

Alkaloid Venom Weaponry of Three *Megalomyrmex* Thief Ants and the Behavioral Response of *Cyphomyrmex costatus* Host Ants

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Abstract Social parasites exploit other societies by invading and stealing resources. Some enter protected nests using offensive chemical weaponry made from alkaloid-based venom. We characterized the venoms of three *Megalomyrmex* thief ant species (*M. mondabora*, *M. mondaboroides*, and *M. silvestrii*) that parasitize the fungus-growing ants, and developed an ethogram to describe host ant reactions to raiding *M. mondaboroides* and *M. silvestrii* parasites. We compared piperidine, pyrrolidine, and pyrolizidine venom alkaloid structures with synthetic samples from previous studies, and describe the novel stereochemistry of *trans* 2-hexyl-5-[8-oxononyl]-pyrrolidine (**3**) from *M. mondabora*. We showed that workers of *Cyphomyrmex costatus*, the host of *M. mondaboroides* and *M. silvestrii*, react to a sting by *Megalomyrmex* parasites mainly with submissive behavior, playing dead or retreating. Host submission also followed brief antennal contact. The behavior of *C. costatus* ants

observed in this study was similar to that of *Cyphomyrmex cornutus*, host of *M. mondabora*, suggesting that the alkaloidal venoms with pyrrolidines from *M. mondabora*, piperidines from *M. mondaboroides*, and pyrolizidines from *M. silvestrii* may function similarly as appeasement and repellent allomones against host ants, despite their different chemical structure. With the use of these chemical weapons, the *Megalomyrmex* thief ants are met with little host resistance and easily exploit host colony resources.

Keywords Parasitism · Solenopsidini · Attini · Chemical defense · Semiochemical · Coevolution · Appeasement substance · Tolerance behavior

Introduction

Many plants and animals deter predation and parasitism by biosynthesizing or sequestering toxic alkaloids. Dietary alkaloid sequestration is common in some herbivorous insects (Termonia et al. 2002) as well as poison dart frogs that consume alkaloid-producing arthropods such as ants and mites (Daly 1995; Saporito et al. 2004). Myrmicinae ants biosynthesize a variety of alkaloids in their venom, providing the ants with a competitive advantage due to the toxicity and repellency functions (Anderson et al. 1991). Ants belonging to the Tribe Solenopsidini (e.g., *Solenopsis*, *Carebarella*, *Monomorium*, *Megalomyrmex*) biosynthesize piperidine, pyrroline, pyrrolidine, indolizidine, piperidine, and pyrolizidine alkaloids (Escoubas and Blum 1990) and readily disperse them during aggressive and competitive interactions (Adams and Traniello 1981; Adams et al. 2013; Obin and Vander Meer 1985).

Social parasitism, when one social species takes advantage of resources (e.g., food, brood) of another society (Hölldobler

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and Wilson 1990), has appeared independently several times in Hymenoptera (ants, bees, wasps). Within the solenopsidine clade, there exist guest-ant and thief-ant parasites that are not closely related to their host and that produce workers to tend their own brood. Although all social parasites are resource exploiters, guest ants also benefit from living within the host nest environment and in extreme cases will defend the nest against other competing parasites acting as an interspecific soldier caste (e.g., *Megalomyrmex symmetochus*) (Adams et al. 2013). In contrast, thief ants simply raid nests of other ants for resources, and they swiftly overwhelm the host colony to steal stored food and host brood.

Social parasites must successfully invade the host colony without being killed; they accomplish this either by avoiding detection (stealth) or by taking the host colony by force (Akino 2008; Lenoir et al. 2001). The weaponry used by social parasites can have many functions that are not necessarily mutually exclusive. Parasite semiochemicals may confuse their host colony (propaganda allomone), lower host aggression (appeasement allomone), or repel threatening host ants (repellent allomone) (Lenoir et al. 2001 and refs therein). *Megalomyrmex*, *Monomorium*, and *Solenopsis* social parasites use their alkaloid based venom during host colony infiltration, usurpation, and integration (Adams and Longino 2007; Adams et al. 2000b, 2012, 2013; Blum et al. 1980). The pyrrolizidine-based venom of *Megalomyrmex symmetochus* serves not only as a repellent allomone but also as a so-called propaganda allomone that confuses other ant species (Adams et al. 2013). An unsymmetrical 2,5-dialkylpyrrolizidine found in *Solenopsis fugax* and *Monomorium pharaonis* aids these ants in successfully stealing brood from diverse ant species by functioning as a repellent (Blum et al. 1980; Hölldobler 1973). Finally, piperidine alkaloids are found in a number of *Solenopsis* thief-ant species, but the function of these specific alkaloids is unknown (Gorman et al. 1998; Jones et al. 1984, 1996). Because piperidine alkaloids are toxic to bacteria, fungi, and insects and likely repel other ants, their functions may include protection against both disease and arthropod enemies (Jouvenaz et al. 1972; Li et al. 2012; Rashid et al. 2013). The high volatility of alkaloids allows the semiochemicals to be dispensed as an aerosol, which can be used against other ants without physical contact, thus providing yet another advantage to using alkaloid based chemical weapons (Adams and Traniello 1981; Adams et al. 2013; Obin and Vander Meer 1985).

Megalomyrmex (Formicidae, Solenopsidini) social parasites exploit fungus-growing or “attine” ants [Formicidae, Attini, used here in the narrow sense, not the broad sense of (Ward et al. 2015)] which are members of a complex species network involving a mutualistic fungal cultivar (Mueller et al. 1998) and diverse bacteria associated with the integument and alimentary canal of the ants, as well as the fungus garden (Barke et al. 2010; Currie et al. 1999; Ishak et al. 2011;

Mueller 2012; Schoenian et al. 2011; Seipke et al. 2011). The over 230 described fungus-growing ant species consume the fungus garden they grow, and propagate it vertically between generations when starting new colonies. A number of non-host ant species profit from the complex microhabitat within a fungus-growing ant nest. These associates range from mutualistic (Adams et al. 2013), to commensal (Rabeling et al. 2007), to social parasites (Boudinot et al. 2013; De Souza et al. 2007; Dijkstra and Boomsma 2003; Schultz et al. 1998). Although most of the 44 described *Megalomyrmex* species are free-living predators, about one quarter are social parasites that specialize in consuming the fungus garden and attine brood (Boudinot et al. 2013). These social parasites are associated with a range of attine species in multiple genera of the tribe.

There is a distinct group of rare *Megalomyrmex* species that associate with the fungus-growing ants in a manner similar to the *Solenopsis* thief ants, which exploit their hosts by nesting in close proximity and conducting periodic raids. *Megalomyrmex mondabora* colonies live in cavities within the arboreal nest structure built by their *Cyphomyrmex cornutus* host ants (Adams and Longino 2007). *Megalomyrmex mondaboroides* colonies live beside their *C. costatus* and *Apterostigma goniodes* host nests in the soil and in the laboratory, *Megalomyrmex silvestrii* colonies live near their *C. costatus* host colonies (Boudinot et al. 2013). All these species share a “thief like” host exploitation strategy, conducting organized raids where a few individuals enter the host colony, graze quickly on the fungus garden, and flee with host brood (Boudinot et al. 2013).

