Quantitative Analysis of Alkaloidal Constituents in Imported Fire Ants by Gas Chromatography

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ABSTRACT: A method based on silica gel chromatography and GC-MS/GC-FID analyses was developed for the quantitation of alkaloidal compounds in imported fire ants, *Solenopsis richteri*, *S. invicta*, and their hybrid found in the southern United States. The *cis* and *trans* alkaloids from fire ant body extracts were successfully separated by silica gel chromatography, identified by GC-MS, and quantitated by GC-FID. Piperideine compounds were eluted together with the *cis* and *trans* piperidines, but were well-resolved on a nonpolar GC column. Eight pairs of piperidine isomers and 12 piperideines were quantitated. The ratios of *trans* alkaloids to corresponding *cis* isomers ranged from 87 to 378:1 in *S. invicta* and were significantly higher than in *S. richteri* and hybrid ants. The results were discussed in relation to the evolution of fire ant venom alkaloids and their role as host location cues for parasitic *Pseudacteon* phorid flies.

KEYWORDS: *quantitation, venom, piperidine, Solenopsis richteri, Solenopsis invicta, hybrid*

INTRODUCTION

In the southern United States, the four main species of *Solenopsis* fire ants are *S. geminata*, *S. xyloni*, *S. richteri*, and *S. invicta*. The first two species are native to the southeastern coastal plain of Florida to Texas and south through Central America to northern South America.[1] However, the last two species were introduced into the United States from South America through the port of Mobile, AL, in the early 20th century. All four species produce venoms containing various 2-methyl-6-alkyl (or alkenyl) piperidines,[2−5] and, significantly, the venom of each species possesses a characteristic alkaloidal "fingerprint".[6−8] These alkaloids are species-specific as the venom of each species has groups of similar compounds either dominating or almost completely lacking. Chemical profiles of venom alkaloids have been widely used as a taxonomic tool to define fire ant populations.[8]

Hybrid fire ants, *S. richteri* × *S. invicta*, which were discovered many years after the introduction of their parent species, were indistinguishable from the parent species solely on the basis of the morphological characters. Vander Meer et al.[9] first presented chemical evidence of the natural occurrence of hybridization between the two imported fire ant species as hybrids exhibited intermediate gas chromatographic profiles of both piperidine alkaloids and cuticular hydrocarbons.[10−12]

In addition to *cis* and *trans* 2-methyl-6-alkyl (or alkenyl) piperidines, a series of $\Delta^{1,2}$- and $\Delta^{1,6}$-2-methyl-6-alkyl (or alkenyl) piperideines have been recently identified from imported fire ant workers and alate queens.[13−18] Furthermore, $\Delta^{1,6}$-piperideines in the imported fire ants, *S. richteri*, *S. invicta*, and hybrids, showed significant interspecific variations very similar to those of the *trans* 2,6-dialkylpiperidines.[14]

Previous studies have mainly focused on identification of alkaloidal components from *Solenopsis* fire ants by using a GC-MS technique. To the best of our knowledge, however, no

proper analytical method has been developed that permits a detailed quantitative analysis of fire ant venom alkaloids. In the present study, GC-FID was used for quantitation of venom alkaloids because of low detection limits, wide linearity range, and general utility for various analytes. GC-MS was used for discrimination of alkaloidal components instead of quantitative analysis because the MS detector response that requires calibration for each individual compound is less uniform and less convenient for quantitation purposes compared to the FID. A primary goal of this work was to establish a routine analysis procedure to determine alkaloidal components of *Solenopsis* fire ants. A comparative study of the venom alkaloids in *S. richteri*, *S. invicta*, and hybrid fire ants was undertaken to further assess the distribution of these 2,6-disubstituted piperidines and piperideines. The results may contribute to an understanding of the evolution of fire ant venom alkaloids and mechanisms of host specificity and preference in *Pseudacteon* phorid flies, which are important biological control agents of imported fire ants.

