Comparing electroantennogram and behavioral responses of two *Pseudacteon* phorid fly species to body extracts of Black, Red and Hybrid imported fire ants, *Solenopsis* spp.

Li Chen [a,b,*], Samuel A. Ochieng [c], Xiaofang He [b,d], Henry Y. Fadamiro [b]

[a] *State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, The Chinese Academy of Sciences, Beijing 100101, PR China*
[b] *Department of Entomology & Plant Pathology, Auburn University, Auburn, AL 36849, USA*
[c] *Institute of Agricultural and Environmental Research, Tennessee State University, McMinnville, TN 37110, USA*
[d] *Department of Entomology, South China Agricultural University, Guangzhou 510642, PR China*

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ABSTRACT

Several phorid fly species were introduced to the southern United States for biological control of the invasive imported fire ants, *Solenopsis richteri* (Black), *Solenopsis invicta* (Red), and their Hybrid *S. richteri* × *S. invicta* (Hybrid). It has been previously reported that the Jaguariuna biotype of *Pseudacteon tricuspis* and the Formosan biotype of *Pseudacteon curvatus* could distinguish among the three fire ant species with greater preference for Hybrid and Red fire ants. We hypothesized that phorid flies might use host derived chemical cues to differentiate ant species. To determine possible differential olfactory sensitivity of phorid fly species to different fire ant species, we compared electroantennogram (EAG) and behavioral responses of both sexes of *P. tricuspis* and *P. curvatus* to body extracts of Black, Red and Hybrid fire ants. As worker sizes of Black and Hybrid fire ants used in this study were much larger than that of Red fire ant (the average weight for Black, Red and Hybrid workers was 1.707, 0.863, 1.223 mg per ants, respectively), at doses of 0.01, 0.1, 1 worker equivalent, body extracts of Black and Hybrid fire ant elicited significantly greater EAG response in both sexes of *P. tricuspis* than that of Red fire ant. Similarly, the EAG response in female *P. curvatus* to body extract of Black fire ant was significantly greater than to body extract of Red fire ant. To eliminate worker size influence on EAG response in phorid flies, we conducted a second EAG study using a dose of 1 mg ant equivalent (body extract from 1 mg of worker). No difference in EAG responses was recorded to body extract obtained from the same amount of workers among the three fire ant species (we consider viable Hybrid fire ant as a species in this paper), suggesting that worker size differences contributed to difference in EAG response in the first EAG study. In both EAG studies, male *P. tricuspis* showed significantly greater EAG responses than male *P. curvatus* to all three fire ant species. In four-way olfactometer bioassay, worker body extracts of all three fire ant species were equally attractive to *P. tricuspis* and *P. curvatus* (i.e. both phorid fly species did not show any preferences among the three fire ant species). Together, the results of the EAG and behavior studies suggest that parasitic phorid flies utilize host derived non-polar compounds from worker ants extracted out by hexane for host location but not for host preference, since both fly species are not able to distinguish among the body extracts of the three fire ant species. Future study will investigate possible involvement of polar compounds and/or non-chemical cues in mediating host preference by phorid flies.

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1. Introduction

Parasitoids may use visual, olfactory or acoustic cues associated with their hosts for host location and acceptance (Vinson, 1984; van Alphen and Jervis, 1996). Although physical factors are important, chemicals often are the major stimulus for parasitoids.

Parasitic wasps have been shown to locate hosts using olfactory cues derived from plants, host insects, or an interaction between herbivores and their plant host (Bruce et al., 2005). Similar to parasitic wasps, parasitic flies use various types of cues to locate hosts (Vinson, 1985; Feener and Brown, 1997; Morehead and Feener, 2000; Köhler and Lakes-Harlan, 2001). Female *Microdon mutabilis* hoverflies use a host derived cue, methyl 6-methylsalicylate, to identify *Formica lemani* colonies (Schönrogge et al., 2008). The two products of the mandibular glands of *Paraponera clavata*, 4-methyl-3-heptanone and 4-methyl-3-heptanol, which are produced in the mandibular glands of numerous ant species and

* Corresponding author at: State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, The Chinese Academy of Sciences, Beijing 100101, PR China. Tel./fax: +86 10 64807780.
E-mail address: chenli@ioz.ac.cn (L. Chen).

serve as alarm pheromones in some species, attract the parasitoid *Apocephalus paraponerae* (Diptera: Phoridae) (Brown and Feener 1991; Feener et al. 1996).

