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Highlights

- Host chemical profile has complex features unmatched by parasites.
- Parasite odour profile consists of only a few long-chained cuticular hydrocarbons.
- Venom alkaloids dominate parasite chemical profile.
- Nonparasitized colonies are aggressive against parasites, parasitized colonies are not.
- Parasite uses a 'chemically insignificant' strategy and defensive weaponry.

https://doi.org/10.1016/j.anbehav.2018.02.021

Please cite this article in press as: Neupert, S., et al., Host colony integration: *Megalomyrmex* guest ant parasites maintain peace with their host using weaponry, Animal Behaviour (2018), https://doi.org/10.1016/j.anbehav.2018.02.021

Animal Behaviour xxx (2018) 1–9

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Host colony integration: *Megalomyrmex* guest ant parasites maintain peace with their host using weaponry

Stefanie Neupert [a], Alexandria DeMilto [b], Falko Drijfhout [c], Simon Speller [c], Rachelle M. M. Adams [b, d, *]

[a] University of Konstanz, Konstanz, Germany
[b] The Ohio State University, Columbus, OH, U.S.A.
[c] Keele University, Keele, Staffordshire, U.K.
[d] University of Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 1 September 2017
Initial acceptance 23 October 2017
Final acceptance 11 January 2018
Available online xxx
MS. number: A17-00707R

Keywords:
aggression
alkaloid
cuticular hydrocarbon
host–parasite system
Megalomyrmex symmetochus
nestmate recognition
resistance
Sericomyrmex amabilis
submission
tolerance

Social parasites exploit resources of other social species, to the detriment of their host. In order to enter and integrate in a host colony, social parasites must avoid being detected as a non-nestmate. The parasites, therefore, use one or a combination of chemical strategies: (1) producing recognition cues that match host's (mimicry), (2) acquiring recognition cues from the hosts or its nest (camouflage), (3) not producing recognition cues (insignificance) and/or (4) using substances for confusing, suppressing or appeasing the host (weaponry). In this study, we investigate the integration strategy of *Megalomyrmex symmetochus* ants into colonies of the fungus-growing ant *Sericomyrmex amabilis*. We compared the chemical odour profiles of parasitized and nonparasitized *S. amabilis* colonies with the profiles of the parasites. Additionally, we conducted behavioural assays, where we introduced a single ant, being either a nestmate, a conspecific non-nestmate or a parasite into an arena with five *S. amabilis* workers and scored the behaviour of the latter ants. The chemical analysis revealed that the social parasites have distinct odour profiles and share only one hydrocarbon with its host, have a low overall abundance of cuticular hydrocarbons and have high concentrations of venom-derived alkaloids. In behavioural experiments, we found that workers of nonparasitized colonies fight against parasite intruders, whereas workers of parasitized colonies treat introduced parasites (from their own and from another parasitized colony) similar to their conspecific nestmates. All workers (parasitized or not) show more submissive behaviour towards parasitized workers and parasites than towards nonparasitized workers. The chemical analysis of odour profiles suggests that the parasites use a chemical insignificance strategy. Furthermore, the chemical and behavioural data suggest that the parasites use weaponry to maintain an amiable association with their host ants. We discuss the biological significance of the lack of aggression in *S. amabilis* workers from parasitized colonies.

Sophisticated mechanisms have evolved to protect social insect colonies (e.g. ants, some bees and wasps) from invasion (Breed & Bennett, 1987; Gamboa, Reeve, & Pfennig, 1986; Hölldobler & Wilson, 1990). Therefore, a successful exploitation of social insect colonies requires strategic evasion of organized defence tactics. The recognition of colony members is a fundamental component of being social, and thus allows amiable social groupings and loyalty between colony members. The nestmate recognition system of social insects is predominantly chemically based and allows individuals to discriminate between members from their own colony (nestmates) and members from a different colony (non-nestmates). In ants, nestmate recognition is based on long-chained species-specific cuticular hydrocarbons (CHCs) present on the exoskeleton (Brandstaetter, Endler, & Kleineidam, 2008; Lahav, Soroker, Hefetz, & Vander Meer, 1999; Martin & Drijfhout, 2009). CHCs are thought to have evolved primarily as protection against desiccation (Lockey, 1988), and gained secondarily a function for identification of colony membership, where neighbouring colonies of the same species have the same CHCs, which only differ in quantity (d'Ettorre & Lenoir, 2009). The odour profile of a colony is not only genetically determined, but also influenced by environmental factors, like nest

* Correspondence: R. M. M. Adams, The Ohio State University, Department of Evolution Ecology, and Organismal Biology, 1315 Kinnear Road, Columbus, OH 43212, U.S.A.
E-mail address: adams.1970@osu.edu (R. M. M. Adams).

https://doi.org/10.1016/j.anbehav.2018.02.021
0003-3472/

material and diet (Jutsum, Saunderes, & Cherrett, 1979; Liang & Silverman, 2000), and thus varies over time (Vander Meer et al., 1989). It is generally assumed that an ant compares the perceived odour profile (label) of an encountered ant with an internal neuronal representation (template) of its own colony odour, a process called label-template matching (van Zweden & d'Ettorre, 2010). When label and template are similar, the encountered ant will be recognized as a nestmate; if mismatched, the encountered ant is recognized as a non-nestmate and may be attacked (Vander Meer & Morel, 1998). Since the colony odour changes over time, the neuronal template needs to be updated as well (Vander Meer et al., 1989). Social parasites, defined as a social organism that exploits another social organism, avoid host attack by circumventing detection or through host domination (Buschinger, 2009). Social parasites overcome host detection by using one or more chemical strategies: mimicry, camouflage, insignificance and/or weaponry (Akino, 2008; Lenoir, d'Ettorre, Errard, & Hefetz, 2001). Some parasites can produce nestmate recognition cues that match the host's chemical profile (chemical mimicry) while others can acquire them by exposure to the nest environment or host individuals (chemical camouflage). An impressive example for these two strategies is the caterpillar of the butterfly *Maculina rebeli* that parasitizes *Myrmica* ant colonies (Akino, 2008; Nash, Als, Maile, Jones, & Boomsma, 2008). The caterpillars produce host recognition cues and are carried by ant workers into the nest. Later they acquire additional hydrocarbons within the nest, making their CHC profile nearly identical to their host's (Akino, Knapp, Thomas, & Elmes, 1999). Similarly, social parasite wasp CHC profiles change to match colony-specific host odour following infiltration and during host colony integration (Sledge, Dani, Cervo, Dapporto, & Turillazzi, 2001). Other parasite species lack an abundant CHC profile, when recognition cues are absent the parasites appear to be chemically 'invisible' to the host (i.e. chemical insignificance). This strategy is used during host colony infiltration by the social parasitic ant *Acromyrmex insinator*. The parasites avoid host colony aggression by producing fewer hydrocarbons relative to their host and bearing increased n-alkane levels (Nehring, Dani, Turillazzi, Boomsma, & d'Ettorre, 2015). Besides circumventing detection, parasites can also produce chemicals to attack or confuse the host, disrupting nestmate recognition and host defence behaviour (chemical weaponry). This strategy is used in the slave-making ant species *Polyergus rufescens*. The usurping queen uses secretions from its Dufour's gland as an appeasement allomone (Mori, Grasso, Visicchio, & Le Moli, 2000) or repellent (d'Ettorre, Errard, Ibarra, Francke, & Hefetz, 2000).

