

1 For: *Naturwissenschaften*

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3 **Fire ant venom alkaloids act as key attractants for the parasitic phorid fly,**

4 ***Pseudacteon tricuspis* (Diptera: Phoridae)**

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18 **Abstract** The phorid fly, *Pseudacteon tricuspis* Borgmeier, is an introduced parasitoid of  
19 imported fire ants, *Solenopsis* spp., in the United States. Although the assumption that phorid  
20 flies use fire ant alarm pheromones for host location is probably true, we demonstrated in a  
21 previous study the possible involvement of other ant semiochemicals in the response of *P.*  
22 *tricuspis* to fire ants. This study was conducted to determine the glandular sources and identity  
23 of the semiochemicals mediating this interaction. First, we tested the electroantennogram  
24 (EAG) response of *P. tricuspis* to extracts of key body parts and glands of workers of the red  
25 imported fire ant, *S. invicta* Buren. The results confirm that the poison (venom) gland/sac is the  
26 key source of compounds which elicited strong antennal activity in *P. tricuspis*. Follow-up  
27 studies were conducted by using a combination of bioassay-guided fractionation and  
28 behavioral bioassays to test the hypothesis that attraction of this parasitoid to fire ants is  
29 mediated by venom alkaloids. The results confirm the response of *P. tricuspis* to  
30 physiologically relevant amounts of the two venom alkaloid fractions (*cis* and *trans* alkaloid  
31 fractions) of *S. invicta*. Further analysis by coupled gas chromatography-electroantennogram  
32 detection (GC-EAD) revealed nine venom alkaloid components including two novel  
33 2,6-dialkylpiperideines that elicited significant antennal activity in *P. tricuspis*. This is the first  
34 demonstration of the role of venom alkaloids of ants as attractants for their natural enemies. We  
35 propose a semiochemical-mediated host location mechanism for *P. tricuspis* involving both  
36 alarm pheromones and venom alkaloids. The ecological significance of these findings,  
37 including the attraction of male *P. tricuspis* to fire ant venom alkaloids, possibly for mate  
38 location, is discussed.

39 **Keywords** *Pseudacteon tricuspis* · Fire ant · *Solenopsis invicta* · host location · GC-EAD ·

40 Venom · Alkaloid

41

42 **Introduction**

43 Two invasive fire ant species (Hymenoptera: Formicidae) were accidentally introduced  
44 from South America into the southern United States (U.S.) early in the past century. The black  
45 imported fire ant, *Solenopsis richteri* Forel was introduced in the 1910s followed by the  
46 introduction of the red imported fire ant, *S. invicta* Buren in the early 1930s. Hybridization  
47 between the two species has also been documented in some parts of the southern U.S. (Wilson  
48 1958; Vander Meer and Lofgren 1988). Currently, imported fire ants infest more than 320  
49 million acres (~ 129.5 million ha) (Williams et al. 2003) in 13 southern states and Puerto Rico  
50 (APHIS 2007), and are estimated to be responsible for almost \$7 billion annually in damage,  
51 repair costs, medical care, and control costs (Lard et al. 2006). Fire ants have also become a  
52 global problem, having recently been reported in many other countries (Chen et al. 2009). The  
53 observations that population densities of fire ants are 5-10 times higher in the southern U.S.  
54 than in their native South America (Porter et al. 1992) led to the search for effective native  
55 natural enemies in South America for classical biological control of imported fire ants in the  
56 U.S. (Porter and Gilbert 2004).

57 *Pseudacteon* phorid flies (Diptera: Phoridae) are solitary and specific parasitoids of fire  
58 ants in their native South America (Porter 1998a). Four species have been introduced since the  
59 1990s as classical biological control agents of imported fire ants in the southern U.S. (Porter et  
60 al. 1995). The most widely distributed species are *P. tricuspis* Borgmeier and *P. curvatus*  
61 Borgmeier (Porter and Gilbert 2004; Gilbert et al. 2008). Both phorid fly species have been

62 successfully established in most release sites in the southern U.S. and are dispersing at a rate of  
63 10-20 km per year (Thead et al. 2005; Pereira and Porter 2006). Although several aspects of the  
64 behavior and biology of *Pseudacteon* phorid flies have been investigated (Porter 1998b;  
65 Morrison 2000), little is known about their host location mechanisms.

66 Olfaction has been suggested as the long range cue used by *Pseudacteon* phorid flies in  
67 locating host fire ants (Gilbert and Morrison 1997; Orr et al. 1997; Porter 1998b; Morrison and  
68 King 2004). The current assumption is that phorid flies use fire ant alarm pheromones to locate  
69 ant workers (Vander Meer and Porter 2002; Morrison and King 2004; Morrison and Porter  
70 2006). However, there are no published studies which provided direct evidence for fire ant  
71 alarm pheromones as the primary or sole host location cues in parasitic phorid flies.  
72 Furthermore, research on the identification of fire ant alarm pheromones has produced little  
73 results (Wilson 1962). The possible involvement of other fire ant semiochemicals, such as  
74 venom alkaloids, in mediating interactions between fire ants and their natural enemies has not  
75 been explored. Fire ant workers produce a series of alkaloids in their poison (venom) glands,  
76 which are stored in the poison sac and dispensed through the sting apparatus (MacConnell et al.  
77 1971). These alkaloids function primarily in defense, colony hygiene, and food procurement  
78 (Obin and Vander Meer 1985), and also have physiological functions such as antibacterial,  
79 antifungal, phytotoxic, insecticidal, and hemolytic properties (Blum et al. 1958; Javors et al.  
80 1993). Fire ant venom consists of a complex mixture of 2-methyl-6-alkylpiperidines (Brand et  
81 al. 1973; MacConnell et al. 1976; Blum et al., 1992), and several recently identified novel  
82 2,6-dialkyl- $\Delta^{1,2}$ -piperideines and 2,6-dialkyl- $\Delta^{1,6}$ -piperideines (Chen and Fadamiro 2009a, b).  
83 The relative proportions of these alkaloids in the venom may differ qualitatively between fire

84 ant species (Brand et al. 1973; Deslippe and Guo 2000; Chen and Fadamiro 2009a, b),  
85 suggesting that venom alkaloids may provide host-specific signals to *Pseudacteon* phorid flies  
86 and other specialized natural enemies of ants.