Pseudacteon phorid flies (Diptera: Phoridae) are parasitoids of ants, and many species are specific to imported fire ants, *Solenopsis* spp. (Porter, 1998a). Several phorid fly species have been introduced to the southern United States for biological control of the invasive imported fire ant complex consisting of *Solenopsis richteri* Forel, *Solenopsis invicta* Buren and their Hybrid (*S. richteri × S. invicta*) (which are shortened as "Black", "Red" and "Hybrid", respectively) (Gilbert et al., 2008; Callcott et al., 2011). The first two released species, *Pseudacteon tricuspis* Borgmeier and *Psuedacteon curvatus* Borgmeier, have successfully established in most release sites and dispersed at a rate of *ca* 20 km per year (Porter et al., 2004; Thead et al., 2005; Pereira and Porter, 2006; Vazquez et al., 2006; Henne et al., 2007; Porter, 2010), and the established populations appear to be as highly host specific as pre-release populations and pose no obvious threat to nontarget species (Vazquez and Porter, 2005; Morrison and Porter, 2006). A biotype of *P. tricuspis* well established in north Florida was originally collected from Jaguariuna, Brazil in 1996 on Red fire ant (Porter and Alonso, 1999; Porter et al., 2004). Most *P. tricuspis* released in the United States were Jaguariuna flies (Callcott et al., 2011). A biotype of *P. curvatus* collected from *S. richteri* near Las Flores, Argentina was released to control populations of Black and Hybrid fire ants in Alabama, Mississippi and Tennessee (Graham et al., 2003; Vogt and Streett, 2003; Parkman et al., 2005). This biotype failed to establish seven times when released on Red fire ant in Gainesville, Florida (2000–2001) (Graham et al., 2003). A second biotype of *P. curvatus* collected from *S. invicta* near Formosa, Argentina was released on Red fire ant in Arkansas, Florida, Georgia, Louisiana, North Carolina, Oklahoma, Puerto Rico, South Carolina, and Texas (Vazquez et al., 2004, 2006; Gilbert et al., 2008; Callcott et al., 2011).

Phorid flies showed an attack preference for introduced fire ants over native fire ants (Porter et al., 1995a; Porter, 1998b, 2000; Morrison and Gilbert, 1999; Porter and Alonso, 1999; Morrison, 2000a). Las Flores biotype of *P. curvatus* strongly preferred *S. richteri* and Hybrid when each was tested against *S. invicta* (Porter and Briano, 2000). Multiple choice flight bioassay further demonstrated that the Jaguariuna biotype of *P. tricuspis* and the Formosan biotype of *P. curvatus* could distinguish among the three fire ant species with greater preference for Hybrid fire ant and Red fire ant (He and Fadamiro, 2009). Although preference of females of the Formosan biotype of *P. curvatus* was not as conclusive, the data on hovering duration showed a clear preference of females of this biotype for Red fire ant and Hybrid fire ant over Black fire ant. As Red fire ant is the natural host of the Jaguariuna biotype of *P. tricuspis* (Porter and Alonso, 1999), it is not surprising that this *P. tricuspis* biotype shows preference for Red fire ant over Black fire ant. Similarly, a significant preference of the Formosa biotype of *P. curvatus* for its natural host would not have been surprising as this biotype was collected in South America also on Red fire ant (Vazquez et al., 2004; Vazquez and Porter, 2005). Previous studies have demonstrated that both male and female *P. tricuspis* phorid flies are behaviorally attracted to *S. invicta* odors including venom alkaloids and alarm pheromones and show significant electroantennogram (EAG) response to these odors (Chen and Fadamiro, 2007; Chen et al., 2009; Sharma et al., 2011). Therefore, it is believed that ant chemical cues mediate host location in phorid flies. Studies have revealed distinctively qualitative and quantitative differences among the three imported fire ant species in the chemical profiles of their cuticular hydrocarbons, venom alkaloids, and trail pheromones which are species-specific in the three imported fire ant species (Vander Meer et al., 1985; Ross et al., 1987; Vander Meer and Lofgren, 1989; Chen et al., 2010). These distinctive differences

in chemical profiles are expected to play a key role in host preference by phorid flies. To better understand the mechanism underlying host preference in phorid flies, we address the following questions: (1) Are there any differences among the body extracts of the Black, Red and Hybrid fire ants in eliciting an electroantennogram (EAG) response in two phorid fly species? (2) Are phorid flies differentially attracted to the body extracts of the Black, Red and Hybrid fire ants? As phorid flies behaviorally respond to fire ant odors, we hypothesize that phorid flies utilize host derived chemical cues for host preference and show differential attraction to body odors among the three fire ant species.

To test the above hypothesis, in the present study we compared the EAG and behavioral (four-choice olfactometer) responses of *P. tricuspis* and *P. curvatus* to body extracts of the Black, Red and Hybrid imported fire ants. We selected hexane as extraction solvent because species-specific non-polar compounds including cuticular hydrocarbons, venom alkaloids and trail pheromones in *Solenopsis* fire ants were reported to be readily extracted out by hexane (Vander Meer and Lofgren, 1989). Both sexes of the two phorid fly species were tested to determine any interspecific differences in response.