The expected evolutionary response of hosts towards social parasites can be either in the form of resistance or tolerance, both adaptive solutions to parasite exploitation. Parasite resistance may involve direct host aggression towards the parasite, preventing a successful attack. It may also involve a hierarchical sequence of resistance behaviours that occur over time (Kilner & Langmore, 2011). In other circumstances, hosts use a 'tolerance' strategy to minimize detrimental fitness impacts of parasites, in other words, it is better to consent than to risk death (Svensson & Råberg, 2010). Tolerance behaviour would be expected in host species that lack an effective defence (e.g. toxic poison, strong mandibles) or species that do not recognize the parasite as a threat but instead as a harmless nestmate.

The social parasitic ant species *Megalomyrmex symmetochus* (Formicidae: Solenopsidini) (Wheeler, 1925) parasitizes the fungus-growing ant *Sericomyrmex amabilis* (Formicidae: Attini: Attina) (Wheeler, 1925) by living within the nest and consuming the brood and fungus garden of the host (Adams et al., 2013; Adams et al., 2013; Bruner, Wcislo, & Fernández-Marín, 2014; Bruner et al., 2014; Wheeler, 1925). The interactions between the host and parasites are typically amiable. However, aggression has been observed in field and laboratory colonies when the two species are producing sexuals (Boudinot, Sumnicht, & Adams, 2013). In addition, the parasite workers chew the wings from the host female sexuals (i.e. gynes), prohibiting these individuals from dispersing. The parasite workers are armed with toxic alkaloid venom that can kill the host and the hosts' enemies (Adams et al., 2013). In contrast, *S. amabilis* workers do not appear to have a toxic venom, but are capable of biting off legs and antennae from their opponents (Adams et al., 2013; Boudinot et al., 2013). Their reaction to threat is often crypsis, during which ants tuck their antennae and head under and play dead, similar to other attine host species (Adams, Jones, Longino, Weatherford, & Mueller, 2015).

The infiltration strategy (i.e. initiation of the association) and integration strategy (i.e. maintenance of the association) of *M. symmetochus* parasites into colonies of *S. amabilis* are currently unknown. In this study, we investigate the integration strategy of *M. symmetochus* parasites using chemical analysis and a behavioural approach. If host colonies and parasites have similar CHC profiles (i.e. mimicry or camouflage), then we predicted that workers from a parasitized colony would not react to the parasites but workers from a nonparasitized colony would react aggressively, just as they would to a conspecific non-nestmate. If the parasites' CHCs are very low in abundance, then we predicted that the host as well as nonparasitized ants would behave as if they did not detect the parasites (i.e. chemical insignificance). If the CHC profile of the parasite is not similar to their host's and causes a behavioural reaction by *S. amabilis* workers from parasitized and nonparasitized colonies, it would suggest a weaponry strategy. We found no evidence for mimicry or camouflage. In contrast, our chemical analysis suggests that the parasites use a chemical insignificance strategy. Furthermore, the chemical and behavioural data support the hypothesis that the parasites use weaponry to maintain an amiable association with their host ant species *S. amabilis*.

METHODS

Study Animals

Sericomyrmex amabilis colonies or subcolonies (referred to as colonies hereafter) were collected along Plantation Road in Gamboa (9°9′36″N, 79°44′24″W) and on Barro Colorado Island (9°9′36″N, 79°50′24″W), Republic of Panama between 2011 and 2013. Queenright and queenless laboratory colonies can be kept alive for years under humid laboratory conditions as long as they forage to feed their garden (R. M. M. Adams, personal observation). In our experiments, 16 laboratory host colonies contained *M. symmetochus* social parasites (referred to as parasitized colonies) and 19 *S. amabilis* colonies were without parasites (nonparasitized colonies).

Creating CHC Extracts

We randomly collected workers from parasitized and nonparasitized colonies (host and parasites, if present) from their nestboxes into filter paper-lined petri dishes, allowed them to acclimate, then froze them over night at −80 °C (colonies and species were kept in separate dishes). For each extract, we selected three workers and put them in a 2 ml vial with ca. 100 µl of pentane (cuticular wash). The vials were gently agitated for 1 min using a vortex machine with a slow rotating speed. After waiting 10 min and gently shaking the vial again, the solution was moved to a 200 µl insert and evaporated. The samples were frozen until chemically analysed. In total, we created 19 host ant extract samples from four parasitized colonies, 36 ant extracts of eight

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nonparasitized colonies and 24 extracts of parasites from five different parasitized colonies.

