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Task-Related Variation of Cuticular Hydrocarbon Profiles Affect Nestmate Recognition in the Giant ant *Dinoponera quadriceps*

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Abstract Recognition seems to be one of the more remarkable characteristics of social groups. In social insects, cuticular hydrocarbons are important for colonial recognition by providing a chemical signature for colony members. The acceptance threshold model predicts that colony members will accept conspecifics when the levels of nest mate cues dissimilarities are below the acceptance threshold. We tested the hypothesis that the encounter of a guard ant worker with a nurse may cause a delay in the process of recognition, because nurses from distinct colonies may share greater amount of chemical compounds. *Dinoponera quadriceps* guard workers decreased their effectiveness to recognize nurses rather than did to foragers. Alien foragers received significantly more bites and other stronger acts than non-nestmate nurses when they were experimentally introduced. In addition, guards took significantly more time to react against non-nestmate nurses than against alien foragers. Analysis of the cuticular hydrocarbon profiles corroborated our behavioural analysis that nurses from distinct colonies overlap greater amount of cuticular hydrocarbons.

Keywords Chemical similarity · cuticular hydrocarbons · ponerinae · division of labor

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Introduction

Nestmate recognition in social insects is of primary importance for colony development, organization and maintenance (Hölldobler and Wilson 1990, reviewed in D'Ettorre and Lenoir 2009 and van Zweden and D'Ettorre 2010). Workers of many species of ants can also discriminate conspecific and allospecific individuals, potential competitors and enemies from nest mates (Zhi-bin et al. 2000). Colony discrimination in ants that prevents the acceptance of non-nest mates into the nest and maintains high intra colony relatedness is consistent with kin selection theory (Breed and Bennett 1987; Hölldobler and Wilson 1990; Vander Meer and Morel 1998).

Growing evidence indicates that nest mate discrimination signals are encoded by cuticular lipids, particularly hydrocarbons (Singer 1998; Martin and Drijfhout 2009a; Van Zweden and D'Ettorre 2010). Biochemical investigations have shown that insect cuticular hydrocarbons (CHCs) are synthesized internally in oenocytes (Blomquist and Dillwith 1985). The CHCs consist of a homologous series of long straight-chain saturated alkanes, which can be modified by the addition of methyl groups attached to the chain or the introduction of one of more double bonds (Lockey 1988; Jackson and Morgan 1993). These modifications are responsible for the very rich and complex CHC profiles within insect societies.

Variation of cuticular hydrocarbons according to tasks was mainly characterized in ants (reviewed in Provost et al. 2008). For example, foragers and patrollers of the harvester ant *Pogonomyrmex barbatus* had a higher proportion of alkanes on their cuticle than nest maintenance workers (Wagner et al. 1998). Further studies confirmed that foragers were stimulated to work when glass beads coated with cuticular extracts of patrollers were inserted in the nest (Greene and Gordon 2003). It meant that inactive foragers perceived new incoming individuals by their chemical cues rather them behavioral interactions.

Dinoponera quadriceps, known in Brazil as "falsa tocandira", is a giant queenless ant with one mated worker or gamergate monopolizing reproduction, but intracolony conflict is common because a small number of workers compete for access to reproduction while most individuals have no chance of reproducing directly (Monnin et al. 2003). Division of labor is performed by monomorphic workers, which develop a hierarchical dominance after colony founding. Low-ranking workers are generalists that perform foraging and nest maintenance, while higher-rankers, involved in dominance interactions, are more specialized in tasks related to brood care (Monnin and Peeters 1999; Nascimento et al. 2012). In a previous study, we investigated the ability of *D. quadriceps* workers to discriminate nestmate from alien eggs based on the cuticular hydrocarbon composition. We confirmed that workers involved in brood care were able to distinguish nestmate from non-nestmate eggs, while callow and forager workers were incapable of doing so (Tannure-Nascimento et al. 2009).