2. Materials and methods

2.1. Insects

P. tricuspis (Jaguariuna biotype from Brazil) and *P. curvatus* (Formosa biotype from Argentina) flies used in this study were reared on workers of the Red fire ant, *S. invicta*, at Biological Control Rearing Facility of Division of Plant Industry, Florida Department of Agriculture & Consumer Services, part of a USDA-APHIS funded fire ant biological control program in Gainesville, Florida, USA as previously described (Porter et al., 1997). Parasitized fire ant worker heads were received in batches and held in a plastic jar (25×13 cm) with a lid until emergence. Newly-emerged flies were removed with an aspirator, sexed immediately (by using the presence or absence of the distinct female ovipositor), and placed in groups of 2 individuals of opposite sex in a 6-cm diameter plastic Petri dish. Petri dishes were kept in an incubator at 25 ± 1 °C, LD 14:10 h and $70 \pm 5\%$ r.h. Sugar (25% sucrose) solution and water were provided in the Petri dishes as previously described (Chen et al., 2005). Adult phorid flies utilized in the experiments were 1–2 days old.

Black (*S. richteri*), Red (*S. invicta*) and Hybrid (*S. invicta × S. richteri*) fire ants used for extraction were collected from southwest Tennessee and north Mississippi. Mounds were removed and the ants separated from the soil by flooding (Jouvenaz et al., 1977). Ants were raised in the laboratory using sugar solution with some crickets provided.

2.2. Extraction

For each ant species, workers were frozen at −20 °C for 15 min and then extracted with hexane under laboratory conditions for 24 h. Before extraction, the number of ant workers (having similar size for a given species) was counted and the total fresh weight was obtained with an electronic balance. The average weight for Black, Red and Hybrid workers was 1.707, 0.863, 1.223 mg per ant, respectively. The supernatant of each extraction was withdrawn into a glass vial and then split into two parts. As appropriate, an additional amount of hexane was added to part one of each extract to result in 0.1 worker equivalent per μL (WE/μL), and used for both EAG test and behavioral assays. Further dilutions were made to give 10^{-4}, 10^{-3}, 10^{-2} WE/μL solutions for the dose-response study. Part two of the original extract was adjusted with

addition of an appropriate amount of hexane to result in 0.1 mg ant equivalent per μL solutions, and used for EAG test. These solutions were kept in a freezer at −20 °C until use.

2.3. Electroantennogram (EAG) recordings

The EAG techniques used in this study were same as previously described (Chen and Fadamiro, 2007). Briefly, glass capillaries (1.1 mm I.D.) filled with Ringer solution were used as electrodes. The reference electrode was connected to the neck of an isolated head of an adult phorid fly while the recording electrode was connected to the cut tip of the antenna (flagellum) in males or the cut tip of the arista in females. Chlorinated silver–silver chloride junctions were used to maintain electrical contact between the electrodes and input of preamplifier. The analog signal detected through a probe (INR-II, Syntech®, the Netherlands) was processed with a data acquisition controller (IDAC-4, Syntech®, the Netherlands), and later analyzed with EAG 2000 software (Syntech®, the Netherlands) on a PC.

A ten-microliter aliquot of each solution was applied to a piece of filter paper strip (7 × 40 mm, Whatman® No. 1) and later inserted into a glass Pasteur pipette constituting an odor cartridge. The control stimulus was a similar pipette containing a filter paper strip impregnated with a 10 μL aliquot of hexane. The odor stimuli were provided as 0.2 s puffs of air into a continuous humidified air stream at 1000 mL/min generated by an air stimulus controller (CS-55, Syntech®, the Netherlands). At least 2 min was allowed between successive stimulations for antennal recovery.

In the first experiment, we tested the EAG response of female and male flies to four doses of fire ant worker body extract (0.001, 0.01, 0.1, 1 WE) for a total of 12 odor stimuli treatments (4 doses × 3 species). Hexane was used as control. A test series of stimuli for each dose were randomly presented to an antennal preparation (individual fly). Recordings were obtained from 12 individuals for each fly sex and dose (of ant body extract) combination.

In the second experiment, we tested the EAG response of female and male flies to 1 mg ant equivalent of fire ant worker body extract, in other words, body extract prepared from 1 mg of ant worker. This test procedure was the same as the first experiment except for odor differences. The purpose of this experiment was to determine any differences in EAG responses to body extracts prepared from same amount of ant workers for three fire ant species (we consider viable Hybrid fire ant as a species in this paper). Therefore we were able to evaluate the influence of worker size on EAG responsiveness in P. tricuspis and P. curvatus.

2.4. Behavioral bioassay

The behavioral tests were carried out in a four-choice olfactometer. The apparatus consisted of a central chamber (20 cm long × 20 cm wide × 20 cm high) connected to four cylindrical glass jars or "arms" (19 cm long × 11 cm wide). The orifices of the olfactometer were connected through Teflon-glass tube connectors to four pumps on an air delivery system equipped with a vacuum pump (ARS Inc., Gainesville, FL). Purified air was drawn at a constant rate of 200 mL/min through each of the four arms and removed by suction via the vacuum pump through the central orifice of the olfactometer at the rate of 1000 mL/min. The olfactometer was housed in a large paper box (65 × 65 × 43 cm) whose top was left open. The inside surface of the large paper box was covered with white paper, and all outside edges were strengthened with wood stake frames. Two fluorescent tubes (25 W) were placed on the top of the large paper box across its middle line to ensure that every odor chamber was receiving equal illumination.