We describe the raiding behavior of *M. mondaboroides* and *M. silvestrii* and the *C. costatus* host responses. We hypothesize that *Megalomyrmex* thief ants use species-specific, alkaloid weaponry as appeasement, propaganda, and/or repellent allomones. We analyzed the alkaloid compounds of the three thief ant species and compared our results with the published alkaloids of three free-living and one guest ant *Megalomyrmex* species.

Methods and Materials

Ant Collection and Care Between 2003 and 2011, four colonies of *Megalomyrmex mondabora* were collected near El Ceibo Research Station, Costa Rica and in 2001 and 2010 two live colonies of *M. mondaboroides* were collected in the Republic of Panamá. In all cases, samples for chemical analysis were taken immediately after colony collection. The *M. mondaboroides* colony collected near El Llano was associated with *Apterostigma goniodes*. The second colony was collected from a creek embankment on Pipeline Road near Gamboa and was associated with a *C. costatus* colony. This *M. mondaboroides* colony has remained alive for over 4 years

in the laboratory by living with a *C. costatus* host subcolony, feeding on the garden and supplemented weekly with ant brood from *Sericomyrmex* and *Acromyrmex* colonies.

In 2009 and 2010, two colonies of *M. silvestrii* were collected. The first was collected on Barro Colorado Island by Adam Kay and was kept alive on an agar, sucrose, and protein diet for a few weeks before samples were taken for chemical analysis. The second colony was collected on Pipeline Road near Gamboa, Republic of Panamá and was preserved directly into an unknown percentage of ethanol in a plastic vial. Therefore, this sample could only partially confirm compounds of the other collection. With the exception of the latter collection, all other samples for chemical analysis were collected in 1 ml glass vials containing enough 100 % methanol (~50 μ l) to cover the 3–10 ants that were added to the vial with clean forceps. See Online Resource 1 for more collection details. Voucher specimens are deposited at the entomological collections of the National Museum of Natural History of the Smithsonian Institution (Washington, DC) and in the Longino research collection, Department of Biology, University of Utah (Salt Lake City, Utah).

The two live colonies used for behavioral study were transferred to Copenhagen, Denmark and kept in an environmentally controlled rearing room at a constant temperature of 25 °C and relative humidity of 60–70 %. The colonies were housed in clear plastic chambers with a moist plaster bottom (0.5 cm thick). Lids were not removed during video recordings to avoid behavioral disturbance.

Preparation of Ethyl 2-Acetyl-9-Hydroxynonanoate (9)

Freshly distilled ethyl acetoacetate (4.4 ml, 35 mmol) was added dropwise to 30 ml of EtOH in which 0.8 g of sodium had been dissolved, followed by the addition of a solution of 4.9 g (25 mmol) of 7-bromoheptanol (Kang et al. 1985) in 20 ml of EtOH. The mixture was heated to reflux overnight under a drying tube. Upon cooling, the mixture was neutralized with 10 % HCl, and the solvent was removed *in vacuo*. The residue was partitioned between ether and water, and the ether extracts were dried over anhydrous MgSO₄, filtered, and the solvent was removed to provide 5 g of **9** that was >85 % pure and suitable for the next step. Small amounts of **9** were purified by Kugelrohr distillation at 0.1 mm Hg. ¹H NMR (400 MHz, CDCl₃) δ 4.15 (2H, q, J=7.2 Hz), 3.58 (2H, t, J=7.0 Hz), 3.36 (1H, t, J=7.6 Hz), 2.18 (3H, s), 1.9–1.75 (3H, complex m), 1.50 (2H, m), 1.27 (8H, complex m), 1.32 (3H, t, J=7.2 Hz); EIMS *m/z* 226 [M⁺-18] (0.5), 172 (1), 143 (15), 138 (19), 130 (70), 101 (40), 88 (13), 84 (22), 73 (40), 55 (30), 43 (100). HRMS *m/z* 226.1554 ([M-H₂O]⁺), calcd for C₁₃H₂₂O₃, 226.1569.

Preparation of 2-Methyl-2-[8-Oxoocetyl]-1,3-Dioxolane (10)

A mixture containing 4.8 g (19.6 mmol) of

9 and 1.5 g of H₃BO₃ was stirred and heated in an oil bath at 170 °C for 2 h. Upon cooling, the mixture was taken up in ether and carefully neutralized with NaHCO₃. The ether extracts were dried over anhydrous MgSO₄, filtered, and the solvent was removed to provide 4 g of crude (80 % pure) 10-hydroxy-2-decanone EIMS *m/z* 172 [M⁺] (0.5), 97 (10), 96 (15), 71 (28), 58 (100), 55 (40), 43(95). This product was dissolved in 100 ml of benzene containing 5 ml of ethylene glycol and 0.2 g of *p*-toluenesulfonic acid, and the mixture was heated under reflux under a Dean-Stark trap until all separation of water ceased. The mixture was cooled, washed with saturated NaHCO₃, dried over MgSO₄, and after filtration, the solvent was removed to provide 2-methyl-2-[8-hydroxyoctyl]-1,3-dioxolane nearly quantitatively, ¹³C NMR (100 MHz, CDCl₃) δ 110.45, 64.80 (2C), 63.09, 39.38, 32.92, 29.99, 29.57, 25.86, 25.81, 24.19, 23.91; EIMS *m/z* 215 [M-1⁺] (0.5), 201 [M-15⁺] (12), 87 (100), 69 (5), 55 (10), 43 (33). A solution containing 1.3 g (6 mmol) of 2-methyl-2-[8-hydroxyoctyl]-1,3-dioxolane in 3 ml of CH₂Cl₂ was added to a mechanically stirred mixture of 2 g of pyridinium chlorochromate and 0.5 g of NaOAc in 20 ml of CH₂Cl₂. After 2 h, the usual work up (Corey and Suggs) provided 0.8 g of **10** that was ca. 80 % pure by GC/MS. Small amounts of **10** were purified by kugelrohr distillation at 0.1 mm Hg. ¹H NMR (400 MHz, CDCl₃) δ 9.73 (1H, t, J=1.6 Hz), 3.90 (4H, m), 2.31 (2H, t, J=7.2Hz), 1.59 (4H, m), 1.35–1.25 (11H, m); ¹³C NMR (100 MHz, CDCl₃) δ 179.93, 110.42, 64.79 (2C), 39.34, 34.23, 29.80, 29.38, 29.15, 24.85, 24.19, 23.90; EIMS *m/z* 213 [M-1⁺] (0.5), 199 [M-15⁺] (11), 87 (100), 67 (3), 55 (8), 43 (28); HRMS *m/z* 199.1234 ([M-CH₃]⁺), calcd for C₁₁H₁₉O₃, 199.1334.