The acceptance threshold model (Sherman et al. 1997; Reeve 1989) predicts that colony members will accept conspecifics when levels of cue similarities are below the acceptance threshold. In addition, a single individual is likely to be accepted in a nest entrance when its odors match with the guard's internal template. On the other hand, non-nestmates can also be accepted in higher rates due to overlapping distribution of cuticular compounds. However, recent studies support that the presence of an undesirable odor is more expected to elicit the rejection responses (Leonhardt et al. 2007;

Guerrieri et al. 2009). In fact, the antennae sensilla of *Camponotus japonicus* responded to a non-nestmate compound rather than a familiar nestmate compound (Ozaki et al. 2005).

Cuticular hydrocarbons also vary according to the task and function of every worker in the colony (Martin and Drijfhout 2009b; Ferreira-Caliman et al. 2010). In stingless bees, foragers presented higher proportions of mainly alkanes and alkenes than nurse bees (Ferreira-Caliman et al. 2010). In ants, we would expect that the encounter of a guard worker with a nurse present a delay in the process of nestmate or non-nestmate discrimination compared to the recognition with a forager because inside-nest workers from distinct colonies may overlap chemical compounds in a higher proportion than foragers. In this work we predict that *D. quadriceps* guard workers are less efficient in recognizing non-nestmate nurses as compared to non-nestmate foragers.

Methods

Colonies and Rearing

Four colonies of *D. quadriceps* were collected in São Cristóvão (10 °55′56″ S, 37 ° 09′23″ W), Sergipe State, Brazil and transferred to the laboratory. Colonies were housed in plastic boxes (45 × 35 × 10 cm) with internal chambers and connected to the foraging arena by a plastic tube. To avoid a possible effect of distinct food on CHCs (Liang and Silverman 2000), all colonies were fed three times a week with the same diet of mealworm larvae and sugar water was offered ad libitum.

Behavioural Assays

Nestmate recognition in *D. quadriceps* was tested using different groups of workers. We previously performed 30 h of observation in each colony to identify nurses, guards and foragers. These preliminary observations were carried out during 6 h a day (5 days in total), and the main activities were recorded (foraging, brood caring, nest maintenance and defence). Before the experiments all individuals were marked with paint (Magic® Opaque color nº 551) on their thorax and abdomen.

We conducted bioassays by introducing an alien identified nurse or forager into the foraging arena of a discriminator colony. We used a small glass cage (12 × 12 × 10 cm) to settle the introduced individual (30 min). Behavioural interactions were registered during encounters between a guard ant and the introduced worker (nestmate or non-nestmate). We only started an experimental trial when at least two guard workers were close to the nest entrance. For each trial a nestmate forager was used as control group predicting that the nestmate foragers would be recognized by guards and accepted. Interactions between the workers were observed for five minutes, scored and ranked as follows: 1 = approach without interaction, 2 = antennation, 3 = aggression (lunging, pulling or biting), and 4 = fight (prolonged aggression). Scores 3 and 4 were regarded as aggressive behaviours. Latency time of recognition was registered when the introduced ant was perceived by a guard, received the first contact until the final decision (acceptance or rejection). Only a single trial was

performed per day/colony. We normalized for variation by dividing the number of aggressive responses by the total number of interactions. Blind observations were made regarding the identity of the introduced worker (alien or nestmate).

The data were analysed with a generalised mixed-effects models (GLMM) for a binomial response-variable and both discriminator and source colony as random-effects (Bolker et al. 2009). Source and discriminator colonies and ant function were entered into the analysis as the independent variables and response (acceptance or rejection) as the dependent variable (Neter et al. 1996). We contrasted the frequencies of colonies' aggressive responses toward nurses and foragers, nestmates and non-nestmates among using a *G*-test with a Yates correction (Sokal and Rohlf 1995). We also verified whether the latency time of the recognition between the two types of non-nest mate workers differed in each discriminator colony (Wilcoxon test for paired comparison).

