

Hierarchical perception of fertility signals and nestmate recognition cues in two dolichoderine ants

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Received: 26 February 2009 / Revised: 14 April 2009 / Accepted: 14 April 2009 / Published online: 9 May 2009
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Abstract In social insects, queens are likely to “honestly” inform their nestmates of their presence and fertility status through pheromonal communication. Cuticular hydrocarbons (CHCs) have been reported to be effective nestmate discriminators and strongly suspected to act as fertility signals, at least in some species. The use of the same chemical bouquet (i.e., the CHC profile) to convey two fundamentally different information seems puzzling. However, a recent threshold model proposes a hierarchy in the discriminating processes, i.e., fertility signals can only be perceived if nestmate recognition has been reached (Le Conte and Hefetz, *Annu Rev Entomol* 53:523–542, 2008). Here, we developed a simple behavioral bioassay based on chemical recruitment toward a queen placed outside the nest in two dolichoderine ants (*Linepithema humile* and *Tapinoma erraticum*), which allowed us to investigate the interplay between fertility signaling and colonial recognition. Using queen corpses of various origins (nestmates or aliens) and physiological states (fertile or infertile; mated or unmated), we demonstrated that nestmate recognition cues clearly override fertility signals under our experimental conditions. Indeed, while nestmate infertile queens were largely ignored by the workers, nestmate fertile queens (mated or not) induced worker recruitment, whereas alien fertile queens did not and were aggressed by the workers.

Keywords Queen pheromone · Fertility · Nestmate recognition · Virgin egg-laying queens · Queen retrieval

Introduction

Organization of the insect societies mostly relies on chemical communication among nestmates (Wilson 1971). Especially, regulation of reproduction in ants is almost completely mediated by the use of pheromones originating from the breeders that can affect both the behavior (releaser effect; Vander Meer and Alonso 1998) and the physiology (primer effect; Le Conte and Hefetz 2008) of nestmates. Indeed, it has been demonstrated that queen primer pheromones are involved, for instance, in the inhibition of nestmates reproduction (e.g., Fletcher and Blum 1983; Vargo and Passera 1991; Dietemann et al. 2005) or in the regulation of sexual production (e.g., Vargo and Passera 1991; Boulay et al. 2007). Releaser pheromones, on the other hand, are usually involved in queen recognition processes (attraction/aggregation, see Vander Meer and Alonso 1998) which allow workers to preferentially take care of the most fertile queens (e.g., Vander Meer et al. 1980; Cariou-Etienne and Passera 1993; Sommer and Hölldobler 1995; Ortiús and Heinze 1999; Hannonen et al. 2002).

Despite the apparent difference in their mode of action, these two pheromonal effects are often interconnected since primer pheromones may also affect behavior (Le Conte and Hefetz 2008). For example, it is argued that queen pheromones can be perceived as “honest” fertility signals that are used by the workers to orient their behavior or/and physiology. According to Keller and Nonacs (1993), breeders are more likely to “honestly” inform for their fertility level than manipulating workers into helping them. This means that workers are able to accurately assess the fertility of their queens and react accordingly by feeding and grooming them and by preventing their own (i.e., self-policing) and other workers reproduction (i.e., worker policing). Consistently, numerous studies point out the role of queen fertility in the regulation of worker reproduction (reviewed in Vargo 1998; Le Conte and Hefetz 2008).

Communicated by J. Heinze

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In the last decades, attention has been paid to cuticular hydrocarbons (CHCs) as nestmate discriminators and putative queen pheromones (Howard and Blomquist 2005; Monnin 2006; Hefetz 2007). The use of CHCs as both colony specific cues and fertility signals raises questions about how different cuticular profiles can be maintained in a same nest despite the odor homogenization predicted by the *gestalt* model (Crozier and Dix 1979), broadly accepted to explain colonial odor specificity in ants (Lenoir et al. 1999). The question is particularly relevant when the differences between fertile and infertile individuals concern a large number of CHCs in the cuticular profile, as it is the case in *Linepithema humile* (de Biseau et al. 2004). Another difficulty is to explain why the fertile individuals bearing CHCs that are absent from the worker profiles are not identified as non nestmates. Le Conte and Hefetz (2008) suggest that either the CHCs related to fertility do not serve as recognition cues or that the fertile individuals are recognized by other signals. They also propose a third hypothesis based on perception thresholds. This model implies a hierarchy in the discriminating processes, e.g., the fertility signal may only be perceived if nestmate recognition is reached.

