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The ectoparasitoid *Scleroderma guani* (Hymenoptera: Bethyridae) uses innate and learned chemical cues to locate its host, larvae of the pine sawyer *Monochamus alternatus* (Coleoptera: Cerambycidae)

Zhen Li^{1,†}, Baishu Li^{2,†}, Zhenjie Hu³, J. P. Michaud⁴, Jie Dong⁵, Qingwen Zhang¹, and Xiaoxia Liu^{1,*}

Abstract

In Asia, the pine sawyer beetle *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is the most important vector of the pinewood nematode *Bursaphelenchus xylophilus* Steiner and Buhner (Aphelenchida: Parasitaphelenchidae), the causal agent of pine wilt disease, and the ectoparasitoid *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethyridae) is the most important natural enemy of this pest. Efficient host location is critical to parasitoid fitness, and chemical cues are key factors guiding the host searching process. This study was conducted to elucidate the odor cues guiding host location in *S. guani* and the impact of previous host experience on this behavior. Tests were conducted in which *S. guani* oriented to different odor resources associated with *M. alternatus* and its habitat, and components of these volatiles were analyzed with gas chromatography/mass spectrometry. Orientation to various odors also was investigated using adult *S. guani* subjected to different adult experiences. Female *S. guani* could easily distinguish *M. alternatus* larvae from other odor resources. Hydrocarbons were the main components of volatiles derived from *M. alternatus* larvae and were absent in volatiles from wood and frass associated with *M. alternatus* larvae. Female *S. guani* oriented most strongly to 1st and 3rd instars of *M. alternatus*. Prior experience of host odor without oviposition decreased orientation of *S. guani* towards the host, and the host used for rearing had no impact on subsequent odor orientation by adult *S. guani*. Volatile hydrocarbons emanating from host larvae might be the key to host location by adult *S. guani*, and experience with hosts appears to reinforce behavioral responses. The present results may be useful references for improving augmentative biological control using *S. guani* against *M. alternatus* and other forest pests.

Key Words: volatile hydrocarbon; experience; host location; biological control

Resumen

En Asia, el escarabajo cerambycido *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) es el vector mas importante del nematodo *Bursaphelenchus xylophilus* Steiner and Buhner (Aphelenchida: Parasitaphelenchidae), el agente causal de "pine wilt disease", y el ectoparasitoide *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethyridae) es el enemigo natural mas importante de este plaga. Localizacion eficiente del huesped es critical al capacidad biologica del parasitoide y indicaciones quimicas son factores claves guiando el proceso. Este estudio fue conducido para elucidar los claves guiando el proceso de localizacion de huesped por *S. guani* y el efecto de experiencia en este comportamiento. Pruebas fue conducido en lo cual *S. guani* se oriento a varios olores asociados con *M. alternatus* y su habitat y componentes de estes quimicas volatiles fue analizado por "gas chromatography/mass spectrometry." Orientacion a varios olores fue tambien investigado usando adultos de *S. guani* condicionados con diferentes experiencias adultos. Hembras de *S. guani* pudieron distinguir olores de larvas de *M. alternatus* desde los otros ofrecidos. Hidrocarbones fuerone los componentes principales de compuestos volatiles derivado de larvas de *M. alternatus* y estos fueron aucente en volatiles de madera y excremento asociado con aquellas larvas. Hembras de *S. guani* orientaron mas fuertemente a larvas de *M. alternatus* de primera y tercera etapa. Experiencia previa de olor de huespede sin oportunidad de postura reducion orientacion de *S. guani* hasta olor de su huesped, y el huesped de crianza not tenia efecto en la orientacion de *S. guani* adultos hacia olores. Hidrocarbones volatiles emanando de larvas huespedes poderon ser clave en el proceso de localizacion de huesped por *S. guani* adultos y experiencia con huespedes aparece resforzar esta comportamiento. Los resultados actuales pueden ser referencias útiles para mejorar el aumento en el control biológico usando *S. guani* contra *M. alternatus* y otras plagas forestales.