MATERIALS AND METHODS

Reagents and Standard. HPLC grade *n*-hexane used for extraction and purification of the venom alkaloids was purchased from CNW Technologies GmbH (Düsseldorf, Germany). Anhydrous sodium sulfate, acetone, and triethylamine purchased from Beijing Chemical Reagent (Beijing, China) were of analytical reagent grade. Acetone and triethylamine were redistilled before use. Silica gel (300–400 mesh) for column chromatography was obtained from the Qingdao Marine Chemical Factory (Qingdao, China). 4-(2,6-
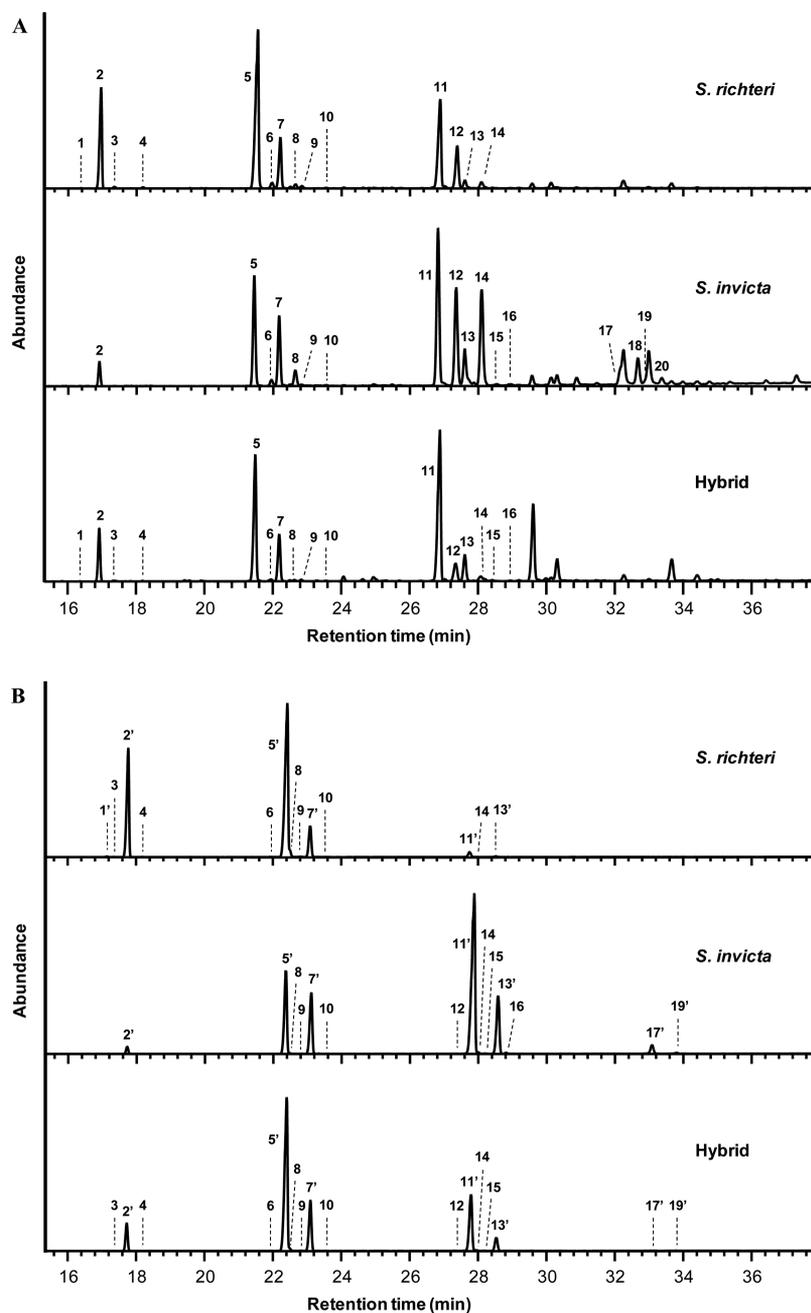
Figure 1. Typical GC-FID traces of the (A) *cis* and (B)*trans* alkaloid fractions.

Dimethyl-1-piperidinyl)-1-phenyl-1-butanone purchased from Sigma-Aldrich (St. Louis, MO, USA) was used as external standard for GC-FID analysis.

Collection and Identification of Fire Ant Colonies. Colonies of the black imported fire ant, *S. richteri* Forel, the red imported fire ant, *S. invicta* Buren, and their hybrid, *S. richteri* × *S. invicta*, were used in this test. We collected colonies of *S. richteri* in northern Mississippi and western Tennessee and colonies of *S. invicta* in Alabama (Auburn University campus) in mid-November 2011. At the same time, hybrid fire ant colonies were collected from northern Alabama and northeastern Mississippi, where hybrids are known to occur almost exclusively. Each colony containing workers, alates, broods, and queens was transferred in 1 gallon Rubbermaid plastic jars coated with Fluon to prevent escape. All colonies were reared in the laboratory and fed with 10% sugar water and crickets.

The identity of fire ant colonies was confirmed to be *S. richteri*, *S. invicta*, or hybrid fire ants by GC analysis using both alkaloid and hydrocarbon characters as previously reported.[17,19] Briefly, about 50 workers selected randomly from each colony were soaked in 1 mL of hexane for 2 h. A small aliquot (1 μL) of the hexane extract was injected into an Agilent 7890A GC equipped with a 30 m × 0.25 mm i.d., 0.25 μm, HP-5ms capillary column (Agilent Technologies) in splitless mode. Nitrogen was used as carrier gas at a flow rate of 2 mL/min. The injection port and detector temperatures were set at 270 and 280 °C, respectively. The GC oven was programmed at a rate of 15 °C/min from 90 to 270 °C, with a 2 min initial time and a 16 min final holding time.

