind-tunnel attraction of Guatemalan potato moth, *Tecia solanivora*, males to five calling females, mating disruption dispensers, and a combination of both. Asterisks show significant differences ($P<0.05$) in the various behaviors between treatments and control

females or mating disruption dispensers, showing that the behavioral mode of action of the two blends is similar.

That the behavioral mode of action to these two blends is similar, might be due to the main pheromone component alone being sufficient for attracting males (Bosa et al., 2005, 2006). Nonetheless, the behavioral effect of the two blends is not identical. Differences in male attraction to female-blend and off-blend mating disruption dispensers in potato fields confirmed that males can discriminate between the two blends. In addition, males took longer to become

Table 2 Times to activation, take-off, and initiation of upwind flight in a wind tunnel for males of Guatemalan potato moth, *Tecia solanivora*, in response to 5 calling females, with and without synthetic mating disruption dispensers present. Males were either naive, or pre-exposed to female-blend or off-blend pheromone for 24 hr, and tested either 1 or 24 hr after removal of the pre-exposure stimulus. Males were tested individually. Numbers followed by the same letter are not different ($P>0.05$), according to Wilcoxon rank sum tests. Asterisks indicate differences ($P>0.05$) between naïve and pre-exposed males, according to a paired *t*-test

Lure		Pre-exposure treatment	Activation [S]	Take off [S]	Upwind flight [S]
Calling females	Dispenser	(Time after stimulus removal)			
5 Females		– [b]	6.8±0.9 a	1.7±0.2 a	
–	Female-blend	–	9.8±2.9 b	12.1±2.6 b	
–	Off-blend	–	27.5±4.8 c	15.7±4.9 b	
5 Females	Female-blend	–	13.2±3.8 cd	15.1±5.0 b	
5 Females	Off-blend	–	42.8±6.3 d	13.9±3.4 b	
5 Females [a]	–	– [c]	9.2±2.7	8.9±1.7	44.4±5.9
”		Female-blend, 1 hr [a]	30.9±6.1*	21.0±5.6 *	42.7±6.3
5 Females [a]	–	– [c]	22.8±4.2	14.9±2.4	43.0±5.9
”		Off-blend, 1 hr [b]	39.1±6.6 *	16.3±3.2	38.8±9.8
5 Females [b]	–	– [c]	14.0±4.3	8.0±1.5	54.8±7.5
”		Female-blend, 24 hr [b]	8.1±2.1	8.3±1.6	46.6±7.2
5 Females [b]	–	– [c]	29.4±7.2	9.9±2.5	41.1±6.4
”		Off-blend, 24 hr [b]	15.4±4.7	8.9±1.7	52.7±7.6 *

[a] Naive and pre-exposed males were tested simultaneously to allow for fluctuations in female pheromone release

[b] Naive males

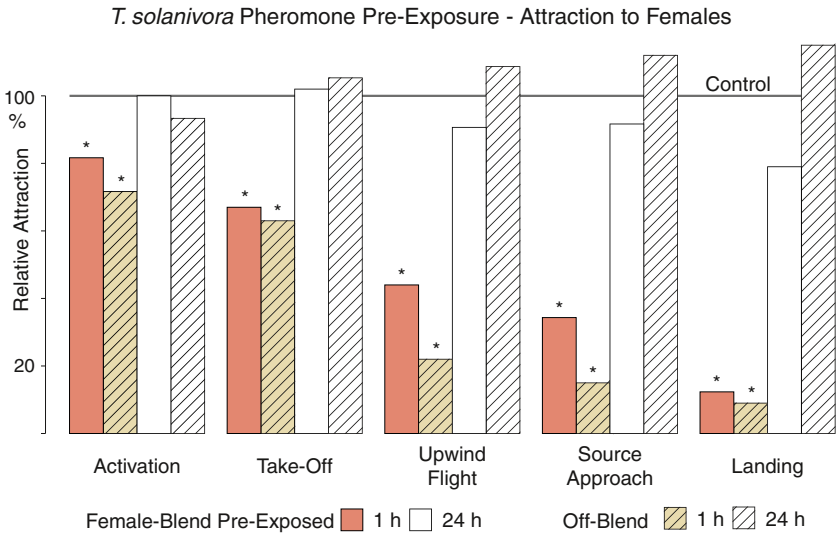

Fig. 3 Wind-tunnel attraction of Guatemalan potato moth, *Tecia solanivora*, males to five calling females, 1 and 24 hr after a 24-hr pre-exposure to a mating disruption dispenser, containing either the female blend or an off-blend. Asterisks show significant differences ($P<0.05$) in the various behaviors between treatments and control

activated and fly upwind in the presence of off-blend dispensers, compared to female-blend dispensers, in accord with visual observations in the mesh house showing lesser flight activity in the presence of the off-blend treatment.

False-trail following, to either of the two dispenser formulations, is not an important contributing behavioral mechanism, because we observed low upwind flight attraction rates in the wind tunnel and mesh house. However, males may respond differently to high dispenser-release rates in the field. Field-trap captures indicated that false-trail following could occur in response to female-blend dispensers, although significant captures of insects were recorded only within the off-blend treatment or the control field. Males may also become attracted to, but interrupt their upwind flights in the vicinity of, dispensers, without becoming trapped (e.g., Carde et al., 1977).

Camouflage of pheromone plumes released by calling females, in addition to sensory fatigue, also may be be a contributing mechanism, as evidenced by a strong reduction of male attraction to calling females in the wind tunnel, induced by one mating disruption dispenser. Camouflage and sensory overload are non-competitive mechanisms, leading to successful communication disruption in several species (Schmitz et al., 1997; Judd et al., 2005; Miller et al., 2006; Stelinski et al., 2008). Our results show that both mechanisms may interact in *T. solanivora*, but it is not possible to dissociate camouflage and sensory overload without further physiological studies at the antennal and brain level, which is now experimentally possible, even in small moths such as Oriental fruit moth or codling moth (Piñero et al., 2008; Trona et al., 2010).

Exposure to either female- or off-blend dispensers induced a significant sensory overload, due to either peripheral adaptation and/or central habituation, that persisted for at least 1 hr after males were placed into a pheromone-free atmosphere. Sensory overload is, therefore, an important contributing mechanism that is likely to affect males within pheromone-treated fields. This pre-exposure effect was, however, reversible; males exposed to the off-blend became more responsive after 24 hr in clean air than males that had been exposed to the pheromone blend. Increased behavioral sensitivity after a first exposure to female pheromone has been reported for the cotton leafworm *Spodoptera littoralis* (Anderson et al., 2003, 2007).

Reduced responsiveness, as a consequence of exposure to high pheromone concentrations, is important in mating disruption of other species (Rumbo and Vickers, 1997; Schmitz et al., 1997; Stelinski et al., 2003). Treatment of large areas should produce a better effect, as movement of males between treated and untreated plots, or flights along borders, will offset the effect of sensory overload.

Off blends have been shown to be effective for mating disruption (Minks and Cardé, 1988; Bengtsson et al., 1994; Evenden et al., 1999). In the case of *T. solanivora*, use of the off-blend is advantageous in that synthesis costs are substantially lower than for manufacturing a real female blend. The long-term use of blends deviating from the female blend may, however, lead to resistance. The small tea tortrix *Adoxophyes honmai* (Lepidoptera: Tortricidae) became resistant to mating disruption after long-term use of a single component of its four-component pheromone blend. Resistance was overcome by employing the natural blend (Mochizuki et al., 2002; Mochizukii et al., 2008; Tabata et al., 2007a, b). One strategy to counteract resistance in *T. solanivora*, while also improving the efficacy of mating disruption control, might be to combine off-blend air permeation with mass trapping using female-blend dispensers (see Fig. 1). Availability of a female attractant also could contribute to the development of sustainable control of this important potato pest. A female lure, based on volatiles identified from flowering potato plants (Karlsson et al., 2009), is currently under development.

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Pheromonal Mediation of Intraseasonal Declines in the Attractivity of Female Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis*

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Abstract During the breeding season, female red-sided garter snakes (*Thamnophis sirtalis parietalis*) produce and express a sexual attractiveness pheromone that elicits male courtship behavior. Composed of a homologous series of saturated and monounsaturated methyl ketones, this pheromone is expressed in female skin lipids. Recent studies have shown that the sexual attractivity of unmated female garter snakes declines as the breeding season progresses. Here, we investigated whether temporal changes in the quantity and/or quality of the female sexual attractiveness pheromone are responsible for the observed loss of attractivity. Female red-sided garter snakes were collected immediately following spring emergence and held under natural conditions for the duration of the breeding season. Behavioral experiments confirmed that unmated females become significantly less attractive to males within two weeks of emergence from hibernation. Additionally, these females had lower estradiol concentrations at two weeks post-emergence. Subsequent chemical analyses revealed qualitative variation between the pheromone profiles of newly emerged females and those of females at two weeks post-emergence. Together, these results support the hypothesis that changes in the female sexual attractiveness pheromone are responsible for declining post-emergence female attractivity in garter snakes.

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Introduction

Mate attraction is often a costly endeavor that may be energetically expensive (Vehrencamp et al., 1989) or increase an individual's risk of mortality (Mougeot and Bretagnolle, 2000; Shine et al., 2004). Thus, for many species, attracting potential mates on a constant basis would not be beneficial, particularly at times when environmental conditions are less favorable for reproduction (e.g., colder temperatures, less abundant food resources). Mate attraction, therefore, tends to be limited to discrete breeding seasons with reproductive signals varying seasonally such that individuals attract the opposite sex only at certain times of the year. For example, many vertebrates vary their physical appearance [e.g., American goldfinch (*Carduelis tristis*; McGraw, 2004); striped plateau lizard (*Sceloporus virgatus*; Weiss, 2006)] or behavior patterns between breeding and non-breeding seasons [e.g., green anole lizard (*Anolis carolinensis*; Neal and Wade, 2007); golden-collared manakin (*Manacus vitellinus*; Day et al., 2007)].

Interseasonal variation also occurs with respect to chemically-mediated mate attraction as a number of species vary the concentration and/or composition of chemical signals between breeding and non-breeding seasons [e.g., Australian tree frog (*Litoria splendida*; Wabnitz et al., 2000); red-sided garter snake (*Thamnophis sirtalis parietalis*; LeMaster and Mason, 2001a); ringtailed lemur (*Lemur catta*; Scordato et al., 2007)]. Changes in vertebrate chemical signals that occur intraseasonally, such as during the course of a single breeding season, are more poorly

understood. Nevertheless, intraseasonal variation in chemical signaling can have important implications for signal producers by helping to further minimize the time they are subject to fitness costs associated with mate attraction.

The red-sided garter snake presents an opportunity to study intraseasonal variation in a vertebrate pheromone. The female sexual attractiveness pheromone of this species has been identified chemically as a homologous series of long-chain saturated and monounsaturated methyl ketones (Mason, 1992, 1993), thus allowing for both quantitative and qualitative chemical comparisons. Further, mate recognition relies extensively upon male detection of the pheromone, which elicits stereotypical male courtship behaviors that only occur in a reproductive context and are easily recognized by observers (Crews et al., 1984; Mason et al., 1989; LeMaster and Mason, 2002). Production of the pheromone, which is sequestered within the skin lipids of the female's dorsal surface, appears to be under estrogen control, as exogenous estrogen treatment induces both attractivity in females and expression of female-like pheromone profiles in males (Crews, 1976, 1985; Kubie et al., 1978; Parker, 2010). Previous studies have found the chemical profile of this pheromone to exhibit interseasonal variation, as well as both inter- and intrapopulational variation (LeMaster and Mason, 2001a, 2002, 2003). Intraseasonal variation has been hypothesized to occur (O'Donnell et al., 2004; Shine et al., 2005a), but to date no studies have investigated this hypothesis.

In a series of behavioral experiments, Shine et al. (2003a, 2005a) and O'Donnell et al. (2004) observed that female red-sided garter snakes, including unmated females, are less attractive to males as time progresses following emergence from hibernation. Shine et al. (2005a) demonstrated that the apparent decline in attractivity occurs relatively rapidly and independently of changes in male behavior. Further, Shine et al. (2005a) tested male courtship responses to isolated female skin lipid extracts and found males to exhibit more intense courtship toward the lipids of females newly emerged from hibernation. Based on their respective behavioral observations, both Shine et al. (2005a) and O'Donnell et al. (2004) suggested that changes in the chemical profile of the female sexual attractiveness pheromone during the course of the breeding season could be responsible for declining female attractivity. However, no chemical analyses were performed in either of these studies, and it has yet to be confirmed that the pheromone indeed exhibits intraseasonal variation.

Our study investigated whether the chemical profile of the red-sided garter snake sexual attractiveness pheromone exhibits the intraseasonal variation necessary to account for the observed decline in female attractivity. To test this hypothesis, we first performed behavioral experiments to confirm there was a decrease in attractivity. Subsequently, we conducted chemical analyses of the sexual attractiveness pheromone to examine potential changes in its quantity and/or quality. Given the apparent role of estrogen in pheromone expression, we hypothesized that pheromonal changes would be accompanied by hormonal variation; thus, we also assayed plasma estradiol concentrations. If the loss of attractivity results from changes in the sexual attractiveness pheromone profile, then we would expect a correlation between male behavior and variation in (1) overall pheromone concentration and/or (2) concentration of individual pheromone components when compared between newly emerged and post-emergence females. Further, as pheromone expression is believed to be estrogen regulated, we would expect the decline in female attractivity to be associated with decreased estradiol concentrations.

Methods and Materials

Study Population Red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected during the 2003 spring breeding season in the Interlake region of Manitoba, Canada (50°31'58"N; 97°29'71"W). The field site, located in an abandoned gravel quarry, contains an underground hibernaculum where approximately 35,000 garter snakes spend the winter months (Shine et al., 2006). Spring emergence marks the beginning of an annual cycle similar to that of other local populations of the species: participation in a brief mating period at the den site, migration to the summer feeding grounds, and then an autumn return to the den (Gregory, 1977).

Experimental Animals All female garter snakes utilized were collected immediately upon emergence from the hibernaculum to ensure they were unmated. Also, as pheromone composition is correlated with female body size (LeMaster and Mason, 2002), females of similar snout-vent length (SVL) were utilized (SVL: 60–66 cm). At the beginning of our experiment (week zero), a group of females (N=27) was collected and subsequently divided into 3 treatment groups corresponding to 3 time points: week zero, week one, week two. One group, week zero newly emerged females (N=8), were immediately bled for hormone sampling and then euthanized for pheromone collection. Females in the other treatment groups, hereafter referred to as 1 wk post-emergence females (N=9) and 2 wk post-emergence females (N=10), were kept in outdoor arenas [1×1×1 m; constructed of nylon cloth (Moore et al., 2000)] for 1 and 2 wk, respectively, at which time they were tested in behavioral trials. Additional newly emerged females, referred to as week one and week two newly emerged females, were collected at the week one and week two time points (N=7 and 9, respectively) and tested in behavioral trials on

those days. These newly emerged females served as controls to demonstrate that female attractivity declines independently of changes in male behavior.

Immediately prior to behavioral experiments, groups of actively courting males (~500 / group) were indiscriminately collected from the hibernaculum. Subsets of these males were randomly selected and utilized in behavioral trials the day of their collection, marked with a non-toxic pen, and released back at the den upon completion of that day's experiments. Thus, a new group of males was used at each time point. All procedures used in this study were approved by the Oregon State University Animal Care and Use Committee (protocol number: 2661) and the Manitoba Wildlife Animal Care Committee (protocol number: 2002–06). This research complied with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was carried out under the authority of Manitoba Wildlife Scientific Permit WSP 03009.

Courtship Trials Courtship trials were carried out in the previously described arenas which have been used extensively for similar behavioral studies within this species (e.g., Mason and Crews, 1985; Moore et al., 2000; Shine et al., 2000a; LeMaster and Mason, 2002). To limit confounding effects due to weather conditions, an array of ten arenas was set up, allowing multiple trials to be conducted simultaneously. Behavioral trials were performed on 2 d separated by a 1 wk interval: week one (N=16 trials: 7 trials of week one newly emerged control females; 9 trials of 1 wk post-emergence females), and week two (N=19 trials: 9 trials of week two newly emerged control females; 10 trials of 2 wk post-emergence females). For each behavioral trial, 10 actively courting males were placed into an arena and allowed to acclimate for 5 min. A female, randomly-selected from one of the appropriate treatment groups, then was placed into the arena. Snakes were allowed to interact undisturbed for 5 min after which time the number of males actively courting the experimental female was counted by an observer blind to the treatment. To be considered actively courting, a male had to be observed chin-rubbing or tail-searching along the dorsum of the female. These behaviors are seen only in a reproductive context in this species and are thus indicative of male sexual behavior (Crews et al., 1984).

The behavioral methods utilized in this study were similar to those used by Shine et al. (2005a) with modifications to allow for pheromone collection. First, we attempted to prevent mating, as it is known to immediately reduce female attractivity via the male's deposition of a copulatory pheromone (Whittier et al., 1985; Shine et al., 2000b; Mendonça and Crews, 2001). The presence of the copulatory pheromone potentially could confound behavioral and chemical analyses, thus mating was prevented by placing adhesive tape over the cloaca of each female and limiting the interaction time to a single 5 min period. Despite these precautions, two matings occurred during the experiments. The mated female trials, both in the 1 wk post-emergence treatment group, were excluded from all subsequent statistical analyses. Second, whereas Shine et al. (2005a) conducted simultaneous choice tests, males in each trial of our study were presented with a single female to prevent the potential intermingling of pheromones from the different female treatment groups.

Hormone Analysis: Blood Sampling and Radioimmunoassay Blood samples (approximately 200 μl; collected with heparinized 1-cm^3 syringes and 25-g needles) were obtained from the caudal veins of zero week newly emerged females (N=8), 1 wk post-emergence females (N=7), and 2 wk post-emergence females (N=10); 1 wk and 2 wk post-emergence samples were collected immediately following behavioral trials. The two mated females were excluded from the hormone analysis. Blood samples were stored on ice until return to the field station, where they were centrifuged to separate the plasma. Plasma samples then were stored at −4°C until return to Oregon State University, where they were stored at −80°C until further analysis.

The plasma samples were analyzed for estradiol concentrations following direct radioimmunoassay procedures described in detail by Lutterschmidt et al. (2004) and Lutterschmidt and Mason (2005). The methods used for direct radioimmunoassay of plasma estradiol were previously validated for female red-sided garter snakes by Lutterschmidt and Mason (2009). Briefly, 100 μl of each plasma sample were spiked with 2,000 cpm of tritiated steroid (Amersham Biosciences, Piscataway, NJ, USA) and incubated for 18–24 hr to determine individual sample recovery. Steroids were extracted twice from each plasma sample with anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm (37°C) water bath. Hormone extracts then were reconstituted in 500 μl of phosphate-buffered saline and incubated overnight at 4°C. To determine the percent hormone recovery for each plasma sample, a 50-μl aliquot was removed from each reconstituted sample and placed in a scintillation vial. Following incubation in toluene-based scintillation fluid for 12 h, the radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter. The resulting data were compared to the radioactivity of the initial spike of tritiated steroid and used to calculate the percent sample recovery obtained during the extraction procedure for each individual plasma sample.

The remainder of each reconstituted sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were incubated with 12,000 cpm tritiated steroid (2,4,6,7,16,17-[^{3}H]Oestradiol, Amersham Biosciences, Piscataway, NJ, USA). Samples and maximum binding tubes also were incubated with 100 μl

of antiserum at 4°C for 18–24 h (estradiol antibody E-6006 from Wein Laboratories, Inc., Succasunna, NJ, USA). Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials, incubated in toluene-based scintillation fluid, and the radioactivity of each sample quantified in a scintillation counter.

All estradiol samples were analyzed in a single hormone assay. Mean sample recovery was 72% (± 1.9 SE), well within the range of accepted estradiol extraction efficiencies (e.g., Lynch et al., 2006; Taylor et al., 2004; Almli and Wilczynski, 2009; Lutterschmidt and Mason, 2009; Lutterschmidt et al., 2009). All hormone concentrations were corrected for individual recovery variation. The intra-assay coefficient of variation was 7.7%. Limits of detectability were approximately 1 pg/ml for estradiol. In some instances (<14% of the samples), estradiol concentrations were below the limits of detectability. To retain these samples in our statistical analyses, and because of the high sensitivity of our estradiol assay, we assigned each undetectable plasma sample the limit of detectability (i.e., 1 pg/ml).

Pheromone Collection Based on the behavioral results, and in addition to the week zero newly emerged females (N=8) euthanized at the beginning of the study, we also euthanized subsets of the week two newly emerged females (N=8) and 2 wk post-emergence females (N=8). Snakes were euthanized via an overdose of Brevital sodium (methohexital), then placed individually in glass beakers (500-ml), covered with 100 ml of 100% hexane (C_6H_{12}), and allowed to soak overnight (Mason et al., 1989, 1990). Excess solvent was eliminated from the resulting skin lipid extracts under reduced pressure by rotoevaporation at 35°C. The remaining residues were weighed on a digital scale (Ohaus Adventurer Pro AV 264), resuspended in 5 ml of fresh hexane, and stored at −20°C in 9-ml glass vials with polyethylene lined caps.

In order to isolate the methyl ketones that comprise the sexual attractiveness pheromone, skin lipid extracts were fractionated using column chromatography (as described by Mason et al., 1989). In brief, skin lipid extracts were loaded onto glass columns (11 mm ID) packed with alumina (activity III); the columns were eluted with 30 ml of hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity (LeMaster and Mason, 2002). For each sample, we collected the fractions containing the appropriate methyl ketones (fractions 5 and 6); excess solvent was removed by rotoevaporation (35°C). The resulting residues were weighed on a digital scale, resuspended in 2 ml of fresh hexane, and stored at −20°C in 9-ml glass vials with polyethylene lined caps until further analysis.

Pheromone Analysis To examine qualitative variation in pheromone expression, we determined the number and relative concentrations of structurally unique methyl ketones expressed by each female. The methyl ketones were identified using a Hewlett Packard 5890 series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m×0.22 mm ID; Hewlett Packard); helium was used as the carrier gas. Oven temperature was initially held at 70°C for 1 min, then increased by 30°C/min to 210°C, where it was held for 1 min, then increased by 5°C/min to 310°C, where it was held for 5 min. After identifying the methyl ketones, we used peak integration to calculate the relative concentrations of individual methyl ketones in each sample. Compounds and peak areas were identified using ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph–mass spectrometer. Variations in pheromone quantity were examined by calculating the amount of methyl ketones (to the nearest μg/ml) expressed per unit skin surface area (μg/cm^2) for each female. Multiplication of snout–vent length by mid-body circumference was used to determine a general index of skin surface area for each female (Mason et al., 1990). To determine the concentration of methyl ketones, an external standard of known concentration (methyl stearate–10 μg/ml; 0.5 μl aliquot) was injected into the GC/MS with each sample.

Statistical Analysis Statistical analyses were performed using Jandel SigmaStat software (version 3.11, Systat Software, Inc.) and R (v.1.8-8). Student's *t*-tests were used to compare female SVL and mass among treatment groups in the behavioral trials. Differences in the proportions of males courting females in the various treatments were examined using *Chi-square* analyses (Zar, 1999). As the propensity for male courtship behavior changes over the course of the breeding season (O'Donnell et al., 2004), courtship only was compared within specific time points. Estradiol levels, after being log-transformed to correct for non-normality, were compared using one-way analysis of variance (ANOVA) with Tukey's *post hoc* tests used to make pairwise comparisons.

The SVL and mass of females used in pheromone analyses were compared between treatments with one-way ANOVA, which was also used to examine differences in the amounts of skin lipids and methyl ketones expressed by females. Fisher's exact tests were used to compare differences in the proportions of females expressing each individual methyl ketone with Tukey-type *post hoc* tests used to make necessary pairwise comparisons (Zar, 1999). Differences in the relative concentrations of individual methyl ketones were analyzed using the Multi-Response Permutation Procedure (MRPP) in the vegan package for R with pairwise comparisons carried out by the same procedure but

with a new group excluded each time (McCune et al., 2002; Parker and Mason, 2009). MRPP is a non-parametric multivariate analysis used to detect differences among groups (McCune et al., 2002; Mielke and Berry, 2007). Coordinates for a non-metric multidimensional scaling plot to visually represent differences in individual pheromone profiles were also generated with the vegan package. All graphics were created in SigmaPlot (version 9.01; Systat Software, Inc.).

Results

Behavioral Experiments Snout-vent length (SVL) and mass did not differ significantly between week one newly emerged females and one week post-emergence females (Student's *t*-tests; SVL: $t=-1.042$, $P=0.318$; mass: $t=1.414$, $P=0.183$) or between week two newly emerged females and two week post-emergence females (Student's *t*-tests; SVL: $t=-0.384$, $P=0.706$; mass: $t=1.065$, $P=0.302$). Two week post-emergence females were courted by a lower proportion of males than were week two newly emerged females tested on the same day ($\chi^2=4.686$, *d.f.* = 1, $P=0.030$, Fig. 1). There was no difference in the proportion of males courting 1 wk post-emergence females compared to week one newly emerged females ($\chi^2<0.001$, *d.f.* = 1, $P=1.000$, Fig. 1).

Hormone Analysis Estradiol concentrations varied significantly between treatments (one-way ANOVA, $F=4.532$, $P=0.022$, Fig. 2). Pairwise comparisons (Tukey's tests) indicated that estradiol concentrations were lower in 2 wk post-emergence females compared to week zero newly emerged females ($P=0.017$). Despite similar estradiol concentrations between 1 wk and 2 wk post-emergence females ($P=0.328$), the estradiol concentrations of 1 wk post-emergence females did not differ from those of week zero newly emerged females ($P=0.378$). This lack of a statistically significant difference may be due to relatively low sample sizes ($N=8$, 7, and 10 for zero week newly emerged, 1 wk post-emergence, and 2 wk post-emergence females, respectively) and consequent low power.

Pheromone Quantity Neither SVL nor mass differed between the week zero newly emerged females, week two newly emerged females, and 2 wk post-emergence females from which pheromone samples were collected (one-way ANOVA; SVL: $F=2.671$, $P=0.093$; mass: $F=0.968$, $P=0.396$). The total amounts of skin lipids (mg) extracted from females did not vary significantly between treatments (one-way ANOVA, $F=3.021$, $P=0.070$, Table 1). Once variation in skin surface area was taken into account, the average concentration ($\mu g/cm^2$) of methyl ketones expressed on the skin surface of females also did not differ between treatments (one-way ANOVA, $F=1.723$, $P=0.203$, Table 1).

Pheromone Quality Following GC/MS analysis of the methyl ketone fractions, eighteen structurally unique long-chain methyl ketones were identified across the samples. Of these, nine were identified as long-chain saturated methyl ketones, ranging in size from 394 to 506 daltons; the remaining nine were identified as long-chain ω-9 *cis*-unsaturated methyl ketones, ranging in size from 420 to 532 daltons (Fig. 3). For sixteen of the methyl ketones, the proportion of females expressing a particular compound did not differ between treatments (Fisher's exact tests, $P>0.05$). Two compounds, 420 dalton and 434 dalton methyl ketones, were expressed by a

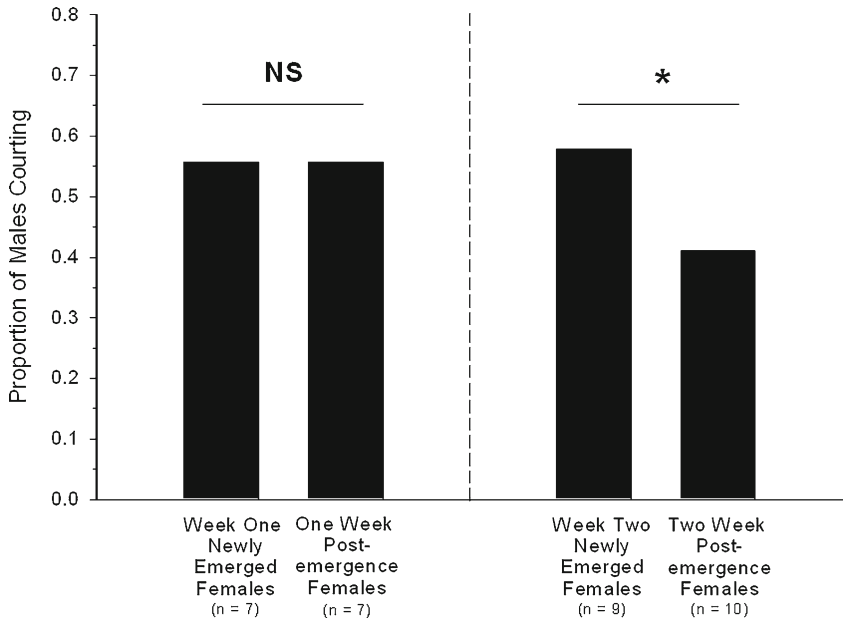

Fig. 1 Total proportion of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) courting females at two time points during the breeding season. Females were newly emerged from hibernation or were post-emergence females having been out of hibernation for either one or two weeks. * $P<0.05$

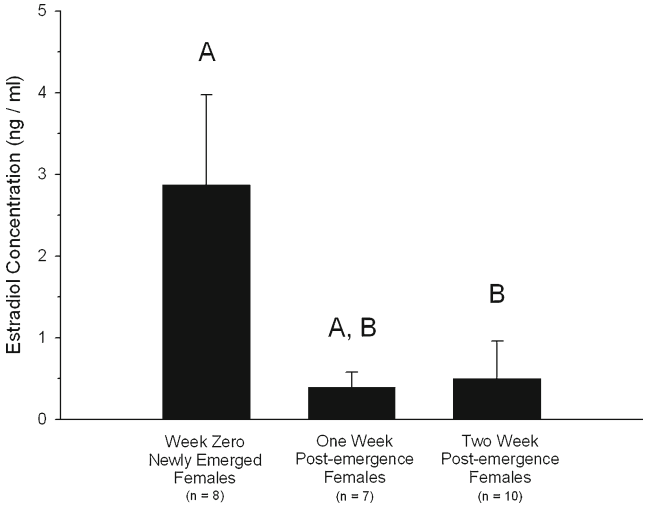

Fig. 2 Mean (+ SE) plasma estradiol concentrations of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) newly emerged from hibernation, 1 wk post-emergence, and 2 wk post-emergence. Bars followed by different letters are statistically significant at $P<0.05$

greater proportion of females in the 2 wk post-emergence treatment (Tukey-type pairwise comparisons, $P<0.001$).