Preparation of 2-[8, 11-Dioxoheptadecyl]-2-Methyldioxolane (11)

A mixture of 1.35 g (9.6 mmol) of 1-nonen-3-one (Jones et al. 1991a), 1.76 g of **10**, and 0.6 g of 5-(2-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride was treated with 3 ml of freshly distilled triethylamine and refluxed overnight under an argon atmosphere. The mixture was cooled, diluted with ether, filtered, and the solvent removed *in vacuo*. Kugelrohr distillation (100–150 at 0.1 mm Hg) provided 2.0 g of **11**. ¹³C NMR (100 MHz, CDCl₃) δ 208.90, 208.85, 109.15, 63.58 (2C), 41.87, 41.83, 38.15, 34.98 (2C), 30.56, 28.64, 28.32, 28.09, 27.85, 23.02, 22.78 (2C), 22.69, 21.47, 13.02; EIMS *m/z* 354 [M⁺] (1), 339 [M-15⁺] (10), 293 (5), 269 (2), 225 (1), 223 (2), 213 (2), 197 (2), 184 (1), 169 (2), 157 (5), 115 (6), 99 (5), 87 (100), 43 (35); HRMS *m/z* 339.2499 ([M-CH₃]⁺), calcd for C₂₀H₃₅O₄, 339.2468.

Preparation of 2-Hexyl-5-[8-Oxononyl]-Pyrrolidine (3)

A solution containing 0.2 g (0.57 mmol) of **11**, 0.1 g of NH₄OAc, 0.1 g (2.7 mmol) NaCNBH₃, and 2 drops of 10 % NaOH in 5 ml of methanol was stirred overnight at room

temperature. The solvent was removed *in vacuo*, and a small sample was partitioned between water and ether. GC/MS analysis of this aliquot revealed two major components with identical mass spectra corresponding to both isomers of the ethylene ketal of **3**. EIMS m/z 338 [$M-1^+$] (1), 324 (7), 254 (46), 224 (8), 210 (21), 154 (100), 87 (93), 82 (18), 43 (35); HRMS m/z 324.2935 ($[M-CH_3]^+$), calcd for $C_{20}H_{38}NO_2$, 324.2903. The remainder of the reaction mixture was quickly acidified with 10 % HCl, and after 10 min neutralized with solid $NaHCO_3$, and extracted with ether (2×10 ml). The ether was dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to provide 0.15 g of a 1:1 mixture of the stereoisomers of pyrrolidine **3** that was 71 % pure by GC/MS, with GC retention time 23.52 min, GC-FTIR ν_{max} 2933, 2866, 1731 (s), 1459, 1360, 1160 cm^{-1} ; and 23.61 min, GC-FTIR ν_{max} 2933, 2866, 1731 (s), 1460, 1362, 1119 cm^{-1} ; respectively, and which exhibited identical mass spectra. EIMS m/z 294 [$M-1^+$] (1), 238 (2), 236 (2), 210 (71), 194 (10), 180 (5), 154 (100), 82 (32), 69 (21), 43 (36); HRMS m/z 210.1820 ($[M-C_6H_{13}]^+$), calcd for $C_{13}H_{24}NO$, 210.1854. The GC-FTIR, EIMS and retention time of the second eluting isomer of **3** were identical to those of the last eluting alkaloid in *M. mondabora*. Treatment of the mixture with a small amount of $NaBH_4$, followed by acidification, neutralization, and ether extraction provided a pair of isomeric pyrrolidines whose mass spectra were identical to that obtained from the $NaBH_4$ of the *M. mondabora* extract.

Chemical Analysis Gas Chromatography/Mass Spectrometry (GC/MS) was carried out in the EI mode using a Shimadzu QP-5000 GC-MS or a Shimadzu QP-2010 GC-MS equipped with an RTX-5, 30 m \times 0.25 mm i.d. column. The instrument was programmed from 60 to 250 $^\circ C$ at 10 $^\circ$ /min. Vapor phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an Hewlett-Packard 5890 gas chromatograph fitted with a 30 m \times 0.25 mm RTX-5 amine column. NMR spectroscopy was carried out in $CDCl_3$ solutions using a Varian Mercury 400 NMR spectrometer. HRMS was performed on a JEOL SX102 instrument in the positive-ion fast-atom bombardment mode using a direct probe and a Waters LCT Premier Time of Flight instrument in the electrospray (ESI) mode.

Behavioral Analysis Raids by *M. mondaboroides* and *M. silvestrii* colonies are rarely observed in the laboratory and have never been behaviorally examined until now. However, behavioral interactions between *M. mondabora* and its host have been described previously (Adams and Longino 2007). We conducted an ethological study and focused on the behavioral reactions of *C. costatus* host ants to *M. mondaboroides* and *M. silvestrii* parasites (i.e., special purpose ethogram). Raids were video-recorded in the laboratory while the parasites were living near host subcolonies. The raid

of *M. mondaboroides* (RMMA100624-18) lasted for 66 min beginning with a single *Megalomyrmex* approaching the host garden. The camera was zoomed to allow the whole garden to remain in view, and many ants could be scored at the same moment in time. The recording captured the entire raid from a stationary perspective looking down on the garden. The *M. silvestrii* (AKJune2010) raid was recorded for the last 26 min of the raid starting when five *Megalomyrmex* ants were on the host garden. The recording was not always stationary, and the view shifted from a top perspective to a side view, and there were 10 short intervals (totaling 2 min 57 s) where the ants were out of focus. Only interactions on the surface of the gardens for both raids could be scored. Statistical analysis is not appropriate for this dataset because we observed only a single raid for each species pair.

We focused on host behavior just after contact and after a parasite sting or gaster flag. Contact between workers of the two species was initiated by one or both species (i.e., ant approaches and reaches out with antennae toward another ant), and ended when one of the ants moved away. Which species initiated an interaction and the immediate series of responses between the two workers were scored in the order they were observed. Following an interaction, three main host behavior categories were scored: 1) submission, when the host ant either played dead (i.e., ant remained still with a downward tilt of the head, antennae retracted into the scrobes, and the gaster tucked) or quickly moved away (i.e., ant steps backward or abruptly changes direction away from the parasite); 2) aggression, (i.e., host ant bit near or on the parasite); and 3) other, (i.e., no abrupt change in body position and no visible reaction, although mandibles may be agape). Biting was not always visible due to the angle of the host ant mandibles. Therefore, biting was sometimes scored based on the larger action of the host ant's body (i.e., abrupt motion toward the parasite). Not surprisingly, stinging behavior by the host ant was not observed, as fungus-growing ants have vestigial stings (Wheeler 1907).