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

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## 99 **Materials and methods**

### 100 **Insects**

101 *Pseudacteon tricuspis* were reared on workers of *S. invicta* at the fire ant rearing facility of  
102 the USDA-APHIS-PPQ-CPHST Laboratory/Florida DPI, Gainesville, Florida, U.S. as  
103 described by Porter et al. (1997). Parasitized fire ant worker heads were received in batches and  
104 held at  $25 \pm 1$  °C, LD 14:10 h and  $70 \pm 5\%$  R.H. in a plastic jar (25 × 13 cm) with a lid until  
105 emergence. Newly-emerged flies were removed with an aspirator, and placed in groups of 2

106 individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish. Petri  
107 dishes were kept in an incubator at the above conditions. Sugar (25% sucrose) solution and  
108 water were provided in the Petri dishes as previously described (Chen et al. 2005). Adult phorid  
109 flies utilized in the experiments were 1-day old.

110 The colonies of *S. invicta* used in this study were collected on the campus of Auburn  
111 University (Auburn, Alabama, U.S.) in spring 2007. Ants were collected by transferring about  
112 1 L of dirt from each mound into 1-gallon Rubbermaid plastic jars coated with Fluon® (ICI,  
113 Wilmington, DE) to prevent escape. Ant colonies collected were considered monogyne based  
114 on colony size, worker size, and presence of only one queen during collection (Greenberg et al.  
115 1985; Morel et al. 1990). They were maintained in the laboratory at the above conditions (for  
116 phorid flies) and fed 10% sugar water and crickets.

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

119 *Solenopsis invicta* workers were chilled to -20 °C for 15 min before their body parts were  
120 separated using micro dissecting scissors. The head and abdomen were further dissected into  
121 different parts, based on the results of a previous study in which extracts of the head and  
122 abdomen elicited the highest EAG response in *P. tricuspis* (Chen and Fadamiro 2007). The  
123 head was subdivided into three parts: antennae, mandibles, and postpharyngeal gland. The  
124 antennae and mandibles (with the mandibular gland) were first separated from the head, and  
125 then the postpharyngeal gland was removed from the remaining part. Since all the glands in the  
126 abdomen of *S. invicta* workers are located posterior of the 4th segment, the abdomen was first  
127 separated into two parts between the 4th and 5th segments: gland part and non-gland part. The

128 poison (venom) gland/sac was then removed from the gland part. The remaining gland part was  
129 considered to be gland part without the poison gland/sac (i.e. other abdominal glands such as  
130 Dufour's gland, pygidial gland) (Billen 1990). Thus, the abdomen was separated into three  
131 parts: non-gland part, poison gland/sac, and gland part without the poison gland/sac. To avoid  
132 contamination among parts, dissections were conducted without saline. A set of 50 workers  
133 were individually dissected into head and abdomen parts, and another set of 50 workers were  
134 individually dissected into the different gland parts. All body parts and glands were extracted  
135 by soaking in hexane (HPLC grade) for 24 h. The final concentration of all extracts was  
136 adjusted to 0.1 worker equivalent (WE) per  $\mu\text{l}$  (Chen and Fadamiro 2007). All samples were  
137 kept in a freezer at  $-20\text{ }^{\circ}\text{C}$  until used.

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

140 Extraction and isolation of venom alkaloids from *S. invicta* was performed as previously  
141 described (Chen and Fadamiro 2009a, b). Workers ( $\sim 5\text{ g}$  or 2500 workers of different sizes)  
142 were killed by freezing and the cuticular compounds were extracted by soaking in hexane ( $\sim 10$   
143 ml) for 24 h. A 0.4 ml aliquot of the extract was loaded onto a silica gel (0.75 g in Pasteur glass  
144 pipette) column and eluted with hexane containing increasing amounts of acetone (ranging  
145 from hexane:acetone ratio of 50:1 to 10:1). The chemistry of each collection (*ca* 1 ml) was  
146 analyzed by gas chromatography (GC) on a Shimadzu GC-17A equipped with a flame  
147 ionization detector (FID). The dimension of the capillary column used was as follows:  
148 Rtx<sup>®</sup>-1MS, 30 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  (Restek, Bellefonte, PA). The injector was operated  
149 in the splitless mode with split opened after 2 min. Helium was used as carrier (1 ml/min) and

150 make-up gas. The GC program used was as follows: injection at 90 °C, increase at 15 °C/min  
151 up to 270 °C and hold at this temperature for 16 min. The collections were pooled based on  
152 changes observed in the GC chromatograms of each collection. The final volume of each  
153 fraction (pooled collections) was adjusted to 1 ml by evaporating part of hexane under a mild  
154 flow of nitrogen and stored in separate glass vials (2 ml) in a freezer at -20 °C.

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

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

167 The electroantennogram (EAG) techniques used in this study were same as previously  
168 described (Chen and Fadamiro 2007). Briefly, glass capillary (1.1 mm i.d.) filled with Ringer  
169 solution was used as electrodes. The reference electrode was connected to the neck of an  
170 isolated head of a *P. tricuspis* female, and the recording electrode was connected to the cut tip  
171 of the arista. Chlorinated silver-silver chloride junctions were used to maintain electrical

172 contact between the electrodes and input of preamplifier. The analog signal was detected  
173 through a probe (INR-II, Syntech<sup>®</sup>, the Netherlands), captured and processed with a data  
174 acquisition controller (IDAC-4, Syntech<sup>®</sup>, the Netherlands), and later analyzed with a software  
175 (EAG 2000, Syntech<sup>®</sup>, the Netherlands) on a personal computer.

