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ORIGINAL PAPER

Fire ant venom alkaloids act as key attractants for the parasitic phorid fly, *Pseudacteon tricuspis* (Diptera: Phoridae)

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Abstract The phorid fly, *Pseudacteon tricuspis* Borgmeier, is an introduced parasitoid of imported fire ants, *Solenopsis* spp., in the USA. Although the assumption that phorid flies use fire ant alarm pheromones for host location is probably true, we demonstrated in a previous study the possible involvement of other ant semiochemicals in the response of *P. tricuspis* to fire ants. This study was conducted to determine the glandular sources and identity of the semiochemicals mediating this interaction. First, we tested the electroantennogram response of *P. tricuspis* to extracts of key body parts and glands of workers of the red imported fire ant, *S. invicta* Buren. The results confirm that the poison (venom) gland/sac is the key source of compounds which elicited strong antennal activity in *P. tricuspis*. Follow-up studies were conducted by using a combination of bioassay-guided fractionation and behavioral bioassays to test the hypothesis that attraction of this parasitoid to fire ants is mediated by venom alkaloids. The results confirm the response of *P. tricuspis* to physiologically relevant amounts of the two venom alkaloid fractions (*cis* and *trans* alkaloid fractions) of *S. invicta*. Further analysis by coupled gas chromatography–electroantennogram detection revealed nine venom alkaloid components including two novel 2,6-dialkylpiperideines that elicited significant antennal activity in *P. tricuspis*. This is the first demonstration of the role of venom alkaloids of ants as attractants for their natural enemies. We propose a semiochemical-mediated host location mechanism for *P. tricuspis* involving both alarm pheromones and venom alkaloids. The ecological significance of these findings, including the attraction of male *P. tricuspis* to fire ant venom alkaloids, possibly for mate location, is discussed.

Keywords *Pseudacteon tricuspis* · *Solenopsis invicta* · Host location · GC-EAD · Venom · Alkaloid

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Introduction

Two invasive fire ant species (Hymenoptera: Formicidae) were accidentally introduced from South America to southern USA early in the past century. The black imported fire ant, *Solenopsis richteri* Forel was introduced in the 1910s followed by the introduction of the red imported fire ant, *S. invicta* Buren in the early 1930s. Hybridization between the two species has also been documented in some parts of southern USA (Wilson 1958; Vander Meer and Lofgren 1988). Currently, imported fire ants infest more than 320 million acres (~129.5 million ha) (Williams et al. 2003) in 13 southern states and Puerto Rico (APHIS 2007) and are estimated to be responsible for almost $7 billion annually in damage, repair costs, medical care, and control costs (Lard et al. 2006). Fire ants have also become a global problem, having recently been reported in many other countries (Chen et al. 2009). The observations that population densities of fire ants are five to ten times higher in southern USA than in their native South America (Porter et al. 1992) led to the search for effective native natural enemies in South America for classical biological control of imported fire ants in the USA (Porter and Gilbert 2004).

Pseudacteon phorid flies (Diptera: Phoridae) are solitary and specific parasitoids of fire ants in their native South America (Porter 1998a). Four species have been introduced

since the 1990s as classical biological control agents of imported fire ants in southern USA (Porter et al. 1995). The most widely distributed species are *P. tricuspis* Borgmeier and *P. curvatus* Borgmeier (Porter and Gilbert 2004; Gilbert et al. 2008). Both phorid fly species have been successfully established in most release sites in southern USA and are dispersing at a rate of 10–20 km/year (Thead et al. 2005; Pereira and Porter 2006). Although several aspects of the behavior and biology of *Pseudacteon* phorid flies have been investigated (Porter 1998b; Morrison 2000), little is known about their host location mechanisms.

Olfaction has been suggested as the long range cue used by *Pseudacteon* phorid flies in locating host fire ants (Gilbert and Morrison 1997; Orr et al. 1997; Porter 1998b; Morrison and King 2004). The current assumption is that phorid flies use fire ant alarm pheromones to locate ant workers (Vander Meer and Porter 2002; Morrison and King 2004; Morrison and Porter 2006). However, there are no published studies which provided direct evidence for fire ant alarm pheromones as the primary or sole host location cues in parasitic phorid flies. Furthermore, research on the identification of fire ant alarm pheromones has produced little results (Wilson 1962). The possible involvement of other fire ant semiochemicals, such as venom alkaloids, in mediating interactions between fire ants and their natural enemies has not been explored. Fire ant workers produce a series of alkaloids in their poison (venom) glands, which are stored in the poison sac and dispensed through the sting apparatus (MacConnell et al. 1971). These alkaloids function primarily in defense, colony hygiene, and food procurement (Obin and Vander Meer 1985) and also have physiological functions such as antibacterial, antifungal, phytotoxic, insecticidal, and hemolytic properties (Blum et al. 1958; Javors et al. 1993). Fire ant venom consists of a complex mixture of 2-methyl-6-alkylpiperidines (Brand et al. 1973; MacConnell et al. 1976; Blum et al., 1992), and several recently identified novel 2,6-dialkyl-$\Delta^{1,2}$-piperideines and 2,6-dialkyl-$\Delta^{1,6}$-piperideines (Chen and Fadamiro 2009a, b). The relative proportions of these alkaloids in the venom may differ qualitatively between fire ant species (Brand et al. 1973; Deslippe and Guo 2000; Chen and Fadamiro 2009a, b), suggesting that venom alkaloids may provide host-specific signals to *Pseudacteon* phorid flies and other specialized natural enemies of ants.