Chemical Analyses

As previously used to *Dinoponera quadriceps* (Monnin et al. 1998), cuticular hydrocarbons of individual workers (nurses and foragers) were sampled with a Supelco 7-μm polydimethylsiloxane fiber, designed to extract compounds of high molecular weight. Following the current rules for SPME quantification (Romeo 2009), we used the same conditions and a single fiber to analyse the compounds of individuals. Ants were anesthetized for several minutes in a refrigerator (4 °C) for complete immobilization. The fiber was carefully rubbed against the thorax and abdomen for 2 min, and then desorbed in the injection port of a gas chromatograph for 4 min, for GC-MS analysis.

Each sample was analyzed by gas chromatography coupled with mass spectrometry (Shimadzu, model QP2010plus, Tokyo, Japan), equipped with a DB-5MS capillary column, a 30 m fused silica column Rtx-5 (30 m×0.37 m×0.5 μm) covered with poly (5 %-biphenyl-95 % dimethyl) siloxane, using helium as the carrier gas. Later the fiber was inserted in the injector port (splitless mode with an injector temperature of 280 °C and a detector temperature of 300 °C) of a gas chromatograph for desorbtion (column temperature increased from 70 °C to 300 °C at 5 °C/min, then isothermic at 300 °C for 20 min). Individual mass spectra were compared with synthetic compounds and Wiley library data (McLafferty et al. 2000).

Principal components analysis (PCA) was used to define the main compounds peaks to be compared. Compounds missing in most individuals of an analyzed group as well as compounds contributing less than 5 % to the first two factors, as indicated by the PCA, were excluded from the statistical analysis. The relative concentrations of the compounds used in the discriminant analysis were readjusted to 100 %. Following this, a stepwise discriminant function analysis was used to observe if combinations of variables could be useful in the predicting group. In this method, variables are successively added to the model based on the higher F to enter values, adding no more variables when the F ratio is no longer significant. Wilks' λ values were used to verify the individual contribution of each variable to the model. The Wilks' λ statistic for the overall discrimination is computed as the ratio of the determinant of the within-groups variance/covariance matrix to the determinant of the total variance/covariance matrix.

When this value is close to 1.0, then the residual is high and the variable is not a good discriminator, while a value closer to 0 means that the residual is low and the variable is a good discriminator (Rao 1973). To avoid errors in compositional sample data, the area under each peak was transformed according to the following formula:

$$Z = \ln[Ap/g(Ap)],$$

where Z is the standardized peak area for individual (forager/nurse), Ap is the area under the peak, g(Ap) is the geometric mean for each individual compound group (Aitchison 1986). All analyses were carried out using Statistica 7.0 (Statsoft, Inc.).

Results

Behavioural Interactions

In total, we made 482 experimental introductions of nurse/forager and nestmate/non-nestmate workers. Table 1 shows that all tested variables provided significant contribution for discrimination. As predicted, guard workers from the four discriminator colonies exhibited agonistic behaviours toward 258 out of 360 non-nestmate workers introduced (total average of 71.66 % of rejection). In contrast, guard workers neither rejected nor exhibited aggressive behaviour toward their mate foragers (control group) during the 122 experimental trials (100 % of acceptance). The most aggressive behaviours (coded as 3 and 4) were most performed toward non-nestmate foragers than to nurse workers (Fig. 1).

Comparing each discriminator colony, we found that guard workers from colony 2 rejected 62.12 % of non-nestmates. For this colony, alien foragers received significantly more bites and other stronger acts than non-nestmate nurses when they were experimentally introduced (G-test = 4.17, $p<0.05$). Guards from colony 2 rejected foragers from colony 8 and colony 5 in a higher rate level than from colony 3 (85.71 %, 81.25 % and 75 %, respectively). In contrast, guards of colony 3 did not discriminate between the two groups of non-nestmate workers, but rejected 73.43 % of all alien individuals (G-test = 0.32, $p>0.05$).

