



## Queen venom isosolenopsin A delivers rapid incapacitation of fire ant competitors



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### ABSTRACT

Fire ant venom contains insecticidal alkaloids named ‘solenopsins’. Whilst species-specific differences are reported, little attention has been given to caste-specific venom adaptations. The venom of fire ant queens has remained particularly poorly studied, though studies have shown it to be strikingly similar across different species, in being primarily composed of the alkaloid isosolenopsin A, regardless of the chemical configuration in workers. We predict that this is the evolutionary outcome of stabilising selection, implying that a shared mechanism is responsible for the conserved venom composition among fire ant queens. The present investigation tests whether venom plays a role in nest founding, when queens must succeed in isolation in the field against competitor species. Here, we report that fire ant queen venom and isosolenopsin A are faster to incapacitate alien ants than the venom of fire ant workers. Representative sympatric competitor ant species were selected and exposed on their heads to the venom of workers and queens of the invasive fire ant species *Solenopsis invicta* and *S. geminata*. Queen venom was found to incapacitate rival foragers quicker than worker venom. The effects were reproduced using synthetic solenopsins, establishing that solenopsin A analogues are particularly effective contact neurotoxins. Overall, the venom of *S. invicta* is more lethal than of *S. geminata*, regardless of the incapacitation speed. We believe these are fundamental aspects of the chemical ecology of the invasive ants which remain overlooked, and emphasise the need for further studies into the venom biology of founding queens.

### 1. Introduction

Venom chemistry is shaped by the need for survival. The ecological diversity of ants is mirrored by an untapped diversity of venom toxins (Touchard et al., 2016). Although ants figure amongst the most abundant extant venomous arthropods, with about 16,000 extant species (Antwiki, 2015), venom chemistry is known for only a few species. For instance, the red imported fire ant *Solenopsis invicta* Buren is a particularly well-studied species of remarkable aggressiveness, capable of delivering painful stings (Tschinkel, 2006). Fire ant venom is composed of a complex mixture of alkaloids (> 90% v/v) (Fox, 2014), known as ‘solenopsins’, of marked insecticidal action (Blum, 1988; Lai et al., 2010). The structure of the most widely studied compound solenopsin A (aka *trans*-C11:0) is depicted in Fig. 1.

Caste specialisation is central to the biology of eusocial animals. However, venom caste specialisation has remained a largely unstudied topic. Fire ant workers present marked species-specific ratios of solenopsin analogues (Brand et al., 1973a,b). The venom of fire ant gynes

(i.e. winged females, or queens) is similar across different species (Tschinkel, 2006) as e.g. described with *S. invicta* (Brand et al., 1973a), *S. richteri* (Brand et al., 1973b), and two cryptic species within the nominal taxon *S. saevissima* (Fox et al., 2012). Fire ant gyne venoms capitalise on analogues of solenopsin A (Fig. 1), mainly on isosolenopsin A (henceforth ‘*cis*-C11’) (Brand et al., 1973a,b; Tschinkel, 2006). The reason behind this fixed chemical configuration has remained unclear (Brand et al., 1973a,b; Shi et al., 2015).

Nest founding is regarded as the most fragile stage in the life cycle of fire ant colonies (Tschinkel, 2006), as newly mated queens issuing from a nuptial flight must seek shelter to found new colonies. They must survive for approximately 5 weeks in absence of workers to protect them (Tschinkel, 2006; Markin et al., 1972). During this period, rival ant species are a major obstacle for the establishment of new fire ant colonies (Vinson and Rao, 2004; Rao and Vinson, 2004).

Within this context this investigation aimed at defining the underlying mechanisms behind the composition and effects of fire ant queen venom. Results demonstrate a remarkably fast incapacitating effect

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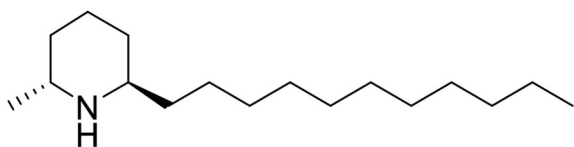


Fig. 1. Solenopsins are piperidinic alkaloids found in some ants species within *Solenopsis* and *Megalomyrmex*, hereby represented by the chemical structure of the fire ant venom alkaloid solenopsin A (formula  $C_{17}H_{35}N$ ).

from intoxication by fire ant gynes against rival ant species, where the major compound isosolenopsin A is responsible for the observed effects.