In a recent study, we confirmed using behavioral and electroantennogram (EAG) bioassays, that host location by phorid flies is mediated by fire ant semiochemicals (Chen and Fadamiro 2007). Our results also suggest that venom alkaloids may play an important role in this interaction. This present study was carried out to (1) determine the glandular sources and chemical identity of the fire ant semiochemicals that elicited response in *P. tricuspis* and (2) provide new insights on the possible role of fire ant venom alkaloids as attractants for phorid flies. First, we tested the EAG response of *P. tricuspis* to extracts of key body parts and glands of *S. invicta* workers. The results indicate that the poison (venom) gland/sac is the main source of biologically active compounds. Next, we tested by using a combination of bioassay-guided fractionation techniques and behavioral bioassays the hypothesis that attraction of the parasitoid to fire ants is mediated by host venom alkaloids.

Materials and methods

Insects

Pseudacteon tricuspis were reared on workers of *S. invicta* at the fire ant-rearing facility of the USDA-APHIS-PPQ-CPHST Laboratory/Florida DPI, Gainesville, Florida, USA as described by Porter et al. (1997). Parasitized fire ant worker heads were received in batches and held at 25±1°C, LD 14:10 h, and 70±5% RH in a plastic jar (25×13 cm) with a lid until emergence. Newly emerged flies were removed with an aspirator and placed in groups of two individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish. Petri dishes were kept in an incubator at the above conditions. 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-day old.

The colonies of *S. invicta* used in this study were collected on the campus of Auburn University (Auburn, Alabama, USA) in spring 2007. Ants were collected by transferring about 1 L of dirt from each mound into 1-gal Rubbermaid plastic jars coated with Fluon® (ICI, Wilmington, DE) to prevent escape. Ant colonies collected were considered monogyne based on colony size, worker size, and presence of only one queen during collection (Greenberg et al. 1985; Morel et al. 1990). They were maintained in the laboratory at the previously mentioned conditions (for phorid flies) and fed 10% sugar solution, water, and crickets.

Dissections and extraction of body parts/glands from *S. invicta*

Solenopsis invicta workers were chilled to −20°C for 15 min before their body parts were separated using microdissecting scissors. The head and abdomen were further dissected into different parts, based on the results of a previous study in which extracts of the head and abdomen elicited the highest EAG response in *P. tricuspis* (Chen and Fadamiro 2007). The head was subdivided into three parts: antennae, mandibles, and postpharyngeal gland. The antennae and mandibles (with the mandibular gland) were first separated from the head, and then the postphar-

yngeal gland was removed from the remaining part. Since all the glands in the abdomen of *S. invicta* workers are located posterior of the 4th segment, the abdomen was first separated into two parts between the 4th and 5th segments: gland part and non-gland part. The poison (venom) gland/sac was then removed from the gland part. The remaining gland part was considered to be gland part without the poison gland/sac (i.e., other abdominal glands such as Dufour's gland and pygidial gland) (Billen 1990). Thus, the abdomen was separated into three parts: non-gland part, poison gland/sac, and gland part without the poison gland/sac. To avoid contamination among parts, dissections were conducted without saline. A set of 50 workers were individually dissected into head and abdomen parts, and another set of 50 workers were individually dissected into the different gland parts. All body parts and glands were extracted by soaking in hexane (HPLC grade) for 24 h. The final concentration of all extracts was adjusted to 0.1 worker equivalent (WE) per microliter (Chen and Fadamiro 2007). All samples were kept in a freezer at −20°C until used.

Extraction, isolation, and identification of venom alkaloids from *S. invicta*

Extraction and isolation of venom alkaloids from *S. invicta* was performed as previously described (Chen and Fadamiro 2009a, b). Workers (~5 g or 2,500 workers of different sizes) were killed by freezing, and the cuticular compounds were extracted by soaking in hexane (~10 ml) for 24 h. A 0.4 ml aliquot of the extract was loaded onto a silica gel (0.75 g in Pasteur glass pipette) column and eluted with hexane containing increasing amounts of acetone (ranging from hexane/acetone ratio of 50:1 to 10:1). The chemistry of each collection (*ca* 1 ml) was analyzed by gas chromatography (GC) on a Shimadzu GC-17A equipped with a flame ionization detector (FID). The dimension of the capillary column used was as follows: Rtx®-1MS, 30 m×0.25 mm i.d., 0.25 μm (Restek, Bellefonte, PA). The injector was operated in the splitless mode with split opened after 2 min. Helium was used as carrier (1 ml/min) and make-up gas. The GC program used was as follows: injection at 90°C, increase at 15°C/min up to 270°C and hold at this temperature for 16 min. The collections were pooled based on changes observed in the GC chromatograms of each collection. The final volume of each fraction (pooled collections) was adjusted to 1 ml by evaporating part of hexane under a mild flow of nitrogen and stored in separate glass vials (2 ml) in a freezer at −20°C.