Palabras Clave: hidrocarbones volatiles; experiencia; localizacion de huesped; control biológico

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Pine wilt disease (PWD) has caused devastating damage to pine forests worldwide, especially in Japan and China (Aikawa et al. 2006), and the combined timber losses and management costs have exceeded US\$ 4 billion annually in China alone (Li et al. 2011). The pine wood nematode *Bursaphelenchus xylophilus* Steiner and Buhner (Aphelenchida: Aphelenchoididae) is the pathogenic agent of PWD. *Bursaphelenchus xylophilus* has high pathogenicity and can cause the death of infected pines within several months (Han et al. 2007; Qiu et al. 2013). The pine sawyer beetle *Monochamus alternatus* Hope (Cerambycidae: Lamiinae) has been identified as the most important vector of the pine wood nematode in Asia (Ding et al. 2001), and management of *M. alternatus* is regarded as the key to control of PWD in China (Xu et al. 2008). Because *M. alternatus* larvae are solitary wood-boring insects living under pine bark, they are protected from contact pesticides and are very difficult to control (Wang & Yang 2008). Although the quick removal of all infected trees provides effective control of wood-boring insects, it is not a feasible tactic for *M. alternatus*-mediated, *B. xylophilus*-infected pines. *Bursaphelenchus xylophilus* can be transmitted to a dying tree, newly cut log, or living pine during feeding or oviposition by *M. alternatus* (Jones et al. 2008), and symptoms can be very difficult to detect in early stages. Once symptoms are evident, pine trees die soon thereafter (Jones et al. 2008). Thus, coevolved natural enemies that can effectively locate and parasitize the concealed larvae are among the best alternatives for sustainable long-term prevention of PWD.

The ectoparasitoid *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethyloidea) is indigenous to China and has been used widely for biological control of forest pests as it can parasitize the larvae and pupae of more than 50 species of wood-boring insects belonging to 22 families in 3 orders (Chen & Cheng 2000). This wasp has been considered the most effective biological control agent of *M. alternatus* and, consequently, *B. xylophilus* (Wang 2004; Xu et al. 2008). Parasitism of up to 81.3% of *M. alternatus* larvae has been reported in years of *S. guani* releases, and mortality of *B. xylophilus*-infected pines has been reduced by as much as 98.0% (Xu et al. 2002). Females of *S. guani* search for hosts by crawling quickly around tree trunks. Once they find the frass of *M. alternatus* on pine bark, they follow it to the host tunnel where they are able to locate *M. alternatus* larvae under bark in complete darkness (Yao & Yang 2008). Laboratory studies report that *S. guani* is an idiobiont (Lu et al. 2013) with a series of stereotypical host selection behaviors performed in a characteristic sequence (Zhang et al. 2004; Hu et al. 2012). Females of *S. guani* prefer to parasitize mid-instars; late instars have strong defensive behaviors and are difficult to parasitize, whereas early instars are vulnerable to mortality during parasitism (Ma et al. 2010). Because larvae of *M. alternatus* live under pine bark in dark galleries filled with sawdust and frass, the mechanism by which *S. guani* locates and selects suitable hosts warrants clarification.

Parasitoid wasps may use olfactory, visual, or acoustic signals to locate concealed hosts and parasitize them (Hou & Yan 1997; da Silva Torres et al. 2005; Wang & Yang 2008; Wölfling & Rostás 2009). Parasitoids evolve to focus on the most reliable of available cues for host location and may exhibit preferences for a particular host species or odor based on previous experience (van Alphen & Vet 1986; Vet & Dicke 1992; Papaj et al. 1994; Takasu & Lewis 2003; Fatouros et al. 2008; Li et al. 2009). In the present study, 3 main questions were addressed: 1) What is the most attractive chemical cue that guides *S. guani* females in host location? 2) Which instar of *M. alternatus* is the most attractive? 3) Does experience affect subsequent host location behavior by *S. guani* females? In order to answer these questions, *S. guani* females were permitted to choose between various habitat-derived odor sources and different instars of *M. alternatus* larvae, and the components of these odors were analyzed by gas chromatography/mass spectrometry (GC/MS). Wasp odor preferences also were

investigated in *S. guani* that had been reared on different hosts. It was reasoned that clarification of the kairomones used by *S. guani* in host location, and the role of learned cues, would improve our understanding of the tritrophic relationship among host pine, *M. alternatus*, and *S. guani* and potentially contribute to improved biological control of *M. alternatus* with *S. guani*.

