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Nestmate discrimination in *Leptothorax crassispinus* (Hymenoptera: Formicidae)

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ABSTRACT. Nestmate discrimination in individual ants *Leptothorax crassispinus* was investigated. In short-time dyadic experiments workers reliably distinguished non-nestmates from nestmates both of those immediately after collection, and those cultivated with equal food, temperature and illumination. However, discrimination became not apparent after rinsing body surface with soap solution. Nestmate discrimination occurred also in an initial stadium of experimental mixing of two factions from unrelated colonies. After several hours of mixing the ability to distinguish their own queen decreased and subsequently both colonies fused and eliminated one queen. After mixing of groups of non-nestmate workers aggression gradually disappeared without trophallaxis.

KEY WORDS: nestmate discrimination, discriminatory behaviour, colony odour, fusion of colonies, *Leptothorax crassispinus*

INTRODUCTION

The ability of individuals to distinguish nestmates from non-nestmates is an important mechanism which preserves the integrity of any social insects colony. Conspecific and allospecific intruders – social parasites, predators etc. can be recognised and opposed. Allospecific strangers are almost always violently attacked, though conspecific ones may be treated with a wide variety of responses, from adoption, to extreme hostility (Hölldobler & Willson 1990).

It is presently generally accepted that ant nestmate recognition systems involve specific chemical compounds, signals or cues (see Terminology) recognisable by all the members of a colony (e.g. Hölldobler & Michener 1980, Hölldobler & Carlin 1987). These substances, discriminators, form a typical body odour. The existence of such an odour has been known since the beginning of 20th century (e. g. Fielde 1904), but its sources are investigated and discussed till now. The two main models of nestmate recognition have been postulated – individualistic and *Gestalt* (Crozier & Dix 1979). In individualistic recognition, which is hypothesised e.g. for some ponerine ants (Lenoir et al. 2001b), each individual bears its own genetically determined odour. In *Gestalt* recognition, found in e.g. in various species of

contacts (Lenoir et al. 2001 a,b). Cuticular hydrocarbons appear to be the most common discriminators (e.g. Bonavita-Cougourdan et al. 1996, 1997; Lahav et al. 1999; Meskali et al. 1995a,b), but other substances, e.g. polar cuticular lipids (Franks et al. 1990) are also considered. Nestmate recognition frequently leads to discriminatory behaviour, e.g. the aiming of altruistic acts preferentially at nestmates and aggressive acts against non-nestmates.

The purpose of the present paper is the investigation of nestmate discrimination in individual ants *Leptothorax* (*Myrafant*) *crassispinus* Karawajev, 1926. *Leptothorax crassispinus* is an obligatory monogynous species remarkable for fusing unrelated colonies in conditions of nest-site scarcity (Tichá 1992; Tichá & Štys 2002). Laboratory experiments have shown that this fusion is always accompanied by an elimination of one of the two queens which thus results in the formation of a genetically heterogeneous monogynous colony (op.cit.). This situation appears to be an evolutionary enigma, because it is not probably adaptive for the workers from a colony of the eliminated queen. Such workers have no related queen in the mixed colony, but are likely to be inhibited in laying their own unfertilised eggs by the presence of a queen which is common in *Leptothorax* species (cf. Heinze et al. 1997). Thus the question how strong is the nestmate recognition in *L. crassispinus* arises. The same phenomenon is recorded by Foitzik & Heinze (1998) in *Leptothorax (Myrafant) nylanderi* (Förster, 1850) and interpreted as "intraspecific slavery" – the workers of the eliminated queen serve in the second colony (Foitzik & Heinze 1998, 2000). Previous study reports that some colonies of *Leptothorax crassispinus* avoided the fusion, retained their autonomy and recognised nestmates from non-nestmates after experimental mixing of colonies (Tichá & Štys 2002). However, neither nestmate recognition in individual ants nor recognition during the fusion of colonies has been investigated yet.

METHODS

Terminology

Nestmate recognition – in this article, the general ability to distinguish nestmates from non-nestmates with or without behavioural discrimination of these classes, observable or unobservable

Nestmate discrimination – discriminatory behaviour, direction of altruistic acts preferentially towards the nestmates, observable

Discriminator – generally a mediator of nestmate recognition, the signal or the cue (see below), a set of discriminators forms a specific colonial odour

Signal – the discriminator adaptive for the sender (Shellmann-Reeve 1997)

Cue – incidental by-product of the sender's action functioning as a discriminator (op.cit.)