Three major fractions were obtained and were subsequently analyzed by gas chromatography–mass spectrometry (GC-MS) using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with an HP-5 MS capillary column (30 m×0.25 mm i.d., 0.25-μm film thickness). Mass spectra were obtained using electron impact (EI; 70 eV). The GC oven temperature was programmed from 90°C (isothermal for 2 min) to 210°C at 15°C/min, then to 280°C at 2°C/min, and held for 10 min. Injection temperature was set at 270°C, and transfer line temperature was set at 280°C. The first fraction was cuticular hydrocarbons, and the remaining two fractions were alkaloids based on comparison with published GC profiles of fire ant venom alkaloids. Alkaloids were identified by analysis of their mass spectra, as well as by comparison of diagnostic ion fragments with published data on *Solenopsis* fire ants.

Electroantennogram response of *P. tricuspis* to extracts of *S. invicta* body parts/glands

The EAG techniques used in this study were the same as those previously described (Chen and Fadamiro 2007). Briefly, glass capillary (1.1 mm i.d.) filled with Ringer solution was used as electrodes. The reference electrode was connected to the neck of an isolated head of a *P. tricuspis* female, and the recording electrode was connected to the cut tip of the arista. Chlorinated silver–silver chloride junctions were used to maintain electrical contact between the electrodes and input of preamplifier. The analog signal was detected through a probe (INR-II; Syntech®, The Netherlands), captured, and processed with a data-acquisition controller (IDAC-4; Syntech®, The Netherlands), and later analyzed with a software (EAG 2000; Syntech®, The Netherlands) on a personal computer.

A 10 μl aliquot of each solution (which translated to ~one WE) was applied to a piece of filter paper strip (7× 40 mm, Whatman® no. 1). After allowing for solvent evaporation, the impregnated filter paper strip was inserted into a glass Pasteur pipette constituting an odor cartridge. The solvent control was hexane. The stimuli were provided as 0.2 s puffs of air into a continuous humidified air stream at 1,000 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. We tested the EAG response of *P. tricuspis* females to extracts of fire ant body parts and glands. For the head part/glands, a test series of stimuli were applied to an antennal preparation (individual fly) using the following order: hexane (solvent control), antenna, mandible, post-pharyngeal gland, and head. For the abdominal parts/glands, a test series of stimuli were applied to an antennal preparation (individual fly) using the following order: hexane, non-gland part, gland part without the poison gland/sac, poison gland/sac, and abdomen. In general, the order of presentation of the extracts was based on the order of EAG activity, from low to high, as determined in preliminary tests. Recordings were obtained from six individuals for each test series (head or abdomen parts/glands) of stimuli. Data

were analyzed by using analysis of variance followed by Tukey-Kramer Honestly Significant Difference (HSD) comparison test to compare EAG responses to different odor stimuli ($P<0.05$; JMP® 7.0.1, SAS Institute 2007).

Electroantennogram response of *P. tricuspis* to chemical fractions from *S. invicta* body extracts

Silica gel chromatography fractions were tested for *P. tricuspis* EAG activity using the method previously described. Hexane was added to 0.4 ml of the original *S. invicta* whole-body extract (same amount loaded to silica gel column) to achieve a volume of 1 ml (same volume as the fractions), which served as the standard stimulus. The stimuli consisted of a 10 µl aliquot of each fraction (which translated to ~one WE). Recordings were obtained from six females. Based on the results of a previous study which showed greater EAG responses for female *P. tricuspis* than for conspecific males (Chen and Fadamiro 2007), only females were used in EAG tests to determine biological activity of the extracts/fractions. EAG response to hexane solvent control was deducted from the EAG amplitudes elicited by the test fractions. Normalization was done by dividing the corrected EAG amplitude of the test fraction with the corrected EAG amplitude of the standard stimulus (whole-body extract). Normalized EAG data were analyzed as described above.