Chemical Analysis of CHC Extracts

Before the gas chromatography-mass spectrometry (GC-MS) analysis, 10 µl of hexane was added to each vial (C22 standard was added to a subset of the samples). Then using an autosampler, 3 µl of extract were injected (split-less) into a GC (Agilent 7890) coupled to MS (Agilent 5975C). The components were separated on a nonpolar column (ZB-5HT, 30 m × 0.32 mm, film thickness 0.25 µm). The carrier gas was helium with a constant flow of 1 ml/min. Chromatograms were recorded with Agilent Chemstation using the following temperature program: (1) constant temperature of 100 °C for 1 min, (2) increasing temperature at 30 °C/min to 250 °C, (3) increasing temperature at 4 °C/min to 360 °C and (4) constant temperature at 360 °C for 2 min. Chromatograms were analysed using Enhanced ChemStation version 2005 (MSD ChemStation, Agilent Technologies, Santa Clara, CA, U.S.A.). Peaks were aligned manually to ensure identical compounds were compared between runs. Peak areas of individual cuticular hydrocarbons were quantified with respect to the total CHC area within single chromatograms. CHCs were identified based on diagnostic ions, and mass spectra were compared with entries of the NIST11 library.

Calculation of Cuticular Surface

To compare the total CHC abundance between the host and the parasite species (insignificance hypothesis), we used only the samples with C22 standard (nonparasitized host samples: $N = 15$; parasitized host samples: $N = 16$; parasite samples: $N = 11$). We calculated the sum CHC area for each chromatogram and normalized the area to the standard peak area. To take into account potential differences in body surface area between the two species (since individuals with a small total body surface area may automatically have a lower total abundance of CHCs), the body surface area was estimated for parasite and host species individuals (for detailed information on calculation of the surface area, see Supplementary material 1, Methods). We used a similar approach as described in Kroiss, Schmitt, and Strohm (2009). We measured length, width and height of the three body parts: head, thorax (mesosoma) and gaster of 10 individuals per species with a stereo microscope equipped with an eyepiece reticle. From these measures, we approximated the surface area of each individual's body parts using corresponding geometric shapes. We calculated the total surface area, by adding the surface areas of the three body parts. We compared the surface areas of the two species using a two-sample *t* test. We then calculated the total CHC peak area per chromatogram corrected with respect to body size of the corresponding species.

Principal Component Analysis for CHC Profiles

We also compared the similarity of the CHC profiles between the host and the parasite (mimicry and camouflage hypothesis) using a principal component analysis (PCA), based on all chromatograms. We used the peak areas of the CHCs normalized to the total CHC peak area within each chromatogram as input. Furthermore, we compared the CHC profiles between parasitized and nonparasitized host colonies with another PCA. For quantification of the differences, we calculated the average pairwise Bray–Curtis distance based on the relative abundance of CHCs (normalized to the overall CHC area of each chromatogram) between all samples. Bray–Curtis distances were calculated with the 'bcdist' function in R (v.3.4.0) and range from zero to one. A value of zero represents a complete matching between two data points, while a value of one represents a complete separation in the *n*-dimensional space.

Behavioural Assay

For one trial, five *S. amabilis* workers (referred to as 'focal ants' hereafter) were selected from either a parasitized or nonparasitized colony and directly put into a small petri dish (35 mm in diameter) with a piece of fungus garden (ca. 100 mm^3). This setting, resembling the nest environment, should provide a social context for the focal ants, ensuring realistic colony defence responses (Kleineidam, Heeb, & Neupert, 2017). We allowed the focal ants to acclimate for at least 5 min. Then, another ant was introduced into the dish and is referred to as the 'stimulus ant' hereafter. The stimulus ant was either a *S. amabilis* nestmate, a conspecific *S. amabilis* non-nestmate or a heterospecific *M. symmetochus* parasite. New focal ants and *S. amabilis* stimulus ants were used in each trial, but it was necessary to use some parasite stimulus ants multiple times (due to a low number of parasite ants). The behaviour of all five focal ants in response to the stimulus ant was videorecorded for 5 min and later scored blindly (the person was unaware of colony origins for focal and stimulus ants). Solomon Coder (Version: beta 15.11.19 by András Péter) was used to record the behaviour of the focal ants following an interaction. An interaction was identified when a stimulus ant and a focal ant were within an antennal length distance from each other or when they touched. The behaviours were scored as being from one of several behavioural categories (Table 1). The behaviours 'short antennation', 'prolonged antennation', 'opening mandibles', 'carrying' and 'biting/pulling' were used for the statistical analysis of aggression. The behaviours 'turning' and 'head tucking' were used for the statistical analysis of submission. Sometimes, a focal ant did not show an obvious reaction or a change in behaviour after being contacted by a stimulus ant, or in some cases, head and mandibles of a focal ant were not visible, and therefore no discernible behaviour could be noted. Both cases were classified as 'unknown'.

We performed the trials with nine different pairings of focal and stimulus ants. The different pairings were necessary to disentangle whether the type of stimulus ant accounted for differences in behaviour of focal ants, or whether the type of focal ant (being from a parasitized or nonparasitized colony) also explained some of the differences. In two pairings, we tested focal ants from parasitized and nonparasitized colonies against their conspecific nestmates as stimulus ants (pairings 1 and 2, respectively). In four pairings, we tested the behaviour of focal ants from parasitized and nonparasitized colonies against conspecific non-nestmates from parasitized and nonparasitized colonies (parings 3, 4, 5 and 6). In the remaining three pairings, we tested the behaviour of focal ants against parasites. Focal ants from parasitized colonies were tested against their own parasites (pairing 7) and also against parasites from a foreign colony (pairing 8), while focal ants from nonparasitized colonies were tested only against foreign parasites (pairing 9), as by definition, they do not have parasites in their colony. In our experiments, we used queenright and queenless colonies for both parasitized and nonparasitized colonies. If the queen status affects nestmate recognition in the corresponding individuals (focal and stimulus ants), it would influence our experiments by increasing the variance in the data within all pairings.