In terms of the relative concentrations of individual methyl ketones, pheromone profiles varied between treatments (MRPP, $A=0.118$, $P=0.002$, Fig. 4a). Week zero newly emerged females and week two newly emerged females did not differ from one another (MRPP, $A=-0.035$, $P=0.895$) although each newly emerged group was different from the two week post-emergence females (MRPP; $A=0.167$, $P<0.001$ and $A=0.149$, $P<0.001$, respectively). When plotted on a non-metric multi-dimensional scaling plot, the pheromone profiles of 2 wk post-emergence females clustered together and were distinct from the profiles of newly emerged females (Fig. 4b).

Discussion

In accordance with previous work by Shine et al. (2005a), the results of our behavioral trials indicate the attractivity of female red-sided garter snakes declines following emergence from hibernation. Subsequent chemical analyses revealed that the pheromone profiles of newly emerged females were significantly different from those of females that were two weeks post-emergence. Specifically, we observed differences in the proportions of females expressing certain methyl ketones as well as changes in the relative concentrations of individual methyl ketones. These results indicate that the female sexual attractiveness pheromone exhibits variation following emergence that may account for the intraseasonal decline in attractivity. Furthermore, estradiol levels were significantly lower in two week post-emergence females than in week zero newly emerged females, supporting the hypothesis that estrogen has a role in mediating the change in pheromone expression.

Based on the current and previous studies, it is clear that the attractivity of female red-sided garter snakes declines intraseasonally. However, the biological significance of the decline has yet to be thoroughly investigated despite the proposals of interesting hypotheses. Shine et al. (2005b), for example, suggested that a decline in female attractivity following emergence could benefit female fitness by limiting the amount of time they are subject to intensive courtship. The vigorous courtship characteristic of this species can be costly for females as it increases the risk of mortality (Shine et al., 2001, 2003b, 2004) and impedes dispersal to the summer feeding grounds, reducing the time females are able to feed (Shine et al., 2005b). Alternatively, declining female attractivity could be beneficial to male fitness: by directing courtship toward newly emerged females, which are relatively weak and less able to resist copulation attempts, males increase their chances of successfully mating (Shine et al., 2005a).

The decline in female attractivity observed in the current study occurred independently of changes in male behavior. Two week post-emergence females elicited less courtship than did week two newly emerged females tested on the same day, indicating that a post-emergence change occurred with respect to females. Male courtship behavior did not differ between week one newly emerged females and one week post-emergence females; however, this may be a reflection of testing conditions. As males in our study were presented with only a single female, it is possible that the attractivity of one week post-emergence females had not declined greatly enough for males to forgo the opportunity

Table 1 Snout-vent length, mass, total skin lipids, and overall methyl ketone concentration (per unit skin surface) for female red-sided garter snakes (*Thamnophis sirtalis parietalis*) in three treatment groups from which pheromone samples were collected. Values represent the mean ± SE

Female treatment group	Snout-vent length (cm)	Mass (g)	Skin lipids (mg)	Methyl ketones (μg/cm^2)
Week zero newly emerged ($N=8$)	64.1±0.4	94.0±3.6	19.38±1.66	1.53±0.35
Week two newly emerged ($N=8$)	62.9±0.7	87.3±3.5	19.50±1.20	2.88±0.96
Two week post-emergence ($N=8$)	62.7±0.3	90.1±3.2	24.75±2.27	3.24±0.61
Statistical analysis (ANOVA)	$F=2.671$, $P=0.093$	$F=0.968$, $P=0.396$	$F=3.021$, $P=0.070$	$F=1.723$, $P=0.203$

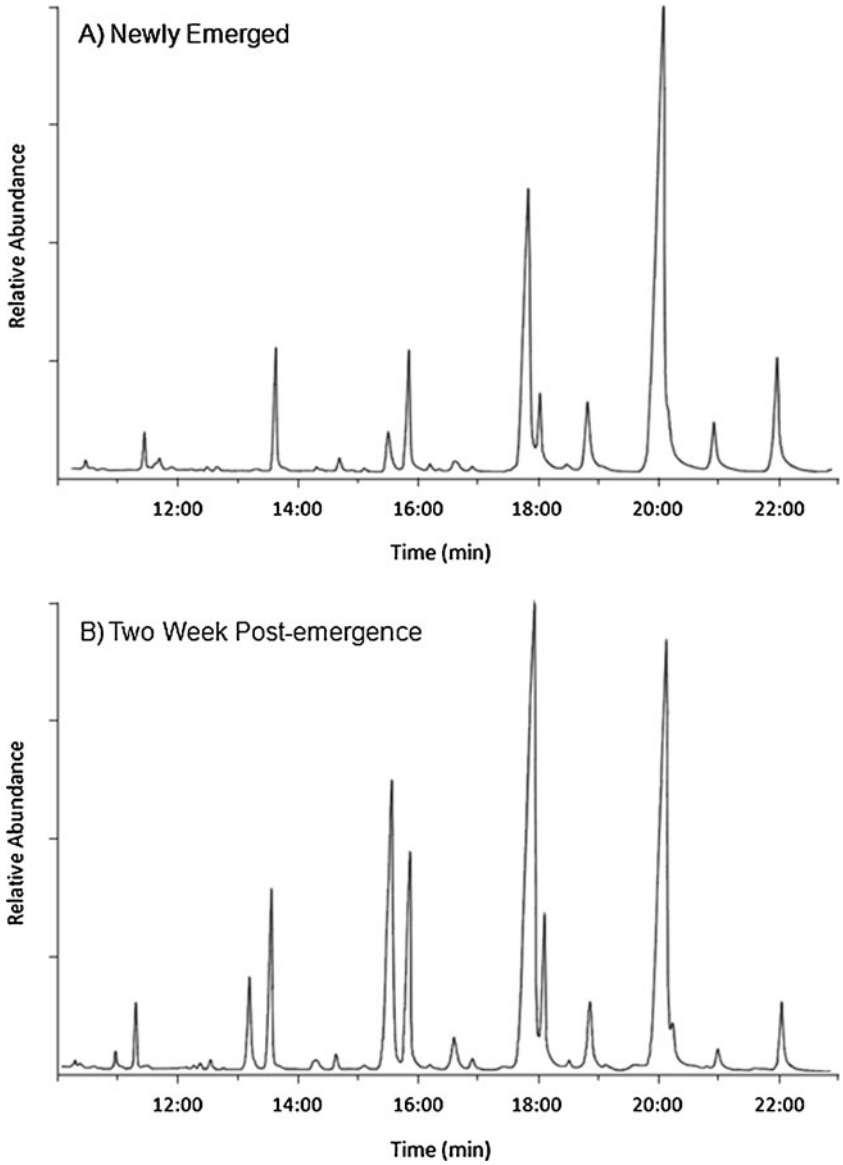

Fig. 3 Chromatograms depicting representative pheromone profiles of **a**) zero week newly emerged and **b**) 2 wk post-emergence female red-sided garter snakes (*Thamnophis sirtalis parietalis*)

to mate when no alternative female was present. Also of note is that we are describing an intraseasonal decline in attractivity distinct from that which occurs in female garter snakes after mating. The post-mating loss of attractivity results from the male's deposition of a copulatory pheromone, which reduces male courtship (Whittier et al., 1985; Shine et al., 2000b; Mendonça and Crews, 2001). In the current study, mating was prevented during behavioral trials in order to avoid the confounding effects of this mating-induced loss of attractivity.

Since the chemical identification of the female sexual attractiveness pheromone, a number of studies have demonstrated the importance of methyl ketones in garter snake reproduction (Mason et al., 1989, 1990; LeMaster et al., 2001; LeMaster and Mason, 2001b, 2002, 2003; Parker, 2010). Males are not only able to discriminate among females with qualitatively different methyl ketone profiles (LeMaster and Mason, 2002, 2003), but also can discern differences among methyl ketone profiles presented as isolated samples devoid of other female cues (Mason et al., 1989, 1990; LeMaster and Mason, 2002). Similar to previous studies that demonstrated that methyl ketone profiles vary according to species, population, season, and body size (LeMaster and Mason, 2001a, 2002, 2003; Mason and Parker, 2010), our results show variation in the relative concentrations of individual methyl ketones between treatments. In addition, we found that two methyl ketones (420 and 434 daltons, respectively) were expressed by a greater proportion of two week post-emergence females. This was somewhat unexpected as variation in the proportion of females expressing particular methyl ketones has not been documented previously.

As both methyl ketones differentially observed in post-emergence females were compounds present in low

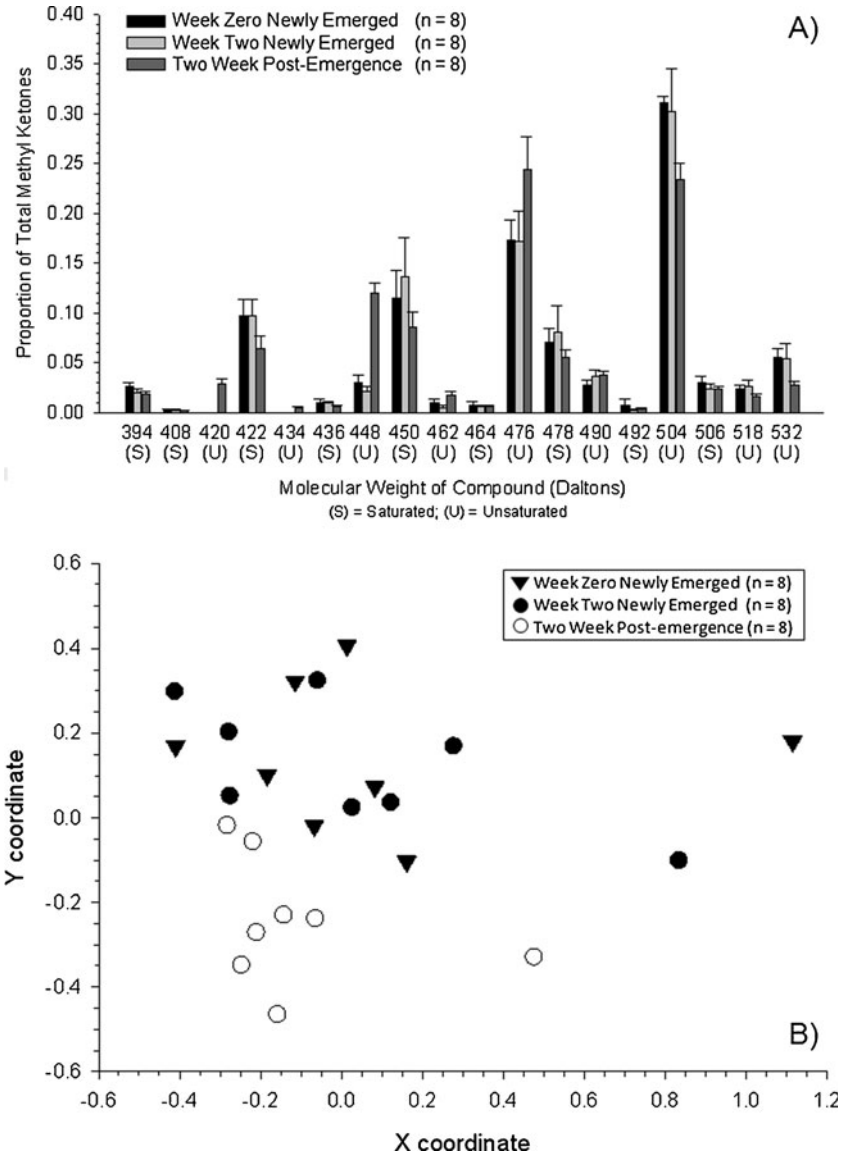

Fig. 4 **a** Relative proportions of individual methyl ketones expressed in the skin lipids of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) newly emerged from hibernation and females 2 wk post-emergence. Bars represent mean + SE. **b** Non-metric multidimensional scaling plot of individual pheromone profiles for newly emerged and 2 wk post-emergence females. Each point represents the pheromone profile of an individual female

concentrations in garter snake skin lipids (LeMaster and Mason, 2002, 2003), it is possible that they were also present in newly emerged females, but at levels below our threshold of detectability. Indeed, although the difference was not significant, post-emergence females had a higher overall concentration of methyl ketones in their skin lipids compared to newly emerged females. Such an upregulation of lipid-based compounds is not surprising considering that, in addition to serving as semiochemicals, skin lipids also play a role in reducing transcutaneous water loss (Lillywhite and Maderson, 1982; Burken et al., 1985; Mason et al., 1987), a function particularly relevant to garter snakes upon emergence from hibernation.

Whether newly synthesized or simply upregulated, the 420 and 434 dalton methyl ketones may indicate compounds that have inhibitory effects on male courtship behavior similar to, but less transitory than, the effect of the aforementioned copulatory pheromone. Alternatively, it is possible that these methyl ketones have little or no role in the loss of female attractivity, which may instead be mediated by other changes in the relative concentrations of individual methyl ketones. As the exact contribution of each methyl ketone to female attractivity is not known and it was not feasible to test synthetic methyl ketones in the current study, the precise nature of the chemical variation that may be involved in the loss of female attractivity is unclear. However, our overall finding that methyl ketone profiles vary between newly emerged females and post-emergence females, which correlates with the results of behavioral trials, is evidence that the intraseasonal decline in female attractivity is chemically mediated. Despit this evidence, we recognize that future studies presenting male snakes with synthetic methyl ketones will be necessary to demonstrate

definitively the role of the pheromone in mediating intraseasonal declines in female attractivity.

The physiological mechanisms that underlie the intraseasonal variation in the sexual attractiveness pheromone remain unclear although the results of our hormone analysis suggest the involvement of estrogen. Two week post-emergence females had lower estradiol than did newly emerged females, lending support to previous studies indicating that the female sexual attractiveness pheromone of the red-sided garter snake may be estrogen regulated (Crews, 1976; Mason and Crews, 1985). Precisely how estrogen may regulate the pheromone is not known; however, prior investigations have suggested that estrogen could have a role in maintaining the pheromone in female skin lipids following emergence (Garstka and Crews, 1985; Mendonça and Crews, 1996). The results of the current study support this hypothesis: the decline in female attractivity and the pheromonal changes were accompanied by a decrease in estrogen, as would be expected if indeed estrogen is responsible for pheromone maintenance.

Instead of, or perhaps in addition to, being hormonally regulated, intraseasonal variation in the female sexual attractiveness pheromone may be impacted by environmental conditions. This seems particularly likely given that pheromone profiles did not differ between week zero newly emerged females and week two newly emerged females indicating that changes occur only after females have emerged from the hibernaculum. Indeed, changes in environmental conditions have been shown to affect the cuticular hydrocarbon profile of the harvester ant (*Pogonomyrmex barbatus*). Wagner et al. (2001) found that harvester ant workers, which spend most of their time inside the nest, developed higher proportions of saturated, unbranched hydrocarbons in their cuticle upon exposure to conditions found outside the nest (i.e., higher temperatures, lower humidity). Emerging from winter hibernation, female red-sided garter snakes experience a similar change in environment as the external conditions are warmer and drier than those of the den interior (Shine et al., 2005a). Thus, it is possible that qualitative variation in the pheromone profiles of newly emerged and two week post-emergence females is a direct reflection of the different amounts of time the snakes were exposed to environmental conditions outside the den. Further work will be required to explore this hypothesis.

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Chemical Signals of Elephant Musth: Temporal Aspects of Microbially-Mediated Modifications

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Abstract Mature male African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants exhibit periodic episodes of musth, a state in which serum androgens are elevated, food intake typically decreases, aggressiveness often increases, and breeding success is enhanced. Urine is a common source of chemical signals in a variety of mammals. Elephants in musth dribble urine almost continuously for lengthy periods, suggesting that the chemicals in their urine may reveal their physiological condition to conspecifics. We investigated the volatile urinary chemicals in captive male elephants using automated solid phase dynamic extraction (SPDE) and gas chromatography–mass spectrometry (GC-MS). We found higher levels of alkan-2-ones, alkan-2-ols, and some aromatic compounds in urine from males in musth than in urine from non-musth males or from females. Levels of ketones and alcohols increased as the urine aged, likely due to microbial metabolism of fatty acids. Protein-derived aromatic metabolites also increased in abundance after urination, likely due to microbial hydrolysis of hydrophilic conjugates. We suggest that microbes may play an important role in timed release of urinary semiochemicals during elephant musth.

Key Words African elephant · *Loxodonta africana* · Asian elephant · *Elephas maximus* · Microbial metabolism · Mammalian chemical signals · Musth · Solid phase dynamic extraction (SPDE)

This manuscript is dedicated to the late Dr. L. E. L. "Bets" Rasmussen.

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Introduction

Compared to insects, the number of known mammalian chemical signals is relatively small (Albone, 1984; Burger, 2005; Sorensen and Hoye, 2010). Two insect pheromones that have also been identified as chemical signals in Asian elephants (*Elephas maximus*) are *Z*-7-dodecen-1-yl acetate and frontalin. The former is a urinary signal of impending ovulation (Rasmussen et al., 1997), and the latter is found in the temporal gland secretion (TGS) of mature males in the periodic state of musth (Rasmussen and Greenwood, 2003). Characteristics of musth include elevated serum androgens (specifically testosterone and dihydrotestosterone), heavy drainage from the temporal glands, urine dribbling, often reduced feeding, increased searching for mates, enhanced

success in competition for breeding, and usually increased aggression (Poole, 1987; Schulte and Rasmussen, 1999; Ganswindt et al., 2005). Musth male elephants emit distinctive odors that are easily detected from a great distance by conspecifics as well as by humans (Poole, 1987, 1989; Hollister-Smith et al., 2008).

The pungent musth secretions and excretions can serve as vehicles for semiochemicals produced by physiological changes (Ganswindt et al., 2005). Asian and African (*Loxodonta africana*) elephants have well-developed primary and secondary (vomeronasal) olfactory systems that are used to detect these semiochemicals (Rasmussen and Schulte, 1998; Lazar et al., 2004). African elephant musth urine trails evoke strong inspection by conspecifics (Poole, 1987), consistent with reports that vertebrate urine includes chemical signals (Albone, 1984). Male African elephants can differentiate between the urine of female conspecifics in the luteal and follicular phases of estrus (Bagley et al., 2006) and between urine from musth and non-musth males (Hollister-Smith et al., 2008).

Using gas chromatography–mass spectrometry (GC-MS), Rasmussen and Wittemyer (2002) analyzed the urine of wild African elephants and found that the following alkan-2-ones were present in significantly higher concentrations in musth than in non-musth urine: propanone (acetone), butanone, pentan-2-one, hexan-2-one, 3-ethylpentan-2-one, heptan-2-one, and nonan-2-one. Rasmussen and co-workers also found volatile ketones emanating from the temporal gland orifices (prior to drainage), TGS, breath, and urine of captive and wild Asian musth elephants (Rasmussen et al., 1990; Perrin et al., 1996; Rasmussen and Perrin, 1999).

Our previous research successfully employed automated solid phase dynamic extraction (SPDE) coupled with GC-MS to analyze volatile organic compounds in female African elephant secretions and excretions (Goodwin et al., 2005, 2007). We noted that frontalin and the brevicomins increased in abundance when samples of female African elephant urine were allowed to remain at room temperature for several days after thawing (Goodwin et al., 2006). That observation led us to hypothesize that the levels of volatile organic compounds in musth and non-musth male urine would also increase during room temperature incubation. Our predictions were as follows: (1) compound abundances would be lower in non-musth than in musth urine, as demonstrated by Rasmussen and Wittemyer (2002) for alkan-2-ones; (2) compound abundances in urine incubated at room temperature would increase over time; and (3) if changes in urine composition are dependent on microbial metabolism, then compound abundance would not change in samples that were centrifuged and filtered to remove microbes before room temperature incubation.

Methods and Materials

Elephant Urine Sources All elephants used in this research were examined routinely by veterinarians and were in good health at the times of urine collection. The adult male urine used in this study was provided by four facilities and four elephants. Musth status was characterized by heavy drainage from the temporal glands, urine dribbling, and a pungent odor from the secretions and excretions. Male elephant ages at the time of urine collection, as well as dates of collection and analysis, are shown in Table 1. Urine from young male elephants that had not experienced musth was obtained from three individuals, ages 2.5, 5, and 7.5 years at the time of sampling. For female African elephants, GC-MS data files from a previous study were used (Goodwin et al., 2006). The data were obtained from samples of female urine collected at the time of the unique anovulatory luteinizing hormone surge (LH1; four samples representing 4 elephants), the ovulatory surge (LH2; six samples representing 5 elephants), and the mid-luteal phase (four samples representing 4 elephants).

Sample Collection, Transport, and Storage Urine samples were collected mid-stream in clean stainless steel bowls, and transferred to clean glass containers. The samples were frozen within 30 min after collection on dry ice or in a −70 or −80°C freezer, and shipped on dry ice to Hendrix

Table 1 Adult male elephant urine sources and bacterial counts (M = musth; NM = non-musth)

Sample	Collection date	Age (yrs) at collection	Day 1 analysis[a]	Bacterial count (CFU/ml)[b]
African #1 M	6/26/2008	29	10/31/2008	N/A[c]
African #1 NM	9/30/2008	29	7/7/2010	N/A[c]
African #2 M	3/22/2010	30	6/30/2010	4,090
African #2 NM	9/1/2009	29	6/30/2010	2,680
African #3 M	11/3/2009	19	6/29/2010	2,730
African #3 NM	3/21/2010	20	6/29/2010	14,300
Asian M	4/30/2009	43	6/30/2009	10,000
Asian NM	6/4/2009	43	9/2/2009	3,140

[a]Day of thawing and first SPDE/GC-MS analysis

[b]Bacteria were cultured and counted three times and the mean is reported

[c]Data are not available

College, Conway, AR, USA. Samples were stored in a −70°C freezer and thawed in a water bath (approximately 37°C) immediately before analysis by SPDE/GC-MS (www.chromtech.de).

Sample Preparation for Analysis Urine samples were thawed and divided into aliquots as needed. These were routinely analyzed by SPDE/GC-MS on the day of thawing (D1), as well as on the 3rd day after thawing (D3; approximately 48 h later). For experiments designed to assess microbially-mediated metabolism, glassware and plasticware were autoclaved at 18 psi and 121°C for 20 min. On the 1st day of thawing, an aliquot of urine was centrifuged at 10–15,000 × *g* for 2 min. The supernatant was passed through a 0.22 μm sterilizing filter to remove insolubles, including microbes. The effectiveness of this procedure was verified by our inability to culture bacteria in centrifuged and filtered urine compared to the significant bacterial growth in samples of the same urine that were not centrifuged and filtered. The following designations for urine samples are used: (1) UN—untreated; (2) CF—centrifuged and filtered; (3) M—musth; (4) NM—non-musth.

Solid Phase Dynamic Extraction (SPDE)/Gas Chromatography–Mass Spectrometry (GC-MS) A 1.0 ml aliquot of urine and a small Teflon®-coated stir bar were sealed in a 20 ml screw-top vial with a threaded, metallic septum cap (silicone/PTFE layered septum; www.autosamplerguys.com). Multiple samples were programmed to run automatically using the Combi PAL robot and associated SPDE hardware and software (www.chromtech.de). The SPDE needle was internally coated with activated charcoal (Carboxen®)-polydimethylsiloxane (AC-PDMS). After incubating the stirred sample at 37°C for 15 min, the headspace was sampled for 13 min (200 up-and-down one ml strokes of the syringe). Desorption of extracted analytes was at 250°C in the GC inlet. Cryo-focusing was not necessary because the compounds of interest appeared as distinct peaks on the chromatograms under our experimental conditions. GC/MS analyses were conducted using an Agilent 6890N GC and 5973N Mass Selective Detector. The capillary GC column was an Equity 1 (bonded; polydimethylsiloxane), 60 m×0.32 mm ID, 1 μm film thickness (Supelco cat. No. 28058U). The GC oven was programmed to hold for 2 min at 35°C, followed by ramping to 180°C at 3.75°C/min where it was held for 5 min before ramping at 20°C/min to a final temperature of 250°C where it was held for 10 min. The mass spectrometer was programmed to collect 3.09 scans/s over the mass range of 30–500 amu. The identities of all compounds were verified by comparisons of retention times and mass spectra to commercial standards except for 1-phenylpropan-2-one, which is a controlled substance. Identifications were confirmed by comparison to the NIST mass spectral library. Compound areas were converted into relative values normalized to the value for the lowest abundance observed. Compound abundances indicate relative quantities of compounds across samples, but should not be used to infer absolute quantities.

Culturing Bacteria from Elephant Urine Frozen (−70°C) musth and non-musth urine samples from 3 male elephants (2 African and 1 Asian) were thawed to quantitate bacteria by a spread plate technique. A sterile bent glass rod was used to evenly spread 0.1 ml of urine over a tryptic soy agar plate that was incubated 48 h at 37°C before counting colonies. For comparison, a mid-stream sample was collected in a sterile container from a different mature, healthy, non-musth male Asian elephant, and was kept cold but not frozen. The sample was inoculated on blood agar a few hours after collection, and was incubated in 10% CO_2 at 37°C for 48 h. Inoculation and incubation were repeated after the urine had remained at the ambient temperature for 48 h. An aliquot of the fresh urine was centrifuged at 1,000 rpm for 10 min, the supernatant was decanted, and the sediment was examined for white blood cells by microscopy (40X).

Research Approval All animal-related experiments were approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent at all participating institutions.

Results

Adult Male Elephants We examined the levels of small alkan-2-ones and alkan-2-ols as well as a few simple aromatic compounds in the urine of male and female elephants. In general, samples contained both the ketone and corresponding alcohol, although for pentan-2-one the alcohol was absent. The target compounds were absent or present at very low levels in centrifuged and filtered musth and non-musth samples on the day of thawing (data not shown). In general, compounds were most abundant in untreated, day three musth samples from 3 African elephants and 1 Asian elephant although for the African elephants, nonan-2-ol, decan-2-ol, and undecan-2-ol were more abundant in untreated day 1 musth urine (Tables 2 and 3). The sum of the abundance of each of these alcohols and its corresponding ketone is largest in untreated day 3 musth urine (Table 2). Many of the compounds could not be detected in the non-musth urine samples (Tables 2 and 3).

