Supporting Information

Adams et al. 10.1073/pnas.1311654110

SI Text

Study System

Natural History of *Sericomyrmex amabilis*. Like the other species in the derived "higher attine" clade (1), *S. amabilis* cultivates an obligatorily associated leucocoprineaceous fungus (Movie S1). It is grown in multiple soil chambers (up to 12 in this study) distributed shallowly, both horizontally and vertically, at a depth of ∼7 cm. In Panama, colonies can be found in forested habitats with thin canopy cover or at forest edges where sunlight penetrates (2). Nests have multiple entrances leading directly into the leaf litter. Pulling away leaves exposes narrow passages in which *S. amabilis* ants and their social parasite, *Megalomyrmex symmetochus*, can be observed. Parasitized and nonparasitized colonies were collected between 2009 and 2011 from the canal region and El Llano in Panama (Table S1). At the Plantation Road site near Gamboa (9° 4' 42.30" N 79° 39' 35.64" W), the parasitism rate was 78% ($n = 9$) in 2010 and 67% ($n = 9$) in 2011.

Natural History of *M. symmetochus*. *M. symmetochus* is a guest ant social parasite (i.e., a xenobiont) (3) with a range from Nicaragua to Peru that is obligatorily associated with *S. amabilis* colonies (4). Workers are distributed throughout the host garden chambers, but are more concentrated in the chamber containing the parasite queen and brood (Table S2). Similar to workers of *Megalomyrmex wettereri* (5) and *Megalomyrmex adamsae* (6), *M. symmetochus* workers manipulate the fungal substrate to construct cavities with small entrances (Movie S2), isolating the guest ant queen(s) and brood from their host ants. Although interactions between the host and parasite workers are typically amiable, aggression can be observed between the two species at the start of the rainy season when they both produce sexuals.

Natural History of *Gnamptogenys hartmani*. *Gnamptogenys hartmani* is a nocturnal and subterranean predatory poneroid ant that has been collected from Louisiana to northern South America (7, 8). It is a specialist agro-predator with usurpation strategies effective against fungus-growing ant species in the genera *Trachymyrmex* and *Sericomyrmex* in Panama (9). *G. hartmani* occurs sympatrically with *S. amabilis* at various sites in the Panama Canal region (e.g., Plantation Road, Pipeline Road, Gamboa Forest) and has been collected year after year by several attine researchers since 1999, although little information on its biology has been published to date (9, 10).

The *G. hartmani* colony used in this study was collected from a shallow ditch near Pipeline Road (Table S1) in a patch of disturbed secondary forest inhabited by *Trachymyrmex*, *Sericomyrmex*, and *Myrmicocrypta* attine ant species. The colony was occupying a usurped *Trachymyrmex zeteki* or *T. cf zeteki* nest with three chambers. Fungus gardens were found in the two deepest chambers in which the *G. hartmani* colony resided (queen, approximately 250 workers, and brood). No host ants were found, but both *T. zeteki* and *T. cf zeteki* have a characteristic nest structure with an auricle entrance (11), so host ant identification to this level was unambiguous despite the fact that the nest entrance was unkempt and the large opening was filled with soil, as is typical of *G. hartmani*-occupied *Trachymyrmex* nests.

Methods and Results

Guest Ant Venom Function. Staged raids were conducted to observe host and guest ant reactions to a *G. hartmani* scouts. Three remarkable observations were made:

i) In host colonies without guest ant parasites, *G. hartmani* killed *S. amabilis* workers at a very high rate, although some raiding workers with missing legs and antennae were seen after an altercation (Fig. S1*A*).

ii) In colonies with guest ant parasites, the *M. symmetochus* workers quickly appeared in large numbers on the top of the fungus garden when a scout of *G. hartmani* entered the colony, whereas the host ants hid or fled (Movie S1). Later observations revealed that this was likely because *M. symmetochus* workers recruit sisters from their scattered fungal cavities soon after *G. hartmani* workers enter the fungus garden (Movie S2).

iii) *G. hartmani* workers were observed attacking one another after interactions with an *M. symmetochus* guest ant (Fig. S1*B* and Movie S2).

