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Behavioral and Chemical Correlates of Long-Term Queen Adoption in the Facultative Polygynous Ant *Ectatomma tuberculatum*

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Abstract In ants, queen adoption is a common way of achieving secondary polygyny but the mechanisms involved are little known. Here we studied the process of long-term adoptions of alien queens in the facultative polygynous ant *Ectatomma tuberculatum*. In eight out of 10 successful adoption experiments, all the introduced queens showed similar behavior and fecundity as the resident queens even after

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2 months, indicating complete integration into the colony. Chemical analysis revealed that the cuticular hydrocarbon profiles of resident and introduced queens were clearly distinct from those of workers and that they did not change after adoption. We propose that queen-specific cuticular hydrocarbon profile may represent a reliable signal of queen's fertility and discuss about the evolution of high level of queen acceptance in *E. tuberculatum*.

Keywords Formicidae · secondary polygyny · nestmate recognition · cuticular hydrocarbons · fertility signal

Abbreviations

Qr	resident queen
Qa	adopted queen
SPME	solid phase micro extraction
GC/MS	gas chromatography mass spectroscopy
CHC	cuticular hydrocarbon
PCA	principal component analyses
DA	discriminant analysis
MSMD	mean squared Mahalanobis distance

Introduction

The evolution of sociality in insects has been enlightened by inclusive fitness theory of Hamilton (1964) which emphasizes that relatedness between individuals can promote cooperation in certain circumstances. Queen number per colony has thus been the subject of numerous studies since polygyny is likely to decrease within-colony relatedness demoting cooperation (Keller 1993; Bourke and Franks 1995). However, in several cases polygyny was shown to be compatible with kin selection theory (Nonacs 1988; Pamilo 1991; Keller 1995). First, nestmate queens can be related and thus lead to a relatively high relatedness between nestmate workers (Pamilo and Rosengren 1984; Douwes et al. 1987; Pamilo 1991; Lipski et al. 1992; Trontti et al. 2005). Second, polygyny facilitates larger colony size (*i.e.* worker force) thus increasing overall colony productivity (Rosengren and Pamilo 1983; Rissing et al. 1989). Furthermore, pleometrosis (colony foundation by multiple queens) enhances the success rate of colony foundation compared with haplometrosis (colony foundation by a single queen) (Herbers 1993; Bourke and Heinze 1994). Polygyny can either be achieved by pleometrosis which leads to primary polygyny, or by queen adoptions which result in secondary polygyny (Rissing and Pollock 1988). Although secondary polygyny is common in ants (Hölldobler and Wilson 1977, 1990) only a few studies investigated the dynamics of queen acceptance and the mechanisms by which queen number per colony is regulated (Bennett 1988; Fortelius et al. 1993; Stuart et al. 1993; Evans 1996; Sundström 1997; Mori and Le Moli 1998; Hora et al. 2005; Souza et al. 2005; Vásquez and Silverman 2008). Moreover, most of the studies on secondary polygyny were short-term and overlooked the possibility that queens might be rejected at a later stage of the introduction. Most studies focused on workers' ability to discriminate between reproductive females depending on their nest of origin (nestmate or non-nestmate) and on their mating status, with varying, species specific, results. For example, no discrimination occurred between mated and virgin females neither in *Formica podzolica* (Bennett 1988) nor in *F. lugubris* (Fortelius et al. 1993), but in *F. truncorum* mated females were discriminated against at greater rates than virgin females when co-introduced into the colony (Sundström 1997). Moreover, monogynous and polygynous colonies of *F. truncorum* showed differences in the propensity of adopting queens, which was proposed to be associated with differences in dispersal strategies found in each population (Sundström 1997). Queen adoption was also studied in two facultative polygynous ant species, *Myrmica tahoensis* (Evans 1996) and *Ectatomma tuberculatum* (Hora et al. 2005), colonies of which coexist as monogynous or polygynous in the same population, the latter likely resulting from queen adoptions (*i.e.* secondary polygyny). In both above-mentioned studies, alien mated queens were introduced into monogynous colony and the queens' behavior was monitored for a short time post-introduction (15–19 and 8 days, respectively). Although adoptions were considered as successful, in both species the resident queens behaved differently than the introduced queens in showing greater brood attendance. Queen adoption was also reported in the slave-making ant *Formica sanguinea* (Mori and Le Moli 1998) and in the monogynous leaf-cutting ant *Acromyrmex subterraneus molestans* (Souza et al. 2005). Recently, Vásquez and Silverman (2008) showed that queen acceptance in the invasive Argentine ant *Linepithema humile* depends on resident queen number and not adopted queen fecundity.

