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Authors

Mathis, Kaitlyn A
Tsutsui, Neil D

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Cuticular Hydrocarbon Cues Are Used for Host Acceptance by *Pseudacteon* spp. Phorid Flies that Attack *Azteca sericeasur* Ants

Kaitlyn A. Mathis[1,2] · Neil D. Tsutsui[2]

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Abstract Parasitoids often use complex cues to identify suitable hosts in their environment. Phorid fly parasitoids that develop on one or a few host species often use multiple cues, ranging from general to highly specific, to home in on an appropriate host. Here, we describe the hierarchy of cues that *Pseudacteon* phorid flies use to identify *Azteca* ant hosts. We show, through behavioral observations in the field, that phorid flies are attracted to two cryptic *Azteca* species, but only attack *Azteca sericeasur* (Hymenoptera: Formicidae: Dolichoderinae). To test whether the phorid flies use cuticular hydrocarbons (CHCs) to distinguish between the two *Azteca* taxa, we first documented and compared cuticular hydrocarbons of the two *Azteca* taxa using gas chromatography/mass spectrometry. Then, using cuticular hydrocarbon-transfer experiments with live ants, we characterized the cuticular hydrocarbons of *A. sericeasur* as a short-range, host location cue used by *P. lasciniosus* (Diptera: Phoridae) to locate the ants.

✉ Kaitlyn A. Mathis
kmathis@email.arizona.edu

1   Department of Ecology and Evolutionary Biology, University of Arizona, 1041 East Lowell Street, Tucson, AZ 85721, USA

2   Department of Environmental Science, Policy, and Management, University of California, Berkeley, 130 Mulford Hall #3114, Berkeley, CA 94702-3114, USA

Introduction

Identifying suitable hosts in a complex environment is a key challenge for parasitoids. Many parasitoids have evolved to use cues from their host or the host's environment to locate and correctly identify hosts (Askew 1971; Godfray 1994; van Lenteren 1981; Vinson 1976). Highly specific parasitoids that develop on one or a few host species often require the use of several cues, ranging from general to highly specific, to home in on a preferred host. For example, parasitoids may first use a general cue shared by many insects to define a search area in which they may successfully find their hosts. Then, once the parasitoid is within the appropriate search area, it may need to use more specific cues to distinguish more finely between similar insects. This fine-scale differentiation may require the parasitoid to distinguish among closely related species, or between viable hosts and unsuitable, previously parasitized, hosts. This complex host selection process can be categorized into five general and sometimes overlapping steps: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination, and (e) host regulation (Mathis and Philpott 2012).

Dipteran parasitoids in the family Phoridae frequently use social insects as hosts (Brown and Feener 1991; Disney 1994; Feener et al. 1996; Morehead and Feener 2000). Phorid fly parasitoids first locate hosts and then hover over a chosen target before diving down to insert an egg beneath the insect's exoskeleton (Consoli et al. 2001; Disney 1994; Feener and Brown 1997; Porter 1998). For phorid flies that parasitize ants, host selection cues often include ant pheromones. Pheromones are effective host location cues for parasitoids because they are both detectable and reliable: ants living in high densities produce large volumes of volatile pheromones when disturbed, and these pheromones often are highly conserved among closely related taxa. Once a phorid parasitoid

has located a potential host using long-range cues, oviposition is triggered by the detection of appropriate host-acceptance cues. Short-range cues, such as movement, host size, and contact chemicals, have all been implicated as triggers in phorid fly oviposition (Chen et al. 2009; Gazal et al. 2009; Gilbert and Morrison 1997; Pesquero et al. 1996; Porter et al. 1995; Silva et al. 2008; Wuellner et al. 2002).

Throughout the New World tropics, several species of *Pseudacteon* phorid flies parasitize *Azteca* ants. Three species of phorid flies, *P. lasciniosus*, *P. planidorsalis*, and *P. pseudocercus* parasitize *Azteca sericeasur* ants within the same region in Chiapas, Mexico (Brown and Philpott 2012). However, workers in the genus *Azteca* are notoriously difficult to distinguish from one another (Longino 2007). Indeed, *A. sericeasur* co-occurs with another, nearly identical, species of *Azteca* (currently undescribed, but referred hereafter and on Ant Web (www.antweb.org) as *Azteca* JTL020, J. Longino, personal communication), yet phorid flies that parasitize *A. sercieasur* do not parasitize *A.* JTL020 (Mathis, personal observation). Previous work has shown that phorid flies that parasitize *A. sericeasur* are attracted to the ant's alarm pheromone, which is produced in their pygidial gland. The phorid flies then use movement of an individual ant to home in on a host (Mathis et al. 2011). Here, we show that the phorid flies are attracted to the pygidial gland contents and to movement of both *A. sercieasur* and *A. JTL020*. This begs the question: if these workers are so similar, and the flies are attracted to both species of ants, how do phorid flies distinguish between them to oviposit only in *A. sericeasur*?