When a host ant initiated contact, her mandibles were often agape, potentially allowing alarm pheromones to be dispensed (Crewe and Blum 1972). This was termed "open mandible threat" in former studies (Carlin and Hölldobler 1987; Hölldobler and Wilson 1990) but was placed in the "other" category here because it did not appear to influence the behavior of the parasite and, therefore, we could not conclude it was an aggressive act. It is equally plausible that the ant was emitting alarm substances (e.g., 3-octanol) for conspecifics (Crewe and Blum 1972). Open mandibles, just after the initiation of contact by the host ant, was recorded when possible (16 out of 29 events in *M. mondaboroides* hosts and 13 out of 15 events by *M. silvestrii* hosts). Parasite behavior also was categorized, allowing us to describe host behavioral response to neutral parasite actions (i.e., contact with antennae or in close proximity) and aggressive parasite actions. "Gaster

flagging” is an ant behavior described in other solenopsisidine species that allows the ants to dispense venom alkaloids (Adams and Traniello 1981; Obin and Vander Meer 1985). Here, we define it as the vibration of the abdomen (mainly the gaster) held at a 45 degree angle where the stinger may or may not hold a droplet of venom (Fig. 1a). A “side-swipe sting” is where the ant positions her gaster toward her opponent from the side (Fig. 1b). A “gaster-tuck sting” is where the gaster is tucked under the body and between the legs of the ant (Fig. 1c). In both types of stinging behavior the spatulate shaped stinger is likely to be loaded with venom that can be smeared onto the host ant. Hereafter, we define gaster flagging

and both stinging behaviors as antagonistic alkaloid dispensing behaviors.

During the raids, alkaloid dispensing behavior events were counted for each interaction regardless of whether the host reaction could be viewed or not, so the number of these events is not equivalent to the total number of host responses. If the *Megalomyrmex* parasite exhibited these behaviors more than once during a contact period, the behavior was counted only once. The number of times the parasite gaster flagged or stung as a response to a host bite was also reported. At a moment of contact, the number of visible host and parasite ants was counted. These numbers were averaged to illustrate the host and parasite ratio for each raid.

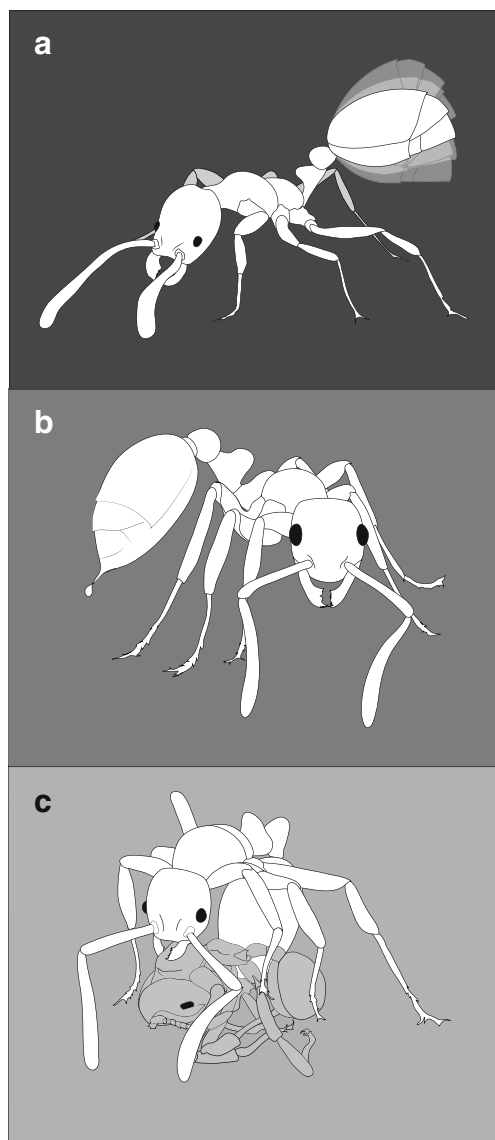


Fig. 1 Parasite alkaloid dispensing behaviors. **a** Gaster flagging—vibrating gaster held at approximately a 45 degree angle, **b** Side-swipe sting—gaster waves from the side in the direction of the host ant, **c** Gaster tucking sting—gaster tucked under body and between legs and directed at the host ant (in grey)

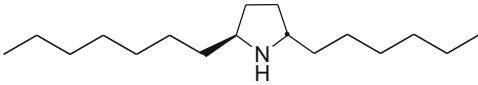
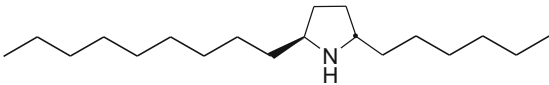
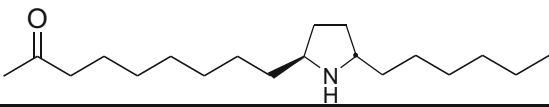
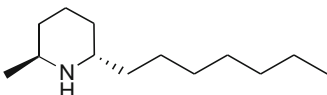
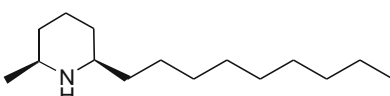
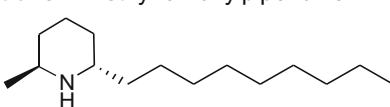
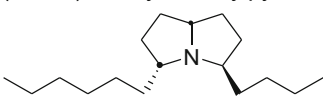
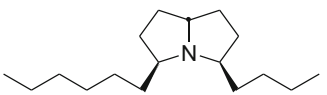
Results

Ant Alkaloid Identification The methanol extracts of *M. mondabora* (colony codes: RMMA030213-07, RMMA030213-09, RMMA050625-01, RMMA110324-01) contained on average three alkaloids in a 33:1:16 ratio. The first and second of these had mass spectra and gas chromatographic retention times identical to those of authentic samples of *trans*-2-heptyl-5-hexylpyrrolidine (**1**) (Jones et al. 1991a) and *trans*-2-hexyl-5-nonylpyrrolidine (**2**) (Jones et al. 1982a) (Table 1; Fig. 2). The third compound (**3**) had an EIMS m/z 294 [$M-1^+$] (1), 238 (2), 236 (2), 210 (71), 194 (10), 180 (5), 154 (100), 82 (32), 69 (21), 43 (36), and an absorption at 1731 cm^{-1} in its GC-FTIR spectrum. Treatment of the mixture with a small amount of NaBH_4 , followed by acidification, neutralization, and ether extraction changed the mass spectrum of the last eluting alkaloid to EIMS m/z 296 [$M-1^+$] (1), 282 (2), 212 (20), 194 (12), 180 (1), 154 (100), 82 (16), 69 (28), 55 (26), 45 (36), 43 (36), 41 (42).

The methanol extracts of *M. mondaboroides* contained three alkaloids with a mass spectra base peak at $m/z=98$ (100) (Table 1; Fig. 2). These alkaloids were identified as *trans*-2-heptyl-6-methylpiperidine (**4**), *cis*-2-methyl-6-nonylpiperidine (**5**), and *trans*-2-methyl-6-nonylpiperidine (**6**), and direct comparison with synthetic samples (Jones et al. 1982b) confirmed their structures. All samples contained mainly (**6**) but differed in the second compound. The ants associated with *A. goniodes* (HF010330-50) had (**4**) and (**6**) in a 1:19 ratio, whereas the *C. costatus* associates (RMMA100624-22) had (**5**) and (**6**) in a 7:26 ratio.