Queen discrimination leading to adoption or rejection probably involves the use of olfactory cues. While the usurpation and subsequent adoption of queens of parasitic ants was demonstrated to rely on chemical cues and chemical deception (Allies et al. 1986; Topoff et al. 1988; Lenoir et al. 1997), no chemical data exists, to our knowledge, pertaining to the adoptions of conspecific queens in the context of secondary polygyny. One factor that might intervene in queen adoption is colony insularity, *e.g.* acceptance or rejection of queen based on similarity of recognition cues (Vander Meer and Morel 1998; Lenoir et al. 1999). Alternatively, queens might be recognized as such in having caste specific odors, in addition to colonial identity. These separately, or in concert may affect the process of adoption. Finally, the resident queens may acquire the colony odor through mechanisms that results in Gestalt colony odor (Crozier and Dix 1979; Soroker et al. 1994; Lenoir et al. 2001). This is supported by findings in *M. tahoensis* in which workers groomed adopted queens at higher rates after adoption compared to that occurring in naturally polygynous colonies (Evans 1996).

Here, we studied some of the behavioral and chemical correlates of queen adoption in the facultative polygynous ant *Ectatomma tuberculatum*. In this species, colony and population genetic study showed that limited female dispersal and polydomy are associated with genetic viscosity (Zinck et al. 2007). This suggests that females seeking adoption are likely to be related to resident queens and it may explain secondary polygyny evolution in this species. Since cuticular hydrocarbons were demonstrated as serving both as nestmate and queen recognition cues (reviewed in Monnin 2006; Le Conte and Hefetz 2008) we focused our chemical analyses on these compounds. We determined whether adopted queens were behaviorally integrated over long term (*i.e.* after 2 months), and we studied the dynamic of cuticular hydrocarbon profiles of both the resident and adopted queens, and that of host colony workers during adoption. We also discuss the ultimate causes of queen adoption in connection with colony insularity and nestmate recognition in *E. tuberculatum.*

Materials and Methods

Colony Collection and Ant Maintenance

Ten polygynous and ten monogynous colonies of *E. tuberculatum* were collected in Buerarema and Itabuna, Bahia state in Brazil. Given that *E. tuberculatum* is polydomous we ascertained that the adopted and resident queens were neither nestmates nor related queens by selecting colony-pairs that were at least 50 m apart (adoptions 1–6 from Itabuna; Table 1) (Zinck et al. 2007). The colonies used for adoptions 7 to 10, which were also used for chemical analyses, corresponded to cross-adoptions between two different populations (*i.e.* Buerarema and Itabuna) located 17 Km apart (Table 1). Colonies were maintained in the laboratory in artificial nests made of plaster-of-Paris connected to an outside arena at 28±2°C and 70±2 % RH. They were fed twice a week with frozen crickets and honey. The experiments were started after an acclimatization period of 2–3 months to these laboratory conditions.

Behavioral Assay

Monogynous host colonies containing the queen (resident queen, Qr), individually marked workers, and brood were placed in new nests for 1 week for acclimation. In order to standardize colony size among the experiments we reduced the number of workers per colony to 64 (monogynous colonies of *E. tuberculatum* generally contain around 200 workers (Hora et al. 2005)). Before introducing an alien queen into these monogynous host colonies, the behavior of the resident queen was scored during 5 days by performing ten scan-samplings per day for each nest ($n=500$).

Table 1 Summary of Experimental Queen Adoptions and the Origin of Queen Colonies Used for Adoptions

	Monogynous colony (resident queen)	Polygynous colony (adopted queen)
Adoption 1–6	Itabuna	Itabuna
Adoption 7[a,b]	Buerarema	Itabuna
Adoption 8[a,b]	Buerarema	Itabuna
Adoption 9[a]	Itabuna	Buerarema
Adoption 10[a]	Itabuna	Buerarema

[a] indicate adoption experiments used for chemical analysis

[b] indicate adoption experiments that failed

Behavioral items consisted of queen's oviposition, brood attendance by the queens, and queen grooming by workers (Hora et al. 2005). Prior to the alien queen introduction (adopted queen, Qa) we removed the brood of the resident queen, so that all the brood at the end of the experiment (*i.e.* 2 months later) corresponded to eggs laid after the adoption. Alien queens used for the adoption experiments were mature mated queens (recognized by their matte cuticle (Hora et al. 2008)) that came from polygynous colonies. Queen adoptions were performed as described in Hora et al. (2005) by introducing the alien queen into the exterior arena of the host colony. Behavioral observations were conducted during the first and eighth weeks post-introduction by scan sampling as described above.

