J Chem Ecol (2017) 43:869–880
DOI 10.1007/s10886-017-0880-6

What are the Mechanisms Behind a Parasite-Induced Decline in Nestmate Recognition in Ants?

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Received: 16 March 2017 / Revised: 11 August 2017 / Accepted: 14 August 2017 / Published online: 25 August 2017
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Abstract Social insects have developed sophisticated recognition skills to defend their nests against intruders. They do this by aggressively discriminating against non-nestmates with deviant cuticular hydrocarbon (CHC) signatures. Studying nestmate recognition can be challenging as individual insects do not only vary in their discriminatory abilities, but also in their motivation to behave aggressively. To disentangle the influence of signaling and behavioral motivation on nestmate recognition, we investigated the ant *Temnothorax nylanderi*, where the presence of tapeworm-infected nestmates leads to reduced nestmate recognition among uninfected workers. The parasite-induced decline in nestmate recognition could be caused by higher intra-colonial cue diversity as tapeworm-infected workers are known to exhibit a modified hydrocarbon signature. This in turn may broaden the neuronal template of their nestmates, leading to a higher tolerance towards alien conspecifics. To test this hypothesis, we exchanged infected ants between colonies and analyzed their impact on CHC profiles of uninfected workers. We demonstrate that despite frequent grooming, which should promote the transfer of recognition cues, CHC profiles of uninfected workers neither changed in the presence of tapeworm-infected ants, nor did it increase cue diversity among uninfected nestmates within or between colonies. However, CHC profiles were systematically affected by the removal of nestmates and addition of non-nestmates, independently from the ants' infection status. For example, when non-nestmates were present workers expressed more dimethyl alkanes and higher overall CHC quantities, possibly to achieve a better distinction from non-nestmates. Workers showed clear task-specific profiles with tapeworm-infected workers resembling more closely young nurses than older foragers. Our results show that the parasite-induced decline in nestmate recognition is not due to increased recognition cue diversity or altered CHC profiles of uninfected workers, but behavioral changes might explain tolerance towards intruders.

Keywords Cuticular hydrocarbons · Nestmate recognition · Parasite-induced changes · Sociobiology · Tapeworm · *Temnothorax nylanderi* · Formicidae

Introduction

Social insects form impressive and highly organized societies. They developed numerous lines of defense to protect their resources from exploitation and to maintain colony integrity. Nestmate recognition enables social insects to distinguish nestmates from foreign individuals and therefore represents an important, first line of defense against various intruders. Access to the nest and its resources is only given to nestmates. To this end cuticular hydrocarbons (CHCs) serve as cues for nestmate recognition (Lahav et al. 1999; Wagner et al. 2000; Dani et al. 2001; Akino et al. 2004; Dani et al. 2005). Recognition cues are heritable (Beye et al. 1998), but can be obtained from the environment as well (Heinze et al. 1996; Liang and Silverman 2000). Qualitative differences in hydrocarbons facilitate recognition between species, while

Electronic supplementary material The online version of this article (doi:10.1007/s10886-017-0880-6) contains supplementary material, which is available to authorized users.

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conspecifics rather differ in the quantitative profile of hydrocarbons (van Zweden and d'Ettorre 2010). The '*Gestalt*' model postulates the formation of a colony-specific odor among nestmates (Crozier and Dix 1979), which may promote recognition by reducing the possibility of errors (Reeve 1989). Through the exchange of CHCs via passive physical contact, allogrooming and trophallaxis (Soroker et al. 1995; Leonhardt et al. 2016), nestmate cues are homogenized. However, a complete homogenization may not be desired as the CHC profile contains more information beside colony identity, such as fertility (Dietemann et al. 2003), age (Wakonigg et al. 2000; Cuvillier-Hot et al. 2001), sex (Kleeberg et al. 2017) and task specialization (Greene and Gordon 2003; Kather et al. 2011). Moreover, the CHC composition shifts with parasite infections (e.g. Trabalon et al. 2000; Salvy et al. 2001; Baracchi et al. 2012; Csata et al. 2017), and nestmates use the altered signals to detect and contain infections by social immunity measures (Cremer et al. 2007; Richard et al. 2008; McDonnell et al. 2013).

Nestmate recognition is generally composed of three parts – *signaling, perception* (sensory perception and neuronal processing) and the *behavioral motivation to act* (Newey et al. 2010). However, the precise underlying mechanisms remain poorly understood and may differ between social insect species. It is commonly agreed that during perception, recognition cues of another individual are compared to one or several neuronal templates (Leonhardt et al. 2007; Newey 2011). Encountered individuals can be identified as foreign and may elicit aggression by the recipient when the other's profile differs in the amount of cues (Lenoir et al. 2001; Cini et al. 2009; Di Mauro et al. 2015) or contains novel compounds (Guerrieri et al. 2009). Aggression assays are commonly used as proxies for nestmate recognition (Roulston et al. 2003), whereby the level of aggression is assumed to reflect recognition abilities: strong behavioral responses imply good discriminatory skills, while low aggression signifies diminished nestmate recognition. However, the behavioral component of nestmate recognition is not simply a response to the level of dissimilarity in recognition cues, but rather sensitive to the context. For instance, ecological factors such as the quality of the nest and familiarity with the opponent can influence the decision to behave aggressively (Heinze et al. 1996; Tanner and Adler 2009). Moreover, the presence of nestmates (Tanner and Adler 2009) and the number of queens in a colony as well as the relatedness between individuals (Morel et al. 1990) can be linked to different levels of aggression. Finally, individuals of the same colony can vary in their aggressive response (Newey et al. 2010). Differences in morphology and behavior (Sturgis and Gordon 2012; Larsen et al. 2014), and consequently differential experience of workers with intruders but also nestmates can contribute to intra-colonial variation in aggression (Esponda and Gordon 2015). These examples reflect the complexity of nestmate recognition and the challenge to understand which component is altered when recognition tests show changed behavioral responses towards non-nestmates.