Discriminatory behaviour – in this article aiming of amicable acts preferentially to nestmates and aggressive acts against non-nestmates

***Gestalt* odour** – a colonial odour which is collectively formed and shared by all the members of an ant colony. It contains colonial discriminator (mostly cuticular substances) and is permanently upgraded by their exchange within the colony by allogrooming, trophallaxis or other physical contact

Synonymy

Leptothorax crassispinus was treated as *Leptothorax nylanderi* (Förster, 1850) till half of ninetieth years of 20th century. Then Seifert (1995, 1996) separated from "*L. nylanderi*" its East European populations as the new species, *Leptothorax slavonicus* Seifert, 1995. Into this populations also Czech and Slovak populations belong. Later Radchenko (2000) revealed that *Leptothorax slavonicus* is a junior subjective synonym of *Leptothorax (Myrafant) crassispinus*.

Collecting of the ants and cultures

A small myrmicine ant *Leptothorax crassispinus* inhabits mainly deciduous, mixed and pine woods of temperal zone. Its colonies regularly contain several dozen individuals, which establish their nests in the small cavities, such as pieces of rotten wood, acorns and another material laying on the ground.

All 127 colonies, from which experimental individuals were used, were collected in the period 1997–2002. The colonies came from Czech, Moravian and Slovak localities – Prague, Znojmo, Mohelno and Piešťany (see Tichá 1992; Tichá & Štys 2002).

Complete colonies, retained in their nest-sites, were placed into plastic bags and transferred to the laboratory. They were then transferred into plastic formicaria (dimensions 100 x 75 x 25 mm). Breeding took place at a temperature of 20 °C; in long-term cultures were hibernated in 2–5 °C from the beginning of December to the half of March. Diet contained sugar, honey, gelatine, white of egg and water.

Dyadic experiments took place in glass chambers, dimensions 20 x 10 x 3 mm, with movable counter. Group experiments were placed into the plastic vials, dimensions: l = 50 mm, d = 30 mm.

Experimental fusions took place in circular plastic boxes 55 mm in diameter with moist-filter paper flooring.

EXPERIMENTS

1. Discrimination of non-nestmates

Dyadic experiments tested the discriminatory behaviour of individual ants *Leptothorax crassispinus*, the influence of cultivation on nestmate discrimination, and the occurrence of colonial discriminators on body surfaces. Experiments based on the confrontation of pairs of workers, either non-nestmates or nestmates, would reveal if the interaction with non-nestmates significantly differed from the interaction with nestmates. The experiments, lasting

10 minutes, were recorded on video or directly observed and analysed. There were 90 replications.

Individual ants were placed in opposite halves of the experimental chamber separated by a counter. After removing the counter their behaviour was monitored and both amicable interactions – allogrooming and trophallaxis – and aggressive interaction – ritualised or actual attacks – were observed and noted. The statistically significant differences in behaviour of non-nestmates and nestmates were then sought. Results were statistically surveyed by Whitney – Mann – test (Statistica 4.5.).

Experiments had three designs:

a) Freshly collected ants. The experiments tested nestmate discrimination in freshly collected ants; 15 times with non-nestmates and 15 times with nestmates.

b) Cultures. The experiments tested nestmate discrimination after a culture of food, temperature and illumination. Cultivation was at least for 1 month, 15 with non-nestmates and 15 with nestmates.

c) Rinsing the body surface. The experiments tested the occurrence of colonial discriminators on the body surface. The ants were carefully rinsed in 10 % soap solution (water, sodium tallowate, sodium cocoate, glycerin, sodium chloride, sodium sulfate, sodium carbonate, titanium dioxid, tetrasodium EDTA, etidronic acid). The soap was chosen for its ability to remove apolar matter, to which cuticular substances mostly belong, and for its gentleness, essential for use in vivo again 15 times with non-nestmates, 15 with nestmates.

2. Experimental fusion

These experiments were designed to reveal how long nestmate discrimination lasts after the fusion of colonies. Freshly collected queens with fifty nestmate workers were taken from two unrelated colonies and the ants were individually marked by the cutting the last tarsomere or by lacquer paint (see Tichá & Štys 2002). These factions were mixed and observed for 15 minutes 4 times a day. (To increase the probability of fusion a small formicarium without broken space was chosen.) This experiment was replicated twice and lasted 5 days.