Whole body extracts of the three fire ant species at a concentration of 0.1 WE/μL were compared with a solvent control (hexane).

Each stimulus (or control) was delivered as 10-μL sample impregnated on filter paper strips (1 × 1 cm, Whatman® No. 1). After allowing for solvent evaporation (∼15 s), the filter paper strip was inserted into its designated olfactometer arm. The ant body extracts (treatments) were tested. One-day old female and male phorid flies were separated under a microscope and then chilled in a refrigerator for 15 min before introduced into the central chamber. Female or male flies (40 for P. tricuspis, and 30 for P. curvatus) were introduced into the central chamber all at one time. The flies were observed continuously for 15 min, and those found in each arm were counted and removed. Flies that did not walk into any of the arms within 15 min were scored as "nonresponders". After each test, the olfactometer was cleaned with hexane and acetone and the arms were rotated (90°) to minimize positional effect. Bioassay tests were replicated 8 times for each sex.

2.5. Statistical analyses

For analysis, EAG response to the solvent control was deducted from the EAG amplitudes elicited by the test extractions. Corrected EAG data were first analyzed by using the standard least squares fit model (SAS Institute, 2004) to determine the effects of fly sex, fire ant species, dose, and interactions among these factors on the EAG response in each fly species. Further analysis of EAG data was performed by using analysis of variance (ANOVA) followed by Tukey–Kramer HSD for multiple comparisons to compare EAG responses to all fire ant odor stimuli and fly sex combinations (SAS Institute, 2004). Absolute EAG responses to all three fire ant species were then compared between both sexes of P. tricuspis using the Student's t-test ($P < 0.05$; SAS Institute, 2004). The mean numbers of flies in each odor chamber were compared by ANOVA, and means were separated by the Tukey–Kramer HSD test at the 95% confidence level as well.

3. Results

3.1. Electrophysiological responses to fire ant body extracts

Standard least squares modeling revealed significant effects of fly sex, fire ant species and dose of fire ant extract on EAG responses of P. tricuspis, resulting in significant sex × dose and ant × dose interactions (Table 1). Since sex exerted a significant effect on EAG response, we used student's t-test to determine significance of EAG responses between both fly sexes to each fire ant species. Both female and male P. tricuspis showed similar dose-dependent EAG responses to all three fire ant species (Fig. 1A and B). In general, the EAG responsiveness in females was significantly higher than that in males. At a dose of 1 WE, Red fire ant elicited relatively, but not significantly, greater EAG in females than in males

Table 1
Standard least squares model testing for effects of sex, mating, test compound, dose, and interactions of these variables on absolute EAG responses of two parasitoid species.

Source of variation	P. tricuspis			P. curvatus		
	DF	F	P	DF	F	P
Sex[a]	1	69.39	<.0001	1	50.16	<.0001
Ant[b]	2	21.14	<.0001	2	4.79	0.0090
Dose[c]	3	291.57	<.0001	3	131.86	<.0001
Sex × ant	2	2.14	0.1190	2	2.31	0.1011
Sex × dose	3	23.63	<.0001	3	31.34	<.0001
Ant × dose	6	4.97	<.0001	6	2.05	0.0601
Sex × ant × dose	6	0.99	0.4286	6	1.48	0.1865

[a] Phorid fly sex.
[b] Fire ant species.
[c] Dose of fire ant extracts.
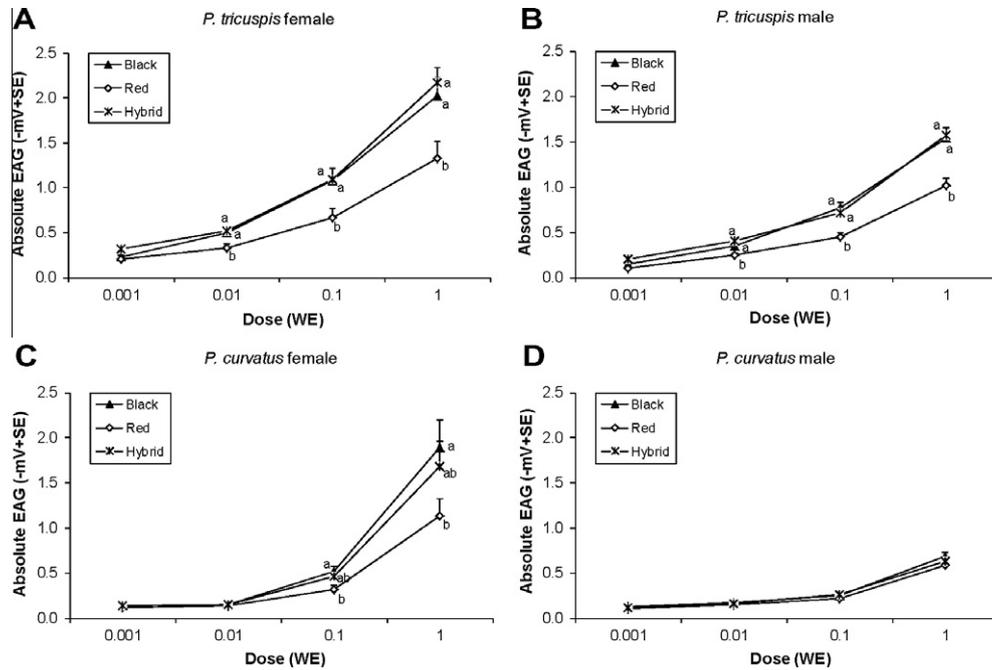
Fig. 1. EAG dose responses of *P. tricuspis* to body extracts of Black (= *S. richteri*), Red (= *S. invicta*), Hybrid (= *S. richteri* × *S. invicta*) fire ants (*n* = 12/fly species/sex). Means for same dose having no letter in common are significantly different (*P* < 0.05, Tukey–Kramer HSD test).