Methods. To investigate *M. symmetochus* venom function and its behavioral effects on *G. hartmani*, we conducted a pairwise interaction study. A single naïve *G. hartmani* worker was placed in a small (35-mm) Petri dish and allowed to acclimatize for at least 15 min. Then another *G. hartmani* worker was gently held down while an *M. symmetochus* worker, held in forceps, was rubbed against it for 1 min (Fig. S1*C*). Care was taken to ensure that mainly the gaster of the *M. symmetochus* worker made contact, and that venom was visible in droplets at the end of the sting. This methodology ensured that the *G. hartmani* individual received multiple smears from the sting, as is seen in natural altercations. The stung individual was then placed in the Petri dish with a naïve nestmate, and behavior was videorecorded for 1 h (Fig. S1*D*) and later scored. An equal number of control experiments ($n = 6$) were also conducted, in which the second *G. hartmani* individual was not stung, but rather was rubbed with empty soft forceps.

Results. Aggression. We observed distinct differences in behavior between the stung and control individuals and identified the naïve ant as the aggressor in four of the six treatment replicates. Aggressive interactions were defined as one ant gaster flexing toward the other and sometimes biting (Fig. S1*B*). No aggressive interactions were observed in the control experiments.

Avoidance/Attraction. Times of contact were divided by the duration of the experiment and converted to percentages. Experiment duration ranged from 16 to 52 min (average, 46 min). The percentage of time that the two workers were in close contact between treatments was compared using Welch's t test, given the significantly different variance between the two treatments (Levene's test, $F_{1,10} = 22.6$, $P = 0.0008$). The shortest duration was in a treatment replicate in which the stung ant died shortly after an aggressive interaction with her sister, whereas no other experimental ants died for up to 24 h after the experiment.

Defense Efficiency in Two-Species Interactions (Fig. 2). To examine the interaction between a single *G. hartmani* scout worker and two, three, four, six or eight *S. amabilis* or *M. symmetochus* worker opponents/defenders, we used arenas made of 35-mm Petri dishes lined with moist filter paper. Defenders were allowed to acclimatize to the new arena for ≥15 min, after which a single starved *G. hartmani* worker was placed in the arena and videorecorded for 1 h. Observations were then made opportunistically for 24 h, after which surviving ants were separated by species and monitored under similar conditions for another 24 h. No ant was used in more than one trial. Replicates were named by colony ID (i.e., Mb, Mc, Md, and Me; Table S1). Each trial

type was replicated once for each of the experimental colonies Mb, Mc, Md, and Me; however, owing to no or low interactions, some *Sericomyrmex* replicates were excluded (i.e., Mb3S, Me3S, and Me8S), because we were testing the combat abilities of the two opponent types against a single *G. hartmani* invader. In those trials, the *S. amabilis* ants escaped attack by hiding on the lid of the arena, an area that the *G. hartmani* were incapable of occupying.

The duration of fights between *S. amabilis* and *G. hartmani*, during which the two ants were visibly biting and stinging each other, were measured in four trials (Mb2S, Mb4S, Me2S, and Me4S) from two colonies (Mb and Me) that included six different ants (Mb2SA, Mb2SB, Mb4SA, Me2SA, Me2SB, and Me4SA). A single *S. amabilis* worker was seen to engage in as many as five locked interactions with *G. hartmani* workers before dying. The times of 11 interactions were averaged to provide a duration length of engagement.

Three-Species Interactions, Survival, and Raider Mutilation (Fig. 3). Subcolonies were set up in 54-mm Petri dishes lined with moist filter paper, using 18 *S. amabilis* workers and a small amount of fungus garden (ca. 1.5 cm in diameter). Trials included three treatments with zero, three, or six *M. symmetochus* workers and were replicated using separate subcolonies derived from three parasitized colonies (Mb, Mc, and Md). The ants were allowed to acclimatize in the Petri dish for ≥24 h before two starved *G. hartmani* workers were introduced. Behavior was videorecorded for 1 h, and dead ants were collected and counted after 1 h and 24 h. The bodies of *G. hartmani* workers were analyzed under a 20× dissecting microscope, and missing legs and antennae (collectively termed "extremities") were counted (up to a total of 16) to quantify the degree of damage and determine the possible cause of death. Each ant was used in only one trial.