3. Trophallaxis

Group experiments had to show in the event of trophallaxis – the possible methods of transfer of colonial discriminators – if this appeared among conspecific non-nestmates after mixing. Five worker nestmates were fed with the sugar solution marked by yellow food dye (E 102). Similarly, five nestmates from an unrelated colony were fed with sugar solution marked by the blue food dye (E 133). They were then mixed and examined four times a day. Trophallaxis would be indicated by the green colour of the stomach content. The marked food was easy detectable through the translucent body wall. This experiment was replicated 7 times and lasted 5 days.

The test workers were randomly chosen from the colonies in all the experiments and probably included both experienced and inexperienced ants. Thus individual variability in discriminatory ability was not excluded.

RESULTS

1. Discrimination of nestmates (Tables 1–3, Fig. 1)

Upon removing the counter ants first continued the exploration of their surroundings, which had begun immediately after their placement into the experimental chamber, and also groomed themselves (selfgrooming). The workers noticed each other very soon; that was mostly followed by the contacts including antennation, allogrooming and both ritualised (wide opening of mandibles against partner) and actual (tugging, biting etc.) attacks. Trophallaxis appeared exceptionally. The speed of reaction and the number of interactions varied individually. In some experiments the exploration prevailed over all the other activities including ant interaction.

a) Freshly collected ants. Freshly collected ants (30 experiments, 15 on non-nestmates, 15 on nestmates) performed all analysed interactions. Allogrooming occurred in 2 experiments on non-nestmates and in 8 experiments on nestmates. Trophallaxis did not appear in non-nestmates and occurred in 4 experiments on nestmates. Ritualised attacks were observed in 11 experiments on non-nestmates and in 6 experiments on nestmates. Actual attacks occurred in 7 experiments on non-nestmates but in no experiment on nestmates. There were no significant differences in the number of allogroomings, trophallaxis and actual attacks between the non-nestmates and nestmates. The number of **ritualised attacks was significantly higher in non-nestmates.** For details see Table 1and Fig. 1.

b) Cultures. The ants from the laboratory cultures (30 experiments, 15 on non-nestmates, 15 on nestmates) reacted similarly to the freshly collected ones. Allogrooming occurred in 2 experiments on non-nestmates and in 10 experiments on nestmates. Trophallaxis was not observed in these experiments. Ritualised attacks were observed in 14 experiments on non-nestmates and in 2 experiments on nestmates. Actual attacks appeared in 4 experiments on non-nestmates and in no experiment on nestmates. There were **significant differences in the number of allogroomings and ritualised attacks between the non-nestmates and nestmates**. The number of trophallaxis and actual attacks did not differ significantly. For details see Table 2 and Fig. 1.

c) Rinsing the body surface. The ants that rinsed by the soap solution (30 experiments, 15 on non-nestmates, 15 on nestmates) tended to spend a longer time selfgrooming and more frequently performed antennation than the ones in former experiments. Allogrooming occurred in 1 experiment on non-nestmates and in 4 experiments on nestmates. Trophallaxis was not observed in these experiments. Ritualised attacks were observed in 13 experiments on non-nestmates and in 10 experiments on nestmates. Actual attacks occurred in 3 experiments on non-nestmates and in no experiment on nestmates. **No significant differences were found in the number of analysed interactions between the non-nestmates and nestmates.** For details see Table 3 and Fig. 1.

2. Experimental fusion

After the mixing factions of colonies, the ants performed to standard behaviour, e.g. exploration, initial fights, temporary separation of colonies, fusion, and elimination of one

queen. (For a detailed study of experimental fusion see Tichá 1992; Tichá & Štys 2002.) Initially, fights appeared almost without exception among non-nestmate workers. The queens were passive. In the first experiment the fights ceased within 20 hours and the ants gathered into two clusters containing its queen and her nestmates. They remained in this state three days. Then the aggression among workers did not renew. Within next ten hours **both the colonies fused** and **one of the two queens was found decapitated**. (The decapitation itself was not observed, since it took place between the examinations.) **Workers of both colonies agglomerated around the surviving queen and groomed her**. There was no observed aggression against the queen, nor among original impossible non-nestmates. In the second experiment the ants initially clustered around their queens for the first six hours. Until next eight hours **one queen was abandoned without any grooming or trophallaxis on the periphery of formicarium**. She did not return among the other ants and one of **her nestmates** even repeatedly came before her and **performed ritualised attacks against her**. **The second queen was surrounded by workers from both the colonies. Alien workers groomed her** in addition to her own nestmates and all these ants behaved towards her without aggression. This situation lasted about twenty hours, by then the abandoned queen was dead which again happened between examinations. The corpse was eaten by nearby ants. Therefore it was not possible to determine if she was killed or if she died by another reason. **In both experiments after the reduction in the number of queens to one all ants lived in one colony and behaved peacefully and practically without aggression.**

3. Trophallaxis

The transfer of marked food between non-nestmates was not observed during experiments. In four experiments the groups of workers from unrelated colonies refused to merge, in three experiments they fused after initial fights and then lived peacefully together without trophallaxis among non-nestmates until the termination of these experiments.