Analysis of Behavioural Data

Sericomyrmex amabilis ants respond to various perturbations with a crypsis defence strategy (submission). Submission is therefore not unique to ant–ant interactions; however, it is a recognized defence behaviour in many attine ant species (Adams et al., 2015;

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Table 1
Ethogram of behavioural responses from focal ants towards stimulus ants used in statistical analyses

Behavioural response	Description	Analysis type
Short antennation	Focal ant's antennae move towards or touch the stimulus ant for <3 s	Aggression
Prolonged antennation	Focal ant antennation lasting >3 s	Aggression
Opening mandibles	Focal ant opens her mandibles in response to the stimulus	Aggression
Carrying	Focal ant displaces a stimulus ant by dragging or lifting it with her mandibles for >2 s	Aggression
Biting/pulling	Focal ant bites at or on the stimulus ant's body, including pulling at one of her extremities	Aggression
Turning	Focal ant changes direction (>45 degrees) after close contact with stimulus ant	Submission
Head tucking	Focal ant retracts her antennae into her scrobes and tucks her head down for at least 2 s	Submission

Wheeler, 1925). Since submission behaviour is not typically linked to nestmate recognition, we investigated the submissive behaviours head tucking and turning independently from the other behavioural responses (e.g. short antennation, prolonged antennation, opening mandibles, carrying, biting/pulling).

The behavioural data were analysed with respect to the differences in aggression behaviour between the nine different pairings. We ran a multinomial logistic regression model (a model with a categorical outcome variable) on the aggression data. This kind of analysis allowed us to account for differences in the number of encounters per trial by investigating the proportion of a single behaviour within each trial. Furthermore, it enabled us to account for repetitions of trials from the same colony (nonindependent data). For each trial, we counted the total number of occurrences of short antennation, prolonged antennation, opening mandibles, carrying and biting/pulling. We used these counts as the multinomial response variable. In the model, we included an explanatory variable for the specific pairing (1–9). The colony origin of focal ants and stimulus ants were added as random effects. The parameters of the multinomial logistic regression model were estimated in a Bayesian framework using Markov chain Monte Carlo simulations. We ran OpenBUGS from within R using the 'R2OpenBUGS' package (Sturtz, Ligges, & Gelman, 2005) and generated random initial values, then ran three Markov chains for 100,000 iterations. The burn-in was set to 10,000 and the chain was thinned by 10 (i.e. sampled every 10th iteration) to reduce autocorrelation. We assessed convergence graphically and by the R-hat values (i.e. MCMC convergence statistic), which were always close to one (Brooks & Gelman, 1998). From the Markov chains, we extracted the means using them as estimates and the 2.5% and 97.5% quantiles to describe the 95% credible intervals (Gelman, Carlin, Stern, & Rubin, 2004). In contrast to the null hypothesis testing in frequentist methods (via P values), Bayesian statistics allow the assessment of significance through probabilities of meaningful hypotheses (measure of certainty). We tested whether there were differences in the proportions of behavioural responses (e.g. short antennation, prolonged antennation, opening mandibles, carrying, biting/pulling) within a trial between two pairings. Therefore, we compared the proportion of a specific behaviour by calculating the percentage of simulated values from the posterior distribution that were larger (or smaller) in one pairing compared to another pairing. We considered a difference between the proportions of a specific behaviour between the two pairings to be statistically significant if the calculated percentage was larger than 0.98 (certainty of 98%).

We also investigated the differences of submission responses between the nine different pairings. We used a binomial generalized linear mixed model (GLMM) with submission (head tucking and turning) as the binary response variable. Again, this kind of analysis allowed us to account for repetitions of trials from the same colony (nonindependent data). Whenever submission (head tucking or turning) occurred within one trial, the trial was counted as submissive. If no submission occurred, the trial was counted as nonsubmissive. Additionally, we included the specific pairing (1–9) as explanatory variables in the model. We added origin of the focal ants as well as origin of the stimulus ant as the two random effects. Furthermore, we used a Bayesian framework in order to reveal certainty measures for the parameter values. In contrast to other methods, Bayesian methods are the only exact way to draw inferences from GLMMs (Bolker et al., 2009). We used an improper prior distribution (flat prior) and directly simulated 5000 values from the posterior distribution of the model parameters by using the 'sim' function of the R package 'arm' (Gelman & Hill, 2007). For each pairing, we used the fitted values as estimates and the 2.5% and 97.5% quantiles from the simulated model parameters as the upper and lower limits of the 95% credible intervals. We tested for differences in submission probabilities between two pairings by comparing the probabilities of submissive behaviour. We calculated the proportion of simulated values from the posterior distribution that were larger (or smaller) in one pairing compared to another pairing. We considered a difference between the probability of submission between two pairings to be statistically significant if the calculated proportion was larger than 0.98 (certainty of 98%). The statistical analysis was done using R 3.4.0 (R Core Team, 2017).

RESULTS

Chemical Analysis

To determine odour profiles, we analysed the chemical content of cuticular washes. The GC-MS analysis showed that the CHC profiles differed between the host and parasite ants (Fig. 1). Together, the *M. symmetochus* parasites and *S. amabilis* workers from parasitized and nonparasitized colonies revealed 33 different CHC compounds (Fig. 1, Table 2). Both *S. amabilis* workers from parasitized and nonparasitized colonies had 24 hydrocarbons, ranging from C29 to C37, and shared the same CHC profile (Table 2). In contrast, the CHC profile of *M. symmetochus* parasites ranged from C33 to C39 with 10 distinct compounds. Among those compounds were possibly several rarely occurring methyl-branched dienes (branched C35:2, C37:2 and C39:2). The reason why we are not certain is that peaks 20, 21, 27, 28, 32 and 33 had profiles of the mass spectra up to m/z 100 that looked very much the same as alkadienes compared to corresponding mass spectra in the NIST11 library. However, we also found indications that they were possibly branched alkadienes because there were a few ions at the higher mass end (m/z 200–420) that were more elevated, but this could also be due to higher ion abundances. We found only a single hydrocarbon (the alkene C35:1, Fig. 1, Table 2) that was shared between the host and parasites.