We hypothesised that stabilising selection impeded diversification of solenopsins composition in the queen caste across the different fire ant species. In such a scenario, queen venom would be playing a central role in fire ant biology.

## 2. Materials and methods

### 2.1. Animals

Colonies of the crazy ant *Paratrechina longicornis* L., acrobat ant *Crematogaster dohrni* Mayr, and ghost ant *Tapinoma nr. melanocephalum* F. were obtained from a fire ant-invaded area in Guangdong, P.R. China. These species are locally abundant, and represent ant genera typically observed in fire ant-infested areas elsewhere, e.g. in South America (personal experience of EGPF). Mature colonies of *S. invicta* and *Solenopsis geminata* F. were obtained from Beihai, Guangxi, P.R. China, and separated in the lab from the soil according with methods described in details elsewhere (e.g. Fox et al., 2013).

Fire ant species identification was based on characters proposed by Pitts et al. (2005), mainly: the presence or absence of a medial frontal streak in major workers; the presence or absence of a true soldier worker; and the body colour of males. Furthermore, local *S. geminata* are easily distinguishable from *S. invicta* within China based on colour patterns and the aggressiveness of major workers (supplementary images of majors available in Fox et al., 2018), in addition to differences in nest size and structure (Tschinkel, 2006). Species identifications were further supported by the chemical profiles (e.g. Fox et al., 2012; Shi et al., 2015). Voucher specimens are deposited at HSK University China, Hong Kong, P.R. China.

The ant colonies were conditioned inside ca. 50 × 25 cm plastic boxes with internal walls painted with anti adherent Fluon to prevent the insects from escaping. They were fed adult commercial *Locusta migratoria* L. every two or three days and provided with water ad libitum. Colonies were reared at fluctuating room conditions between 25 and 30 °C and relative humidity 55–90%, photoperiod L:D 14:10.

### 2.2. Venom extraction, purification, fractions

Venom from fire ants was extracted and fractions from *S. invicta* workers and gynes obtained from the conditioned colonies following methods detailed elsewhere (Fox et al., 2013; Shi et al., 2015, respectively). In short, colonies maintained in the laboratory as above described were first inspected to manually sort numerous gynes (> 1000) and workers (> 60,000). These were then immersed in pure HPLC-grade *n*-hexane with 1: 5 clean water for 12 h (Fox et al., 2013), after which the solvent was recovered and concentrated for subsequent purification of alkaloids and fractions through graded elutions with hexane: acetone in silica-gel columns (Shi et al., 2015). Through this method we obtained about 600 mg of pure alkaloid mixture, and about 200 mg of purified *cis* and *trans* fractions of each caste.

### 2.3. GC-MS analysis

Crude venom from fire ants was sampled from a single droplet from

the stinger to the inner wall of a 0.5 mm ID capillary tube and dissolved in 10 µg/mL nicotine in hexane as internal standard solution for relative dosage of peaks. Venom fractions (pure alkaloids, *cis*- and *trans*-alkaloids) were collected with a metal pin and similarly dissolved for relative analysis of peaks. GC-MS injections were performed as described in Fox et al. (2018) into an Agilent GC-7890B system coupled with an MS-5977B MSD by manual 1-µl injections. Obtained chromatograms were analysed with OpenChrom 1.2.0 Community Edition. External controls were nicotine and a mixture of hydrocarbons. Obtained GC-MS chromatograms and the method files can be found in Fox et al. (2018).

### 2.4. Synthetic alkaloids

Synthetic racemic solenopsins (Table S1) were purchased from WuXi AppTec (ShangHai, China) after synthesis according with Pianaro et al. (2012) for *cis* isomers and Herath and Nanayakkara (2008) for *trans* isomers. Synthetic racemic nicotine was purchased from Sigma. ‘Synthetic venoms’ were produced mimicking natural venoms by mixing the main components (i.e. those present at > 10% of the natural extract) present in gynes’ venom using precision syringes according to their relative proportions observed in *S. invicta* and *S. geminata*. Raw chromatograms of synthetic venoms and nicotine are available from Fox et al. (2018).

### 2.5. Venom bioassays

#### 2.5.1. Venom application

Methods were designed to simulate the natural effects of topical applications of venom. It should be emphasised that fire ants cannot introduce the stinger into the body of other hard-bodied insects of similar size because of insufficient leverage, as they must first bite in order to position their stinger. Therefore, fire ants fight other ants with their mandibles, and employ venom by contact application.

