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Sensory Aspects of Trail-Following Behaviors in the Asian Longhorned Beetle, *Anoplophora glabripennis*

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Abstract *Anoplophora glabripennis* has a complex suite of mate-finding behaviors, the functions of which are not entirely understood. These behaviors are elicited by a number of factors, including visual and chemical cues. Chemical cues include a male-produced volatile semiochemical acting as a long-range sex pheromone, a female-produced cuticular hydrocarbon blend serving as a sex-identification contact pheromone, and a recently identified female-produced trail sex pheromone that is followed by mate-seeking males. However, the sensory appendages and sensilla on these appendages used to detect the trail sex pheromone are unknown. We evaluated the ability of virgin male *A. glabripennis* to follow a sex pheromone trail after removal of the terminal four antennal segments and/or the maxillary and labial palps using a two-choice behavioral bioassay. We also tested the ability of males to follow the trail sex pheromone using volatile pheromone cues only, without physical contact with the pheromone. Results indicate that the palps are primarily responsible for sensing the pheromone, with males lacking palps unable to respond behaviorally to the trail sex pheromone. Under the conditions of this study, males could not follow the sex pheromone trail without direct contact, suggesting that olfaction may not be involved in detection of this pheromone. However, we did not determine to what degree the trail pheromone chemicals can volatilize under our experimental conditions. This work is important in elucidating the behaviors and sensory structures involved in mate-finding

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by this species on host trees, and these studies may help determine whether the trail sex pheromone has applications for monitoring and management.

Keywords *Anoplophora glabripennis* · trail following · sex pheromones · gustatory detection · mate-finding behavior

Introduction

Trail pheromones are chemical signals deposited on a substrate used by many animal species to communicate with nearby conspecifics (Greenfield 2002). Trails serve a variety of functions, including foraging, mate location, orientation, and aggregation. The most well-known examples are ant foraging trails that lead workers from their colonies to food sources. Many other social insects employ foraging-recruitment trails, including termites and certain species of bees and wasps (Greenfield 2002; Czaczkes et al. 2015). Similarly, larvae of some gregarious lepidopteran species, particularly of the family Lasiocampidae (e.g. Colasurdo and Despland 2005), follow trails to remain aggregated as they move to food sources (Costa and Pierce 1997).

Many other animal species use pheromone trails to locate mates, including copepods (Kiørboe 2007), polychaetes (Ram et al. 2008), gastropods (Ng et al. 2013), and vertebrates such as the garter snake, *Thamnophis sirtalis parietalis* (LeMaster and Mason 2001; Shine et al. 2005). In these cases, trail pheromones are produced by females and followed by males. Because this type of trail pheromone is emitted by only one sex and causes a behavioral response – in these cases attraction – in the other sex, this type of trail pheromone functions as a sex pheromone. Sexual dimorphism in signaling indicates that trail following is likely used for mate finding rather than for resource-based recruitment. We suggest that these trails should be called "trail sex pheromones" to distinguish them from other types of trails.

Trail sex pheromones used in mate location have been reported in arthropods, such as the Scorpionida (e.g. Melville et al. 2003) and Opiliones (e.g. Willemart and Hebets 2012). Males of the hemipteran species *Orius sauteri* are more attracted to leaves on which females have walked (Nakashima and Hirose 1999), and females of congeneric *O. insidiosus* are known to produce a non-volatile chemical trail that is followed by mate-seeking males (Aldrich et al. 2007).

Until recently, the only putative sex-specific chemical trail pheromone reported in beetles was in the cerambycid *Nadezhdiella cantori,* but the chemical composition was not identified and behavioral evidence was unclear (Wang et al. 2002). More recently, a female-produced trail sex pheromone was identified for the Asian longhorned beetle (*Anoplophora glabripennis*) (Hoover et al. 2014), an invasive, xylophagous pest introduced into North America and Europe from China (Dodds and Orwig 2011). *Anoplophora glabripennis* is highly polyphagous, attacking a broad range of hardwood tree species, including those in the genera *Acer*, *Salix*, *Populus*, *Betula,* and *Ulmus* (Hu et al. 2009; Haack et al. 2010; Dodds and Orwig 2011). Nowak et al. (2001) estimated that ALB has the potential to eliminate 35 % of the urban tree canopy, valued at $669 million, if it spreads across the U.S.

Anoplophora glabripennis exhibits several behaviors that are not well understood. Mate-finding behaviors are especially complex and are elicited through different stimuli

and other factors, which include age- and nutrition-based sexual maturity Hu et al. 2009, visual cues (e.g. Wang et al. 2003), and chemical signaling (Meng 2014). Chemical communication is especially important, allowing the insect to locate mates and host plants from a distance. Male *A. glabripennis* produce a long-range volatile sex pheromone identified by Zhang et al. (2002) that attracts primarily virgin females (Nehme et al. 2014; Meng et al. 2014). However, the pheromone blend is only weekly attractive, which appears to be due at least in part to the unstable aldehyde component of the lure (Nehme et al. 2014). When in proximity to a conspecific, males identify possible mates and rivals through contact, via a hydrocarbon blend produced in the cuticle (Zhang et al. 2003). Additionally, sexually mature females produce a trail sex pheromone, which is applied to a substrate by the female as she walks and is followed by both virgin and mated males (Hoover et al. 2014).