Here, we studied a model that allows us to disentangle the different causes of altered nestmate recognition. The ant *Temnothorax nylanderi* commonly serves as an intermediate host for the tapeworm parasite *Anomotaenia brevis* (Trabalon et al. 2000). Infected adult workers exhibit a fundamentally different phenotype including alterations in behavior (Scharf et al. 2012), morphology (Trabalon et al. 2000; Scharf et al. 2012), survival (Beros et al. 2015) and their CHC profile (Trabalon et al. 2000). When colonies contain tapeworm-infected nestmates, uninfected workers show reduced aggression towards non-nestmate conspecifics (Beros et al. 2015). This effect can be experimentally induced by the addition and removal of infected ants (Beros et al. 2015). A possible explanation for this reduced nestmate recognition is that the deviant hydrocarbon profiles of infected ants increase the quantitative diversity in recognition cues in parasitized ant colonies (i.e. having tapeworm-infected nestmates) and consequently widens the ants' neuronal template (Errard et al. 2006; Leonhardt et al. 2007). This could result in a higher tolerance towards individuals with aberrant hydrocarbon signatures. In this case, changes in aggression towards non-nestmate conspecifics should be due to a higher variation in recognition cues. This scenario resembles the higher acceptance rate of non-nestmates due to habituation to aberrant hydrocarbon signatures as shown in artificial mixed-species colonies (Errard 1994). Alternatively, aggression towards non-nestmates could change due to physiological stress caused by the parasite infection, thus reducing the behavioral motivation to act aggressively.

To differentiate whether changes in nestmate recognition are due to higher cue diversity, we experimentally studied whether potential differences in the cue signature of parasitized and unparasitized colonies are directly related to the presence of infected workers. We exchanged infected workers between colonies, and analyzed hydrocarbon profiles after two months of exchange. As a control, we exchanged uninfected workers between colonies to assess effects to removal of nestmates vs. addition of non-nestmates. We expected that uninfected workers from naturally and experimentally parasitized colonies show CHC profiles different from colonies lacking infected workers. Furthermore, we were interested how worker transfer between colonies changes CHC profiles and CHC diversity within each colony. Here, we expected a higher intra-colonial hydrocarbon diversity in parasitized ant colonies. Additionally, we investigated the hydrocarbon profiles of behavioral castes (i.e. nurses, foragers) from naturally parasitized and unparasitized colonies, and our experimental colonies. The presence of infected workers in the colony might affect workers of specific behavioral castes to varying degrees. Ant scouts, foragers and nurses possess different

hydrocarbon profiles (Bonavita-Cougourdan et al. 1993; Greene and Gordon 2003). Since workers of the same or of different castes regularly exchange CHCs during trophallaxis and allogrooming (Vienne et al. 1995; Leonhardt et al. 2016), worker castes which differ most strongly from infected workers might show the strongest CHC changes after exposure to infected workers. On the other hand, infected workers are long-lived (Beros et al. 2015) and foragers are usually older than nurses (Mersch et al. 2013). Hence, we either expected to find more age-related similarities between foragers and infected workers, or alternatively, nurses could resemble infected workers due to their frequent social interactions and spatial proximity (Scharf et al. 2012).

Material and Methods

Study System, Colony Collection and Maintenance

Temnothorax nylanderi occurs in deciduous forests throughout Europe and inhabits small, protected cavities in acorns and sticks on the forest floor. Ant colonies were collected from different locals in the vicinity of the cities Mainz and Rüdesheim (Germany) from May to June 2014 (Supplementary Table 1). Colonies were transported in Ziploc bags to the laboratory and transferred to artificial observation nests (i.e. pre-cut cavity between two glass slides; 50 × 10 × 3 mm^3), which were housed in plastered boxes (100 × 100 × 30 mm^3). Ants were kept in a climate chamber at 20 °C:16 °C (12 L:12D cycle) and were provided with honey and pieces of crickets once a week. Access to water was unlimited and nest boxes were moistened if necessary. Colonies were counted after moving to the new nest. We included the number of queen, brood and workers - differentiating between brown and yellow workers in parasitized colonies (Beros et al. 2015).

Dataset a – Field Data: Cuticular Hydrocarbon Profiles of Field Colonies

Tapeworm-infected *T. nylanderi* workers possess a modified CHC profile (Trabalon et al. 2000), and frequent allogrooming and trophallaxis could alter the CHC profile of their nestmates (Scharf et al. 2012), potentially explaining the hampered nestmate recognition of parasitized colonies (Beros et al. 2015). To analyze this, we sampled an uninfected forager, an uninfected nurse each from 21 naturally parasitized and 23 naturally unparasitized colonies, and sampled one infected worker from 21 parasitized colonies. Uninfected workers were selected based on their spatial position (Modlmeier et al. 2012): foragers were collected from outside the nest, whereas nurses were defined as those that cared for the brood inside the nest. *Anomotaenia brevis*-infected workers can be reliably recognized by the bright yellow coloration of their cuticle (Beros et al. 2015). However, it is difficult to assign them to either the forager or the nurse caste, because infected workers engage less in colony tasks (Scharf et al. 2012). When assigned according to their position on the brood pile (Scharf et al. 2012), infected workers take after nurses, but when grouped by age they resemble more the older foragers.

Dataset b – Experimental Data: Manipulation of Ant Colony Parasitism Status

Given the low aggression towards non-nestmate conspecifics (Foitzik et al. 2007; Beros et al. 2015), *Temnothorax nylanderi* colonies can be easily manipulated by adding or removing workers (Beros et al. 2015). Indeed, within areas of high population density, unrelated colonies will regularly merge (Foitzik and Heinze 1998, 2001). To investigate the influence of infected workers on the hydrocarbon profile of their nestmates, we manipulated colony composition. For this 'worker exchange experiment', we used 80 queenright colonies from a single population (see Supplementary Table 1). Twenty parasitized and 60 unparasitized colonies were assigned to four different treatments (I – IV) (Fig. 1), in which we either removed or added workers. We removed all yellow workers (3–14 individuals) from 20 parasitized colonies (treatment I: originally parasitized, currently unparasitized; 'donor colonies'). Workers originating from a single 'donor' colony were then added to an unparasitized 'receiver' colony (treatment III: originally unparasitized, currently parasitized; $N = 20$). Colonies of treatment II and IV served as controls for the removal and addition of ant workers. Hence, we removed workers from a total of 20 unparasitized colonies (s 3–13 workers; treatment II: originally unparasitized, currently unparasitized; 'donor colonies') and added them to an alien unparasitized colony (treatment IV: originally unparasitized, currently unparasitized; 'receiver colonies', $N = 20$). We chose to remove and add only nurses as they are more likely to remain inside the nest. This allowed us the comparison between colonies of treatment III (infected workers added) and IV (uninfected workers added). Colonies that received non-nestmates (treatment III & IV) were matched in terms of colony size and the added number of workers. Moreover, westandardized the intra-colonial infection rate to ~13% infected workers per colony (12.72 mean ± 0.01), which resembles the infection rate found in the field (Scharf et al. 2012). To facilitate manipulations, colonies were anaesthetized with CO_2 for a few seconds before workers were either removed or added. Before introducing workers to a new colony, all ants, including infected workers ($N = 291$) were tagged with colored wires (ELEKTRISOLA, 0.02 mm) to be reliably identified and distinguished from native colony members. After manipulation, experimental colonies of all four treatments were kept at 8 °C for ten days to reduce the likelihood of