In this study, we identified a three-step hierarchy of cues that phorid flies use to identify host ants. Using bioassays and behavioral observations, we confirmed that phorid flies are attracted to both *A. sericeasur* and *A.* JTL020 pygidial gland compounds and will hover over both taxa, but do not attack *A.* JTL020. We then characterized the cuticular hydrocarbons (CHCs) of the two *Azteca* species, and identified them as short-range host location cues used by at least one species of phorid fly to locate *A. sericeasur* ants. Finally, given that both *A. sericeasur* and *A.* JTL020 attract phorid flies, we tested whether two species of phorid fly, *P. lasciniosus* and *P. planidorsalis*, use CHCs to discriminate between their host (*A. sericeasur*) and *Azteca* JTL020.

Methods

Study Site We conducted all fieldwork on a shaded coffee plantation, Finca Irlanda, in the Soconusco region of Chiapas, Mexico (15° 11′ N, 92° 20′ W) between July 2012 and March 2013, in both the wet and dry seasons. Finca Irlanda is approximately 280 ha in size, located at an elevation between 950 and 1150 m, and receives approximately 4500 mm of precipitation per year. *Azteca sericeasur* is the

most dominant species of the ca. 60 species of arboreal ants that occur on the farm (Philpott 2005). *Azteca sericeasur* builds carton nests on the trunks of shade trees within the coffee plantation, where their colonies tend to be distributed in patches (Perfecto et al. 2014). *Azteca* JTL020 also builds large carton nests on the trunks of shade trees within the coffee plantations, but these nests are much less common (Mathis, unpublished data).

Pygidial Gland Bioassays To confirm whether phorid flies are attracted by the alarm pheromone of both *A. sericeasur* and *A.* JTL020, we prepared three treatment solutions: 1) 1 ml of pesticide-grade hexanes, 2) 20 crushed *A. sericeasur* pygidial glands in 1 ml of hexanes, and 3) 20 crushed *A.* JTL020 pygidial glands in 1 ml of hexanes. We then placed treatment solutions in 2-dram open glass vials along with a filter paper wick at 22 field sites. All field sites were at least 25 m apart within the coffee farm, at the base of trees that contained an *A. sericeasur* nest. At each site, we placed the treatment solution vial on the ground with leaf litter removed from the surrounding area. After opening a vial, we observed a 10 cm² area surrounding the vial for 15 min and used an aspirator to collect flies that arrived at the observation area. We later identified the flies and calculated the total number of flies from each species collected at each site with each treatment type. Only two of the three species of phorid fly, *P. lasciniosus* and *P. planidorsalis*, were present in sufficient numbers to compare among trials. We tested for differences among treatment types using a two-way analysis of variance (ANOVA), and made pairwise comparisons between treatment types using Tukey's *post-hoc* tests.

Behavioral Observations We collected behavioral data on the parasitism of *Azteca* by *Pseudacteon* by placing 10 ant workers (either *A. sericeasur* or *A.* JTL020) in shallow plastic dishes with Fluon-coated sides (Northern Products Inc., Woonsocket, Rhode Island, USA). We then placed these dishes near *A. sericeasur* nests to record phorid parasitism. Phorid attack rates on ants are density-dependent, and the frequency of attacks attenuates sharply at approximately 1 m from *Azteca* nests (Philpott et al. 2009). Twenty trees containing strong *A. sericeasur* colonies, each separated by at least 30 m, were used as trial sites. During each observation, we recorded phorid fly arrivals, hover behaviors, and attacks on ants within the plastic containers for 20 min. We recorded every time that a phorid fly entered the area directly above the plastic container, and all phorid fly hover behaviors. We defined hover behaviors as any time a fly hovered <3 cm over a single ant worker and followed it (including events when the fly touched the ant without ovipositing). We also recorded phorid attacks, which were considered to be any time a phorid

fly dove to parasitize an ant, causing the ant to recoil from the impact of oviposition.