Because these beetles dwell in large trees, the trail sex pheromone that lead males to potential mates facilitates finding a receptive female; this pheromone blend is not produced in sufficient amounts or in the correct ratio until females are sexually mature (Hoover et al. 2014). Also a pheromone that can act as both a contact and short-range volatile signal would provide a significant advantage to mate-seeking males trying to locate the trail. Although many trail pheromones elicit responses through direct contact, some ants respond to trail pheromones without contact, indicating pheromone volatility (Morgan 2009). Beyond these studies of ant species, evidence for responses to volatile trail pheromones in other insect groups is lacking.

Based on apparent "tasting" behaviors observed during trail pheromone bioassays conducted previously (Hoover et al. 2014), we hypothesized that the maxillary and/or labial palps are involved in trail pheromone detection via contact chemoreception.

Methods

To determine which sensory structures are involved in detection of the female produced sex trail pheromone, two bioassays were conducted. Bioassay I involved testing the males' ability to follow trails after the surgical removal of both sets of palps plus the last four antennal segments, removal of both sets of palps leaving the antennae intact, or removal of the last four antennal segments leaving the palps intact. We reasoned that the last four antennal segments would be the ones potentially playing a significant chemosensory role in the detection of the trail sex pheromone because they are moved through the air as males walk on a branch. In single cell recordings we found that the several most-distal antennal segments harbored numerous types of sensilla housing many types of differentially tuned olfactory sensory neurons responding to a wide range of plant-related terpenes and aliphatic molecules (J. Wei, Q. Zhou, L. Hall, M. Domingue, K. Hoover, K. Shields, T. C. Baker, unpublished). It thus seemed logical that extirpation of these segments with their multitude of olfactory sensory neurons should be likely to impair sex pheromone trail following were they to house sufficient numbers of neurons tuned to the trail pheromone.

Bioassay II evaluated the ability of intact males to follow a trail without having direct contact with the pheromone. In this assay, procedures from Bioassay I were followed without antennal or palp extirpation, and with the introduction of a wire mesh barrier that prevented the beetles from actually touching the pheromone trail. Because it

is possible that even these relatively high-molecular-weight, low volatility pheromone components can volatilize, it is important to also determine if the trail pheromone can be followed without physical contact with the pheromone components.

Beetles Adults were obtained from the *A. glabripennis* colony maintained in a USDA-approved quarantine facility at Penn State University, University Park, PA. Larvae were reared on artificial diet (Keena 2005) and adults were maintained separately in ventilated glass jars and fed on *Acer rubrum* twigs, which were changed once per week, which is routine for colony rearing. The rearing facility was kept at ~23 °C with ~30–57 % humidity, and overhead fluorescent lighting remains on from 8 am to 5 pm daily. Virgin males 20–52 days post-eclosion were selected in groups of eight from the colony based on randomization of I.D. number. Only male beetles were used because females do not follow the trail sex pheromone (Hoover et al. 2014).

Treatments For Bioassay I, individuals were assigned one of four treatments based on randomization of I.D. number. Treatments were as follows: 1) maxillary and labial palps were removed at the joint most distal to the head (designated RP for removed palps) using dissecting scissors sterilized using an alcohol lamp; 2) the four most distal segments of the antennae were removed (designated RA for removed antennae); 3) both sets of palps plus the antennal segments were removed (designated RAP for removed palps and antennae); and 4) control beetles, which were subjected to a sham-operation treatment by rubbing dissecting scissors on the palps and the distal antennal segments at the same locations as the areas that were removed in treated beetles. All beetles were secured in a plastic tube and placed in a freezer at −20 °C for 6 min to slow their movements prior to surgical treatment. Following treatment, beetles were replaced in their respective glass jars, where they were allowed approximately 24 h to recover and resume normal behavior before bioassays were performed.

Bioassay Procedures A behavioral bioassay was devised using the two-choice design described by Hoover et al. (2014). This assay uses twigs of a host species oriented vertically in the shape of a Y (Fig. 1). This arrangement provides only two possible choices for insects walking up the twig and takes advantage of the natural behavior of the beetles to walk upward.

Norway maple (*Acer platanoides*) twigs were obtained from a location at approximately 40 49′ 04.82″ N, 77 52′ 23.34″ W. Twigs were selected based on the symmetry of the arms of the Y in the twig, with a main branch splitting into two branches at an angle between ~70 and ~110 degrees, but as close to ~90 degrees as could be obtained. Twigs were ~20 cm in length with each branch being ~10 cm long.