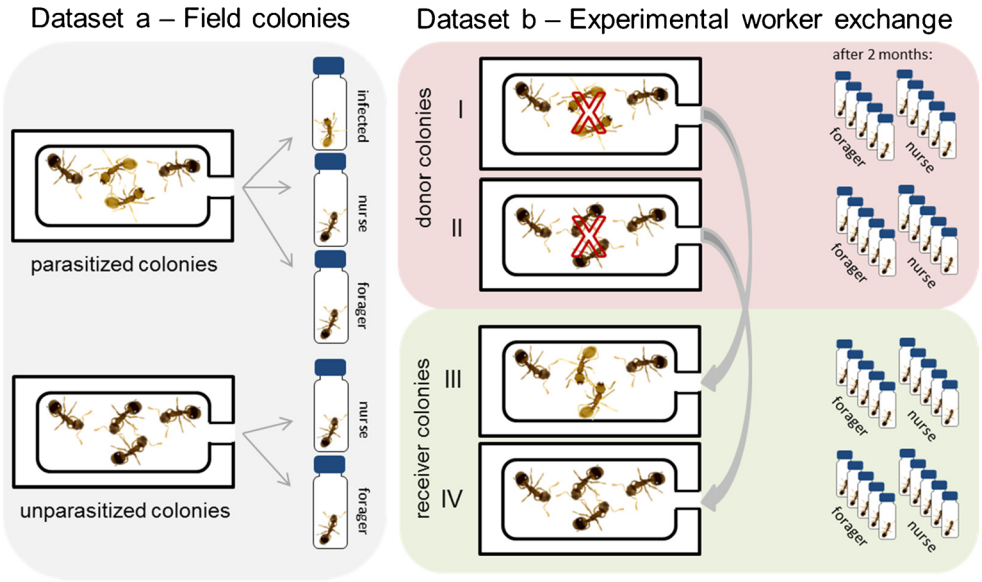

Fig. 1 Data sampling and experimental set-up. From field colonies we sampled one nurse and one forager from each colony type (unparasitized & parasitized) and additionally an infected worker from parasitized *T. nylanderi* colonies. All infected workers from naturally parasitized colonies (i.e. treatment I) were removed and added to naturally unparasitized colonies of treatment III. As controls we used naturally unparasitized colonies assigned to treatment II from which we removed identical numbers of workers and added them to naturally unparasitized colonies of treatment IV. Two months after the exchange, we sampled from each colony five native foragers and five native nurses

worker rejection. During this time, receiver colonies (treatment III & IV) were checked on a daily basis, and if necessary, expelled wire-marked ants were reintroduced by carefully pushing them back to the colony with forceps and closing the nest entrance with a tissue until the next day. After ten days, colonies were moved to a climate chamber at 20 °C for the next 46 days such that the experimental manipulation lasted for a total of 56 days.

Dataset b – Experimental Data: Ant Sampling for Chemical Analyses

The experiment ended 56 days after manipulation when we sampled 10 native, uninfected workers (five nurses and five foragers) from each colony. Ants were individually frozen in glass vials at −20 °C. For our chemical analyses of individuals from treatment III ($N = 16$) and IV ($N = 16$), we only used those colonies in which non-nestmates were well integrated into the colony. From six donor colonies ($N_{\text{treatment I}} = 5$; $N_{\text{treatment II}} = 1$), we did not take samples for chemical analyses as these colonies consisted of less than ten workers, which showed no behavioral differentiation and hence impeded us to reliably distinguish workers according to their behavioral task.

Chemical Analyses via GC-MS

CHC of single ants were extracted in ~0.5 ml of hexane for 10 min. During extraction, 100 ng *n*-C18 was added as internal standard (Mas et al. 2009). The extracts were concentrated to ~20 μl, and we injected 5 μl into a gas chromatograph coupled to a mass selective detector (GC-MS) (Agilent Technologies, GC: Agilent 7890A, MSD: Agilent 5975) equipped with a HP5-MS column (30 m × 0.25 mm; coating: 0.25 μm). Injection was performed in the split-less mode at 250 °C, using helium as carrier gas with a constant flow of 1.2 ml min. Oven temperature was set at 150 °C for 3 min, followed by a two-step temperature increase from 150 to 250 °C at 30 °C min and 250–300 °C at 2 °C min, where the temperature remained constant for 2 min. Masses were scanned in the range 40–500 amu at an ionization voltage of 70 eV. Data were acquired using the software MSD Chemstation E.02.02 (Agilent). We quantified the relative amounts of CHC based on peak areas, and made sure that none of the sample runs contained any overcharged peaks. Hydrocarbons were identified using their retention indices and diagnostic ions. Using this method, a total of 108 samples from field colonies and 526 samples from the worker exchange experiment were analyzed.

Statistical Analyses

For both datasets we assessed differences in CHC profiles between (*a*) infected and uninfected workers from field colonies, and (*b*) between foragers and nurses from the four experimental colonies, using a permutational multivariate analysis of variance (PERMANOVA, 999 permutations) based on the Bray-Curtis similarity in the software Primer 6.0 & PERMANOVA (Primer-E Ltd.). The model on the field data included the *worker type* (infected worker, uninfected nurse, uninfected forager) and the *field parasitism status* (parasitized,

unparasitized), with interactions allowed. For dataset *b*, we constructed a model including *behavioral caste* (nurse, forager), *experimental treatment* (donor, receiver colonies) and *exchanged worker type* (infected, uninfected worker) as well as their interactions as fixed predictors. To account for colony identity, we added *colony ID* as a random factor, which was nested in *experimental treatment* and *exchanged worker type.*

In both datasets we additionally performed analyses on the abundance of specific substance classes and hydrocarbon characteristics. To elucidate differences in CHC composition of infected and uninfected workers from the field data (dataset *a*), and to evaluate whether the addition and removal of infected workers affected the CHC composition of nestmates (dataset *b*), we analyzed in each dataset the following traits, each as the dependent variable in separate linear mixed models (LME): (*i*) the mean chain-length of CHCs, (*ii*) the total amount of CHCs, (*iii*) the proportion of straight-chain alkanes (*n*-alkanes) as well as the proportion of (*iv*) mono-, (*v*) di- and trimethyl alkanes;the two hydrocarbon classes were pooled since *T. nylanderi* only possesses five trimethyl alkanes. For the field data, we used *worker type* (infected, nurse, forager) and *field parasitism status* (parasitized, unparasitized) as fixed predictors, with interactions allowed. For the worker exchange experiment, we included *behavioral caste* (nurse, forager), the *experimental treatment* (donor, receiver colony) and *exchanged worker type* (infected, uninfected workers) as fixed predictors, with interactions allowed. Again, the colony ID (nested in *experimental treatment* and *exchanged worker type*) was entered as a random factor.