176 A 10  $\mu$ l aliquot of each solution (which translated to  $\sim 1$  WE) was applied to a piece of filter  
177 paper strip (7  $\times$  40 mm, Whatman<sup>®</sup> no. 1). After allowing for solvent evaporation, the  
178 impregnated filter paper strip was inserted into a glass Pasteur pipette constituting an odor  
179 cartridge. The solvent control was hexane. The stimuli were provided as 0.2 s puffs of air into a  
180 continuous humidified air stream at 1000 ml/min generated by an air stimulus controller  
181 (CS-55, Syntech<sup>®</sup>, the Netherlands). At least 2 min was allowed between successive  
182 stimulations for antennal recovery. We tested the EAG response of *P. tricuspis* females to  
183 extracts of fire ant body parts and glands. For the head part/glands, a test series of stimuli were  
184 applied to an antennal preparation (individual fly) using the following order: hexane (solvent  
185 control), antenna, mandible, postpharyngeal gland, and head. For the abdominal parts/glands, a  
186 test series of stimuli were applied to an antennal preparation (individual fly) using the  
187 following order: hexane, non-gland part, gland part without the poison gland/sac, poison  
188 gland/sac, and abdomen. In general, the order of presentation of the extracts was based on the  
189 order of EAG activity, from low to high, as determined in preliminary tests. Recordings were  
190 obtained from 6 individuals for each test series (head or abdomen parts/glands) of stimuli. Data  
191 were analyzed by using analysis of variance (ANOVA) followed by Tukey-Kramer HSD  
192 comparison test to compare EAG responses to different odor stimuli ( $P < 0.05$ ; JMP<sup>®</sup> 7.0.1,  
193 SAS Institute 2007).

194

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

196 Silica gel chromatography fractions were tested for *P. tricuspis* EAG activity using the  
197 method described above. Hexane was added to 0.4 ml of the original *S. invicta* whole body  
198 extract (same amount loaded to silica gel column) to achieve a volume of 1 ml (same volume as  
199 the fractions), which served as the standard stimulus. The stimuli consisted of a 10  $\mu$ l aliquot of  
200 each fraction (which translated to  $\sim$  1 WE). Recordings were obtained from 6 females. Based  
201 on the results of a previous study which showed greater EAG responses for female *P. tricuspis*  
202 than for conspecific males (Chen and Fadamiro, 2007), only females were used in EAG tests to  
203 determine biological activity of the extracts/fractions. EAG response to hexane solvent control  
204 was deducted from the EAG amplitudes elicited by the test fractions. Normalization was done  
205 by dividing the corrected EAG amplitude of the test fraction with the corrected EAG amplitude  
206 of the standard stimulus (whole body extract). Normalized EAG data were analyzed as  
207 described above.

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

210 A four-choice olfactometer was used to test the behavioral response of both sexes of *P.*  
211 *tricuspis* to the three major fractions (cuticular hydrocarbons, *cis* alkaloids and *trans* alkaloids)  
212 obtained from body extracts of *S. invicta* workers. The apparatus consisted of a central  
213 chamber (20 cm long  $\times$  20 cm wide  $\times$  20 cm high) connected to four cylindrical glass jars or  
214 ‘arms’ (19 cm long  $\times$  11 cm wide). The orifices of the olfactometer were connected through  
215 Teflon-glass tube connectors to four pumps on an air delivery system equipped with a vacuum

216 pump (ARS, Inc, Gainesville, FL). Purified air was drawn at a constant rate of 200 ml/min  
217 through each of the four arms and removed by suction via the vacuum pump through the central  
218 orifice of the olfactometer at the rate of 1000 ml/min. The apparatus was positioned under a  
219 fluorescent light source (~ 100 lux) for uniform lighting. The three major fractions (cuticular  
220 hydrocarbons, *cis* alkaloids and *trans* alkaloids) were compared with a solvent control (a  
221 mixture of hexane and acetone in the ratio of 20:1) in two separate experiments. Each stimulus  
222 (or control) was delivered as 10- $\mu$ l sample impregnated on filter paper strips (1  $\times$  1 cm,  
223 Whatman<sup>®</sup> no. 1). After allowing for solvent evaporation (~ 15 s), the filter paper strip was  
224 inserted into its designated olfactometer arm. The fractions (treatments) were tested at a dose  
225 equivalent to 1 WE (Experiment 1) and 10 WE (Experiment 2). The release rates of the  
226 treatments from the vials were not quantified. For each test, 20 female or male flies (1-day old)  
227 were released at the top of the central chamber. The flies were observed continuously for 15  
228 min and those found in each arm were counted and removed. Flies that did not walk into any of  
229 the arms within 15 min were scored as “non responders”. After each test, the olfactometer was  
230 cleaned with hexane and acetone and the arms were rotated (90°) to minimize positional effect.  
231 Each experiment was replicated 20 times per sex. All tests were conducted at 25  $\pm$  1 °C,  
232 40-60% R.H., and between 12:00 to 16:00 h, the time of day for high phorid fly activity  
233 (Pesquero et al. 1996). For each experiment, data on number of flies attracted were normally  
234 distributed and thus were analyzed using analysis of variance followed by the Tukey-Kramer  
235 HSD comparison test ( $P < 0.05$ ; JMP<sup>®</sup> 7.0.1, SAS Institute 2007).