Behavioral response of *P. tricuspis* to major fractions from *S. invicta* body extract

A four-choice olfactometer was used to test the behavioral response of both sexes of *P. tricuspis* to the three major fractions (cuticular hydrocarbons, *cis* alkaloids, and *trans* alkaloids) obtained from body extracts of *S. invicta* workers. 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 1,000 ml/min. The apparatus was positioned under a fluorescent light source (~100 lux) for uniform lighting. The three major fractions (cuticular hydrocarbons, *cis* alkaloids, and *trans* alkaloids) were compared with a solvent control (a mixture of hexane and acetone in the ratio of 20:1) in two separate experiments. 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 fractions (treatments) were tested at a dose equivalent to one WE (experiment 1) and ten WE (experiment 2). For each test, 20 female or male flies (1-day old) were released at the top of the central chamber. 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 "non-responders". After each test, the olfactometer was cleaned with hexane and acetone and the arms were rotated (90°) to minimize positional effect. Each experiment was replicated 20 times per sex. All tests were conducted at 25±1°C, 40–60% RH, and between 12:00 to 16:00 h, the time of day for high phorid fly activity (Pesquero et al. 1996). For each experiment, data on number of flies attracted were normally distributed and thus were analyzed using analysis of variance followed by the Tukey-Kramer HSD comparison test ($P<0.05$; JMP® 7.0.1, SAS Institute 2007).

Gas chromatography–electroantennogram detection response of *P. tricuspis* to venom alkaloid fractions of *S. invicta*

Based on the results which showed that only the *cis* and *trans* alkaloid fractions elicited significant EAG and behavioral responses in *P. tricuspis*, both alkaloidal fractions were subjected to coupled gas chromatography–electroantennogram detection (GC-EAD) analyses with *P. tricuspis* females to detect biologically active peaks (components) in each fraction. A 3-µl sample (which translated to ~0.3 WE) of the *cis* or *trans* alkaloid fraction was injected into a Shimadzu GC-17A, which was programmed as previously described. The column effluent was split 1:1, with one part going to the FID of the GC and the other through a heated (220°C) transfer line (Syntech®, Hilversum, Netherlands) into a humidified airstream (1,000 ml/min) directed at the antenna preparation. The antenna preparation and EAG set-up were same as previously described for EAG. The antennal and FID signals were amplified and recorded simultaneously using software (GC-EAD Pro, Syntech®, Hilversum, The Netherlands) on a personal computer. At least five successful GC-EAD runs were obtained for each fraction, and traces were overlaid on the computer monitor to determine which GC peaks consistently yielded EAD responses. GC-EAD active peaks were identified by comparison with GC-MS data as previously described.

Results

Electroantennogram response of *P. tricuspis* to extracts of *S. invicta* body parts/glands

Extracts of *S. invicta* workers' whole head elicited strong EAG response (1.70±0.15 mV) in *P. tricuspis*, significantly

greater than the EAG elicited by any of the head parts/glands or hexane (solvent control; F=52.72, df=4, P< 0.0001). The EAG response evoked by extract of the mandibles (0.61±0.11 mV) was significantly greater than that evoked by extracts of the antennae (0.24±0.04 mV), postpharyngeal gland (0.23±0.03 mV), or hexane (0.23± 0.03 mV), but significantly less than the EAG elicited by extract of the whole head. However, EAG responses to extracts of the antennae or postpharyngeal gland were not significantly greater than EAG response to hexane.

For the abdomen extracts, *P. tricuspis* showed significantly greater EAG responses to extracts of the whole abdomen (1.74±0.09 mV) and poison gland/sac (1.81±0.04 mV) than to extracts of the non-gland part (0.48±0.04 mV), gland part without the poison gland/sac (1.14±0.03 mV), or hexane (0.13±0.02 mV; F=87.25, df=4, P<0.0001; Fig. 1a). The gland part without the poison sac (i.e., other abdominal glands) elicited a response significantly lower than the poison gland/sac but higher than the non-gland part or hexane. Extract of the non-gland part elicited only a slight but not significant EAG response, compared with hexane.

Electroantennogram response of *P. tricuspis* to chemical fractions from *S. invicta* body extracts

Silica gel chromatography of *S. invicta* body extract yielded five fractions. The first fraction was cuticular hydrocarbons. No GC peaks were visible in the GC chromatographs of the second and fourth fractions. GC-MS analysis showed that the third fraction was *cis* alkaloids and the fifth fraction was *trans* alkaloids. The chemical characteristics of both alkaloidal fractions have been published recently (Chen and Fadamiro 2009b). The first (cuticular hydrocarbons), second, and fourth fractions failed to elicit significant EAG response in *P. tricuspis*. However, both the third (*cis* alkaloids) and fifth (*trans* alkaloids) fractions evoked significant EAG response in *P. tricuspis*, with the *trans* alkaloid fraction eliciting the greatest EAG activity (F= 43.73, df=4, P<0.0001; Fig. 1b).