Sample Preparation for Quantitative Analyses. A total of 32 colonies were analyzed in this study, including 8 colonies of *S. richteri*, 8 colonies of *S. invicta*, and 16 colonies of hybrids. Alkaloids were extracted and isolated from fire ant samples as previously reported.[15−17] For each colony, ant workers (1 g) were killed by freezing and extracted with hexane (enough hexane to cover ant bodies, ~3.5 mL) for 24 h. The extract was dried over anhydrous

Table 1. Quantitation of Alkaloids from *Solenopsis* Fire Ants

peak	structure	name	amount (μg/g $\pm$ SE)		
			S. richteri	*S. invicta*	hybrid
cis alkaloids					
1		*cis*-C11:1[a]	+[b]		
2		*cis*-C11	81.16 $\pm$ 12.28 a	1.48 $\pm$ 0.30 b	17.78 $\pm$ 2.76 b
5		*cis*-C13:1	213.52 $\pm$ 33.97 a	16.25 $\pm$ 1.88 c	85.78 $\pm$ 7.73 b
7		*cis*-C13	46.63 $\pm$ 7.59 a	8.99 $\pm$ 1.28 b	24.30 $\pm$ 3.76 b
11		*cis*-C15:1	76.91 $\pm$ 11.71 ab	26.29 $\pm$ 3.26 b	151.84 $\pm$ 23.85 a
13		*cis*-C15	10.58 $\pm$ 1.72 b	6.26 $\pm$ 0.82 b	21.53 $\pm$ 3.02 a
17		*cis*-C17:1		0.75 $\pm$ 0.15	
19		*cis*-C17		0.27 $\pm$ 0.05	
trans alkaloids					
1'		*trans*-C11:1	10.37 $\pm$ 1.73		
2'		*trans*-C11	1851.85 $\pm$ 322.12 a	87.15 $\pm$ 11.76 b	510.58 $\pm$ 58.98 b
5'		*trans*-C13:1	5618.02 $\pm$ 628.26 a	1997.26 $\pm$ 195.72 c	3844.62 $\pm$ 326.78 b
7'		*trans*-C13	809.73 $\pm$ 96.40 b	1293.62 $\pm$ 141.51 a	966.70 $\pm$ 92.04 ab
11'		*trans*-C15:1	284.56 $\pm$ 48.61 c	4257.75 $\pm$ 315.05 a	1485.38 $\pm$ 162.63 b
13'		*trans*-C15	44.79 $\pm$ 12.77 c	1470.16 $\pm$ 152.36 a	332.38 $\pm$ 50.30 b
17'		*trans*-C17:1		223.45 $\pm$ 34.64 a	17.36 $\pm$ 2.91 b
19'		*trans*-C17		24.89 $\pm$ 3.40 a	7.04 $\pm$ 1.28 b

Table 1. continued

peak	structure	name	amount (μg/g ± SE)		
			S. richteri	*S. invicta*	hybrid
piperideines					
3	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_{10}$CH$_3$	$\Delta^{1,6}$-C11	5.94 ± 0.74 a		0.42 ± 0.09 b
4	H$_3$C—N=(CH$_2$)$_{10}$CH$_3$	$\Delta^{1,2}$-C11	4.09 ± 0.63 a		0.25 ± 0.05 b
6	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_3$CH=CH(CH$_2$)$_7$CH$_3$	$\Delta^{1,6}$-C13:1	15.82 ± 1.34 a	2.75 ± 0.56 c	8.14 ± 1.05 b
8	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_{12}$CH$_3$	$\Delta^{1,6}$-C13	5.99 ± 0.81 a	4.73 ± 0.80 ab	3.39 ± 0.39 b
9	H$_3$C—N=(CH$_2$)$_3$CH=CH(CH$_2$)$_7$CH$_3$	$\Delta^{1,2}$-C13:1	18.54 ± 2.16 a	2.30 ± 0.26 c	7.43 ± 0.93 b
10	H$_3$C—N=(CH$_2$)$_{12}$CH$_3$	$\Delta^{1,2}$-C13	4.14 ± 0.42 a	2.50 ± 0.30 b	3.27 ± 0.38 ab
12	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_5$CH=CH(CH$_2$)$_7$CH$_3$	$\Delta^{1,6}$-C15:1	57.86 ± 7.67 b	26.73 ± 3.29 b	122.30 ± 15.64 a
14	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_{14}$CH$_3$	$\Delta^{1,6}$-C15	17.97 ± 2.96 b	21.81 ± 3.69 b	54.86 ± 4.44 a
15	H$_3$C—N=(CH$_2$)$_5$CH=CH(CH$_2$)$_7$CH$_3$	$\Delta^{1,2}$-C15:1		8.03 ± 0.82	5.87 ± 0.76
16	H$_3$C—N=(CH$_2$)$_{14}$CH$_3$	$\Delta^{1,2}$-C15		4.36 ± 0.31 a	0.88 ± 0.14 b
18	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_7$CH=CH(CH$_2$)$_7$CH$_3$	$\Delta^{1,6}$-C17:1		5.91 ± 1.11	
20	H$_3$C$^{\prime\prime}$—N—(CH$_2$)$_{16}$CH$_3$	$\Delta^{1,6}$-C17		1.46 ± 0.24	

[a]Carbon numbers have been widely used to represent the trivial names of alkaloids found in *Solenopsis* fire ants, e.g., in *cis*-C11:1, with 11 referring to the chain length and 1 referring to the number of double bonds.[6] [b]Trace amount.