Raid Preference Choice: Colonies with Guest Ants vs. Colonies Without Guest Ants (Fig. 4). ***Methods.*** The *G. hartmani* colony was given a choice between parasitized and nonparasitized *S. amabilis* colony pairs that were approximately size-matched based on host worker number, garden mass, and nest box volume. Four pairs—Mb+Sb, Mc+Sc, Md+Sd, and Me+Se (Table S1)—were tested 11, 24, 29, and 23 times, respectively. Olfactometers (i.e., y-tubes) were composed of a bifurcating core made of white polyethylene plastic with a transparent acrylic plastic lid, with a 3.85-cm-long trunk and two 4-cm long branches (Fig. S2). Three transparent acrylic plastic tubes were added to each opening of the core to extend the length by 6 cm. The tubes had an inner diameter of 11.5 mm and a core diameter of 15 mm. The bottom of each tube was lined with moist filter paper, which was changed after every trial. Each colony pair was tested several times alternating the position of the parasitized relative to the nonparasitized colony. A wire mesh screen was set at the entrance of each of the *S. amabilis* colonies to allow airflow and only minimal physical interaction between *G. hartmani* scouts and *S. amabilis* workers. All parts (12 tubes, four cores, and eight wire meshes) were cleaned with 96% ethanol and randomized between trials.

Two types of trials were conducted between July 4 and September 27, 2011. The first set was allowed to run for 30 min or until a clear raid started and then waned. A total of 53 trials took place over 21 d, with 2 trials excluded from analysis owing to lack of activity. The second set of 32 trials also took place over 21 d, but these trials were allowed to run between 1.5 and 6 h. All trials were scored blindly from videorecordings to assess whether or not raids had occurred. *Gnamptogenys* recruitment behavior was as described in previous studies (9, 12–15). Strong recruitment to one side of the bifurcation was preceded by scout trail-laying behavior, excited nestmate interactions, sporadic tandem running, and a constant trail of foragers (Fig. S2 and Movie S3). Trail-laying behavior was observed in real time during the 30-min trials. In 10 cases, recruitment was somewhat ambiguous (i.e., some recruitment to both sides and/or alternating between the bifurcation arms), and a blind observer determined whether to keep the sequence as a trial or discard it as uninformative.

The direction in which and time that the first ant passed the bifurcation of the y-tube could be recorded in most trials ($n = 76$; Table S3). Direction data were used first to determine whether there was an experimental bias for the left, right, parasitized, or nonparasitized side of the y-tube. The interval between the first ant passing the bifurcation and the subsequent start of a raid was averaged across repeated measures and used as a measure of overall colony activity and motivation of the *G. hartmani* scouts. ***Results.*** In all but two trials, a *G. hartmani* worker entered the y-tube within 4 min after the start of the experiment ($n = 76$; Table S3). Two trials were excluded for lack of activity, but most of the trials resulted in scout investigations only. Out of 10 recruitment events deemed ambiguous (i.e., recruitment to both sides and/or crossing between arms), a blinded observer chose to keep three as being informative and to discard the other seven. Twenty-one of the 23 raiding trials resulted in a preference for colonies without guest ants (Fig. 4).

We conducted two types of trials (long and short), and summary statistics illustrate little difference between them. There was a marginally significant bias toward the left ($\chi^2 = 4.26$, $P = 0.04$) when the long and short trials were grouped, but this did not bias the results, given the aforementioned randomization (Fisher's exact test for heterogeneity, $P = 0.596$). The *G. hartmani* scouts first investigated the colony with *M. symmetochus* social parasites in 55% of the trials overall (42 of 76) and in 65% of the trials resulting in raids (15 of 23), neither of which was significantly different from 50% ($\chi^2 = 0.842$, df = 1, $P = 0.285$ and $\chi^2 = 2.13$, df = 1, $P = 0.094$, respectively). This finding illustrates that the decision to recruit to host colonies with or without *M. symmetochus* parasites was not clearly associated with which side of the bifurcation a scout investigated first.