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237 GC-EAD response of *P. tricuspis* to venom alkaloid fractions of *S. invicta*

238 Based on the results which showed that only the *cis* and *trans* alkaloid fractions elicited  
239 significant EAG and behavioral responses in *P. tricuspis*, both alkaloidal fractions were  
240 subjected to coupled gas chromatography-electroantennogram detection (GC-EAD) analyses  
241 with *P. tricuspis* females to detect biologically active peaks (components) in each fraction. A  
242 3- $\mu$ l sample (which translated to  $\sim 0.3$  WE) of the *cis* or *trans* alkaloid fraction was injected  
243 into a Shimadzu GC-17A, which was programmed as described above. The column effluent  
244 was split 1:1, with one part going to the flame ionization detector (FID) of the GC and the other  
245 through a heated (220°C) transfer line (Syntech, Hilversum, Netherlands) into a humidified  
246 airstream (1000 ml/min) directed at the antenna preparation. The antenna preparation and EAG  
247 set-up were same as described above for EAG. The antennal and FID signals were amplified  
248 and recorded simultaneously using software (GC-EAD Pro, Syntech, Hilversum, The  
249 Netherlands) on a personal computer. At least five successful GC-EAD runs were obtained for  
250 each fraction, and traces were overlaid on the computer monitor to determine which GC peaks  
251 consistently yielded EAD responses. GC-EAD active peaks were identified by comparison  
252 with GC-MS data as described above.

253

## 254 **Results**

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

256 Extracts of *S. invicta* workers whole head elicited strong EAG response ( $1.70 \pm 0.15$  mV)  
257 in *P. tricuspis*, significantly greater than the EAG elicited by any of the head parts/glands or  
258 hexane (solvent control) ( $F = 52.72$ ,  $df = 4$ ,  $P < 0.0001$ ). The EAG response evoked by extract  
259 of the mandibles ( $0.61 \pm 0.11$  mV) was significantly greater than that evoked by extracts of the

260 antennae ( $0.24 \pm 0.04$  mV), postpharyngeal gland ( $0.23 \pm 0.03$  mV), or hexane ( $0.23 \pm 0.03$   
261 mV), but significantly less than the EAG elicited by extract of the whole head. However, EAG  
262 responses to extracts of the antennae or postpharyngeal gland were not significantly greater  
263 than EAG response to hexane.

264 For the abdomen extracts, *P. tricuspis* showed significantly greater EAG responses to  
265 extracts of the whole abdomen ( $1.74 \pm 0.09$  mV) and poison gland/sac ( $1.81 \pm 0.04$  mV) than to  
266 extracts of the non-gland part ( $0.48 \pm 0.04$  mV), gland part without the poison gland/sac ( $1.14 \pm$   
267  $0.03$  mV), or hexane ( $0.13 \pm 0.02$  mV) ( $F = 87.25$ ,  $df = 4$ ,  $P < 0.0001$ ; Fig. 1a). The gland part  
268 without the poison sac (i.e. other abdominal glands) elicited a response significantly lower than  
269 the poison gland/sac but higher than the non-gland part or hexane. Extract of the non-gland part  
270 elicited only a slight but not significant EAG response, compared to hexane.

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

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

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

Data from the four-choice olfactometer experiment 1 (1 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 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 to solvent control.

In the second experiment (10 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 experiments, suggesting that the behavioral response to the alkaloid fractions is not dose dependent.

304 GC-EAD response of *P. tricuspis* to venom alkaloid fractions of *S. invicta*

305 The EAG and behaviorally active third (*cis* alkaloid) and fifth (*trans* alkaloid) fractions  
306 were further analyzed by GC-EAD to determine and identify biologically active peaks. At least  
307 seven peaks from the third fraction and three peaks from the fifth fraction consistently elicited  
308 GC-EAD response in *P. tricuspis* (Figs. 3a, b). The chemical identities of most of these peaks  
309 have been recently published (Chen and Fadamiro 2009a, b). The GC-EAD active peaks 1, 2, 3,  
310 4, 5, 6, 8 were identified based on mass spectrum data and GC retention times as *cis* C<sub>11</sub>, *trans*  
311 C<sub>11</sub>, *cis* C<sub>13:1</sub>, *cis* C<sub>13</sub>, *trans* C<sub>13:1</sub>, *cis* C<sub>15:1</sub>, *cis* C<sub>15</sub>, respectively. In addition, two novel peaks (7  
312 and 9) were also GC-EAD active (Figs. 3a, b), and were identified as  
313 2-methyl-6-(6-pentadecenyl)- $\Delta^{1,6}$ -piperidine and 2-methyl-6-*n*-pentadecyl- $\Delta^{1,6}$ -piperidine,  
314 respectively (Chen and Fadamiro 2009a, b). We used  $\Delta^{1,6}$ -C<sub>15:1</sub> and  $\Delta^{1,6}$ -C<sub>15</sub>, respectively to  
315 represent these two novel alkaloids.

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## 317 **Discussion**

318 Our results from the electrophysiological and behavioral experiments provide strong  
319 evidence for the involvement of venom alkaloids in mediating interactions between fire ants  
320 and parasitic phorid flies. In the first experiment, *P. tricuspis* exhibited high EAG response to *S.*  
321 *invicta* abdominal extracts. Although the non-gland part and the gland part without the poison  
322 gland/sac elicited slight EAG responses, the poison gland/sac alone elicited the highest EAG  
323 response, comparable to that elicited by the whole abdomen. The active components of the  
324 gland part without the poison gland/sac may have been derived from the following three  
325 sources: 1) other glandular sources, such as Dufour's gland, pygidial gland, and Pavan's gland

326 (Billen 1990); 2) sting apparatus containing venom components; and 3) contamination from  
327 the poison gland/sac during dissection. Altogether, the results indicate that the poison (venom)  
328 gland/sac is the major source of biologically active compounds (relative to *P. tricuspis* EAG  
329 response) in the abdomen.