Materials and Methods

INSECT PREPARATION

Larvae of *M. alternatus* ($n \geq 200$) were collected as 2nd and 3rd instars from infested pine trees in Hangzhou City, Zhejiang Province, China, in Jul 2006. The larvae were isolated in plastic vials (5.0 cm height \times 3.0 cm diameter), held in complete darkness under ambient temperature in our laboratory, and reared on an artificial diet (provided by the Chinese Academy of Forestry). Following adult emergence, beetles were transferred to cages and provided with fresh pine branches as described by Song et al. (2008). After 2 wk, 1st and 2nd instars of *M. alternatus* could be found under the bark of branches near oviposition scars. Mixtures of wood and frass were collected from their larval galleries and stored at -20°C for subsequent analysis.

Adults of *S. guani* were obtained from a colony maintained on larvae of *Saperda populnea* (L.) (Coleoptera: Cerambycidae) on the Beijing Xishan Forest Farm. Pupae of the mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were used as a factitious host for rearing *S. guani* in all experiments. Mealworm larvae were purchased from the Beijing Guanyuanqiao pet market and reared on artificial diet (Zhang & Liu 2005) at 26.0°C , a 16:8 h L:D photoperiod, and 70% RH until pupation. Pupae of *T. molitor* (100–160 mg) were held at 0.0°C for 48 h before use in order to immobilize them for parasitism. Pupae were then placed into glass vials (10.0 cm height \times 3.0 cm diameter) with mated females of *S. guani* in a parasitoid-to-pupa ratio of 2:1. Each vial contained a cotton ball with 10% honey solution and was plugged with a segment of cotton tampon. Parasitoids were removed after cocoons formed, and vials were held in a climate-controlled chamber under the same environmental conditions described above until adult parasitoid emergence.

Parasitoids were reared on larvae of *M. alternatus* by confining 3 mated *S. guani* females with 1 third instar (25–30 mm long) in a glass vial (5.5 cm height \times 2.0 cm diameter) at 8.0°C for 48 h. A 10% honey solution was provided on a ball of cotton, and the vials were plugged with cotton tampons. Insects were then kept in a climatic chamber at 27.0°C , a 16:8 h L:D photoperiod, and 60% RH for adults collection. Once the 2nd generation of *S. guani* adults emerged, mated females were collected, kept in glass vials (5.5 cm height \times 2.0 cm diameter) at 25°C , a 16:8 h L:D photoperiod, and 60% RH, and supplied with 10% honey solution for 5 to 6 d to ensure oogenesis before use in experiments.

RESPONSES OF NAÏVE *S. GUANI* TO HOST-DERIVED ODORS

Mated female adults of *S. guani* (6 d old post-emergence) had no contact with any host (i.e., they were naïve) when they were tested in a glass Y-tube olfactometer modified according to de Kogel et al. (1999). Tests were carried out at 25.0°C in a dark room with a fluorescent lamp hanging directly above the experimental apparatus. A black sheet was used to cover the whole apparatus to prevent access to other visual stimuli. All tests were performed between 9:00 and 10:30 a.m. or between 3:30 and 5:00 p.m., when the parasitoid is most active. Naïve *S. guani* females, 1 at a time, were released into the main arm and walked

upwind to choose between 2 odor sources. A clock was started when each wasp entered the branch point of the main arm and direct observations were made for 5 min. Choices were recorded when a wasp walked more than 1.0 cm beyond the branch point and remained there for at least 10 s, or reached the end of the branch arm; otherwise no choice was recorded. Sixty repetitions were conducted for each set of odor choices, and each wasp was used only once. The Y-tube was reversed after each observation and replaced with a new tube after 10 observations. All tubes were cleaned with 95% ethanol before reuse. The following odor sources were tested: 10.0 mg of the wood diet used for feeding of *M. alternatus* (W), 10.0 mg of mixtures of wood diet and frass of *M. alternatus* (WF), 10.0 mg of sawdust produced by *M. alternatus* larval boring (S), 10 larvae of *M. alternatus* starved for 3 h (L), and a clean air control (A). WF and S were obtained from habitat occupied by 10 *M. alternatus* larvae for 1 wk.