Female and Immature Male African Elephants Of the alkan-2-ones and alkan-2-ols listed in Tables 2 and 3, only

Table 2 Comparison of selected compounds in musth and non-musth urine of three male African elephants under various conditions. The data are derived from mean peak areas from GC-MS total ion chromatograms (Supplemental Material S1) normalized to the value for non-musth CFD3 pentan-2-one, the compound of lowest abundance.[a] The maximum relative value for each compound is indicated in bold. Unless otherwise noted, means are based on data from all three elephants

	Non-musth			Musth		
Compound	CFD3[b]	UND1[c]	UND3[d]	CFD3[b]	UND1[c]	UND3[d]
pentan-2-one	1.00[e]	–	2.26[e]	82.3	28.8	**120**
hexan-2-one	–	–	–	57.0	22.0[f]	**102**
hexan-2-ol	–	–	–	–	–	**2.52[e]**
heptan-2-one	–	–	15.0[f]	27.0	16.9[f]	**242**
heptan-2-ol	–	–	–	–	–	**7.40[e]**
octan-2-one	–	–	21.6	–	7.42[f]	**302**
octan-2-ol	–	–	3.81[e]	–	2.42[e]	**23.4[f]**
acetophenone	4.67[f]	2.67[e]	39.8	49.9	22.9	**81.3**
4-methylphenol	3.34[f]	12.0	178	763	1,260	**11,400**
nonan-2-one	2.50[e]	3.66[e]	33.6	52.0	421	**7,830**
nonan-2-ol	–	7.01[e]	4.88[e]	113	**312**	280
decan-2-one	–	–	–	1.74[e]	49.0	**988**
decan-2-ol	–	–	–	3.66[e]	**30.9**	16.4[f]
4-ethylphenol	–	4.52[e]	17.8[f]	127	185	**723**
undecan-2-one	–	–	3.33[f]	–	183	**3,110**
undecan-2-ol	–	–	1.88[e]	14.5[e]	**120**	95.5

[a]A dash indicates non-detectable or inadequate amount for accurate integration, [b]Centrifuged and filtered, day 3 after thawing, [c]Untreated, day 1 after thawing, [d]Untreated, day 3 after thawing, [e]$N=1$, [f]$N=2$

three were detected in any of the 14 female urine samples: pentan-2-one (9 of 14 samples), heptan-2-one (3/14), and octan-2-ol (1/14). Of the aromatic compounds, the most abundant and widespread were 4-methylphenol (11/14) and acetophenone (10/14), with 4-ethylphenol detectable only in two samples. For the 3 young male African elephants that had not exhibited any signs of musth, no urinary alkan-2-ones or alkan-2-ols were detected.

Quantifying Bacteria from Elephant Urine Results from plate count experiments to quantify bacteria are reported in Table 1 as colony forming units per ml (CFU/ml) because some bacteria were found in clusters rather than singly. The data are probably conservative because of the difficulty in counting tightly packed colonies. The bacterial counts reported in Table 1 do not show any obvious musth-related trends.

Culturing Bacteria from Fresh, Unfrozen, Mid-stream, Non-Musth Urine There were no white blood cells in the sample of male Asian elephant urine, indicating that no urinary tract infection was present. Cultures begun on the day of

Table 3 Comparison of selected compounds in musth and non-musth urine of a male Asian elephant under various conditions. The data are derived from peak areas from GC-MS total ion chromatograms (Supplemental Material S1) normalized to the value for non-musth UND1 octan-2-one, the compound of lowest abundance.[a] The maximum relative value for each compound is indicated in bold

	Non-musth			Musth		
Compounds	CFD3[b]	UND1[c]	UND3[d]	CFD3[b]	UND1[c]	UND3[d]
heptan-2-one	2.78	–	5.51	9.52	5.21	**33.0**
heptan-2-ol	–	–	–	–	–	**5.90**
octan-2-one	3.34	1.00	9.83	–	–	**13.1**
octan-2-ol	–	–	–	–	–	**7.17**
acetophenone	11.3	1.30	198	40.4	18.8	**1,330**
4-methylphenol	–	–	1,048	142	428	**1,230**
nonan-2-one	–	–	7.94	2.23	11.9	**326**
nonan-2-ol	–	–	–	1.68	23.6	**93.7**
1-phenylpropan-2-one	–	–	–	84.8	42.0	**92.8**
decan-2-one	–	–	–	–	–	**9.22**
decan-2-ol	–	–	–	–	–	**9.13**
4-ethylphenol	–	–	11.6	–	7.39	**44.6**
undecan-2-one	–	–	12.1	–	–	**42.6**
undecan-2-ol	–	–	9.86	–	–	**9.81**

[a]A dash indicates non-detectable or inadequate amount for accurate integration, [b]Centrifuged and filtered, day 3 after thawing, [c]Untreated, day 1 after thawing, [d]Untreated, day 3 after thawing

collection yielded a few colonies of unidentified bacteria species. Incubation of an aliquot of the urine at the ambient temperature for 48 h yielded heavy bacterial growth.

Discussion

Chemical Signals of Musth and Bacterial Metabolism Rhodes et al. (1982) detected alkan-2-ones in the urine of diabetic rats and proposed their genesis from decarboxylation of β-keto-acids, as in the well-known formation of acetone from fatty acid-derived acetoacetate. Rasmussen suggested that alkan-2-ones in musth male elephant secretions and excretions are indicative of elevated fatty acid catabolism (Rasmussen et al., 1990; Rasmussen and Perrin, 1999).

Bacterial involvement in production of mammalian chemical signals is well-known (Albone et al., 1977; Albone, 1984; Zechman et al., 1984), as is bacterial production of alkan-2-ones (Dickschat et al., 2005; Schulz and Dickschat, 2007). Alkan-2-ols likely are derived by NADH- or NADPH-assisted enzymatic reduction of the corresponding ketone (Scheline, 1973; Schulz and Dickschat, 2007). Intestinal microflora can exert a large effect on mammalian blood and urinary metabolites, especially those from amino acids (Williams et al., 2002; Wikoff et al., 2009). Acetophenone may be derived from phenylalanine, whereas 4-methylphenol (*p*-cresol), and 4-ethylphenol may result from tyrosine catabolism (Curtius et al., 1976; Martin, 1982; Schulz and Dickschat, 2007).

Urinary phenols are likely excreted as either their hydrophilic sulfate or glucuronide conjugates or both, as demonstrated in humans and other mammals (Vanholder et al., 1999; Crespin et al., 2002). Bacterial hydrolysis of such phenolic conjugates has been described previously (Scheline, 1973), and is a reasonable explanation for increased levels of phenols in aged urine. Microbially-mediated modifications have been proposed as proximate causes for the enhanced signaling bioactivity of aged urine samples over that of fresh ones (Albone, 1984, pp. 186–187).

In an earlier study, we noted that levels of volatile organic compounds were higher in aged urine of female African elephants than in fresh urine (Goodwin et al., 2006). Therefore, in the present study, we anticipated increases in the abundances of alkan-2-ones, alkan-2-ols, and aromatic compounds (Tables 2 and 3) in musth male urine. On the day samples were thawed, these compounds were elevated in untreated musth urine compared to untreated non-musth urine. On the third day after thawing, compound abundances were increased relative to the amount on day one for the same sample and also for musth compared to non-musth urine (Tables 2 and 3).

We detected bacteria in the elephant urine, with no clear trends in bacterial counts between musth and non-musth samples (Table 1). The elephants in this study were healthy. Therefore, the microbes likely are from contamination in the distal urethra and periurethral area by skin, soil, and/or feces (van Hoven et al., 1981; Clemens and Maloiy, 1982). Wiedner et al. (2009) cultured bacteria from mid-stream urine of healthy female Asian elephants, and attributed the bacteria to contamination. Variation in the level of microbial contamination among the urine samples in our study (Table 1) may be a consequence of sample collection on different dates and in different facilities.

Compounds were less abundant in aseptic aged urine than in untreated aged urine (Tables 2 and 3), indicating possible microbial activity. However, in some cases the abundance of pentan-2-one, hexan-2-one, heptan-2-one, and acetophenone in the centrifuged and filtered musth day 3 samples was higher than in untreated musth samples on the day of thawing. Inadvertent reintroduction of microbes to the centrifuged and filtered urine samples might lead to these observations, but one would expect to see an increase in all compound abundances in that case. Analysis of more samples may clarify relationships between fresh and aseptic samples.

The relative paucity of alkan-2-ones and alkan-2-ols in the urine of young males, cycling females, and non-musth adult males supports the suggestion by Rasmussen and Wittemyer (2002) that these compounds are characteristic urinary markers of musth in mature male elephants. However, only untreated, freshly thawed female and immature male urine samples were analyzed in our study. Effects of microbial action on aged samples may provide additional insight into urinary semiochemicals of elephants at various stages of sexual maturity.

Timed Release of Chemical Signals after Urination Mammalian secretions or excretions contain chemical signals that may persist changed or unchanged in the environment for a period of time after release. Changes may be a consequence of microbial action or slow release from a carrier protein. The Asian elephant pre-ovulatory pheromone (*Z*-7-dodecen-1-yl acetate) is bound to albumin in the urine, and is transferred to an "odorant binding protein" in the trunk mucus of a conspecific as part of the flehmen response that ultimately transports the chemical signal to the vomeronasal organ (Lazar et al., 2004). Slow release of volatile chemical signals from proteins in urine scent marks of male house mice (*Mus domesticus*) has been demonstrated, along with a suggestion that conspecifics may be able to tell from a distance whether these airborne signals emanate directly from the donor, or from the urinary scent marks (Hurst et al., 1998; Novotny, 2003).

Alberts (1992) characterized the "temporal parameters" of vertebrate chemical communication in terms of two related design systems as follows: "…. signal fade-out time,

the elapsed time from deposition until the signal is no longer detectable; and signal rise time, the time until the signal reaches maximum spatial range." Poole (1987) speculated that constant or intermittent urine dribbling in musth male African elephants may function as a source of semiochemicals to conspecifics over a period of time. When the musth male urinates, microbes can continue to produce chemical signals in the deposited urine until the nutrient source is depleted and the signals fade out, thus informing conspecifics when the male was at a specific location. As the male moves about dribbling urine, similar time-release information will be deposited. Therefore, we propose that microbes may play an important role in the temporal release of urinary semiochemicals during elephant musth.

Summary and Future Studies We have shown that the production of alkan-2-ones, alkan-2-ols, and several aromatic compounds continues exogenously in musth male elephant urine in a process that appears to be mediated by microbes. This phenomenon suggests the possibility of continued chemical signaling to conspecifics over several days after urination. In addition, this process could also lead to the demise of a meaningful signal, which could be valuable in that new, older, and no longer relevant signals would be distinguishable. We are conducting additional experiments to more clearly define the role of microbes in the production of chemical signals in musth male elephant urine.

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Composition of Extrafloral Nectar Influences Interactions between the Myrmecophyte *Humboldtia brunonis* and its Ant Associates

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Abstract Ant–plant interactions often are mediated by extrafloral nectar (EFN) composition that may influence plant visitation by ants. Over a 300 km range in the Indian Western Ghats, we investigated the correlation between the EFN composition of the myrmecophytic ant-plant *Humboldtia brunonis* (Fabaceae) and the number and species of ants visiting EFN. EFN composition varied among *H. brunonis* populations and between plant organs (floral bud vs. young leaf EFN). In general, EFN was rich in sugars with small quantities of amino acids, especially essential amino acids, and had moderate invertase activity. In experiments at the study sites with sugar and amino acid solutions and with leaf or floral bud EFN mimics, dominant EFN-feeding ants differentiated between solutions as well as between mimics. The castration parasite *Crematogaster dohrni* (northern study site) was the least selective and did not exhibit any clear feeding preferences, while the largely trophobiont-tending non-protective *Myrmicaria brunnea* (middle study site) preferred higher sucrose concentrations and certain essential/non-essential amino acid mixtures. The mutualistic *Technomyrmex albipes* (southern study site) preferred sucrose over glucose or fructose solutions and consumed the leaf EFN mimic to a greater extent than the floral bud EFN mimic. This young leaf EFN mimic had low sugar concentrations, the lowest viscosity and sugar:amino acid ratio, was rich in essential amino acids, and appeared ideally suited to the digestive physiology of *T. albipes*. This preference for young leaf EFN may explain the greater protection afforded to young leaves than to floral buds by *T. albipes*, and may also help to resolve ant–pollinator conflicts. The differential response of dominant ants to sugar, amino acids, or solution viscosity suggests that plants can fine-tune their interactions with local ants via EFN composition. Thus, EFN can mediate local partner-choice mechanisms in ant–plant interactions.

Key Words Dolichoderinae · Extrafloral nectar · Amino acids · Extrafloral nectar sugars · Invertase · Myrmecophyte · Partner-choice mechanism

Introduction

Ant-plants provide food and/or housing to their ant partners in exchange for protection from herbivores (Davidson and McKey, 1993; Heil and McKey, 2003) and/or for provision of limiting nutrients such as nitrogen (Wagner and Nicklen, 2010). Food resources offered to ants consist of carbohydrate-rich extrafloral nectar (EFN) as well as protein- and/or lipid-rich food bodies (Heil and McKey, 2003). While food bodies occur mostly in specialized and stable interactions between plants and obligate ants, EFN is offered in both obligate and facultative interactions (Blüthgen et al., 2000; Heil et al., 2005; Rudgers et al., 2010). Extrafloral nectar is usually

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carbohydrate-rich and nitrogen-poor (Baker et al., 1978; Blüthgen and Fiedler, 2004).

Since arboreal ants differ in their biology, digestive physiology and nutrient requirements (Davidson, 1997; Davidson et al., 2004; Kay, 2004; Cook and Davidson, 2006), ant species differ in their responses to EFN based on its qualitative and quantitative composition (Heil et al., 2005, 2009; González-Teuber and Heil, 2009a, b). Specifically, ant preferences for EFN solutions of different compositions may be dictated by ant responses to sugar and amino acid identity or concentration, or to ratios of sugar and amino acids, or to non-additive interactions between these components (Davidson and Cook, 2008). For example, ants that do not possess the enzyme invertase, which converts sucrose to glucose and fructose, may be averse to sucrose-rich solutions (Heil et al., 2005). Ants that do not have gut symbionts to synthesize essential amino acids (Cook and Davidson, 2006) may be attracted to solutions rich in essential amino acids. Ant species may develop preferences for sugars or amino acids characteristic of familiar sources of nutrition (González-Teuber and Heil, 2009b).

Variation within a plant in quality and quantity of floral nectar (Lanza et al., 1995; Herrera et al., 2006) impacts plant reproductive success through its effect on pollinators. The effects of differences in EFN composition within a plant, especially EFN produced on different plant structures, e.g., young leaves vs. floral buds, have rarely been examined (Keeler, 1977). Extrafloral nectar secretion may interfere with pollination by attracting pollinator-intimidating ants (Ness, 2006), thus increasing the ecological costs of EFN production (Heil and McKey, 2003).

While intraspecific variation in floral nectar has been described (Nicolson, 2007), less is known about variation in EFN (Rudgers and Gardener, 2004; González-Teuber and Heil, 2009b). Geographical variation in the quality and quantity of EFN may influence the type and frequency of ants visiting EFN-bearing plants (Blüthgen et al., 2000; Rudgers et al., 2010), and thus mediate locality-specific interactions between ants and plants (Rudgers and Gardener, 2004; Rudgers et al., 2010). Variation in EFN quality and in ant responses could set up a mosaic of interactions between plants and ants (Chamberlain and Holland, 2008).

In this study, we used the myrmecophytic plant *Humboldtia brunonis* Wallich (Fabaceae) to examine how geographical variation in EFN chemistry and related physical properties such as viscosity affected EFN consumption by locally available ant species. This allowed us to evaluate whether EFN composition influences the identity and abundance of ants that are attracted to this ant-plant. Ant preferences for nutrients usually have been tested with simple aqueous solutions, often under laboratory conditions (Lanza, 1988; Tinti and Nofre, 2001; Blüthgen and Fiedler, 2004). Therefore, we also tested ant feeding preferences at the study sites using such simple solutions. We further examined ant response to complex mixtures such as natural EFN by using EFN mimics under natural situations because such interactions as well as the impact of EFN viscosity have not been fully explored (Koptur and Truong, 1998; Kost and Heil, 2005; González-Teuber and Heil, 2009b). *Humboldtia brunonis* is ideal for an investigation into questions related to the impact of EFN composition on ant–plant mutualisms because it is an unspecialized myrmecophyte that is host to a diversity of ant species throughout its geographical range, which spans 300 km within the Indian Western Ghats (Gaume et al., 2005a,b, 2006; Shenoy and Borges, 2010). Using experiments conducted in the natural setting, we asked: 1. What is the sugar:amino acid ratio in *H. brunonis* EFN, i.e., is EFN carbohydrate-rich and amino acid-poor? 2. Is there geographical variation in EFN composition across the range of this ant-plant? 3. Does the composition of young leaf (YL) EFN differ from that of floral bud (FB) EFN? 4. Are traits of nectar composition (sugar or amino acid concentration, sugar:amino acid ratios, caloric value, viscosity or familiarity) correlated with ant visitation to EFN? Do patterns vary between ant species at the different sites?

Methods and Materials

Study System and Sites *Humboldtia brunonis* (Fabaceae: Caesalpinioideae) is a dominant understorey ant-plant that houses ants in swollen, hollow internodes called domatia. It is distributed in low elevation, wet evergreen forests (11°10′N to 13°45′N) of the Indian Western Ghats (Ramesh and Pascal, 1997). EFN is produced on the sepals of floral buds, young leaves, reniform stipules, and occasionally from the stalk of the inflorescence, but mostly from young leaves and flower buds. Flowering occurs in the dry season (December–April), as does production of young leaves and maximum ant activity (Basu, 1997). Extrafloral nectar chemistry and ant preferences for it were examined at three sites matched for elevation: (i) Agumbe Reserve Forest (13°31′N, 75°5′E; 633 m asl); (ii) Sampaji Reserve Forest (12°29′N, 75°35′E; 665 m asl); (iii) Solaikolli within Brahmagiri Wildlife Sanctuary (12°4′N, 75°49′E; 651 m asl).

Geographical Variation in EFN Volume, Chemical Composition and Invertase Activity Extrafloral nectar from bagged (using fine cloth mesh) and ant-excluded (for 24 h) floral bud nectaries of a single inflorescence (FB) or all four leaflets of each young leaf (YL) was pooled to determine volume and composition (Agumbe: N=10 FBs and 11 YLs; Sampaji: N=7 and 9; Solaikolli: N=11 and 8; each FB or YL sample was from a different plant). Inflorescences and young leaves were bagged in the morning between 0900–1200 h. Owing to its high viscosity, EFN was diluted with known volumes of HPLC-grade distilled water for collection and stored with 50 µl of HPLC-grade methanol

at −10°C until further analysis. Sugars and amino acids in EFN were derivatized to their trimethyl silylated forms (Kost and Heil, 2005) prior to GC-MS analysis (Agilent-HP GC model 6890N, MS model 5973N) using an HP-5 MS column with the following temperature program: 50°C for 2 min isothermal, 10°C min^{-1} to 120°C, and 5°C min^{-1} to 250°C for 5 min isothermal. The GC was operated in the split mode with a split-ratio of 10:1. A mixture containing 1 mg of each of commercial (Sigma-Aldrich, India) sugars (sucrose, glucose, fructose, inositol, galactose, mannose, arabinose, raffinose) and of each of the 20 naturally occurring amino acids was derivatized using 1 ml of N-methyl-N-(trimethyl silyl) trifluoro acetamide in 2 ml pyridine and used as a reference standard for the GC-MS analysis. All sugar and amino acid concentrations are reported in g/100 ml, and all sugar:amino acid ratios are reported as mass ratios. Invertase activity (μg glucose $\mu l^{-1} min^{-1}$) was measured in separately collected samples of FB and YL EFN (Heil et al., 2005). These EFN samples were not diluted with water, to minimize disruption of nectary cells and release of invertase (Pate et al., 1985).

Differential Utilization of Solutions by Ants in Cafeteria Assays We modified the experimental design of Blüthgen and Fiedler (2004) to test responses of ants to simple standard sugar solutions, to sugars mixed with amino acids, or to complex solutions mimicking EFN compositions (Table 1). At each site we chose 10 *H. brunonis* trees separated by a distance of at least 8 m to minimize pseudoreplication of ants from the same colony. On these 10 trees, at 1.5 m above the ground, we placed 5 replicate sets of 2 ml plastic vials containing the test solutions. Each tube had a cotton wick that delivered the solution to ants. Vials in each set were randomly ordered to minimize location effect. After setting up the experimental vials between 1100–1200 h, we counted ant workers of the different species that visited the wicks of each solution at five times during the next 24 h (1500 h, 1800 h, 2100 h, next morning 1100 h, and next afternoon 1300 h). We refilled vials at least 1 h before each count, if required. Since EFN is a complex secretion containing sugars (mainly sucrose, glucose, and fructose) and amino acids (Baker et al., 1978), we used sugars and amino acids in our experiments in ranges normally available to ants from plant secretions or honeydew. Plain water solutions were not offered in these experiments since the EFN of *H. brunonis* is the only major source of extrafloral liquids for ants at these sites in the dry season.

Cafeteria Assays Using Standard Solutions of Sugars and Amino Acids We explored the preference of ants for three sugars at different concentrations, and for different mixtures of essential and non-essential amino acids. In Experiment 1, ants selected between equal concentration solutions of sucrose, glucose, or fructose. Solutions with sugar concentrations

Table 1 Summary of experiments performed to test utilization by ant species of nectar solutions at each site

Experiment	Aim	Brief Description	Result
Experiment 1	To determine if ants prefer certain sugars over others only at particular concentrations	Choice between solutions of sucrose, glucose, or fructose at 4 different concentrations, one concentration tested at a time	Sucrose was preferred sugar but at different concentrations for two ant species, *Technomyrmex albipes* and *Crematogaster dohrni*
Experiment 2	To determine if ants prefer sugar solutions offering high or low caloric rewards irrespective of sugar identity	Choice between equicaloric solutions of sucrose, glucose, and fructose at low or high concentrations, one concentration tested at a time	Castration parasite *C. dohrni* unresponsive to caloric value; *Myrmicaria brunnea* preferred hexoses within same caloric class; *T. albipes* most responsive to sugar identity
Experiment 3	To determine whether ants prefer only certain concentrations of each sugar	Choice between 4 concentrations of sucrose, glucose, or fructose, one sugar tested at a time	Variable response
Experiment 4	To determine whether ants prefer solutions containing only essential amino acids over those containing non-essential amino acids or various combinations of essential and non-essential amino acids	Choice between 6 solutions containing essential and non-essential amino acids in various combinations	Only *T. albipes* showed preference for essential amino acids over non-essential amino acids
Experiment 5	To determine whether ants prefer familiar (from their own site) EFN mimics over unfamiliar ones (from other sites)	Choice between EFN mimics of all sites (familiar and unfamiliar), with either floral bud or young leaf EFN mimics tested at a time	Familiarity did not influence choice of EFN mimics, but sugar concentration, sugar:amino acid ratios and viscosity did, especially for *T. albipes*
Experiment 6	To determine whether ants prefer floral bud EFN over leaf EFN at each site	Choice between mimics of floral bud and young leaf EFN of a familiar site only	*C. dohrni* exhibited no preference; *M. brunnea* preferred FB over YL EFN; *T. albipes* preferred YL over FB EFN

ranging from 5% to 50% were tested. In Experiment 2, we presented ants simultaneously with equicaloric solutions of sucrose, glucose, or fructose. We compared mixtures supplying 0.197 kcal/ml (equivalent to 5% w/v sucrose or 10% w/v of the monosaccharides glucose or fructose) to mixtures supplying 0.788 kcal/ml (equivalent to 20% sucrose or 40% w/v glucose or fructose). In Experiment 3, ants selected between several concentrations of a single sugar. Solutions with sugar concentrations ranging from 5% to 50% were tested. In Experiment 4, ants selected between 15% sucrose solutions containing different combinations of essential and non-essential amino acids each at a concentration of 0.7 mg/ml, which is the average single amino acid concentration in EFN. We assumed that the essential amino acids for ants are the same as the essential amino acids for mammals (Davidson et al., 2004). Three essential amino acid solutions were used: E1 (arg, leu, met, thr, and val), E2 (his, isoleu, lys, phe, and try), and E (all 10 essential amino acids). The non-essential amino acid solutions were prepared as N1 (asn, glu, gly, pro, and tyr), N2 (ala, asp, cys, gln, and ser), and N (all 10 non-essential amino acids). Some of these solutions were used as mixtures (E1N1, E2N2, E1N2, and E2N1) to provide functional group diversity. All six solutions E, N, E1N1, E2N2, E1N2, and E2N1 were simultaneously presented to ants at each site.

Cafeteria Assays Using Solutions Mimicking Young Leaf and Floral Bud EFN We compared the preferences of ants for solutions that mimicked YL and FB EFN from *H. brunonis* at the three study sites (Table 1). We prepared EFN-mimicking solutions that represented the inter- and intra-site variability in composition and concentration (high or low concentrations of sugars and amino acids) of FB and YL EFN (Supplemental Table 1, Supplemental Fig. 1). In Experiment 5, we simultaneously offered ants at each site FB EFN mimics corresponding to that site (familiar EFN) and that of other sites (non-familiar EFN) followed by a separate test with familiar and non-familiar YL EFN mimics. In Experiment 6, we offered ants at each site a choice between mimics of FB and YL EFN from that site (familiar EFN only).

We measured the viscosity of all FB and YL EFN mimics at 20, 30, and 40°C with a rotational (stress/strain) rheometer (TA Instruments AR1000N) to determine if viscosity of a solution with a particular chemical composition is a correlate of ant preference for EFN.

Statistical Analyses The sugar and amino acid compositions of FB and YL EFN types were compared using permutational (non-parametric) multivariate analyses of variance (MANOVA) since the data were not normally distributed. This was done via the Adonis function of the Vegan package in the software R (Anderson, 2001; Oksanen et al., 2011). The Euclidean distance measure and a total of 999 permutations were employed for each comparison. By using this procedure, we examined effects of site and EFN type (FB vs. YL EFN) as well as interaction effects of these variables on EFN composition. Pairwise *post-hoc* comparisons of these data were performed using the Mann-Whitney *U* test. EFN volumes and invertase levels were compared between FB and YL EFN types using a non-parametric Kruskal-Wallis ANOVA. All data on ant visitation to simultaneously presented solutions were analyzed for normality using the Shapiro-Wilks test. None of the distributions were normal and could not be rendered normal by any procedure. Therefore, non-parametric Kruskal-Wallis tests followed by Mann-Whitney *U* tests with appropriate Bonferroni corrections or Wilcoxon matched pairs tests were employed. All univariate non-parametric tests were performed using the software package STATISTICA '99 Edition, Kernel Release 5.5 A. These tests were performed to statistically identify solutions that were utilized to a greater extent by ants over others presented in the various cafeteria assays. In this paper, although data are not normally distributed, we have elected to depict means and standard errors in the figures for ease of graphical representation.

Results

Geographical Variation in EFN Volume, Chemical Composition and Invertase Activity There was within- and between-site variation in EFN composition. In all cases, EFN contained more sugar than amino acids, with total sugar:total amino acid mass ratios varying from 37.0 for FB to 86.0 for YL EFN (samples pooled across sites) (Table 2). Across sites, floral buds and young leaves produced the same volume of EFN (5 μl) within 24 h ($H_{5,\ 56}$ =3.524, P=0.611; Table 2). Twelve sugars were found in EFN (Table 2, Supplemental Table 2). Sucrose, glucose, and fructose were dominant in both types of EFN (Table 2). In general, the concentrations of sucrose and glucose were significantly higher in FB EFN than in YL EFN, pooled across all sites and also within sites (Table 2). In EFN samples collected from all sites, glucose and fructose concentrations were not significantly different from each other (Wilcoxon matched pairs test: Z=1.17, N=28 pairs, P=0.24). Invertase was present in EFN and its activity in FB and YL EFN did not differ across sites ($H_{5,\ 29}$ =6.71, P=0.243; Table 2).