Extraction, Application, and Analysis of *Azteca* Cuticular Hydrocarbons (CHCs) We performed CHC-transfer experiments with living ants to test whether species-specific CHCs are used as host recognition cues by *Pseudacteon* phorid flies. We collected *A. sericeasur* and *A.* JTL020 CHCs by rinsing 10 frozen ant workers in approximately 1.5 ml of hexane for 10 min. We filtered this extract through a silica column constructed from a glass pipette filled with silica gel (70–230 μm mesh, Fisher Scientific), rinsed the column with 1 ml of hexane, and collected the extract in glass vials. We evaporated the extracts under argon or nitrogen while swirling the vial, thus coating the walls of the vial with a layer of CHCs. These coated vials were used immediately for behavioral assays.

We treated individual live ants by first placing them in 4-dram vials containing 0.1–0.15 g of clean silica gel (70–230 mesh, Fisher Scientific), and subsequently tapped the vial for 30 s to remove some of the ant's CHCs (Choe et al. 2012). We removed ants from the silica vials, placed them in a CHC-coated vial, and vortexed them for 90 s to transfer CHCs. These ants were allowed to recover from vortexing (5 min) before the behavioral assays were conducted. One CHC-coated vial was used to treat 1 individual. We stored a subset of treated ants at −20 °C for later CHC extraction and GC/MS analysis. We treated worker ants with either CHCs from nestmates, as a negative control, or CHCs from the other *Azteca* species, as an experimental treatment. The negative control addresses the potential role of altering overall CHC concentration and controls for possible effects of handling. Ants were used immediately in bioassays after treatment to prevent any potential replacement of treatment CHCs with newly secreted CHCs (however, ants were maintained alive for at least 24 h after the experimental treatment). This method did not injure the treated ants, and is similar to CHC-transfer methods used by Torres et al. (2007), in which living Argentine ants, *Linepithema humile*, were treated with CHCs from nestmates and non-nestmates.

For GC/MS analysis, CHC extracts using one frozen ant worker were prepared as described in the previous section. After silica filtration, solutions were placed in autosampler vials with glass inserts, evaporated under nitrogen, and subsequently re-eluted with 60 μl hexane. Cuticular hydrocarbon extracts then were stored at −20 °C until use. Two microliters of this solution were injected into a Finnigan Trace MS+ gas chromatograph/mass spectrometer equipped with a DB-5 capillary column (30 m × 0.32 mm X 0.25 μm, Agilent Technologies, CA, USA). Extracts were analyzed by using splitless injection and a column oven temperature program that started at 100 °C (held for 1 min), increased by 20 °C.min^{-1} to 150 °C, and then increased by 5 °C.min^{-1} to 325 °C. before being held for 5 min. Injector and transfer line temperatures were kept at 325 °C and 280 °C, respectively. Individual hydrocarbon peaks were identified by comparing mass spectra and retention times with those of synthetic standards, studying fragmentation patterns, and Kovat's retention indices of the peaks, and also by matching with previously published spectra.

Before performing CHC-transfer bioassays, we compared CHC profiles of 10 individual untreated workers to those of 10 individual workers treated with CHCs using pair-wise comparisons (2 *Azteca* species, 4 comparisons in all) to determine if these ant taxa differed in CHC profile. To examine the effects of CHC-transfer treatments on the overall CHC profiles of both *A. sericeasur* and *A.* JTL020 workers, we applied extracted CHCs to ants and then re-extracted the treated ant CHCs and analyzed them by GC/MS. We compared the CHC profiles of these treated ants to the profiles of untreated ants. To visualize the relationships between profiles of untreated and treated ants, we performed a two-dimensional Non-Metric Multidimensional Scaling (NMDS) (R Development Core Team 2013).