Chemical Analysis

Chemical characterization of queen and worker cuticular hydrocarbons was done both using solvent extraction and solid phase micro extraction (SPME) using a polydimethylsiloxane, 7 μm bonded fiber (Arthur and Pawliszyn 1990). Initial chemical analyses of queen and worker profiles were performed by combined gas chromatography mass spectroscopy (GC/MS) (Perkin Elmer, at the EI mode with turbo mass electron energy of 70 eV). Compounds identification was deduced from their respective fragmentation patterns. Further characterization of cuticular hydrocarbons was done by GC (Varian 3900) equipped with a 30-m-long DB-5 fused silica capillary column and flame ionization detection. The fiber was desorbed into the column for 5 min (injector and column temperatures set at 280°C and 100°C, respectively; Helium was used as carrier gas at a flow rate of 28.57 cm/s). Thereafter, the column was temperature programmed from 100–250°C at 20°C/min and then to 320°C at 3°C/min with a final hold of 5 min. Peak integration was done with Varian system control (version 6.20). Sampling of resident and introduced queens as well as five randomly selected workers of each colony was done by SPME prior to introduction and 1 and 8 weeks post-introduction. Fiber loading was achieved by rubbing it for 10 min against the last intersegmental membranes of the gaster. Replicates of queen profiles were achieved by repeated sampling ($n=5$) at approximately 45 min intervals between sampling. For workers, replicates consisted of profiles that were obtained by sampling once each of five workers.

Statistical Analyses

Queens' behaviors were compared by using nonparametric tests (StatXact 3.1). Oviposition and brood attendance were compared by paired permutation tests using the queen (adopted *versus* resident) and the week (week 1 *versus* week 8) as variables. Statistical analysis of the number of grooming received by resident *versus* adopted queens was also done by paired permutation tests. For all the permutation based analyses, the units permuted between classes were single observations.

For comparing between cuticular hydrocarbon (CHC) compositions of the various queens and workers we first subjected the quantifiable CHC (marked with an asterisk in the legend of Fig. 2) to a Principal Component Analyses (PCA), to reduce the number of variables (CHC) in the subsequent Discriminant Analysis (DA) (Heinze et al. 2002). The first five factors of the PCA that explained a high proportion of the variance (92.32% and 93.38% for the experimental adoption 9 and 10, respectively) were used to perform standard DA. To investigate chemical differences between adopted and resident queens' profiles, we compared the mean squared Mahalanobis distance (MSMD) between each replicate of the hydrocarbon profile of adopted queens and the centroid of the corresponding resident queens for each adoption experiment. Similarly, to study the chemical differences between workers and adopted or resident queens, we compare the MSMD between workers and queens in each colony, prior queen adoption. Kruskall-Wallis and permutation tests (StatXact 3.1) were then conducted to make comparison between these Mahalanobis distances.

Results

Behavioral Observation of Queen Adoption

Eight out of the ten introduced queens were adopted by the host colonies and two queens were rejected. In the eight successful adoptions, neither the resident workers nor the resident queen were aggressive toward the adopted queen. There were no significant differences in queen's brood attendance at any time of the experiment between the adopted and resident queens (Paired permutation tests: $p=0.297$ and $p=0.109$ for weeks 1 and 8 post-introduction, respectively) (Table 2). Although the resident queens tended to lay more eggs than the adopted queens 8 weeks after adoption (Table 2), no significant difference was found in oviposition between them neither 1 week nor 8 weeks post-introduction (Paired permutation tests: $p=0.313$ and $p=0.063$, respectively). Figure 1 depicts worker behavior towards the resident or introduced queens. The number of grooming events towards the adopted queens was significantly higher than that towards the resident queens during the first week after adoption (Permutation test: $p=0.043$) but both equally subsided in the 8th week post-adoption and were not significantly different from each other (Permutation test: $p=0.866$) (Fig. 1).