Finally, we analyzed within-colony variation for field colonies (dataset *a*), and between- and within-colony variation among experimental colonies (dataset *b*). For each field colony, we calculated the Bray-Curtis distance between forager and nurse of unparasitized colonies and compared it to the average distance between forager and nurse, forager and infected worker, and nurse and infected worker of parasitized colonies using a *t* test. For experimental colonies, between-colony variation was compared between the four treatments using PERMDISP (Primer). To analyze within-colony variation, we calculated the average distance from the colony centroid (output of the PERMDISP command in Primer). We included both foragers and nurses, but repeated the analysis for nurses and foragers separately to control for effects of caste differences. These values (one per colony, $N = 61$) were then compared between treatments using a linear model. All linear models were constructed in R v 2.15.2 (R Core team 2012).

Results

We found a total of 37 saturated CHC peaks on the cuticle of *T. nylanderi* ants (Table 1), consisting of seven *n*-alkanes, 30 monomethyl, 20 dimethyl as well as five trimethyl alkanes. All hydrocarbons were shared among the three worker types (infected workers, nurses, foragers), with differences in the relative abundance of 28 hydrocarbons (Table 1). Foragers differed strongly from infected workers and nurses (Table 1), whereas the latter two were more similar. Yet, we found significant differences between infected workers and nurses in one n-alkane and seven methyl-branched alkanes (Table 1).

Chemical Analyses of Field Colonies

As infected workers are frequently groomed by their uninfected nestmates and grooming leads to CHC transfer (Soroker et al. 1995), we firstly investigated whether the hydrocarbon profile of uninfected workers was affected by the parasitism status of the colony. Interestingly, the chemical profile of uninfected foragers and nurses from parasitized colonies did not differ from workers of the same caste from unparasitized colonies (Supplementary Table 2).

However, *worker type* had a strong influence on the CHC profile, showing differences between infected workers, nurses and foragers (PERMANOVA: Pseudo-$F_2 = 8.96$, $p < 0.001$). The CHC profile of foragers differed most strongly from infected workers (PERMANOVA pairwise contrast: $t = 4.01$, $P = 0.001$) and nurses ($t = 2.99$, $p = 0.001$), but also between infected ants and nurses ($t = 1.79$, $P = 0.015$; Fig. 2). Compared to foragers, the chemical profile of infected workers (LME: $t = -4.73$, $P < 0.001$) and nurses (LME: $t = -4.24$, $P < 0.001$) was composed of shorter-chained hydrocarbons (Fig. 3a). Moreover, infected workers and nurses had both proportionally more *n*-alkanes (LME: infected: $t = 4.37$, $P < 0.001$; nurses: $t = 4.27$, $P < 0.001$), but less mono- and di/trimethyl alkanes than foragers (LME: monomethyl alkanes $_{\text{infected vs. forager}}$: $t = -2.18$, $P = 0.032$; monomethyl alkanes $_{\text{nurses vs. forager}}$: $t = -2.00$, $P = 0.048$; di/trimethyl alkanes $_{\text{infected vs. forager}}$: $t = -3.21$, $P = 0.002$; di/trimethyl alkanes $_{\text{nurses vs. forager}}$: $t = -3.25$, $P = 0.002$); (Fig. 3c). On the other hand, foragers had generally more hydrocarbons on their cuticle than nurses ($t = 3.17$, $P = 0.002$), while infected workers did neither differ from foragers ($t = 1.32$, $P = 0.189$) or nurses ($t = -1.23$, $P = 0.220$; Fig. 3b). Despite these distinct differences between worker types, parasitized and unparasitized colonies did not differ in the average chemical distance between workers (*t* test: $t_{30.9} = 1.31$, $P = 0.20$).

Chemical Analyses of Worker Exchange Colonies

Contrary to our expectations, only few differences in hydrocarbon chemistry were observed between colonies that received infected or uninfected ants (Supplementary Table 3). Thus, the infection status of added or removed workers played a minor role (all $P > 0.29$; Supplementary Table 3). In contrast, most differences were found between the two behavioral

Table 1 Cuticular hydrocarbons of *Temnothorax nylanderi* workers from field colonies (parasitized and unparasitized colonies)