Volatile Chemical Analyses. ***Methods.*** We analyzed all three species to detect volatile chemical components that could play roles in the behavioral interactions between species. The results of *S. amabilis* analyses have been published elsewhere (16), whereas the *G. hartmani* and *M. symmetochus* compounds reported here have not been described previously. All samples were extracted in methanol and analyzed by GC-MS following established methods (16). For *G. hartmani*, both whole ants and trisected samples (head, mesosoma, and gaster separately) were analyzed. For *M. symmetochus*, six pooled whole-body samples of 5–20 workers from colony RMMA100610-03/Me were analyzed, and the ratios of the alkaloids present were calculated. Venom gland extractions were also analyzed to confirm that the alkaloids came from that gland.

Volatile alkaloids were investigated using head-space analysis with a solid-phase microextraction (SPME) fiber assembly carboxen/polydimethylsiloxane (57318 SUPELCO; Sigma-Aldrich) that was exposed for ∼5 h to either 8 *M. symmetochus* workers from colony Me or 10 *S. amabilis* ants and fungus garden from a control colony (RMMA100611-03) without *M. symmetochus* guest ants, in a carbon-filtered air-flushed glass vial. After exposure, the SPME fiber was introduced into the injection port of an Agilent Technologies 6890N gas chromatograph equipped with a HP-5MS capillary column (Agilent 19090S-433: 30 m, 250 μm, 0.25 μm) and coupled to an Agilent Technologies 5975 inert mass selective detector with 70 eV electron impact ionization. After an initial hold at 60 °C for 1 min, the temperature was increased gradually to 250 °C for 10 min, to 300 °C for 3 min, and then to 320 °C for 10 min. The spit-splitless injector ran in

splitless mode with a carrier gas of helium flowing at 1 mL min^{-1}. Compounds were identified based on their mass spectra and analyzed with ChemStation v. D. 02.00.237. Before and after sample injection, the fiber was analyzed to detect any alkaloids in the air or on the GC-MS column. All nonalkaloid peaks were ignored.

Results and discussion M. symmetochus. (5Z,8E)-3-Butyl-5-hexylpyrrolizidine and (5E,8E)-3-butyl-5-hexylpyrrolizidine were found in all six *M. symmetochus* samples in a 59%:41% ratio (Fig. S3). The headspace sample indicated a >99%:<1% ratio, however. The difference is likely due to the level of volatility of (5Z,8E)-3-butyl-5-hexylpyrrolizidine compared with the (5E,8E)-3-butyl-5-hexylpyrrolizidine isomer, with the nonbonding electrons covered more extensively by the two alkyl side chains in the (5E,8E)-isomer. In this ring system, the nonbonding electrons and the alkyl side chains are all on the same side of the cis-fused pyrrolizidine ring system. The stereochemistry of the (5E,8E)-3-butyl-5-hexylpyrrolizidine was first described when the compound was discovered in *Megalomyrmex modestus* (17), but function was not addressed in that study.

Our behavioral and chemical analyses indicate that *M. symmetochus* synthesizes a potent chemical weapon that is effective against both the *S. amabilis* host and the *G. hartmani* raider. The pyrrolizidine isomers are toxic, induce submissive behaviors, and repel *S. amabilis.* A similar response was observed when the alkaloids were used against *G. hartmani*. In addition, the pyrrolizidines disrupt nestmate recognition and induce *G. hartmani* sisters to attack and kill one another. The chemicals used by social parasites that cause this kind of social disruption are known as "propaganda allomones" (18, 19). Our findings do not exclude the possibility that other *M. symmetochus*-derived compounds or behavioral interactions induced some of the observed responses in *G. hartmani*; however, the avoidance of parasitized colonies in the y-tube experiment (Fig. 4), along with the reactions of *G. hartmani* nestmates to their experimentally stung (i.e., alkaloid-smeared) nestmates (Fig. S1*B*), strongly suggest that the 3-butyl-5-hexylpyrrolizidine alkaloids produced in the *M. symmetochus* sting gland are responsible for killing and disrupting nestmate recognition in *G. hartmani* workers.