The two adoptions that failed (Adoption 7 and 8, Table 1) ended in the death of the newly introduced queens. In these two cases, the workers initially transported the introduced queen from the foraging area into the nest like the queens in the successful introductions, but afterwards they continuously assaulted the newly

Table 2 Mean Occurrence of Oviposition and Brood attendance by Resident (Qr) and Adopted (Qa) Queens in Successful Adoptions ($n=8$) Before the Adoption, 1 week After and 8 weeks After the Adoption

Behavior occurrence	Before adoption	1 week after adoption		8 weeks after adoption	
Queen	Qr	Qr	Qa	Qr	Qa
Oviposition	2.6±2.6	1.6±0.7	2.9±2.2	3.9±2.1	2±0.8
Brood attendance	31.4±7.9	26±8.6	29.4±9.7	41.4±5.7	45.9±2.8

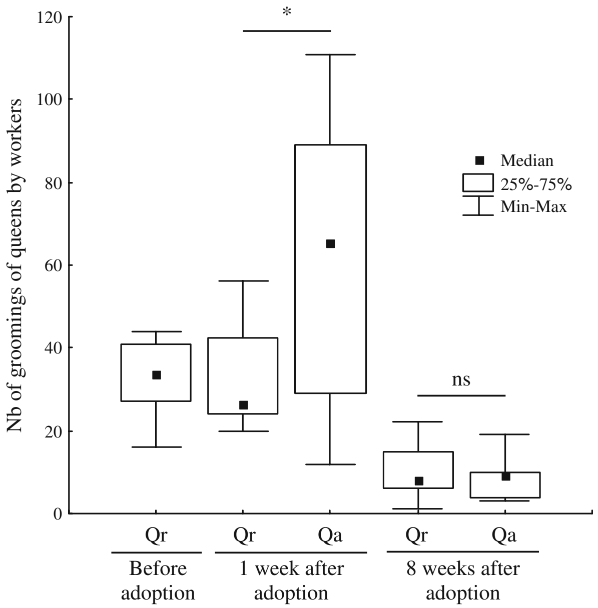

Fig. 1 Grooming by *E. tuberculatum* workers of resident (Qr) and adopted (Qa) queens before the adoption, and 1 week and 8 weeks post-adoption. Levels of significance are indicated by * if $p<0.05$, ** if $p<0.01$, *** if $p<0.001$ and ns if they were not significant.

introduced queen, culminating in her death within the 24 h. The resident queens did not participate in these aggressions, but behaved normally.

Chemical Investigation of Queen Adoption

Figure 2 presents the cuticular hydrocarbon profiles of queens and workers. Queens profile was identical to that previously described (Hora et al. 2008), and the ratio of heptacosane to nonacosane indicates that the queen presented was mated. The profile of workers is drastically different from that of queens. The major mated-queen compound, heptadecane, occurs only as traces in workers. Also the amount of nonacosane, the second major queen component is greatly reduced in workers. On the other hand, the major worker peak is a mixture of 11-; 13-; and 15-methylnonacosane, and the second largest component is a similar mixture of methylhentriacontane. Two other pronounced worker components are 12,14 dimethyloctacosane and hentriacontene (double bond position undetermined). To test the hypothesis that queen adoption is accompanied by chemical changes in cuticular hydrocarbon composition, we compared the profiles of adopted queens, resident queens and host workers (Fig. 3). As expected from the qualitative chemical differences between queens and workers, these were completely separated in the discriminant analysis, irrespective of colonial origin. However, neither the profiles of queens nor those of workers appeared to change significantly following an alien queen introduction. This refutes the hypothesis that queen adoption is accompanied by convergence of the adopted queen profile with that of the queen or workers from the host colony.