Peaks	Substance	Kovats index	Relative abundance, mean ± *SE (%)*			*p* value	
			Infected (I)	Nurses (N)	Foragers (F)	I – N - F	I - N
1	nC25	2500	0.38 ± 0.05	0.52 ± 0.08	0.59 ± 0.08		
2	nC26	2600	0.12 ± 0.01	0.14 ± 0.02	0.14 ± 0.01		
3	nC27	2700	5.95 ± 0.69	5.46 ± 0.73	4.06 ± 0.27		
4	*11;13-MeC27*	2731	0.63 ± 0.09	0.63 ± 0.07	1 ± 0.11	*0.046*	
5	*7-MeC27*	2741	0.08 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	*0.008*	
6	**5-MeC27**	2749	0.41 ± 0.05	0.32 ± 0.04	0.42 ± 0.06		**0.028**
7	11,15-;11,17-DiMeC27	2762	0.1 ± 0.02	0.08 ± 0.01	0.09 ± 0.01		
8	**3-MeC27**	2772	3.24 ± 0.45	2.53 ± 0.27	2.86 ± 0.23		**0.006**
9	nC28	2800	0.45 ± 0.05	0.3 ± 0.02	0.38 ± 0.03		
10	*6-MeC28*	2842	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	*0.009*	
11	*4-MeC28*	2857	0.43 ± 0.06	0.32 ± 0.03	0.46 ± 0.04	*0.042*	
12	***nC29***	2900	1.52 ± 0.18	0.91 ± 0.08	1.04 ± 0.07	*0.014*	**0.002**
13	*11;13;15-MeC29*	2932	2.09 ± 0.24	2.24 ± 0.24	3.93 ± 0.45	*0.028*	
14	*7-MeC29*	2941	0.23 ± 0.03	0.19 ± 0.02	0.24 ± 0.02	*>0.0001*	
15	***5-MeC29***	2951	0.28 ± 0.04	0.18 ± 0.03	0.21 ± 0.02	*0.029*	**0.006**
16	*11,17-DiMeC29*	2961	0.49 ± 0.09	0.36 ± 0.04	0.54 ± 0.06	*0.011*	
17	**3-MeC29**	2975	0.73 ± 0.11	0.49 ± 0.09	0.55 ± 0.05		**0.002**
18	***5,15-DiMeC29***	2980	0.2 ± 0.05	0.11 ± 0.01	0.2 ± 0.05	*0.003*	**0.014**
19	***3,15-DiMeC29***	3012	0.4 ± 0.05	0.27 ± 0.03	0.39 ± 0.05	*0.009*	**0.007**
20	*11;12;13;14;15-MeC30*	3031	0.26 ± 0.03	0.29 ± 0.03	0.52 ± 0.06	*0.021*	
21	*3,7,11;3,7,15-TriMeC29*	3037	0.12 ± 0.03	0.08 ± 0.01	0.13 ± 0.03	*<0.0001*	
22	4;2-MeC30; 11.15;13.17-DiMeC30	3061	0.12 ± 0.02	0.13 ± 0.02	0.25 ± 0.04		
23	*3-MeC30*	3072	0.07 ± 0.02	0.06 ± 0.03	0.06 ± 0.01	*0.021*	
24	*nC31*	3100	0.13 ± 0.03	0.06 ± 0.01	0.09 ± 0.01	*0.020*	
25	*11;13;15-MeC31*	3131	1.25 ± 0.18	1.44 ± 0.18	2.89 ± 0.35	*<0.0001*	
26	*11,15-;13,17-DiMeC31;* *9,17-DiMeC31;* *11,15,19-TriMeC31*	3152 3162 3180	1.15 ± 0.25	1.02 ± 0.12	1.81 ± 0.19	*>0.0001*	
27	*3,15; 3,17-DiMeC31*	3202	0.22 ± 0.04	0.18 ± 0.02	0.28 ± 0.04	*0.0417*	
28	*12;14;16-MeC32*	3231	0.08 ± 0.02	0.1 ± 0.02	0.23 ± 0.03	*<0.0001*	
29	***8,16-DiMeC32***	3237	0.52 ± 0.1	0.35 ± 0.05	0.66 ± 0.1	*0.043*	**0.049**
30	*12,16-DiMeC32*	3255	0.14 ± 0.05	0.11 ± 0.01	0.26 ± 0.03	*<0.0001*	
31	*13;15;17-MeC33*	3330	0.4 ± 0.09	0.48 ± 0.07	1.21 ± 0.16	*<0.0001*	
32	*13,17-;13,19-DiMeC33*	3355	0.93 ± 0.24	0.89 ± 0.09	1.69 ± 0.18	*0.0001*	
33	*9,17-;9,19-DiMeC33*	3361	0.46 ± 0.1	0.56 ± 0.09	1.11 ± 0.16	*>0.0001*	
34	*11,15,19-TriMeC33*	3379	0.32 ± 0.12	0.26 ± 0.03	0.44 ± 0.05	*0.005*	
35	*nC34*	3400	0.09 ± 0.02	0.08 ± 0.01	0.13 ± 0.02	*0.033*	
36	*11/13/15,17/19/21-DiMeC35**	3558	1.65 ± 0.4	1.57 ± 0.15	3.1 ± 0.37	*>0.0001*	
37	*11,15,19-TriMeC35*	3575	0.25 ± 0.07	0.21 ± 0.03	0.36 ± 0.05		

The table shows identified substances, the Kovats index as well as the mean relative abundance (±SE) in infected workers (I), nurses (N) and foragers (F). Significant differences between all three worker types are given in italics. Differences between infected workers and nurses are represented in bold

*combination of 1st and 2nd methyl group unknown

castes (i.e. nurses and foragers) and the experimental treatment (i.e. donor and receiver colonies), and/or the interaction of those two factors (Supplementary Table 3). After the experimental exchange of *T. nylanderi* workers, foragers and nurses still showed clear differences in their chemical profiles (PERMANOVA: Pseudo-F_1 = 20.98, $P < 0.001$), and this difference partially depended on the experimental treatment (interaction: *behavioral caste* x *experimental treatment*:

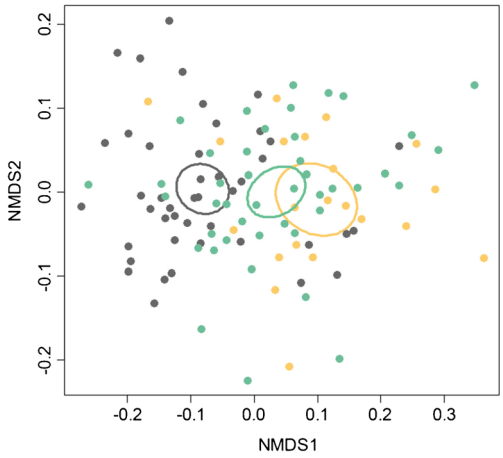

Fig. 2 NMDS plot showing cuticular hydrocarbon resemblance of uninfected foragers (grey dots), uninfected nurses (green dots) and tapeworm-infected *T. nylanderi* (yellow dots). Resemblance is based on the Bray-Curtis Similarity and stress value is 0.14. Ellipses represent 95% of confidence intervals

$F_1 = 2.98$, $P = 0.021$). Foragers and nurses from receiver colonies differed more in their hydrocarbon profiles ($t = 3.774$, $P = 0.001$) than those from donor colonies ($t = 3.194$, $P = 0.001$); (Supplementary Fig. 1).