ORIENTATION TO HOST INSTARS

First, third, and overwintering fifth instars of *M. alternatus* were all tested versus clean air with naïve, 6-d-old *S. guani* females that were isolated after emergence without any host or odor experience. The same protocol was used as described above for other odor sources.

VOLATILE COLLECTION AND ANALYSIS

Volatiles were collected separately from 10 *M. alternatus* 3rd instars that had been starved for 3 h and from 10.0 mg samples of the wood diet and frass mixture, using a dynamic headspace air collection device (QC-1, Beijing Municipal Institute of Labor Protection, Beijing, China) as in the study of Tang et al. (2012). Tenax-TA absorbent (150 mg) was connected to an oven bag (Wegmans Food Market, USA) where the volatiles were delivered. Air was delivered into the collection device after passage through activated charcoal and distilled water and through the bag containing odor sources. Volatile samples were collected for 24 h, at 25.0 °C in the dark with an airflow rate of 50 mL/min and subsequently analyzed using thermal-desorption cold-trap injector GC/MS (TCT-GC/MS) (CP-4010 PTI/TCT, Varian, Bergen op Zoom, Belgium; Thermo Finnigan Voyager GC/MS with Trace 2000 GC, ThermoQuest, United Kingdom). The TCT had a trap injection temperature of 200.0 °C, a trap temperature of -130.0 °C, a desorption temperature of 250.0 °C, and a desorption time of 10 min. A DB-5 Low Bleed Column (60 m × 0.32 nm × 0.5 μm) was used for GC/MS analysis, with a column temperature of 40.0 °C for 3 min increasing at 6.0 °C/min to 270.0 °C. Helium was used as carrier gas, the separator temperature was 280.0 °C, and the scan range of the MS monitor was 19 to 435 m/z for ion monitoring. The relative abundance and total ion current of MS for each sample were thus obtained. Compound identification was conducted by comparing the retention time of each component in the NIST 11 Mass Spectral Library (NIST/2011/EPA/NIH). The relative ratio of each compound in samples was calculated using the area normalization method.

EXPERIENCE AND ODOR SELECTION BY *S. GUANI*

Females of *S. guani* were subjected to various pre-trial experience treatments that included 1) no odor or oviposition experience (N); 2) contact with sawdust collected from the *M. alternatus* larval habitat (S); or 3) exposure to volatiles of *M. alternatus* larvae, but without oviposition (L). Naïve *S. guani* females (as described above) were held in glass vials (5.5 cm height × 2.0 cm diameter) sealed with fine mesh gauze and placed into cages together with 10 starved 3rd instars of *M. alternatus* (L) or 10.0 mg of sawdust (S) for 30 min. Wasps could not contact host larvae or sawdust samples directly but were exposed to only their odors. These wasps were used in olfactometer tests which

offered *M. alternatus* larvae versus clean air as described above. *Scleroderma guani* colonies were reared separately either on pupae of *T. molitor* or larvae of *M. alternatus* for at least 5 generations and were then tested for effects of the natal host on odor responses. Females ($n = 60$) of *S. guani* from each colony were then offered a choice between the odor of 10 pupae of *T. molitor* (TP) versus 10 larvae of *M. alternatus* (ML).

STATISTICAL ANALYSES

Cochran's *Q* test was used to compare the relative preference of *S. guani* females for different odor sources, using SPSS Statistics 17.0 (SPSS Inc., Chicago, USA). Stacked vertical bars were constructed to depict differences in volatile components between different extracted samples with SigmaPlot 10.0 (Systat Software, Inc., California, USA).