In this study, we developed a behavioral bioassay to easily test the existence of a fertility queen releaser pheromone in two dolichoderine ant species. *L. humile* (the Argentine ant) and *Tapinoma erraticum* (the erratic ant) are both polygynous and mass-recruiting (Beckers et al. 1989) species, using chemical trails to recruit their nestmates toward food sources or new nesting sites. A previous work has shown that workers of *L. humile* are also able to recruit toward queens located outside the nest, allowing them to return to the nest by following the chemical trail laid by the workers (Aron 1992). This queen retrieval behavior, although poorly investigated in ants (Glancey et al. 1983; Aron 1992), allows to measure in a clear *all-or-nothing* way the response of workers toward any individual discovered outside the nest: They recruit or they do not. We therefore developed a simple bioassay based on this queen retrieval behavior and the observation of worker aggressiveness in order to evaluate the ability of workers to recognize individuals according to their fertility and colonial identity.

Materials and methods

Ant colonies

L. humile

Large numbers of *L. humile* ants were collected at Port Leucate (France) and at Sant Cugat del Vallès, near

Barcelona (Spain); these two populations belonging to different supercolonies (Giraud et al. 2002). The ants were grouped in stock colonies (hundreds of queens and thousands of workers) and maintained under standard laboratory conditions (Passera et al. 1988a). In order to obtain alate females, some colonies from the French population were dequeened since only queenless colonies of *L. humile* are able to produce sexuals (Passera et al. 1988b). Queen pupae were removed from these colonies and reared in small units of 200–300 tending workers to obtain virgin queens. Some of these virgin queens became egg layers after several weeks (Passera and Aron 1993).

We set up 25 artificial nests, each housing five mature queens and 1.5 cm³ of workers (ca. 800–1000 ants), from the stock colonies of Port Leucate. Each of these nests was then placed in a transparent plastic box (27×26×8.5 cm) and was regularly fed with sugar water and pieces of dead maggots. Despite originating from the same supercolony, these nests were treated as independent nests for statistical analysis.

T. erraticum

Ten colonies of *T. erraticum* were collected in the vicinities of Vireux-Molhain (French Ardennes) and ten in the vicinities of Tours (France). Some colonies ($N=15$) were collected during swarming period so that sexuals were also present. Alate females were removed from these nests and reared in small units with a hundred of tending workers. After several weeks (10–30 days), some of these gynes started laying eggs (Meudec 1979).

We set up 30 artificial nests, each housing two mature queens and about 700–800 workers from the same collected colony (one to three artificial nests were set up from each colony according to its size). The nests were placed in the same conditions as for *L. humile* (see above). For statistical analysis, all these nests were considered as independent units. Remaining queens were used to study queen retrieval and were housed with the remaining nestmate workers.

Study of queen retrieval

Experimental design

The experimental units were set up in order to test the ability of queens at various physiological states to induce a recruitment trail. The transparent plastic box containing the artificial nest was placed on a circle (8.5 cm diameter) divided into 12 30° sectors drawn on a blank paper sheet (see Fig. 1). A queen was killed by freezing (−20°C) for 10 min and allowed to thaw for another 10 min. The corpse was then introduced in the experimental unit in the center of the circle (at 10–11 cm from the nest entrance). Activity in the plastic box was video recorded 15 min before and

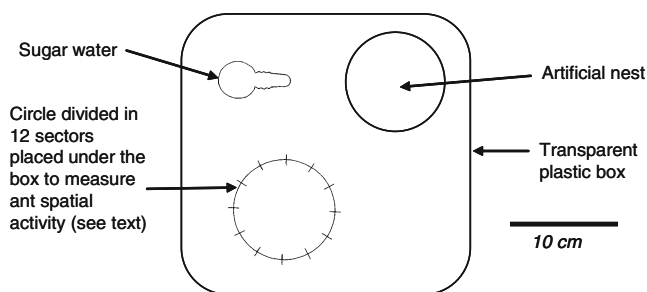


Fig. 1 Experimental design containing artificial nest

15 min after the introduction of the corpse. The eliciting of a directional recruitment between the nest and the corpse was evaluated by counting the number of workers crossing each of the 12 arcs of the circle during 15-min periods.