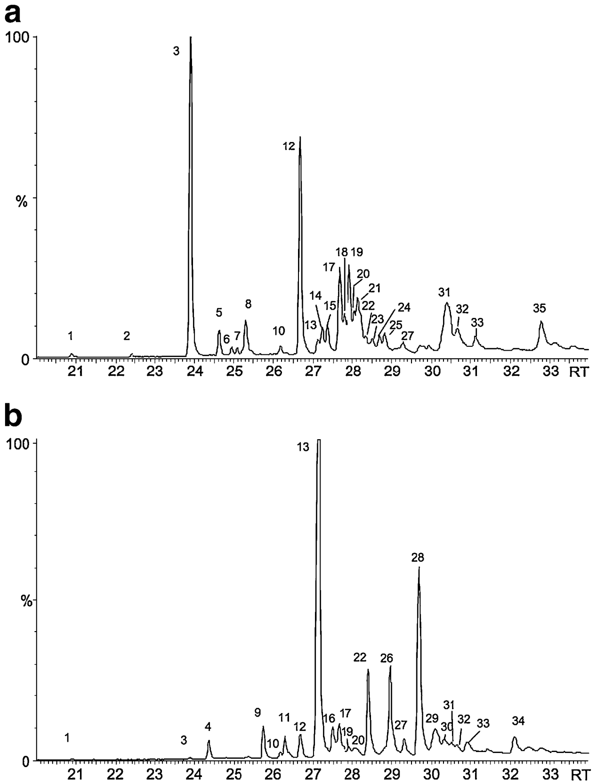

Fig. 2 Chromatograms of *E. tuberculatum* worker (**a**) and queen (**b**). Peak numbers correspond to the following compounds. The compounds marked with an asterisk were utilized for the PCA. 1) Pentacosane; 2) Hexacosane*; 3) Heptacosane*; 4) 11-+13-Methylheptacosane; 5) 5-Methylheptacosane*; 6) 3-Methylheptacosane*; 7) 5,9,-Dimethylheptacosane; 8) Octacosane*; 9) 11,13,15-Trimethylheptacosane; 10) 2-Methyloctacosane*; 11) Nonacosene; 12) Nonacosane*; 13) 11+13+15-Methylnonacosane*; 14) 7-Methylnonacosane*; 15) 5-Methylnonacosane*; 16) 11,15+13,15-Dimethylnonacosane; 17) 3-Methylnonacosane*; 18) 7,9 +7,11-Dimethylnonacosane; 19) 5,23-Dimethylnoacosane*; 20) 5,11-Dimethylnoacosane; 21) 8-Methyltriacontane*; 22) 12,14 Dimethyltriacontane*; 23) 8,12-Dimethyltriacontane; 24) 7,17,23-Trimethylnonacosane; 25) 5,15,23-Trimethylnonacosane; 26) Hentriacotene; 27) 15 +17-Methylhentriacontene; 28) 11-+13-Methylhentriacontane*; 29) 11,15 +13,15-Dimethylhentriacontane; 30) 5,7-Dimethylhentriacontene; 31) 7,11,13-Trimethylhentriacontane; 32) 5,11,13-Trimethylhentriacontane; 33) 12 +14+16-Methyldotriacontane; 34) 11 +13 +15 +17-Methyltritriacontane; 35)?

We also wished to examine whether disparity of profiles between the introduced and resident queens is at the basis of acceptance or rejection of the introduced queens. To that effect we compared the mean squared Mahalanobis distances (MSMD) between pairs of adopted and resident queens in each experiment. Significant differences were found among all adoption experiments (MSMD±SE=15.8±7.5, Adoption 7; 175.4±25.0, Adoption 8; 124.5±21.6, Adoption 9; and 12.3±5.8, Adoption 10) (Kruskall-Wallis test: H=32.12, N=40, p<0.001), but no significant difference were found between mean squared Mahalanobis distances in failed adoptions (MSMD±SE=95.6±81.9, Adoption 7 and 8) and in successful adoptions (MSMD±SE=68.4±58.3, Adoption 9 and 10) (Permutation test: p=0.421). Cuticular hydrocarbon profiles of adopted queens