(t = 1.53, P = 0.1407). For same fly sex, both Black and Hybrid fire ants elicited significantly greater EAG responses than Red fire ant at the highest dose (Fig. 1A and B). There was no significant difference in response to Black and Hybrid fire ants across all test doses.

Similar to the results obtained for *P. tricuspis*, significant effects of fly sex, fire ant species, and dose of fire ant extract, as well as significant interaction of sex × dose were recorded on the EAG responses of *P. curvatus* (Table 1). Significantly greater EAG responses were recorded for females compared to males in response to all three fire ant species at 1 WE dose (*P* values of Student's *t*-test for Black, Red and Hybrid were 0.0006, 0.0112, 0.0013, respectively). In dose-response analysis, Black fire ant elicited higher EAG response in *P. curvatus* females than Red fire ant to two high doses (0.1 and 1 WE), and Hybrid fire ant elicited no significant EAG response as compared to either Red or Black fire ant at these two high doses (Fig. 1C), whereas there was no significant difference in EAG response in *P. curvatus* males to the three fire ant species at same dose (Fig. 1D).

3.2. Comparing EAG response profiles of the two fly species

When the data for both fly species were analyzed together using standard least squares modeling, significant effects of fly species

Table 2
P values of Student's *t*-test analysis to compare EAG responses of two *Pseudacteon* fly species to body extracts of three fire ant species at different doses.

| Dose (WE)	Ant species	Female	Male
0.001	Black	0.1634	0.6961
	Red	0.1048	0.9877
	Hybrid	<.0001	0.0285
0.01	Black	<.0001	0.0003
	Red	0.0019	0.0061
	Hybrid	<.0001	<.0001
0.1	Black	<.0001	<.0001
	Red	0.0023	<.0001
	Hybrid	<.0001	<.0001
1	Black	0.7294	<.0001
	Red	0.4716	0.0002
	Hybrid	0.1477	<.0001

(F = 143.06, df = 1, P < 0.0001), fly sex (F = 94.92, df = 1, P < 0.0001), fire ant species (F = 398.85, df = 1, P < 0.0001), and dose of fire ant extract (F = 28.91, df = 1, P < 0.0001) were recorded. To compare EAG response of the same sex of both parasitoids to each fire ant species, we further analyzed EAG data for all doses by using Student's *t*-test (*P* < 0.05; SAS Institute, 2004). At the three low doses (Hybrid only at 0.001 WE), *P. tricuspis* showed significantly greater EAG response than *P. curvatus* to all three fire ant species (Table 2). At the highest dose 1 WE, female *P. tricuspis* and *P. curvatus* showed similar EAG response to all three fire ant species, whereas male *P. tricuspis* had significantly greater EAG responses than male *P. curvatus* to all three fire ant species (Table 2).

3.3. Comparing EAG response of each phorid fly species to 1 mg ant equivalent extract of three fire ant species

We tested EAG response of each sex of each fly species to 1 mg ant equivalent extracts of all three fire ant species. Data were compared by using Tukey–Kramer HSD test. No significant difference was recorded in EAG responses to different fire ant species for any one of fly species and sex combinations (e.g., F = 0.82, df = 2, P = 0.4488 for *P. tricuspis* female, Fig. 2). The absolute EAG amplitudes in both sexes of *P. tricuspis* and female *P. curvatus* were in the range of 1.5–2.0 – mV. The EAG response in male *P. curvatus* was much lower, and less than half of that in above three fly species and sex combinations.