Essential (E) and non-essential (NE) amino acids were found in EFN (Table 2). In general, the total concentrations of E and NE amino acids in FB and YL EFN were not significantly different from each other across and within sites (Table 2). However, Agumbe FB EFN had the highest concentration of amino acids of all types of EFN examined; this EFN thus had the lowest sugar:amino acid ratio. FB EFN from Sampaji had the highest ratio owing to its low amino acid concentration (Table 2). Among YL EFNs, that from Sampaji also had the highest sugar:amino acid ratio, while that from Solaikolli had the lowest (Table 2). The

Table 2 Volume, concentration of important sugars, total amino acid concentration, and invertase activity in EFN of floral bud inflorescences and young leaves of *Humboldtia brunonis* from three sites [Mean±SD (Median)] [y]

EFN source	Floral Bud EFN				Young Leaf EFN			
SITE	Pooled across sites	Agumbe	Sampaji	Solaikolli	Pooled across sites	Agumbe	Sampaji	Solaikolli
	N=28	*N*=10	*N*=7	*N*=11	*N*=28	*N*=11	*N*=9	*N*=8
Volume (μl)/24 h	5.0±6.6 (3)	5.1±3.2 (5)	3.3±3.9 (2)	5.9±9.7 (3)	4.8±5.6 (3)	6.0±7.6 (5)	3.9±4.4 (2.5)	4.0±3.2 (2.5)
Sugar:Amino Acid Ratio	37	17	1475	131	86	50	323	34
Sucrose	23.0±10.9 (20.0) [a]	30.2±12.2 (34.8)[a]	14.8±3.5 (12.9)	21.7±8.8 (21.0) [a]	7.0±6.0 (5.3) [b]	5.4±2.9 (5.0) [b]	11.0±8.8 (9.6)	4.8±3.2 (4.2) [b]
Glucose	21.5±15.6 (20.7) [a]	13.6±8.2 (16.4) [a]	15.5±22.8 (6.9)	32.5±8.1 (30.9) [a]	9.5±7.6 (8.1) [b]	2.8±1.8 (2.3) [b]	14.9±7.9 (16.2)	12.8±4.9 (13.7) [b]
Fructose	16.9±15.5 (14.8)	27.2±14.6 (30.8) [a]	15.7±11.3 (16.6)	8.4±13.8 (0)	11.8±13.2 (7.1)	4.8±3.4 (3.9) [b]	24.3±15.5 (17.4)	7.5±9.2 (4.2)
Total sugars	69.6±32.4 (55.8) [a]	81.7±39.4 (95.0) [a]	59±37.2 (54.3)	65.4±18.5 (60.2) [a]	34.5±28.5 (21.2) [b]	15.1±9.5 (11.4) [b]	64.6±29.6 (66)	27.3±13.3 (21.7) [b]
Invertase activity (μg glucose $\mu l^{-1} min^{-1}$)	0.1±0.1 (0.04) [n=14]*	0.2±0.2 (0.1) [n=5]	0.01±0.01 (0.004) [n=5]	0.1±0.1 (0.1) [n=4]	0.03±0.05 (0.02) [n=14]	0.02±0.01 (0.03) [n=5]	0.1±0.1 (0.1) [n=4]	0.004±0.003 (0.002) [n=5]
Total essential amino acids	0.5±0.7 (0)	1.4±0.8 (1.5) [a]	0	0.05±0.2 (0)	0.1±0.3 (0)	0.2±0.2 (0.01) [b]	0	0.3±0.4 (0)
Total non-essential amino acids	1.2±3.6 (0)	2.8±5.7 (0.05)	0.006±0.01 (0)	0.4±1.4 (0)	0.1±0.4 (0)	0.09±0.3 (0)	0.03±0.01 (0)	0.3±0.6 (0)
Total amino acids [z]	1.9±4.2 (0)	4.7±6.1 (2.3) [a]	0.04±0.1 (0)	0.5±1.7 (0)	0.4±0.8 (0)	0.3±0.3 (0.2) [b]	0.2±0.7 (0)	0.8±1.2 (0)

Concentrations of all sugars and amino acids are reported in g/100 ml; concentrations of minor sugars and all amino acids are available in Supplemental Table 2; [y] values followed by different letters in comparisons between floral bud and young leaf EFN, within a site or pooled across sites, are significantly different at $\alpha=0.05$ (Mann-Whitney *U* tests; only significantly different pairs are so indicated); * number of EFN samples used to measure invertase activity; [z] the values for total amino acids are not equal to the sum of total essential and total non-essential amino acids in this table owing to the presence of two unidentified compounds that may be unusual amino acids (see Supplemental Table 2)

concentrations of individual amino acids were the same in FB and YL EFN across sites with the exception of alanine which was higher in YL EFN (Supplemental Table 2). Within sites, most essential amino acid concentrations were higher in FB EFN at Agumbe, while those of phenylalanine (E) and alanine (NE) were higher in YL EFN at Solaikolli (Table 2, Supplemental Table 2). Of the 20 normally occurring amino acids, the essential amino acids arginine, lysine and histidine and the non-essential asparagine, cysteine and glutamic acid were not detected (Supplemental Table 2). FB EFN from Agumbe and YL EFN from Solaikolli had amino acid compositions, 7 E+4 NE and 5 E+5 NE, respectively, that were more diverse, especially with regard to essential amino acids, than other FB and YL EFNs (Supplemental Table 2).

We evaluated overall differences between nectar types at the various sites by considering sucrose, glucose, and fructose, as well as essential and non-essential amino acids (Table 3). We found a significant effect of site and nectar type as well as a significant interaction effect between site and nectar type (Table 3). A separate multivariate analysis using the three main sugars but not the amino acids gave the same results including the same amount of explained variation (Table 3). There was a significant site, nectar type, and interaction effect with essential amino acids but not with non-essential amino acids (Table 3), indicating that essential amino acids play a significant role in EFN composition at some sites (Table 2). These results taken together indicate that while FB and YL EFNs can be different, the extent and nature of the variation is site-specific. Therefore, locally-specific interactions with ants is possible.

Table 3 Permutational (non-parametric) multivariate analyses of variance to compare nectar composition of floral bud (FB) and young leaf (YL) extrafloral nectar (EFN) types at the different sites

	df	*F*	R^2	*P*
All major sugars (sucrose, glucose and fructose)+essential and non-essential amino acids				
Site	2, 55	6.818	0.1426	<0.001
EFN type	1, 55	18.65	0.1950	<0.001
Site*EFN type	2, 55	6.676	0.1396	<0.001
Only major sugars (sucrose, glucose and fructose)				
Site	2, 55	6.919	0.1436	<0.001
EFN type	1, 55	18.98	0.1970	<0.001
Site*EFN type	2, 55	6.768	0.1405	<0.001
Only essential amino acids				
Site	2, 55	14.02	0.2393	<0.001
EFN type	1, 55	9.648	0.0824	0.002
Site*EFN type	2, 55	14.73	0.2515	<0.001
Only non-essential amino acids				
Site	2, 55	1.455	0.0496	0.249
EFN type	1, 55	2.603	0.0443	0.123
Site*EFN type	2, 55	1.590	0.0542	0.242

Differential Utilization of Solutions by Ants in Cafeteria Assays A total of 36 ant species fed on the different aqueous solutions at the three sites, and these ants differed in their presence and relative abundance across sites (Shenoy and Borges, 2010). Since the most abundant and agressive ant species feeding on these solutions were also the most abundant consumers of EFN of *H. brunonis* (Shenoy and Borges, 2010), we present the results of differential consumption of various solutions for only these ant species. Additionally, these dominant species were recorded in large enough numbers to provide adequate sample sizes for statistical purposes. These dominant ants were the myrmicines *Crematogaster dohrni* Mayr (Agumbe) and *Myrmicaria brunnea* Saunders (Sampaji), and the dolichoderine *Technomyrmex albipes* Smith (Solaikolli). The results we present for each ant species could be site-specific, and this must be taken into consideration when interpreting them. Throughout this paper, results for *C. dohrni* are presented only for Agumbe, for *M. brunnea* only for Sampaji, and for *T. albipes* only for Solaikolli.

Cafeteria Assays Using Standard Solutions of Sugars and Amino Acids Overall, the cafeteria choice assays demonstrated that the preferences of ant species for nutrients may depend on the available choices in the experimental system. However, some results were consistent for assays with simple sugar and amino acid solutions and those with EFN mimics; those results are highlighted.

When presented simultaneously with low (5% or 20%) concentrations of sucrose, glucose, or fructose, *C. dohrni* and *T. albipes* preferred sucrose compared to glucose or fructose (Experiments 1a–1 d, Table 1; Fig. 1a and c). *Myrmicaria brunnea* did not discriminate between sugars at any concentrations (Experiments 1a–1 d; Fig. 1b).

The ant species at the three sites differed in their responses to equicaloric solutions of sucrose, glucose, and fructose. *Crematogaster dohrni* consumed all solutions equally (Experiments 2a–2b; Fig. 1d). *Myrmicaria brunnea* preferred glucose and fructose solutions over those of sucrose at both caloric levels (Experiments 2a–2b; Fig. 1e). *Technomyrmex albipes* preferred solutions of glucose over those of sucrose or fructose at low caloric values (Experiment 2a; Fig. 1f). However, *T. albipes* preferred sugars in the order sucrose>glucose> fructose at high caloric values (Experiment 2b; Fig. 1f). Therefore, *C. dohrni* appeared equally attracted to all sugars providing the same caloric value (low or high), while *M. brunnea* when faced with a choice of a disaccharide (sucrose) or a monosaccharide (glucose or fructose) chose the monosaccharide, indicating a general preference for monosaccharides rather than a preference for a particular sugar. *Technomyrmex albipes* appeared to be more responsive to sugar identity than *C.*

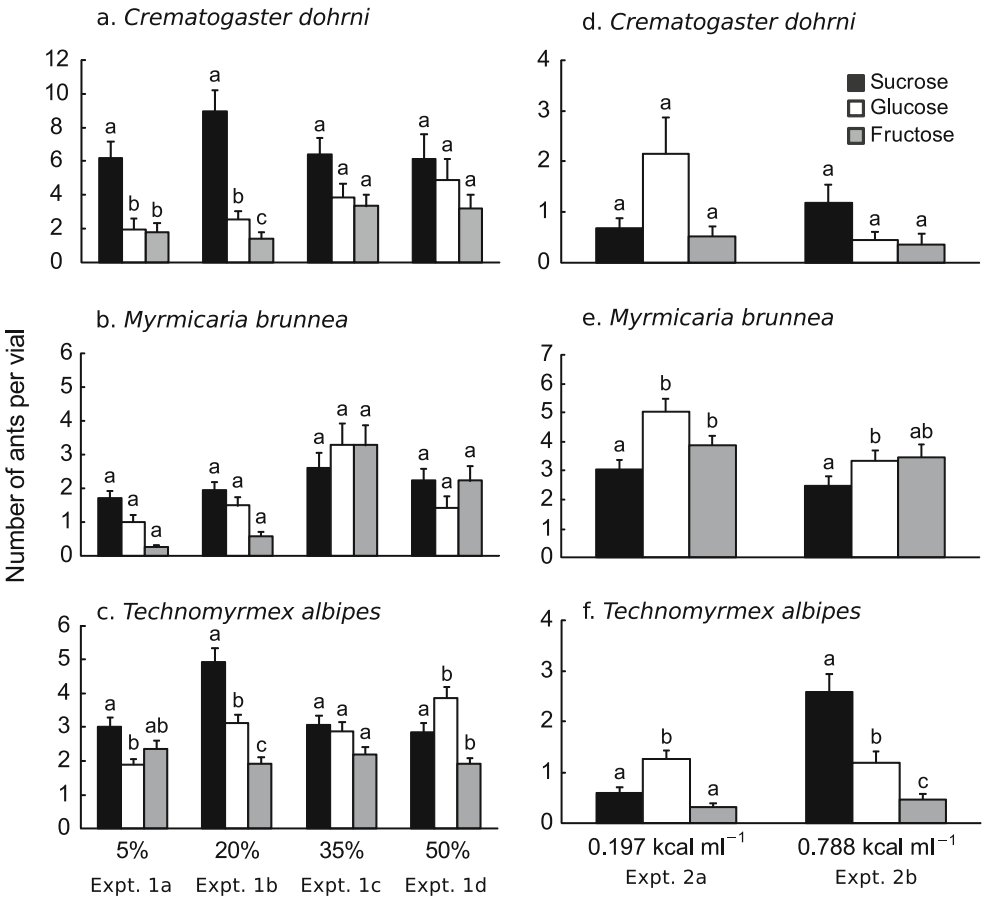

Fig. 1 Effect of sugar concentration. a-c, Ant visits to vials of different sugars at the same concentration **a**: *Crematogaster dohrni* in Agumbe; **b**: *Myrmicaria brunnea* in Sampaji; **c**: *Technomyrmex albipes* in Solaikolli. d-f, Ant visits to vials of equicaloric low or high sugar concentrations **d**: *Crematogaster dohrni* in Agumbe; **e**: *Myrmicaria brunnea* in Sampaji; **f**: *Technomyrmex albipes* in Solaikolli. Ant visitation was quantified as mean number of ants per sugar solution at each sampling point. Means+SE followed by the same letter within the same experiment are not significantly different (Kruskal-Wallis tests followed by Mann-Whitney *U* tests after appropriate Bonferroni correction)

dohrni and *M. brunnea* by preferring glucose at low caloric values and sucrose at high caloric values.

Ant species differed in their response when simultaneously presented with solutions containing the same sugar at different concentrations (Experiment 3, Table 1). *Crematogaster dohrni* preferred solutions with higher concentrations of sugars (Experiments 3a–3c; Fig. 2a). *Myrmicaria brunnea* preferred the highest available concentration of sucrose (Fig. 2b). *Technomyrmex albipes* consumed only higher concentration solutions of glucose to a significantly greater extent over lower concentrations (Fig. 2c).

In experiments with solutions containing mixtures of sugars and amino acids, *C. dohrni* consumed all solutions equally (Experiment 4; Fig. 3a), while *M. brunnea* preferred solutions with amino acid groups E1N1, E1N2, and E2N1 (Fig. 3b). Only *T. albipes* significantly utilized the solution containing all essential amino acids (solution E) to a greater extent over other solutions (Fig. 3c). *Technomyrmex albipes* thus exhibited greater responsiveness to essential amino acid

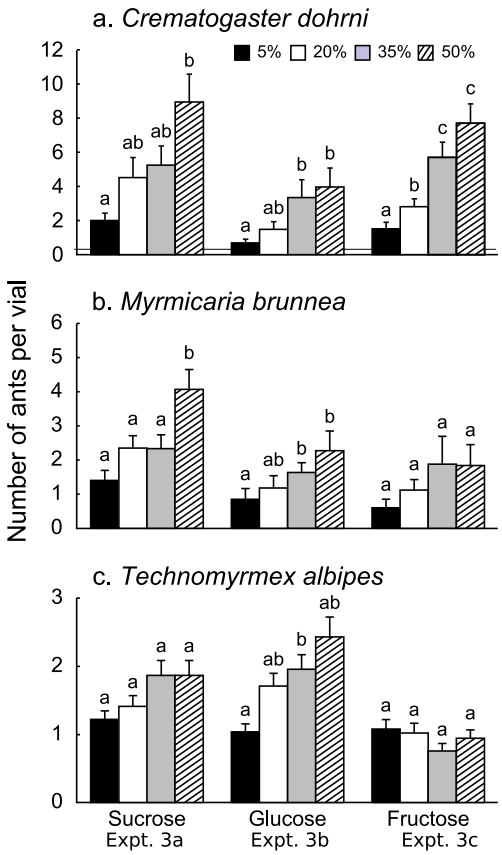

Fig. 2 Ant visits to vials of different concentrations of the same sugar presented simultaneously **a**: *Crematogaster dohrni* in Agumbe; **b**: *Myrmicaria brunnea* in Sampaji; **c**: *Technomyrmex albipes* in Solaikolli. Ant visitation was quantified by mean number of ants per sugar solution at each sampling point. Means+SE followed by the same letter within the same experiment are not significantly different (Kruskal-Wallis tests followed by Mann-Whitney *U* tests after appropriate Bonferroni correction)

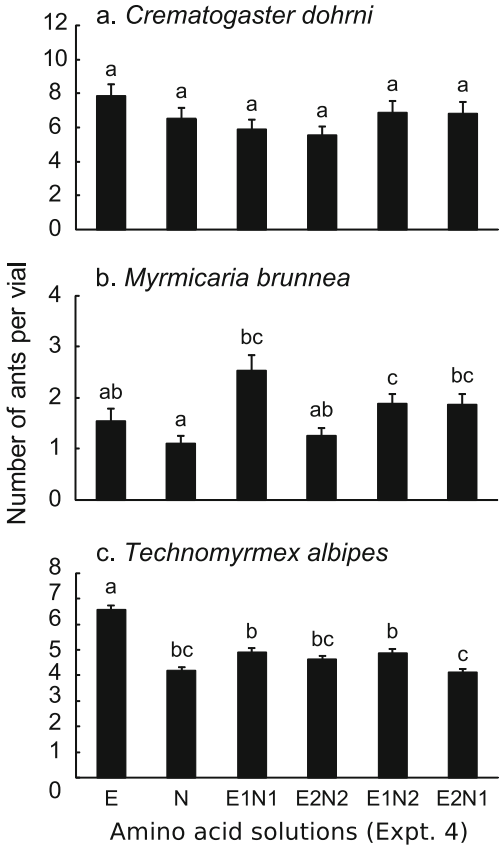

Fig. 3 Ant visits to vials containing solutions of different combinations of amino acids **a**: *Crematogaster dohrni* in Agumbe; **b**: *Myrmicaria brunnea* in Sampaji; **c**: *Technomyrmex albipes* in Solaikolli. Means+SE followed by the same letter are not significantly different (Kruskal-Wallis tests followed by Mann-Whitney *U* tests after appropriate Bonferroni correction)

contents (which could be their specific dietary targets) compared to *C. dohrni* and *M. brunnea*.

Cafeteria Assays Using Solutions Mimicking Familiar and Non-Familiar Floral Bud EFN Familiarity did not influence consumption of FB EFN mimics. When presented simultaneously with FB EFN mimics from familiar and non-familiar sites, *C. dohrni* did not differentiate between mimics of the familiar location (Agumbe) and unfamiliar locations (Sampaji, Solaikolli) (Fig. 4a). Similarly, *M. brunnea* did not differentiate between mimics of familiar (Sampaji) and unfamiliar (Agumbe, Solaikolli) locations (Fig. 4b) (Experiment 5a). *Technomyrmex albipes* preferred the non-familiar mimics of low concentration FB EFN from Agumbe or Sampaji over the familiar mimics of FB EFN from Solaikolli (Fig. 4c). The mimics of low concentration FB EFN that were preferred by *T. albipes* had neither the highest amino acid concentration of the FB EFN mimics nor the lowest sugar:amino acid ratio (Supplemental Table 1) but had the lowest viscosity (Fig. 5a). Furthermore, the mimic of Sampaji FB EFN preferred by *T. albipes* did not

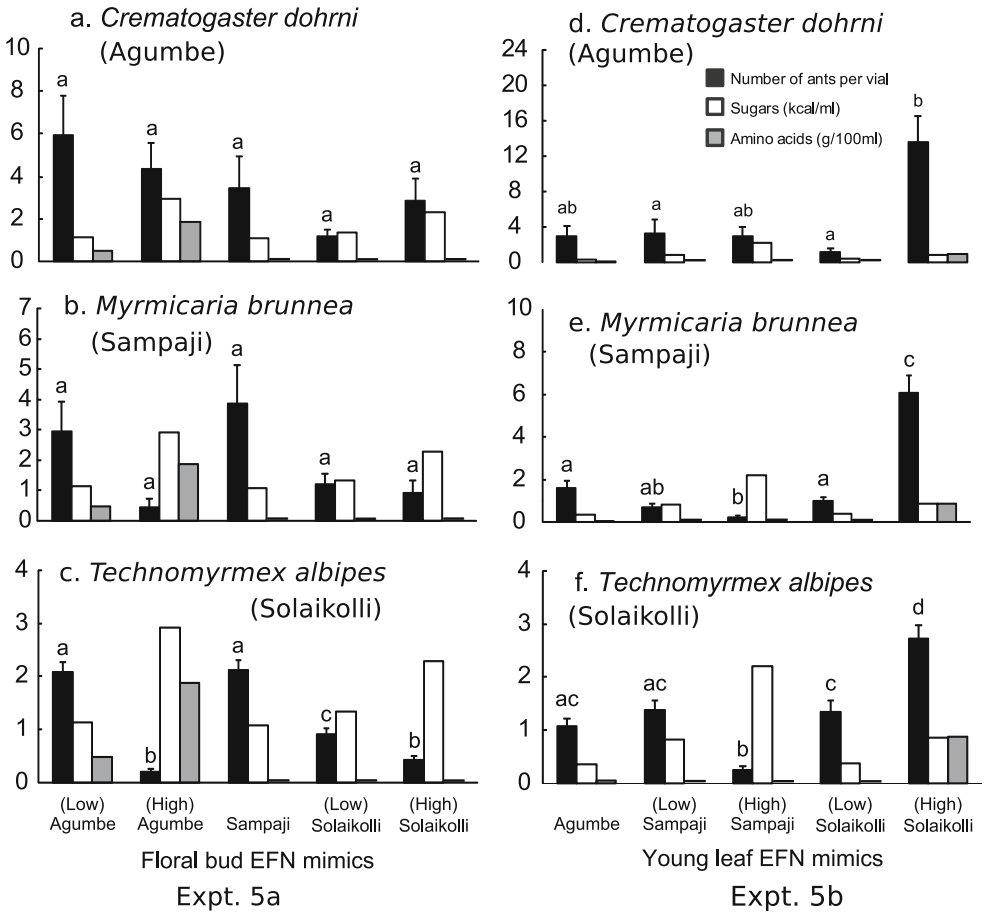

Fig. 4 Ant visits to vials containing solutions that mimic EFN and caloric content and amino acid content for each tested solution. a-c, floral bud EFN mimics **a**: *Crematogaster dohrni*; **b**: *Myrmicaria brunnea*; **c**: *Technomyrmex albipes.* d-f, young leaf EFN mimics **d**: *Crematogaster dohrni*; **e**: *Myrmicaria brunnea*; **f**: *Technomyrmex albipes*. Means+SE showing the number of ants per vial followed by the same letter are not significantly different (Kruskal-Wallis tests followed by Mann-Whitney *U* tests after appropriate Bonferroni correction)

contain any amino acids, but had a low sugar content similar to that of the low concentration Agumbe FB EFN (Supplemental Table 1, Fig. 4c). These results are consistent with those from Experiments 1 and 2 (Fig. 1c, f) in which sugar concentrations up to 20% were preferred by this ant.

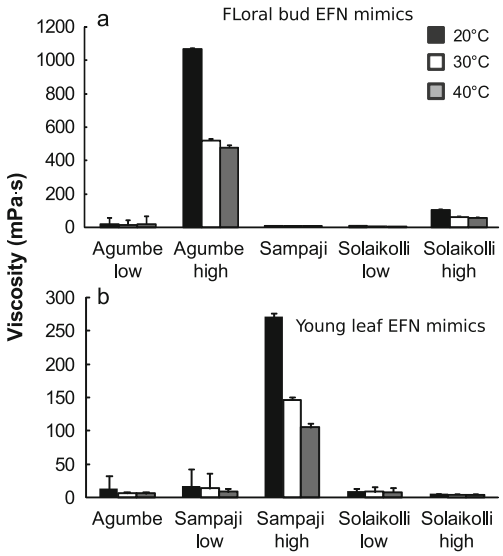

Fig. 5 Viscosity of solutions that mimic **a:** floral bud EFN and **b**: young leaf EFN produced by *Humboldtia brunonis* at the three sites. Mean+SE (*N*=10 trials per solution)

Cafeteria Assays Using Solutions Mimicking Familiar and Non-Familiar Young Leaf EFN Familiarity did not influence consumption of YL EFN mimics. When presented simultaneously with mimics of YL EFN from familiar and non-familiar sites, all three ant species preferred the mimic of high concentration YL EFN from Solaikolli over all others (Experiment 5b; Fig. 4d, e, f). *Technomyrmex albipes* clearly did not prefer the non-familiar mimic of high concentration YL EFN from Sampaji (Fig. 4f). This EFN mimic had the highest sugar concentration and viscosity among YL EFN mimics (Figs. 4f, 5b). The mimic of high concentration YL EFN from Solaikolli that was preferred by all three ant species had the lowest ratio of sugars to amino acids of all YL EFN mimics (Supplemental Table 1) and also the lowest viscosity (Fig. 5b).

Cafeteria Assays Using Solutions Mimicking Familiar Floral Bud and Young Leaf EFN In a choice between solutions mimicking FB or YL EFN from their native site, *C. dohrni* utilized all solutions equally (Experiment 6; Fig. 6a). *Myrmicaria brunnea* preferred FB mimics over high concentration YL EFNs (Fig. 6b). These FB mimics had sugar concentrations in the 40% range and no amino acids (Supplemental

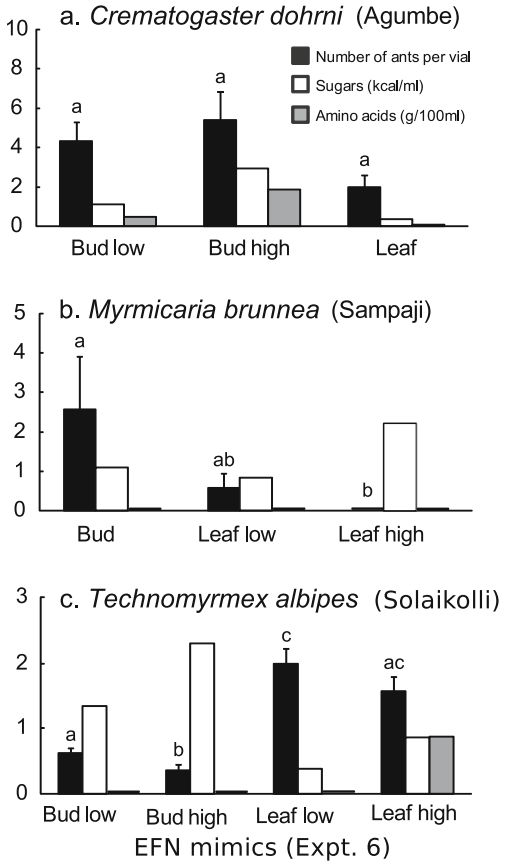

Fig. 6 Ant visits to vials containing solutions that mimic floral bud and young leaf EFN from each site **a**: *Crematogaster dohrni* in Agumbe; **b**: *Myrmicaria brunnea* in Sampaji; **c**: *Technomyrmex albipes* in Solaikolli. Means+SE showing the number of ants per vial followed by the same letter are not significantly different (Kruskal-Wallis tests followed by Mann-Whitney *U* tests after appropriate Bonferroni correction)

Table 1). These results are consistent with those of Experiment 3 in which *M. brunnea* preferred 35% and 50% sucrose and glucose solutions (Fig. 2b). *Technomyrmex albipes* preferred mimics of either high or low concentration YL EFN over FB EFN mimics (Fig. 6c). These YL EFNs had lower sugar levels than those of FB EFNs (Supplemental Table 1). These results are also consistent with those from Experiments 1 and 2 (Fig. 1c, f) in which sugar concentrations up to 20% were most utilized by this ant. Among the four solutions presented in this test, only the high concentration YL EFN from Solaikolli had amino acids (Supplemental Table 1).

Differences in Viscosity of the EFN Mimics The viscosity of the solution mimicking high concentration FB EFN from Agumbe (1065 mPa s) was 400- to 600-fold higher than that of the other FB EFN mimics (Fig. 5a). The viscosity of the solution mimicking high concentration YL EFN from Sampaji (270 mPa s) was 50- to 200-fold higher than that of other YL EFN mimics (Fig. 5b). The high viscosities are a result of high sugar concentrations (Table 2, Supplemental Table 1) resulting in crystalline EFN in some cases. For comparison, note that glycerin has a viscosity of about 1500 mPa s while the viscosity of motor oils varies between 100–500 mPa s.

Discussion

EFN Chemistry in H. brunonis Relative to Other Plants Overall, the sugar composition of *H. brunonis* EFN was similar to that of other plants (Baker et al., 1978; Koptur, 1992). However, the total amino acid concentration in some sites (Table 2) was at the higher end of the EFN range reported in the literature (3×10^{-5} to 2.67 g/100 ml) with most other plant species having EFN amino acid concentrations in the range of 3×10^{-2} g/100 ml (Koptur, 1979; Inouye and Inouye, 1980; Caldwell and Gerhardt, 1986; Heil et al., 2000; Blüthgen et al., 2004). Consequently, the sugar:amino acid ratios in *H. brunonis* EFN were lower than in EFNs of most plants (Davidson and Cook, 2008; Ness et al., 2009). This could be because *H. brunonis* is a legume and is not nitrogen limited (McKey, 1994), or because it is a myrmecophyte that does not produce food bodies but provides amino acids to its resident ants via EFN thus improving their nutritional balance and ensuring their fidelity. The invertase activity of *H. brunonis* EFN (Table 2) is similar to that of the EFN produced by generalist (0.01–0.09 μg glucose $\mu l^{-1} min^{-1}$) as compared to specialized (0.73–1.52 μg glucose $\mu l^{-1} min^{-1}$) ant-*Acacia* plant species (Heil et al., 2005), suggesting a generalist interaction between *H. brunonis* and its EFN-consuming ants.