Cuticular Hydrocarbon Transfer Behavioral Assays To test the response of phorid flies to ant CHCs, 10 workers treated with either nestmate (negative control) or foreign CHCs (treatment) were placed in plastic containers for behavioral assays. These assays included 20 trials for each of the four treatments: a) *A. sericeasur* painted with nestmate CHCs, b) *A. sericeasur* painted with *A.* JTL020 CHCs, c) *A.* JTL020 painted with nestmate CHCs, and d) *A.* JTL020 painted with *A. sericeasur* CHCs. During field seasons in 2011 through 2013, we observed phorid fly parasitism of ants in Fluon-coated plastic dishes for 20 min at the same 20 trial sites described above. During each observation, we recorded the number of phorid fly attacks on ant workers within the plastic containers. Phorid flies hovering over individual ants frequently will touch ants without ovipositing; therefore, an "attack" was characterized by any contact a phorid made with an ant that caused the ant to recoil from the force of oviposition. After their first attack, phorid flies were collected and returned to the laboratory for species identification. Only two of the three species of phorid flies, *P. lasciniosus* and *P. planidorsalis*, were present in sufficient numbers to compare across trials.

Results

Pygidial Gland Bioassays In pygidial gland bioassays, all three species of phorid flies were attracted to the pygidial gland extracts of both *A. sericeasur* and *A.* JTL020, but were not attracted to the control hexane (Fig. S1; ANOVA; *P. lasciniosus*: $F_{2, 63} = 19.84$, $P < 0.001$; *P. planidorsalis*: $F_{2, 63} = 21.86$, $P < 0.001$). Furthermore, although phorid flies

were attracted to solutions of the pygidial gland extracts of both ant taxa, each of the three phorid species arrived less frequently to bioassays using *A*. JTL020 pygidial gland extract (Fig. S1; ANOVA; *P. lasciniosus*: $F_{2, 63}$ = 19.84, P < 0.01; *P. planidorsalis*: $F_{2, 63}$ = 21.86, P < 0.01).

Behavioral Observations In initial observations, phorid flies behaved differently toward *A. sericeasur* workers and *Azteca* JTL020 workers. While phorid flies arrived to containers with either ant taxon, they arrived much less frequently during observations of *A*. JTL020 (Fig. 1a; ANOVA; $F_{1, 143}$ = 20.01, P < 0.001). Similarly, we observed phorid flies hovering over both *A. sericeasur* and *A*. JTL020, but phorid flies hovered over *A*. JTL020 workers less frequently than over *A. sericeasur* workers (Fig. 1b; ANOVA; $F_{1, 143}$ = 20.01, P < 0.03). Interestingly, although phorid flies arrived to behavioral observations of *A*. JTL020 and hovered over workers, none of the phorid flies attacked these ants during our behavioral observations. In contrast, phorid flies frequently attacked *A. sericeasur* workers (Fig. 1c; ANOVA; $F_{1, 143}$ = 10.15, P < 0.003). These results indicate that the phorid flies were able to distinguish between these two taxa when at close range, despite their initial attraction to *A*. JTL020 worker ants.

GC/MS Profiles of *Azteca* Ants Analysis of hexane extracts of CHCs from both *A. sericeasur* and *A*. JTL020 showed that workers from these species have distinctly different chemical profiles. For the two species, *A. sericeasur* and *A*. JTL020, we identified 10 and 13 CHC peaks, respectively, each representing at least 1 % of the total area of all compounds (Table 1). Compounds consisted of straight chain alkanes, monomethyl alkanes, and, on *A. sericeasur*, some dimethyl alkanes. Compounds had chain lengths from 21 to 29 carbons,

with *A. sericeasur* containing, on average, compounds with longer carbon chains than *A*. JTL020. Five peaks (n-C_{23}; n-C_{25}; C27; n-C_{26}/10-MeC_{26}/12-MeC_{26}/14-MeC_{26}; n-C_{27}; 11-MeC_{27}/13-MeC_{27}) were found in both species. Representative chromatograms of CHCs obtained from each species are depicted in Fig. 2. Profiles of treated ants more closely resembled their treatment chemotype than their original chemotype with very little "bleed through" of the original CHCs (Fig. 2). Our observations are supported by a two dimensional NMDS analysis, which showed that ants treated with *A. sericeasur* or *A*. JTL020 CHCs clustered with untreated *A. sericeasur* and *A*. JTL020 ants, respectively (Fig. 3; stress coefficient = 0.061, indicating a good fit between distance data and the two-dimensional rendering).