Behavioral response of *P. tricuspis* to venom alkaloid fractions of *S. invicta*

Data from the four-choice olfactometer experiment 1 (one WE dose) showed a significant difference in the behavioral responses of *P. tricuspis* females (F=137.54; df=3; P<0.001) and males (F=91.07; df=3; P<0.001) to the treatments (Fig. 2a). Both sexes were significantly more attracted to the *cis* and *trans* alkaloid fractions than to the cuticular hydrocarbon fraction or solvent control (a mixture of hexane and acetone in the ratio of 20:1). Females showed a slightly but significantly greater attraction to the *cis* alkaloid fraction than to the *trans* alkaloid fraction, but

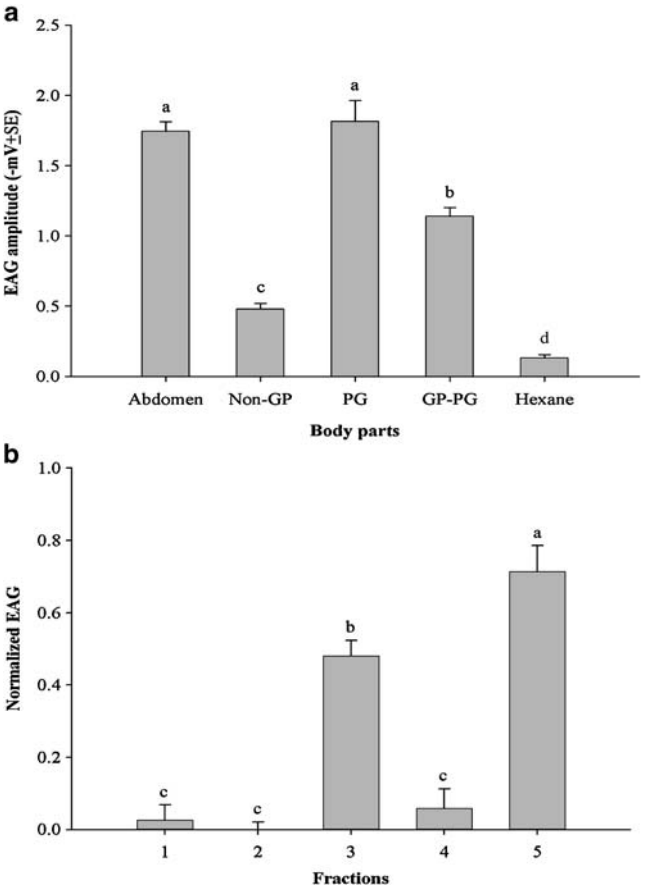

Fig. 1 EAG response of female *P. tricuspis* to extracts of *S. invicta*: **a** absolute EAG response to extracts of *S. invicta* abdomen and its different parts (*Non-GP* non-gland part, *PG* poison gland/sac, *GP-PG* gland part without the poison gland/sac), and **b** normalized EAG response to fractions obtained from silica gel chromatography of *S. invicta* body extract. Note: *Fractions 1*, *3*, and *5* are cuticular hydrocarbons, *cis* alkaloids, and *trans* alkaloids, respectively. Means (±SE) followed by different letters are significantly different (P<0.05, Tukey-Kramer HSD test)

no significant differences were recorded in the responses of males to both alkaloid fractions. Also, the cuticular hydrocarbon fraction did not elicit greater responses in both sexes compared with solvent control.

In the second experiment (ten WE dose), *P. tricuspis* females and males were also significantly attracted to both the *cis* and *trans* alkaloid fractions of *S. invicta* body extract (Fig. 2b). Significantly, more females were attracted to the *cis* alkaloids and *trans* alkaloid fractions than to the cuticular hydrocarbon fraction or solvent control (F= 111.39; df=3; P<0.001). However, no significant differences were recorded in the response of females to both alkaloid fractions; mean numbers of females attracted to the *cis* alkaloid fraction versus the *trans* alkaloid fraction were not significantly different. Similar results were recorded also for males (F=68.86; df=3; P<0.001). In general, similar numbers of flies responded to the treatments in both