A blend of the two major components of the trail pheromone, 2-methyldocosane (2-Me-C22) and (*Z*)-9-tricosene (Z9-C23) were synthesized as described previously (Hoover et al. 2014). Only these two "major" pheromone components were used because the addition of two other "minor" components did not have an effect on virgin male choice behavior in a previous study (Hoover et al. 2014). Equal aliquots of 1.25 μg/μl 2-methyldocosane and 7.5 μg/μl (Z)-9-tricosene were combined in the naturally occurring ratio of 1:6 and diluted with hexane (95 % purity, J.T. Baker Chemicals, Center Valley PA) to yield a blend of the major components at a concentration of 0.0125 μg/μL. For each replicate, we applied ~500 μl of the pheromone blend to one branch of the Y-twig from crotch to tip using a

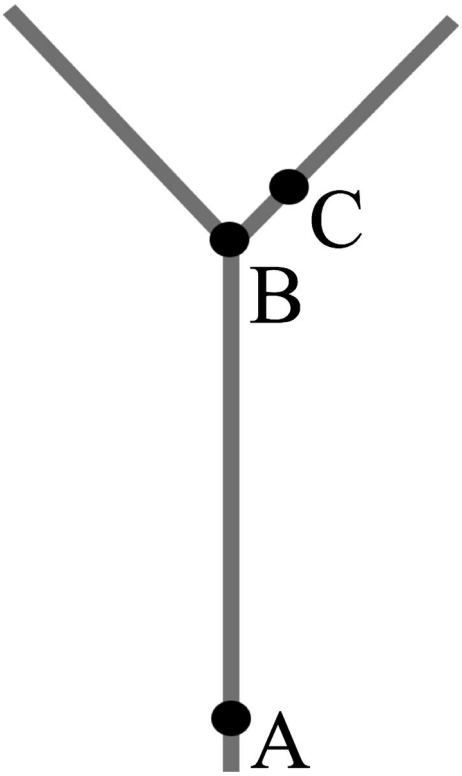

Fig. 1 Reference points for recording time to trail (seconds from **A** to **B**), time to choice (seconds from **B** to **C**), and total assay time (seconds from **A** to **C**), where points represent the beetle's location on a Y-twig during each assay

paintbrush. We randomly determined the branch that received the pheromone blend by tossing a coin. Using a clean brush, we applied ~500 μl hexane to the other branch; both branches were allowed to dry for 10 min under a laminar flow hood. The prepared Y-twig arrangement was then secured upright in a sturdy base.

For Bioassay I, beetles were treated in groups of eight due to colony availability and timing constraints. A freshly prepared Y-twig was used for each group. The bioassay was replicated 14 times, with the intended number of beetles per treatment being 28 (see Table 1).

The room for bioassays was illuminated by two banks of two fluorescent lights each. Each light used standard T-8 bulbs. The bank of lights was directly over the bioassay arena, at the halfway point between each end of the bank of lights, to ensure lighting was not directional.

Table 1 For Bioassay I, the number of beetles that died before being tested and the number of beetles that did not make a choice are listed by treatment.

	RA	RP	RAP	CTRL
No choice	2	2	3	2
Died	2	3	5	0

All tests were performed between 13:00 and 16:00, when the adults were most active (personal observation). Each beetle was placed at the base of the twig and allowed 10 min. to walk from the bottom of the twig until it chose one branch to walk on. For a choice to be recorded, palpation and/or antennation at the crotch of the twig (where the pheromone trail and hexane control trail met) had to occur. If a beetle moved too quickly up the twig without making contact with the crotch of the branch, the beetle was placed again at the bottom of the twig and the test was repeated. If this occurred more than twice with the same beetle, the beetle was allowed 3 min. to rest in its jar before it was given one final attempt. If a beetle did not walk on either branch of the Y by the third attempt, the beetle was recorded as having made no choice, and was discarded from the statistical analyses.

For Bioassay II, the prepared twig was secured inside a wire mesh cage constructed using thrips-proof screen material (150 X 150 μm holes) at a distance of ~4 mm between the twig surface and the front mesh, with the apparatus then being secured upright as in Bioassay I. We chose a distance of 4 mm because this distance was just beyond the reach of the palps and we did not observe beetles antennating the twig in Bioassay I. Procedures for this bioassay, including the dosage used on the applied trail, were otherwise identical, except all the males possessed intact palps and antennae. The bioassay was replicated seven times, such that a total of 54 intact male beetles were tested.

After the initial bioassay, we returned the beetles to their containers to resume normal behavior. After 48 h had elapsed, we repeated the bioassay, again using a blend of the two major pheromone components, but this time they were applied at the 100-fold higher concentration of 1.25 μg/μl to increase the chances of achieving a behaviorally active airborne concentration of volatilized trail sex pheromone.

Choice & Time A choice was recorded when all six legs of the male were on one of the two arms of the twig. "Total assay time" was recorded as the time it took the beetle to walk the full distance of the twig, i.e., from when a beetle had all six legs on the base of the twig (point A, Fig. 1), walked up to the crotch, and made a choice, which was recorded when all six legs were on the chosen arm of the twig (point C, Fig. 1). The "time to trail" was recorded as the time it took for the beetle to walk up the twig from the starting point until it made contact with the pheromone trail at the crotch of the twig (A- B, Fig. 1). The "time to choice" was recorded as the elapsed time from when the male made contact with the crotch of the twig (B, Fig. 1) to the time all six legs were on the chosen arm of the branch (C, Fig. 1). A beetle that remained on the twig for 10 min. Without making a choice was recorded as having made no choice and was not included in the analyses. Table 1 provides the details for beetles that made no choice.