The rejection of non-nestmates in the colony 5 was observed in 80 % of the results. Introduced foragers received significantly more aggressive acts than nurses (Fig. 2; G-test = 4.55, $p<0.05$). Guards also showed distinct behavioural responses toward foragers from experimental colonies, colony 3 and colony 8 (colony 2: 90 %, colony

Table 1 Results of a general linear mixed model to test for the effect of discriminator colony (2, 3, 5 and 8), workers' function (nurse or forager) and colony (nestmate or non-nestmate) on the recognition responses

	Effect (F/R)	Deviance	d.f.	F	p
Model	Fixed	37.78	1	77.03	0.0014
Discriminator colony	Random	1.99	3	5.09	0.0018
Source of ant	Random	23.87	3	182.77	<0.0001
Function of ant	Fixed	1.52	1	11.65	0.0007
Error		47.54	481		

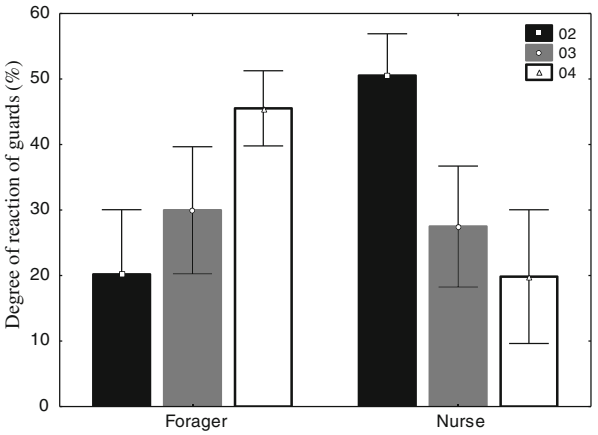

Fig. 1 Frequency of aggressions (Mean±SE) of *D. quadriceps* guards towards non-nestmate foragers and nurses during experimental encounters. Black bars (code 2) = antennation; gray bars (code 3) = moderate aggression and white bars (code 4) = fight

3: 100 %, and colony 8: 60 % of very aggressive acts). Guard workers of colony 8 rejected about 60 % of non-nestmates. Non-nestmate foragers were attacked more often than nurses (G-test = 4.20, $p<0.05$). Foragers from distinct colonies did not showed distinctness between introduced non-nestmate foragers (colony 2: 42.8 %, colony 3: 45 % and colony 5: 57.14 %).

The behavioural responses of introduced nurses and foragers toward a non-nestmate guard were also distinct. Nurses behaved quietly and passively trying to escape from the guard or enter the foreign nest while foragers were always very hostile against the guard. In the latter confrontations, both ants usually grab the opponent's mandibles trying to sting her body. However, resident guards were always very aggressive after non-nestmate recognition.

In three out of four colonies non-nestmate nurses were usually recognized after a longer period of time. Guards took significantly more time to react against non-nestmate nurses

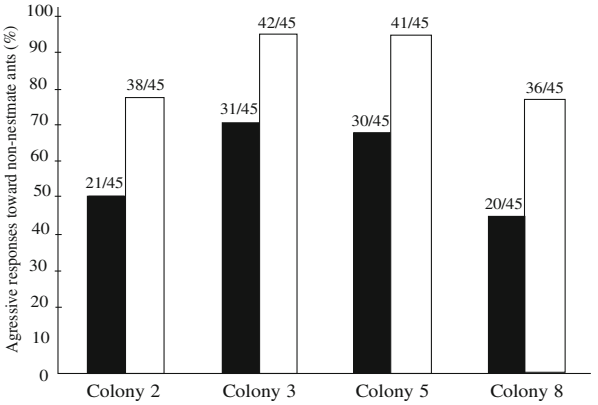

Fig. 2 Pooled comparison of aggressive responses of guard ants towards introduced non-nestmate nurses (*black bars*) and foragers (*white bars*) in *D. quadriceps*. Numbers on the top of bars show rejected/total introduced individuals

than against alien foragers (nurses x foragers: Colony 2: 12.8±1.2 s×7.2±0.4 s, Z=2.24, p<0.05; Colony 3: 11.2±0.5 s×7.3±0.4 s, Z=2.67, p<0.01; Colony 5: 9.8± 0.7 s×6.1±0.3 s, Z=2.4, p=0.01; Fig. 2). On the other hand, guards of colony 8 showed similar responses toward nurses and foragers (14.0±1.4 s×9.6±1.2 s, Z=1.23).