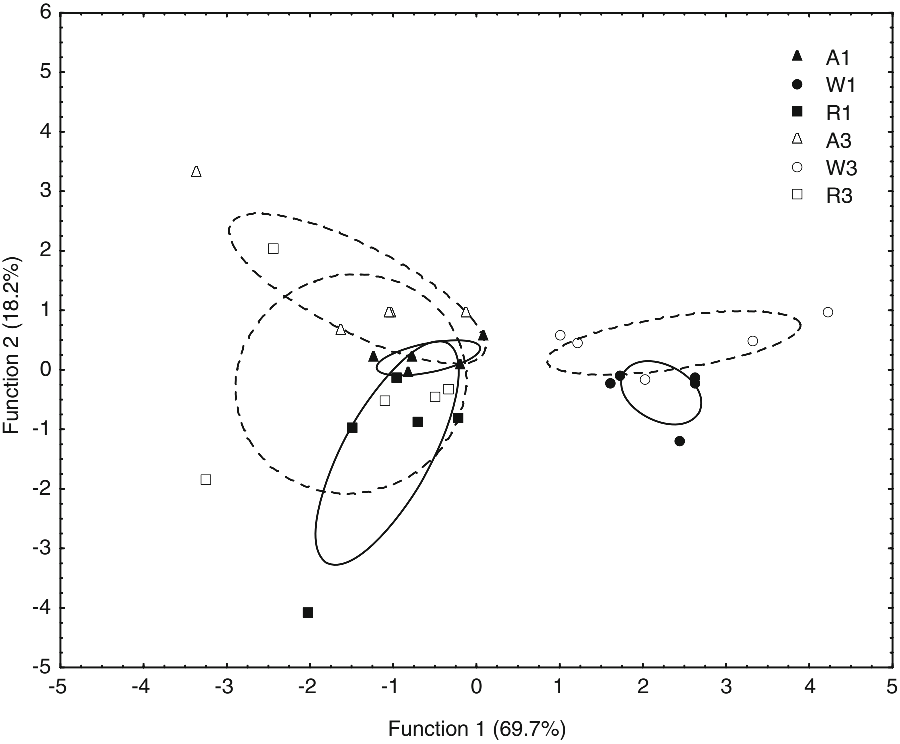

Fig. 3 Discriminant analysis of the cuticular hydrocarbon profiles of the workers (W), the resident (R) and the adopted (A) queens, before adoption (W1, R1 and A1), and 8 weeks after adoption (W3, R3 and A3) in the experimental adoption 9. Ellipses represent 95% confidence limits. The percentages of variance explained by each of the two discriminant functions are provided in parenthesis on the axis labels.

were thus not found to be more similar to those of resident queens in successful adoption experiments, compared to those of rejected and resident queens in failed adoption experiments. Moreover, the mean squared Mahalanobis distance between workers and adopted queens was found to be significantly less important than between workers and resident queens among all adoption experiments (MSMD± SE=34.3±21.8, Workers - Qa; 63.7±48.3 Workers - Qr) (Permutation test: $p=0.002$). However this result was only due to chemical differences between workers and queens in a single experimental adoption (Table 3, Adoption 8). Cuticular hydrocarbon profiles of workers were thus not found to be more similar to those of resident queens than adopted queens.

Discussion

Our results revealed that long-term queen adoptions can occur in *Ectatomma tuberculatum* resulting in functional polygyny, which is consistent with the existence of secondary polygyny in this species (Hora et al. 2005; Zinck et al. 2007). While queen adoption was already demonstrated in *E. tuberculatum* (Hora et al. 2005), this

Table 3 Mean Squared Mahalanobis Distance (MSMD±SE) Between Workers and Adopted (Qa) or Resident (Qr) Queens for Each Experimental Adoption, Before The Adoption

Experimental adoption	MSMD±SE		Permutation test
	Workers - Qa	Workers - Qr	
Adoption 7[a]	19.1±9.0	23.1±9.6	ns
Adoption 8[a]	30.3±9.4	133.2±25.3	0.008
Adoption 9	65.6±15.1	75.0±15.6	ns
Adoption 10	22.3±11.8	23.4±11.2	ns

[a] indicate adoption experiments that failed

ns means not significant

was short-term experiments (8 days only), which cannot exclude the possibility of rejection at a later stage. For example, in *Formica sanguinea* out of ten queens introduced, five were rejected immediately, but two more were killed 2 weeks after adoption, and only three lasted long enough to be considered as successful adoptions (Mori and Le Moli 1998). In the study presented here, both resident and adopted *E. tuberculatum* queens behaved similarly even 2 months after adoption. This study thus shows that short-term experimental queen adoptions can be performed to accurately study secondary polygyny phenomenon, at least in *E. tuberculatum*. Moreover, similar egg-laying rates of resident and adopted queens indicate that they equally participated in reproduction. Furthermore equal egg-laying rates likely leads to equal worker production since oophagy (*i.e.* egg eating) by queens or workers does not exist in polygynous colonies from this population (Hora et al. 2005) and workers do not show nepotistic queen care behavior (Zinck et al. 2009).