Results

RESPONSES OF NAÏVE *S. GUANI* TO HOST-DERIVED ODORS

Naïve *S. guani* females expressed no preference between odor pairs that lacked *M. alternatus* larvae (Fig. 1): (W vs. WF, $Q = 0.176$, $P = 0.674$; S vs. A, $Q = 0.209$, $P = 0.647$; WF vs. A, $Q = 0.020$, $P = 0.888$; W vs. A, $Q = 0.556$, $P = 0.456$). However, host larvae were preferred to clean air ($Q = 6.149$, $P = 0.013$), to wood diet ($Q = 21.333$, $P < 0.001$) and to sawdust ($Q = 5.255$, $P = 0.022$, $n = 55$).

VOLATILE COMPONENTS OF ODOR SOURCES

Fourteen compounds were identified in volatiles derived from *M. alternatus* larvae (Fig. 2), including 11 different hydrocarbons (nonane 3.23%, decane 1.61%, limonene 9.08%, dodecane 5.44%, tetradecane 7.83%, dotriacontane 9.25%, ethylbenzene 2.70%, 1,3-dimethyl benzene 5.70%, biphenyl 7.97%, octane 1.58%, and hexadecane 11.39%), 2 alcohols (dimethyl hexadecanol 2.18% and borneol 16.02%), and 1 aldehyde (nonaldehyde 16.05%). Collectively, hydrocarbons were the main components and comprised 65.75% of the total. Six compounds

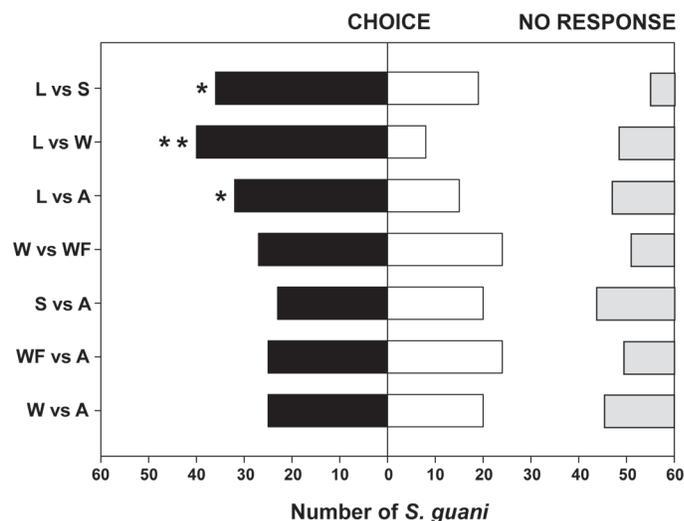


Fig. 1. Numbers of naïve *Scleroderma guani* females responding to various odor sources presented in pairs in a Y-tube olfactometer. W = wood diet, WF = mixture of wood diet and *Monochamus alternatus* frass, S = sawdust from *M. alternatus* galleries, L = 10 *M. alternatus* 3rd instars, and A = clean air. *: $P \leq 0.05$, **: $P \leq 0.01$. Numbers indicate numbers of wasps responding.