Behavior Notes on observed behaviors were made during Bioassay I. Videos were taken for each beetle tested in Bioassay I, although video footage for two of the dates (4 replicates) was lost. Video footage was viewed and behaviors were recorded as occurring or not occurring for each beetle. Behaviors were defined as follows:

1) "Palpation": one or both sets of palps, maxillary and/or labial, came into contact with the twig surface.
2) "Backward antennae": the antennae were held over the elytra such that the angle between them was visually estimated at 45° or less. This resulted in the appearance of the antennae being held straight backward along the dorsum of the body.

3) "Mouthing": one or both of the following behaviors occurred A) widely opening the mandibles around the twig surface without chewing, and B) extension of the labrum.

Statistical Analyses

All statistics were performed using RStudio version 0.99.489 (RStudio, Inc. 2015).

Bioassay I Choice Analysis Data were analyzed by logistic regression to determine if removal of the antennae, palps, or both affected the proportion of males that chose the trail pheromone in comparison to the control beetles. The presence or absence of the last four segments of the antennae and/or palps were used as predictors of branch choice. A Hosmer-Lemeshow test was performed to assess Goodness-of-fit.

Bioassay I Time Analysis Times required to complete a defined portion of the bioassay were fitted to general linear models. Time to choice (seconds from B to C, Fig. 1), time to trail (seconds from A to B, Fig. 1), and total assay time (seconds from A to C, Fig. 1) were log-transformed (base *e*). For each model, the absence of the last four antennal segments and/or palps were used as predictors of time to choice and total assay time, as well as time to trail. Two data points that were extreme outliers were removed from the final analysis.

Bioassay I Behavioral Analysis Data on times to complete a particular portion of the trail following were fitted to general linear models, with the presence of mouthing and/or backward antennae behavior (holding antennae straight back over the elytra) used as predictors of time to choice and total assay time, as well as time to trail.

Logistic regression was used to determine whether there was a correlation between the backward antennae behavior and choice. Within the removed-palp group, mouthing behavior was analyzed by logistic regression to determine if mouthing was associated with an increased likelihood of correctly choosing the trail sex pheromone.

Bioassay II A binomial test was used to determine if the percentage of beetles choosing the branch with the trail sex pheromone was statistically different than the 50 % expected if choice was made at random.

To determine whether physical contact with the trail sex pheromone is necessary for detection, we used a 2 × 2 contingency analysis using the Bioassay I data for intact virgin males ($n = 24$) as the positive control. This was compared against the results for intact males that completed the bioassay without physical contact. Because the presence of the mesh barrier was not expected to have a positive effect on choice, one-tailed *P*-values were obtained using Fisher's exact test.

Results

Seventy-three percent of intact, sham-operated control beetles chose the trail sex pheromone over the hexane control (Fig. 2). Beetles having their maxillary and labial palps removed showed no preference for the pheromone, with 48 % choosing the trail over the

hexane control. A similar result was seen in beetles having both palps and antennal segments removed; 48 % chose the pheromone. For beetles that had only antennal segments removed, 67 % preferred the pheromone trail to the control (Fig. 2); this result was not statistically significant compared with the intact control group (log odds −0.15, $P = 0.72$). The removal of maxillary and labial palps significantly decreased the ability of males to follow the trail sex pheromone compared to beetles possessing intact palps (log odds −0.94, $P = 0.03$). Because removal of antennae did not significantly affect choice, for cases in which removal of palps did have an effect, the RA group was analyzed separately in subsequent analyses, whereas the RP and RAP groups were combined. A Hosmer-Lemeshow Goodness-of-fit test showed no significant difference between our data and the model (Hosmer-Lemeshow $\chi^2 = 0.06$, d.f. = 8, $P = 1$).

Removal of maxillary and labial palps (RP and RAP treatment groups) significantly increased the total assay time (+0.78 ± 0.0096 s, $P < 0.001$), time to trail (+0.60 ± 0.0086 s, $P < 0.01$), and time to choice (+0.44 ± 0.014 s, $P < 0.01$). Overall, beetles moved more slowly with their palps removed (Table 2). Removal of the last four antennal segments only (RA treatment group) had the opposite effect; these beetles moved significantly more quickly, with decreased total assay time (−0.36 ± 0.019 s, $P = 0.0046$) , time to trail (−0.21 ± 0.017 s, $P = 0.015$), and time to choice (−0.26 ± 0.022 s, $P = 0.016$).

Beetles possessing both intact palps and antennae walked up the twigs with their maxillary palps in constant contact with the twig surface. Forty-three percent of beetles with palps removed exhibited the mouthing behavior (Fig. 3). No beetles with intact palps exhibited this behavior.