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

340 The results of the olfactometer bioassays, which demonstrated the strong attraction of both  
341 sexes of *P. tricuspis* to physiologically relevant amounts (i.e. 1 worker equivalent) of the *cis*  
342 and *trans* alkaloid fractions of *S. invicta* body extract but not to the cuticular hydrocarbon  
343 fraction, provided the final evidence for the involvement of fire ant venom alkaloids in host  
344 location by *P. tricuspis*. The results also suggest that fire ant cuticular hydrocarbons are not  
345 utilized as cues by *P. tricuspis*. The result which demonstrated attraction of male *P. tricuspis* to  
346 fire ant venom alkaloids is also interesting. In parasitoids, the female is the sex primarily  
347 involved in host location, and *P. tricuspis* females are attracted to fire ant workers presumably

348 for egg laying. Attraction of *P. tricuspis* males to fire ant workers is probably secondary and  
349 may be related to mate location. In *P. tricuspis*, both sexes are attracted to fire ants and mating  
350 occurs while females are searching for ant workers to attack (Porter 1998b). Furthermore,  
351 evidence for a sex pheromone in this species is lacking. Thus, it is possible that male *P.*  
352 *tricuspis* have evolved to use fire ant semiochemicals (e.g., venom alkaloids) as mate location  
353 cues.

354 To our knowledge, this is the first report on the role of venom alkaloids in mediating  
355 interactions between fire ants and their natural enemies. There are only a few examples of the  
356 role of insect defensive secretions as attractants (kairomones) for natural enemies (Köpf et al.  
357 1997; Al-Abassi et al. 2001; Conti et al. 2003; Zvereva and Rank 2004; Laumann et al. 2009).  
358 Al-Abassi et al. (2001) reported on the response of the ladybird parasitoid, *Dinocampus*  
359 *coccinellae* Schrank (Hymenoptera: Braconidae) to toxic alkaloids from its host, *Coccinella*  
360 *septempunctata* L. (Coleoptera: Coccinellidae). Similarly, *Megaselia opacicornis* Schmitz  
361 (Diptera: Phoridae), a fly parasitoid in the same family as *Pseudacteon* phorid flies, was  
362 reported to use as host location cues the larval defensive secretions of its host, *Chrysomela*  
363 *lapponica* L. (Coleoptera: Chrysomelidae) (Zvereva and Rank 2004). However, we are not  
364 aware of any previous reports on the use of ant venom alkaloids as host location cues by their  
365 natural enemies.

366 A series of *cis* and *trans* alkaloid isomers including C<sub>11</sub>, C<sub>13:1</sub>, C<sub>13</sub>, C<sub>15:1</sub>, C<sub>15</sub>, C<sub>17:1</sub>, C<sub>17</sub> had  
367 been previously identified from *S. invicta* venom (Brand et al. 1972; MacConnell et al. 1976;  
368 Blum et al. 1992). We have also established in a previous study the chemical identities of the  
369 two EAG active venom alkaloid fractions obtained from fire ant body extracts, as containing

370 the above 2,6-dialkylpiperidines (*cis* and *trans* alkaloid isomers) and novel  
371 2,6-dialkylpiperideines (Chen and Fadamiro 2009a, b). The biological activity of both venom  
372 alkaloid fractions was also confirmed by our GC-EAD results which showed that *P. tricuspis*  
373 responded selectively to only nine venom alkaloid components. Among the *cis* alkaloids, *P.*  
374 *tricuspis* responded to *cis* C<sub>11</sub>, *cis* C<sub>13:1</sub>, *cis* C<sub>13</sub>, *cis* C<sub>15:1</sub>, *cis* C<sub>15</sub> but not to *cis* C<sub>17:1</sub>, *cis* C<sub>17</sub>.  
375 Among the *trans* alkaloids, *P. tricuspis* responded only to *trans* C<sub>11</sub> and *trans* C<sub>13:1</sub>. It also  
376 responded to two novel 2,6-dialkylpiperideines ( $\Delta^{1,6}$ -C<sub>15:1</sub> and  $\Delta^{1,6}$ -C<sub>15</sub>).

377 *Pseudacteon tricuspis* females showed slightly greater attraction to the *cis* alkaloid  
378 fraction at low dose (1 WE), but the *trans* alkaloid fraction elicited the greatest response in  
379 EAG tests. The amount of *trans* alkaloids in *S. invicta* workers is at least ten times more than  
380 that of *cis* alkaloids (unpublished data), and this may explain the relatively greater EAG  
381 response elicited by the *trans* alkaloid fraction. Nonetheless, the results together suggest that  
382 the biologically active compounds are probably shared by both fractions, but the behavior data  
383 also suggest that the *cis* alkaloid fraction is more potent. The GC-EAD results showed that  
384 2-methyl-6-*n*-pentadecyl- $\Delta^{1,6}$ -piperideine or  $\Delta^{1,6}$ -C<sub>15</sub> (peak 9) is a new EAG active compound  
385 which was detected in both *cis* and *trans* alkaloid fractions. Further studies are necessary to  
386 synthesize this novel alkaloid and confirm its biological activity, as well as the activity of other  
387 key venom alkaloid components.