DISCUSSION

The experiments showed a good ability by freshly collected individuals of *Leptothorax crassispinus* to distinguish nestmates from non-nestmates. The recognition system functions reliably in the identification of con-specific intruders during the short-term encounters. Hence, fusions of colonies are not enabled by the total absence of colonial recognition.

Nestmate discrimination appeared even after cultivating experimental ants under the identical diet, temperature and illumination conditions. This may point to a genetic component to colonial recognition in *L. crassispinus*, which is supposed in various other ants as well (reviewed e.g. Lenoir et al. 1999). However, although the ants used in experiments were cultivated at least one month in persisted artificial nests, they had been collected in the field and it is not entirely sure whether any influence of original nest material. We must take into account that some environmental component may play a role in *L. crassispinus* colonial

Table 1. Interactions of freshly collected ants. Explanations: NE – serial number of experiment, AG – allogrooming, T – throphallaxis, RA – ritualised attack, A – attack, NNM – non-nestmates, NM – nestmates

NE	AG		T		RA		A	
	NNM	NM	NNM	NM	NNM	NM	NNM	NM
1	0	0	0	0	4	0	1	0
2	0	0	0	0	18	0	20	0
3	0	0	0	1	31	1	9	0
4	1	1	0	0	0	4	1	0
5	0	5	0	2	1	0	0	0
6	0	0	0	0	8	0	1	0
7	0	0	0	0	0	1	0	0
8	0	0	0	0	0	1	0	0
9	0	4	0	0	3	0	3	0
10	1	0	0	0	8	4	0	0
11	0	5	0	0	0	0	0	0
12	0	4	0	0	5	1	2	0
13	0	2	0	1	2	0	0	0
14	0	3	0	0	7	0	0	0
15	0	7	0	3	7	0	0	0

Mann-Whitney:
U = 60,5 Z = -2,15686 p = 0,031024
U = 82,5 Z = -1,244342 p = 0,213383
U = 51 Z =-2,550901 p = 0,010749
U = 52,5 Z = -2,488684 p = 0,012827

Table 2. Interactions of ants cultivated on identical diet, temperature and illumination at least 1 month. Explanations: NE – serial number of experiment, AG – allogrooming, T – throphallaxis, RA – ritualised attack, A – attack, NNM – non-nestmates, NM – nestmates

NE	AG		T		RA		A	
	NNM	NM	NNM	NM	NNM	NM	NNM	NM
1	0	1	0	0	5	1	0	0
2	0	0	0	0	2	0	7	0
3	0	0	0	0	5	0	2	0
4	0	2	0	0	7	0	0	0
5	0	2	0	0	5	0	0	0
6	0	1	0	0	1	0	0	0
7	1	2	0	0	5	0	0	0
8	0	0	0	0	5	0	1	0
9	0	1	0	0	2	0	1	0
10	1	0	0	0	0	0	0	0
11	0	2	0	0	6	0	0	0
12	0	0	0	0	6	0	0	0
13	0	2	0	0	13	0	0	0
14	0	3	0	0	14	0	0	0
15	0	2	0	0	7	2	0	0

Mann-Whitney:
U = 45,5 Z = -2,779031 p = 0,005455
U = 112,5 p = 0
U = 11 Z = -4,210024 p = 2,56E-05
U = 82,5 Z = -1,244342 p = 0,027

Table 3. Interactions of ants rinsed by the soap solution. Explanations: NE – serial number of experiment, AG – allogrooming, T – throphallaxis, RA – ritualised attack, A – attack, NNM – non-nestmates, NM – nestmates