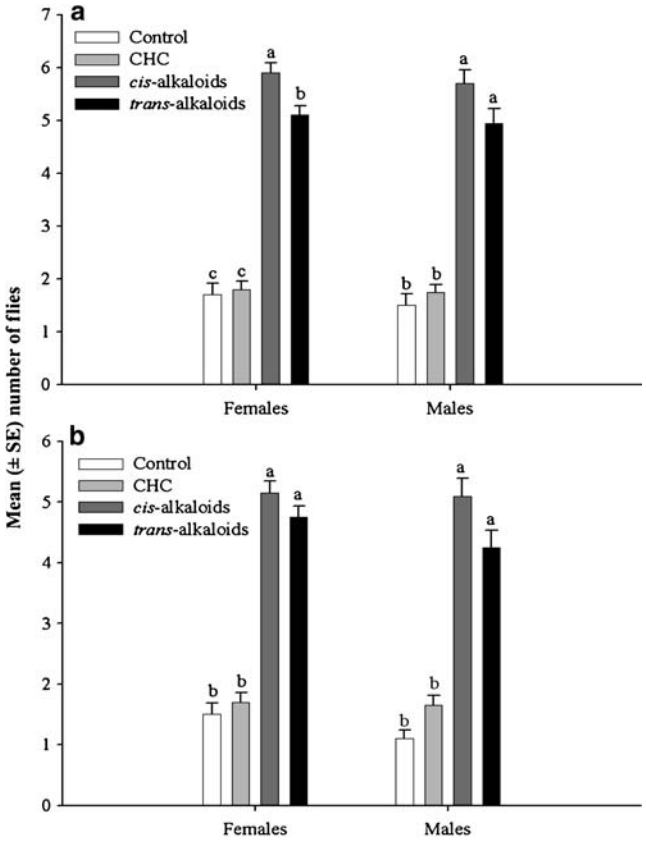

Fig. 2 Response of female and male *P. tricuspis* in a four-choice olfactometer to major chemical (*CHC* cuticular hydrocarbons, *cis* alkaloid, and *trans* alkaloid fractions) obtained from *S. invicta* body extracts: **a** one worker equivalent (*WE*), and **b** ten WE. Note that control is a mixture of hexane and acetone in the ratio of 20:1. Figure shows mean (±SE) number of flies attracted per 15 min. Means for the same sex having no letter in common are significantly different ($P<0.05$, Tukey-Kramer HSD test)

experiments, suggesting that the behavioral response to the alkaloid fractions is not dose dependent.

Gas chromatography–electroantennogram detection response of *P. tricuspis* to venom alkaloid fractions of *S. invicta*

The EAG and behaviorally active third (*cis* alkaloid) and fifth (*trans* alkaloid) fractions were further analyzed by GC-EAD to determine and identify biologically active peaks. At least seven peaks from the third fraction and three peaks from the fifth fraction consistently elicited GC-EAD response in *P. tricuspis* (Fig. 3a, b). The chemical identities of most of these peaks have been recently published (Chen and Fadamiro 2009a, b). The GC-EAD active peaks 1, 2, 3, 4, 5, 6, and 8 were identified based on mass spectrum data and GC retention times as *cis* C_{11}, *trans* C_{11}, *cis* $C_{13:1}$, *cis* C_{13}, *trans* $C_{13:1}$, *cis* $C_{15:1}$, and *cis* C_{15}, respectively. In addition, two novel peaks (seven and nine) were also GC-EAD active (Fig. 3a, b) and were identified as 2-methyl-6-(6-pentadecenyl)-$\Delta^{1,6}$-piperideine and 2-methyl-6-*n*-pentadecyl-$\Delta^{1,6}$-piperideine, respectively (Chen and Fadamiro 2009a, b). We used $\Delta^{1,6}$-$C_{15:1}$ and $\Delta^{1,6}$-C_{15}, respectively to represent these two novel alkaloids.

Discussion

Our results from the electrophysiological and behavioral experiments provide strong evidence for the involvement of venom alkaloids in mediating interactions between fire ants and parasitic phorid flies. In the first experiment, *P. tricuspis* exhibited high EAG response to *S. invicta* abdominal extracts. Although the non-gland part and the gland part without the poison gland/sac elicited slight EAG responses, the poison gland/sac alone elicited the highest EAG response, comparable to that elicited by the whole abdomen. The active components of the gland part without the poison gland/sac

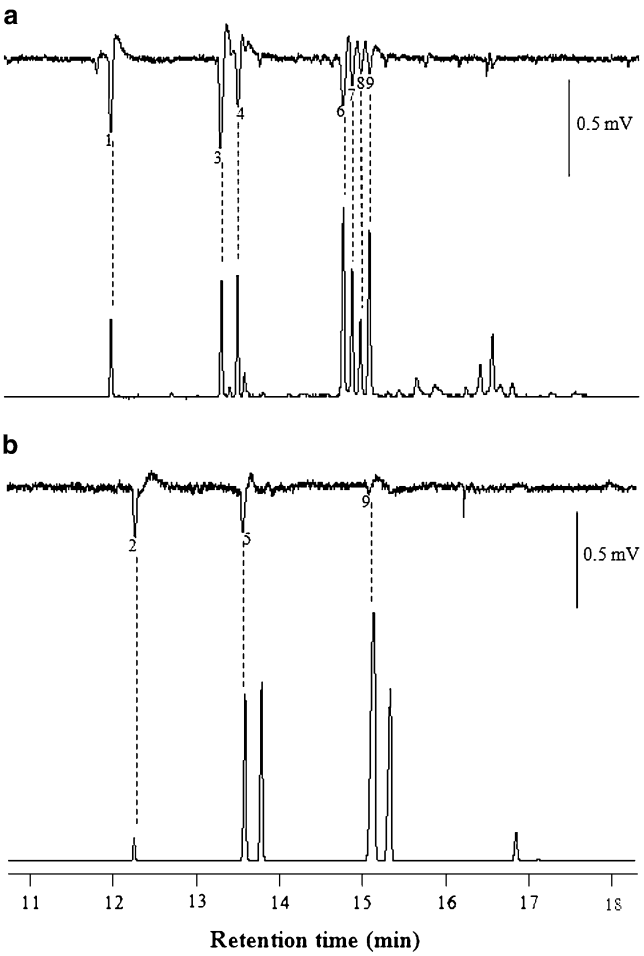

Fig. 3 GC-EAD responses of *P. tricuspis* female antennae to alkaloidal fractions of *S. invicta* body extract: **a** the third fraction (*cis* alkaloids), and **b** the fifth fraction (*trans* alkaloids)