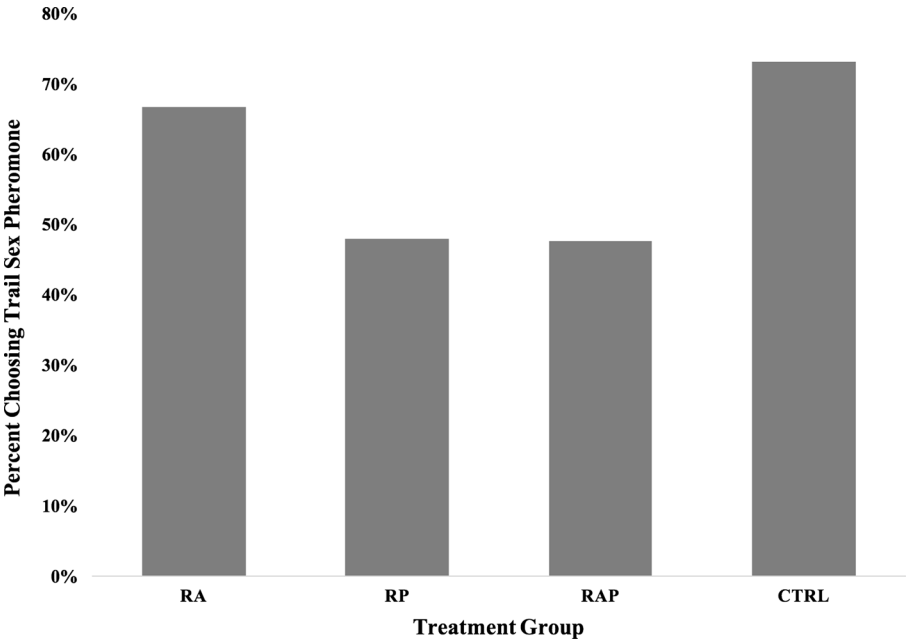

Fig. 2 Percentage of beetles that chose the trail sex pheromone over the hexane control by treatment: removed antennal segments (RA; $n = 24$), removed palps (RP; $n = 23$), removed palps plus antennal segments (RAP; $n = 20$) and intact control (CTRL; $n = 26$)

Table 2 Effects of treatment on time to choice, total assay time, and time to trail, determined using generalized linear models. Effects on time are listed in seconds. Mean time ± SEM and *P*-values are provided. Statistically significant values ($P < 0.05$) are indicated by a different letter after the mean within the same column

	RA only ($n = 19$)	RP, RA/RP ($n = 44$)	Control ($n = 20$)
Mean seconds to choice	5.9	19.8	8.6
Effect, time to choice	-0.26 ± 0.022 ($P = 0.016$)[a]	+0.44 ± 0.014 ($P < 0.001$)[a]	
Mean seconds to finish	13.6	48.6	18.7
Effect, total assay time	-0.36 ± 0.019 ($P = 0.005$)[a]	+0.78 ± $9.6e^{-3}$ ($P < 0.001$)[a]	
Mean seconds to trail	7.8	23.6	10.1
Effect, time to trail	-0.21 ± 0.017 ($P = 0.015$)[a]	+0.60 ± $8.6e^{-3}$ ($P < 0.001$)[a]	

Within the RP and RAP groups, mouthing behavior did not affect choice of the pheromone compared with the control arm of the Y-twigs (log odds 0.059, $P = 0.93$). Across all treatment groups mouthing behavior was associated with increased total assay time (+0.36 ± 0.022 s, $P < 0.01$) time to reach the trail (+0.36 ± 0.029 s, $P = 0.015$), and time to make a choice (effect of +0.43 ± 0.022 s, $P < 0.01$). Thus, beetles exhibiting mouthing behavior walked more slowly (Table 3). However, within the RP and RAP treatment groups only, mouthing was not significantly associated with increased total assay time, time to trail, or time to choice.

While the removal of palps was associated with the backward antennae behavior (log odds 1.16, $P = 0.02$), removal of antennal segments had no significant effect on this behavior (Fig. 3). Across all treatment groups, backward antennae behavior was associated with increased total assay time and time to trail, but not with time to choice (Table 3).

In Bioassay II, in which a wire mesh barrier prevented contact with the substrate (and thus with the trail sex pheromone), beetles did not show a preference for the pheromone over the hexane control (Fig. 4). At a pheromone concentration of 0.0125 μg/μL, ~48 % of males chose the pheromone trail (CI 0.347, 0.620). A 100-

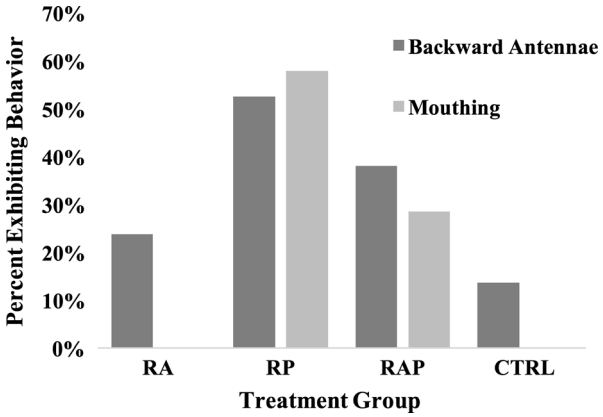

Fig. 3 Percentage of beetles exhibiting mouthing and/or backward antennae behaviors by treatment group: removed antennal segments (RA; $n = 24$), removed palps (RP; $n = 23$), removed palps and antennal segments (RAP; $n = 20$), and intact control (CTRL; $n = 26$)

Table 3 Effects of mouthing and backward antennae behaviors on time to choice, total assay time, and time to trail, determined using generalized linear models. Effects on time are listed in seconds, log-transformed (base *e*). Significant values ($P < 0.05$) are indicated by an asterisk (*).