Categories of queens tested

The following categories of queens were used in each of our experiments:

- Nestmate young virgin nonegg-laying queens, less than 1 week after emergence (their wings were gently cut off to mimic the morphology of dealated queens)
- Nestmate virgin egg-laying queens
- Nestmate mated egg-laying mature queens
- Nestmate mated egg-laying mature queens previously rinsed with ca. 3 mL of hexane for 10 min and allowed to dry for ten more minutes
- Alien mated egg-laying mature queens (queens from a different supercolony in *L. humile* and from a different nest in *T. erraticum*)

In *L. humile*, we also used nestmate virgin nonegg-laying queens at the time of dealation. Indeed, virgin queens of this species always remove their wings after several weeks (Passera and Aron 1993). This was not observed in *T. erraticum* in which old virgin queens can still be alate after months (Meudec 1979; Cournault, personal observation).

Mating and egg-laying status of queens

To check for their fertility, queens were placed alone in a glass tube half-filled with water for 24 h. They were considered as egg layers if they had laid at least one egg within this period. Moreover, after the experiments, all queens were dissected in order to verify their mating and their fertility status. Dissections confirmed that nestmate virgin egg-laying queens were unmated and that mated egg-laying mature queens had their spermatheca full of sperm. They also revealed that egg-laying queens (mated or not) had developed ovaries with signs of former egg-laying activity (i.e., yellow bodies).

Aggressiveness toward queen corpses

Behavior of workers toward queen corpses was noted. We considered that workers attacked a queen corpse if they bit it and pulled its legs and antennae off.

Results

In all cases, the total number of ants crossing the circle was significantly different after than before the introduction of the queen corpse ($P < 0.0001$ for each of the 11 paired t test, t range = 5.22–10.55, $df = 24$). However, in both *L. humile* and *T. erraticum*, this increase of activity (ratio N_a/N_b of the total numbers of ants crossing the circle perimeter after (N_a) and before (N_b) the introduction of the queen corpse) was much higher using egg-laying queens, mated or not, than in all other experimental conditions (Fig. 2).

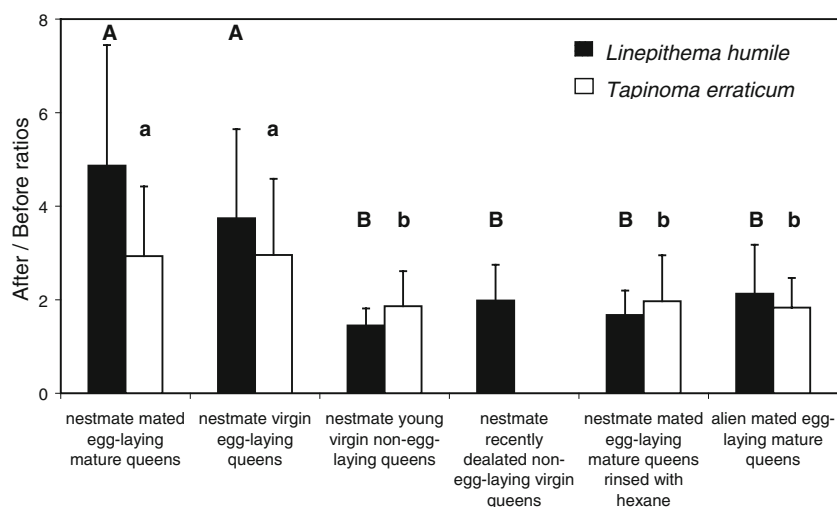
In the control experiments (absence of queen corpse), no recruitment trail (revealed by an unambiguous column of workers walking between the nest and the queen corpse) was observed between the center of the circle and the nest. Moreover, the maximum number of ants crossing one of the 12 sectors never exceeded 15% of the cumulated number of ants crossing the entire circle. By contrast, less than 5 min after the introduction of a nestmate mated egg-laying mature queen, a trail between the corpse and the nest was always observed (except for one replicate in *L. humile*). The sector crossed by the column of workers always drove more than 15% of the cumulated number of ants crossing the circle (Table 1; Fig. 3). Hence, we therefore considered that a queen corpse successfully induced a recruitment if it elicited the formation of a clearly defined column of ants that drove more than 15% of the total number of ants crossing the circle during the 15 min following the introduction of the corpse.