The parasite workers had a smaller amount of CHCs on their cuticle than the host species (Fig. 1a). To control for the differences of body size, we calculated the body surface area of host and parasite workers. The parasites had on average an 18.8% smaller body surface area than the host workers (two-sample t test: $t_{17.965} = 4.2911$, $P < 0.001$). However, when we compared the

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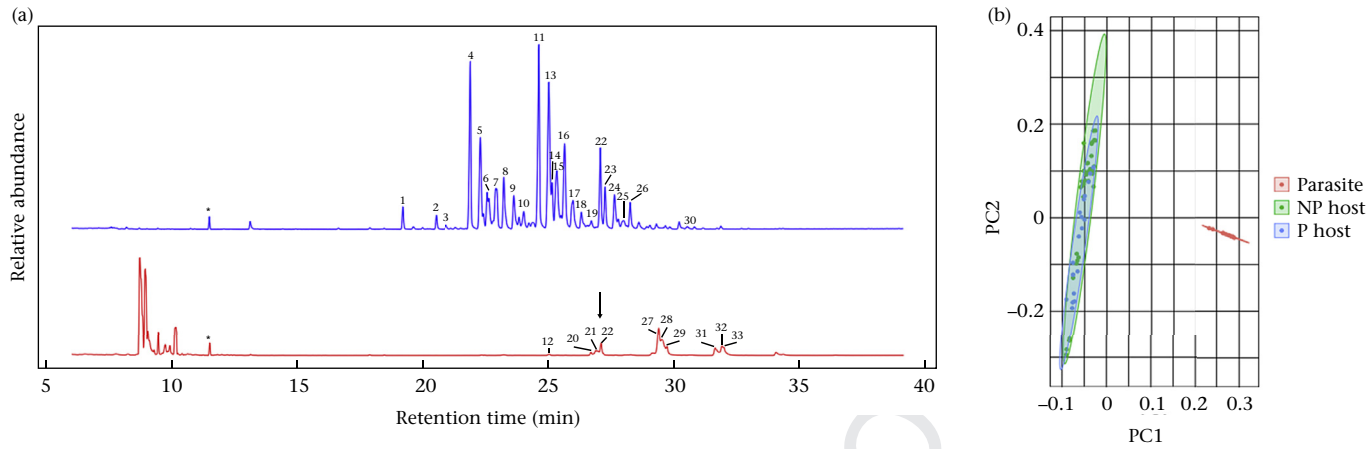

Figure 1. Odour profile differences between hosts and parasites. (a) Example chromatograms of *S. amabilis* and *M. symmetochus* odour profiles. The upper trace is a sample of *S. amabilis* workers from a parasitized colony and the lower trace is a sample of *M. symmetochus* parasite workers. Peaks with retention times of 8–10 min are parasite venom alkaloids. The peak marked with an asterisk refers to the C22 standard (11.5 min). The relative abundance of the two profiles were adjusted to the C22 standard to make them comparable. Numbers at the peaks refer to the peak numbers in Table 2. Peak 22 (arrow) is the alkene hydrocarbon that is shared between host and parasite. (b) Comparison of cuticular hydrocarbon (CHC) profiles between host and parasites using a principal component analysis (PCA). We used the peak areas of the CHCs normalized to the total CHC peak area within each chromatogram as input. Visualized are the first two principal components explaining 68.1% of the total variance. Parasite profiles were separated mainly along the first principal component (PC1); nonparasitized and parasitized *S. amabilis* host (NP host and P host, respectively) profiles were separated mainly along the second principal component (PC2).

Table 2
Mean relative abundances (±SD) of cuticular hydrocarbons in the chemical profiles of the host S. amabilis and its social parasite *M. symmetochus*

Peak no.	RT (min)	Compound name	*S. amabilis* from nonparasitized colonies *N*=36	*S. amabilis* from parasitized colonies *N*=19	*M. symmetochus* parasite *N*=24
1	19.2	C29	1.36 ± 0.54	0.97 ± 0.44	x
2	20.5	C30	1.27 ± 0.47	0.95 ± 0.42	x
3	20.9	14,16MeC30	1.05 ± 0.4	0.7 ± 0.23	x
4	21.9	C31	13.73 ± 5.72	12.99 ± 4.33	x
5	22.3	13,15MeC31	11.42 ± 3.02	11.41 ± 2.56	x
6	22.7	11,19DiMeC31; 13,17DiMeC31; 15,19DiMeC31	5.3 ± 0.94	3.85 ± 1.24	x
7	22.9	3MeC31	3.41 ± 2.13	4.9 ± 2.45	x
8	23.3	C32	4.61 ± 1.01	5.08 ± 1.51	x
9	23.6	12,14,16,18MeC32	3.95 ± 0.84	4.52 ± 1.03	x
10	24.0	4MeC32	2.39 ± 0.49	2.72 ± 0.59	x
11	24.6	C33	16.22 ± 6.54	12.98 ± 4.97	x
12	25.0	11,13MeC33	x	x	1.73 ± 0.25
13	25.1	13,15MeC33	16.13 ± 2.17	15.08 ± 2.83	x
14	25.2	Unknown	3.34 ± 2.42	4.8 ± 4.19	x
15	25.4	13,19DiMeC33; 15,21DiMeC33; 17,21DiMeC33; 11,21DiMeC33	9.26 ± 1.86	7.89 ± 2.86	x
16	25.6	3MeC33	5.25 ± 2.82	4.93 ± 1.85	x
17	26.0	C34	2.92 ± 1.85	1.93 ± 1.04	x
18	26.3	12,14,16MeC34	1.83 ± 0.66	1.68 ± 0.22	x
19	26.7	12,22DiMeC34	1.68 ± 0.55	1.36 ± 0.32	x
20	26.7	C35:2 (possibly branched)	x	x	45.24 ± 49.88
21	26.9	C35:2 (possibly branched)	x	x	3.55 ± 0.49
22	**27.1**	**C35:1**	**2.37 ± 0.91**	**3.32 ± 1.46**	**18.18 ± 25.79**
23	27.2	C35	2.17 ± 0.94	1.68 ± 0.87	x
24	27.6	13,15,17MeC35	2.08 ± 0.66	2.88 ± 0.78	x
25	28.0	3MeC35	3.29 ± 1.7	3.16 ± 1.53	x
26	28.2	?,13DiMeC35	0.66 ± 0.53	1.04 ± 0.53	x
27	29.3	C37:2 (possibly branched)	x	x	29.65 ± 11.4
28	29.5	C37:2 (possibly branched)	x	x	22.86 ± 3.68
29	29.7	C37:1	x	x	12.55 ± 7.4
30	30.6	13,15MeC37	1.06 ± 0.59	1.31 ± 0.33	x
31	31.6	Possibly C39:3	x	x	7.57 ± 1.61
32	31.9	C39:2 (possibly branched)	x	x	8.84 ± 1.35
33	32.0	C39:2 (possibly branched)	x	x	7.29 ± 1.12

Peak no. = number of the peaks in Fig. 1a; RT = retention time; *N* = sample size. The shared hydrocarbon across all three categories is indicated in bold. Compounds that were found in <0.5% on average or absent from >90% of the samples were omitted (x). Unknown branching site is indicated in with '?'.