Behavioral Assays with CHC Transfers Phorid flies arrived to behavioral assays in comparable numbers for all treatments (Fig. 4; ANOVA; $F_{3, 76}$ = 0.74, P > 0.5). However, phorid flies attacked *A. sericeasur* ants treated with nestmate CHCs more than all other treatments (Fig. 4; ANOVA with Tukey *post hoc*; $F_{3,7\ 6}$ = 6.486, P < 0.001). Interestingly, when phorid attacks were broken down by species, although both *P. planidorsalis* and *P. lasciniosus* attacked *A. sericeasur* workers treated with nestmate CHCs, *P. planidorsalis* phorid flies also attacked *A*. JTL020 workers treated with *A. sericeasur* CHCs and *A. sericeasur* treated with *A*. JTL020 CHCs (Fig. 5; ANOVA with Tukey *post hoc*; $F_{3, 76}$ = 2.086, P = 0.109). Thus, it appears that *P. lasciniosus* relies more heavily on CHCs as recognition cues for host choice before attacking an ant (Fig. 5; ANOVA with Tukey *post hoc*; $F_{3, 76}$ = 6.275, P < 0.001).

Discussion

The results illustrate that a hierarchy of different cues is used by a parasitoid to identify and parasitize its host. We demonstrate that phorid flies are attracted to both *A. sericeasur* and *Azteca* JTL020 and will hover over both taxa, but will attack only *A. sericeasur*. Additionally, although *A. sericeasur* and *Azteca* JTL020 are nearly identical morphologically and share both chemical and movement cues that attract phorid flies, these *Azteca* taxa differ in their CHC composition. For phorid flies, particularly *P. lasciniosus*, these CHCs play a role as a short-range cue in host recognition. Our CHC-transfer experiments show that *P. lasciniosus* phorid flies attacked *A. sericeasur* ants treated with nestmate CHCs more than they attacked ants treated with *A*. JTL020 CHCs, thus indicating that the presence of *A. sericeasur* hydrocarbons is a short-range cue used in host choice. However, *P. lasciniosus* flies did not attack *A*. JTL020 ants treated with *A. sericeasur* CHCs despite these two ant species being nearly morphologically identical. This result may be due to *P. lasciniosus* phorid flies
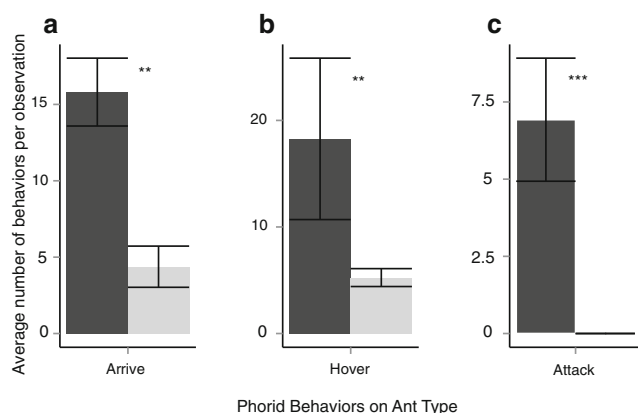
Fig. 1 Plot of average number of phorid behaviors per observation in the field with either *Azteca sericeasur* ants (*black bars*) or *Azteca* JTL020 ants (*grey bars*). Plots showing (**a**) number of phorid flies arriving to arena, (**b**) number of phorid fly hover behaviors in the arena, and (**c**) number of phorid fly attack behaviors in the arena. *Asterisks* represent significance level (* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001)

Table 1 Cuticular hydrocarbons of untreated and treated *Azteca sericeasur* (*SER*) and *Azteca* JTL020 ants as described in Fig. 2. All hydrocarbons are measured as average percent composition ± standard deviation