However, worker behavior towards the adopted queen differed from that towards the resident queen. There was significantly greater grooming of the adopted compared to the resident queens during the first week of adoption. But this subsided in the eighth week post-adoption. Similar worker propensity to groom newly introduced queens was also found in the facultative polygynous ant *Myrmica tahoensis* (Evans 1996). The possibility that workers may have attempted to homogenize colony odor after the introduction of a new queen in the colony (Crozier and Dix 1979) was refuted by the chemical analyses. We attribute the increased interest in the adopted queen to the novelty of its odor, and the decline in interest to odor familiarization. Alternatively workers may have been attracted to the queen specific substances as was shown for honeybees (Slessor et al. 1988; Katzav-Gozansky et al. 2001), and the fire ant *Solenopsis invicta* (Vander Meer and Alonso 2002).

Queen chemical specificity is also supported by the chemical analyses. Workers lacked completely heptacosane and had only minor amounts of nonacosane, the queen's major components. The discriminant analyses also revealed that worker and queen clustering was according to caste not colony origin. Furthermore similar chemical distances between workers and adopted or resident queens (in 3 over 4 experimental adoptions) also suggest the existence of a caste signature. Caste-specific cuticular hydrocarbon profiles are rather common among social insects, and in many species they are correlated with ovarian activity supporting their postulated role as fertility signals (reviewed in Monnin 2006; Hefetz 2007; Le Conte and Hefetz 2008). In *E. tuberculatum* mated and virgin queens show significant differences in proportions of heptacosane and nonacosane that could allow worker assessment of queen mated status (Hora et al. 2008). Our results on the stability of adopted queen cuticular hydrocarbon profiles over time, in spite of numerous worker grooming, therefore suggest that queen-specific cuticular hydrocarbon profile may correspond to a reliable signal of queen's fertility in *E. tuberculatum*.

Dyadic aggression tests and field experiments had revealed that *E. tuberculatum* workers are characterized by an open recognition system (Zinck et al. 2008). Such low insularity may explain the high rate of success (80%) of long-term adoptions of alien completely unrelated queens (*i.e.* non-nestmate and unrelated). However as a result of polydomy and limited queen dispersal (Zinck et al. 2007) *E. tuberculatum* queens seeking adoption are likely to be related to the workers making the decision to accept them or not in the colony. Workers may thus commonly accept any alien queen presenting a signal of fertility, in order to avoid costly rejection errors (Reeve 1989). Moreover, even if adoption is of an unrelated female, the decrease in within-nest relatedness may be outweighed by the benefits of increased colony development (Rosengren and Pamilo 1983; Rissing et al. 1989). Indeed the presence of multiple queens per colony allows it to reach larger colony size (*e.g.* Hora et al. 2005), which can increase in turn colony productivity (Herbers 1984; Walin et al. 2001) especially as genetic diversity among matrilines can increase division of labor efficiency (Carlin et al. 1993; Blatrix et al. 2000). In *E. tuberculatum*, Hora et al (2005) showed that polygynous colonies contain more workers than monogynous colonies and that queen number is correlated with the number of worker. However, in polygynous colonies, the authors did not find any correlation between queen number and colony size, which suggests that queen adoption may not increase per capita productivity in polygynous colonies. As a consequence, productivity benefits of polygyny would not explain the acceptance of unrelated alien queens in some colonies of *E. tuberculatum*, but one should keep in mind that in nature, queens seeking adoption are expected to be related to some extent to the workers and resident queens (discussed above). Finally, as it was postulated in *F. truncorum* by Sundström (1997) queen adoptions, independently of the level of relatedness, likely enhances the ability of the colony to reproduce by budding and thus to monopolize the entire habitat. It is therefore possible that queen adoptions in *E. tuberculatum* may have been favored because of its effects on maintaining ecological dominance (Zinck et al. 2007).

Our results show that high queen acceptance threshold in *E. tuberculatum* colonies generally lead to multiple-queen colonies and increased colony size (Hora et al. 2005). High genetic viscosity and particular constraints of the mosaic of arboreal ants that characterize Neotropical habitats likely favored secondary polygyny in *E. tuberculatum*, facilitating ecological dominance (Zinck et al. 2007). Here we propose that caste-specific cuticular hydrocarbon profiles which could correspond to reliable signal of queen fertility may favor queen adoptions, while colony-specific cuticular hydrocarbon profiles could rather result in higher rejection rate and lower queen acceptance. As a consequence natural selection may have selected for caste and fertility signaling rather than colony signature due to their respective effects on queen adoptions and the consequences on *E. tuberculatum* ecological dominance.

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