3.4. Behavioral responses to fire ant body extracts

The data from the four-choice olfactometer bioassay showed that the numbers of phorid flies present in odor chambers was significantly higher than the number of flies present in the control (hexane) chamber (Fig. 3). *P. curvatus* was much less active than *P. tricuspis*. Approximately 25% of *P. tricuspis* flies and 50% of *P. curvatus* flies in the central flight chamber, that had not made a choice at the time of data collection, had usually settled along the edges or remained on the floor of the central flight chamber. In all four fly species and sex combinations, the numbers of flies attracted to the odor chambers were significantly higher than the
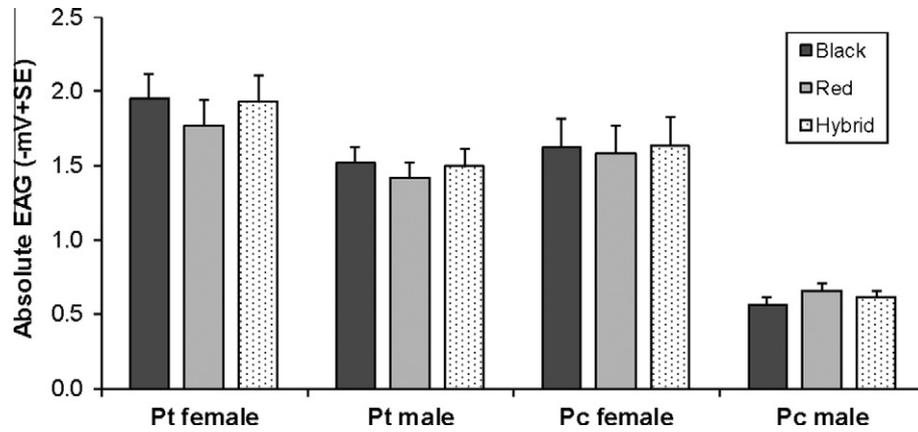
Fig. 2. EAG responses of two *Pseudacteon* fly species to 1 mg ant equivalent extracts of Black (= *S. richteri*), Red (= *S. invicta*), Hybrid (= *S. richteri* × *S. invicta*) fire ants (*n* = 12/fly species/sex). Pt, *P. tricuspis*; Pc, *P. curvatus*.
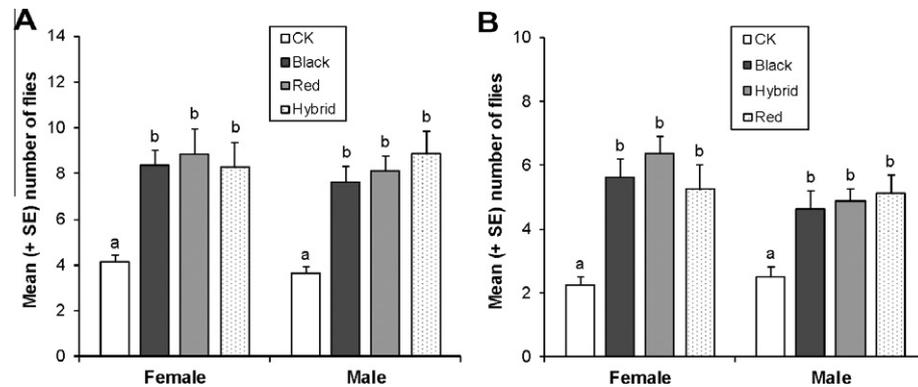
Fig. 3. Response of females and males of two *Pseudacteon* fly species in a four-choice olfactometer bioassay to body extracts obtained from the three fire ant species. (A) *P. tricuspis*, (B) *P. curvatus*. Figure shows mean (±SE) number of flies attracted in 15 min. Means having no letter in common are significantly different (*P* < 0.05, Tukey–Kramer HSD test).

number attracted to control (e.g., *F* = 6.67, *df* = 3, *P* = 0.0015 for *P. tricuspis* female). However, no significant difference was recorded in the number of flies present in the odor chamber among the three fire ant species. The results suggested that there was no preference of phorid flies to all three test fire ant odors.

4. Discussion

4.1. Fly species differences in EAG responses

P. curvatus is a small-sized phorid fly species, whereas *P. tricuspis* is a medium-sized phorid fly species. Antennae of female and male *P. curvatus* are much smaller than that of female and male *P. tricuspis* (Chen and Fadamiro, unpublished data). At the three low doses, two fly species showed similar trend in EAG responses to all three fire ant species. Unexpectedly, at 1 WE dose, we did not record any significant difference in EAG response between female flies of two fly species, even though females of *P. tricuspis* showed slightly greater, but not significantly different, EAG response than females of *P. curvatus* to all three fire ant species. In contrast, males of *P. tricuspis* showed significantly greater EAG response than males of *P. curvatus* to all three fire ant species at 1 WE dose. Different trends in EAG response in females and in males of the two fly species may imply differences in the antennal morphology and/or ultrastructural differences in antennal sensilla between *P. tricuspis* and *P. curvatus*. Males of *P. tricuspis* were observed to be attracted to disturbed fire ants, but males of *P. curvatus* were not (Wuellner et al., 2002). Significant lower

EAG responses by *P. curvatus* males than *P. tricuspis* males to fire ant body extracts may interpret this observation.