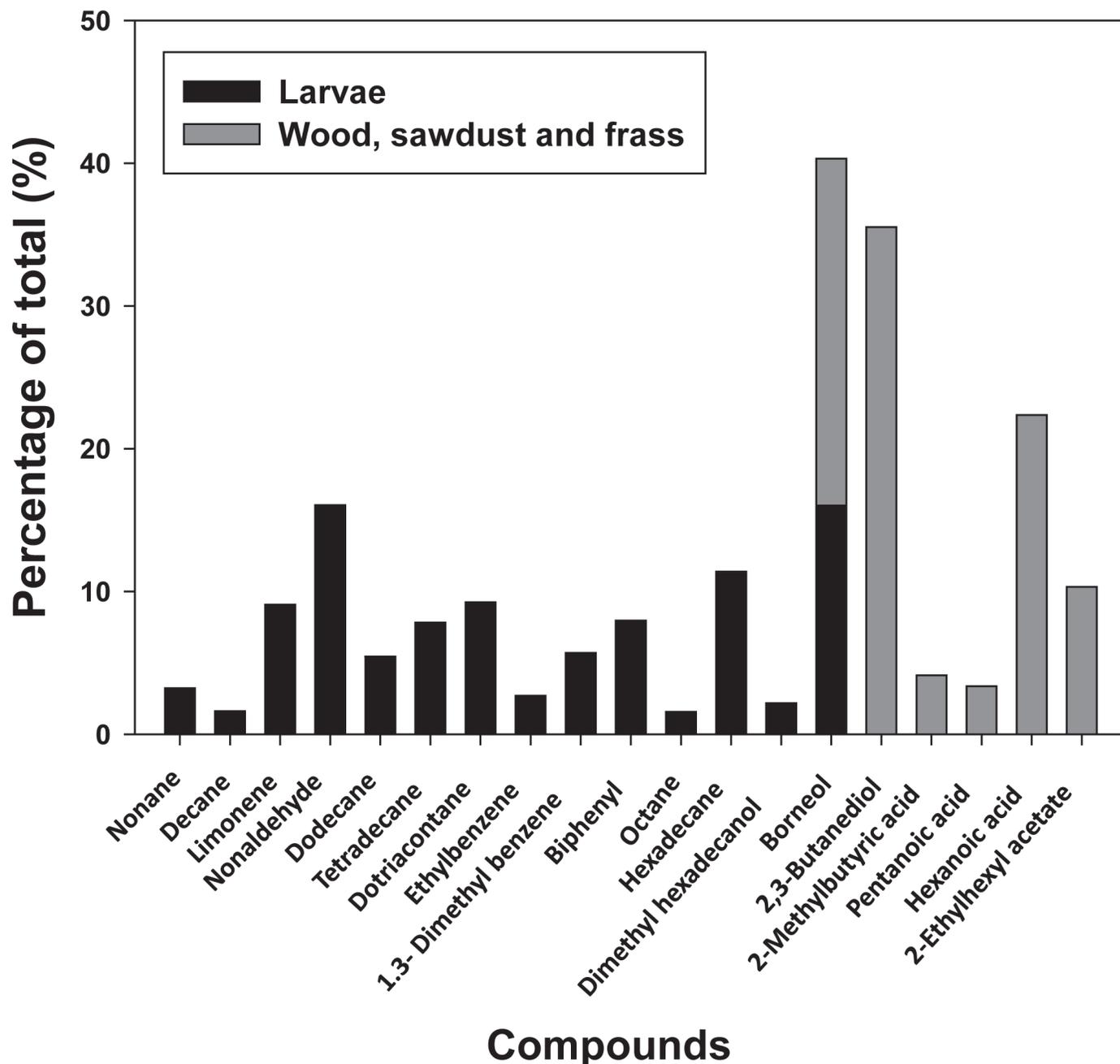


Fig. 2. Relative proportions of organic compounds identified in headspace volatiles from *Monochamus alternatus* larvae and a mixture of wood diet, sawdust, and frass.

were detected in volatiles from mixtures of wood diet and frass of *M. alternatus* larvae, including 2 alcohols (borneol 24.30% and 2,3-butanediol 35.53%), 3 organic acids (2-methylbutyric acid 4.13%, pentanoic acid 3.36%, and hexanoic acid 22.36%), and 1 ester (2-ethylhexyl acetate 10.33%), of which the alcohols comprised the largest proportion of the total (59.83%), followed by the acids (29.85%). Borneol was detected in both volatile samples but was present in higher proportion in the volatiles of wood and frass than in those of larvae.

ORIENTATION TO HOST INSTARS

Females of *S. guani* showed no preference for the odor of overwintering *M. alternatus* 5th instars compared with clean air ($Q = 0.021$, $P =$

0.884), but did respond to odors of 1st instars ($Q = 5.898$, $P = 0.015$) and even more strongly to those of 3rd instars ($Q = 11.520$, $P = 0.001$; Fig. 3).