Ants in this study were anesthetized prior to manipulation using either CO<sub>2</sub> (*P. longicornis*, *T. melanocephalum*) or placing on ice packs (*C. dohrni*, *Solenopsis* spp.). Further details on the manipulation of each species is provided in Fox et al. (2018).

As bioassays were designed to emulate natural conditions, venom fractions had to be applied in volumes equivalent to venom droplets released by the ants. Venom droplet sizes from fire ant workers and gynes were measured with Measure Pictures v.1.0, and volume estimated by extrapolation to a perfect sphere using the formula

$$V = \frac{4\pi r^3}{3}$$

The effects of the venom of fire ant workers and gynes on other ants were assessed using two different approaches: (i) direct single droplet application using live excised gasters, and (ii) using venom alkaloid fractions or synthetic alkaloids as described in section 2.2 and 2.4. Droplets of venom fractions and synthetic compounds were applied using a finely stretched capillary tube attached with silicon glue to a glass precision 1-µl syringe (supplementary videos and images with further details in Fox et al., 2018). Single droplets were used and calibrated under a stereomicroscope with a micrometric eyepiece to ca. 20–30 nL, as estimated from diameter, while gently pressing the syringe plunger. This volume was selected to better emulate fire ant stings as based on Brand et al. (1973a). Pictures of venom droplets were measured. Droplets were applied either on the head, typically between the frons and mouthparts, unless stated otherwise; e.g. antennae. Intoxication was assessed by perceived altered behaviour (e.g. rubbing, self-venom application, agitation) which was always followed by a state of trembling paralysis, knockdown (section 2.5.2), and ultimately death (section 2.5.3).

Supplementary videos and other resources related to this article can be found at <https://doi.org/10.1016/j.toxicon.2018.11.428>.

**Table 1**

Perceived behavioural alterations within 1 h following topical venom application, venom fractions, and synthetic solenopsins from *Solenopsis* fire ants on three different species of competitor ants, given as tentative frequencies annotated during observations. Short videos examples of behavioural reactions are provided in Fox et al. (2018).

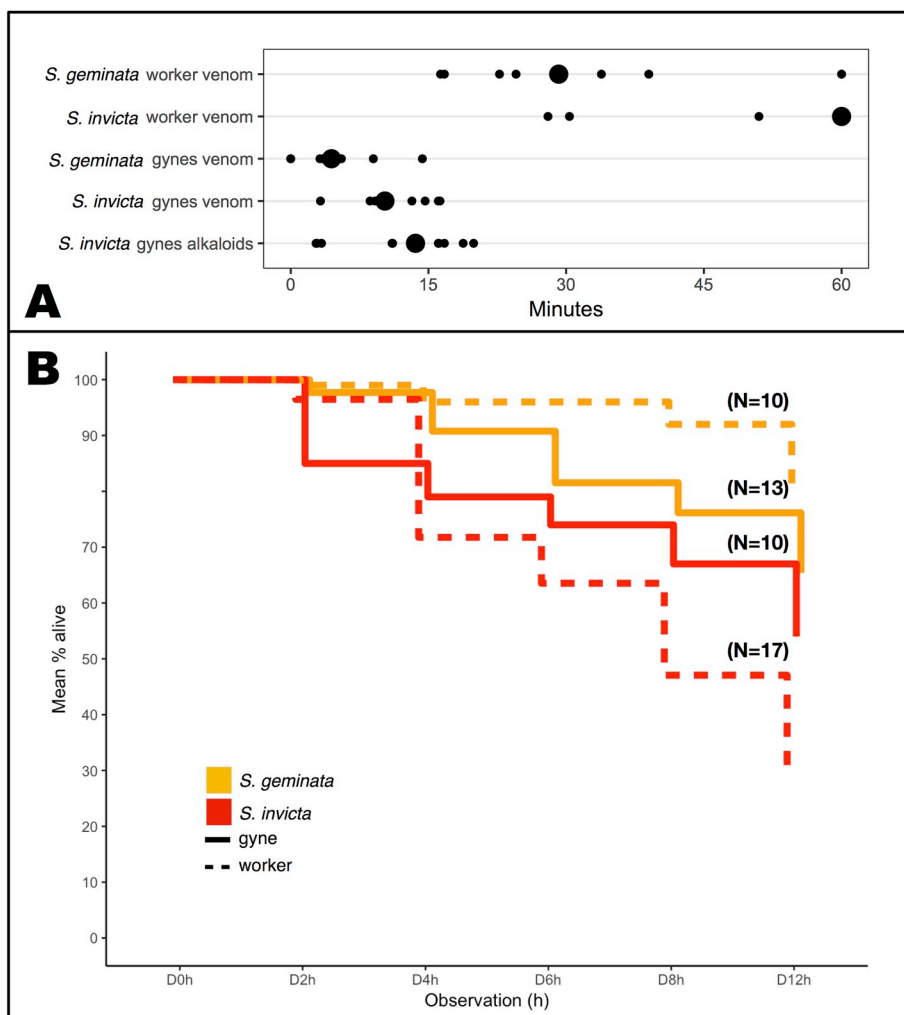
	Agitation	Rubbing	Detoxification	Grooming	No reaction
<b>Crude Venom</b>					
Gyne <i>S. geminata</i>	+	+		+	+++
Gyne <i>S. invicta</i>	+	++	+	++	
Worker <i>S. geminata</i>	+	+	+	++	+
Worker <i>S. invicta</i>		++	++	++	
<b>Cis Fraction</b>					
Gynes <i>S. invicta</i>	+	++	+	++	
Workers <i>S. invicta</i>	+			++	+++
C11		++	++	++	
C13	+++		+		++
C15	+++				+++
<b>Trans fraction</b>					
Gynes <i>S. invicta</i>		++		++++	
Workers <i>S. invicta</i>	+	+++	+	+	
C11	+	+++	++		
C13		+++	++	+	
C15	+	++		+	++
Nicotine	+	++	++	+	