There was between- and within-site variation in EFN composition and significant interaction effects between site and EFN type. The causes for such variation may include microhabitat heterogeneity in soil, humidity, and exposure to microbes that produce invertase and other enzymes that alter EFN composition (Inouye and Inouye, 1980; Koptur, 1994; Heil et al., 2005). The highly viscous EFN at Agumbe, for example, may result from the low canopy cover and resulting evaporation.

Partner-Choice Mechanisms: Role of EFN Composition in Maintaining Ant–Plant Associations *Humboldtia brunonis* produces EFN only during the dry season when ant activity is high (Basu, 1997). Such a brief period of EFN availability may preclude long-term specialization by ants (Schemske, 1982) on *H. brunonis* EFN. In this system, therefore, the short-term partners are likely to be those ant species that discriminate between plants or plant parts through the quality of the seasonal EFN they provide. Our results indicate that in these species, familiarity with the composition of seasonally available EFNs did not influence their utilisation by local ants, unlike some other ant species that are specialized on nectar sources such as honeydew (Völkl et al., 1999; Tinti and Nofre, 2001) or EFN (González-Teuber and Heil, 2009b). We found

a single dominant ant species at each site feeding on EFN or on experimental solutions. Of these, *C. dohrni* at Agumbe is a castration parasite of *H. brunonis* (Gaume et al., 2005a), *M. brunnea* at Sampaji is largely a tender of homopterans and a non-predatory scavenger (Gaume et al., 2005a), while the dolichoderine *T. albipes* at Solaikolli is a mutualist and provides protection to *H. brunonis* from herbivores (Gaume et al., 2005a; 2005b; Shenoy and Borges, 2010).

The biology of the ants was consistent with their particular nectar preferences. The castration parasite *C. dohrni* appeared least selective in all tests and did not appear to have specific dietary targets or constraints under the experimental conditions. Whether castration parasitism is coupled with nutrient opportunism in ant–plant interactions is unknown. The largely trophobiont-tending non-protective *M. brunnea* was attracted to solutions with high sucrose concentrations. It is possible that since trophobiont-tending provides access to sufficient quantities of very dilute sugars in honeydew (Woodring et al., 2004; Detrain et al., 2010), *M. brunnea* values only high sugar concentration EFN sources. The mutualistic dolichoderine *T. albipes* demonstrated definite feeding preferences as well as constraints. Dolichoderines such as *T. albipes*, unlike myrmicines, are constrained by their proventricular anatomy to consume more liquids than solids (Davidson, 1997; Davidson et al., 2004). *Technomyrmex albipes* had a clear preference for dilute solutions with low viscosity, and also those with the lowest sugar:amino acid ratios. Whether the viscosity constraint results from its proventricular anatomy is unknown. Furthermore, *T. albipes* preferred solutions with essential amino acids. It is possible that *T. albipes* does not harbor endoysmbionts that synthesize essential amino acids, as occurs in specialized plant-ants such as *Dolichoderus* (Schroder et al., 1996; Cook and Davidson, 2006). These constraints could explain why *T. albipes* preferred those EFN mimics that had sugars in the lower concentration range, lower viscosities, and that contained essential amino acids. The YL EFN mimic from Solaikolli that was most strongly preferred by *T. albipes* was also the one that had the largest number of amino acids of which most were essential amino acids (Supplemental Information Table 1). We suggest that these essential amino acids could be the specific dietary targets (*sensu* Simpson and Raubenheimer, 1996) that *T. albipes* seeks in plant-based exudates. The preferential foraging by *T. albipes* on individual plants with suitable EFN could also result in greater patrolling or nesting on these plants, and may explain the increased fitness of certain individual trees occupied by *T. albipes* (Gaume et al., 2005b).

Our study also suggests that the chemical composition of EFN can reduce ant–pollinator conflict, at least in the mutualist *T. albipes* via its decreased utilization of floral bud EFN. Spatial, temporal, and chemical resolution of ant–pollinator conflicts via floral attributes is known (Raine et al., 2002; Nicklen and Wagner, 2006), but the role of the chemical composition of EFN in ant–pollinator conflict is unexplored. *Technomyrmex albipes* preferred leaf over floral bud EFN mimics suggesting that EFN composition can play a pivotal role in minimizing possible antagonistic interactions between ants and pollinators. *Technomyrmex albipes* protects young leaves to a greater extent than floral buds resulting in 24.5% reduction of herbivory on patrolled leaves vs. 16% reduction on patrolled floral buds (Shenoy and Borges, 2010). This difference in protection of plant parts may be due to differential utilization of floral and leaf EFN resulting from their chemical differences. Differential protection of foliar compared to reproductive structures was found in one other ant-plant system (Palmer and Brody, 2007) and was attributed to the fact that only leaves afforded the ants with nutrition and/or housing in the form of domatia.

Our study has shown that local variation in dominant ant species coupled with local variation in plant reward attributes may lead to a local and seasonal protection mutualism between ants and plants. We demonstrated: (i) the ability of mutualistic ant species to choose between food sources that differ in quality; (ii) how this ability to choose could lead to selection of trees or of particular plant tissues producing suitable EFN; and (iii) how ant preference for specific EFN compositions could be based on anatomical/physiological constraints, sugar:amino acid ratios or specific dietary targets but not on familiarity of nutrients. These findings improve our understanding of the extent to which EFN composition can influence the identity and abundance of ants that are attracted to EFN and thereby alter the nature of ant–plant interactions.

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Herbivore-Mediated Effects of Glucosinolates on Different Natural Enemies of a Specialist Aphid

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Abstract The cabbage aphid *Brevicoryne brassicae* is a specialist herbivore that sequesters glucosinolates from its host plant as a defense against its predators. It is unknown to what extent parasitoids are affected by this sequestration. We investigated herbivore-mediated effects of glucosinolates on the parasitoid wasp *Diaeretiella rapae* and the predator *Episyrphus balteatus*. We reared *B. brassicae* on three ecotypes of *Arabidopsis thaliana* that differ in glucosinolate content and on one genetically transformed line with modified concentrations of aliphatic glucosinolates. We tested aphid performance and the performance and behavior of both natural enemies. We correlated this with phloem and aphid glucosinolate concentrations and emission of volatiles. *Brevicoryne brassicae* performance correlated positively with concentrations of both aliphatic and indole glucosinolates in the phloem. Aphids selectively sequestered glucosinolates. Glucosinolate concentration in *B. brassicae* correlated negatively with performance of the predator, but positively with performance of the parasitoid, possibly because the aphids with the highest glucosinolate concentrations had a higher body weight. Both natural enemies showed a positive performance-preference correlation. The predator preferred the ecotype with the lowest emission of volatile glucosinolate breakdown products in each test combination, whereas the parasitoid wasp preferred the *A. thaliana* ecotype with the highest emission of these volatiles. The study shows that there are differential herbivore-mediated effects of glucosinolates on a predator and a parasitoid of a specialist aphid that selectively sequesters glucosinolates from its host plant.

Keywords *Arabidopsis thaliana* · *Brevicoryne brassicae* · *Diaeretiella rapae* · *Episyrphus balteatus* · Parasitoid wasp · Predator · Sequestration

Introduction

Plants have evolved a wide array of traits that confer resistance to herbivores (Karban and Baldwin, 1997; Schoonhoven et al., 2005). Specialists that are adapted to feeding on plants containing specific secondary metabolites, however, often use these compounds for their own benefit, e.g., as oviposition or feeding stimulants (van Loon et al., 1992; Gabrys and Tjallingii, 2002). Some concentrate metabolites actively taken up from host plants in special tissues or organs. This sequestration can make these herbivores unpalatable to natural enemies (Duffey, 1980; Müller, 2009).

Brassicaceous plants contain glucosinolates (GLS) that, upon damage by chewing herbivores, become exposed to the plant enzyme myrosinase that hydrolyzes GLS, resulting in toxic compounds such as (iso)thiocyanates and nitriles that negatively affect a wide variety of generalist herbivores

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(Halkier and Gershenzon, 2006; Hopkins et al., 2009). Phloem-feeding herbivores, however, can ingest GLS without bringing these compounds into contact with plant myrosinases (Andreasson et al., 2001). Thus, aphids prevent the formation of toxic hydrolysis products of most GLS (de Vos et al., 2007; Kim and Jander, 2007). The cabbage aphid *Brevicoryne brassicae* is a specialist that uses GLS as feeding stimulants (Gabrys and Tjallingii, 2002), and sequesters GLS from its food plants (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Kos et al., 2011). It contains an endogenous myrosinase, which is stored separately from the GLS (Jones et al., 2001; Bridges et al., 2002; Francis et al., 2002). Upon predator attack, sequestered GLS come in contact with the aphid myrosinase, resulting in the formation of toxic hydrolytic products. Negative effects of this sequestration have been reported for aphid predators, such as ladybird beetles, hoverflies, and lacewings (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Chaplin-Kramer et al., 2011; Kos et al., 2011). Most predators kill their prey immediately and feed on multiple individuals during their development. Parasitoids, in contrast, develop inside a single host individual, and koinobiont parasitoids allow the host to continue to grow and feed after parasitization (Godfray, 1994). Parasitoids are probably differentially affected by sequestration of GLS in *B. brassicae* compared to predators, but this rarely has been investigated. Le Guigo et al. (2011) compared the fitness of the solitary endoparasitoid *Diaeretiella rapae* when developing in *B. brassicae* that were feeding on host plant species with different foliar GLS concentrations. Parasitoid performance did not correlate with foliar GLS concentrations. In that study, GLS concentrations in the aphids were not analyzed. It has been shown previously that *B. brassicae* sequesters GLS selectively (Kabouw et al., 2011; Kos et al., 2011). It is still unknown to what extent *D. rapae* performance is affected by GLS sequestration in *B. brassicae*.

GLS not only affect performance of natural enemies of herbivores feeding on brassicaceous plants, but also their behavior. Formation of volatile GLS breakdown products, resulting from herbivore feeding, increase attraction of several specialist parasitoids (Bradburne and Mithen, 2000; Blande et al., 2007; Mumm et al., 2008). Effects of volatile GLS breakdown products on the behavior of generalist predators are largely unknown.

Our objective was to investigate herbivore-mediated effects of GLS on the performance and the behavior of the parasitoid *D. rapae* and the predacious hoverfly *Episyrphus balteatus*. These species represent two different groups within carnivorous insects and are two of the most important natural enemies of *B. brassicae*. To obtain aphids that differ in their sequestered GLS concentrations, we reared them on three ecotypes of *Arabidopsis thaliana* that differ qualitatively and quantitatively in their GLS content (Houshyani et al., in press). Additionally, a genetically transformed line was created to produce higher concentrations of foliar aliphatic (methionine-derived) GLS compared to the wild-type plants. We compared the performance of *B. brassicae* on these different ecotypes/lines, analyzed the GLS in the phloem and in the aphids feeding on it, and determined the performance of *E. balteatus* and *D. rapae* when feeding on these aphids. Additionally, we studied parasitoid and predator preference behavior in response to aphid-induced volatile organic compounds emitted by the different plant ecotypes/lines.

Methods and Materials

Plant Material and Growth Conditions Three *Arabidopsis thaliana* (L.) Heynh. ecotypes were selected, based on their maximal divergence in metabolite profiles (qualitative and quantitative composition of the mix of metabolites) (Houshyani et al., in press). Columbia (Col)-0 was provided by Dr. P. Reymond (Lausanne, Switzerland); Cape Verde Island (Cvi) was obtained from the European *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk/, Cvi = N8580); and Eringsboda (Eri) was collected in Sweden by members of the Laboratory of Genetics, Wageningen University (Eri-1 = CS22548).

To produce plants with higher foliar levels of aliphatic GLS, we over-expressed the transcription factor HAG1/MYB28 in *A. thaliana* ecotype Col-0 (Houshyani et al. unpublished data, see also Supplemental Material Online Resource 1). This transcription factor represents a key component in the regulation of aliphatic GLS biosynthesis in *A. thaliana* (Gigolashvili et al., 2007). T2 generation seeds of one successfully transformed line (hereafter named Col-0-MYB28) were used in the experiments.

Arabidopsis thaliana seeds were surface-sterilized overnight by vapor phase sterilization and inoculated on a growth medium (purified agar 0.8%+2.2 gl^{-1} 0.5 MS+ vitamins; pH 6; containing 30 $\mu g\ ml^{-1}$ kanamycin to select transformed seedlings). After 4 d of stratification at 4°C, plates were transferred to a growth chamber at 21±2°C, 50–70% relative humidity (RH) and a 8:16L:D photo regime, with a light intensity of 200 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD).

Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diam) containing autoclaved soil (80°C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times a week, and the soil was treated weekly with entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) to control infestation by larvae of sciarid flies. Plants used were 6–7 wk-old, and remained in the vegetative state during experiments.

Insect Rearing *Brevicoryne brassicae* L. (Hemiptera: Aphididae) were reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus). *Episyrphus balteatus* de Geer

(Diptera: Syrphidae) pupae were provided by Koppert Biological Systems and kept in gauze cages (67 × 50 × 67 cm). Adults emerging from the pupae were provided with water, a *B. brassicae*-infested *B. oleracea* plant, organic sugar grains, and bee-collected pollen provided by Koppert Biological Systems. *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) was reared in gauze cages (30 × 40 × 60 cm) containing *B. brassicae*-infested *B. oleracea* plants. Wasps were provided with water and honey. The *B. brassicae* and *D. rapae* culture originated from individuals obtained from *B. oleracea* in the vicinity of Wageningen (The Netherlands) in 2008. All insect species were reared at 22±2°C, 60–70% RH and a 16:8 h L:D photo regime.

GLS and Primary Metabolites in Phloem of Aphid-Infested Plants After the aphid performance experiment ended, phloem of aphid-infested plants was collected for chemical analysis. We used 8 mM EDTA, following the procedure described in Kos et al. (2011). Four fully-grown leaves of each plant were placed with their petiole for 5 min in the EDTA solution to remove any plant chemicals from the incision. Then, leaves were placed for 4 h in a new vial with 200 μl EDTA solution, under dark conditions. Using this method, a small amount of mesophyll fluids was inherently collected as well. Following incubation, the EDTA solution was collected from the vials, and each vial was rinsed with 50 μl EDTA, resulting in a sample of 250 μl per leaf. The EDTA solution of four plants (16 leaves) was pooled to form one sample of 4 ml, resulting in five replicates per ecotype/line. Leaves were dried at 80°C for 3 d and weighed on an analytical balance (Mettler-Toledo PM200, Tiel, The Netherlands). Phloem samples were frozen at −80°C immediately after collection, freeze-dried, and re-suspended in 2 ml 8 mM EDTA. Half of the collected phloem sample was used for GLS extraction, and half for soluble carbohydrate and amino acid extraction. To extract GLS from the phloem, we used the protocol described by Kos et al. (2011). GLS were separated using high-performance liquid chromatography (HPLC) as described previously by van Dam et al. (2004) and Kabouw et al. (2010). GLS detection was performed with a photodiode array detector set at 229 nm as the integration wavelength. Different concentrations of sinigrin (2-propenylGLS; Acros, NJ, USA) were used as external standard. The retention times of the GLS compounds can be found in Supplemental Material Online Resource 2. The correction factors at 229 nm from Buchner (1987) and the European Community (1990) were used to calculate the concentrations of the GLS. DesulfoGLS peaks were identified by comparison of HPLC retention times and ultraviolet spectra with standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology, Jena, Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels, Belgium, code BCR-367 R).

Soluble carbohydrates (50 μl from the one ml sample) and amino acids (50 μl) were extracted and analyzed as described previously by van Dam and Oomen (2008). For the carbohydrates, we used a "10 ppm" reference solution containing 54.9 μM sorbitol and mannitol, 29.21 μM trehalose, sucrose, and melibiose, and 55.51 μM glucose and fructose. This reference solution was diluted to obtain 7.5, 5, and 2.5 ppm calibration standards to obtain a reference curve. To obtain a reference sample containing the 20 most common amino acids, the Sigma AA S 18 amino acid standard (Sigma, St Louis, MO, USA) containing 17 amino acids was supplemented with asparagine, glutamine, and tryptophane (2.5 μmoles ml^{-1} each). This reference solution was diluted to obtain calibration standards ranging from 1 to 8 μM for each amino acid, except for cysteine, which had a range of 0.5–4 μM. For both the carbohydrates and the amino acids, an additional standard was injected after every 10 samples to check for deviations of retention times and the calibration curve.

Dynamic Headspace Collection of Volatiles from Aphid-Infested Plants Six-to-seven week-old *A. thaliana* plants were infested with 100 *B. brassicae* nymphs of mixed instars 3 d prior to headspace collection. Dynamic headspace collection was carried out in a climate chamber at 20± 2°C. Plants were removed from pots, and the soil was wrapped with aluminum foil. Three plants were placed together in a 2.5 l glass jar. Volatiles were collected by sucking air out of the jar at a rate of 90 ml min^{-1} for 3 h through a stainless steel cartridge (Markes, Llantrisant, UK) containing 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, MI, USA). Foliar fresh weight of the plants in each pot was measured after volatile collection. For each ecotype/line, 8–11 replicate samples were collected (8 Cvi, 10 Eri, 9 Col-0, 11 Col-0-MYB28).

Headspace samples were analyzed by using a Thermo Trace Gas Chromatography Ultra (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer (MS) (Supplemental Material Online Resource 3). The peak area of each compound was expressed per unit plant fresh weight.

Identification of Compounds Identification of compounds was based on comparison of mass spectra with those in the NIST 2005, Wiley and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) also were used as additional criterion for confirming the identity of the compounds. Relative quantification (peak areas of individual compounds) was performed using a single (target) ion, in selected ion monitoring (SIM) mode (see Supplemental Material Online Resource 4 for detailed information on the identification methods for each compound).

Plant Morphology and Foliar GLS Concentrations of Uninfested Plants We quantified two plant morphological characteristics, trichome density and plant biomass, because these might influence aphid and natural enemy performance and behavior. For 10 uninfested plants per ecotype/line, we measured foliar biomass and counted the number of trichomes in a 25 mm^2 area in the central part of the abaxial side of the 6th or 7th youngest leaf by using a microscope (Leitz Dialux 20 EB, Wetzlar, Germany; magnification 40×).

For foliar GLS analysis, we harvested all leaf material of 10 uninfested plants per ecotype/line. Samples were frozen at −80°C immediately after collection, freeze-dried, weighed (approximately 100 mg) into micro-centrifuge tubes, and ground to a fine powder.

GLS were extracted and purified from the leaves by using a methanol extraction (van Dam et al., 2004; Kabouw et al., 2010). GLS were separated and detected as described above for the phloem samples.

Insect Performance Individual plants with insects were confined to cylindrical plastic containers (height 13 cm; diam 11 cm) with a gauze lid. Experiments were performed in a climate chamber at 21±2°C, 50–70% RH and a 8:16 L:D photo regime for *B. brassicae* and 16:8 L:D for *E. balteatus* and *D. rapae*. The light intensity at plant level was 200 μmol m^{-2} s^{-1} PPFD. Plants were watered once a week.

Aphid Performance Several 6-wk-old plants of each ecotype/line were inoculated with 10 adult aphids per plant. After 24 h, adult aphids were removed, and the produced offspring were allowed to develop for 3 d until they reached the second instar (L2). Three L2 nymphs were transferred to each of 20 *A. thaliana* plants per ecotype/line, the same ecotype/line as the one on which these nymphs had been feeding before. Until the adult stage, survival of nymphs was recorded daily. The fastest developing adult was kept on the plant, while the other adults were removed. Alate (winged) adults (ca. 5% of all adults) were excluded from the experiment as these contain lower concentrations of GLS than apterous (wingless) aphids (Kazana et al., 2007). The development time until first reproduction ($=T_d$) of the remaining adult was recorded, and the adult fresh weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany). Adults were allowed to feed on plants and produce offspring, and after a certain number of days (equivalent to T_d), the number of offspring ($=N$) produced by the adult was counted. The estimated intrinsic rate of population increase (r_m) was calculated for each aphid using the formula: $r_m = 0.738 \times (\ln N)/T_d$ (Karley et al., 2002).

Aphid GLS Concentrations After the aphid performance experiment ended, aphids on the 4 plants that were used to obtain one phloem sample (described above) were removed and pooled into one sample. GLS were extracted similarly to the method used for the leaves.

Predator Performance Female *E. balteatus* from the stock rearing were allowed to lay eggs on Brussels sprouts plants infested with *B. brassicae.* After hatching, neonate larvae were transferred to *A. thaliana* plants that had been infested by 10 adult *B. brassicae* from the stock rearing 1 wk earlier. Larvae were allowed to develop on the plants until pupation. Pupae were checked once a day for eclosion of adults. Survival, larva-to-adult development time, sex, and adult dry weight were determined. Newly eclosed adults were frozen, dried to constant weight at 80°C for 3 d, and then weighed on a microbalance. We determined the performance of 35 larvae per *A. thaliana* ecotype/line, one larva per plant.

Parasitoid Performance Aphid mummies containing a *D. rapae* pupa were collected from the stock rearing, and reared until adult parasitoid eclosion. Adult parasitoids were provided with water and honey, allowed to mate, and used for parasitisation when they were 2–4 d-old. Second instar (3-d-old) *B. brassicae* nymphs that had been feeding on one of the *A. thaliana* ecotypes/lines were exposed individually to mated female parasitoids on an aphid-infested leaf until parasitisation was observed (i.e., when the female inserted her ovipositor into the nymph). Four parasitized nymphs were transferred to one *A. thaliana* plant of the same ecotype/line as the one on which these aphids had been feeding before. In total we tested 22 plants per *A. thaliana* ecotype/line. Mummies were collected from plants, and after eclosion, parasitoid sex was determined, and egg-to-adult development time and adult dry weight were measured, as described for the predator. The percentage of successful parasitism of *B. brassicae* by *D. rapae* was calculated per plant by dividing the number of *D. rapae* adults by the total number of *B. brassicae* nymphs that survived (either until the adult stage or until *D. rapae* eclosion) on each plant.

Predator and Parasitoid Preference The preference of predators and parasitoids for volatiles from an ecotype/line was investigated in two-choice bioassays. We tested the ecotypes against each other, and Col-0-MYB28 against Col-0. Plants were treated similarly as described above under *Dynamic Headspace Collection of Volatiles from Aphid-infested Plants.*

Predator Oviposition Preference Mated female hoverflies from the stock rearing were used in the behavioral assays when they were 2–3-wk-old. Females were transferred to a plastic cage (30 × 30 × 30 cm) containing one aphid-infested plant of two different ecotypes/lines, and 10% sugar solution. Females were allowed to oviposit on the plants for

24 h. The number of eggs deposited on each plant was counted. Replicates with females that did not lay any eggs were eliminated from the analysis. For each plant combination, at least 22 replicates with ovipositing females were obtained.

Parasitoid Preference for Aphid-Induced Plant Volatiles Parasitoid behavior was assessed in a Y-tube olfactometer in a climatized room at 22±2°C as described by Bukovinszky et al. (2005). Compressed air was filtered over charcoal and split into two air streams each at a flow of 2 $l min^{-1}$. Each air stream was led through a 5 l glass jar that contained 4 aphid-infested plants of one of the two ecotypes/lines of a test combination. Each air stream was then led into one of the two arms of the Y-tube. The olfactometer was illuminated from above with artificial light at an intensity of 60 $\mu mol\ m^{-2}\ s^{-1}$ PPFD.

Naïve, mated 2-d-old *D. rapae* females were allowed to oviposit for 1 h in aphids feeding on one of the two ecotypes/lines of a combination (equally divided among the tested wasps) to increase their host-searching behavior. Experienced wasps were released individually at the base of the Y-tube, and their preference for one of both odor sources was recorded. A choice was recorded when a wasp crossed a finish line drawn one cm before the end of each arm, and did not return to the junction within 15 s. Wasps that did not make a choice within 15 min were considered as non-responsive and were omitted from the statistical analysis. Four or five new sets of plants were used for each test combination. For every new set of plants, 20 wasps were tested. After every 10 wasps, the position of the odor sources was exchanged to compensate for any asymmetry in the set-up.

Statistical Analyses Analyses were performed in SPSS for Windows (18th edition, Chicago, IL, USA), unless indicated otherwise. If variables were log-transformed to obtain normality and equal variance, this is indicated in the relevant table of the Results section. To test the effect on the continuous variables, such as development time and body weight, we used ANOVA followed by *post-hoc* Tukey-tests for pair-wise ecotype comparisons and *t*-tests for comparisons between Col-0 and Col-0-MYB28. If assumptions on normality and equal variance were violated, Kruskal-Wallis tests with *post-hoc* Mann–Whitney *U* tests with a Holm's sequential Bonferroni correction were used for pair-wise ecotype comparisons, and Mann–Whitney *U* tests for comparisons between Col-0 and Col-0-MYB28. Survival and the percentage of successful parasitism were calculated per plant (plant was used as the experimental unit), and differences in these variables among ecotypes and between Col-0 and Col-0-MYB28 were analyzed by logistic regression in GenStat (13th edition, VSN International, UK). If over-dispersion was observed, the data were corrected for this by using estimated dispersion instead of fixed dispersion. T-probabilities were calculated to test pair-wise differences between means.

To test whether an equal number of predator eggs was laid on each ecotype/line in a test combination, Wilcoxon matched-pairs signed-rank tests were used. To test whether an equal number of parasitoid wasps chose either ecotype/line in a test combination in the Y-tube olfactometer, *Chi-square* tests were used. Effects of parasitoid experience on the preference of the wasps was tested by logistic regression in GenStat.

To determine whether there were differences in volatile profiles and aphid GLS profiles among the ecotypes and between Col-0 and Col-0-MYB28, we used multivariate discriminant analysis Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12th edition, Umetrics, Umeå, Sweden) (Eriksson et al., 2006). For the volatiles, the variable importance in the projection (VIP) was calculated. Variables with a VIP value higher than 1 are most influential for the discrimination among the ecotypes/lines (Eriksson et al., 2006). For volatile compounds with a VIP higher than 1, the difference among the ecotypes and between Col-0 and Col-0-MYB28 was analyzed as described above for the continuous variables. Partial Least Squares Projections to Latent Structures (PLS) in SIMCA-P, a multivariate method for regression analysis, was used to test the relationship between a) metabolite profiles in the phloem and performance of aphids feeding on those plants, and b) the GLS profile in the phloem and in the aphids feeding on those plants. For the latter PLS-analysis, only the GLS compounds that were found in both the phloem and the aphids, as well as the total GLS, total aliphatic GLS and total indole GLS, were included. To pre-process data, metabolite concentrations were log-transformed, mean-centered, and scaled to unit variance. To test whether concentrations of individual GLS compounds or classes in the phloem and aphids were correlated, we used Spearman's correlation test. PLS analyses of the relationships between aphid GLS concentrations and predator/parasitoid performance could not be performed, as we measured these variables in separate experiments. Note that in the Results section 'aliphatic GLS' refers to the total of all aliphatic GLS compounds that were detected, 'indole GLS' refers to the total of all indole GLS compounds, and 'total GLS' refers to the total of all GLS compounds (aliphatic and indole GLS combined).