sodium sulfate, concentrated to 0.5 mL under a mild stream of N$_2$, and then loaded onto a 17 mm (o.d.) chromatography column (20 g of silica gel). The column was eluted with hexane containing 2% triethylamine and stepwise increasing amounts of acetone (hexane/acetone ratio ranging from 50:1 to 10:1) to obtain the alkaloids. The chemistry of each collection (ca. 2 mL) was analyzed by gas chromatography (GC) on an Agilent 7890A GC equipped with a flame ionization detector (FID) under the same GC conditions described above. The collections were pooled on the basis of changes observed in the GC chromatograms of each collection to obtain two major alkaloid fractions: *cis* and *trans* alkaloid fractions. The two alkaloid fractions of each colony were concentrated to a certain volume for GC-MS and GC-FID analyses. As imported *Solenopsis* fire ant workers contain large amounts of *trans* alkaloids compared to corresponding *cis* alkaloids, the final volume of the *trans* alkaloid fraction was 20 times more than that of the *cis* alkaloid fraction prior to GC injection to achieve proper concentrations of *trans* alkaloids for

quantitation, that is, 2 mL for the *cis* alkaloid fraction and 40 mL for the *trans* alkaloid fraction.

A stock solution of standard compound, 4-(2,6-dimethyl-1-piperidinyl)-1-phenyl-1-butanone (2000 ng/μL), was prepared and then diluted to a series of concentrations ranging from 0.39 to 400 ng/μL. All dilutions were transferred to the GC autosampler, and 1 μL of each dilution was used for GC-FID analysis.

GC-MS and GC-FID Analysis. GC-MS analyses of alkaloid fractions were performed on an Agilent 7890A GC coupled to a 5975C mass selective detector, with an HP-5ms capillary column as described above. Helium was used as carrier gas at a flow rate of 1 mL/min. Injections (1 μL) were made in the splitless mode at an injector temperature of 230 °C. The GC oven temperature was programmed from 90 °C (isothermal for 1 min) to 160 °C at 10 °C/min, then to 250 °C at 3 °C/min, and held for 2 min. Total run time was 40 min. The transfer line temperature was set at 250 °C. Mass spectra were obtained using electron impact (EI, 70 eV). The chemical identities of alkaloids were determined by analysis of their mass spectra, as well as

by comparison of diagnostic ion fragments with published results on *Solenopsis* fire ants.[15−18]

Quantitative chromatographic analyses were carried out with the Agilent 7890A GC equipped with an FID. Nitrogen at a flow rate of 1 mL/min was used as the carrier gas. The GC conditions including the dimensions of capillary column, oven temperature program, and injection operation were the same as those for GC-MS. The detector temperature was set at 260 °C.

Statistical Analysis. A standard curve was calculated by linear regression analysis. The concentrations of the identified venom alkaloids were calculated against the standard curve. The absolute abundances of the alkaloids were analyzed using the PROC GLM procedure of one-way analysis of variance (ANOVA) followed by the Tukey−Kramer HSD comparison test ($P < 0.05$) to establish significant differences among the three fire ant species (we consider hybrid as a species in this study).[20] A PROC TTEST procedure ($P < 0.05$) was used to establish significant difference between, if applicable, any two of the three fire ant species.

RESULTS AND DISCUSSION

Fire ant venom alkaloids were readily extracted out of ant worker bodies by hexane. As previously reported,[15−17] the venom alkaloids of fire ant extract were separated into two alkaloid fractions (*cis* and *trans* alkaloid fractions) by silica gel chromatography, with the *cis* stereoisomers always being eluted before the corresponding *trans* stereoisomers. The two alkaloid fractions were then subjected to GC-MS analysis, and the chemical identities of alkaloidal components were determined by comparing mass spectrum and retention time with published data.[15−17] This routine procedure can be applied to the analysis of alkaloids from any *Solenopsis* or related alkaloid-containing ant species.

Typical GC-FID traces of the *cis* and *trans* alkaloid fractions of fire ant samples are shown in Figure 1. Both GC patterns of the two alkaloid fractions are species-specific. Almost all of the peaks show interspecific variations. Furthermore, both *cis* and *trans* alkaloids from hybrids show characteristics intermediate to those of the two parental species. A total of eight pairs of *cis* and *trans* alkaloids, those being **1−1′**, **2−2′**, **5−5′**, **7−7′**, **11−11′**, **13−13′**, **17−17′**, and **19−19′**, were identified by GC-MS on the basis of mass spectra with characteristic base peak *m/z* 98, molecular ion [M⁺], and [M − 15]⁺,[2,3,5,6] meaning that there were eight pairs of diastereoisomers isolated from the three fire ant species (Table 1). Peaks 2, 5, 7, 11, 13 and their corresponding *trans* stereoisomers were found in all ant samples. However, peaks 1 and 1′ were not found in *S. invicta* samples, whereas peaks 17, 19, and their corresponding *trans* stereoisomers were not found in *S. richteri* samples. Peaks 17′ and 19′ were detected in trace amounts in the *trans* alkaloid fraction of hybrids, but their counterparts in the *cis* alkaloid fraction of hybrids were not detectable.