Chemical Analyses

Analyses of cuticular hydrocarbons showed that all workers of a single colony presented a higher similarity of cuticular lipids (Table 2). However, as predicted, nurse workers belonging to different colonies cannot be discriminated by their chemical profiles. Although discriminant analysis showed that about 97 % of workers were correctly assigned in their respective predicted groups (Fig. 3), an overlap of chemical profiles was observed for nurses across the colonies. The main compounds discriminating nurse workers from outside-nest workers were 11-, 15-Dimethyl C_{29} ($F_{1,60}$=6.58, p<0.001), C_{23} ($F_{1,60}$=3.82, p<0.01), 9- Methyl C_{31} ($F_{1,60}$=3.67, p<0.05), 3- Methyl C_{27} ($F_{1,60}$=3.12, p<0.05).

Discussion

In the present study, we demonstrated that guard workers of *D. quadriceps* were able to reject alien workers and accept nestmates, but they needed significantly more time to recognize alien nurses. This suggests that the recognition cues of nurses may not be very distinct from colony to colony at least in *D. quadriceps.* These results were reinforced by the time that guards spend to make the decision to accept or reject a non-nestmate nurse after its first contact with antennae. Indeed, as expected we found a higher similarity in hydrocarbons between nurses than between foragers from different nests, which could be an indication of the presence of task related CHCs (Wagner et al. 1998, 2001; Kather et al. 2011).

Table 2 Chemical distances estimated by multivariate analyses of cuticular compounds from nurses (NRS) and foragers (FOR) of the analyzed colonies (Col). Data revealed that nurses from distinct colonies presented a higher chemical similarity compared to foragers

	NRS (Col 3)	NRS (Col 5)	NRS (Col 8)	FOR (Col 3)	FOR (Col 5)	FOR (Col 8)	FOR (Col 2)
NRS (Col 2)	10.88 ns	14.45 ns	15.99 ns	18.67*	20.13*	35.22 **	10.12 ns
NRS (Col 3)		19.66 ns	17.47 ns	9.76 ns	17.64 *	58.01**	13.77*
NRS (Col 5)			11.80 ns	29.78 *	18.20 ns	60.38 **	31.02**
NRS (Col 8)				33.95 *	27.90 **	46.40 **	28.13**
FOR (Col 3)					21.27 **	56.44 ***	10.31 ns
FOR (Col 5)						49.14 **	59.83**
FOR (Col 8)							52.11***

ns not significant, * p< 0.05, ** p<0.01, *** p<0.001

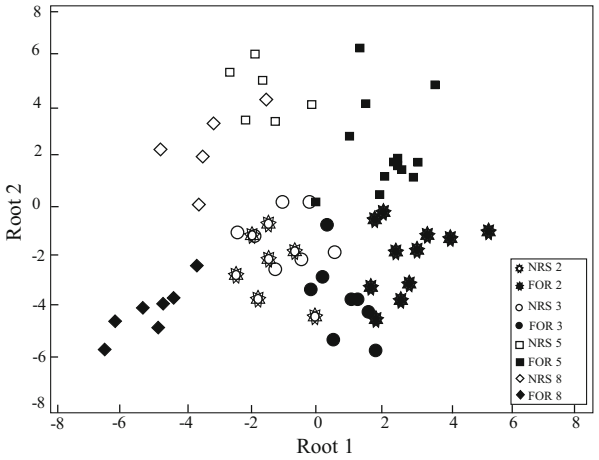

Fig 3 Discriminant analysis of *D. quadriceps* nurses (NRS) and foragers (FOR) based on their cuticular hydrocarbon profiles. Root 1 and Root 2 encompass respectively 35 % and 23 % of explained variation of the relative percentage of chemical compounds from the analyzed workers