may have been derived from the following three sources: (1) other glandular sources, such as Dufour's gland, pygidial gland, and Pavan's gland (Billen 1990); (2) sting apparatus containing venom components; and (3) contamination from the poison gland/sac during dissection. Altogether, the results indicate that the poison (venom) gland/sac is the major source of biologically active compounds (relative to *P. tricuspis* EAG response) in the abdomen.

Fire ant venom contains about 90–95% water insoluble alkaloids and a small amount of protein (MacConnell et al. 1971; Baer et al. 1979). Since it is relatively easier to obtain venom alkaloids from *S. invicta* body extract than from the poison gland extract, we utilized silica gel column chromatography to purify active constituents of *S. invicta* whole-body extract and to locate venom alkaloids by EAG-guided fractionation. Of the five chemical fractions obtained, only the *cis* alkaloids (third fraction) and *trans* alkaloids (fifth fraction) elicited significant EAG responses in *P. tricuspis*. The cuticular hydrocarbon fraction (first fraction) did not evoke an EAG response in *P. tricuspis*. This is in agreement with our data which showed no EAG response of *P. tricuspis* to extract of the postpharyngeal gland, a glandular source of cuticular hydrocarbons in fire ants (Vander Meer et al. 1982).

The results of the olfactometer bioassays, which demonstrated the strong attraction of both sexes of *P. tricuspis* to physiologically relevant amounts (i.e., one WE) of the *cis* and *trans* alkaloid fractions of *S. invicta* body extract but not to the cuticular hydrocarbon fraction, provided the final evidence for the involvement of fire ant venom alkaloids in host location by *P. tricuspis*. The results also suggest that fire ant cuticular hydrocarbons are not utilized as cues by *P. tricuspis*. The result which demonstrated attraction of male *P. tricuspis* to fire ant venom alkaloids is also interesting. In parasitoids, the female is the sex primarily involved in host location, and *P. tricuspis* females are 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. In *P. tricuspis*, both sexes are attracted to fire ants and mating occurs while females are searching for ant workers to attack (Porter 1998b). Furthermore, evidence for a sex pheromone in this species is lacking. Thus, it is possible that male *P. tricuspis* have evolved to use fire ant semiochemicals (e.g., venom alkaloids) as mate location cues.

To our knowledge, this is the first report on the role of venom alkaloids in mediating interactions between fire ants and their natural enemies. There are only a few examples of the role of insect defensive secretions as attractants (kairomones) for natural enemies (Köpf et al. 1997; Al-Abassi et al. 2001; Conti et al. 2003; Zvereva and Rank 2004; Laumann et al. 2009). Al-Abassi et al. (2001) reported on the response of the ladybird parasitoid, *Dinocampus coccinellae* Schrank (Hymenoptera: Braconidae) to toxic alkaloids from its host, *Coccinella septempunctata* L. (Coleoptera: Coccinelidae). Similarly, *Megaselia opacicornis* Schmitz (Diptera: Phoridae), a fly parasitoid in the same family as *Pseudacteon* phorid flies, was reported to use as host location cues the larval defensive secretions of its host, *Chrysomela lapponica* L. (Coleoptera: Chrysomelidae) (Zvereva and Rank 2004). However, we are not aware of any previous reports on the use of ant venom alkaloids as host location cues by their natural enemies.

A series of *cis* and *trans* alkaloid isomers including C_{11}, $C_{13:1}$, C_{13}, $C_{15:1}$, C_{15}, $C_{17:1}$, and C_{17} had been previously identified from *S. invicta* venom (Brand et al. 1972; MacConnell et al. 1976; Blum et al. 1992). We have also established in a previous study the chemical identities of the two EAG active venom alkaloid fractions obtained from fire ant body extracts, as containing the above 2,6-dialkylpiperidines (*cis* and *trans* alkaloid isomers) and novel 2,6-dialkylpiperideines (Chen and Fadamiro 2009a, b). The biological activity of both venom alkaloid fractions was also confirmed by our GC-EAD results which showed that *P. tricuspis* responded selectively to only nine venom alkaloid components. Among the *cis* alkaloids, *P. tricuspis* responded to *cis* C_{11}, *cis* $C_{13:1}$, *cis* C_{13}, *cis* $C_{15:1}$, and *cis* C_{15} but not to *cis* $C_{17:1}$ and *cis* C_{17}. Among the *trans* alkaloids, *P. tricuspis* responded only to *trans* C_{11} and *trans* $C_{13:1}$. It also responded to two novel 2,6-dialkylpiperideines ($\Delta^{1,6}$-$C_{15:1}$ and $\Delta^{1,6}$-C_{15}).