In both alkaloid fractions, two series of piperideines were found. $\Delta^{1,6}$-Piperideines, **3**, **6**, **8**, **12**, **14**, **18**, and **20**, were identified by characteristic mass ions *m/z* 96, 111, [M⁺], and [M − 15]⁺, whereas $\Delta^{1,2}$-piperideines, **4**, **9**, **10**, **15**, and **16**, were identified by characteristic mass ions *m/z* 96, 97, 110, [M⁺], and [M − 15]⁺.[6,13,15−17] As reduction of these piperideines to piperidine was proposed to be the final step of biosynthesis of piperidine alkaloids in *Solenopsis* fire ants, the absolute configuration of piperideines was established as (2*R*) or (6*R*).[15,21,22] GC trace of the *cis* alkaloid fraction had distinctive $\Delta^{1,6}$-piperideine peaks, mainly 12, 14, and 18 (for instance, in *S. invicta*), but fairly visible $\Delta^{1,2}$-piperideine peaks. GC traces of the *trans* alkaloid fraction also had $\Delta^{1,6}$-piperideine and $\Delta^{1,2}$-

piperideine peaks in trace amounts. The intermolecular hydrogen bonding between nitrogen (−N=) of piperideine and hydrogen (H−N<) of piperidine might cause the inseparability of piperideines and piperidines and the slow elution of piperideines in the *trans* alkaloid fraction. The fact that we did not detect any alkaloidal components in the chromatographic collections between the *cis* and *trans* alkaloid fractions also supports this interpretation. The GC profile of piperideines in the *cis* alkaloid fraction of hybrids showed intermediate characteristics similar to those of the *cis* and *trans* alkaloids.

The external standard method was used to obtain the regression equation. We used log-transformed data to construct a calibration curve, $\log(A) = 0.9894 \log(C) + 1.2551$, where A is the peak area and C is the concentration of the standard compound (ng/μL). The selected piperidine standard showed good linearity ($r^2 = 0.9996$, $P < 0.001$) in relatively wide concentration ranges. Then the obtained calibration curve was used to determine alkaloidal components in all ant samples (Table 1).

Among the *cis* alkaloids, *cis*-C13:1 (peak 5) in *S. richteri* had the highest amount, 231.52 ± 33.97 μg/g, of ant workers. All three ant species had large amounts of shared *trans* alkaloids. *trans*-C13:1 (peak 5′) was quantitatively the most dominant component with 5618.02 ± 628.26 μg/g in *S. richteri*, whereas *trans*-C15:1 (peak 11′) was quantitatively the major component with 4257.75 ± 315.05 μg/g in *S. invicta*. These two components were both predominating in hybrids. The amounts of piperideines were all relatively low in the three ant species. In most cases, the amounts of these piperideines were under 10 μg/g of worker ants. The results of the statistical analysis showed that significant differences were detected for all peaks with only one exception, $\Delta^{1,2}$-C15:1 (peak 15), suggesting that there exists interspecific variation for all *cis* and *trans* alkaloids and piperideines. For any given alkaloidal component, the amount in hybrids was always intermediate to that of *S. richteri* and *S. invicta*. Although in some cases there was statistical nondifference between hybrids and the parental species, the amount of hybrids was always numerically either higher or lower than that of any parental species. Clearly, wide variation in alkaloid chemistry in hybrids resulted in the statistical indifference. This is why, in the current study, the number of hybrid colonies investigated was 2 times higher than that of the parental species. Peaks 11−14, however, were exceptions showing that these were higher in the hybrid than in both parental species. This inconsistency complicated the alkaloid chemistry of hybridization.

It has been previously hypothesized that the venom of ancestral *Solenopsis* species was simple (i.e., contained only a few components) and the evolution of venom alkaloids would have begun with a condition in which the venom contained abundant C11 and minute traces of C13, with the *cis* isomers dominating and the *trans* isomers at a much lower level.[23] The evolution of the alkaloidal compounds in *Solenopsis* could involve two steps: a switch from a probable normal production ratio of *cis*- and *trans*-C11 (**2** and **2′**), to a thermodynamically highly unfavorable decrease in *cis* and an increase in *trans* isomers and further evolution of abundant *trans*-C13 (**7′**) and *trans*-C13:1 (**5′**) and then the addition of *trans*-C15 (**13′**), *trans*-C15:1 (**11′**), *trans*-C17 (**19′**), and *trans*-C17:1 (**17′**), accompanied by a reduction in the production of *trans*-C11 (**2′**).[16,23,24] We first calculated the mean ratio of *trans* to *cis* alkaloids for *S. richteri*, *S. invicta*, and their hybrid (Table 2).