The methanol extracts of *M. silvestrii* contained one major component with intense ions at $m/z=194$ (80 %) and 166 (100) and a minor component with an identical mass spectrum (Table 1; Fig. 2). These were identified as (5E,8E)-3-butyl-5-hexylpyrrolizidine (**7**) and (5Z,8E)-3-butyl-5-hexylpyrrolizidine (**8**), respectively, by direct

Table 1 Venom alkaloids of *M. mondabora*, *M. mondaboroides* and *M. silvestrii* parasites

	<i>Megalomyrmex</i> species and venom compounds	MS fragments [<i>m/z</i> (relative %)]	Citation
<i>M. mondabora</i>			
1	<i>trans</i> -2-heptyl-5-hexylpyrrolidine 	253(1, M ⁺), 168(85), 154(100)	(Jones et al. 1991a)
2	<i>trans</i> -2-hexyl-5-nonylpyrrolidine 	281(1, M ⁺), 196(85), 154(100)	(Jones et al. 1982a)
3	<i>trans</i> -2-hexyl-5-[8-oxononyl]-pyrrolidine 	294 (1, M ⁺ -1), 210(71), 194 (10), 154(100)	This work
<i>M. mondaboroides</i>			
4	<i>trans</i> -2-heptyl-6-methylpiperidine 	197(1, M ⁺), 98(100)	(Jones et al. 1982b)
5	<i>cis</i> -2-methyl-6-nonylpiperidine 	225(1, M ⁺), 98(100)	(Jones et al. 1982b)
6	<i>trans</i> -2-methyl-6-nonylpiperidine 	225(1, M ⁺), 98(100)	(Jones et al. 1982b)
<i>M. silvestrii</i>*			
7	(5 <i>E</i> ,8 <i>E</i>)-3-butyl-5-hexylpyrrolizidine 	251(1, M ⁺), 194(85), 166(100)	(Jones et al. 1991a)
8	(5 <i>Z</i> ,8 <i>E</i>)-3-butyl-5-hexylpyrrolizidine 	251(1, M ⁺), 194(85), 166(100)	(Jones et al. 1991a)
	unknown	251(M ⁺)	This work

*Compound 1 is also found in *M. silvestrii* in trace amounts

comparison with synthetic samples (Jones et al. 1991a). Trace amounts of (1) and an unidentified compound (*m/z* 251) also were present. The final ratio of compounds (1), (7), (8), and *m/z*=251 is 6:82:7:5 (AKJune2010; the second colony, GB2009, could only be used for confirmation of the alkaloids present).

We found eight venom alkaloids in the three social-parasitic ant species *M. mondabora*, *M. mondaboroides*, and *M. silvestrii* (Table 1; Fig. 2). All of these alkaloids were identified by direct comparison with synthetic samples available from previous studies, with the exception of the ketopyrrolidine (3) from *M. mondabora*. The mass

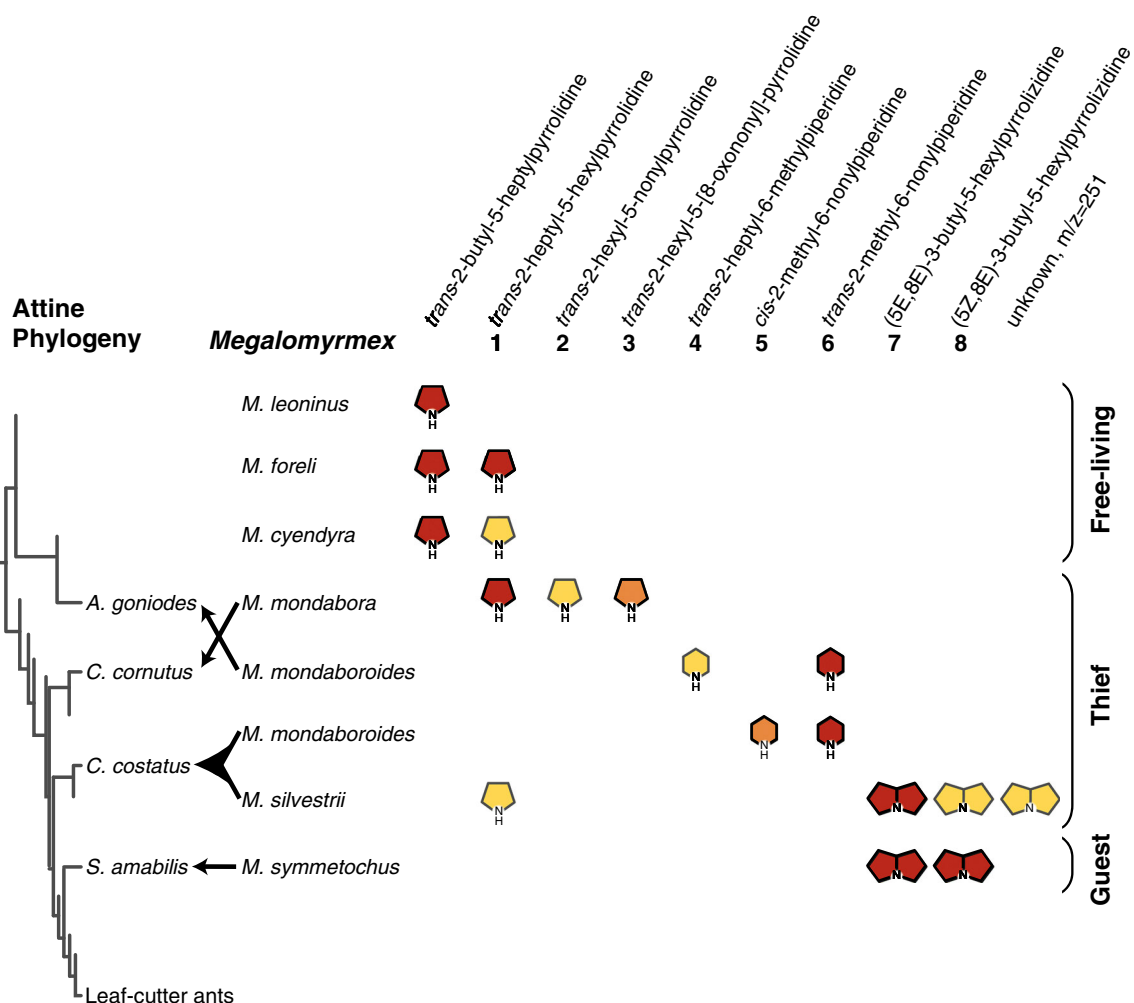
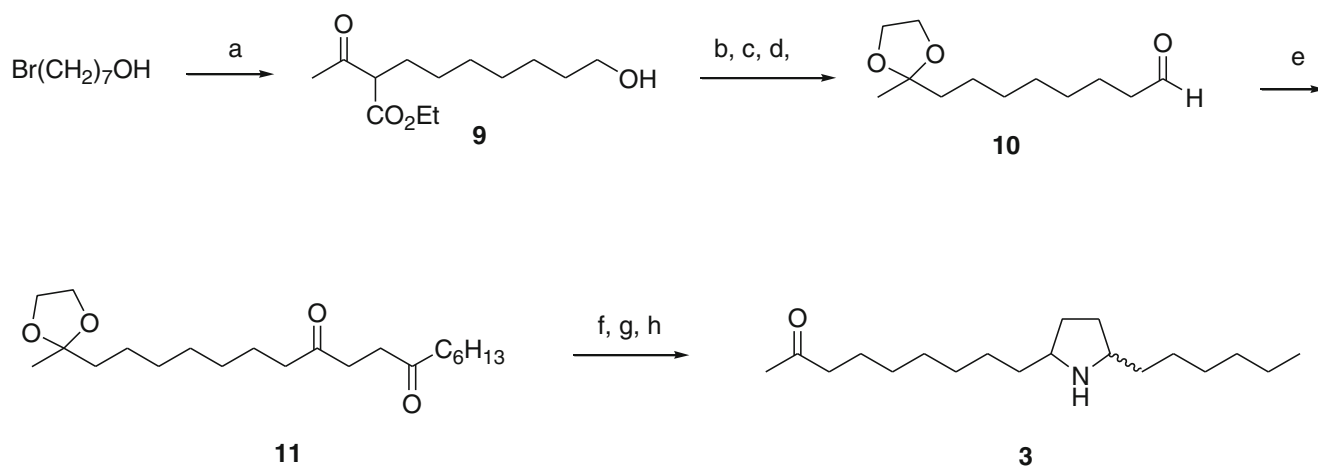