EXPERIENCE AND ODOR SELECTION BY *S. GUANI*

Female *S. guani* exposed to volatile chemicals from host larvae without opportunity to oviposit showed no significant orientation to *M. alternatus* larvae ($Q = 1.923$, $P = 0.166$), but those exposed to odors of sawdust did ($Q = 5.255$, $P = 0.022$). Naïve females did orient to *M. alternatus* larvae ($Q = 11.520$, $P = 0.001$), but the number of non-responding individuals was also highest in this test (Fig. 4).

Females of *S. guani* showed no preference for either host type whether they were reared on pupae of *T. molitor* pupae (TP, $n = 48$, $Q =$

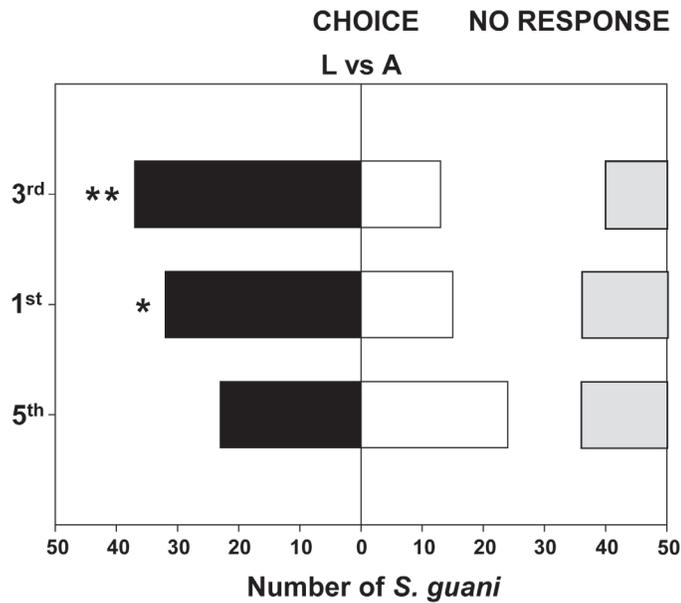


Fig. 3. Numbers of naïve *Scleroderma guani* females responding to the odor of 10 *Monochamus alternatus* 1st, 3rd, or 5th instars (L) versus clean air (A) in a Y-tube olfactometer. *: $P \leq 0.05$, **: $P \leq 0.01$.

0.750, $P = 0.386$) or on *M. alternatus* larvae (ML, $n = 52$, $Q = 0.692$, $P = 0.405$), although those reared on the factitious host were less responsive in the apparatus.

Discussion

Naïve *S. guani* females responded to odors of *M. alternatus* larvae, but not to other odor sources from the larval habitat. Neonate *M. alternatus* larvae feed on phloem in the endothelial layer of the pine

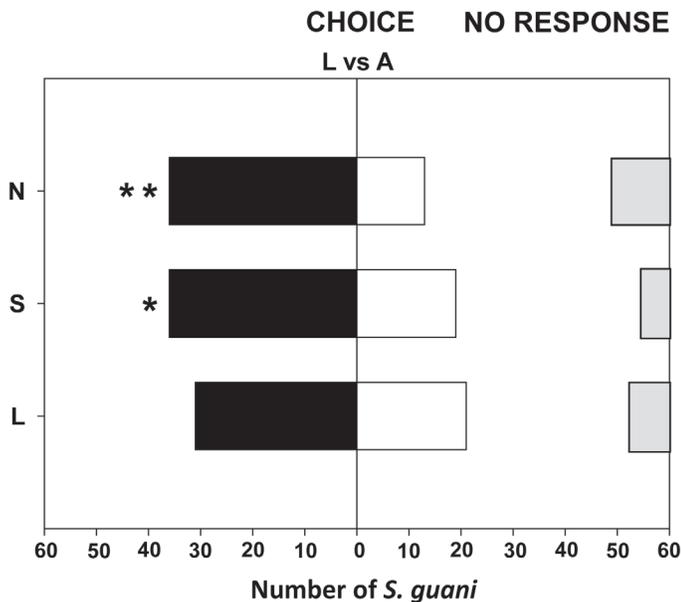


Fig. 4. Numbers of *Scleroderma guani* females responding to the odor of *Monochamus alternatus* 3rd instars (L) versus clean air (A) in a Y-tube olfactometer. Females were either naïve (N), previously exposed to gallery sawdust (S), or with previous exposure to the odor of *M. alternatus* larvae (L). *: $P \leq 0.05$, **: $P \leq 0.01$.