Table 2. Mean Ratio of *trans* Alkaloids to *cis* Alkaloids

no. of carbons	S. richteri	S. invicta	hybrid
unsaturated side chain			
C11	×[a]		
C13	32.33 ± 6.14 b	133.13 ± 16.92 a	47.76 ± 4.43 b
C15	4.00 ± 0.71 b	176.79 ± 21.22 a	13.31 ± 2.40 b
C17		377.65 ± 90.05	×
saturated side chain			
C11	28.31 ± 6.80 b	87.37 ± 24.23 a	38.21 ± 7.60 b
C13	20.37 ± 3.35 b	160.20 ± 22.06 a	50.25 ± 5.68 b
C15	4.17 ± 0.71 b	261.47 ± 38.62 a	17.65 ± 2.78 b
C17		110.61 ± 21.65	×
both unsaturated and saturated side chains			
C11	28.47 ± 6.84 b	87.37 ± 24.23 a	38.21 ± 7.60 b
C13	30.10 ± 5.44 b	142.70 ± 17.92 a	48.00 ± 4.49 b
C15	3.99 ± 0.68 b	192.47 ± 22.82 a	13.86 ± 2.46 b
C17		305.88 ± 73.42	×

[a]Only *trans* alkaloids were detected.

The ratios of *trans* to *cis* alkaloids in *S. invicta* were significantly greater than in *S. richteri*, and the ratios in hybrids were numerically but not significantly greater than in *S. richteri*, indicating that this character in hybrids was intermediate to both parental species. The ratios for combined alkaloids with the same length of unsaturated and saturated side chains in *S. invicta* ranged from 87.37 to 305.88, suggesting that *trans* alkaloids were extremely predominant in this ant species. Brand et al.[23] gave rough estimates of the ratio of *trans*-C11 (**2'**), to *cis*-C11 (**2**), collected from 10 individuals of *S. richteri* and of *S. invicta*, which were all 10:1. In this study, we present data calculated from 1 g of pooled ant samples. The ratios of *trans*-C11 (**2'**), to *cis*-C11 (**2**), for *S. richteri* and *S. invicta*, were 28.31 ± 6.80, and 87.37 ± 24.23, respectively, which were much

greater than the reported data.[23] The venoms of several species native to the United States, *Solenopsis aurea*, *S. xyloni*, and *S. geminata*, consisted mainly of *cis*-C11 (**2**) and *trans*-C11 (**2'**). The ratio of *trans*-C11 (**2'**), to *cis*-C11 (**2**), in pooled samples of the first two species was about 1:4, whereas it was 1:1.2−1.5 in the latter species.[7,8,23,25] On this basis, *S. aurea*, *S. xyloni*, and *S. geminata* would appear to be the more primitive species, whereas *S. richteri* and *S. invicta* are relatively newer species. The venom of *Solenopsis eduardi* (a close relative of *S. geminata*) collected from Colombia consisted of > 98% *trans*-C11 (**2'**) and about 1% of the corresponding *cis* isomer (**2**),[8] which represented a major shift from *cis*-C11 (**2**) to *trans*-C11 (**2'**). Furthermore, the ratios of *trans* alkaloids to *cis* alkaloids for *S. invicta* were significantly higher than for *S. richteri*, suggesting that *S. invicta* is a much newer species than *S. richteri*.

Second, we calculated the total alkaloids with the same side-chain length at the 6-position of the piperidine and/or piperideine ring (Table 3). The *cis* alkaloids and piperideines were minor components, whereas the *trans* alkaloids were major components. Furthermore, the *cis* alkaloids and piperideines in *S. richteri* were relatively higher than in *S. invicta*. The *trans* alkaloids C11 (**2'**) and C13 (**5'** and **7'**) were predominant in *S. richteri*, whereas the *trans* alkaloids C13 (**5'** and **7'**) and C15 (**11'** and **13'**) were predominant in *S. invicta*. Hybrids showed intermediate characteristics between the two parental species. However, there were two exceptions, *cis*-C15 (including *cis*-C15 (**13**) and *cis*-C15:1 (**11**)) and piperideines C15 (including **12**, **14**, **15**, and **16**). Hybrids had significantly greater amounts of *cis*-C15 (**11** + **13**) and piperideines C15 (**12** + **14** + **15** + **16**) than *S. richteri* and *S. invicta*, which possibly resulted from wide variations in alkaloid chemistry among hybrid colonies. Data from Table 3 clearly demonstrated the dominance of *trans*-C13 (**5'** + **7'**) along with the addition of *trans*-C15 (**11'** + **13'**) and *trans*-C17 (**17'** + **19'**), accompanied by a reduction in the production of *trans*-C11 (**2'**).

Table 3. Total Amounts of Alkaloids with the Same Side Chain Length at the 6-Position of the Piperidine Ring