Fig. 2 Pyrrolidines, piperidines, and pyrrolizidines found in the venom of *Megalomyrmex* ants. The fungus-growing ant phylogeny is drawn from Schultz and Brady (2008), highlighting the phylogenetic position of the host ant species of parasitic *Megalomyrmex* ants (indicated by arrows). *M. leoninus*, *M. foreli* (= *M. latreillei*) and *M. cyendra* are free-living predators and have previously been reported to contain only pyrrolidine alkaloids (Jones et al. 1991a, 1999). *M. mondabora*, *M. mondaboroides*, *M. silvestrii*, and *M. symmetochus*, are social

parasites. *M. mondabora*, *M. mondaboroides*, *M. silvestrii* are thief ants. *M. symmetochus* is a guest ant parasite that produces two isomers of 3-butyl-5-hexylpyrrolizidine (Adams et al. 2013). *Megalomyrmex* parasites are ordered by the class of alkaloids found in their venom. Shapes indicate the ring structure and the color, the quantities of compounds relative to each other within a sample(s) and species (Yellow/light grey=5–6 %; Orange/grey=21–33 %; Red/black=50–95 %)

spectrum and GC-FTIR of (3) suggested a carbonyl group in the C-9 side chain, and borohydride reduction suggested a ketone at the penultimate carbon of the C-9 carbon chain whose reduction provided an alcohol showing a loss of CH_3 (m/z 282) and a CH_3CHOH fragment at (m/z 45). An authentic sample of *cis* and *trans*-2-hexyl-5-[8-oxononyl]-pyrrolidine (3) was prepared (Scheme 1), and the natural pyrrolidine had identical GC-FTIR, mass spectrum and retention time as the second eluting isomer. In 2,5-disubstituted pyrrolidines, the *trans* isomer is the second eluting isomer, and does not have a shoulder on the lower frequency side of the Bohlman region in the GC-FTIR that is observable for the *cis* isomer (Garraffo et al. 1994).

Behavioral Description of Parasite Raids During the raids, the *M. mondaboroides* and *M. silvestrii* parasites arrived at the garden and grazed vigorously, consuming the mycelium by ripping out small bits and masticating them with their mandibles. In some cases, small pieces of garden could be seen in the parasites' mandibles. Both species also exhibited searching behavior including rapid movement about the garden. They either made brief contact with host ants during these searches, or they found host brood, which they rapidly moved to their nesting area. Although rare, alkaloid-dispensing behavior such as gaster flagging (Fig. 1a) occurred at times when heterospecifics were not near the parasites, and occurred on and off the fungus garden. Parasites also tapped the end of their gaster onto the garden, possibly dispensing alkaloids or



Scheme 1 Synthesis of 2-hexyl-5-[8-oxononyl]-pyrrolidine **4**. Reagents: **a** Sodium ethanolate, ethylacetoacetate. **b** H_3BO_3 , 170°C , **c** $(\text{CH}_2\text{OH})_2$, H^+ , **d** PCC/NaOAc, **e** 1-nonen-3-one, thiazolium salt, Et_3N , **f** NaCNBH_3 , NH_4OAc , **g** H^+ , **h** NaHCO_3

other semiochemicals. The most frequent alkaloid dispensing behaviors were the two types of stings directed toward the host ants (i.e., side-swiping, Fig. 1b and gaster tucking, Fig. 1c).

Megalomyrmex mondaboroides **Raid** Over the course of the full *M. mondaboroides* raid (66 min), the host and parasites made contact 67 times. The average host and parasite ant number was 5.4 and 1.5, respectively. The *M. mondaboroides* parasites initiated contact 38 times (57 %), the host initiated contact 23 times (34 %), and both initiated contact simultaneously 6 times (9 %). The host initiated contact with open mandibles 15 out of 16 observable times (mandibles were not in view 13 times). The overall interactions between the two species were aggressive, but the frequency of interactions and aggression level markedly decreased after the first 10 min. The host generally was very active, sometimes chasing the invading parasite.

The behavior of the parasites suggests that they frequently dispense a substance from their sting, either as an aerosol by gaster flagging or as contact venom by stinging or dabbing their gaster on substrate. These are behaviors shared with other alkaloid producing species (Adams et al. 2013; Obin and Vander Meer 1985). Directly following the initiation of contact, the host reacted by actively pursuing the raiders, and bit the parasite 25 % of the time, but there was no obvious reaction in 57 % of the encounters (Fig. 3a). If a host ant snapped her mandibles or bit the parasite, the parasite responded with a sting 72 % of the time (13 of 18 total interactions). The host reacted to an initiation of contact with the parasite submissively in only 18 % of 67 interaction periods, by either playing dead (58 %) or abruptly moving away from the parasite (42 %) (Fig. 3a).

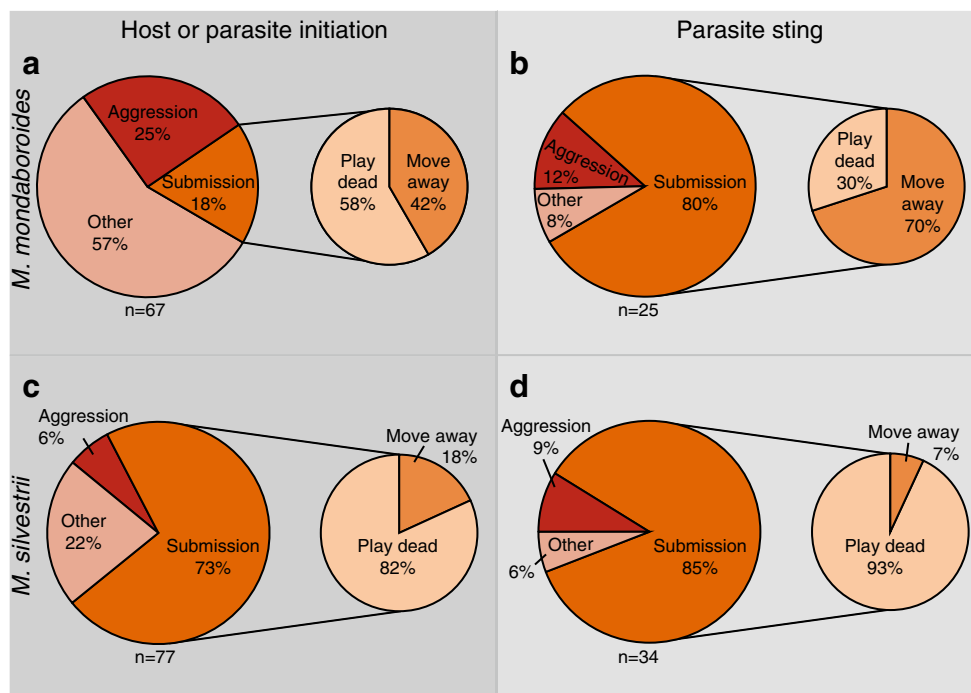
In 25 of 67 interactions, the parasite stung the host at least once during the contact period. The host reacted to a sting with submission 80 % of the time and aggression 12 % of the time