4.2. Differences in EAG responses to different fire ant species and phorid fly host preference

Worker body extracts of all three fire ant species elicited similar dose-dependent EAG responses with 1-WE dose triggering highest EAG amplitudes. In *P. tricuspis*, both females and males show greater EAG responses to Black and Hybrid fire ants than to Red fire ant at most tested doses but the lowest dose (0.001 WE). Clearly, there is no significant difference between Black and Hybrid fire ants across all tested doses, suggesting that Black and Hybrid fire ants may share same compounds attractive to phorid fly. It is also possible that these compounds have similar dosages in Black and Hybrid fire ants. The observed greater EAG response to Black and Hybrid fire ants than to Red fire ant may be due to relatively higher quantities of attractive compounds in Black and Hybrid fire ants reflected by their larger size as compared to Red fire ant. There is no significant difference in EAG response of *P. curvatus* males to extracts of the three fire ant species. This trend is quite different from that in *P. curvatus* females and in both sexes of *P. tricuspis*.

To eliminate size difference in the three fire ant species, we tested EAG responses of two phorid fly species to 1 mg ant equivalent extracts. No difference in EAG responses to 1 mg ant equivalent in the second EAG study was recorded. In contrast, we recorded significant difference in EAG responses to 1 WE extracts

of the three fire ant species in the first EAG study. This nonconformance suggested that ant worker size had a significant effect on the EAG response to the three fire ant species and that ant workers from different species with same weight had same amount of attractive compounds. Previous studies on the chemistry of *S. invicta* worker showed that major components of hexane extract were cuticular hydrocarbons, venom alkaloids, trail pheromones and alarm pheromones (Vander Meer and Lofgren, 1989; Chen et al., 2009; Vander Meer et al., 2010). Cuticular hydrocarbons and trail pheromones have proved not to be EAG-active (Chen and Fadamiro, 2007; Chen et al., 2009). Therefore, venom alkaloids and alarm pheromones were possibly the major EAG-active compounds in the hexane extract (Chen et al., 2009; Sharma et al., 2011). Since *S. invicta* worker contained only about 300 pg of the alarm pheromone component, 2-ethyl-3,6-dimethylpyrazine (Vander Meer et al., 2010), and 1 ng (= 1000 pg) of this compound elicited about 0.4 mV EAG response (Sharma et al., 2011), tiny amount of alarm pheromone in a worker ant was unlikely to contribute as much as venom alkaloids to the EAG response recorded for 1 WE dose. The chemical identity of alarm pheromones in Black and Hybrid fire ants has not yet been explored. As *S. richteri* and *S. invicta* readily hybridize in USA, we assume that the three fire ant species share same alarm pheromone component, 2-ethyl-3,6-dimethyl-pyrazine (Vander Meer et al., 2010). Even though Black, Red and Hybrid fire ants exhibited distinctive difference in their venom alkaloids (Vander Meer et al., 1985; Vander Meer and Lofgren, 1989), phorid flies showed similar EAG responses to the same amount of hexane extracts of the three fire ant species, suggesting that phorid flies might not rely on chemical cues extractable by hexane for host preferences over Red, Black and Hybrid fire ants.

4.3. Behavioral response and host preference

The results of the four-way olfactometer bioassays in this study, which demonstrated the strong attraction of both sexes of *P. tricuspis* and *P. curvatus* to worker body extracts of Black, Red and Hybrid fire ants, provided further evidence for the involvement of chemical cues in host location by phorid flies. The EAG response in both sexes of *P. tricuspis* and *P. curvatus* correlated well to behavioral attraction to fire ant worker body extracts. Hybrid fire ant was apparently as attractive as Black and Red fire ants in behavior test and as responsive as Black and Red fire ants in EAG test even though hybrids are known not to occur in the native range of these flies in South America.

A previous flight chamber bioassay demonstrated that both sexes of *P. tricuspis* could distinguish live ant workers among the three imported fire ant species with greater preference for Hybrid fire ant and Red fire ant over Black fire ant (He and Fadamiro, 2009). Similarly, host preference tests in the field revealed that the Las Flores biotype of *P. curvatus* strongly preferred Black and Hybrid fire ants when tested against Red fire ant (Porter and Briano, 2000). The preference of *P. curvatus* for Black fire ant was understandable given that the Las Flores biotype was originally collected from Black fire ants in South America. In the present study, however, there was no significant difference in attraction (fly numbers) to worker body extracts of all three fire ant species, suggesting that chemical cues which can be readily extracted out with hexane solvent may be utilized by phorid flies only for host location but not for host preference. Venom alkaloids and alarm pheromone have proved to play an important role in mediating attraction of phorid flies to host ant workers (Chen et al., 2009; Sharma et al., 2011). Although the alkaloid pattern is species-specific in *Solenopsis* fire ants, these alkaloids are a homologous series. Antennal sensilla in phorid fly encoding fire ant venom alkaloids may not be able to differentiate the subtle change on the molecular structure (e.g., difference in side carbon-chain). Similar physiolog-

ical responses to venom alkaloids and alarm pheromones shared by the three fire ant species may contribute to the incapability of phorid fly in differentiating body extracts from different fire ant species.