Results

GLS and Primary Metabolites in Phloem of Aphid-Infested Plants Ecotype effect: Total, aliphatic and indole GLS concentrations in the phloem of aphid-infested plants differed among ecotypes (Kruskal-Wallis H, df=2, total: χ^2=6.48, P=0.039; aliphatic: χ^2=8.07, P=0.018; indole: χ^2=10.82, P=0.004), due to both qualitative and quantitative differences (Table 1). Phloem of Cvi had the highest total and aliphatic GLS concentrations, whereas phloem of Eri plants had the highest indole GLS concentrations. Phloem of Col-0

Table 1 Mean (± SE) concentrations of metabolites in the phloem of aphid-infested plants of three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line, and in *Brevicoryne brassicae* aphids reared on these plants

Metabolite			*Arabidopsis thaliana* ecotype			Transformed
			Cvi	Eri	Col-0	Col-0-MYB28
Phloem	Carbohydrates[a]	sorbitol	0.002±0.001	0.005±0.001	0.002±0.002	0.002±0.002
		mannitol	0.008±0.001	0.008±0.002	0.010±0.002	0.014±0.003
		trehalose	0.010±0.009	0.001±0.001	0.002±0.001	0.013±0.008
		glucose	0.392±0.069	0.149±0.020	0.156±0.010	0.216±0.014
		fructose	0.321±0.060	0.097±0.015	0.112±0.008	0.166±0.012
		sucrose	0.988±0.191	2.328±0.305	1.892±0.142	1.949±0.147
		raffinose[b]	0	0.015±0.006	0	0
		Total carbohydrates	1.721±0.312a	2.604±0.340a	2.175±0.139a	2.318±0.148 ns
	Amino acids[a]	arginine	0.095±0.017	0.080±0.011	0.136±0.012	0.157±0.014
		lysine	0.016±0.006	0.024±0.005	0.040±0.006	0.053±0.008
		glutamine	2.474±0.370	2.348±0.292	3.671±0.375	3.552±0.363
		asparagine	0.139±0.022	0.065±0.010	0.032±0.016	0.027±0.014
		alanine	0.026±0.013	0.036±0.005	0.070±0.008	0.099±0.011
		threonine	0.833±0.148	0.313±0.042	0.472±0.037	0.610±0.048
		valine	0.018±0.008	0.036±0.006	0.065±0.011	0.090±0.015
		serine	0.081±0.017	0.065±0.011	0.121±0.013	0.077±0.008
		leucine	0.010±0.006	0	0.008±0.005	0.009±0.006
		methionine	0.001±0.001	0	0.002±0.002	0.001±0.001
		histidine	0.004±0.004	0.012±0.001	0.021±0.003	0.095±0.014
		phenylalanine	0.017±0.004	0.014±0.002	0.033±0.004	0.034±0.004
		glutamate	0.066±0.001	0.040±0.004	0.098±0.009	0.114±0.010
		aspartate	0.058±0.012	0.047±0.009	0.113±0.016	0.091±0.013
		tyrosine	0.007±0.002	0.009±0.001	0.015±0.004	0.020±0.005
		Total amino acids[c]	3.844±0.592a	3.088±0.390a	4.897±0.470a	5.028±0.480 ns
	GLS[d,e]	2-(*S*)-2-hydroxy-butenylGLS	1.958±1.199	0	0	0
		2-propenylGLS	1.208±0.803	1.028±1.028	0	0
		3-butenylGLS	1.030±0.699	0	0	0
		3-methylthiopropylGLS	1.694±0.237	0.384±0.250	0.735±0.051	0.147±0.010
		Total aliphatic GLS	5.889±1.860b	2.426±1.258a	0.915±0.186a	0.147±0.010*
		3-indolylmethylGLS	0.348±0.100	0.705±0.069	0.302±0.068	0.248±0.055
		4-methoxy-3-indolylmethylGLS	0.507±0.073	0.805±0.064	0.309±0.054	0.297±0.052
		Total indole GLS	0.855±0.055a	1.511±0.117b	0.611±0.102a	0.544±0.090 ns
		Total GLS	6.744±1.851b	3.936±1.287ab	1.526±0.253a	0.691±0.086*
Aphid	GLS[e,f]	3-methylsulfinylpropylGLS	0	30.04±17.38	5.61±3.54	2.36±1.49
		4-methylsulfinylbutylGLS	2.13±0.52	5.53±2.77	70.08±35.61	46.96±23.86
		2-propenylGLS	76.59±10.86	7.49±1.35	7.27±2.25	3.05±0.95
		2-hydroxy-4-pentenylGLS	0.57±0.19	0.09±0.09	2.76±1.20	1.16±0.50
		3-butenylGLS	272.87±49.87	1.08±0.86	2.47±1.18	1.04±0.50
		3-methylthiopropylGLS	0.16±0.07	2.19±1.22	0	0
		4-methylthiobutylGLS	0.25±0.12	1.66±0.88	3.16±1.31	1.32±0.55
		3-hydroxypropylGLS	0.42±0.08	99.01±43.23	5.87±3.78	2.47±1.59
		7-methylsulfinylheptylGLS	16.37±2.71	3.72±1.45	6.68±2.67	1.94±0.78
		8-methylsulfinyloctylGLS	210.25±37.20	118.69±45.04	34.49±13.11	2.07±0.79
		Total aliphatic GLS	579.61±98.59b	269.50±111.37ab	138.40±58.95a	62.36±28.58 ns
		3-indolylmethylGLS	29.63±8.45	30.14±18.29	7.20±1.87	9.36±2.43
		4-hydroxy-3-indolylmethylGLS	0.44±0.18	1.11±0.64	0.19±0.19	0.05±0.05

Table 1 (continued)

Metabolite	*Arabidopsis thaliana* ecotype			Transformed
	Cvi	Eri	Col-0	Col-0-MYB28
4-methoxy-3-indolylmethylGLS	0.85±0.11	2.30±0.36	3.16±0.40	2.75±0.35
1-methoxy-3-indolylmethylGLS	0.05±0.04	0.08±0.05	0	0
Total indole GLS	30.98±8.71a	33.63±19.20a	10.54±2.14a	12.16±2.60 ns
Total GLS	610.59±104.11b	303.13±130.08ab	148.94±60.76a	74.52±30.89 ns

N=5 for each sample. For every sample, phloem or aphids collected from four plants were pooled

[a] μmol g^{-1} dry weight leaf

[b] Tentatively identified

[c] Parameter was log-transformed in statistical analysis to obtain normality

[d] nmol g^{-1} dry weight leaf

[e] Glucosinolates (GLS) are grouped according to their biosynthetic origin into indole and aliphatic GLS, and analyses were performed separately for total GLS, aliphatic GLS and indole GLS

[f] μmol g^{-1} dry weight aphids

Statistical tests were performed only for the total carbohydrate, amino acid, aliphatic GLS, indole GLS and total GLS concentrations, not for individual compounds. Different letters denote differences in means among the three ecotypes as analyzed by Mann–Whitney *U*-tests with sequential Bonferroni correction (for GLS) or ANOVA and *post-hoc* Tukey tests (for carbohydrates and amino acids)

*denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analyzed by Mann–Whitney *U*-tests (for GLS) or t-tests (for carbohydrates and amino acids)

Carbohydrates and amino acids have been identified and quantified based on calibration lines for the corresponding authentic standards. The retention times used for identification of each GLS compound can be found in Supplemental Material Online Resource 2. For quantification of GLS sinigrin (2-propenylGLS) was used as the external standard

plants had the lowest concentration of all GLS classes (Table 1). Ecotypes did not differ in total concentrations of carbohydrates and amino acids in the phloem (ANOVA, $P>0.05$ for both analyses), although there were small qualitative and quantitative differences in the concentrations of the individual compounds (Table 1).

Over-expression effect: The phloem of aphid-infested Col-0-MYB28 plants had lower concentrations of total and aliphatic GLS than Col-0 plants, and similar concentrations of indole GLS (Mann–Whitney *U*-test: total: $U<0.001$, $P=0.008$; aliphatic: $U<0.001$, $P=0.008$; indole: $U=7.00$, $P=0.310$; Table 1). This was unexpected, as foliar tissue of Col-0-MYB28 plants had higher concentrations of aliphatic GLS than Col-0 plants (Supplemental Material Online Resource 5). Total concentrations of carbohydrates and amino acids in the phloem did not differ between Col-0 and Col-0-MYB28 plants (Table 1).

Dynamic Headspace Collection of Aphid-Infested Plants Ecotype effect: The three *A. thaliana* ecotypes differed in volatile profiles of aphid-infested plants (4 PLS-DA principal components, $R_2X_{cum}=0.906$, $R_2Y_{cum}=0.836$, $Q_{2cum}=0.682$). The volatile profile of Cvi was high in breakdown products of GLS such as 3-butenyl isothiocyanate, 3-butene nitrile, and 3-methyl-3-butene nitrile. Col-0 plants emitted larger amounts of the sesquiterpenes δ-selinene and daucene, whereas the headspace of Eri plants was high in the ester methyl salicylate (Fig. 1a, b; Table 2).

Over-expression effect: Volatile profiles of Col-0 and Col-0-MYB28 plants could be separated by PLS-DA (4 PLS-DA principal components, $R_2X_{cum}=0.861$, $R_2Y_{cum}=0.932$, $Q_{2cum}=0.612$; Fig. 1c,d). Of the compounds that had a VIP-value higher than 1 in the PLS-DA model, only one compound was emitted in significantly different amounts by Col-0 and Col-0-MYB28 plants: the GLS breakdown product 3-butene nitrile, which was emitted in larger amounts by Col-0-MYB28 plants (Table 2).

Plant Morphology Ecotype effect: Eri plants had a higher biomass than plants of the other ecotypes. Cvi plants had the highest, and Eri plants the lowest trichome density (Supplemental Material Online Resource 5). Over-expression effect: There was no difference in biomass or trichome density between Col-0 and Col-0-MYB28 plants (Supplemental Material Online Resource 5).

Aphid Performance Ecotype effect: Aphid survival did not significantly differ among ecotypes (logistic regression, $P=0.051$, Table 3). Aphid development time, adult weight, number of offspring, and estimated intrinsic rate of population increase (r_m) differed among ecotypes (ANOVA, respectively $F_{2,57}=15.10$, $P<0.001$; $F_{2,57}=30.24$, $P<0.001$; $F_{2,57}=18.62$,

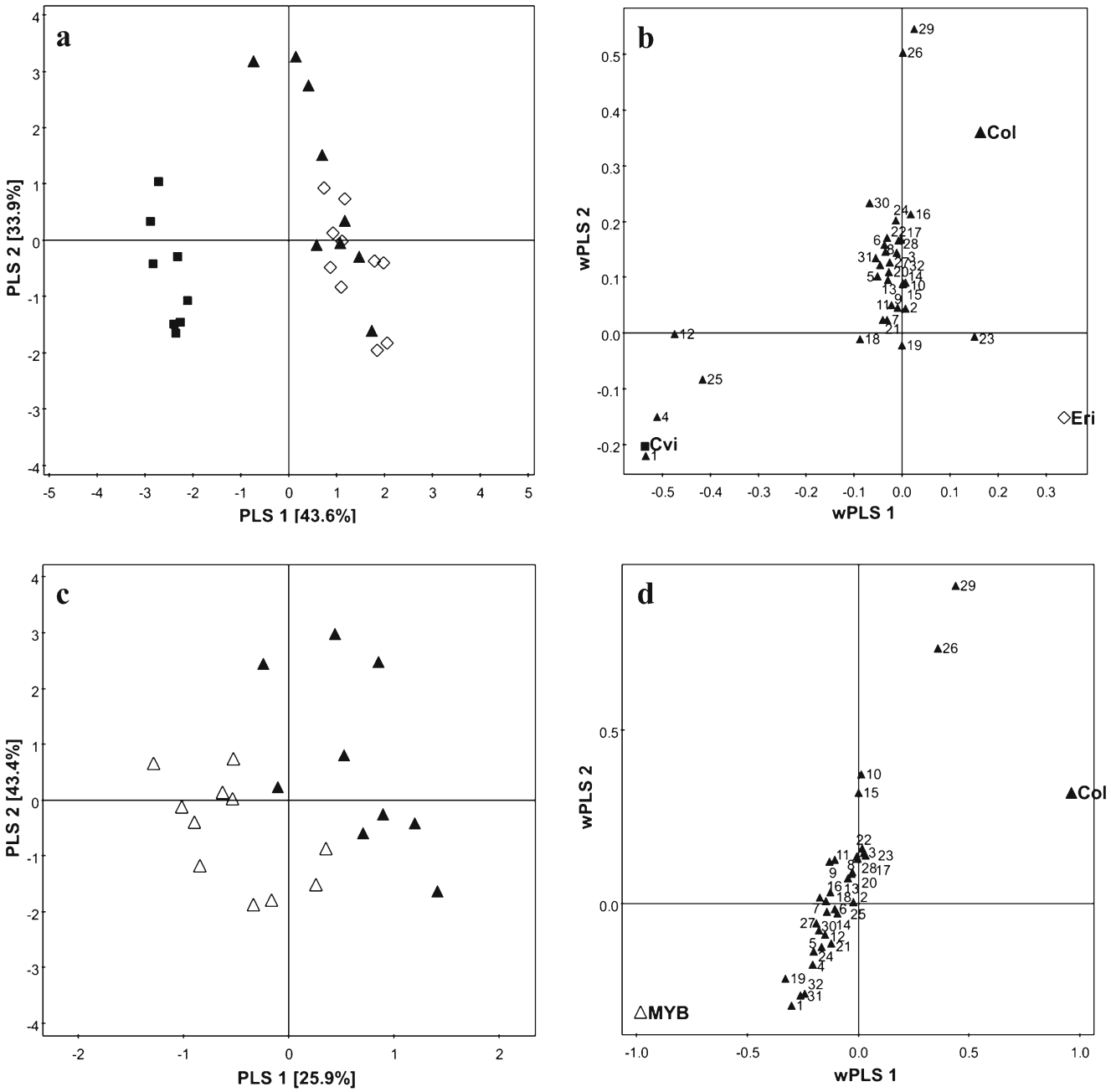

Fig. 1 Projection to Latent Structures-Discriminant Analysis (PLS-DA) score and loading plots of the first two components based on the volatile emission of aphid-infested plants of three *Arabidopsis thaliana* ecotypes (**a** and **b)** and of ecotype Col-0 and the transformed Col-0-MYB28 (**c** and **d)**. Plant ecotypes investigated are Cvi (filled boxes), Eri (open diamonds) and Col-0 (filled triangles); the transformed line is Col-0-MYB28 (open triangles). The score plots (**a** and **c**) show the distinction in volatile profiles of the ecotypes/lines. In brackets the percentage of variation explained is indicated. The loading plots (**b** and **d**) show the contribution of the volatile compounds to the discrimination among the ecotypes/lines. Numbers refer to the volatile compounds listed in Table 2

$P<0.001$; $F_{2,57}=45.66$, $P<0.001$). All aphid performance parameters were higher (development time shorter) on Cvi plants than on plants of the other ecotypes (Table 3).

Over-expression effect: There was no difference in any of the measured performance parameters of *B. brassicae* between Col-0 and Col-0-MYB28 plants ($P>0.05$ for any comparison, Table 3).

All measured aphid performance parameters were significantly positively correlated (development time inversely correlated) with total and aliphatic GLS and several carbohydrates (4 PLS principal components, $R_2X_{cum}=0.757$, $R_2Y_{cum}=0.735$, $Q_{2cum}=0.343$; Fig. 2). Aphid performance parameters were, to a lesser extent, also seemingly positively correlated with indole GLS, but this was significant only for

Table 2 Mean (± SE) amount of volatiles emitted by aphid-infested plants of three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line

No.	Compound	*Arabidopsis thaliana* ecotype			Transformed
		Cvi	Eri	Col-0	Col-0-MYB28
1	3-butene nitrile[a]	7216±1024 b	80±15 a	131±37 a	246±44 *
2	2-pentanone	818±98	1519±746	1118±221	1263±264
3	4-methyl-2-pentanone	263±40	178±27	584±277	375±69
4	3-methyl-3-butene nitrile[a]	5731 ±1059 b	58±11 a	147±57 a	204±40 ns
5	1-pentanol	804±117	399±61	797±148	1130±149
6	2-hexanone	474±73	311±56	626±130	735±118
7	butyl acetate	211±37	174±58	255±103	346±131
8	2-pentyl acetate	35±10	32±10	43±14	47±14
9	styrene[a]	2986±708	3892±1755	3860±1457	6055±2879 ns
10	cumene[a]	424±117	602±300	602±185	622±207 ns
11	isocumene	375±83	474±237	446±168	588±210
12	3-butenyl isothiocyanate[a]	253044±35561 b	5109±1809 a	19333±11465 a	17190±4633 ns
13	hemimellitene	932±116	846±295	1093±277	1182±207
14	p-cymene	231±49	305±112	297±80	384±63
15	limonene	1495±504	1519±467	1924±699	1855±577
16	o-cresol	592±188	539±154	1092±297	1495±392
17	m-cymene	436±53	402±88	772±172	669±77
18	γ-terpinene	101±42	112±58	127±48	112±44
19	linalool[a]	97±24	75±18	85±29	564±433 ns
20	cis-limonene-1,2-epoxide	68±10	47±14	75±13	76±9
21	menthol	853±283	695±275	542±42	942±311
22	1-methylene-1H-indene	2662±624	1595±256	3254±626	2912±372
23	methyl salicylate[a]	177±42 a	749±140 b	459±104 ab	422±100
24	diethyl-2-methylene succinate[a]	1485±218	1324±309	3218±985	3867±614 ns
25	cyclosativene[a]	1041±220 b	27±5 a	46±8 a	60±9
26	daucene[a]	216±109 ab	82±14 a	1483±675 b	242±50 ns
27	γ-elemene[a]	706±271 a	453±124 a	974±384 a	1003±224 ns
28	longifolen	669±168	506±101	968±251	955±171
29	δ-selinene[a]	321±206 a	153±54 a	6053±3112 a	261±44 ns
30	6-methyl-alpha-ionone[a]	369±79	153±25	635±210	661±125 ns
31	lilial[a]	1698±146	930±98	1983±450	3391±460 ns
32	farnesylacetaldehyde[a]	16171±3015	9846±1432	18047±3806	29986±4227 ns

N=8–11 for each ecotype/line. Unit is peak area mg^{-1} fresh weight

Numbers correspond to the numbers in Fig. 1

[a] Statistical tests were performed only for the compounds that had a VIP-value higher than 1 in the PLS-DA model shown in Fig. 1 that included either the three ecotypes (Fig. 1a and b) or Col-0 and Col-0-MYB28 (Fig. 1c and d). The compounds with a VIP higher than 1 are most influential for the discrimination among the ecotypes/lines. Different letters denote differences in means among the three ecotypes as analyzed by Mann–Whitney *U*-tests with sequential Bonferroni correction. * denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analyzed by Mann–Whitney *U*-tests. Compounds have been identified based on the linear retention index (LRI) and mass spectrum, or mass spectrum only (compounds 4, 5 and 22). See Supplemental Material Online Resource 4 for details on identification methods

development time (inversely correlated). Aphid performance parameters were in general not correlated with amino acids, sucrose, or total carbohydrates.

Aphid GLS Concentrations Ecotype effect: Aphids reared on different ecotypes differed in total and aliphatic GLS concentrations (Kruskal-Wallis H, df=2, total: χ^2=6.98, P=0.031; aliphatic: χ^2=6.98, P=0.031), due to both qualitative and quantitative differences (Table 1). Aphids reared on Cvi plants contained the highest, and aphids reared on Col-0 plants the lowest total and aliphatic GLS concentrations (Table 1). There were no differences in indole GLS

Table 3 Mean (± SE) performance characteristics of *Brevicoryne brassicae*, *Episyrphus balteatus* and *Diaeretiella rapae* reared on three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line

Insect species	Performance parameter	*A. thaliana* ecotype[a]			Transformed line[b]
		Cvi	Eri	Col-0	Col-0-MYB28
B. brassicae	Survival until adult stage (%)[c,d]	98 a	92 a	90 a	95 ns
	Development time until first reproduction in days (T_d)[e,f]	8.0±0.1 a	8.4±0.1 a	9.1±0.2 b	9.2±0.2 ns
	Adult fresh weight in mg[f]	0.635±0.025 b	0.471±0.020 a	0.408±0.018 a	0.393±0.015 ns
	Number of offspring (N) in time period equivalent to T_d[e,f]	33.2±1.5 b	22.2±1.2 a	22.2±1.7 a	19.2±1.2 ns
	Estimated intrinsic rate of population increase (r_m)[f]	0.322±0.005 c	0.270±0.006 b	0.250±0.006 a	0.236±0.006 ns
E. balteatus	Survival until adult stage (%)[c]	17 a	40 a	26 a	20 ns
	Larva-to-adult development time in days[e,f,g]	21.5±1.1 c	16.3±0.3 a	18.4±0.6 b	16.7±0.6 ns
	Adult dry weight in mg[e,f,g]	3.30±0.53 a	2.74±0.19 a	2.72±0.16 a	3.25±0.40 ns
D. rapae	Successful parasitism (%)[c,d]	73±5 a	76±5 a	57±7 a	64±7 ns
	Larva-to-adult development time in days[e,f,g]	11.1±0.1 a	11.2±0.1 a	11.1±0.1 a	11.3±0.1 ns
	Adult dry weight in mg[f,g]	0.068±0.002 b	0.058±0.002 a	0.059±0.002 a	0.055±0.002 ns

[a] Different letters denote differences in means among the three ecotypes

[b] ns denotes no significant difference between Col-0 and Col-0-MYB28

[c] Analyzed by logistic regression and *post-hoc* *T*-probability tests

[d] Performance parameter was averaged per plant before statistical analysis

[e] Performance parameter was log-transformed in statistical analysis to obtain normality

[f] Analyzed by ANOVA and *post-hoc* Tukey tests (for the ecotypes), or *t*-test (for the wild-type and transformed Col-0 line)

[g] The data for males and females were combined

among aphids reared on the different ecotypes (Kruskal-Wallis H, $P>0.05$).

Over-expression effect: Aphids reared on Col-0-MYB28 plants had similar concentrations of total, aliphatic and indole GLS to aphids reared on Col-0 plants (Mann–Whitney *U*, $P>0.05$ for every analysis; Table 1).

Correlations Between GLS Profiles in Phloem and in B. Brassicae Aphids In both the univariate Spearman's correlation tests, as well as the multivariate PLS model, concentrations of most of the GLS compounds or classes in the aphids were not significantly positively correlated with their concentrations in the phloem. Positive correlations were, however, significant for total and aliphatic GLS (PLS model: 1 PLS principal component, $R_2X=0.486$, $R_2Y=0.373$, $Q_2=0.280$; Spearman's correlation: 3-butenylGLS: $r_s=0.53$; $P=0.015$; aliphatic: $r_s=0.72$; $P<0.001$; total: $r_s=0.82$; $P<0.001$). The concentration of the indole 4-methoxy-3-indolylmethylGLS seemed to be negatively correlated, although not significantly, between aphids and the phloem they were feeding on (Fig. 3; Spearman's correlation, $P=0.650$).

The contribution of indole GLS to the total concentration of GLS was lower in aphids than in the phloem of the aphid-infested plants (10% indole GLS in aphids compared to 42% indole GLS in the phloem, as averaged over all four ecotypes/lines; see also Table 1). Additionally, the ratio of the indole compounds was different in the aphids from that in the phloem: in the phloem the concentration of 4-methoxy-3-indolylmethylGLS was higher than the concentration of 3-indolylmethylGLS, whereas this was reverse in the aphids (Table 1).

Predator Performance Ecotype effect: Survival of *E. balteatus* to the adult stage did not differ among ecotypes (logistic regression, $P>0.05$, Table 3). Larva-to-adult development time of the hoverflies was affected by plant ecotype and hoverfly sex (ANOVA, ecotype: $F_{2,23}=27.11$, $P<0.001$; sex: $F_{1,23}=8.10$, $P=0.009$). Hoverflies developed slowest on aphids fed on Cvi plants and fastest on aphids fed on Eri plants (Table 3). Averaged over ecotypes, male hoverflies took longer (18.7±0.8 d) to develop into adults than females (17.3±0.5 d). However, the difference in development time between males and females was only significant on Cvi, and not on Col-0 and Eri, resulting in a significant interaction between ecotype and sex ($F_{2,23}=4.65$, $P=0.020$). Adult dry weight was affected by hoverfly sex (ANOVA, $F_{1,23}=6.63$, $P=0.017$), as male hoverflies (3.18±0.23 mg) were heavier than females (2.49±0.13 mg), but not by plant ecotype (Table 3) or the interaction between ecotype and sex (ANOVA, $P>0.05$ for both analyses).

Over-expression effect: There was no difference in survival, development time, or adult dry weight of *E. balteatus*

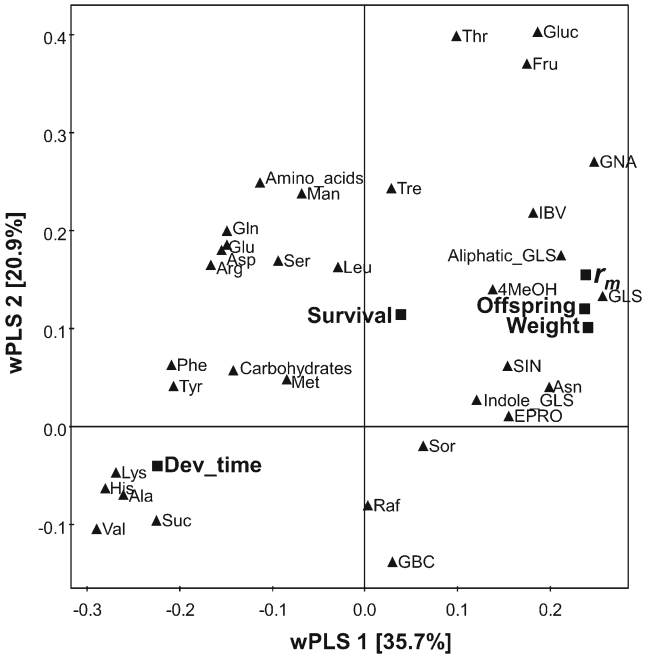

Fig. 2 Loading plot of the first two components of Projection to Latent Structures showing the contribution of each individual compound or compound class measured in the phloem, i.e., glucosinolates (GLS), carbohydrates and amino acids, to the performance of the aphid *Brevicoryne brassicae* in terms of survival, development time, adult weight, number of offspring and estimated intrinsic rate of population increase (r_m). In brackets the percentage of variation explained is indicated. Compound abbreviations: Aliphatic GLS: EPRO = 2-(*S*)-2-hydroxy-butenylGLS (epiprogoitrin), GNA = 3-butenylGLS (gluconapin), IBV = 3-methylthiopropylGLS (glucoiberverin), SIN = 2-propenylGLS (sinigrin). Indole GLS: GBC = 3-indolylmethylGLS (glucobrassicin), 4MeOH = 4-methoxy-3-indolylmethylGLS (4-methoxyglucobrassicin). Carbohydrates: Fru = fructose, Gluc = glucose, Man = mannitol, Raf = raffinose. Sor = sorbitol, Suc = sucrose, Tre = trehalose. Amino acids: Ala = alanine, Arg = arginine, Asn = asparagine, Asp = aspartate, Glu = glutamate, Gln = glutamine, His = histidine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine

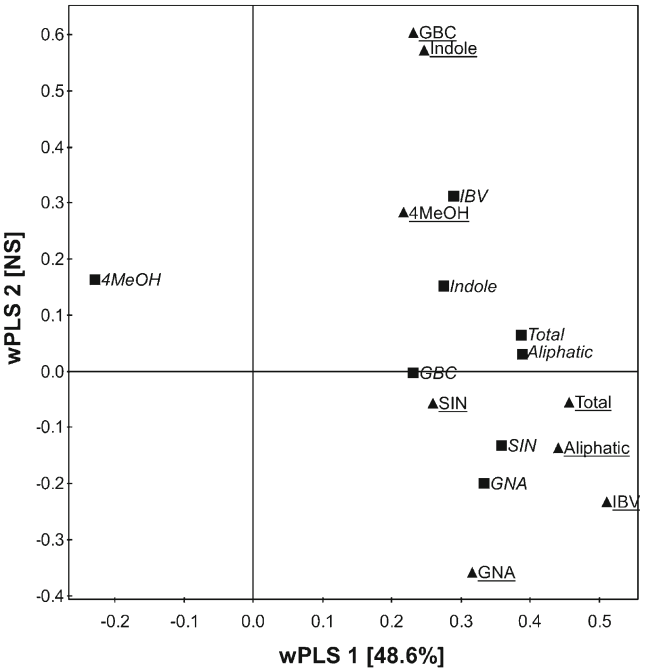

Fig. 3 Loading plot of the first two components of PLS showing the relationship of the concentration of each glucosinolate (GLS) compound or class (aliphatic, indole and total) in the phloem of *Arabidopsis thaliana* (squares, label in *italics*) with the concentration of these compounds or classes in the aphid *Brevicoryne brassicae* (triangles, label underlined) feeding on the phloem. In brackets the percentage of variation explained is indicated. Note that only the first component is significant according to the multivariate model, whereas two components were included to enhance the clarity of the figure. Compound abbreviations: Aliphatic GLS: GNA = 3-butenylGLS (gluconapin), IBV = 3-methylthiopropylGLS (glucoiberverin), SIN = 2-propenylGLS (sinigrin). Indole GLS: GBC = 3-indolylmethylGLS (glucobrassicin), 4MeOH = 4-methoxy-3-indolylmethylGLS (4-methoxyglucobrassicin)

between Col-0 and Col-0-MYB28 ($P>0.05$ for all parameters; Table 3).