	Mouthing	Backward antennae	Neither
Mean seconds to choice	15.7	11.1	9.8
Effect, time to choice	+0.43 ± 0.022 ($P = 0.0041$)*	+0.028 ± 0.017 ($P = 0.23$)	
Mean seconds to finish	48.5	44.1	22.6
Effect, total assay time	+0.36 ± 0.022 ($P < 0.0069$)*	+0.25 ± 0.015($P = 0.0064$)*	
Mean seconds to trail	32.8	33.0	12.7
Effect, time to trail	+0.36 ± 0.029 ($P = 0.015$)*	+0.53 ± 0.017 ($P < 0.001$)*	

fold increase in the pheromone concentration also had no effect on male choice; ~48 % of males chose the pheromone trail at 1.25 μg/μL over the hexane control (CI 0.343, 0.622). When compared to males in contact with the twig surface, males separated from the twig surface by the wire mesh were significantly less likely to choose the pheromone trail at either 0.0125 μg/μl or at the 100-fold higher dose of 1.25 μg/μl ($P = 0.0294$; Fig. 4).

Discussion

For both treatment groups in which palps were removed in Bioassay I, approximately half of the males chose the blend of the trail sex pheromone and half chose the hexane control (Fig. 2), indicating that these males chose randomly and that the palps are essential for detection and response to the trail sex pheromone. Apical pits on the maxillary and labial palps of *A. glabripennis*, described in Meng (2014) contain what were defined as basiconic sensilla; these sensilla appear to be the most likely candidates for housing chemoreceptive neurons responsible for detecting the trail sex pheromone. Similar structures have been found in the cerambycid species *Phoracantha recurva*, whose labial palps contain apical slits densely packed with basiconic sensilla (Faucheux 2013). It is possible that similar structures may be prevalent in the Cerambycidae.

Removal of antennal segments did not have a significant effect on the ability of males to detect the trail sex pheromone (Fig. 2). Wang et al. (2002) reported that male *Nadezhdiella cantori* (Coleoptera: Cerambycidae) only followed trails of female extracts once their antennae appeared to make contact with the trail. However, in their study no observations were made on whether tasting of the substrate while walking occurs, or if mouthparts are involved in trail detection in this species.

The increase in total assay time, time to trail, and time to choice (Fig. 1) seen in beetles with removed palps is likely due to efforts by these beetles to make contact between their remaining mouthparts and the substrate. The decrease in total assay time, time to choice, and time to trail seen in beetles with removed antennae (RA group only) may be a behavioral response to the beetles' inability to obtain olfactory cues from their environment. This behavior is in contrast to intact beetles, which often arrest

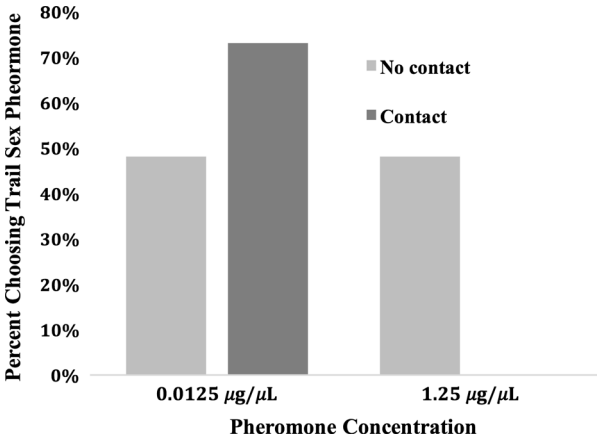

Fig. 4 Percentage of beetles that chose the trail sex pheromone over the hexane control by presence or absence of the wire mesh screen, which prevented physical contact with the pheromone. The intact control group from Bioassay I ($n = 26$) was used for comparison to detection with physical contact. Bioassay II beetles were prevented from direct contact with the pheromone trail by the presence of a wire mesh screen. Groups assayed at two different concentrations are shown for Bioassay II ($n = 56, 54$)k

periodically or change direction toward or away from a particular stimulus, which slows them down.

Mouthing behaviors, which included mandible movement without chewing and extension of the labrum, in beetles lacking palps is likely an attempt to gather as much gustatory information as possible in the absence of the major gustatory organs. These mouthing behaviors were not associated with an increase in choice of the trail sex pheromone in these beetles, suggesting that the non-palp mouthparts are not involved in detection of the pheromone. Mouthing behavior did not affect walking speed within the RP and RAP groups, indicating that this mouthing behavior is not responsible for the increased total assay time, time to trail, and time to choice in beetles with palps removed. The backward antennae behavior may also be a sign of stress in the beetle. Often, beetles will exhibit this behavior while fighting (personal observations). It is also possible that this behavior may be an attempt by the beetle to hold their antennae away from the sensory appendages on their mouth to reduce interference between sensations from the antennae and those from the mouthparts.