Hydrocarbon traits differed mainly between donor and receiver colonies. Ant workers from receiver colonies had higher absolute amounts of hydrocarbons ($t = 2.02$, $P = 0.048$; Fig. 4b), and foragers had marginally higher absolute amounts than nurses (LME: $\chi^2_1 = 3.76$, $P = 0.053$). The proportion of *n*-alkanes was generally lower in workers from receiver colonies ($t = -2.65$, $P = 0.010$), but differences in the proportion of mono- and di/trimethyl-alkanes between the experimental colonies depended also on the behavioral caste (monomethyl: *behavioral caste* x *experimental treatment*: $\chi^2_1 = 7.80$, $P = 0.005$, di−/trimethyl: monomethyl: *behavioral caste* x *experimental treatment*: $\chi^2_1 = 5.56$, $P = 0.018$). Hence, nurses from receiver colonies had the lowest amount of monomethyl alkanes ($t = -6.83$, $P < 0.001$; Fig. 4c), but the highest of di/trimethyl alkanes ($t = 3.44$, $P < 0.001$; Fig. 4c). The mean hydrocarbon chain length differed between behavioral castes (LME: $\chi^2_1 = 5.15$, $P = 0.023$) and experimental treatments (LME: $\chi^2_1 = 26.83$, $P < 0.001$; Fig. 4a). Similar to the effects in the field colony analyses, nurses had shorter-chained hydrocarbons ($t = -2.27$, $P = 0.024$), and colonies that received foreign workers had generally longer-chained hydrocarbons ($t = 5.08$, $P < 0.001$). The infection status of added or removed workers only weakly affected the abundance of di−/trimethyl alkanes (LME: $\chi^2_1 = 5.25$, $P = 0.022$) or mean CHC chain length (LME: $\chi^2_1 = 4.19$, $P = 0.041$), and did not influence any other parameters. Uninfected workers from colonies that once contained infected nestmates or received infected non-nestmates had overall shorter-chained hydrocarbons ($t = -2.03$, $P = 0.047$) and shorter-chained di−/trimethyl alkanes ($t = -2.26$, $P = 0.028$) and overall shorter-chained hydrocarbons ($t = -2.26$, $P = 0.028$; Fig. 5).

Between-colony chemical variation (based on five foragers and five nurses per colony) was highest in colonies from which infected workers had been removed ('donor colonies'; treatment I). They were more variable than those from which uninfected workers had been removed ('donor colonies'; treatment II) (PERMDISP: overall $F_{3,523} = 15.75$, $P = 0.001$; I vs II $t = 3.35$, $P = 0.001$). The variation was the lowest in the two receiver treatments (III and IV; $t > 3.1$, $P < 0.002$ for each pairwise comparison to donor treatments), which did not differ from each other (III vs. IV: $t = 0.15$, $P = 0.88$). Analyses on

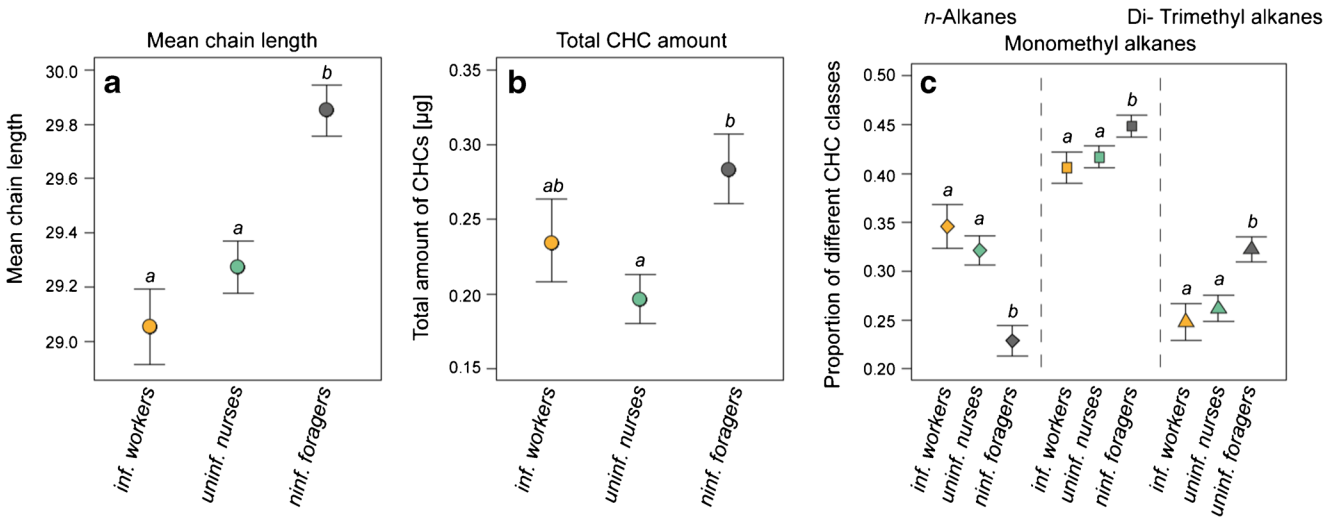

Fig. 3 Cuticular carbohydrate (CHC) profiles of different *Temnothorax nylanderi* ant worker types from field colonies (parasitized and unparasitized colonies) **a** mean chain length of hydrocarbons, **b** total amounts, **c** proportion of *n*-alkanes (diamonds), monomethyl alkanes (squares) as well as di/trimethyl alkanes (triangles). Colors show the three worker types (infected workers = yellow, uninfected nurse = green, uninfected foragers = grey). Plots with same letters are not significantly different

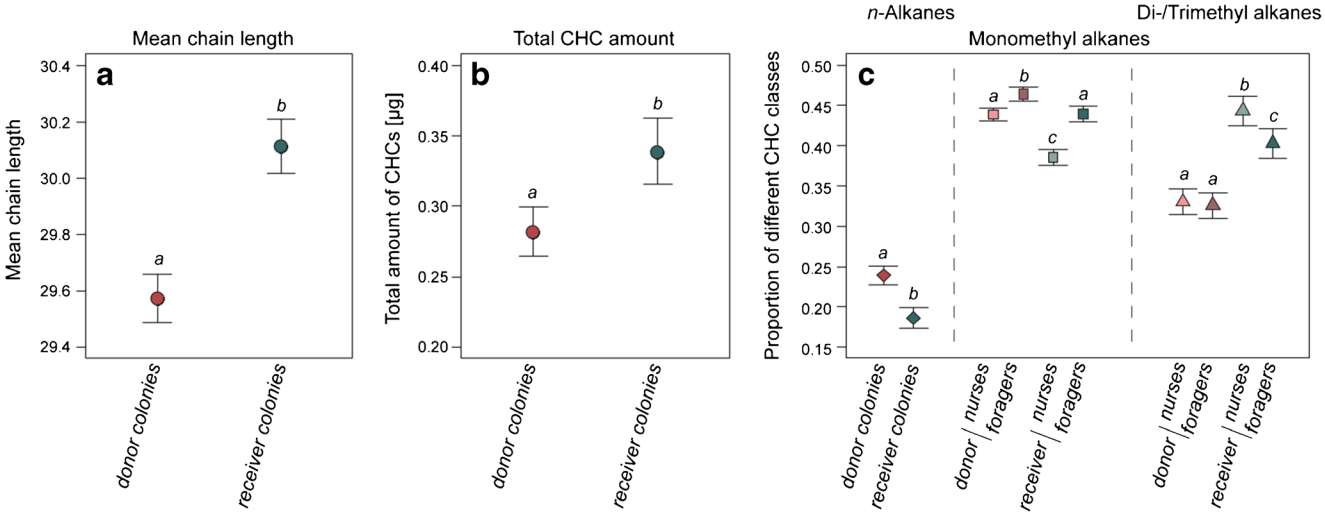

Fig. 4 Cuticular carbohydrate (CHC) profiles of *Temnothorax nylanderi* ant colonies and individuals subjected to different *experimental treatments* **a.** mean chain length of hydrocarbons, **b** total amounts, **c** proportion of *n*-alkanes (diamonds), monomethyl alkanes (squares) as well as di/trimethyl alkanes (triangles). Colors show the two experimental treatments (donor colonies = red, receiver colonies = green). Nurses (light red & green) differed from foragers (dark red & green) only for the complex CHCs. Plots with different letters differ significantly from each other