	amount (μg/g ± SE)		
no. of carbons	S. richteri	S. invicta	hybrid
cis alkaloids			
C11	81.16 ± 12.28 a	1.48 ± 0.30 b	17.78 ± 2.76 b
C13	260.15 ± 40.70 a	25.24 ± 3.01 c	110.08 ± 10.83 b
C15	87.49 ± 13.26 b	32.55 ± 4.04 b	173.36 ± 26.49 a
C17		1.01 ± 0.20	
trans alkaloids			
C11	1862.22 ± 323.69 a	87.15 ± 11.76 b	510.58 ± 58.98 b
C13	6427.74 ± 676.80 a	3290.88 ± 272.96 c	4811.32 ± 345.47 b
C15	329.35 ± 60.03 c	5727.91 ± 416.95 a	1817.76 ± 209.78 b
C17		248.34 ± 37.18 a	24.40 ± 3.86 b
piperideines			
C11	10.03 ± 1.21 a		0.68 ± 0.13 b
C13	44.49 ± 3.24 a	12.29 ± 1.28 c	22.24 ± 2.02 b
C15	75.83 ± 10.36 b	60.93 ± 7.10 b	183.92 ± 18.85 a
C17		7.37 ± 1.32	
total alkaloids[a]			
C11	1953.40 ± 320.55 a	88.63 ± 11.71 b	529.03 ± 60.89 b
C13	6732.39 ± 657.22 a	3328.41 ± 273.44 c	4943.64 ± 352.69 b
C15	492.67 ± 75.13 c	5821.38 ± 420.66 a	2175.04 ± 216.92 b
C17		256.72 ± 37.19 a	24.40 ± 3.86 b

[a]Total alkaloids mean sum of the *cis*, *trans* piperidines and piperideines with the same side chain length at the 6-position of the piperidine and piperideine rings.

Third, we calculated the total amounts of the *cis* and *trans* alkaloids and all alkaloids detected from all three ant species (Figure 2). *S. invicta* had a significantly lower amount of *cis*
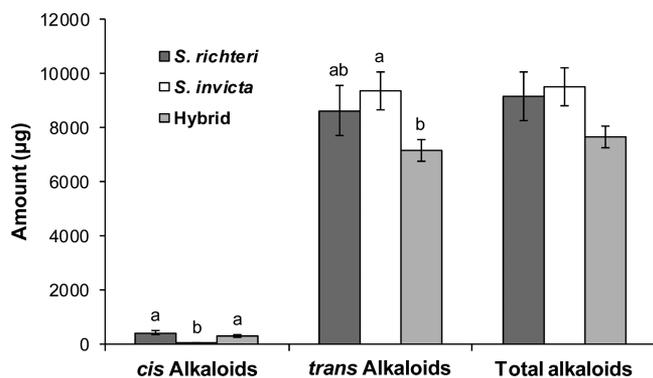

Figure 2. Amounts of alkaloids isolated from 1 g of ant workers. Total alkaloids are the sum of *cis* alkaloids, *trans* alkaloids, and piperideines. Means for same alkaloid type having no letter in common are significantly different ($P < 0.05$, Tukey−Kramer HSD test).

alkaloids than *S. richteri* and hybrids. In contrast, *S. invicta* had a significantly greater amount of *trans* alkaloids than hybrids and a relatively, but not significantly, greater amount than *S. richteri*. Hybrids produced a slightly lower amount of total alkaloids compared to the two parental species. The total amounts of alkaloids produced by 1 g of ant workers of the three species ranged from 8 to 10 mg, meaning that ant workers can produce up to 1% alkaloids of their body weight. The order of total amounts of alkaloids was *S. invicta* > *S. richteri* > hybrids even though the differences were not statistically significant (Figure 2). If the production of venom alkaloids represents competition ability of an ant species, a relatively lower production of total alkaloids in hybrids would decrease their fitness. It has been suggested that hybrid fire ants have reduced relative fitness compared to their parent species due to intrinsic selection.[26] Aggression toward heterospecifics has been used as a measure of fitness among sympatric congeneric species.[27] It was found that *S. invicta* had the lowest aggression threshold, whereas *S. richteri* was the least aggressive.[19] Moderate aggression threshold of hybrids might be related to intermediate chemistry in venom alkaloids shown in this study.

Piperideine alkaloid was first found in *S. xyloni* as $\Delta^{1,2}$-C11 (**4**).[6] Recently, several studies have reported the identification of a series of $\Delta^{1,2}$- and $\Delta^{1,6}$-piperideine alkaloids from the workers and alate queens of the imported fire ants.[13−18] The $\Delta^{1,2}$- and $\Delta^{1,6}$-piperideines have been proposed to function as precursors for biosynthesis of *cis* and *trans* alkaloids in fire ant venom.[15,22] Reduction of $\Delta^{1,6}$-piperideine or $\Delta^{2,3}$-piperideine gave a ratio of the *cis*-C11 to the *trans*-C11 of 4:1.[28,29] The composition of *cis*- and *trans*-C11 (**2** and **2′**), in *S. aurea* matched that of the equilibrium mixture formed during their chemical synthesis in the laboratory. This matchup supports the above hypothesis on the biosynthetic function of piperideines. As the imported *Solenopsis* fire ants have the biosynthetic capacity to synthesize mainly *trans* alkaloids, it is likely that enantioselective enzymes are present in these species which can reduce $\Delta^{1,2}$-piperideines exclusively into (2*R*,6*R*) dialkylpiper-idines and $\Delta^{1,6}$-piperideines mainly into (2*R*,6*R*) and partially into (2*R*,6*S*) dialkylpiperidines. In most cases, the amount of $\Delta^{1,2}$-piperideine was similar to that of corresponding $\Delta^{1,6}$-piperideine (same side-chain length at the 6-position) (data