Young nestmate virgin nonegg-laying queens that were artificially dealated and, in *L. humile*, nestmate virgin nonegg-laying queens at the time of natural dealation never induced a recruitment. By contrast, workers that discovered a nestmate virgin egg-laying queen outside the nest elicited a recruitment in 92% and 84% of the replicates in *L. humile* and *T. erraticum*, respectively (Table 1). The traffic observed was similar to that measured with mated egg-laying mature queens (Fig. 2).

The frequency of recruitment toward nestmate mature egg-laying queens previously rinsed in hexane was drastically reduced to only 20% and 12% in *Linepithema* and *Tapinoma*, respectively (Table 1).

Finally, no recruitment occurred after the introduction of an alien mated egg-laying queen (Table 1). Moreover, these nonnestmate queens, despite their fertility, were always (25

Fig. 2 Ratios of the total numbers of ants crossing the circle perimeter 15 min after and 15 min before the introduction of a queen corpse in the center of the circle placed under the foraging area (see text). Same letters (*capitalized for L. humile*, *small for T. erraticum*) indicate no significant differences in a Dunn's multiple comparison test. Number of replicates is 25 for each of the 11 experiments; nestmate recently dealated nonegg-laying virgin queens were not tested in *T. erraticum* (see text)



out of 25 for each species) aggressed by the workers (Table 1).

In some experiments, workers successfully displaced the queen corpse outside the circle. These replicates were discarded from the results reported above but we noted where the queen was located (i.e., inside the nest or elsewhere in the plastic box) at the end of each experiment. In both species, there is a significant difference ($P < 0.001$ for each species, Fisher's exact test, data pooled) between the treatment of nestmate fertile queens, mated or not, and infertile or washed queens by the workers. The first ones were generally found inside the nest (*L. humile*—18 cases out of 23; *T. erraticum*—21 cases out of 29) whereas the latter ones were mostly located outside

the nest (*L. humile*—18 cases out of 20; *T. erraticum*—19 cases out of 24). Alien queens were never displaced outside the circle within the period of the experiment ($N = 25$ for each species).

Discussion

Despite the use of pseudo-independent groups, our results are unambiguous and confirm that workers of *L. humile* (from the main European supercolony) actively recruit nestmates toward one of their mature queens discovered outside their nest (Aron 1992) and show that this stereotyped collective behavior is also observed in another dolichoderine ant

Table 1 Frequency of recruitments toward queens at different physiological status

	<i>L. humile</i>			<i>T. erraticum</i>		
	% trail	% activity in MFS	% Agg.	% trail	% activity in MFS	% Agg.
No queen (control)	0 (0/125)	10.66±0.80 ^a (125)	—	0 (0/100)	11.77±1.18 ^a (100)	—
Nestmate mated egg-laying mature queens	96 (24/25)	25.04±6.16 ^b (24)	0 (0/25)	100 (25/25)	28.62±7.93 ^b (25)	0 (0/25)
Nestmate virgin egg-laying queens	92 (23/25)	24.28±6.16 ^b (23)	0 (0/25)	84 (21/25)	25.03±5.63 ^b (21)	0 (0/25)
Nestmate young virgin nonegg-laying, queens	0 (0/25)	10.88±0.94 ^a (25)	0 (0/25)	0 (0/25)	11.05±1.11 ^a (25)	0 (0/25)
Nestmate recently dealated nonegg-laying virgin queens	0 (0/25)	10.54±0.75 ^a (25)	0 (0/25)	n.a	n.a	0 (0/25)
Nestmate mated egg-laying mature queens rinsed with hexane	20 (5/25)	11.17±1.00 ^a (20)	0 (0/25)	12 (3/25)	11.48±1.42 ^a (22)	0 (0/25)
Alien mated egg-laying mature queens	0 (0/25)	10.68±1.05 ^a (25)	100 (25/25)	0 (0/25)	11.38±1.05 ^a (25)	100 (25/25)