Chemical similarity between inside-nest workers from different colonies appears to affect the ability of nest mate recognition in *D. quadriceps*. The fact that ants have a colony specific cuticular odour, but at the same time share a nest independent cuticular profile can be explained by a passive accumulation of compounds within the nest. The hydrocarbons are thought to be stored in the postpharyngeal gland and are distributed throughout the colony via allogroming (Soroker et al. 1998). Additionally, it has been observed that inside the nest, alien ants are generally not rejected (Hölldobler and Wilson 1990). This fact also explains why inside the nest, any social parasite will be accepted as a fellow nest mate (D'Ettorre and Heinze 2001). Chemical similarity between individuals from distinct colonies has also been reported in other social insects, such as stingless bees (Nunes et al. 2008).

Members of an insect colony should prefer to help related individuals and avoid aliens. In fact, the mechanism of individual recognition is adaptive in most social insect groups. The acceptance threshold model predicts that colony members will accept conspecifics when levels of nestmate cues dissimilarities are below the acceptance threshold (Reeve 1989). In addition, according to the Gestalt theory (Crozier and Dix 1979), nestmates blend their hydrocarbons to form a common colonial signature. However, in social insects, cuticular hydrocarbons vary according the task the workers perform (Wagner et al. 2001). Compared to nurse workers, most foragers experience higher temperatures, extreme solar radiation and lower humidity, as a result physiological changes of their metabolism may be reflected in distinct cuticular chemical profiles. Therefore, the encounter of a guard worker with a nurse (inside-nest worker) may cause a delay in the process of nestmate recognition due to a homogenous nest environment. Moreover, the model predicts that a recognition system should be adjusted according to the environmental changing conditions (Reeve 1989). The present results showed that the effectiveness of non-nest mate rejection may be related to the higher threshold of discrimination by guard workers toward foragers, and also that the inside-nest individuals (e.g. nurses) may share a similar chemical profile that is largely overlapped between colonies affecting the

reliability of nest mate and non-nest mate recognition. As with chemical identification, the behavioural component of the recipient individual (introduced ant) may trigger different reaction by the receiver (guard ant) depending on the context and its associated decision. In our study, erratic and peaceful reaction of alien nurses yields longer time of guards' response compared to that with an alien forager.

In previous reports, it has been shown that task-specific cuticular hydrocarbons in ants are used to adjust the number of individuals performing foraging activities (Greene and Gordon 2003). In fact, workers of the harvester ant *Pogonomyrmex barbatus* presented distinct cuticular hydrocarbon profiles according to their functional roles (Wagner et al. 1998). Lavine et al. (1990) demonstrated that foragers and callows in *Camponotus floridanus* are differentiated according to their CHC profiles. Task-specific variation in the cuticular hydrocarbons was also found in *Myrmecaria eumenoides* (Kaib et al. 2000). In this species, higher proportions of alkenes and alkadienes were found in foragers and nurses, respectively. However, this is the first study demonstrating that a specific group of workers having distinct cuticular hydrocarbon profiles produce unreliable reaction on the guard workers. Our methods also supports that it is possible to separate castes and colonies based on shared compounds.

The role of a single class or a blend of hydrocarbons as cues for nest mate discrimination has been intensively reported in several species of social insects (see Howard and Blomquist 2005). Recent studies showed that alkenes ($Z9\text{-}C_{n:1}$) are involved in the process of recognition in *Formica* ants (Martin et al. 2008). In *D. quadriceps*, however, the only alkene ($9\text{-}C_{31:1}$) present on cuticle has an important role as a reproductive cue (Monnin et al. 1998). No information about nest mate recognition cues in this species has been reported to date. Our results showed that monomethyl- and dimethyl alkanes significantly discriminated between members of colonies. Further studies will evaluate if nestmate recognition in *D. quadriceps* is mediated by a blend of complex chemicals or by a single class of compound.

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