between-colony variation based on only foragers or only nurses yielded similar results. In contrast, within-colony variation (based on foragers and nurses) did not differ between treatments (LM: $F_3 = 0.42$, $P = 0.74$), and the same was true for variation concerning only foragers or only nurses.

Discussion

In this study we investigated experimentally whether odor mixing between infected and uninfected ant workers leads to a more diverse colony odor in parasitized colonies. This increase in intra-colonial hydrocarbon variance could reduce inter-colonial differences and therefore explain the reduced aggression exhibited by parasitized colonies (Beros et al. 2015). Contrary to our predictions, parasitized colonies were not chemically more diverse. Despite frequent grooming and trophallaxis, behaviors known to mediate hydrocarbon transfer (Vienne et al. 1995), the presence of infected workers did not affect the hydrocarbon profiles of uninfected workers. Interestingly, the removal of nestmates and addition of non-nestmates rather than the infection status of the exchanged workers affected the colony odor. This stands in stark contrast to our earlier behavioral experiments (Beros et al. 2015), in which the removal and addition per se did not change colony aggression, whereas the infection status of the removed or added workers did. One possible explanation for the radical change in chemical signature in response to worker exchange is that ants increase the production of colony-specific recognition cues, which changes the overall colony composition. Yet, overall our findings suggest that aggression in our study species is not simply associated with differences in CHC profiles. A similar conclusion has been reached for the harvester ant *Pogonomyrmex barbatus*, where chemical and behavioral differences between task groups are stronger than those between colonies (Sturgis and Gordon 2012).

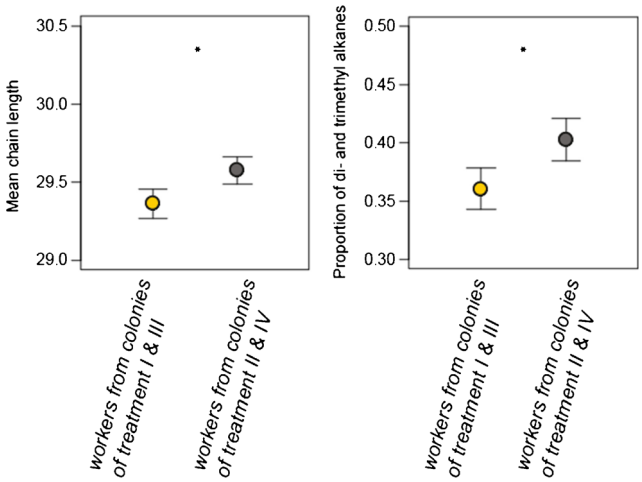

Fig. 5 Cuticular carbohydrate (CHC) profiles of *Temnothorax nylanderi*, showing mean chain length of hydrocarbons and the proportion of di-and trimethyl alkanes of uninfected workers in relation to the addition of infected (yellow colored) and uninfected conspecific workers (grey colored)

No Effect of Infected Workers on CHC Profiles and Cue Diversity

Uninfected workers from parasitized colonies are less aggressive than those from unparasitized colonies (Beros et al. 2015). Ants from artificially mixed and hence, chemically diverse colonies show particularly low aggression towards

non-nestmates (Errard et al. 2006). As such, we hypothesized that the reduced aggression of workers from parasitized colonies of *T. nylanderi* is due to the aberrant profile of infected workers, which should lead to higher cue diversity within these colonies.

The '*Gestalt*' model suggests that frequent interactions (e.g. allogrooming, trophallaxis) between nestmates allow a continuous transfer of hydrocarbons and therefore lead to the formation of a colony-specific odor. In artificial mixed colonies (Stuart 1988; Errard 1994), workers of both species gradually acquired some hydrocarbons from their heterospecific nestmates, probably leading to a broader colony signature and in turn increasing tolerance towards alien individuals. The same is true for naturally mixed colonies of slavemaking ants, in which both slavemakers and hosts obtain hydrocarbons from each other (Bauer et al. 2010; Brandt et al. 2005). As workers of parasitized *T. nylanderi* colonies show high allogrooming and trophallaxis rates towards their tapeworm-infected nestmates (Scharf et al. 2012), which possess a distinct hydrocarbon profile (Trabalon et al. 2000), the cue signature of uninfected workers in our colonies might have shifted towards those of infected workers. However, the hydrocarbon profiles of uninfected nestmates did not change with parasitism status of the colony, nor did workers from either colony type differ in their average chemical distance. These findings imply that the behavioral changes observed in uninfected nestmates of infected individuals (Scharf et al. 2012; Beros et al. 2015), in particular the reduction in aggression, are determined by factors other than hydrocarbon chemistry and colony odor. Chemical compounds other than CHCs can also modify the behavior of social insects. Nest volatiles such as short-chain alkanes have been shown to decrease aggression in *Camponotus fellah* ant workers (Katzav-Gozansky et al. 2008), and also in a parasitic context they play an important role as olfactory cues. The odor of mice infected with transmissible stages of the malaria parasite, *Plasmodium chaubaudii*, enhances the attraction of mosquitos due to the production and release of volatiles (De Moraes et al. 2014). Volatiles were further involved in the interactions between microorganisms and insects via microbial volatile organic compounds (Davis et al. 2013). Thus, the release of volatiles in tapeworm-infected individuals might similarly influence colony aggression, opening up new avenues for research. Moreover, members of social insect colonies are known to alter their behavior when confronted with infected peers and pathogens (Cremer et al. 2007), and may be even able to weigh the risk of disease transmission and damage to their colony (e.g. Heinze and Walter 2010, Rueppell et al. 2010, Bos et al. 2012). The behavioral repertoire is diverse and ranges from antagonistic responses (Richard et al. 2008; Baracchi et al. 2012) to more social care such as increased self- and allogrooming (Aubert and Richard 2008; Walker and Hughes 2009; Konrad et al. 2012), which can be observed in tapeworm-parasitized colonies of *T. nylanderi* as well (Scharf et al. 2012). More investigations are required to understand whether the deviant hydrocarbon profile of infected ants (Trabalon et al. 2000) induces the behavioral changes in their nestmates and whether the altered behaviors have implications for social immunity. A recent study demonstrates that the detection of CHCs from immune-stimulated and diseased honeybees can initiate an immune response in queens (López et al. 2017). Likewise it is possible that the interactions with tapeworm-infected workers alter their nestmates' physiology. Indeed, the gene expression pattern in brains of uninfected *T. nylanderi* workers from parasitized colonies show differences to those living in unparasitized colonies (Feldmeyer et al. 2016). In addition, the higher mortality rate of uninfected workers from parasitized colonies (Beros et al. 2015) implies that caring for infected workers (Scharf et al. 2012) is associated with physiological costs, which might weaken the ants' aggression level.