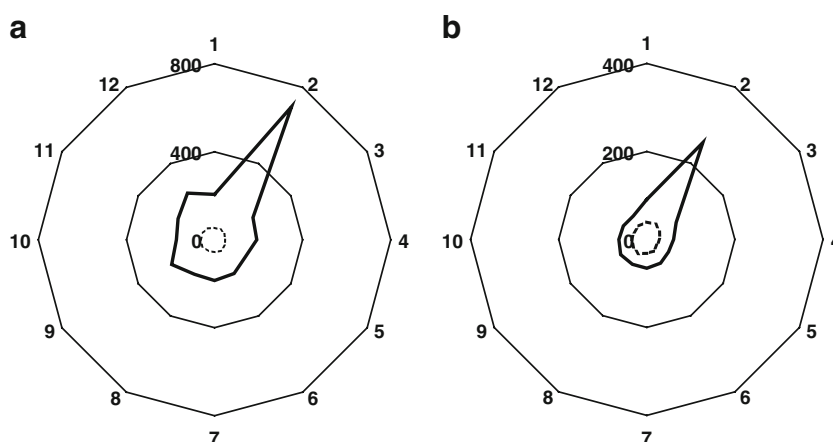
“No queen (control)” represents the 15 min before each queen corpse introduction

% trail percentage (and numbers) of replicates in which a recruitment trail was clearly revealed by a column of workers between the queen's corpse and the nest, % activity in MFS proportion of ants crossing the MFS of a circle drawn around the queen corpse (see text), MFS most frequented sector, n.a data nonavailable, % Agg. proportion of queen corpses attacked by workers (see text)

^a Means ± standard deviation (and numbers of replicates) are given for replicates in which recruitment did not occur

^b Means ± standard deviation (and numbers of replicates) are given for replicates in which recruitment occurred

Fig. 3 Workers spatial activity before (dotted line) and after (bold line) the introduction of a nestmate mated egg-laying mature queen in *L. humile* (a; $n=24$) and *T. erraticum* (b; $n=25$). Radius length indicates mean number of ant crossings through each of the 12 arcs of a circle drawn around the queen (see text). Sector 2 points at artificial nest



species, *T. erraticum*. This queen retrieval recruitment could be related to the frequent nest moving reported in these species (Aron 1992; Meudec 1982), during which the queens, not carried by workers, can be easily lost.

Besides this putative function, recruitment toward queens provides the opportunity to test for nestmate recognition and fertility assessment by workers in a simple bioassay. Our results show that, in both species, recruitment was elicited only when nestmate egg-laying queens, mated or not, were discovered by the workers. Nonegg-laying queens were ignored, although they were recognized as nestmates, as demonstrated by the fact that they were not aggressed by the workers. By contrast, nonnestmate queens, although fertile, never elicited recruitment and were always attacked. Moreover, only nestmate egg-laying queens were observed to be transported to the nest by the workers of both species (confirming previous observations from Aron 1992, in *L. humile*) whereas infertile individuals were moved elsewhere in the foraging area. Collectively, these results strongly suggest that queens of both species produce a pheromone linked to ovarian activity, inducing recruitment behavior in nestmate workers when these queens are detected outside the nest.

Our results are consistent with previous studies that focused on queen recognition in *L. humile*. Vásquez and Silverman (2008) have recently shown that nonnestmate queen adoption is rarely observed, at least in queenright colonies. Among nestmate queens, Cariou-Etienne and Passera (1993) demonstrated that attraction is correlated with fertility, the “queen power”, as they defined attraction/aggregation syndrome (see Vander Meer and Alonso 1998, for a critical review on queen attractive/aggregative pheromones in ants), being strictly related to egg-laying activity. However, their results cannot rule out the possibility that mating is an obligatory prerequisite for the ontogeny of a fertility signal. Actually, former studies usually only considered mated egg-laying queens versus

virgin nonegg-laying queens which makes the distinction between mating and fertility signaling difficult (e.g., Passera 1969; Berndt and Nitschmann 1979; Vargo and Passera 1991). Despite their interest in the understanding of fertility signaling processes in ants, virgin egg-laying queens have rarely been studied. Indeed, results involving virgin egg-laying reproductives mostly come from queenless ants and *Solenopsis invicta*. In queenless ants, workers may mate (= gamergate, Peeters 1991) and thus play the same role as a fertilized queen. In several species, when a worker become dominant and egg layer (before mating), she is able to regulate subordinates reproduction as a fertile gamergate does (e.g., Heinze et al. 2002; Lommelen et al. 2006). In *S. invicta*, it has been demonstrated that virgin egg-laying queens produce queen pheromones, with both releaser and primer effects, as mature mated egg-laying queens do (Vargo 1999; Vargo and Hulseley 2005). The present study, based on a fertility releaser pheromone, and another involving a fertility primer effect in *T. erraticum* queens (Cournault and de Biseau, unpublished data) confirm that fertility rather than mating is signaled by egg-layer individuals (Peeters and Liebig 2009).