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total CHC area normalized to the C22 peak and corrected for the difference in body surface area, we still found significant differences between host and parasite workers. The total CHC area was significantly smaller in parasites than in the hosts (two-sample t test: $t_{15.72} = -6.23$, $P < 0.001$).

The PCA showed that host CHC profiles (from parasitized and nonparasitized colonies) were separated from the parasite profiles, both forming distinct clusters (Fig. 1b, Supplementary material 2, Fig. S1). The chemical profiles between parasitized or nonparasitized hosts and parasites were very different, having a mean Bray–Curtis distance of 0.97 or 0.98, respectively. In contrast, host CHC profiles (from parasitized and nonparasitized colonies) were similar and overlapping, with a mean Bray–Curtis distance of 0.26 between parasitized and nonparasitized host samples. For comparison, the mean Bray–Curtis distance between single parasite samples and single *S. amabilis* (parasitized and nonparasitized) samples was 0.11 and 0.25, respectively. Samples from the same *S. amabilis* host colonies were generally more similar to each other than they were to other colonies (Supplementary material 2, Figs. S1 and S2).

In addition to the CHCs, we found large amounts of volatile compounds in all 24 parasite samples (retention times of 8–10 min in Fig. 1). These included a mixture of the two isomers (5Z,8E)- and (5E,8E)-3-butyl-5-hexylpyrrolizidine alkaloids (Adams et al., 2013), ranging from 16 to 43 µg per ant (T. Jones, personal communication). These isomers are dispensed from the parasite's specialized sting as an aerosol or contact venom (Adams et al., 2013).

Behavioural Analysis

In total, we conducted 348 trials. From all behaviours observed, 65% were used for the aggression analysis and 7% were used for the submission analysis. The missing 28% corresponds to the 'unknown' behaviour category, where either no obvious reaction from the focal ants towards the introduced ants occurred (no reaction: <8%), or the behaviour could not be evaluated because it was out of view (unclear: ca. 20%).

Aggression Analysis

The focal ants encountered the stimulus ant frequently during the 5 min trials, and the proportion of behaviours depended on the kind of pairing (Fig. 2). In the aggression analysis, the highest proportion of behaviours [illegible] short antennation, mandible opening and biting/pulling. We focus only on these behaviours and compare them between the different pairings below.

Short antennation was the most frequent behaviour observed between nestmates (pairings 1 and 2). The mean proportion of short antennation for focal ants from parasitized and nonparasitized colonies was 0.77 and 0.78, respectively (Supplementary material 2, Table S1). In contrast, opening mandibles and biting/pulling were rarely or never seen in focal ants towards nestmate stimulus ants in pairings 1 and 2 (mean proportions: opening mandibles: pairing 1 = 0.06; pairing 2 = 0.13; biting/pulling: pairings 1 and 2 = 0.0). Focal ants encountering a conspecific non-nestmate stimulus ant (pairings 3–6) showed biting/pulling and mandible opening behaviours significantly more often than in nestmate encounters (>98.9% in all comparisons between pairings 1 or 2 and pairings 3–6). When the focal ants originated from a parasitized colony and the stimulus ant was a parasite from the same colony (pairing 7), the proportions of the short antennation, opening mandibles and biting/pulling behaviours were similar as towards their own (conspecific) nestmates (pairing 1). However, when the stimulus ant was a foreign parasite (pairing 8), opening mandibles was shown significantly more often (certainty of >99.9%) compared to their (conspecific) nestmates (pairing 1) and compared to their own parasites (pairing 7 certainty of >99.9%). Focal ants from nonparasitized colonies encountering a parasite (pairing 9), showed more opening mandibles (certainty 97.2%) and biting/pulling (certainty >99.9%) behaviours within a trial than focal ants from a parasitized colony towards foreign parasites (pairing 8). The proportions of opening mandibles and biting/pulling behaviours were similar to the proportions in encounters with conspecific non-nestmates (pairings 5 and 6).

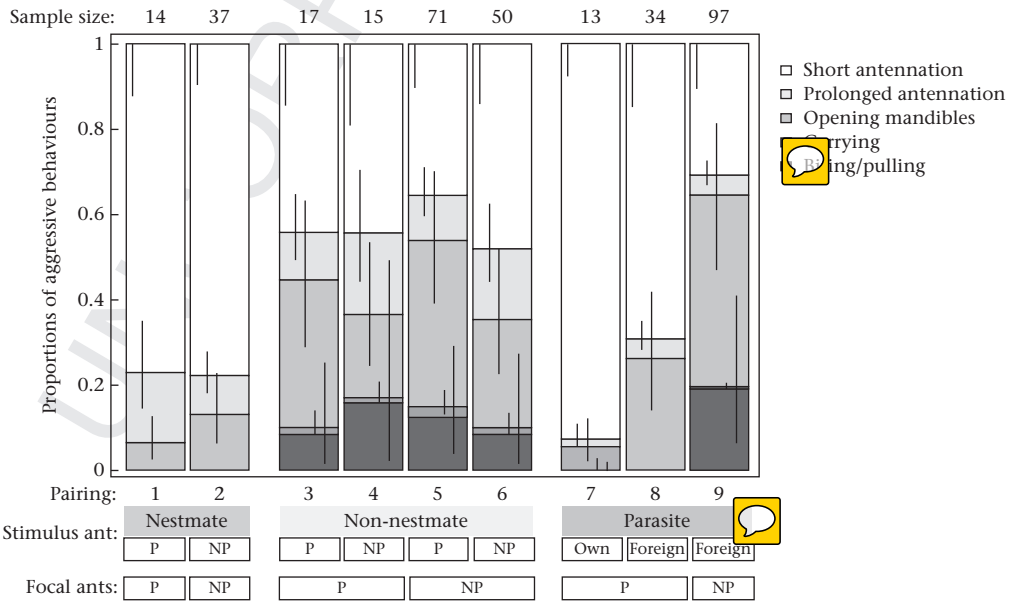

Figure 2. Proportions of single behaviours shown by *S. amabilis* focal ants within a trial. Bars represent the means of the model parameters from the posterior probabilities and lines represent the 95% credible intervals for each behaviour based on the Markov chain Monte Carlo simulations (all means and credible intervals are given in Supplementary material 2, Table S1). Stimulus and focal ants originated from either a parasitized (P) or nonparasitized (NP) colony. Sample sizes are indicated at the top of the figure and specific pairings are numbered directly below.