2.5.2. Tests for incapacitation times by knockdown

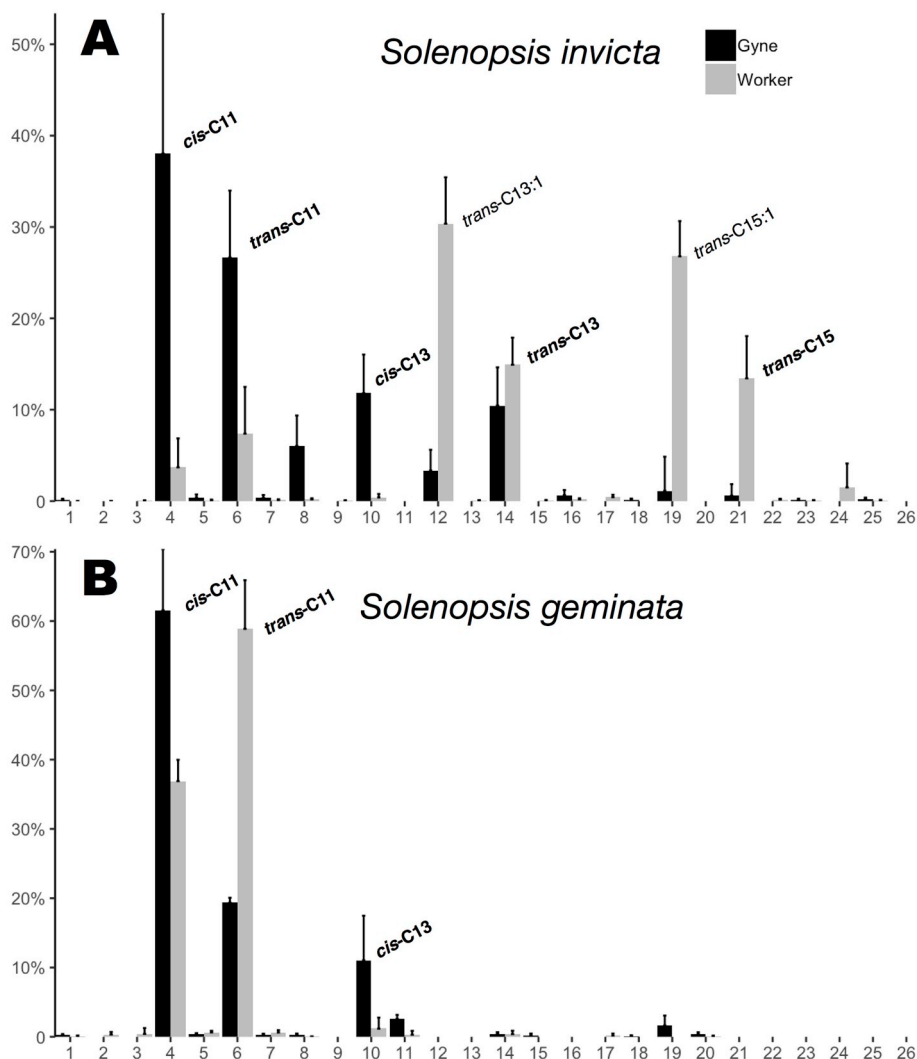
Assayed ants were placed individually in vertically-supported plastic 2 mL eppendorf tubes (n = 10) containing a 0.8 cm-wide rectangular strip of paper provided as a standing substrate. Two digital timers were set from the onset until the end of the tests from which individual KDt times laps were noted down each time an assayed ant lost contact with substrate and fell to the bottom of the tube. At the end of 60 min, all assayed ants were transferred to a Petri dish as described below to monitor mortality after 12 h.