Effect of Removal and Addition of Workers

Although infection status of exchanged workers strongly affected aggression (Beros et al. 2015), we show that it does not influence the hydrocarbon profiles of nestmates. Rather the removal and addition of workers per se had a strong and complex impact on the CHC profile. Colonies that received non-nestmates showed more di−/trimethyl alkanes, but less *n*-alkanes and monomethyl alkanes in their profiles, higher average chain lengths, and higher absolute CHC quantities. As we only analyzed the profiles of native workers, the chemical changes we observed suggest that these workers responded to the presence of foreign individuals by producing more hydrocarbons, and in particular more di−/trimethyl alkanes. These more complex hydrocarbons have been shown to be utilized by bees and ants for nestmate recognition (Dani et al. 2001; Akino et al. 2004; Dani et al. 2005; Guerrieri et al. 2009; Sturgis and Gordon 2012). Further trimethyl alkanes are more easily learned by ants compared to *n*-alkanes and monomethyl alkanes (van Wilgenburg et al. 2011). It is therefore possible that the enhanced production of these structurally more complex hydrocarbons is an adaptive response to signal colony identity more clearly (Sturgis and Gordon 2012), thereby ensuring that altruistic behaviors are directed only to related individuals. The longer chain length may be explained by the need to maintain the viscosity of the CHC layer despite a lower percentage of *n*-alkanes (Gibbs 1995; Gibbs and Pomonis 1995). Surprisingly, the changes in the proportion of hydrocarbon classes due to worker exchange were not accompanied by changes in hydrocarbon variation within colonies. However, between-colony variation was affected. Supposedly, the addition of 13% non-nestmates blurred chemical differences between colonies such that inter-colony variation was lower in receiver colonies.

Differentiation Between Infected Workers, Nurses and Foragers

CHCs of *T. nylanderi* workers were comprised of a mixture of linear and methyl-branched alkanes, which differed in their relative abundance among nurses, foragers and infected workers. These analyses support earlier findings on CHC changes with parasite infections (e.g. Trabalon et al. 2000; Salvy et al. 2001; Richard et al. 2008; Csata et al. 2017), which also revealed shifts in the relative abundance rather than the absence or presence of certain hydrocarbons. Yet, in contrast to Trabalon and colleagues (2000), we differentiated between worker castes and thus can demonstrate that the CHC profiles of infected workers differ more strongly from foragers than from nurses. Moreover, the difference between nurses and foragers was greater than that between nurses and infected workers. As the presence of infected workers did not affect the hydrocarbon profiles of other workers, the chemical resemblance of nurses and infected workers is likely not due to the exchange of hydrocarbons. It is more likely that similarities in physiology, behavior or spatial location in the nest might lead to profile convergence. Due to their high survival rates infected workers likely represent the oldest worker caste in the nest (Beros et al. 2015). Nurses are usually the youngest workers in a colony, while the older workers become foragers (Mersch et al. 2013). Thus, chemical differences between foragers and nurses are not solely due to age – otherwise, infected workers should have resembled foragers more than nurses. The deviant profile of foragers may rather reflect acclimation to their external environment. Being frequently outside the nest foragers experience more desiccation stress. In several social insect species foragers carry more *n*-alkanes than nurses (Wagner et al. 1998, Martin and Drijfhout 2009, Kather et al. 2011, Sturgis and Gordon 2012, Pamminger et al. 2014), which protect better against desiccation than methyl-branched alkanes (Gibbs and Pomonis 1995). In this study, however, *Temnothorax*-foragers had relatively less *n*-alkanes, but carried generally more hydrocarbons and also more information-rich methyl-branched hydrocarbons (Akino et al. 2004). One simple reason could be the stable environmental conditions in the laboratory. A biological more relevant argument is that foragers may need more recognition cues since they are more likely to encounter non-nestmates than nurses. In addition, nurses and infected workers may have acquired hydrocarbons from the brood, which mainly contain *n*-C27 (60–70% of the profile) and 3-MeC27 (12%; unpublished data). These two substances are common in all three behavioral castes, but particularly abundant in infected workers.

The profiles of infected workers differed in the relative abundance of eight compounds from uninfected workers. Interestingly, four out of these eight hydrocarbons (i.e. 5-MeC27, 3-MeC27, 5-MeC29 and 3-MeC29) were found to explain inter-colonial aggression and thus are thought to be important in nestmate recognition in a North American *Temnothorax* species (Jongepier and Foitzik 2016). We found that, overall, worker aggression in parasitized *T. nylanderi* is, however, reduced in the presence of infected workers, but the difference in CHC profile still may explain the occasional attacks against infected workers within their colonies (Scharf et al. 2012).

Conclusions

Our study provides experimental evidence that the presence of infected workers does not lead to higher intra-colonial variation in CHC profiles. Thus, the previously observed decrease in aggression due to the presence of infected workers in the nest must be caused by other factors. CHC chemistry, however, changes with the removal and addition of workers, probably inducing either a change in the transfer of different hydrocarbon classes or in their biosynthesis. Finally, our study emphasizes intra-colonial differences between foragers and nurses, showing that these differences are greater than those between infected and uninfected workers, suggesting that ants are well able to identify the behavioral role of nestmates by their hydrocarbon profile.

Acknowledgements We thank Cecilia Felicia Gall for data collection and Inon Scharf for helpful comments on the manuscript.

Funding This work was funded by the German Research Foundation (DFG; grant numbers: FO 298/15–1 and ME 2842/3–1).

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