When the beetles were excluded from making any kind of contact with the trail sex pheromone by the wire screen, they made a random chose of which branch of the Y to follow, indicating that under these assay conditions, volatiles of the pheromone could not be detected in sufficient amounts to result in trail following (assuming the components have volatility). This highlights the importance of direct contact with the pheromone in evoking trail following for this species. This differs from studies on ants that are capable of using olfaction to follow foraging trails without contact (Morgan 2009; Van Vorhis Key et al. 1981). Because contact appears to be necessary for trail following in male *A. glabripennis*, we suggest that gustation, i.e., contact chemoreception by the palps, is the most likely means of detection of the trail sex pheromone. However, the basiconic sensilla in the apical pits reside on an invaginated surface, and so it is possible that direct contact of these sensilla with the trail is not necessary for trail sex pheromone detection. Indeed preliminary electropalpogram recordings with electrodes contacting

the inner surface of these pits and using puffs of airborne major components of the trail sex pheromone suggest that that the basiconic sensilla have an olfactory capability (L. Hall, A. Myrick, F. Graves, K. Hoover, T. Baker, unpublished).

It is important to note that Hoover et al. (2014) found that only the two major components of the female trail secretions identified as 2-methyldocosane and (*Z*)-9-tricosene were followed by male *A. glabripennis*. Addition of the minor components of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene slightly reduced the percentage of males that chose the pheromone, although this difference was not statistically significant. Thus, we argue that it may not be accurate to refer to (*Z*)-9-pentacosene and (*Z*)-7-pentacosene as components of the trail sex pheromone; instead these compounds may have a different purpose. Since the four-component pheromone blend is repellant to virgin female *A. glabripennis* while the minor components alone are attractive to females (Hoover et al. 2014), the blend of all four components may act as a spacing pheromone for females. This would be advantageous for a species that can reach high population densities, reducing intraspecific competition by indicating where females have recently walked and oviposited. It has been noted in the field that females return to their oviposition sites by retracing their steps, before moving on to another location on the tree to begin chewing the next oviposition site (personal observations). Interestingly, the major components of this pheromone alone produced no significant behavioral response in females. Since virgin females only show an aversion to the full trail pheromone blend, the minor components may be needed to complete the blend required for eliciting a purported spacing behavior.

Understanding how *A. glabripennis* detects the female-produced trail sex pheromone and recognizing the behaviors associated with trail-following will help to elucidate the mechanisms of this portion of the mate-finding process in this species, and perhaps offer insights into trail-following behaviors of other species. Trail pheromones are not known to commonly occur in coleopterans. Notable exceptions to trail following in this group include certain myrmecophilous species that use ant foraging trails to locate colonies (e.g. Quinet and Pasteels 1995; Geiselhardt et al. 2007). However, these species do not produce a pheromone; instead they follow the foraging trail pheromones of ants. Species that produce their own trail pheromones include the gregarious larvae of the weevil *Phelypera distigma* (Fitzgerald et al. 2004); these weevils follow each other's trails to aggregate. To our knowledge, a sex-specific sex trail pheromone has only been reported in one other cerambycid, *Nadezhdiella cantori* (Wang et al. 2002), but the pheromone components were not investigated. It is possible that trail sex pheromones are more prevalent in the Cerambycidae than previously thought.

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References

Aldrich JR, Oliver JE, Shifflet T, Smith CL, Dively GP (2007) Semiochemical investigations of the insidious flower bug, *Orius insidiosus* (say). J Chem Ecol 33:1477–1493

Colasurdo N, Despland E (2005) Social cues and following behavior in the forest tent caterpillar. J Insect Behav 18(1):77–87

Costa JT, Pierce NE (1997) Social evolution in the Lepidoptera: ecological context and communication in larval societies. In: Choe JC, Crespi BJ (eds) The evolution of social behavior in insects and arachnids. Cambridge University Press, Cambridge, pp. 407–442

Czaczkes TJ, Grüter C, Ratnieks LW (2015) Trail pheromones: an integrative view of their role in social insect colony organization. Annu Rev Entomol 60:581–599

Dodds KJ, Orwig DA (2011) An invasive forest pest invades natural environments – Asian longhorned beetle in northeastern US hardwood forests. Can J For Res 41:1729–1742

Faucheux MJ (2013) Mouthpart sensilla of the adult yellow longicorn beetle *Phoracantha recurva* Newman, 1840 (Coleoptera, Cerambycidae, Cerambycinae). Bulletin de l'Institut Scientifique, Rabat, Section Sciences de la Vie 35:83–94

Fitzgerald TD, Pescador-Rubio A, Turna MT, Costa JT (2004) Trail marking and processionary behavior of the larvae of the weevil *Phelypera distigma* (Coleoptera: Curculionidae). J Insect Behav 17(5):627–646

Geiselhardt SF, Peschke K, Nagal P (2007) A review of myrmecophily in ant nest beetles (Coleoptera: Carabidae: Paussinae): linking early observations with recent findings. Naturwissenschaften 94(11):871–894