2.5.3. Tests for mortality

Ants of either *P. longicornis*, *T. melanocephalum*, or *C. dohrni* were grouped (n = 10) inside 7-cm-wide acrilate Petri dishes provided with 2 mL drinking water, a centrifuge tube with cotton, and a 20 cm<sup>2</sup> piece of filter paper as substrate for walking and shelter. Their reactions were observed for 1 h under a ZEISS stereomicroscope followed by a rapid check every 2 h until 12 h. Plates were tipped to the side upon every check and any paralysed individuals failing to hang on to the substrate paper were recorded as either “incapacitated by knockdown” (if still moving) or “dead” (if clearly not moving). Negative controls included ants merely touched with a glass needle containing no chemicals and ants touched with a droplet of acetone. Positive controls included ants tested with synthetic nicotine and synthetic solenopsins.



**Fig. 2.** Speed until incapacitation by knockdown (A) and mean mortality (B) among workers of the crazy ant *Paratrechina longicornis* exposed on the head to a droplet of invasive fire ant venom in natura. Statistical analysis: (A) Each dot is an individual observation (may superpose). Quickest incapacitation is delivered by fire ant queens (chi-squared = 31.9394, df = 4, p-values < 0.01), while the slowest was delivered by *S. invicta* workers (p-values < 0.05) where it occurred; *S. invicta* crude venom had the same effect as *S. invicta* purified alkaloids (p-value = 0.3958). (B) N = numbers of repetitions. The venom of *S. geminata* workers was over time the least lethal of all (p-values = 0.02; 0.00; 0.00) based on the Kruskal-Wallis test (chi-squared = 52.5862, df = 3); the venom of *S. invicta* workers was the most lethal after 12 h (p-values = 0.00, 0.02, 0.00; chi-squared = 22.438, df = 3). Mortality from gynes venom was equivalent overall.



**Fig. 3.** Relative proportions (as % means  $\pm$  standard deviations) of venom alkaloids across queens (gyne) and workers in the fire ants *Solenopsis invicta* and *S. geminata* as identified by GC-MS analysis of venom droplets from individual insects ( $n = 5$ ). Numbers correspond to compounds listed in Table S1. Saturated compounds were synthesised to compare for insecticidal effects; unsaturated solenopsin analogues are more difficult to obtain, and shall be dealt with in a subsequent study focusing on the venom of workers.

## 2.6. Imaging

Pictures of intoxicated ants and venom droplets were taken with a stereomicroscopes ZEISS Stereo Discovery v.20 for images. Videos were taken with a Miniso macro lens clipped to an iPhone 6S, and edited with iMovie 10. Obtained media can be found in Fox et al. (2018).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.toxicon.2018.11.428>.

## 2.7. Statistical analysis

All numeric data was input to R version 3.5.1 for graphical representation of plotted results and general statistical analyses. The following R packages were used: 'plyr', 'reshape2', 'ggplot2', 'Hmisc', 'dunn.test', 'tidyr'. Numbers of dead individuals were compared by non-parametric Kruskal-Wallis at  $\alpha = 0.05$  complemented with Dunn's paired test. Scripts containing all the plot details and raw data are provided in Supplementary Files and Fox et al. (2018).