G. hartmani. GC-MS analysis of the methanol extracts of whole *G. hartmani* revealed the presence of 2,5-dimethyl-3-isopentylpyrazine and 3-indole ethanol (Fig. S3). A subsequent analysis of heads, mesosomas, and gasters found the pyrazine concentrated in the heads and the indole ethanol present in the mesosomas. 2,5-Dimethyl-3-isopentylpyrazine has been found in the mandibular glands of the ponerine ant *Odontomachus brunneus*, where it functions as an alarm pheromone and repellent (20); in that experiment, *O. brunneus* workers became agitated when presented with the pyrazine, whereas *Solenopsis invicta* workers were repelled. 3-Indole ethanol was recently reported as an insect secretion, found in the pygidial glands in the abdomen of the myrmicine ant *Ocymyrmex laticeps* (21), but its function remains unknown. We detected no volatile compounds in abdominal *G. hartmani* samples, suggesting that *G. hartmani* produce venom of a nonvolatile nature, similar to *Gnamptogenys menadensis* (22). Previous descriptions of *G. hartmani* raids (9) and our observations in the present study clearly indicate that this agro-predator uses volatile compounds to make most resident *Trachymyrmex* and *Sericomyrmex* workers abandon their fungus gardens as soon as the first *G. hartmani* workers appear in the nest. Thus, it seems likely that pyrazine mediates this effect, but this awaits direct experimental proof.

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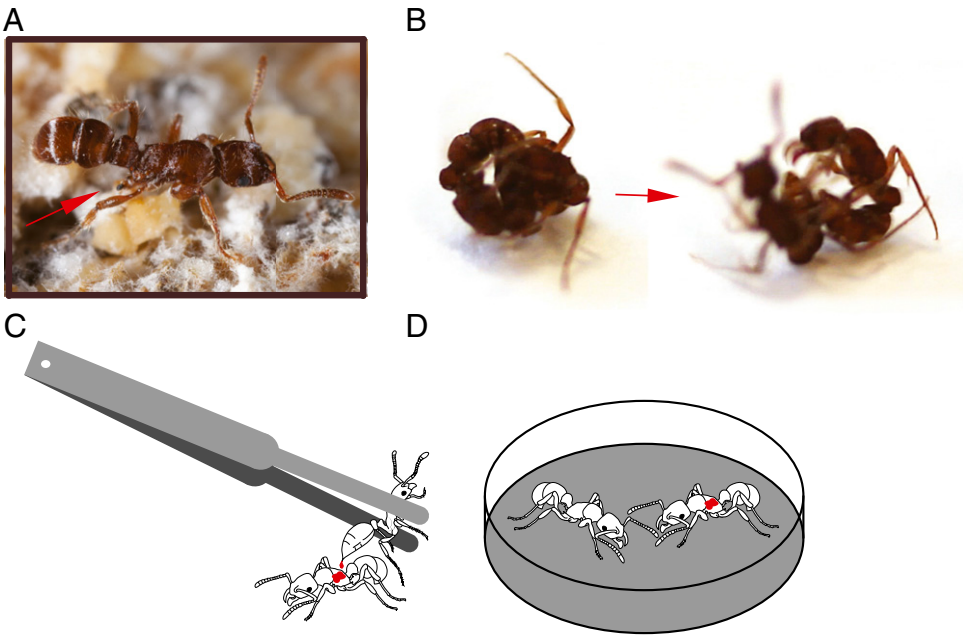


Fig. S1. Guest ant venom function. (*A*) A *G. hartmani* worker missing a leg (red arrow) just after an altercation with an *S. amabilis* host ant, which was killed in the interaction. (*B*) Consecutive video frames of two *G. hartmani* sisters attacking one another after one was stung by an *M. symmetochus* guest ant during an attempted raid. (*C*) A guest ant forced to sting a *G. hartmani* worker (venom colored red for emphasis). (*D*) Stung individual (red) introduced to a naïve *G. hartmani* worker in a 35-mm Petri dish.