388 Venoms of native fire ants in the *geminata* complex, such as *S. geminata* (Fabricius) and *S.*  
389 *xyloni* McCook, also contain large amounts of *cis* and *trans* C<sub>11</sub> (Brand et al. 1972), but  
390 qualitative and quantitative differences have been recorded in the composition of piperidine  
391 alkaloids in the venoms of different species of fire ants (Brand et al. 1972; Brand et al. 1973;

392 Deslippe and Guo 2000; Chen and Fadamiro 2009a, b). Differences in venom alkaloid  
393 composition would likely provide reliable signals for *Pseudacteon* phorid flies to differentiate  
394 among different fire ant species. Thus, we can predict that the venom alkaloids of native *S.*  
395 *geminata* and *S. xyloni* will not elicit significant behavioral response in *P. tricuspis* or other  
396 *Pseudacteon* spp. since these flies show a strong preference for fire ants in the *saevissima*  
397 complex (including *S. richteri*, *S. invicta*, and hybrid *S. richteri* × *S. invicta*) over closely  
398 related *geminata* complex (including *S. geminata*, *S. xyloni*) (Gilbert and Morrison 1997;  
399 Porter 1998a). This prediction is also supported by the results of this present study which  
400 identified at least one novel fire ant alkaloid that may be involved in fire ant-phorid fly  
401 interaction. This novel alkaloid ( $\Delta^{1,6}$ -C<sub>15</sub>) has not been identified in native fire ants in the  
402 *geminata* complex.

403 Our results have clearly demonstrated that fire ant venom alkaloids play a role in  
404 mediating attraction of parasitic phorid flies to fire ant workers. However, other fire ant  
405 semiochemicals such as alarm pheromones and non-chemical cues (e.g., visual cues) may also  
406 be involved in this interaction. This proposition is supported by the results of our first EAG  
407 experiment, which showed significant EAG response of *P. tricuspis* to *S. invicta* whole head  
408 extract and to a lesser extent the mandibles. The mandible has been reported as a source of  
409 alarm pheromones in fire ants (Vander Meer and Alonso 1996). Thus, our data may suggest  
410 also the involvement of alarm pheromones in fire ant-phorid fly interactions, as proposed by  
411 several authors (Vander Meer and Porter 2002; Morrison and King 2004; Morrison and Porter  
412 2006). Based on our results and previous observations by the above authors, we propose a  
413 semiochemical-mediated host location mechanism for *P. tricuspis* involving both alarm

414 pheromones and venom alkaloids. Fire ant alarm pheromones are highly volatile short carbon  
415 chain compounds (alcohols, aldehydes, terpenoids, esters, and heterocycles) in the 100-200  
416 molecular weight range ( Vander Meer and Alonso 1998), and thus may be utilized as long  
417 range host location cues by *P. tricuspis*. In contrast, the larger, longer chain and less volatile  
418 venom alkaloids are probably used as medium/short range host location chemical cues.  
419 Alternatively, fire ant venom alkaloids and alarm pheromones may work synergistically to  
420 attract *P. tricuspis* and other *Pseudacteon* species. This is possible since venom release is part  
421 of the alarm response and probably a component of the complex alarm odor. Venom alkaloids  
422 may also be used by *P. tricuspis* for ant worker discrimination or may have additional functions  
423 other than fire ant location. Recent progress towards the identification of fire ant alarm  
424 pheromones (R. K. Vander Meer, personal communication) and novel venom alkaloids (our  
425 laboratory) will likely provide an opportunity to test the above hypotheses. Our results provide  
426 a strong basis for future systematic studies to unravel the complexity of chemical mediated  
427 interactions between fire ants and their natural enemies, and may have ecological and applied  
428 significance in the utilization of phorid flies as biological control agents for imported fire ants.  
429 Future studies will also investigate the possible role of visual cues in phorid fly host location.  
430 The findings may provide new insights in the evolution of host-parasitoid interactions and  
431 catalyze future research on the indirect ecological roles of toxic chemicals produced by ants  
432 and other social insects, such as the potential use of these chemicals as kairomones by  
433 parasitoids and predators.

434

435 **Acknowledgements**

436 We thank Debbie Roberts and Amy Croft (USDA-APHIS-PPQ-CPHST  
437 Laboratory/Florida DPI, Gainesville, FL, USA) for the supply of adult *P. tricuspis* for this  
438 study, and Dr. Art Appel (Auburn University) for helping to review an earlier version of this  
439 article. This research was funded in part by grants (to HYF) from the Alabama Fire Ant  
440 Management Program and Auburn University Biogrants Program.

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574

575

576 **Figure Legends**

577

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

585

586 Fig. 2 Response of female and male *P. tricuspis* in a four-choice olfactometer to major  
587 chemical (cuticular hydrocarbons = CHC, *cis* alkaloid and *trans* alkaloid fractions) obtained  
588 from *S. invicta* body extracts: (a) 1 worker equivalent (WE); and (b) 10 WE. Note that control  
589 is a mixture of hexane and acetone in the ratio of 20:1. Figure shows mean ( $\pm$  SE) number of  
590 flies attracted per 15 min. Means for the same sex having no letter in common are significantly  
591 different ( $P < 0.05$ , Tukey-Kramer HSD test)