Submission Analysis

Focal ants from parasitized and nonparasitized colonies were significantly more submissive when tested against non-nestmates from a parasitized colony (pairings 3 and 5, respectively) than when tested against a non-nestmate from a nonparasitized colony (pairings 4 and 6, respectively) (certainty for focal ants from parasitized colonies: 99.6%; certainty for focal ants from non-parasitized colonies: 99.4%; Fig. 3). Focal ants from parasitized colonies were less submissive towards parasites (own and foreign, pairings 7 and 8, respectively) than focal ants from nonparasitized colonies (pairing 9) (certainty of 95.6% and 99.9%, respectively). Note that focal ants from nonparasitized colonies approaching a parasite frequently showed head tucking right after they showed opening mandibles (Supplementary video S1).

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anbehav.2018.02.021.

DISCUSSION

In this study, we investigated the integration strategy of the social parasite *M. symmetochus*. We compared CHC profiles of parasite and host species and tested the behaviour of parasitized and nonparasitized colonies against an introduced stimulus ant. We found very different CHC profiles for both ant species, ruling out mimicry and camouflage. The chemical analysis revealed low amounts of CHCs, suggesting that the parasites use an insignificance strategy. Both the chemical profiles of parasites and the behavioural data support the hypothesis that the parasites use weaponry to maintain an amiable association with their host ants.

Support for the Chemical Insignificance Strategy

One of the predictions for the chemical insignificance strategy is a low abundance of CHCs and/or unusually long-chained CHCs compared to the host colony, as it has been shown in other social parasites (Lambardi, Dani, Turillazzi, & Boomsma, 2007). We found support for this strategy as the CHCs of the parasite *M. symmetochus* were less abundant and the CHCs were generally longer-chained than the host CHCs. The muted CHC profiles of the parasites could reflect an evolutionary adaptation making *M. symmetochus* workers stealthy inside the host nest. Alternatively, the insignificant CHC profile could also be a by-product of the parasites' life in the host nest, as the parasite workers do not leave the humid nest environment and, therefore, they lack the need for CHC protection against desiccation. In addition, in the CHC profiles of the parasites, we potentially found a high proportion of methyl-branched alkadienes, which are found in other ant species in interspecific, amicable associations (Menzel & Schmitt, 2012). However, further work is needed to unequivocally determine whether methyl-branched alkadienes are also present in our host–social parasite system. Another prediction for the chemical insignificance strategy is that workers from parasitized and nonparasitized colonies behave as if they do not detect the parasites. Our results are contradictory to this prediction, since we found that workers of nonparasitized colonies responded with similar aggression towards parasites and conspecific non-nestmates. This recognition may be because the workers of non-parasitized colonies can detect either (1) the parasite's CHCs, (2) traces of host-derived CHCs on the cuticle of the parasites (not detectable with our GC-MS analysis), or (3) something else in the parasite's odour profile. Scenarios 1 and 2 are possible, because focal workers from parasitized colonies showed opening mandibles towards foreign parasites significantly more often than towards their own parasites. However, focal ants from parasitized colonies did not escalate to high-level aggression (biting/pulling). Scenario 3 is also likely because the chemical analyses demonstrated that parasite odour profiles not only consist of CHCs, but also volatile alkaloids. The aggressive responses (biting/pulling) in *S. amabilis* workers from nonparasitized colonies towards

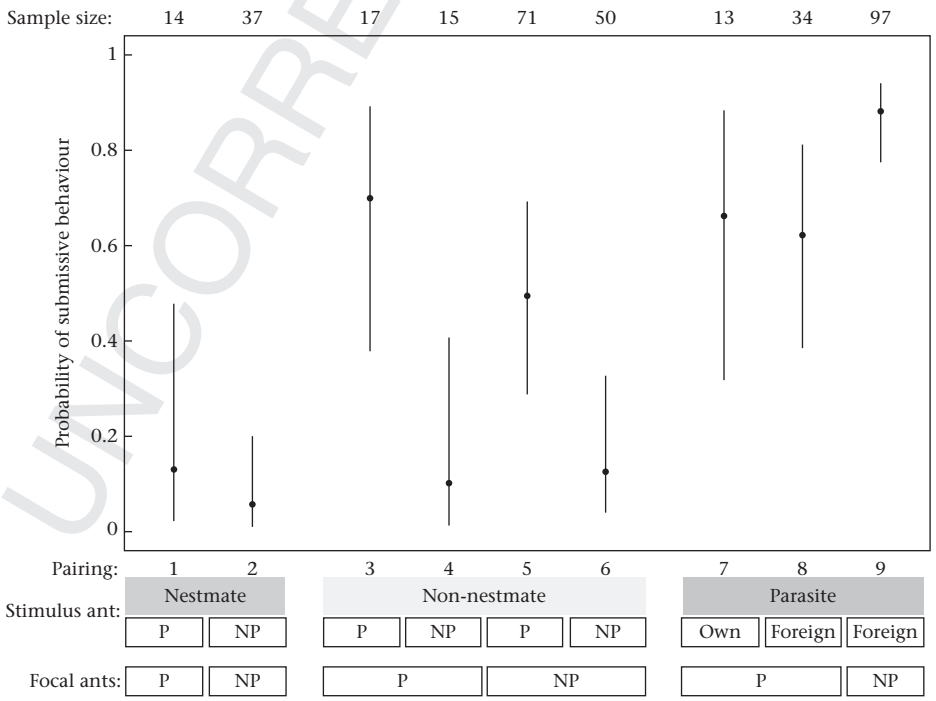

Figure 3. The probability of submission behaviour in *S. amabilis* focal ants within a trial. Dots represent the mean of the model parameters from the posterior probabilities and lines depict the 95% credible intervals for submissive behaviour based on the binomial generalized linear model (parameter estimates and standard errors are given in Supplementary material 2, Table S2). Stimulus and focal ants originated from either a parasitized (P) or nonparasitized (NP) colony. Sample sizes are indicated at the top of the figure and specific pairings are numbered directly below.

parasites were likely due to the parasite venom alkaloids rather than to the parasite CHC profile.