Peak No.	Compound ID	JTL020	JTL020 on SER	JTL020 on JTL020	SER	SER on SER	SER on JTL020
1	n-C_{21}	1.8 ± 0.9	3.0 ± 2.6	1.4 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	n-C_{22}	1.1 ± 1.1	3.5 ± 4.1	1.2 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	n-C_{23}	16.4 ± 2.3	20.7 ± 12.2	19.0 ± 1.8	1.6 ± 3.3	1.5 ± 1.7	4.0 ± 2.4
4	13-MeC_{23}	9.0 ± 0.8	11.9 ± 5.7	8.2 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	3-MeC_{23}	2.2 ± 0.7	4.1 ± 2.7	2.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6	n-C_{24}	3.3 ± 0.6	1.4 ± 2.0	2.2 ± 1.0	0.0 ± 2.5	0.0 ± 0.0	0.0 ± 0.0
7	13 and 15-MeC_{24}	2.6 ± 0.3	5.3 ± 3.5	3.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	3 and 7-MeC_{24}	3.8 ± 0.5	0.6 ± 1.6	1.4 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9	n-C_{25}	16.6 ± 2.6	7.0 ± 6.0	18.1 ± 2.9	9.7 ± 1.3	11.8 ± 1.4	16.2 ± 3.0
10	13 and 15-MeC_{25}	28.3 ± 2.9	28.7 ± 14.5	29.7 ± 4.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
11	13 and 15 and 3-MeC_{25}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.4 ± 1.0	0.3 ± 1.4
12	n-C_{26} and 10 and 12 and 14-MeC_{26}	1.3 ± 0.9	2.8 ± 1.3	0.6 ± 0.7	1.2 ± 1.7	2.1 ± 1.9	3.6 ± 1.8
13	n-C_{27}	4.2 ± 0.7	3.1 ± 5.4	4.0 ± 0.8	52.7 ± 7.0	55.6 ± 6.1	51.4 ± 8.0
14	11 and 13- MeC_{27}	9.5 ± 1.1	7.9 ± 5.9	8.0 ± 1.2	7.9 ± 0.0	5.3 ± 0.0	2.9 ± 3.0
15	6, 16 and 8, 15-diMeC_{27}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.7	4.3 ± 0.4	2.1 ± 1.7
16	10 and 12 and 13 and 14-MeC_{28}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.7	1.1 ± 1.5	0.0 ± 1.1
17	n-C_{29}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13.3 ± 1.7	12.1 ± 1.1	14.9 ± 1.9
18	MeC_{29}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 2.6	3.9 ± 2.7	3.4 ± 4.4
19	7, 15 and 7, 17-diMeC_{29}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 2.1	1.9 ± 2.1	1.1 ± 0.0

being repelled by trace amounts of *A.* JTL020 CHCs remaining on the cuticle of some ants, which can be seen in the two outliers in Fig. 3. Alternatively, these results may indicate that, while CHCs are a necessary cue, these flies may also require an additional synergistic short-range behavioral cue in host selection, such as the body position of the ant or specific types of movement.

While *P. planidoralis* phorid flies attacked ants with *A. sericeasur* CHCs more than ants of other treatments, and did not attack the *A.* JTL020 ants treated with nestmate CHCs, the numbers of attacks were not different. This likely is due to the relatively lower abundance of this species in the field (Reese and Philpott 2012) and the subsequent overall scarcity

of *P. planidorsalis* attacks. Previous work has shown that these species of *Pseudacteon* phorid flies also use the ant's alarm pheromone (originating from their pygidial gland) to locate hosts at a distance, and use movement to home in on individual ants (Mathis et al. 2011).