(Fig. 3b). Most submissive behavior included backing away or moving in another direction (70 %) rather than playing dead (30 %).

Megalomyrmex silvestrii **Raid** The *M. silvestrii* raid shared similarities in parasite behavior with the *M. mondaboroides* raid, but the host reaction was different. Unfortunately, the beginning of the *M. silvestrii* raid was not observed, and videotaping began after 5 parasites were already on the garden. Over the course of the last 26 min of the *M. silvestrii* raid, the number of ants on or near the garden and visible for observation was 3.5 parasites and 5.1 hosts, on average. The host and parasites made contact 91 times: 76 were initiated only by the parasite (84 %), 7 by the host, and another 8 were initiated by both species simultaneously. The host initiated contact with open mandibles 12 out of 13 times (mandibles were not in view 2 times). In 77 of the 91 interactions, the host reaction was observable. Following the initial contact, the host reacted with aggression 6 % of the time, submission 73 % of the time, and with “other” behavior 22 % of the time (Fig. 3c). Unlike the host of *M. mondaboroides*, the *M. silvestrii* host ants often remained still, feigning death following an interaction (82 %) but sometimes abruptly walked away (18 %). Some host ants remained in the same position between interaction periods and were repeatedly stung by the same or different parasites.

The *M. silvestrii* parasites were very aggressive towards their host, stinging the host 41 times out of the 91 interactions viewed. Host reactions were scored only for those interactions where the host could be clearly observed ($N=34$). The host reacted aggressively to a parasite sting 9 % of the time and submissively 85 % of the time, and the majority of the submissive behavior was playing dead (93 %; Fig. 3d). Aggression by the host increased by the end of the raid; when a single parasite remained, the host actively chased the parasite.

Fig. 3 Reactions of *C. costatus* host ants after contact with *M. mondaboroides* or *M. silvestrii* parasites. **a** after the host or *M. mondaboroides* parasite initiates contact, **b** after the *M. mondaboroides* parasite stings the host, **c** after the host or *M. silvestrii* parasite initiates contact, and **d** after the *M. silvestrii* parasite stings the host



Overall, the host only bit the parasite seven times and the parasite responded with stinging four times or abruptly moved away.

Host Recovery from Parasite Stings In raids of both *M. mondaboroides* and *M. silvestrii*, the host ants were stung immediately and repeatedly, yet these individuals exhibited natural garden-tending behavior during and following the raids. The colonies were observed following the raid for 34 min for the *M. mondaboroides* host colony and 2 min for the *M. silvestrii* host colony to assess the health of the host ants. At no time during or following the *M. mondaboroides* and *M. silvestrii* raids, 94 min and 27 min total, respectively, did the host ants exhibit behavior that would indicate suffering or death. These behaviors are recognizable and have been observed in other studies involving *Cyphomyrmex* species (Adams et al. 2000a, 2000b).

Discussion

The three behaviorally similar thief ant species studied here vary greatly in the kind of alkaloids they biosynthesize. The cyclic secondary amine, pyrrolidine (1), with a five-membered ring, is found in both *M. mondabora* and *M. silvestrii* and also occurs in free-living *Megalomyrmex* species (Fig. 2). Pyrrolizidine alkaloids, with two fused five-membered heterocycle rings (7 and 8), are found in *M. silvestrii*, and were also discovered in the guest ant parasite, *M. symmetochus* (Adams et al. 2013). Most surprising are

the piperidines (4), (5), and (6), heterocyclic amines with a six-membered ring, found in *M. mondaboroides*. These are well known as venom components from *Solenopsis* (Jones et al. 1982b) and *Monomorium* ants (Snyder et al. 2009), but this is the first report of their occurrence in a *Megalomyrmex* species. The significance of this observation lies in the different biosynthetic pathways required by piperidines as opposed to pyrrolidines. The polyacetate pathway to the *Solenopsis* piperidines has been established (Leclercq et al. 1996), whereas the biosynthesis of simple 2,5-dialkyl pyrrolidines has not been elucidated, although studies of alkaloids from the formicoid *Tetraponera*, require amino acid as well as acetate precursors (Morgan 2004). Furthermore, a ketone-containing indolizidine has been reported in venom alkaloids from *Myrmecaria* (Francke et al. 1995), as has a solenopsidine (Ward et al. 2015). Pyrrolidine (3) is the first ketopyrrolidine to be observed in *Megalomyrmex*. The 19-carbon 2-hexyl-5-nonylpyrrolidine carbon skeleton is common to North American *Monomorium* species (Jones et al. 1982a). In those ants, the side chains often contain terminal alkenes. In the case of (3), the carbonyl on the penultimate carbon of the nine-carbon side chain may indicate a common biosynthesis, except for the establishment of functionality at the end of the side chain.

Megalomyrmex ants have a range of life styles and use their venom in various ways. Free-living species like *M. leoninus*, *M. foreli* (= *M. latreillei*), and *M. cyendyra* live in logs and shallow nests in the soil (Boudinot et al. 2013; Jones et al. 1999; Longino 2010). They forage in the leaf litter and aggressively protect honeydew-producing insects (Longino

2010). Based on what is known about their natural history, it is likely these ants use their alkaloid-based venom for killing prey, when competing for food, and for colony and symbiont defense (Jones et al. 1991b). Alkaloid-dispensing behaviors, such as gaster flagging and stinging, also have been observed in these and related species (Boudinot et al. 2013; Adams pers. obs.). We contrasted the venom content of free-living species (i.e., *M. leoninus*, *M. foreli*, and *M. cyendyra*) with *M. mondabora* to illustrate that these species biosynthesize the same or similar pyrrolidine alkaloids. This supports the hypothesis that *M. mondabora* is a transitional species that shares similarities with the free-living *Megalomyrmex* species as well as the social parasites. Adams and Longino (2007) reported that *M. mondabora* were observed foraging at night and leaving their nest structure during the day, an unusual observation for *Megalomyrmex* parasites. Perhaps the nutritional needs of these thief ant parasites are not fully met by their fungus-growing ant hosts, and they supplement their diet with other resources. It is likely that *M. mondabora*, similarly to the free-living species, utilize their venom for repelling competitors and/or killing prey. In addition, it is plausible that they use the same compounds to subdue their *C. cornutus* host ants when young queens infiltrate the host colony or when parasites are foraging within the host nest.