Workers from Parasitized Colonies Tolerate Parasites

Previous studies showed that ants and honeybees can maintain several neuronal templates for nestmate odours simultaneously (Breed, Diaz, & Lucero, 2004; Errard, 1994; Greene & Gordon, 2003). Similarly, in our experiments, workers from parasitized colonies may have developed a parallel neuronal template for the parasites' odour and this could explain their tolerance towards their parasites. We exclude the possibility that workers from parasitized colonies are unable to recognize nestmates because we show that *S. amabilis* workers (irrespective of being from a parasitized or nonparasitized colony) were aggressive towards conspecific non-nestmates and not aggressive towards their sisters. Social parasites may affect the nestmate recognition abilities of the host as has been shown in social wasps (Lorenzi, 2003). However, we did not find evidence of this in our host–parasite system.

Interestingly, the long-term association of coexistence between *S. amabilis* and *M. symmetochus* (>7 years) can lead to host rebellions where the host ants turn against the parasites and kill them (Boudinot et al., 2013; R. M. M. Adams, personal observation). This supports the idea that *S. amabilis* workers from parasitized colonies can recognize parasites, but accept them as nestmates. However, when their acceptance threshold shifts due to factors that are still unknown for our host–social parasite system, they can turn aggressive towards the parasites, as has been shown in other species (d'Ettorre, Brunner, Wenseleers, & Heinze, 2004). In the laboratory, host rebellion can result in the death of the parasite colony, but in the field, with the looming threat of other parasites, it may simply be a 'culling' of the parasite worker forces (Boudinot et al., 2013), since hosting *M. symmetochus* parasites can have context-dependent advantages (i.e. host protection against more lethal parasites) (Adams et al., 2013). Workers from nonparasitized colonies are unlikely to have encountered the parasite odour profile (both the CHCs and the alkaloids) before, and therefore recognize the parasites as non-nestmates and react with aggression. The alkaloids originating from the venom gland contain the volatile isomers (5Z,8E)- and (5E,8E)-3-butyl-5-hexylpyrrolizidine in a 59:41 ratio (Adams et al., 2013). The large amount of volatile alkaloids (16–43 µg/ant) suggests that the parasite *M. symmetochus* uses chemical weaponry.

Evidence for Chemical Weaponry Strategy in M. symmetochus

In general, submissive behaviours in fungus-growing ants are not observed between nestmates. Considering the parasite occasionally threatens or even kills host workers (Adams et al., 2013), it is important that the host is behaviourally adapted to avoid an escalation that results in death. Accordingly, in our study, submission by *S. amabilis* focal ants from parasitized colonies was more prevalent towards parasites than towards conspecific nestmates. In focal ants, opening mandibles often preceded head tucking behaviour as they approached the parasite. This observation and the fact that the alkaloids are volatile suggest they are found in the air surrounding the parasites (Adams et al., 2013). Focal ants may perceive the alkaloids in increasing concentrations as they approach the parasite, eliciting a shift in behavioural response from aggression to submission. Furthermore, the high probability of submission in trials with 'alkaloid-exposed' stimulus ants (i.e. *S. amabilis* workers from parasitized colonies or the parasites) also suggests that the parasite-derived alkaloids are not only found on the cuticle of parasites and in the fungus garden (Adams et al., 2013), but also on the cuticle of the host ants. Further work is needed to unequivocally identify that the venom alkaloids facilitates *M. symmetochus* host colony integration.

In this study, we focused on social parasite integration rather than infiltration and found tolerance for social parasites by host workers, especially by individuals that had been exposed to social parasites. From these results, we hypothesize that virgin *S. amabilis* queens originating from colonies that had social parasites might have incorporated the parasite chemical profile (CHCs and alkaloids) into their neuronal template for nestmate recognition. It is possible that these queens could be more likely to accept parasite queens during nest founding than host queens from a non-parasitized colony. Future work on alkaloid dispensing behaviour in the nest environment and the host's propensity for accepting a parasite queen will shed light on invasion tactics and success.

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Acknowledgments

We thank two anonymous referees and the editor for their helpful comments on the manuscript. We are grateful for permit and facilities support from the Smithsonian Tropical Research Institute (STRI). Autoridad Nacional del Ambiente y el Mar (ANAM) provided permission to collect and export Panamanian ants. Jon Shik, Anders Illum, Joanito Liberti helped with colony collections in conjunction with the graduate course, Tropical Behavioural Ecology and Evolution (TBEE) hosted by the Centre for Social Evolution, University of Copenhagen and STRI. We thank the TBEE 2013 students and co-instructors Koos Boomsma and Jon Shik for discussion and support during the fieldwork in Panama and Fränzi Korner-Nievergelt and Tobias Roth for valuable help in the statistical analysis of the behavioural data. We thank Matthew Boot for measuring the body sizes of individual ants. We also thank David Nash for helpful discussion while we designed the behavioural experiments and for comments on the final statistical analysis. R.M.M.A. thanks Patricia d'Ettorre and her postdoctoral advisors, David Nash and Koos Boomsma (University of Copenhagen Marie Curie International Incoming Fellowship, 237266 – ANCEPS, 2010–2013) for fruitful conversations and encouragement. Additional financial support was received from The Ohio State University for R.M.M.A. and A.D. and by the International Max Planck Research School for Organismal Biology and the University of Konstanz for S.N.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anbehav.2018.02.021.