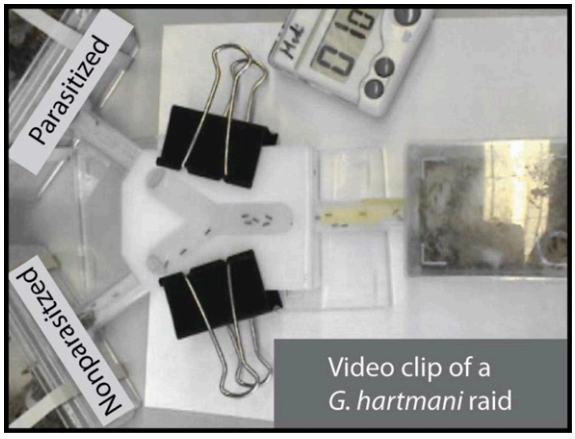


Fig. S2. Frame from a y-tube experiment video (Movie S3), illustrating the experimental setup and a typical recruitment column. The *Gnamptogenys* colony is in the nest box on the right.

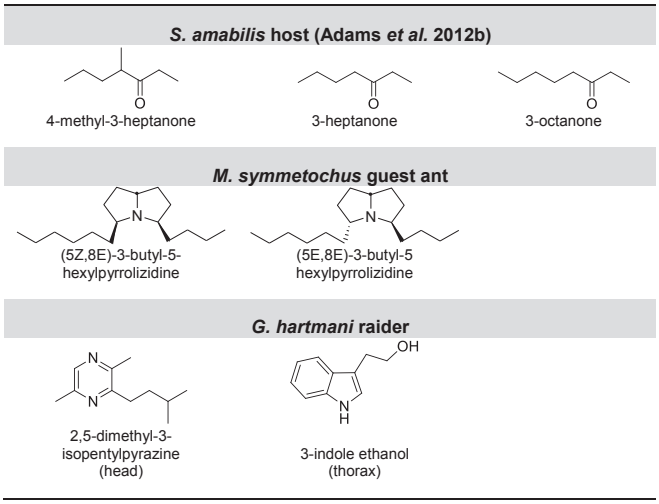


Fig. S3. Volatile chemical compounds found in *S. amabilis* (16), *M. symmetochus*, and *G. hartmani* ants.

Table S1. Collection information

Collector ID/ colony code	Site	GPS data	Species
AI110511-05/Mb	Plantation Road	9°4′42″N 79°39′36″W (111 m)	*M. symmetochus*/*S. amabilis*
AI110515-06/Mc	Plantation Road	9°4′54″N 79°39′33″W (111 m)	*M. symmetochus*/*S. amabilis*
RMMA110529-01/Md	Plantation Road	9°4′42.30″N 79°39′36″W (127 m)	*M. symmetochus*/*S. amabilis*
RMMA100610-03/Me	Plantation Road	9°4′36.30″N 79°39′34″W (105 m)	*M. symmetochus*/*S. amabilis*
HHdFL09####-15/Sb	Gamboa Forest	Unknown	*S. amabilis*
HHdFL10####-01/Sc	Gamboa Forest	Unknown	*S. amabilis*
RMMA100521-14/Sd	El Llano	9°16′46.40″N 78°57′41″W (382 m)	*S. amabilis*
RMMA100611-03/Se	Plantation Road	9°4′36.30″N 79°39′34″W (105 m)	*S. amabilis*
RMMA110510-01	Pipeline Road	9°08′16″N 79°43′24″W (45 m)	*G. hartmani*

Collector ID includes collector's initials, year, month, day, collection number, and experimental ID. Collectors were Anders Illum (AI), Rachelle M. M. Adams (RMMA), and Henrik H. de Fine Licht (HHdFL).