Based on previous studies, and the results presented here, we propose the following hierarchical use of cues in host location, selection, and acceptance for *P. lasciniosus*. First, *A. sericeasur* releases alarm pheromone that attracts phorid flies over a distance. Then, once in visual range, the fly homes in on the movement of an ant and hovers over an individual worker. Finally, *P. lasciniosus* briefly touches a worker, that verifies the ant is *A. sericeasur* through assessing the ant's
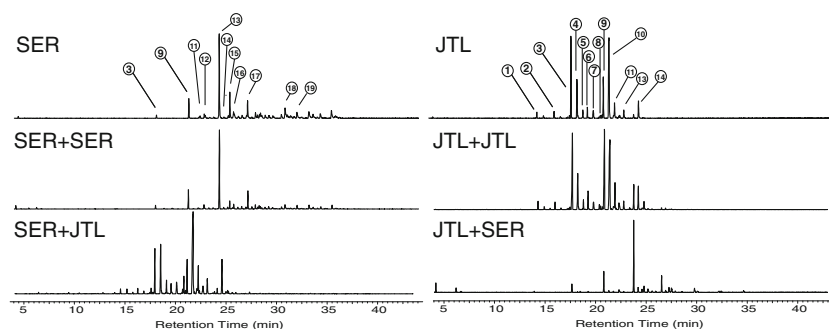
Fig. 2 Representative total ion mass chromatograms of *Azteca* ant cuticular hydrocarbons (CHCs) extracted with hexane, including untreated *Azteca sericeasur* (SER), untreated *Azteca* JTL020 ants (JTL), *A. sericeasur* ants treated with *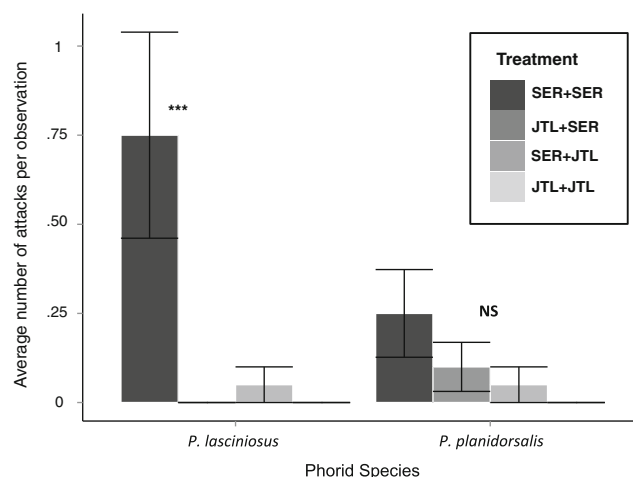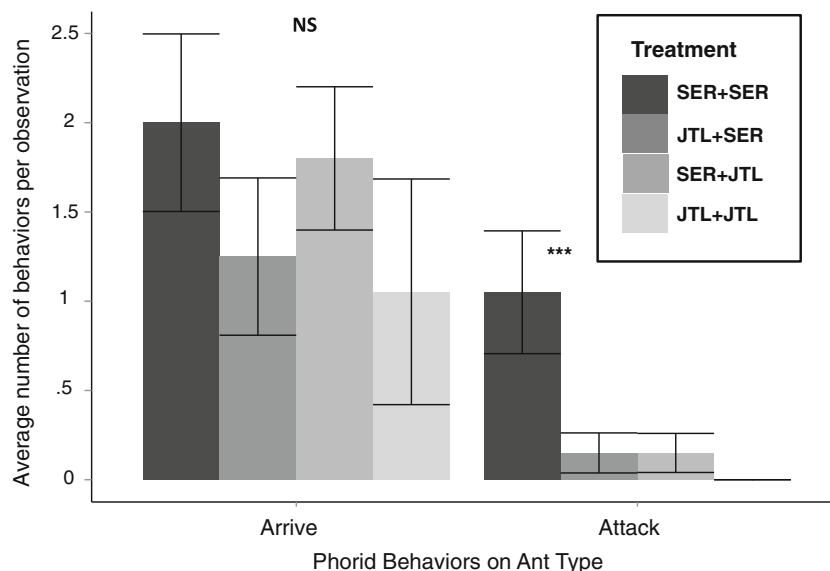A. sericeasur* CHCs (SER + SER), *A.* JTL020 ants treated with *A.* JTL020 CHCs (JTL + JTL), *A.* JTL020 ants treated with *A. sericeasur* CHCs (JTL + SER), and *A. sericeasur* ants treated with *A.* JTL020 CHCs (SER + JTL). In both untreated ant chromatograms, numbers indicate peak numbers seen in Table 1
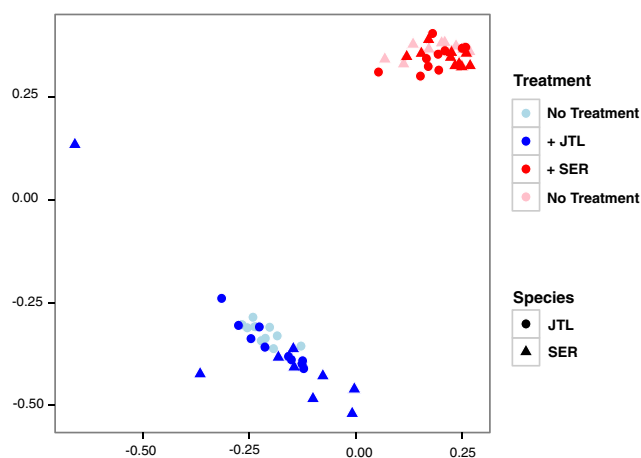
Fig. 3 Two-dimensional Non-Metric Multidimensional Scaling analysis of hydrocarbon profiles shown in Fig. 2. Each symbol represents an individual hydrocarbon profile of one ant worker. Symbol colors represent treatment types as described in Fig. 2