Social parasites enter the protected fortress of their host colony by circumventing the “security system” using disguise or overwhelming the guards by force (Akino 2008; Lenoir et al. 2001). Ants recognize each other by a chemical code based on a profile made of waxy cuticular hydrocarbons detected from the surface of the ants (Martin and Drijfhout 2009). To avoid discovery, parasites often mimic or acquire the profile of their host colony, or reduce their hydrocarbons to undetectable levels (i.e., chemical insignificance) (Guillem et al. 2014). In addition to, or instead of, manipulating nestmate recognition cues, the parasites can use chemical weapons to overpower and confuse the host. This weaponry has been well-described in other social parasites as 1) propaganda substances that confuse or panic the host (Allies et al. 1986; Regnier and Wilson 1971), 2) repellents that discourage dangerous confrontations (Blum et al. 1980; D’Ettorre et al. 2000), and 3) appeasement allomones that pacify the host into submission (Mori et al. 2000).

The three weaponry strategies were investigated by exploring how diverse *Megalomyrmex* thief-ant venoms may be perceived by their *C. costatus* host during colony raids. The alkaloid classes we detected function as propaganda, repellent, and appeasement substances in other ant species. For example, the two pyrrolizidine isomers found in *M. silvestrii* thief ants are used as a propaganda substance by *M. symmetochus* against a competing social parasite (Adams et al. 2013). The pyrrolizidine-based venom confuses *Gnamptogenys* raiders and they attack members of their own raiding party. However, in our study, the piperidine- and pyrrolizidine-

alkaloid-based venoms did not appear to cause panic or confusion among the host workers. Instead, the workers were submissive and avoided the parasites. Even when stung by the parasite, host aggression was much less frequent than submissive behavior (Fig. 3b, d). In another example, *Solenopsis fugax* uses a repellent compound that is similar to compounds found in both free-living *Megalomyrmex* and *M. mondabora* parasites (Blum et al. 1980). *Solenopsis fugax* is a thief ant that lives near other ant species, breaks through the walls, and discharges an alkaloid-based repellent, causing the host colony to abandon brood that the thieves subsequently steal (Blum et al. 1980; Hölldobler 1973). However, as previously reported, the *C. cornutus* host ants rarely respond to their *M. mondabora* parasite aggressively and play dead following contact (Adams and Longino 2007), suggesting that the pyrrolizidine alkaloids (1–3) in this context might pacify or appease the host. To our knowledge, alkaloids have not yet been described to function as appeasement substances, but the aforementioned study and our behavioral observations suggest that they do.

Chemical mimicry or insignificance did not appear to be a strategy employed by the parasites in our study. The *C. costatus* host ants responded aggressively to the parasite invasion, even occasionally biting the *M. mondaboroides* raiders. The host ants appeared distressed, quickly walking around the garden, sometimes with open mandibles, presumably emitting alarm substances from their mandibular glands (Crewe and Blum 1972). The host bites did not deter or harm the parasites, and often were followed quickly by a parasite sting that resulted in a submissive response by the host. For the entirety of the raid, the activity level of the *M. mondaboroides* host ants was high, and they seemed generally to be repelled by the parasites. However, the parasites did induce a submissive response where the host ants played dead, remaining still for several seconds. Initially, this behavior might suggest that the host ants were impaired, but they fully recovered as they typically do after harmless human disturbance (Adams pers. obs.).

The *C. costatus* ants, host of *M. silvestrii*, were not very active, and most interactions were initiated by the parasites. The host ants appeared overall submissive and were most likely to play dead regardless of the type of contact (i.e., initiation or sting; Fig. 3c, d). The beginning of the raid was not observed and, therefore, our results may not reflect the host behavior following the initial invasion, but still, the parasites and their pyrrolizidine venom clearly induced thanatosis (playing dead) in the host ants, a submissive behavior that reflects the defensive strategy of crypsis used by *Cyphomyrmex* ants (Adams and Longino 2007; Adams et al. 2000b).

Our observations on *C. costatus*, host of *M. mondaboroides* and *M. silvestrii*, and previous observations on *C. cornutus*, host ants of *M. mondabora* (Adams and Longino 2007),

suggest that the host species are in various ways tolerating thief ant colony invasions. First, the host ants are rather small (*C. costatus* head length 0.67 mm and *C. cornutus* head length 0.87–0.96 mm) and do not have mandibular strength to remove appendages of the parasites (Adams and Longino 2007), unlike the *Sericomyrmex amabilis* hosts that can bite off the legs and antennae of their *M. symmetochus* guest ant parasite (Boudinot et al. 2013). Second, like other fungus-growing ants (Hermann et al. 1970; Wheeler 1907), the host likely has a vestigial sting. Third, the thief ant venoms have low or no toxicity towards *C. costatus* host workers. Host ants were repeatedly stung by the parasites but recovered by the end of the raid and 2–30 min following it. An alternative response to such an invasion would be to flee the nest with brood and bits of garden just as *C. longiscapus* does when their colony is infiltrated by *M. wettereri* (Adams et al. 2000b) but this was not the case; rather the *C. costatus* ants remained on or near their garden during the raid and offered little resistance. The negative fitness impact of the raids likely influences future reproductive success of the host colony because worker and sexual brood could be stolen and garden mass reduced, but raids do not appear to threaten the host colonies immediate survival.

Parasite tolerance and resistance are two viable host strategies observed in nature (Svensson and Råberg 2010). Both defense strategies come at a cost to the victim and vary from population to population (Ruano et al. 2011). If resistant to a raid, the host may lose workers and other resources in the battle if nest defense fails. If tolerant to a raid, the host loses resources but does not risk bodily injury. Tolerant hosts may adapt to frequent raids by increasing garden mass and brood production, just as magpie birds increase clutch size when living in sympatry with cuckoos (Soler et al. 2001). Tolerance behavior also has been shown in slave-making ant hosts, and the fact that it differs among populations with and without parasite pressure suggests that it could be a counter-defense and a consequence of antagonistic coevolution (Zamora-Munoz et al. 2003).

Future experimental work is needed to determine if the tolerance behavior we observed is consistent across all host colonies despite their evolutionary history or if it varies from population to population. It also would be useful to uncouple parasite behavior and the specific semiochemicals to assess the relative importance of each. One limitation of such studies is the rarity of these small tropical social parasites. On the other hand, the *C. costatus* host is abundant (Shik and Kaspari 2010) and experiments with synthetic compounds could be used for behavioral assays and dose-response experiments. Like *Solenopsis* and *Monomorium* ants, it is becoming apparent that *Megalomyrmex* also are a valuable source of alkaloids. Not only have these natural products enabled ants to procure resources, but their influence on the microbial environment can change the ecological makeup of habitats

(Vander Meer 2012). Our future work will involve additional comparisons across the Solenopsidini to elucidate the evolution of the biosynthetic pathways needed for shifts in venom alkaloid production.

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