592

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

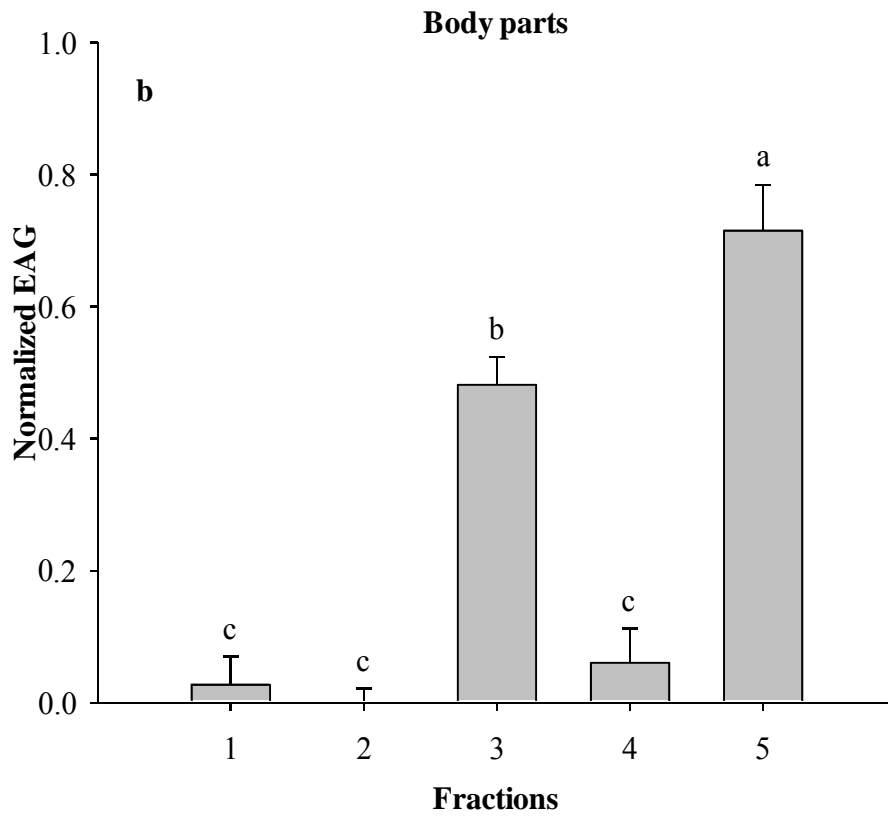
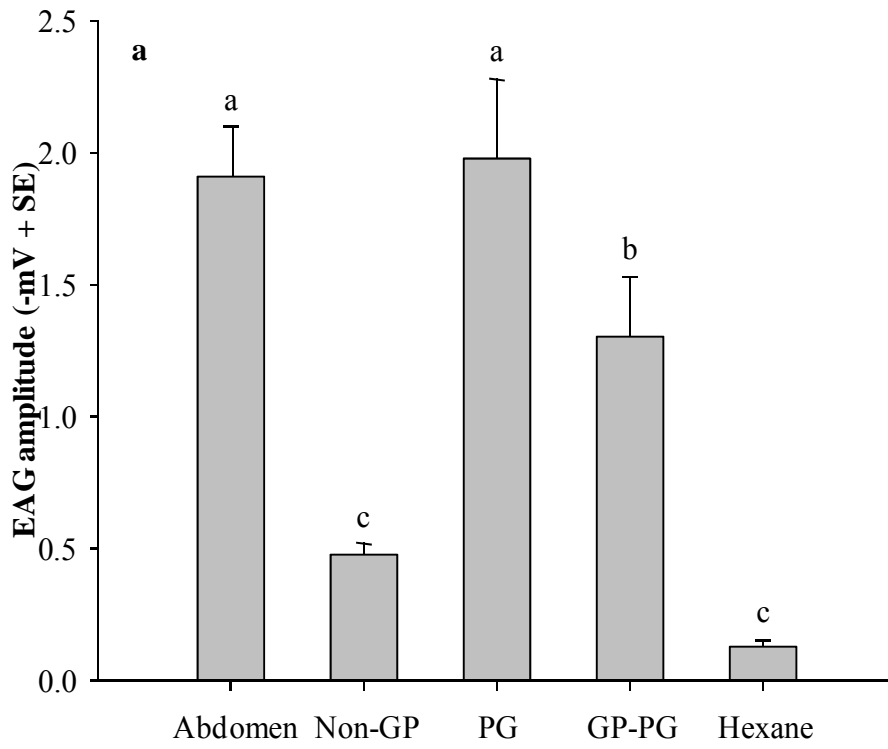
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