NE	AG		T		RA		A	
	NNM	NM	NNM	NM	NNM	NM	NNM	NM
1	0	1	0	0	1	1	0	0
2	0	0	0	0	3	2	0	0
3	0	1	0	0	6	0	1	0
4	1	0	0	0	10	2	4	0
5	0	1	0	0	3	1	0	0
6	0	0	0	0	1	2	0	0
7	0	1	0	0	4	1	0	0
8	0	0	0	0	5	0	0	0
9	0	0	0	0	0	1	1	0
10	0	0	0	0	3	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	1	0	0	0
13	0	0	0	0	3	1	0	0
14	0	0	0	0	2	1	0	0
15	0	0	0	0	1	2	0	0
Mann-Whitney:								
U = 82,5 Z = -0,933257 p = 0,350695		U = 112,5 p = 0		U = 55 Z = -2,384989 p = 0,017085		U = 90 Z = -0,933257 p = 0,350695		

recognition. The strong influence of nest material on nestmate recognition has been recorded in the related ant species *Leptothorax (Myrafant) nylanderi* (cf. Heinze et al. 1996; Foitzik & Heinze 1998). Regarding the similarities of life histories of *L. crassispinus* and *L. nylanderi* we must consider that their nestmate recognition may be based on a similar mechanism.

Moreover, nestmate discrimination decreased, becoming not apparent, after rinsing in soap solution. The abilities to remove discriminators by the soap suggests their apolar nature and location on the body surface. This is in agreement with the opinion of many authors assuming participation of apolar cuticular compounds in ant nestmate recognition (see e.g. Lenoir et al. 1999, 2001 a,b,c). In *Gestalt* model (see Introduction), which is currently regarded as the most common recognition system in ants, the important role of apolar substances of low volatility, mainly hydrocarbons, is considered (e.g. Lenoir et al. 2001 a, b, c). These substances are located in a lipid layer of the cuticle, applied on the body by selfgrooming and spread among nestmates by trophallaxis, allogrooming and other physical contacts (Lenoir et al. 1999, 2001 a, b). They may originate from workers, from the queen or from the environment (Hölldobler & Carlin 1987). The exploration of the nature of colonial discriminators in *L. crassispinus* is a subject for future studies.

As the experimental fusions showed, the colonial discrimination may decrease also when members of different colonies come in very close long-term contact. Immediately after mixing colonies their members easily recognised their nestmates. Within several hours their recognition capability probably decreased, which manifested in the disappearance of

aggression among non-nestmates and attraction of workers by the alien queen. This enabled the fusion of original colonies into one genetically heterogeneous colony and the following reduction of the queen number was apparently a consequence of the monogyny of this species.

Proximately it is explainable probably by the "diffusion" of colonial discriminators among the non-nestmates during their simple physical contact. The mechanism of ant's nestmate recognition is based on the comparison of the individual labels (odour) with the own template (reviewed e.g. in Lenoir et al. 1999, 2001 a). When members of some colony are in close physical contact with non-nestmates and alien discriminators stick to their bodies and vice-versa their own discriminators spread among the strangers, and nestmate recognition may be impaired. This mechanism may enable e.g. the incorporation of some social parasites into the host's society (e.g. Franks et al. 1990).

On the ultimate level the fusions of colonies are hardly explainable. Maybe, it could be interpreted simply as a non-adaptive (see Introduction) and, from the evolutionary view, faulty adoption of an alien queen. On the other hand, the worker reproduction has never been studied in *L. crassispinus*. We do not know if a queen, especially the unrelated one, strongly inhibits the egg-laying by workers and this situation is entirely non-adaptive for workers of the eliminated queen. Nevertheless, the ability of these ants to live in a genetic heterogenous colony supports the assumption that the kinship has been replaced by the nestmateship in ants (see Bourke & Franks 1995; Leinoir et al. 1999).

The easy transfer of colonial discriminators in *L. crassispinus* supports my proximate explanation of the fusions. My experiments with ants fed by the marked food showed that the trophallaxis is not necessary for the disappearance of aggression among non-nestmates. This indicates that the transfer of colonial odour may pass by simple physical contact without trophallaxis. The results of my previous study (Tichá 1992), in which I reported the absence of initial fights in the unrelated colonies *L. crassispinus* mixed under narcosis, suggest a possibility of passive transfer of colonial discriminators in this species. Similarly Buschinger (1967) mixed the colonies of *Leptothorax nylanderi* which had been narcotised before experiments to merge their smell. The transfer of colonial odour via physical contacts without trophallaxis has been noticed also in less related species, *Pachycondyla apicalis* (Latreille, 1802) (cf. Soroker et al. 1998) and *Aphaenogaster senilis* Mayr, 1853 (cf. Lenoir et al. 2001 a, c).

In a connection with unsolved problems concerning the nestmate recognition in *L. crassispinus*, the important tasks for future research have arisen, mainly the investigation of the origin and the chemistry of colonial discriminators.