## 3. Results

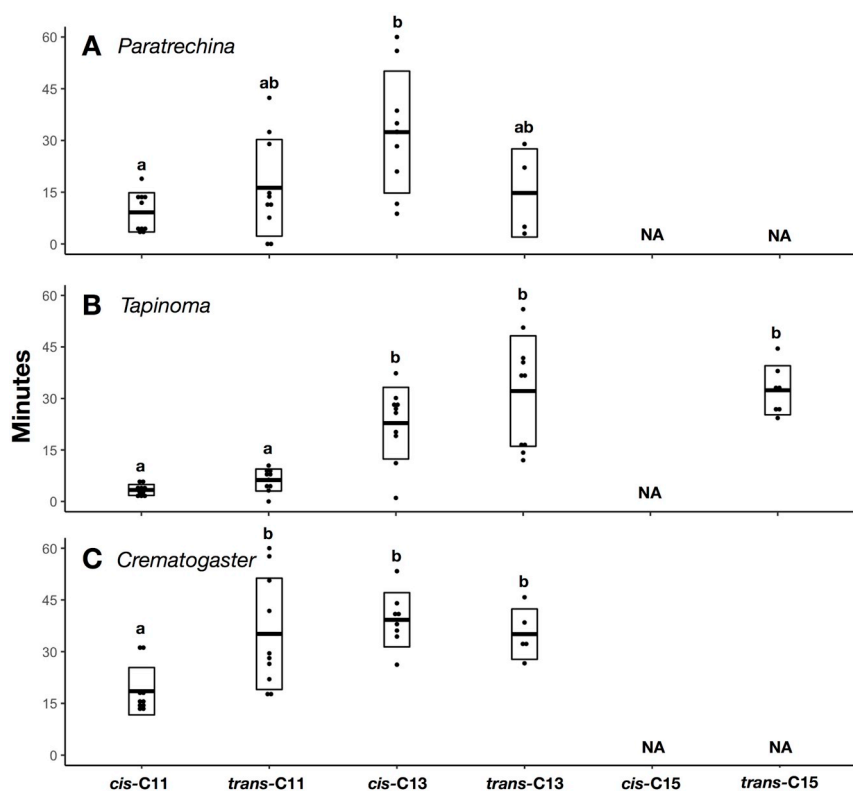
Competitor ant species showed intoxication symptoms shortly following contact with fire ant venom (Table 1). The effects generally

confirm observations by previous authors regarding intoxication of competitors by fire ant worker venoms (Greenberg et al., 2008; LeBrun et al., 2014). In addition, it is worth noting that *Paratrechina longiconis* actively attempted to avoid the application of gyne venom and synthetic cis-C11 as soon as the droplets were formed, suggesting a volatile activity. Workers of all tested species often went motionless for several seconds following application of *S. invicta* gyne venom and *S. geminata* worker venom. *Crematogaster dohrni* workers secreted liquids (i.e. venom and oral fluids, which stained the filter paper in yellow) after remaining motionless for 1 h following contact with either a droplet of *S. invicta* worker venom, isomers of C13, or trans-C15.

For the present investigation we focused on quantifying the remarkably rapid paralytic effect from envenomation by fire ant queen venom (Fig. 1, supplementary videos in Fox et al., 2018). Such complete incapacitation, or knockdown, takes considerably longer, or may not happen at all, following exposure to fire ant worker venom (Fig. 1A). It should be stressed that a higher speed of knockdown incapacitation is not directly associated to increased mortality (Fig. 1B), which was significantly highest from exposure to *S. invicta* workers venom.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.toxicon.2018.11.428>.