Parasitoid Performance Ecotype effect: Plant ecotype did not affect the percentage of successful parasitism of *B. brassicae* by *D. rapae* (logistic regression, $P>0.05$), nor did it affect egg-to-adult development time (ANOVA, $P>0.05$; Table 3). Only adult dry weight was affected by plant ecotype (ANOVA, ecotype: $F_{2,169}=10.16$, $P<0.001$). Adult dry weight was higher on Cvi plants than on plants of the other ecotypes (Table 3). There was no effect of parasitoid sex or the interaction between ecotype and sex for any of the performance parameters ($P>0.05$ for all parameters).

Over-expression effect: There was no difference in the percentage of successful parasitism, development time, or adult dry weight of *D. rapae* between Col-0 and Col-0-MYB28 ($P>0.05$ for all parameters; Table 3).

Predator Oviposition Preference Female *E. balteatus* preferred to oviposit on aphid-infested Eri plants over aphid-infested Col-0 and Cvi plants, and aphid-infested Col-0 plants over aphid-infested Cvi plants (Wilcoxon: Eri vs. Col-0: $Z=2.63$, $N=27$, $P=0.008$; Eri vs. Cvi: $Z=3.44$, $N=22$, $P=0.001$; Col-0 vs. Cvi: $Z=2.12$, $N=29$, $P=0.034$). Females did not differentiate between aphid-infested plants of Col-0 and Col-0-MYB28 (Wilcoxon, $P>0.05$; Fig. 4).

Parasitoid Preference for Aphid-Induced Plant Volatiles Female *D. rapae* preferred volatiles from aphid-infested Cvi plants over volatiles from aphid-infested Col-0 plants (Chi-square, $\chi^2=5.69$, $P=0.017$). Females neither differentiated between volatiles from any of the other ecotype combinations, nor between Col-0 and Col-0-MYB28 (*Chi-square*, $P>0.05$ for every combination; Fig. 5). There was no effect of previous oviposition experience on the preference of the wasps (logistic regression, $P>0.05$ for any combination).

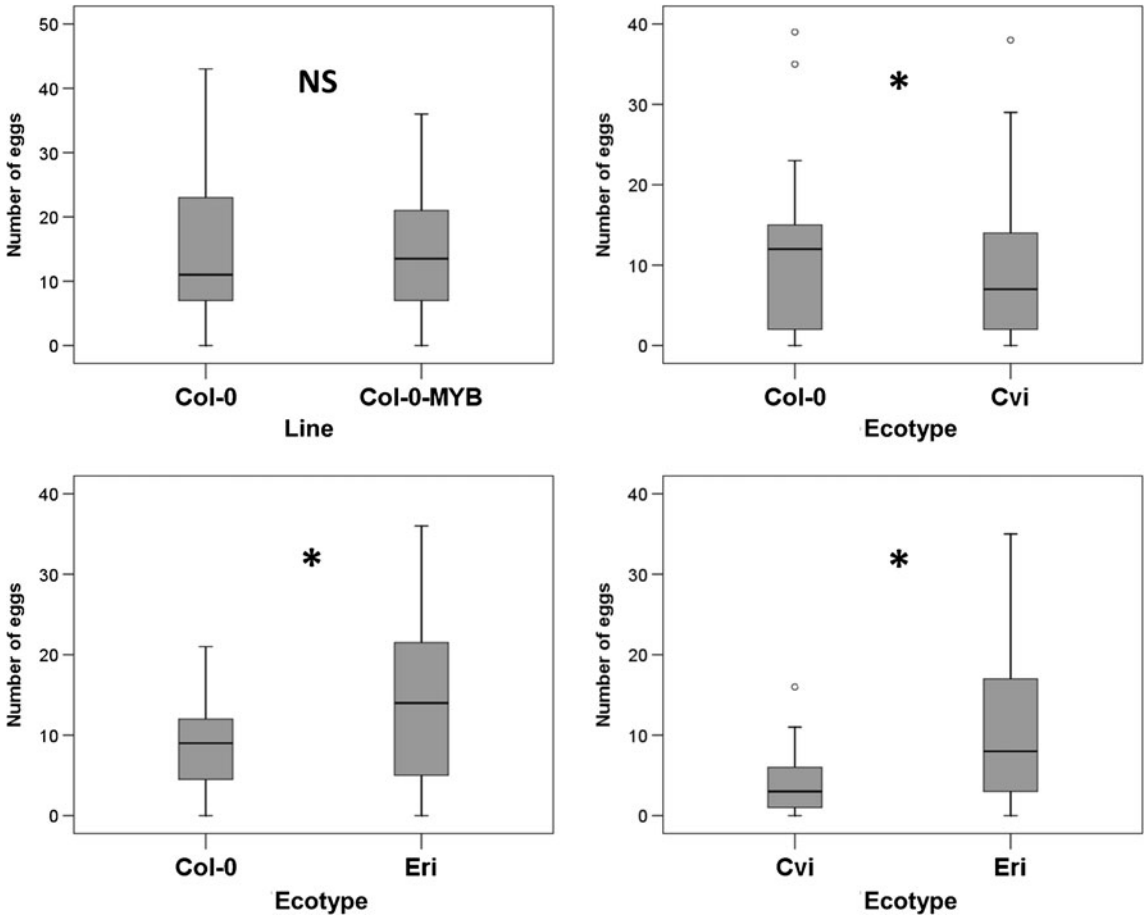

Fig. 4 Oviposition preference of the aphid predator *Episyrphus balteatus* in a two-choice assay with aphid-infested plants of three *Arabidopsis thaliana* ecotypes (Col-0, Cvi and Eri) and one transformed line (Col-0-MYB28). The boxes span the first to third quartile range with the line across the box indicating the median. The whiskers represent the range. Open circles represent outliers. An asterisk indicates a significant difference ($P<0.05$) between the number of eggs deposited on each ecotype/line as analyzed by the Wilcoxon matched-pairs signed-rank test; NS = not significant

Discussion

The performance of *B. brassicae* was best on the *A. thaliana* ecotype with the highest concentrations of aliphatic GLS in the phloem. Furthermore, we found a positive correlation between aliphatic GLS and aphid performance in the multivariate regression analysis. Due to the intercellular path taken by the aphid stylet to the phloem (Tjallingii and Hogen Esch, 1993), aphids can ingest aliphatic GLS from the phloem without bringing these compounds into contact

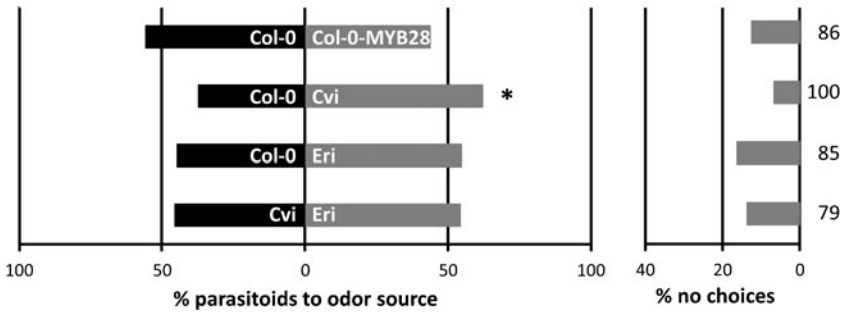

Fig. 5 Responses of *Diaeretiella rapae* females to volatile blends emitted by aphid-infested *Arabidopsis thaliana* ecotypes/lines in a Y-tube olfactometer. Ecotypes investigated are: Col-0, Cvi and Eri; the transformed line is Col-0-MYB28. Each bar represents the percentage of females that made a choice for the indicated odor sources. The percentage of no choice in each experiment and the total number of tested females are indicated on the right. An asterisk indicates a significant preference for one of the two ecotypes/lines in a combination, as analyzed by *Chi-square* tests

with plant myrosinases that are stored in cells adjacent to the phloem (Andreasson et al., 2001). Thus, aphids can prevent the formation of toxic hydrolytic products of aliphatic GLS (de Vos et al., 2007; Kim and Jander, 2007). Together with the observation that *B. brassicae* uses GLS as feeding stimulants (Gabrys and Tjallingii, 2002), our finding of a positive correlation between aliphatic GLS and aphid performance is expected. In contrast to aliphatic GLS, indole GLS are hydrolyzed by aphids into toxic products independently of myrosinase activity (Kim and Jander, 2007; Kim et al., 2008), and negative correlations between the concentrations of indole GLS and performance of *B. brassicae* and other aphid species have been reported (Cole, 1997; Mewis et al., 2005; Kim and Jander, 2007; Kim et al., 2008). Our observation of a slight, but significant, positive correlation between aphid performance and total indole GLS concentrations in the phloem are in disagreement with these latter studies. A possible explanation for this discrepancy is that specific indole GLS may affect aphid performance more strongly than others. The difference in the abundance of specific indole GLS between our study and studies from the literature might be the explanation of differences in effects on aphid performance.

The *A. thaliana* ecotypes did not differ significantly in the concentrations of carbohydrates and amino acids in the phloem, and we did not observe a consistent correlation between aphid performance and concentrations of individual or total carbohydrates and amino acids in the regression analysis. Aphids did not seem to be affected by trichomes, as their performance was best on the *A. thaliana* ecotype with the highest trichome density. We note that we measured trichome density of uninfested plants. It has been demonstrated that feeding by leaf chewers can increase trichome density in *A. thaliana* (Traw and Dawson, 2002), but whether this also is true for aphids is, to our knowledge, unknown.

Brevicoryne brassicae sequestered GLS from the phloem, and total aliphatic GLS concentrations in the phloem were significantly positively correlated with their concentrations in the aphids. In contrast, whereas in the phloem 4-methoxy-3-indolylmethylGLS was the most abundant indole GLS, aphids sequestered this compound in low concentrations compared to its precursor, 3-indolylmethylGLS. This is in accordance with what we reported previously in aphids feeding on *B. oleracea* plants (Kos et al., 2011). Although the concentrations of most aliphatic GLS in phloem were positively correlated with their concentrations in aphids, selective sequestration also was observed for aliphatic GLS. For example, whereas 3-butenylGLS was the least abundant GLS in the phloem of Cvi plants, it dominated in the aphids feeding on these plants. These findings suggests that *B. brassicae* selectively sequestered GLS from the phloem, a phenomenon we previously reported (Kos et al., 2011). The mechanism underlying the selective sequestration of GLS could be that transporters for GLS in the aphid gut wall may be specific. In other GLS-sequestering species, such as the sawfly *Athalia rosae*, selective sequestration of aliphatic GLS has been reported (Müller and Wittstock, 2005; Müller, 2009). Little is known about the specificity of GLS transporters or the specific mechanisms underlying GLS sequestration (Opitz et al., 2010). In our study, aliphatic GLS dominated the profile of sequestered GLS, in agreement with previous work (Kos et al., 2011). Because hydrolysis into toxic products requires myrosinase activity circumvented by aphids (de Vos et al., 2007; Kim and Jander, 2007), aphid performance itself is most likely little affected by high sequestration of aliphatic GLS. Interestingly, aliphatic GLS are degraded more by purified aphid myrosinase, whereas the lowest activities of the aphid myrosinase are observed with indole GLS (Francis et al., 2002). Thus, higher sequestration of aliphatic GLS by *B. brassicae* may lead to higher toxicity to predators, without affecting aphid performance itself. We note that not all GLS detected in the aphids were detected in the phloem, probably because the concentrations of some GLS in the phloem were below the detection limit of the HPLC. However, we cannot rule out that the aphids converted certain GLS from the phloem into other compounds that were subsequently stored in their body.

As expected, over-expressing Col-0-MYB28 plants produced higher foliar concentrations of aliphatic GLS and similar concentrations of indole GLS compared to the wild-type plants. Unexpectedly, we observed that aliphatic GLS concentrations in the phloem were lower in Col-0-MYB28 plants than in Col-0 plants. Probably, GLS biosynthesis in Col-0-MYB28 plants occurred mainly in the mesophyll, and phloem loading of GLS was limited.

Performance of the generalist aphid predator *E. balteatus* was lowest in terms of development time when fed *B. brassicae* aphids that contained the highest aliphatic GLS concentrations (i.e., aphids reared on Cvi). It is likely that this led to highest concentrations of GLS hydrolysis products after breakdown by the aphid myrosinase, as purified aphid myrosinase quickly degrades aliphatic GLS (Francis et al., 2002), although we did not quantify GLS and their hydrolytic products separately. Our results are in agreement with other studies that have reported negative effects of GLS sequestration by *B. brassicae* on the performance of *E. balteatus* (Vanhaelen et al., 2002; Kos et al., 2011) and other aphid predators (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Chaplin-Kramer et al., 2011; Kos et al., 2011). There are several other Brassicaceae-feeding insects that sequester GLS for defense against predators. Similarly to *B. brassicae*, the turnip aphid (*Lipaphis erysimi*) sequesters GLS from phloem into the haemolymph and contains an endogenous myrosinase, a mechanism that is expected to affect negatively predators (Bridges et al., 2002).

Episyrphus balteatus performance did not differ between Col-0 and Col-0-MYB28 plants. This was expected as aphid GLS concentrations did not differ between these plant lines. Hoverfly performance in terms of development time was better when their prey had been feeding on ecotype Eri than on Col-0. However, Eri-fed aphids had higher but not statistically different total GLS concentrations in their phloem from Col-0-fed aphids. This points to the importance of qualitative effects of GLS profiles on hoverfly performance. It was reported previously that differences in *B. brassicae* GLS profiles affect the performance of *E. balteatus* (Kos et al., 2011). Furthermore, haemolymph of *A. rosae* larvae, containing a mix of several GLS compounds, deterred ants and predatory wasps more strongly than the individual major GLS compounds (Müller et al., 2002; Müller and Brakefield, 2003). This suggests that GLS profiles rather than total concentrations influenced these predators, although the stronger deterrence also could have been due to completely different compounds from GLS present in the haemolymph. We cannot rule out effects of other plant or aphid traits on predator performance. Epicuticular plant characteristics, such as leaf waxes and trichomes, may affect predator attachment to the plant (Eigenbrode, 2004). Trichomes have been shown to negatively affect the performance of hoverfly larvae due to entrapment by glandular trichomes, reduced mobility, or falling off the plant (Verheggen et al., 2009). Although we do not know whether this is also true for non-glandular trichomes on *A. thaliana*, the lower trichome density of Eri plants may have contributed to the better performance of hoverfly larvae on this ecotype.

Parasitoid performance in terms of adult weight was best when developing in the largest aphids, containing the highest GLS concentrations. The positive correlation between host size and parasitoid size is in agreement with other studies (Harvey, 2005; Bukovinszky et al., 2008). Our results suggest that the performance of *D. rapae* is not negatively affected by GLS concentrations in the host, supporting the findings of Le Guigo et al. (2011). Although *D. rapae* parasitizes several aphid species, it is the main parasitoid of *B. brassicae* (Bukovinszky et al., 2008), and may be relatively tolerant to GLS. We do not know, however, how *D. rapae* copes with GLS in its host. In fact, there is not much known about detoxification of plant secondary metabolites by parasitoids in general (Ode, 2006; Gols and Harvey, 2009). Negative effects of breakdown products of GLS on *D. rapae* might be prevented by the feeding strategy of the parasitoid larvae. *Brevicoryne brassicae* stores GLS in the haemolymph, but the aphid's myrosinases are stored in the non-flight muscles (Jones et al., 2001; Bridges et al., 2002; Francis et al., 2002). Tissue-feeding endoparasitoids, such as *D. rapae*, consume host haemolymph during most of their larval development and only consume other host tissues shortly before egression (Godfray, 1994; Harvey et al., 2000), thereby possibly preventing the breakdown of GLS into toxic products during the major part of their development. *Diaeretiella rapae* performance did not differ between Col-0 and Col-0-MYB28 plants, which was expected as both aphid size and aphid GLS concentrations did not differ between these lines.

Aphid-infested plants of the three *A. thaliana* ecotypes differed in their volatile profiles. Both the predator (*E. balteatus*) and the parasitoid wasp (*D. rapae)* preferred the ecotype on which their offspring performed best. This demonstrates that preference and performance of these natural enemies are positively correlated, in agreement with other studies (Soler et al., 2007; Gols et al., 2009). The predator always laid fewest eggs on the ecotype within a test combination that had the highest emission of volatile GLS hydrolysis products, suggesting that volatile breakdown products of GLS were repellent for the predators. *Episyrphus balteatus* had access to the aphid-infested plants in the bioassays. We do not know if other plant characteristics or aphid cues also played a role in the selection of an oviposition site by *E. balteatus*. In particular, the preferred ecotype in each test combination had the lowest trichome density. It has been shown previously that adult hoverflies have problems with landing on plants with high trichome densities (Verheggen et al., 2009). In contrast to the predator, the parasitoid preferred volatile cues from the ecotype with the highest emission of volatile GLS hydrolysis products (Cvi), but only when offered in combination with ecotype Col-0. A preference for a high emission of volatile GLS hydrolysis products was expected, as *D. rapae* is known to be attracted to host plants emitting volatile breakdown products of GLS (Read et al., 1970; Bradburne and Mithen, 2000; Blande et al., 2007). Neither the predator nor the parasitoid wasp differentiated between cues from Col-0 and Col-0-MYB28 plants. The relatively small difference in volatile profiles between these lines might not allow olfactory discrimination.

In summary, the four main findings of our study are: 1) The performance of the specialist cabbage aphid *B. brassicae* is positively correlated with concentrations of both aliphatic and indole GLS in the phloem of *A. thaliana* plants; 2) *Brevicoryne brassicae* selectively sequestered GLS from the phloem; 3) The performance of the aphid predator *E. balteatus* is negatively correlated with aphid GLS concentrations. The performance of the aphid parasitoid *D. rapae* is positively correlated with aphid GLS concentrations, probably because the aphids with the highest GLS concentrations have a higher body weight; 4) Both natural enemies prefer the *A. thaliana* ecotype on which their offspring perform best, indicating a positive performance-preference correlation. The predator preferred the *A. thaliana* ecotype with the lowest emission of volatile breakdown products of GLS in each test combination, whereas the parasitoid wasp preferred the *A. thaliana* ecotype with the highest emission of these volatiles, but only in one test combination. Our study shows that there are differential herbivore-

mediated effects of GLS on a predator and a parasitoid of a specialist aphid that selectively sequesters GLS from its host plant.

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Diet Quality Can Play a Critical Role in Defense Efficacy against Parasitoids and Pathogens in the Glanville Fritillary (*Melitaea cinxia*)

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Abstract Numerous herbivorous insect species sequester noxious chemicals from host plants that effectively defend against predators, and against parasitoids and pathogens. Sequestration of these chemicals may be expensive and involve a trade off with other fitness traits. Here, we tested this hypothesis. We reared Glanville fritillary butterfly (*Melitaea cinxia* L.) larvae on plant diets containing low- and high-levels of iridoid glycosides (IGs) (mainly aucubin and catalpol) and tested: 1) whether IGs affect the herbivore's defense against parasitoids (measured as encapsulation rate) and bacterial pathogens (measured as herbivore survival); 2) whether parasitoid and bacterial defenses interact; and 3) whether sequestration of the plant's defense chemicals incurs any life history costs. Encapsulation rates were stronger when there were higher percentages of catalpol in the diet. Implanted individuals had greater amounts of IGs in their bodies as adults. This suggests that parasitized individuals may sequester more IGs, increase their feeding rate after parasitism, or that there is a trade off between detoxification efficiency and encapsulation rate. Larval survival after bacterial infection was influenced by diet, but probably not by diet IG content, as changes in survival did not correlate linearly with the levels of IGs in the diet. However, *M. cinxia* larvae with good encapsulation abilities were better defended against bacteria. We did not find any life history costs of diet IG concentration for larvae. These results suggest that the sequestering of plant defense chemicals can help herbivorous insects to defend against parasitoids.

Keywords Aucubin · Catalpol · Chemical defense · Encapsulation rate · Immunological defense · Iridoid glycosides · *Plantago lanceolata* · *Serratia marcescens* · Tritrophic interactions

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Introduction

Many plants produce secondary metabolites that can be harmful to insects eating the plant, and detoxification can be energetically costly (Berenbaum and Zangerl, 1993; Després et al., 2007; but see Cohen, 1985; Camara, 1997). However, herbivorous insects can sequester plant secondary metabolites (PSM) that are used for defense against natural

enemies such as predators (de la Fuente et al., 1994/1995; Theodoratus and Bowers, 1999; Nishida, 2002), parasitoids (Nieminen et al., 2003; Harvey et al., 2005; Singer et al., 2009), and pathogens (Baden and Dobler, 2009). Thus, the fitness of herbivorous species is affected by the quality of their diet (Ojala et al., 2005; Mody et al., 2007; Lindstedt et al., 2010). Poor diet, in terms of quality and/or quantity, can have detrimental consequences for many life history traits of herbivorous insects including growth rate, development time, pupal mass, fecundity (Ojala et al., 2005; Mody et al., 2007, Lindstedt et al., 2010), immunological defense capacity (Ojala et al., 2005; Klemola et al., 2007), and anti-predator traits (Codella and Raffa, 1995, Grill and Moore, 1998, Lindstedt et al., 2006, 2010).

Insects are defended against parasitoids with encapsulation. Layers of haemocytes and/or melanin encapsulate the parasitoid egg causing the parasitoid to die (Gillespie et al., 1997). Plant chemicals provide the substances necessary to mount the encapsulation reaction (Ojala et al., 2005). Diet protein content, for example, enhances lysozyme-like antibacterial activity and phenoloxidase activity in insects (Lee et al., 2008). The chemical composition of the diet may affect the health of the herbivore also, which then indirectly affects the success of the encapsulation reaction positively (Ojala et al., 2005; Kapari et al., 2006) or negatively (Klemola et al., 2007). The effects of PSM on insects' defense capacity against parasitoids and pathogens are widely recognized (Karban and English-Loeb, 1997; Nieminen et al., 2003; Harvey et al., 2005; Smilanich et al., 2009) However, most published studies have been comparative, and only a few have directly tested the effect of secondary metabolites on insect immunological defenses (Nieminen et al., 2003; Harvey et al., 2005).

Maintaining and mounting immunological responses is costly and involves a trade off with other traits (Cotter et al., 2004a; Rantala and Roff, 2005), such as detoxification and sequestration of chemicals from the diet (Smilanich et al., 2009) and antipredation (Rigby and Jokela, 2000). Thus, the effects of PSM on the efficacy of an immune defense are not necessarily additive. Maximization of immune defense can be detrimental to the herbivore through an increase in the risk of autoimmunity (attack against the herbivore's own tissues) (Rolff and Siva-Jothy, 2003) and thus is likely to be under stabilizing selection (Rolff and Siva-Jothy, 2004). The optimal diet choice is likely to be determined by multiple factors that include the composition of the natural enemy community, diet quality and quantity, and time limitations for development.

We conducted a factorial rearing experiment to test the costs and benefits of host PSM on the performance of the herbivorous Glanville fritillary (*Melitaea cinxia* L.). *Melitaea cinxia* is a specialist and can benefit from PSM as its larvae are heavier and develop faster on diets high in iridoid glycoside (IG) content than larvae reared on low IG diets (Suomi et al., 2002; Harvey et al., 2005; Saastamoinen et al., 2007). The high defense chemical content of the host plant also decreases the parasitism risk of caterpillars in the field: Nieminen et al. (2003) found that larval groups of *M. cinxia* that were feeding on plants with lower catalpol concentrations suffered higher parasitism by the specialist parasitoid *Cotesia melitaearum* (Braconidae: Microgastrinae) (Wilkinson, 1937) than those on high-catalpol plants. In addition to defense against predators and parasitoids, diet quality influences defense against pathogens (Bauce et al., 2002; McVean et al., 2002; Cotter et al., 2011). Baden and Dobler (2009) found that IGs had an inhibitory effect on the growth of *Bacillus thuringiensis*.

We divided *M. cinxia* larvae into five groups that were fed on different diets. The five diets differed significantly in IG-levels. Total IG concentration was lowest in diet 1 and highest in diet 5. Larvae were divided between three different immunological treatments within each diet treatment. In this way, we were able to test: 1) how diet quality (specifically iridoid glycosides) affects larval encapsulation activity (defense against an artificial parasitoid) and defense against the bacterial pathogen *Serratia marcescens* (measured as survival time after infection); 2) whether defenses against parasitoids and pathogens interact (both encapsulation and survival after bacterial infection were measured); and 3) how the IG concentration of host plants affects the life history (growth rate, pupal mass) and anti-predatory (chemical defense) traits of individuals. If IGs enhance defense against parasitoids and pathogens (Nieminen et al., 2003), a diet high in IGs could increase larval encapsulation rate and survival time after bacterial infection. However, if handling IGs is costly, the relative benefits of sequestering IGs would decrease with increasing diet IG content, or a high diet IG content would trade off with other life history traits such as fecundity (Lindstedt et al., 2010).

Methods and Materials

Study Species Melitaea cinxia (Nymphalidae) is found throughout Europe, northern Africa, and in the east from Russia to West Asia. Numbers have declined and distribution has become fragmented in northern Europe in the last decades. *Melitaea cinxia* overwinter gregariously as larvae in winter nests. In spring, the larvae feed gregariously on two host plants: ribwort plantain (*Plantago lanceolata*) (Plantaginaceae) and spiked speedwell (*Veronica spicata* L.) (Plantaginaceae) (Marttila, 2005). Both produce IGs, mainly aucubin and catalpol (Duff et al., 1965; Suomi et al., 2002). Glanville fritillary sequester IGs as larvae, and retain these chemicals later as adults (Suomi et al., 2001, 2003). Iridoid glycosides also are used as oviposition cues (Nieminen et al., 2003; Reudler Talsma et al., 2008).

The caterpillars are parasitized by two specialist parasitoids: the solitary endoparasitoid *Hyposoter horticola* (Ichneumonidae) and the gregarious endoparasitoid *Cotesia melitaearum* (Hanski et al., 1995). *Hyposoter horticola* avoid the larval behavioral defenses of *M. cinxia* by parasitizing first instars while they are still within the eggshell (van Nouhuys and Hanski, 2002). Most individuals of *M. cinxia* individuals encapsulate some of the parasitising eggs and larvae of *C. melitaearum*, and some manage to kill all of them (Saskya van Nouhuys, personal communication).

Rearing of Larvae The *M. cinxia* larvae used were descendants of 23 families collected from the Åland Islands in the autumn of 2006. Three generations were maintained as laboratory stock (Metapopulation Research Group, University of Helsinki).We used post-diapausing fifth instars for the experiment because they grow well individually. All larvae were reared on *P. lanceolata*. The experiment was carried out in the spring of 2008 at the University of Jyväskylä.

Larvae from the different families were divided randomly among the diets to obtain genetically similar groups for the 5 diet treatments. Larvae were placed in group containers for the first 5 day (85–108 larvae per container), and fresh *P. lanceolata* leaves were offered *ad libitum*. Each diet group consisted of 4 group containers. Twenty-eight percent of the larvae woke from diapause, and after 5 day, larvae were reared individually in Petri dishes lined with a filter paper (General Purpose Filter Paper, ø 70 mm, Munktell Filter AB). Fresh food was added *ad libitum*. During the experiment, larvae were kept in an environmental chamber with regulated temperature and light conditions (see supplemental material, Table S1).

Larval growth was monitored during the experiment. Larvae were first weighed 6 day after diapause (i.e., initial weight: mean 13.65 mg±SD 3.93 mg) and once a week after that. Immune defense treatments were performed when larvae reached the weight of 100 mg. Mortality, moulting, pupation, and adult eclosion were recorded every day. Pupae were weighed on the day of pupation.

Diet Treatments The plants used were derived from an artificial selection experiment in which lines of Dutch *P. lanceolata* were selected for high and low leaf IG concentration for four generations: low-IG genotypes were crossed with low-IG genotypes and high-IG genotypes, with high-IG genotypes (Marak et al., 2000). A fifth generation of 16 different crosses then was grown in our greenhouse, and the IG content of each cross was measured at two time points and then ranked. Four low-IG, two intermediate-IG and four high-IG genotype crosses were selected to create five distinct diets for the experiment. Each diet consisted of two, consecutively ranked, plant crosses to ensure enough food per diet. Plants were grown under greenhouse conditions. Larvae were fed with a mixture of leaves of both plant crosses that made up their diet every day.