SUMMARY

Individual workers of *Leptothorax crassispinus* reliably recognise non-nestmates from nestmates during short-term encounters. This discriminatory behaviour occurred in dyadic experiments both after the collection and after the cultivation non-nestmates in equal conditions, but did not reach statistical significance after rinsing the ant in soap solution. The apolar nature

of colonial discriminators and their presence on the body surface was identified. In experimental fusion of individually marked factions of unrelated colonies nestmate discrimination appeared immediately. However, after the passage of time aggression faded and the alien ants behaved as the members of one colony. This process is accompanied by the elimination of one of the two queens. Group experiments on workers fed with marked food have shown that the aggression may disappear without changing of discriminators by trophallaxis.

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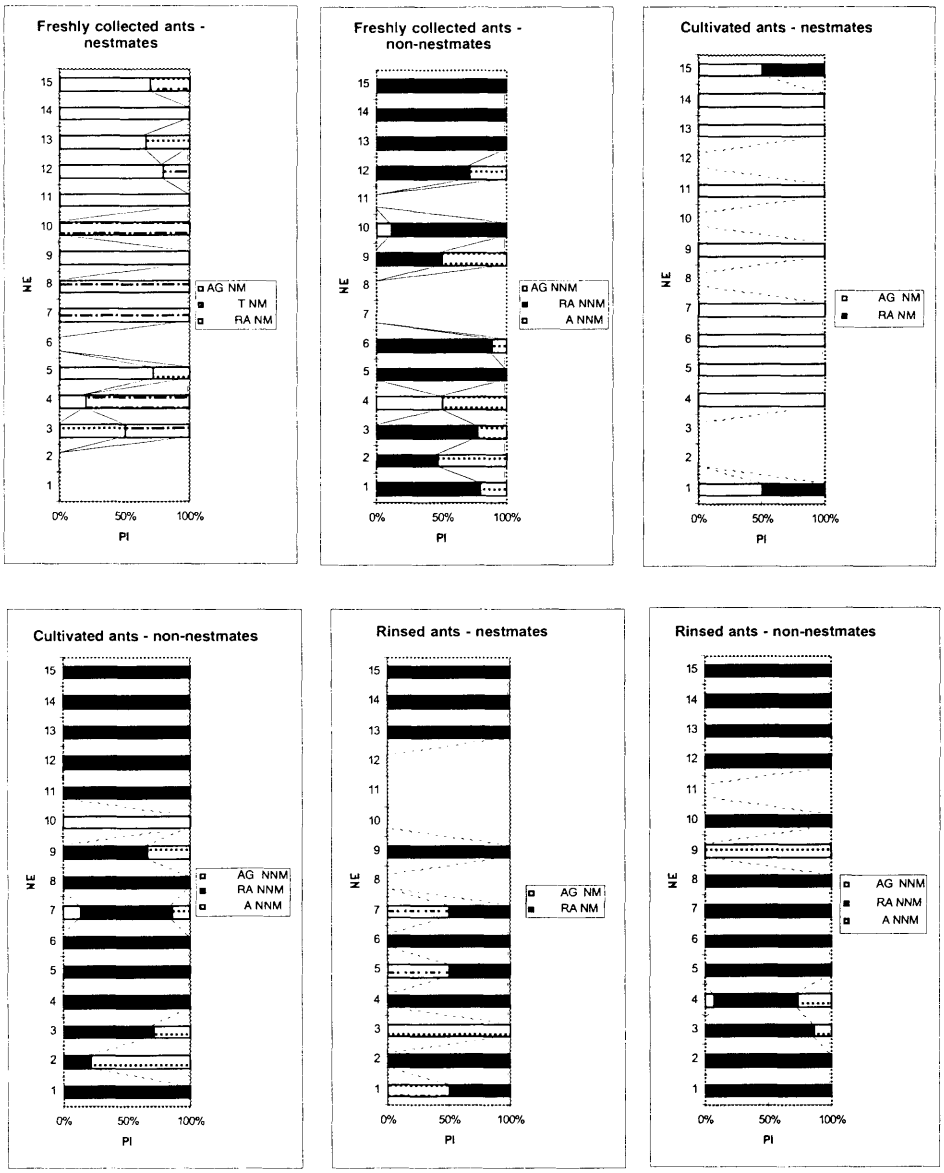

Fig. 1. Interaction with ants. A- number of attacks, RA – number of ritualised attacks, T – number of trophallaxis, AG -number of allogroomings, NNM – non-nestmates, NM – nestmates, NE – serial number of an experiment, PI – percentage of interactions.