Non-polar compounds including cuticular hydrocarbons, venom alkaloids, trail pheromones, and alarm pheromones can be readily extracted out from ant workers. It is possible that some polar compounds, not included in the worker body hexane extract, are responsible for host preference by phorid fly for closely related ant species. Alternatively, non-chemical cues (e.g., visual or vibration cues), or in combination with chemical cues, in short range may be involved in differentiation among closely related host species.

We can rule out the influence of rearing host (*S. invicta* for both phorid fly species) as evidence by a strong preference of the Las Flores biotype of *P. curvatus* for *S. richteri* that was maintained, even after flies had been cultured for 1–2 years (about 8–16 generations) in the lab using exclusively *S. invicta* workers as hosts (Porter and Briano, 2000). The fact that Hybrid fire ant was apparently as attractive as Black fire ant in behavior test and as responsive as Black fire ant in EAG test suggests that the source of this attraction is a qualitative trait that is not diminished in the Hybrid fire ant.

4.4. Potential ecological significance of results

In this study, we utilized the EAG technique to evaluate EAG response of phorid flies to Black, Red and Hybrid fire ants. Although EAG response may not directly relate to the strength of behavioral response, it can provide an indication of antennal response of *Pseudacteon* flies to semiochemicals from fire ants. The fact that there is no significant difference in EAG and behavioral responses of either phorid fly species to all three fire ant species suggests that *P. tricuspis* and *P. curvatus* flies may do well wherever they are released onto Black, Red or Hybrid fire ant populations in southern United States. It is evidenced that the Las Flores biotype of *P. curvatus* from Black fire ants has apparently been able to adapt to Red fire ant populations in southern Mississippi, Alabama, and Georgia (Callcott et al., 2011) even though repeated releases on Red fire ants in Florida failed (Graham et al., 2003). As *P. tricuspis* prefers high temperature (Calcaterra et al., 2005), why the most released Jaguariuna biotype of *P. tricuspis* has not expanded its range into Tennessee could be due to cold temperature where Black fire ants are. Furthermore, *P. curvatus* may be as effective as *P. tricuspis* in regions of the United States where either one of the three fire ant species predominates. *Pseudacteon tricuspis* was abundant and the most commonly distributed species in South America, and the ratio of males to females was about 2:1 in field. It has been observed that both sexes of *P. tricuspis* are attracted to fire ants and mating occurs while females are searching for ant workers to attack (Porter, 1998a), and males of *P. tricuspis* are normally attracted to disturbed fire ant colonies in field (Calcaterra et al., 2005). Although the female is the sex primarily involved in host location, attracted to fire ant workers presumably for egg laying, attraction of *P. tricuspis* males to fire ant workers is probably secondary and may be related to mate location. Therefore, it is possible that male *P. tricuspis* have evolved to use fire ant semiochemicals (e.g., venom alkaloids) as mate location cues.

Male and female phorid flies display similar hovering behavior over fire ants. Males can elicit defensive postures in ants in *Solenopsis* workers as females do (Porter et al., 1995b), and thus reduce their food harvesting capabilities substantially (Morrison, 1999). Phorid fly males show significant EAG and behavioral responses to fire ants as well as females, suggesting that phorid fly males may respond to ant's chemical cues for mating and pose significant impact on field fire ant populations, and may provide more contributions than previously thought to the overall biological control

effects of phorid flies in the field. Male *P. curvatus* show significantly lower EAG response than male *P. tricuspis*, which may explain why higher sex ratio of males to females of *P. tricuspis* were collected in the field in South America than that of *P. curvatus* (Calcaterra et al., 2005). Therefore, *P. curvatus* males may pose less impact in field on fire ant populations than *P. tricuspis* males. As only 3% of ants from a colony are actually parasitized (Morrison et al., 1997), the indirect effects of *Pseudacteon* flies on foraging and interspecific interactions have been extensively studied (Morrison, 2000b; Mehdiabadi et al., 2004; Mottern et al., 2004). Although no obvious impacts on *S. invicta* populations were observed from the introduction of a single *Pseudacteon* species over the temporal and spatial scales measured (Morrison and Porter, 2005), phorid flies hold the premise for their use as biocontrol agents to reduce imported fire ant populations via indirect effects over time.

In conclusion, the results of this study represent an initial attempt to elucidate possible differences in the sensitivity of phorid fly antennae and in behavioral preference of phorid fly individuals to hexane body extracts from the three fire ant species. The fact that there is no significant difference in EAG and behavioral responses of either phorid fly species to all three fire ant species suggests that *P. tricuspis* and *P. curvatus* flies may not use chemical cues extractable by hexane from ant hosts to differentiate closely related host species. Future studies will attempt to test importance of visual cues or a combination of visual cues and chemical cues in mediating phorid fly's host preference.

Acknowledgements

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