The five diet groups varied significantly in IG content (see results). Plants were measured before the experiment (twice: to rank the crosses by their IG levels), four and 9 week after the experiment began, and twice 1 year after the experiment. A subset of the plants was used in a different experiment and also was measured. The six and, for some, seven measuring points that we have for all diets show that the rank order was constant over time, and that there was no correlation between diet IG-content and nutritional quality (see results). We used the IG measurements that were conducted during the experiment (4 and 9 week after the start of the experiment) for all statistical analyses with diet treatments (Table 1A).

Random leaf samples per cross (before the experiment) or per diet treatment were used for IG measurements, which were done with high-performance liquid chromatography (HPLC).

HPLC Analyses Storage of sequestered chemicals, in the body or wings, could have different anti-predator functions. We, therefore, analyzed wings and bodies of adults separately. We used total sequestered chemicals per individual (wings and body) in our final analyses. Butterflies were freeze-dried and individually weighed to measure dry weight. The manually-ground wings and bodies were extracted in 5 ml 70% methanol and left to sit overnight. The crude extract was filtered on Whatman filter papers (no. 4, diam. 90 mm). The filtrate was diluted 10 times with Milli-Q water. We used a Dionex (Sunnyvale, CA) Bio-Lc equipped with a GP40 gradient pump, a CarboPac PA20 3× 30 mm guard column, a CarboPac PA20 3×150 mm analytical column and an ED40 electrochemical detector for pulsed amperimetric detection (PAD) for HPLC analyses. The eluent was NaOH 7%, and the flow rate 0.25 ml/min. A standardization curve for aucubin, catalpol, and glucose of 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ppm was used to calibrate samples.

For diet analyses, all leaves were freeze-dried and ground to a fine powder with a mikro-dismembrator U (B.Braun Biotech international). Finely ground and dry leaf material (25 mg) was extracted in 10 ml of 70% MeOH and shaken overnight (15°C/110 RPM). Filtration and other methods were the same as above.

Nutrient Analyses A subset of plants from all diets (N=16) was used for the nutrient analyses. For P and K analysis, ground leaf samples (250 mg) were digested with a microwave-accelerated method in closed PTFE-vessels. Nine ml of 3 M HNO_3 and 1 ml of 30% H_2O_2 were added to the samples, which were heated using a CEM (Matthews, NC, USA) Mars 5 microwave oven. Total time of the digestion

Table 1 Experimental conditions: A) average concentrations (% dry weight) of aucubin, catalpol, and total iridoid glycosides (aucubin + catalpol) in each diets and B) number of individuals of each diet group in each treatment

A		Average concentrations ± SE		
Diet	No. of larvae	Aucubin	Catalpol	Total IGs
1	108	0.84%±0.65	0.78%±0.56	1.62%±1.20
2	102	1.58%±0.65	1.20%±0.68	2.77%±1.34
3	118	1.72%±0.35	1.23%±0.47	2.96%±0.12
4	115	1.36%±0.18	1.86%±0.25	3.22%±0.43
5	108	1.57%±0.56	1.84%±0.93	3.42%±0.36

B						
Treatment	Diet					
	1	2	3	4	5	Total
Implant	14	15	17	18	16	80
Bacterial infection	16	13	14	14	16	73
Implant + bacterial infection	11	14	15	14	15	69
Control	13	15	15	16	18	77
Water control	13	13	18	14	21	79
Total	67	70	79	76	86	378

program was 33 min, and a maximum pressure of 15 bars for 10 min was used. Digested samples were analyzed by a Perkin-Elmer (Norwalk, CT, USA) model Optima 4300 DV inductively-coupled plasma optical emission spectrometer. For the P analyses, samples were measured at a wavelength of 213 nm. Samples were diluted 10 times for the K analyses, and measured at a wavelength of 766 nm.

The same ground plant samples (5 mg) were weighed in a tin container, with an Elementar (Hanau, Germany) Vario EL III CHN analyser. The C, H, and N amounts of the samples were determined.

Immune Defense Treatments Larvae were divided equally among the five diet treatments according to their weight (mean 13.65 mg±SD 3.93 mg) at the start of the experiment. Mortality over the experiment was high, and some larvae were added to treatments to increase the sample size and ensure equal weight distribution among the treatments. Treatments were implemented after larvae reached a weight of around 100 mg (mean weight=121 mg±17.7 mg (±SD). We investigated: 1) the effects of diet on the life history traits and IG content of the insects (control treatment); 2) the effects of diet on encapsulation activity (implant treatment); 3) the effects of diet on larval survival after pathogen infection (bacterial infection treatment); and 4) the effects of diet on the interaction of pathogen and parasitoid infection (implant + bacterial infection treatment). Survival of larvae was checked once a day in all treatment groups. Individuals from all diet groups participated in the immune defense treatments (Table 1B).

Diet Quality and Life history Traits (control treatment). All larvae were monitored for growth rate, pupal mass, and survival to pupal and adult stages. We also measured the IG concentrations of all adults.

Diet Quality and Encapsulation Activity (Implant treatment). The degree of darkening (melanization) of artificial implants has been used previously as an assay of immunity (König and Schmid-Hempel, 1995; Baer and Schmid-Hempel, 2003; Cotter et al., 2004b; Ojala et al., 2005; Rantala and Roff, 2005; Klemola et al., 2007, 2008). This method measures the strength of the insect host's neutral encapsulation reaction without the added effects of a real parasitoid (Siva-Jothy et al., 2005), giving a relevant quantitative measure of the host's ability to defend against parasitoids

We used a nylon implant (artificial parasitoid; length 4 mm, diam. 0.11 mm) to measure the strength of the encapsulation reaction. Larvae were anesthetized with carbon dioxide before implantation. A small cut was made with a sterilized needle, and two thirds of the implant was inserted into the 2nd segment of the larva. The implant was removed after 6 h, air dried, and photographed. After 6 h, most implants showed detectable encapsulation, and detectable variation in encapsulation rate.

All implants were photographed against white paper from three different angles with 10× magnification, by the same person. The black and white photographs were analyzed with ImagePro Plus 4.0 (Media Cybernetics). One third of each implant was analyzed using the mean grey value of the measured area. The grey value of the background was used to control differences in lighting conditions between photographs. The grey values of implants were corrected by subtracting the grey values of the backgrounds. Encapsulation rate was calculated as the mean difference between the

background and the mean of the three implant images. Higher values (a greater contrast between the dark implant and light background) indicated a stronger encapsulation reaction (i.e., more black melanin around the implant). For more detailed methods of measuring encapsulation see, e.g., Ojala et al. (2005).

Diet Quality and Defense against Pathogens (Bacterial infection treatment). *Serratia marcescens* (Enterobacteriaceae) bacteria were used in the bacterial infection treatment. The *S. marcescens* strain was obtained from the American Type Culture Collection (#13880). *Serratia marcescens* is a cosmopolitan bacterium and a common, opportunistic pathogen with a broad host range that includes plants, nematodes, insects, fishes, and mammals (Grimont and Grimont, 1978). *Serratia marcescens* is present in soil and can invade the haemocoel of insects through wounds (Daly et al., 1998). It is pathogenic to *Drosophila melanogaster* (Flyg et al., 1980) and *Parasemia plantaginis* (Arctiidae) (Friman et al., 2009) when injected into the haemocoel.

Melitaea cinxia larvae were infected by injecting 5 μl of bacterial solution (approx. $1.66*10^6$ bacterial cells) between the 2nd and 3rd segments. The syringe was cleaned with sterilized water between injections. The syringe was filled with pure ethanol between treatment days to ensure sterility. After bacterial infection, infected larvae were kept in a separate environmental chamber (under the same temperature and light conditions) to prevent the spread of infection to control larvae. Survival of the infected larvae was checked four times per day (at 8.00 and 11.00a.m., 2.00 and 5.00p.m.) for the next 5 day and once per day after that.

As a control we injected 5 μl sterile water into 79 larvae. Previous experiments with similar sized larvae show that this amount of water does not cause any hypotonic reactions for the cells of *P. plantaginis* (Friman et al., 2009, 2011). This has been confirmed for *M. cinxia.* Mortality in the water control treatment in 2009 was only 7.9%. The methods were the same as for bacterial infection. Due to a small sample size in 2008, more water control larvae were treated the following summer (2009) under similar experimental conditions (diet, rearing conditions, and methods). Survival of the water control larvae to pupal stage was significantly lower in 2008 than in 2009 (Fisher's exact test: $P=0.002$). However, in both years, the survival in the water control group was significantly higher than that of bacteria-treated larvae (in 2008: all P-values$<$ 0.019, and in 2009: all P-values<0.001). The data sets from both years were pooled to increase the power of the test.

Diet Quality and the Interaction of Pathogen and Parasitoid Infection (implant + bacterial infection treatment). Larvae were implanted and, subsequently, exposed to bacterial pathogens. Encapsulation rate was measured first (for details see implant treatment). Larvae were infected with bacteria within 2 h of implant removal (for details see bacterial infection). We tested the correlation between encapsulation rate and survival after bacterial infection.

Statistical Analyses All statistical analyses were performed with SPSS 15.0 (SPSS Inc.). Differences between the diets were tested with one-way ANOVA using aucubin, catalpol, and total IG as dependent variables, and diet as the factor. The same test was used to test differences in nutritional quality between the diets, with P, K, N, and C as dependent variables, and diet as the factor. Correlations between leaf IG measurements and leaf nutrients were tested with a Spearman correlation. Differences in encapsulation rate between diets were tested with ANCOVA using diet as a fixed factor and larval weight and the time elapsed after moulting as covariates. Linear regression (with stepwise deletion) was used to analyze the effects of diet catalpol and aucubin concentrations on the encapsulation rate. The aucubin concentration of the diet was excluded from the model because it had no significant effect on encapsulation rate ($t=-0.090$, $P=0.929$). Differences in IG concentrations (% from dry weight measured from adults) between implanted (implant treatment) and non-implanted (control) individuals were tested with two-way ANOVA using diet and implant treatment as fixed factors. Spearman's correlation coefficients were used to analyze correlations between encapsulation rate and individual IG concentrations. Correlations between adult IG concentrations and encapsulation rate were analyzed using individuals of the implant treatment only. Implantation had no significant effect on survival after bacterial infection (two-way ANOVA, diet and implantation as fixed factors; implantation: $F_{1,132}=2.113$, $P=0.148$). Therefore, both infection groups (implanted and non-implanted) were pooled for final analyses. Kaplan Meier analysis with log-rank statistics was used to analyze differences in larval survival between diet groups 72 h after bacterial infection. The formula ln pupal mass (mg) / larval development time to pupal stage in days was used to calculate growth (Ojala et al., 2005). Two-way ANOVA was used to test differences in pupal mass and growth rate of control and implanted individuals (no infection) using diet, sex, and implant treatment as fixed factors. All interactions were kept in the model because deleting them did not change the results. Sequestration data were analyzed using non-parametric Spearman's correlation coefficients because IG concentrations were not normally distributed.

Results

Diet Treatments The five diet groups differed significantly in amounts of total IGs and catalpol, based on long-term

measurements of the plants (ANOVA: $F_{total\ IG\ 4,28}$=3.346, P=0.023; $F_{catalpol\ 4,28}$=2.996, P=0.035; $F_{aucubin\ 4,28}$= 2.151, P=0.101). Diet ranking was constant before, during and after the experiment (total IGs as % dry weight given as means of all measurements ± SE; diet 1: 1.26%±0.31, diet 2: 1.87%±0.46, diet 3: 2.02%±0.30, diet 4: 2.62%±0.27, diet 5: 2.76%±0.33). The diets did not differ significantly in their nutritional quality (ANOVA: $F_{P\ 4,15}$=0.732, P=0.589; $F_{K\ 4,15}$=0.650, P=0.639; $F_{N\ 4,12}$=0.360, P=0.830; $F_{C\ 4,12}$= 0.831, P=0.542). Total IGs (all P values>0.491), catalpol (all P values>0.479) and aucubin (all P values>0.714) content were not correlated with the nutritional values of the plants (Table S2, supplemental material).

Diet Quality and Life History Traits Diet type (1–5) did not have significant effects on growth rate (Table 2) or pupal weight. However, pupal weight was higher in females than in males and females had significantly lower growth rates than males. Implanted individuals reached lower pupal masses but implantation did not affect growth rate (Table 2). There were no significant two- or three-way interactions between diet, implant treatment, and sex for pupal mass or growth rate (Table 2). Adult catalpol concentrations correlated positively with pupal mass (r_s=0.336, N=41, P= 0.032), but not with growth rate (r_s=-0.261, N=41, P= 0.099). Adult aucubin concentrations did not correlate with pupal mass (r_s=0.067, N=41, P=0.679) or growth rate (r_s= 0.213, N=41, P=0.180).

Table 2 The effects of diet and gender on *melitaea cinxia* pupal mass and growth rate

Source of variation	*df*	*MS*	*F*	*P*
Pupal mass				
Diet	4	529.159	1.000.	0.414
Implantation	1	4699.981	8.878	0.004
Sex	1	15229.015	28.766	0.000
Diet x implantation	4	787.095	1.487	0.216
Diet x sex	4	915.784	1.730	0.154
Implantation x sex	1	63.309	0.120	0.731
Diet x implantation x sex	4	200.342	0.378	0.823
Error	68	529.414		
Growth rate				
Diet	4	<0.001	0.548	0.701
Implantation	1	<0.001	0.266	0.608
Sex	1	0.007	14.917	0.000
Diet x implantation	4	<0.001	0.703	0.592
Diet x sex	4	<0.001	0.820	0.517
Implantation x sex	1	0.001	1.714	0.195
Diet x implantation x sex	4	<0.001	0.610	0.657
Error	68	<0.001		

The number of larvae that survived to the pupal stage (χ^2=2.169, df=4, P=0.705) or that emerged as adults (χ^2= 1.696, df=4, P=0.791) did not differ among the diets. The immune defense treatments reduced larval survival. Fewer implanted or infected individuals pupated compared to the control group (implanted vs. control: Pearson χ^2=4.866, N= 157, P=0.027, infected vs. control: Pearson χ^2=68.983, N= 150, P<0.001).

Diet Quality and Encapsulation Activity The encapsulation rate (i.e., defense against parasitoids) did not differ among the different diets (ANCOVA: $F_{4,133}$=1.733, P=0.146). Encapsulation rate did not correlate with adult IG concentrations (total IG: r_s=0.275, N=35, P=0.109; catalpol: r_s= 0.250, N=35, P=0.147; or aucubin: r_s=0.220, N=35, P= 0.204). However, the encapsulation rate did increase with increasing diet catalpol concentrations (linear regression, model: R^2=0.043, $F_{1,138}$=6.193, P=0.014, y=30.164+ 8.789× ; catalpol concentration in the diet: t=2.489, P= 0.014) (Fig. 1).

Adult catalpol and total IG concentrations were higher in implanted individuals than in non-implanted individuals (independent t-test catalpol: t=-3.00, df=89, P=0.003; total IG: t=-3.07, df=89, P=0.003) irrespective of variations in their diet catalpol and IG content (Tables 3 and 4). However, diet did have a significant effect on adults' aucubin content (Table 4). Pairwise comparisons with Tukey HSD adjustment showed that adult aucubin concentrations were higher on diet 2 than on diet 4 (P=0.046). There were no significant among-diet differences in the aucubin concentrations of the adults on the other diets (all P-values>0.180).

Diet Quality and Defense against Pathogens Diet affected the survival of larvae infected with *Serratia marcescens* (Kaplan-Meier survival analysis, Log-rank statistics, χ^2= 10.173, df=4, P=0.038). Although there was slightly more

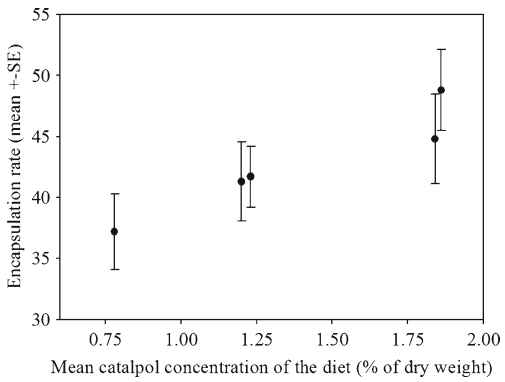

Fig. 1 Catalpol concentrations of the five experimental diets and encapsulation rates of *Melitaea cinxia* larvae after artificial parasitation Error bars show the individuals' mean rate of implant encapsulation (± SE). A higher encapsulation rate indicates a stronger response against an artificial parasitoid. *N=149;* for statistical information see text

Table 3 Average concentrations (±se) (% of dry weights) of total iridoid glycosides (ig) (aucubin and catalpol) in adults according to diet and immune defense treatment

	Diet				
	1	2	3	4	5
Total IG of adults					
Implanted	3.39±0.72	3.47±0.36	3.53±0.39	4.22±0.38	3.40±0.55
Control	4.30±0.48	2.53±0.22	2.60±0.38	2.87±0.40	2.67±0.32
Catalpol of adults					
Implanted	3.30±0.72	3.26±0.35	3.44±0.384	4.15±0.38	3.30±0.53
Control	4.13±0.45	2.44±0.22	2.52±0.36	2.83±0.40	2.59±0.31
Aucubin of adults					
Implanted	0.10±0.02	0.21±0.08	0.10±0.01	0.06±0.01	0.10±0.02
Control	0.17±0.06	0.08±0.02	0.08±0.02	0.04±0.00	0.08±0.02

aucubin in diets 2 and 3 (Table 1A), on which survival was lower, there was no clear trend relating to diet IG concentration (Fig. 2). Pairwise comparisons showed that larval survival was lower on diet 2 than on diets 1, 4, and 5 (all P values<0.032). There were no differences in larval survival among the other diets (all P values>0.072, Fig. 2). Survival time after infection did not correlate with aucubin (r_s=-0.139, N=142, P=0.099) or catalpol (r_s=0.132, N=142, P=0.117) levels in the diet, although the positive correlation between survival time and total diet IG concentration was nearly significant (r_s=0.159, N=142, P=0.058).

The injection method itself did not cause a significant increase in larval mortality. The mortality of larvae injected with water was lower in all diet groups (Pearson χ^2 test for diet 1: P=0.004, 2: P<0.001, 3: P<0.001, 4: P<0.001, 5: P<0.001) than that of larvae injected with bacterial cells.

Table 4 The effects of diet and implant treatment on iridoid glycdoside (ig) (aucubin and catalpol) concentrations of *melitaea cinxia* adults

Source of variation	*df*	*MS*	*F*	*P*
Total IG concentration of adults				
Diet	4	1.925	1.490	0.215
Implant	1	6.845	5.299	0.024
Diet x implant	4	2.334	1.807	0.138
Error	67	1.292		
Catalpol concentration of adults				
Diet	4	2.046	1.660	0.170
Implant	1	6.349	5.149	0.026
Diet x implant	4	2.102	1.705	0.159
Error	67	1.233		
Aucubin concentration of adults				
Diet	4	0.024	2.581	0.045
Implant	1	0.010	1.076	0.303
Diet x implant	4	0.017	1.801	0.139
Error	67	0.009		

Diet Quality and the Interaction of Pathogen and Parasitoid Infection There was a positive correlation between encapsulation rate and survival time after bacterial infection (r_s= 0.331, N=65, P=0.007). Larvae with higher encapsulation rates survived longer after bacterial infection.

Discussion

Host immune defense evolves not only based on direct interaction with pathogens but also in response to interaction with other species, such as predators (e.g., Friman et al., 2009), or in relation to diet quality and quantity (Lill et al., 2002). Here, we showed that diet quality affects the ability of *M. cinxia* to defend against parasitoids (*via* encapsulation activity) and pathogens. We found that encapsulation activity (defense against parasitoids) was positively correlated with defense against pathogens. In contrast to previous studies (Harvey et al., 2005; Saastamoinen et al., 2007), we did not find effects of diet on life history traits in *M. cinxia*. This may be due to the relatively small differences in IG concentration between our diets compared to natural values (Bowers et al., 1992; Fajer et al., 1992). Our results

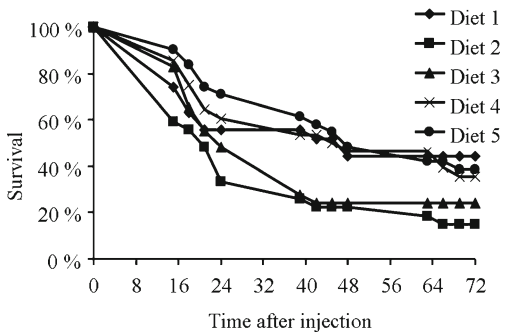

Fig. 2 Survival rates (%) of larvae recorded at different periods after bacterial injection (hours) according to diet (1–5). The concentrations of iridoid glycosides were lowest in diet 1 and the highest in diet 5

suggest that the immunological defense of *M. cinxia* increases with increasing IG concentrations in the diet. These diet-dependent immunological changes are without any life history costs.

Diet catalpol concentration was positively correlated with encapsulation rate. This is consistent with the results of Nieminen et al. (2003) and Reudler Talsma et al. (2011) (but see Harvey et al., 2005) which showed that *M. cinxia* parasitism by the specialist parasitoid wasp *C. melitaearum* was reduced when host-plant catalpol concentrations were high. Nieminen et al. (2003) hypothesized that *C. melitaearum* parasitoids could avoid oviposition of hosts that have high catalpol concentrations in their bodies. Alternatively, the parasitoid larvae could suffer higher mortality when the parasitized hosts have high catalpol concentrations. Our results offer an alternative mechanism: high catalpol concentrations in the diet could directly enhance host immune defense by increasing the larval encapsulation rate. This could happen if catalpol has an antioxidant function (Tundis et al., 2008). Antioxidants can reduce the costs of encapsulation and enable higher encapsulation activity by preventing the damage to the cell that free radicals (produced during encapsulation reactions) can cause (Nappi et al., 1995; von Schantz et al., 1999).

While artificial implants mimicking parasitoids have been widely used as a controlled measurement of the encapsulation reaction (Ojala et al., 2005; Rantala and Roff, 2005; Siva-Jothy et al., 2005; Klemola et al., 2007, 2008), the encapsulation of an artificial parasitoid may not fully correspond to the infection of a natural parasitoid (Klemola et al., 2008). The effects of IGs in the diet of *M. cinxia* on its specialist parasitoids *C. melitaearum* and *H. horticola* have been studied by Harvey et al. (2005) who found that IGs in the host's diet did not affect the development time or adult body mass of *C. melitaearum*. However, increasing the IG content of the hosts' diet shortened the development time of *H. horticola*. Thus, IGs are not necessarily harmful to all parasitoids, and specialist parasitoids have different mechanisms to overcome the host's immune defense system (Poirié et al., 2009). Nevertheless, host encapsulation rate is likely to show the host's defense capacity against generalist parasitoids as well as other foreign objects detected by the individuals' immunological system.

Interestingly, implanted (artificially parasitized) adult individuals had higher amounts of catalpol in their bodies than did non-implanted individuals. Implanted individuals may have sequestered more IGs from their food plants for medicinal purposes (i.e., for self-medication, see, e.g., Singer et al., 2009). Alternatively, implanted individuals may have increased their food consumption leading to a higher accumulation of IGs in their bodies. The higher PSM in implanted individuals also may indicate an energetic trade-off between IG excretion and parasitoid encapsulation rate. Allocation of energy for encapsulation may trade off with excretion of IGs from the body and explain why IG concentrations were higher in implanted individuals. This seems a likely possibility because implanted individuals reached smaller body sizes than non-implanted individuals, suggesting that implantation had costs for developing larvae. Smilanich et al. (2009) found that *Junonia coenia* larvae (specialist herbivores on IG plants) had weaker encapsulation rates when they sequestered higher concentrations of catalpol, indicating negative effects of IGs on encapsulation (Smilanich et al., 2009). Further study, concentrating on the costs of sequestering IGs and the relative importance of different natural enemies on the immune defense of *M. cinxia*, is needed to understand these effects.

Diet also had an effect on the survival of larvae after bacterial infection. Survival was lower on diets 2 and 3 that contained slightly more aucubin. While this difference was not significant, it suggests a detoxification cost for aucubin, which trades off with pathogen defense. With a larger sample size and greater chemical differences among the diet treatments we might have detected these costs. It is also possible that other factors, such as water concentration, nutritional value, or antioxidant content, could have affected larval survival. These have all been shown to affect herbivores' immunological mechanisms (Bauce et al., 2002; Ojala et al. 2005). However, we did not find any nutritional differences between the diets or effects of diet on life history traits, suggesting that the plants were similar in their quality. Variation among diet treatments in our experiment was lower than, for example, in Ojala et al. (2005) where larvae were reared on different plant species.

The encapsulation rate did not trade off with larval survival after bacterial infection. Instead, resistance against both types of enemies correlated positively. A similar result was found in a 2004 study by Rantala and Kortet in which a high encapsulation rate correlated positively with high lytic activity (indicating antibacterial defense) in field crickets (*Gryllus bimaculatus*). Those authors concluded that this result was probably due to the good condition of individuals that allowed them to invest more resources in immune defense (Rantala and Kortet, 2004). We provided larvae with food *ad libitum*, which could offer one explanation for the positive correlation between the two different defenses (encapsulation and antibacterial defense). However, we did not find any significant correlations between our immunological measurements and individual performance (growth rate, pupal weight, or development time) suggesting that good body condition was not the only explanatory factor. It may be that defense against parasitoids and bacteria are genetically linked in *M. cinxia*. Lambrechts et al. (2004) found that high encapsulation rate correlated positively with antibacterial response in mosquito (*Anopheles gambiae*) families, indicating a genetic correlation between these

defenses. Cotter et al. (2004a) found, in contrast, a negative genetic correlation between antibacterial activity and haemocyte density, suggesting a trade off within the immune system in the generalist herbivore *Spodoptera litteralis*, the Egyptian cotton leafworm. Thus, the outcome could depend on insect and pathogen species (Lambrechts et al., 2004). For simplicity, the family effect of immune defense was not tested in this study and, therefore, the possible genetic basis behind the positive correlation remains a topic for future studies.

To understand the evolution of a host's ability to resist infections and parasitoids, it is important to take into account selection from multiple trophic levels as our results emphasize. We conclude that IGs likely benefit *M. cinxia* in the defense against parasitoids because of the positive effect on their encapsulation rate. Moreover, effective defense against parasitoids correlated positively with defense against bacterial pathogens, which could be due to a genetic linkage between these two traits or result from condition-dependent effects. Nevertheless, our results show that the availability of host plants with high IG concentration can be critical for the *M. cinxia* fitness, especially when the risk of parasitism is high. Interestingly, IGs did not cause any life -history costs for the larvae, which suggest that plant defense chemicals can be used to enhance insect immunity without necessarily paying energetic costs. In the future, more studies are needed to understand how other properties of the herbivore diet (e.g., nutritional and antioxidant content) are linked to different immune defense and what role the genetic variation of host plants, insects and parasitoids play in this tritrophic system are essential.

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ERRATUM

Erratum to: Identification, Synthesis, and Bioassay of a Male-Specific Aggregation Pheromone from the Harlequin Bug, *Murgantia histrionica*

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In the original version of this article, the mass spectrum shown in Figure 6 on page 245 is the spectrum of a degradation product from murgantiol, and not of murgantiol itself. The correct mass spectrum for murgantiol is shown below. The authors regret this error.

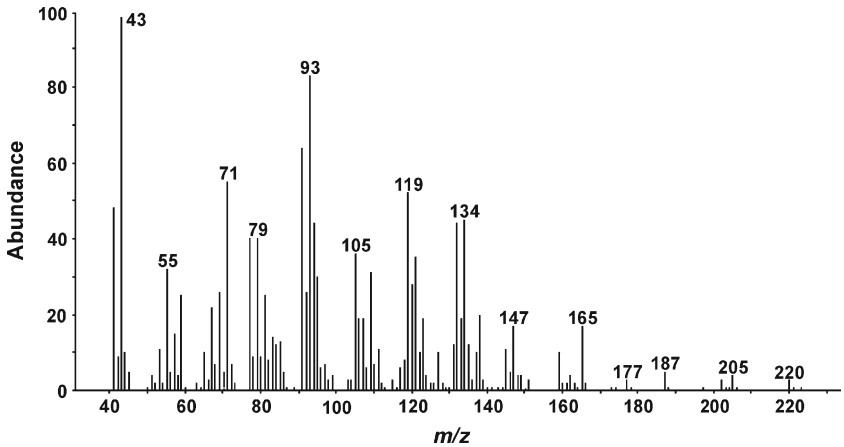

Fig. 6 Electron impact ionization (70 eV) mass spectrum of the *Murgantia histrionica* male-specific compound (murgantiol)

The online version of the original article can be found at http://dx.doi.org/10.1007/s10886-007-9415-x.

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