Pseudacteon tricuspis females showed slightly greater attraction to the *cis* alkaloid fraction at low dose (one WE), but the *trans* alkaloid fraction elicited the greatest response in EAG tests. The amount of *trans* alkaloids in *S. invicta* workers is at least ten times more than that of *cis* alkaloids (unpublished data), and this may explain the relatively greater EAG response elicited by the *trans* alkaloid fraction. Nonetheless, the results together suggest that the biologically active compounds are probably shared by both fractions, but the behavior data also suggest that the *cis* alkaloid fraction is more potent. The GC-EAD results showed that 2-methyl-6-*n*-pentadecyl-$\Delta^{1,6}$-piperideine or $\Delta^{1,6}$-C_{15} (peak 9) is a new EAG-active compound which was detected in both *cis* and *trans* alkaloid fractions. Further studies are necessary to synthesize this novel alkaloid and confirm its biological activity as well as the activity of other key venom alkaloid components.

Venoms of native fire ants in the *S. geminata* complex, such as *S. geminata* (Fabricius) and *S. xyloni* McCook, also contain large amounts of *cis* and *trans* C_{11} (Brand et al. 1972), but qualitative and quantitative differences have been recorded in the composition of piperidine alkaloids in the venoms of different species of fire ants (Brand et al. 1972; Brand et al. 1973; Deslippe and Guo 2000; Chen and

Fadamiro 2009a, b). Differences in venom alkaloid composition would likely provide reliable signals for *Pseudacteon* phorid flies to differentiate among different fire ant species. Thus, we can predict that the venom alkaloids of native *S. geminata* and *S. xyloni* will not elicit significant behavioral response in *P. tricuspis* or other *Pseudacteon* spp. since these flies show a strong preference for fire ants in the *S. saevissima* complex (including *S. richteri*, *S. invicta*, and hybrid *S. richteri* × *S. invicta*) over closely related *S. geminata* complex (including *S. geminata*, and *S. xyloni*) (Gilbert and Morrison 1997; Porter 1998a). This prediction is also supported by the results of this present study which identified at least one novel fire ant alkaloid that may be involved in fire ant–phorid fly interaction. This novel alkaloid ($\Delta^{1,6}$-C_{15}) has not been identified in native fire ants in the *S. geminata* complex.

Our results have clearly demonstrated that fire ant venom alkaloids play a role in mediating attraction of parasitic phorid flies to fire ant workers. However, other fire ant semiochemicals such as alarm pheromones and non-chemical cues (e.g., visual cues) may also be involved in this interaction. This proposition is supported by the results of our first EAG experiment, which showed significant EAG response of *P. tricuspis* to *S. invicta* whole-head extract and to a lesser extent the mandibles. The mandible has been reported as a source of alarm pheromones in fire ants (Vander Meer and Alonso 1996). Thus, our data may suggest also the involvement of alarm pheromones in fire ant–phorid fly interactions, as proposed by several authors (Vander Meer and Porter 2002; Morrison and King 2004; Morrison and Porter 2006). Based on our results and previous observations by the previously mentioned authors, we propose a semiochemical-mediated host location mechanism for *P. tricuspis* involving both alarm pheromones and venom alkaloids. Fire ant alarm pheromones are highly volatile short-chain carbon compounds (alcohols, aldehydes, terpenoids, esters, and heterocycles) in the 100–200 molecular weight range (Vander Meer and Alonso 1998), and thus, may be utilized as long-range host location cues by *P. tricuspis*. In contrast, the larger, longer chain and less volatile venom alkaloids are probably used as medium-/short-range host location chemical cues. Alternatively, fire ant venom alkaloids and alarm pheromones may work synergistically to attract *P. tricuspis* and other *Pseudacteon* species. This is possible since venom release is part of the alarm response and probably a component of the complex alarm odor. Venom alkaloids may also be used by *P. tricuspis* for ant worker discrimination or may have additional functions other than fire ant location. Recent progress towards the identification of fire ant alarm pheromones (R.K. Vander Meer, personal communication) and novel venom alkaloids (our laboratory) will likely provide an opportunity to test the above hypotheses. Our results provide a strong basis for future systematic studies to unravel the complexity of chemical-mediated interactions between fire ants and their natural enemies, and may have ecological and applied significance in the utilization of phorid flies as biological control agents for imported fire ants. Future studies will also investigate the possible role of visual cues in phorid fly host location. The findings may provide new insights in the evolution of host-parasitoid interactions and catalyze future research on the indirect ecological roles of toxic chemicals produced by ants and other social insects, such as the potential use of these chemicals as kairomones by parasitoids and predators.

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