Both species produce the same diversity of venom solenopsins



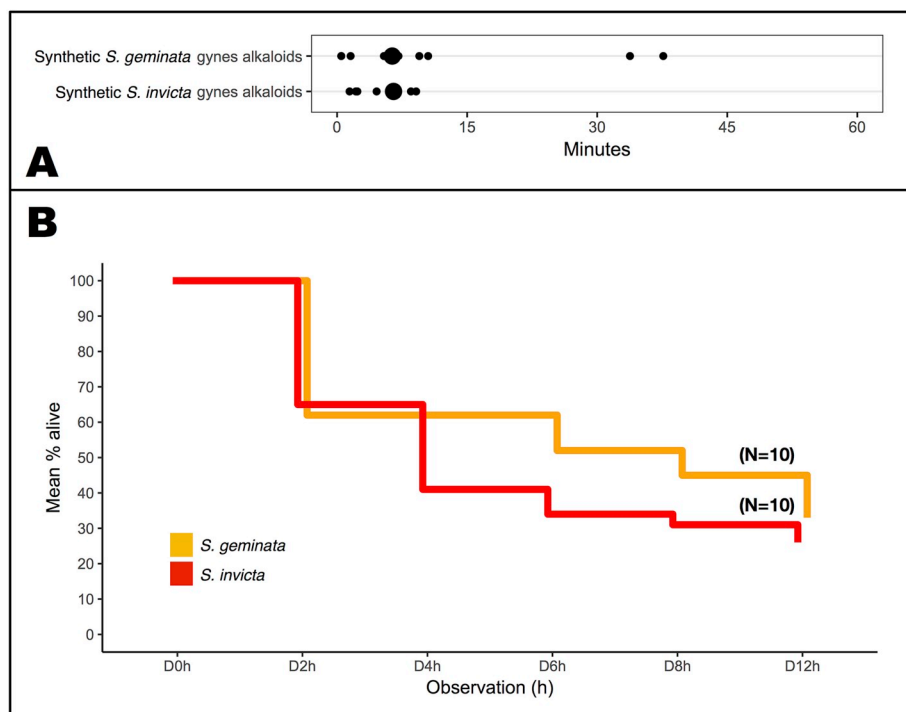
**Fig. 4.** Time until complete incapacitation by knockdown from application of a 20–30 nl droplet of synthetic solenopsins to the heads of ant workers of three representative competitor ant species *P. longicornis*, *T. melanocephalum*, and *C. dorhni*. Box plots lower and upper limits are respectively 25th and 75th percentiles around the mean among groups of 10 treated workers monitored for 1 h; dots are raw data points. Statistical Analysis: The compound *cis*-C11 had significantly stronger effect on all tested species, while other solenopsins analogues had similar overall effects. Statistical analysis was carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple analysis at alpha = 0.05. Obtained *p*-values and R script available from Fox et al. (2018).

(Fig. 2, Table S1) however the relative proportion of *trans* solenopsins isomers, particularly of saturated and insaturated analogues of *trans*-C13 (compounds 12 and 14) and *trans*-C15 (compounds 19 and 21) are most abundant in *S. invicta*. The venom of queens capitalises on *cis*- and *trans*-C11 (Fig. 2, compounds 4 and 6). These observations are in accordance with the reports of other authors (e.g. Brand et al., 1973a, Shi et al., 2015).

Synthetic solenopsins were obtained to reproduce the observed

intoxication effects. Incapacitation times by knockdown using synthetic isosolenopsin A (*cis*-C11) were significantly shorter than other compounds (Fig. 4). Isomers of solenopsin C (C15) mostly did not cause a complete incapacitation (Fig. 3), however, it should be noted that an absence of a visible knockdown within 1 h does not imply the intoxicated ants have recovered.

Mixtures of C11 analogues and C13 (concocted to mimic the venom composition of *S. geminata* and *S. invicta* queen venoms) largely



**Fig. 5.** Time until complete incapacitation by knockdown (A), and the mean mortality (B) of workers of the crazy ant *Paratrechina longicornis* touched on the head with a 20–30 nl droplet of a mixture of synthetic solenopsins mimicking the venoms of invasive fire ant queens. Statistical analysis: Overall the effects of both mixtures of solenopsins were similar as compared by non-parametric Wilcoxon ranked test: (A)  $W = 57$ ,  $p$ -value = 0.6305; (B)  $W = 2215$ ,  $p$ -value = 0.027.



reproduced the observed effects (Fig. 4). Comparison with natural alkaloid fractions further confirms that the observed effects are due to venom solenopsins only (Fig. S1). Rapid incapacitation by knockdown from the venom of fire ant gynes therefore chiefly derives from intoxication by isosolenopsin A, *cis*-C11. Although mortality following contact with pure solenopsins was higher, the general pattern is maintained (compare Figs. 1 and 4). It should be emphasised that the artificial venom mixtures were simplified and did not include any of the minor components, which may have effects other than rapid knock-down, thus this is probably the reason why they were slightly more potent than natural pure alkaloids (compare Figs. 2 and 5). Other solenopsin analogues may be associated with higher mortality, particularly isosolenopsin B (*cis*-C13) (see Fig. S2), which seems to account for the higher mortality from *S. invicta* venom (Fig. 1). Thus, testing synthetic mixtures containing C13 and C15 isomers and unsaturated analogues to simulate the venom of *S. invicta* workers is warranted in future investigations. The observed effects were visually similar (albeit slower) to intoxication by the insecticidal alkaloid nicotine (see supplementary video in Fox et al., 2018), suggesting that intoxication by these alkaloids follows related pathways.