The pheromones involved in fertility signaling in both *L. humile* and *T. erraticum* remain to be unambiguously identified. Cuticular hydrocarbons are good candidates for several reasons: (a) Numerous correlation studies and some direct evidences strongly suggest that they are widely used as fertility signals in ants (reviewed in Howard and Blomquist 2005; Monnin 2006; Le Conte and Hefetz 2008). (b) As in some ponerine ants (e.g., *Pachycondyla inversa*: Heinze et al. 2002; *Gnamptogenys striatula*: Lommelen et al. 2006), CHC profiles of *L. humile* queens differ, both quantitatively and qualitatively, between fertile (mated or not) and infertile individuals (de Biseau et al. 2004). (c) The results reported here show that nestmate egg-laying queens of both species failed to elicit recruitment when they were previously washed in hexane, an

organic solvent known to remove nonpolar compounds (Howard and Blomquist 2005). In the same way, fertile queens of *L. humile* lost their “queen power” when they were previously rinsed in pentane (Cariou and Passera 1990). Direct evidences for the role of CHCs as fertility signals could be obtained by using the bioassay described in the present paper. Dummies covered with CHCs extracted from either infertile or fertile nestmates should respectively elicit indifference or recruitment when discovered outside the nest by the workers.

Recently, the role of CHCs as nestmate recognition cues has been demonstrated in *L. humile* (Greene and Gordon 2007; Torres et al. 2007; Vásquez et al. 2008). Therefore, at least in this species, CHCs could be involved in both colonial odor and fertility labeling. As pointed out by Hefetz (2007), this double function appears contradictory since the first requires odor sharing between all colony members, while the second is based on odor specificity of the fertile individuals in the colony. Recognition of egg-laying queens as nestmates is particularly surprising in *L. humile*, since most hydrocarbons identified in the cuticular profile of fertile individuals of this species are not detected in the CHC profile of infertile nestmates (de Biseau et al. 2004). Le Conte and Hefetz (2008) suggest that this apparent paradox can be resolved either if CHCs coding for fertility do not serve as colonial cues or if the workers react in a hierarchical way to successive levels of recognition cues, colonial identification having priority on fertility assessment. The results reported here, although not in contradiction with the first hypothesis, are consistent with the hierarchical model. In our bioassay, since workers behave differently toward non-nestmate egg-laying queens, nestmate egg-laying queens, and nestmate nonegg-laying queens, we had the opportunity to show that nestmate recognition is required before that fertility signals can trigger the typical recruitment behavior of workers observed in both *L. humile* and *T. erraticum*. Under our experimental conditions, nestmate recognition cues clearly overrode fertility signals.

Interestingly, in *L. humile*, recent results show that alien queens are more likely to be adopted by queenless than queenright colonies (Vásquez and Silverman 2008). Since the same is not true for workers adoption, this suggests that the hierarchy of recognition levels is not fixed but, on the contrary, is related to the social context. In queenright colonies, nestmate recognition is needed before that fertility signals can induce specific behaviors among workers. By contrast, in the absence of egg-laying queens, the workers retain their ability to discriminate between nestmates and nonnestmates, as attested by their aggressiveness toward conspecific alien workers. However, fertility signals seem to be predominant over colonial recognition. This model, based on hierarchical levels of recognition influenced by the social context, needs to be confirmed by more

experimental evidences. The bioassay described here could be useful to further test this model, at least in species in which queen retrieval by recruitment is present.

Acknowledgments LC is grateful to A. Lenoir (*T. erraticum*, Tours, France) and X. Espadaler (*L. humile*, Sant Cugat del Vallès, Spain) for their help in collecting the ants. We thank N.J. Vereecken, O. Hardy, P. Mardulyn, and two anonymous referees for their fruitful remarks on a previous version of the manuscript. LC was supported by a doctoral fellowship from the Belgian “Fonds pour la formation à la Recherche dans l’Industrie et l’Agriculture” (FRIA).

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