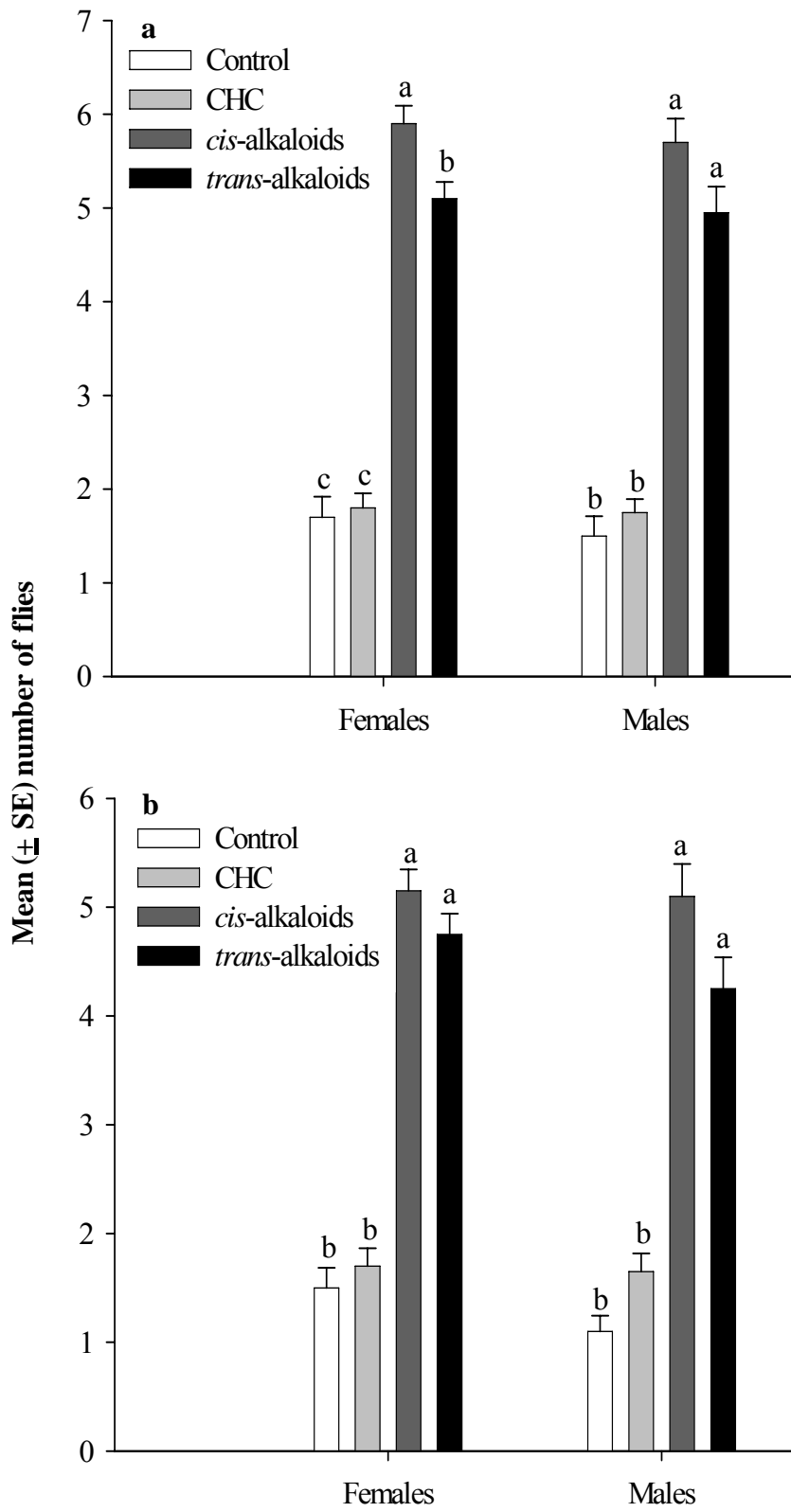
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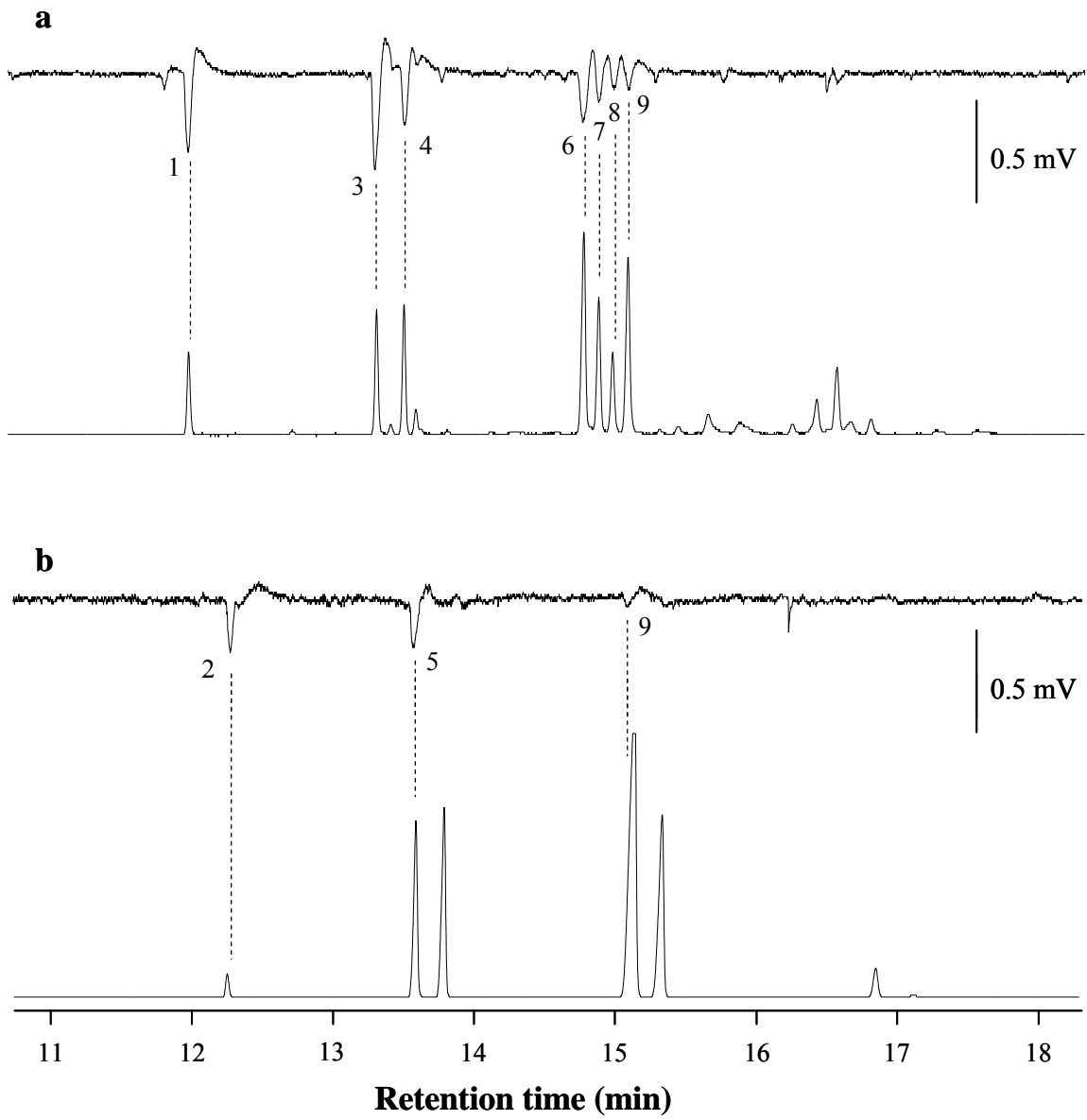
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