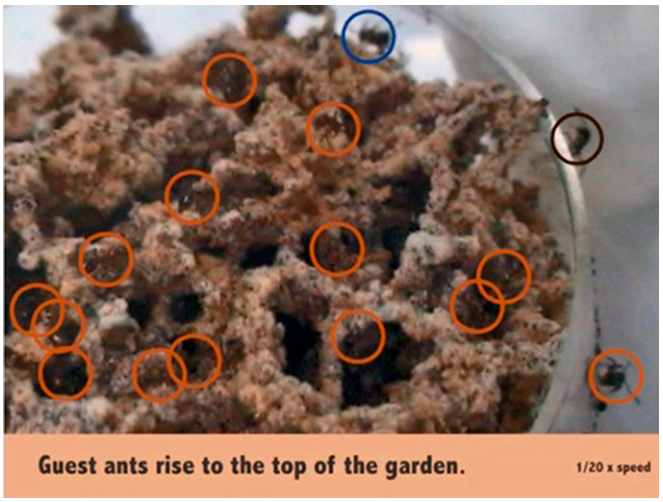
Table S2. Colony counts for AI110511-01, illustrating the distribution of parasite workers throughout the nest chambers

	M. symmetochus			*S. amabilis*		
Chamber	Workers	Queens	Sexuals	Workers	Queens	Sexuals
1	32	0	0	166	0	0
2	122	1	35	189	0	0
3	50	0	6	230	2	0
Total	204	1	41	585	2	0

Table S3. Y-tube summary statistics illustrating colony activity level and the similarly of the short and long trials

		First scout ant passing bifurcation					
Trial type and number of replicates	Tube entry, s, median (range)	Time, s, median (range)	Direction, left/right; χ^2 and P values		Colony type, nonparasitized/ parasitized; χ^2 and P values		Time to raid, s median (range)	Number of raids
Short (30 min) (n = 49)	85 (9–703)	205 (48–985)	30/19	$\chi^2 = 2.47$; $P = 0.12$	25/24	$\chi^2 = 0.02$; $P = 0.89$	1,245 (675–1,718)	8
Long (1.5–6 h) (n = 27)	94 (26–664)	186 (49–997)	17/10	$\chi^2 = 1.81$; $P = 0.18$	9/18	$\chi^2 = 3.00$; $P = 0.08$	7,912 (350–16,091)	15
Short and long (n = 76)	88 (9–702)	198 (48–997)	47/29	$\chi^2 = 4.26$; $P = 0.04$	34/42	$\chi^2 = 0.84$; $P = 0.36$	5,006 (350–16,091)	23

Colony type, y-tube core and extensions, and wire mesh were randomized.

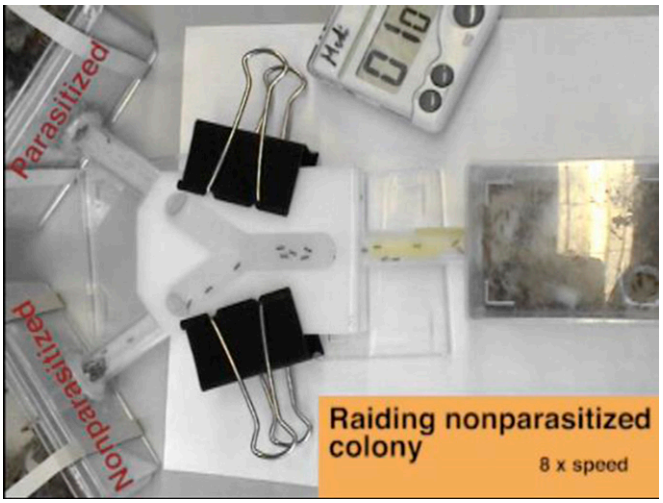


Movie S1. *Megalomyrmex* guest ants (orange circles) were observed rising to the top of the *S. amabilis* fungus garden and releasing volatile alkaloids from their sting soon after a few individuals made contact with a *G. hartmani* scout (brown circle). Only one host worker is seen on the surface (blue circle); the others have fled to lower parts of the garden.

Movie S1

Movie S2. *M. symmetochus* guest ants protect their *S. amabilis* host from *G. hartmani* raiders by recruiting nestmates from their cavity. (Here the cavity is open because it was built against the roof of the nest box.) The guest ants fight the raiders with alkaloid weaponry that is toxic and causes the invaders to attack one another.

Movie S2



Movie S3. *G. hartmani* colony raiding a nonparasitized *S. amabilis* colony in a y-tube choice test.

Movie S3