Because fire ant gynes are bulkier than workers and have a prominently swollen gaster, they are physically unable to gaster-flag or inject venom like workers do. Even when encouraged to sting prey or skin, a gyne's stinger will not penetrate (personal observations by EGPF). Therefore, gynes are apparently limited to using venom by topical application, either on eggs during oviposition (Tschinkel, 2006) or when

attacking other arthropods. The present results demonstrate the venoms of gynes can quickly incapacitate competitor ant foragers, and that the effect is mainly delivered by isosolenopsin A. This trait is likely invaluable during claustral nest foundation, which is a lifecycle challenge shared by all fire ant species, as is the composition of their venom. We are currently working on simulating field scenarios under controlled conditions, to assess a role of solenopsins as repellents and antimicrobials against natural enemies.

In conclusion, the present investigation demonstrates for the first time that the venom of fire ant gynes is particularly effective for the rapid incapacitation of competitor ants, apparently as a result of selected proportions of analogous alkaloids. We believe this is an overlooked aspect in the biology of fire ants which helps explain the ecological and invasive success of the clade.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicon.2018.11.428>.

Table S1

List of alkaloid compounds identified by GC-MS from venom extracts and fractions of fire ants from Guangdong, China.

Compound #	RT (mins)	Trivial name	m/z ...
1	12.07	<i>cis</i> C9:0	98.1; 99.1; 55.1; 210.2; 56.0 ...
2	12.37	<i>trans</i> C9:0	98.0; 55.1; 99.0; 210.2; 56.1 ...
3	13.87	<i>trans</i> C11:1	98.0; 124.1; 111.1; 130.0; 180.2 ...
4	13.93	<i>cis</i> C11:0	98.2; 99.1; 238.3; 55.1; 252.3 ...
5	14.09	Piperideine	111.1; 96.1; 107.1; 124.1; 98.1 ...
6	14.23	<i>trans</i> C11:0	98.2; 99.1; 238.3; 55.1; 56.1 ...
7	14.38	Piperideine	110.1; 97.1; 96.1; 98.1; 152.1 ...
8	15.43	<i>cis</i> C13:1	98.1; 124.1; 111.1; 180.2; 55.1 ...
9	15.59	Piperideine	98.1; 99.1; 224.2; 55.1; 252.2 ...
10	15.65	<i>cis</i> C13:0	98.2; 99.1; 266.3; 55.1; 280.3 ...
11	15.71	Piperideine	111.1; 96.1; 151.1; 55.1; 150.1 ...
12	15.74	<i>trans</i> C13:1	98.1; 124.1; 111.1; 99.1; 55.1 ...
13	15.85	Piperideine	98.1; 96.1; 110.1; 178.1; 97.1 ...
14	15.92	<i>trans</i> C13:0	98.1; 99.1; 55.1; 56.1; 266.3 ...
15	16.00	unknown alkaloid	98.1; 57.1; 71.1; 85.1; 55.1 ...
16	16.98	<i>cis</i> C15:1	98.1; 99.1; 55.1; 154.2; 307.4 ...
17	17.14	Piperideine	111.1; 96.1; 124.1; 152.1; 55.1 ...
18	17.20	<i>trans</i> C15:1	98.2; 99.1; 55.1; 292.3; 56.1 ...
19	17.30	Piperidiene	98.1; 152.1; 110.1; 97.1; 96.1 ...
20	17.32	<i>trans</i> C15:0	98.1; 99.1; 55.1; 56.1; 57.1 ...
21	17.46	Piperidiene	98.1; 99.1; 55.1; 56.1; 207.0 ...
22	17.54	unknown alkaloid	98.1; 207.0; 73.0; 281.1; 99.1 ...
23	18.58	<i>cis</i> C17:0	98.2; 99.2; 55.1; 322.4; 69.1 ...
24	18.70	Piperidiene	98.1; 152.1; 110.1; 97.1; 96.1 ...
25	18.87	<i>trans</i> C17:1	98.1; 99.1; 55.0; 320.3; 56.1 ...
26	18.90	Piperideine	98.1; 99.1; 55.0; 111.1; 96.1 ...

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