shown in Table 1), suggesting that the two reduction routes are equally important in the biosynthesis of alkaloids in the imported fire ants.[15] In contrast, the detection of only $\Delta^{1,2}$-C11 (**4**) in *S. xyloni* together with a higher ratio of *cis*-C11 (**2**) to *trans*-C11 (**2′**) found in this species supported the hypothesis that the $\Delta^{1,2}$-C11 pathway could be more important than the $\Delta^{1,6}$-C11 pathway in more primitive *Solenopsis* species. Therefore, all *Solenopsis* fire ants may utilize similar biosynthetic pathways to produce both *cis* and *trans* alkaloids and even maintain favorable stereochemical composition diverging rather markedly from the thermodynamic equilibrium mixture.[15,16,22] Both $\Delta^{1,6}$-C17:1 (**18**) and $\Delta^{1,6}$-C17 (**20**) were found only in *S. invicta*, suggesting that this species possibly has evolved specific enzymes that could synthesize piperidines with longer and unsaturated side chains associated with greater toxicity and biological advantage.[8]

Pseudacteon phorid flies (Diptera: Phoridae) are parasitoids of ants, and many species are specific to *geminata* and/or *saevissima* species groups of the genus *Solenopsis*.[30] Nineteen of these parasitic flies are specifically associated with the *saevissima* group[31] and generally show an attack preference for imported fire ants over native fire ants.[32−40] Several phorid fly species, *Pseudacteon tricuspis*, *P. curvatus*, *P. litoralis*, *P. obtusus*, and *P. cultellatus*, have been introduced to the southern United States for biological control of the invasive imported fire ant complex in the past two decades.[41] Established populations of *P. tricuspis* have proved to be highly host specific to imported *Solenopsis* fire ants.[42] Previous studies have demonstrated that *P. tricuspis* utilizes fire ant venom alkaloids for host location.[43,44] In addition to five *cis* alkaloids (*cis*-C11 (**2**), *cis*-C13:1 (**5**), *cis*-C13 (**7**), *cis*-C15:1 (**11**), and *cis*-C15 (**13**)) and two *trans* alkaloids (*trans*-C11 (**2′**) and *trans*-C13:1 (**5′**)), two piperideine alkaloids, $\Delta^{1,6}$-C15:1 (**12**) and $\Delta^{1,6}$-C15 (**14**) from *S. invicta* were shown to elicit significant electrophysiological response in *P. tricuspis* by GC-EAD analysis. As shown in Figure 1 and Table 1, the quantities of these two $\Delta^{1,6}$-piperideines were relatively greater than those of the remaining piperideines. $\Delta^{1,6}$-C15:1 (**12**) and $\Delta^{1,6}$-C15 (**14**) from *S. richteri* and hybrids could also trigger a similar GC-EAD response in *P. tricuspis* (unpublished data). Dominance of the *cis* alkaloids suggests possible attraction of *P. tricuspis* to North America-native fire ants such as *S. xyloni* and *S. geminata*. *P. tricuspis* is frequently attracted to disturbed ant colonies, and venom release is part of the alarm response and probably a component of the complex alarm odor.[45] A recent study has proved that *P. tricuspis* was attracted to 2-ethyl-3,6 (or 5)-dimethylpyrazine, a commercially available mixture of fire ant alarm pheromone component (2-ethyl-3,6-dimethylpyrazine) and its isomer.[46] The alarm pheromone and venom alkaloids proved to act in concert to attract phorid flies to fire ant workers.[43] It is conceivable that the highly volatile pyrazine alarm pheromone serves as a general host location cue from long range, whereas the larger and less volatile venom alkaloids may play a major role in host preference and discrimination at close range.[43,46] As pyrazine alarm pheromone is a general odor in *Solenopsis* fire ants (R. K. Vander Meer, personal communication), it seems unlikely that phorid flies use ant alarm pheromone for host preference and discrimination.

Different *Pseudacteon* species display different levels of host specificity. Most fly species showed very low attack rate when tested in laboratory conditions against the native fire ant *S. geminata*. However, *P. curvatus* and *P. borgmeieri* freely attacked both *S. invicta* and *S. geminata*. Both fly species developed in *S.*

geminata but with apparent low success.[47] A recent study has demonstrated that *P. curvatus* preferred the alkaloid mixture (*cis* + *trans* alkaloids), whereas *P. tricuspis* showed relatively greater attraction to the *cis* alkaloids.[43] The alkaloid patterns, including the presence and quantity of individual alkaloid components, the ratios of the *trans* to *cis* alkaloids, and the presence of piperideines, are species-specific for *Solenopsis* fire ants. Attraction of *P. curvatus* and *P. borgmeieri* to *S. geminata* may be mediated by presence of the *cis* alkaloids, which generally predominate in native fire ants. A previous study showed that *P. tricuspis* is able to distinguish among *S. richteri*, *S. invicta*, and hybrid fire ants with greater preference for *S. invicta* and hybrids.[48] It is likely that the ratios of the *cis* and *trans* alkaloids, the presence of piperideines, and the quantitative difference in alkaloids detected in this study might be responsible for the reported host preference in phorid flies.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This article published June 11, 2014 with an error in Figure 1. The correct version published June 17, 2014.