bark, leaving coarse, powdery frass outside feeding locations. As larvae grow, they penetrate deeper into the xylem creating galleries full of sawdust and frass that are sometimes detectable from the bark surface (Tang et al. 2005). We infer that sawdust and frass on the surface of pine bark are important visual cues to searching *S. guani* females. Once in the host gallery, volatiles from sawdust and frass mix with those of *M. alternatus* larvae, but it is the latter that guide *S. guani* to its host.

The hydrocarbons that appeared to be important cues in host location were associated with *M. alternatus* larvae but were absent from the volatiles of wood and frass. Similarly, linear and monomethyl-branched alkanes were the major components of residues left by larvae that were utilized in host location by the wasp *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) (Wölfling & Rostás 2009). Upon host contact, non-volatile hydrocarbons present in insect cuticles can be important in host recognition and elicitation of oviposition behavior (Vinson 1976; Morehead & Feener 1999). We infer that volatile hydrocarbons emanating from the body of *M. alternatus* larvae are probably responsible for guiding host location in *S. guani* females in the darkness of *M. alternatus* larval galleries.

Previous research demonstrated that *S. guani* prefers to parasitize *M. alternatus* larvae in mid-instars (Ma et al. 2010). In the present study, females of *S. guani* responded significantly to 1st and 3rd instars of *M. alternatus*, the latter appearing especially attractive, but did not respond to overwintering 5th instars. Early instars have a thin, soft cuticle and high metabolic activity associated with rapid growth, both of which probably facilitate high rates of volatile emission. In contrast, overwintering 5th instars have a much lower metabolic rate due to energy conservation requirements, and they possess a thick cuticle, both of which probably contribute to low levels of volatile emission and reduce attractiveness to *S. guani*.

After a period of coevolution, female parasitoids may evolve innate preferences for cues associated with their specific host or its microhabitat, while learning experiences continue to fine-tune responses to secondary cues (Menzel 1983; Vet & Groenewold 1990; Vinson 1998; Takasu & Lewis 2003; Giunti et al. 2015). Successful oviposition can serve to reinforce associative learning of secondary cues (Papaj & Vet 1990), whereas the lack of this reward can also change behavioral responses (Papaj 1990; Real 1991). In the present study, naïve *S. guani* females showed the strongest preference for host larvae in olfactometer trials, although they were also the most likely to not respond in tests. Females with sawdust experience also showed no orientation towards host larval odor, although fewer wasps failed to respond. In contrast, exposure to volatiles of host larvae alone did not cause females to respond to these odors in the olfactometer. Some previous studies have shown that parasitoids may prefer host species from which they emerged, although such preferences may be altered by learning experiences (Papaj & Vet 1990; Hoedjes et al. 2011; Giunti et al. 2015). Successful foraging or oviposition experiences may establish or enhance parasitoid responses to host-related cues, whereas an unrewarding experience may cause the reverse effect, as has been demonstrated in *Scleroderma pupariae* Yang and Yao (Hymenoptera: Bethyliidae) (Wei et al. 2013) and *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) (Papaj et al. 1994).

Because *S. guani* females prefer early to mid-instars of *M. alternatus*, the best period for *S. guani* release will be from late summer to autumn, when these particular instars predominate. Later releases could be compensated by increasing the number of females released, as more parasitoids will be needed to obtain the same level of parasitism in an older, larger cohort of *M. alternatus* larvae. Because rearing *S. guani* on *T. molitor* pupae had no impact on subsequent responses to host odors, this species is suitable as a factitious host for mass production of the parasitoid, given its ready commercial availability and

low cost. Further analysis of the activity of volatile hydrocarbons emanating from *M. alternatus* larvae using electrophysiological techniques such as simultaneous GC-electroantennogram observations could further elucidate the specific compounds guiding the host location process in *S. guani*.

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