Fig. 5 Plot of average number of *Pseudacteon lasciniosus* and *Pseudacteon planidorsalis* phorid flies to attack ants in cuticular hydrocarbon transfer experiments. Treatments: untreated *Azteca sericeasur* (SER), untreated *Azteca* JTL020 ants (JTL), *A. sericeasur* ants treated with *A. sericeasur* CHCs (SER + SER), *A.* JTL020 ants treated with *A.* JTL020 CHCs (JTL + JTL), *A.* JTL020 ants treated with *A. sericeasur* CHCs (JTL + SER), and *A. sericeasur* ants treated with *A.* JTL020 CHCs (SER + JTL). Bars are standard deviations. *** = significantly different at $P < 0.05$, NS = not significantly different

CHCs, before ovipositing. The use of the close-range cue may be important for phorid flies because the nature of the initial cues allows for a large number of errors before oviposition. *Azteca sericeasur* often releases alarm pheromone during aggressive encounters with other ant species. If the phorid flies arrive to an area where *A. sericeasur* is interacting with one or more other ant species, and movement is the only other cue required for oviposition, it follows that the phorid flies frequently will make host choice errors. Therefore, it seems likely the flies initially use the movement of ants as a cue to home in and become close enough to test the CHCs of target ants, thus ensuring that they are *A. sericeasur*. As phorid flies are also attracted to the alarm pheromone and movement of *A.* JTL020, it remains unclear whether *A.* JTL020 is an unsuitable host for *P. lasciniosus* or whether the specificity of their short-range cue merely renders *A.* JTL020 invisible to them.

Other work has shown that while *Apocephalus paraponerae* phorid flies may not be attracted to ant species closely related to their hosts, they may be able to develop successfully within them (Brown and Feener 1991; Morehead and Feener 2000). Further investigations rearing *P. lasciniosus* in both *Azteca* taxa would provide information as to whether the flies are compatible with both as hosts.

Here, we identified that *P. lasciniosus* phorid flies require the presence of *A. sericeasur* CHCs as a third cue for successful host selection. However, this still may not be the complete picture of successful parasitism by *P. lasciniosus* or *P. planidorsalis*, as the flies are likely using some kind of

Fig. 4 Plot of average number of phorid flies to arrive and attack ants in cuticular hydrocarbon transfer experiments. Treatments: untreated *Azteca sericeasur* (SER), untreated *Azteca* JTL020 ants (JTL), *A. sericeasur* ants treated with *A. sericeasur* CHCs (SER + SER), *A.* JTL020 ants treated with *A.* JTL020 CHCs (JTL + JTL), *A.* JTL020 ants treated with *A. sericeasur* CHCs (JTL + SER), and *A. sericeasur* ants treated with *A.* JTL020 CHCs (SER + JTL). Bars are standard deviations. *** = significant different at $P < 0.05$, NS = not significantly different

synergistic short-range behavioral cue to locate hosts. Even though phorid flies are attracted to pygidial gland contents and movement of both *A. sericeasur* and *A.* JTL020, phorid flies were less likely to parasitize *A.* JTL020 ants in transfer experiments, indicating that phorid flies could still distinguish *A.* JTL020 ants from *A. sericeasur* regardless of CHC profile. Additionally, behavioral observations using previously parasitized ants have shown that phorid flies prefer to attack unparasitized *A. sericeasur* (K. Mathis, unpublished data). Thus, we may infer that phorid flies that attack *Azteca* ants, as with other phorids, also use some kind of host discrimination cue to determine whether ants have been previously parasitized (Braganca et al. 2009; Feener and Brown 1993).

Using GC/MS analysis, we identified five peaks within the *A. sericeasur* CHC profile that are distinct from that of *A.* JTL020 and may be partly responsible for *P. lascniosus* host choice. Additional CHC-transfer experiments, using synthetic versions of these compounds, will allow us to determine whether it is the presence or absence of one or many of these compounds that acts as a cue to *P. lasciniosus.*

While a few other studies have conducted solid-phase CHC-transfer experiments on live ants (Brandt et al. 2009; Liang and Silverman 2000; Torres et al. 2007), our experiments were the first to remove the original CHC signature with a silica rubbing technique (Choe et al. 2012) prior to CHC transfer. Additionally, our study is the first to use this method to investigate parasitoid host-location cues.

In summary, this study shows that phorid flies are able to distinguish between two cryptic taxa of *Azteca* ants, despite these ants sharing two of the cues the phorid flies use in host location. While *Pseudacteon lasciniosus* uses *Azteca sericeasur* CHCs as a short-range cue directly before oviposition, further studies determining synergistic short-range behavioral cues are needed, in addition to identification of the CHCs that act as the short-range cue.

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