Greenfield MD (2002) Signalers and receivers: mechanisms and evolution of arthropod communication. Oxford University Press

Haack RA, Hérard F, Sun J, Turgeon JJ (2010) Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: a worldwide perspective. Annu Rev Entomol 55:521–546

Hoover K, Keena M, Nehme M, Wang S, Meng P, Zhang A (2014) Sex-specific trail pheromone mediates complex mate finding behavior in *Anoplophora glabripennis*. J Chem Ecol 40(2):169–180

Hu J, Angeli S, Schuetz S, Luo Y, Hajek AE (2009) Ecology and management of exotic and endemic Asian longhorned beetle *Anoplophora glabripennis*. Ag For Entomol 11:359–375

Keena MA (2005) Pourable artificial diet for rearing *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and methods to optimize larval survival and synchronize development. Ann Entomol Soc Am 98:536–547

Kiørboe T (2007) Mate finding, mating, and population dynamics in a planktonic copepod *Oithona davisae:* there are too few males. Limnol Oceanogr 52(4):1511–1522

LeMaster MP, Mason RT (2001) Evidence for a female sex pheromone mediating male trailing behavior in the red-sided garter snake, *Thamnophis sirtalis parietalis*. Chemoecology 11:149–152

Melville JM, Tallarovic SK, Brownwell PH (2003) Evidence of mate trailing in the giant hairy desert scorpion, *Hadrurus arizonensis* (Scorpionida, Iuridae). J Insect Behav 16(1):97–115

Meng PS (2014) Where's that smell? Trapping and sensory biology of the Asian longhorned beetle, *Anoplophora glabripennis*. Master's Thesis. The Pennsylvania State University, University Park

Meng PS, Trotter RT, Keena MA, Baker TC, Yan S, Schwartzberg EG, Hoover K (2014) Effects of pheromone and plant volatile release rates on trapping *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in China. Environ Entomol 43(5):1379–1388

Morgan ED (2009) Trail pheromones of ants. Physiol Entomol 34:1–17

Nakashima Y, Hirose Y (1999) Trail sex pheromone as cue for searching mates in an insect predator Orius Sauteri. Ecolog Entomol 24:115–117

Nehme ME, Trotter RT, Keena MA, McFarland C, Coop J, Hull-Sanders HM, Meng P, DeMoraes CM, Mescher M, Hoover K (2014) Development and evaluation of a trapping system for *Anoplophora glabripennis* in the United States. Environ Entomol 43(3):1034–1044

Ng TPT, Saltin SH, Davies MS, Johannesson KE, Stafford R, Williams GA (2013) Snails and their trails: the multiple functions of trail-following in gastropods. Biol Rev 88:683–700

Nowak DJ, Pasek JE, Sequeira RA, Crane DE, Mastro VC (2001) Potential effect of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) on urban trees in the United States. J Econ Entomol 94(1):116–122

Quinet Y, Pasteels JM (1995) Trail following and stowaway behavior of the myrmecophilous staphylinid beetle, *Homoeusa acuminata*, during foraging trips of its host, *Lasius fuliginosus* (hymenoptera: Formicidae). Insect Soc 42:31–44

Ram JL, Fei X, Danaher SM, Lu S, Breithaupt T, Hardege JD (2008) Finding females: pheromone-guided reproductive tracking behavior by male *Nereis succinea* in the marine environment. J Exp Biol 211:757–765

RStudio Team (2015) RStudio: integrated development for R. RStudio, Incnn, Boston

Shine R, Webb JK, Lane A, Mason RT (2005) Mate location tactics in garter snakes: effects of rival males, interrupted trails and non-pheromonal cues. Funct Ecol 19:1017–1024

Van Vorhis Key SE, Gaston LK, Baker TC (1981) Effects of gaster extract trail concentration on the trail following behavior of the argentine ant, *Iridomyrmex humilis* (Mayr). J Insect Physiol 27:363–370

Wang Q, Zeng W, Chen L, Li J, Yin X (2002) Circadian reproductive rhythms, pair-bonding, and evidence for sex-specific pheromones in *Nadezhdiella cantori* (Coleoptera: Cerambycidae). J Insect Behav 15(4):527–539

Wang B, Lance DR, Francese JA, Xu Z, Jia F, Luo Y, Mastro VC (2003) Orientation of and Asian longhorned beetle, *Anoplophora glabripennis*, toward objects of different shapes and colors. In: Proceedings of the 2002 U.S. Dept. Ag. Interagency Research Forum

Willemart RH, Hebets EA (2012) Sexual differences in the behavior of the harvestman *Leiobunum vittatum* (Opiliones, Sclerosomatidae) toward conspecific cues. J Insect Behav 25:12–23

Zhang A, Oliver JE, Aldrich JR, Wang B, Mastro VC (2002) Stimulatory beetle volatiles for the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky). Z Naturforsch 57:553–558

Zhang A, Oliver JE, Chauhan K, Zhao B, Xia L, Xu Z (2003) Evidence for contact sex recognition pheromone of the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae). Naturwissenschaften 90:410–413