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Immune response affects ant trophallactic behaviour

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ABSTRACT

Sociality is associated with many benefits that have favoured its evolution in social insects. However, sociability also presents disadvantages like crowding of large numbers of individuals, which may favour the spread of infections within colonies. Adaptations allowing social insects to prevent and/or control pathogen infections range from behavioural responses to physiological ones including their immune systems. In a state of infection, social interactions with nestmates should be altered in a way which might prevent its spreading. We simulated a microbial infection in workers of the ant *Camponotus fellah* by the administration of peptidoglycan (PGN) and then quantified their immune response and social interactions. PGN injections as well as control injections of Ringer solution elicited similar production of antibacterial compounds, during 1–4 days after. However, injections of PGN reduced the ability of encapsulation of a nylon implant compared to Ringer controls. The immune challenged workers did not decrease the level of interactions with their nestmates. On the contrary, they devoted more time to trophallaxis. These results are discussed in relation to ant life history traits.

1. Introduction

Ants constitute one of the most important groups of animals worldwide. Their evolutionary success can be attributed largely to their eusocial behaviour. Eusociality is rare among insects and is most frequently observed in insect orders Hymenoptera and Isoptera. Such highly social insects as ants, bees, wasps and termites are able to perform complex activities in their daily lives through mutual cooperation carried out in the context of such tasks as foraging for food and nest material, nest defence or food storing (Bonabeau et al., 1997; Hölldobler and Wilson, 1990; Wilson, 1971). These abilities have allowed them to colonize different habitats and to create large populations. Nevertheless, social insect colonies are exposed to numerous pathogens and diseases, a problem which may menace an entire social group and which becomes more dangerous with growing colony size. Therefore, ants have developed several physiological and behavioural mechanisms to solve or at least to minimize that risk. An important innovation — found exclusively in ants—was the appearance of metapleural glands situated at the extreme posterior corners of the mesosoma. It is known that in some ant species these glands produce highly active antiseptic substances that provide protection against microorganisms for both body surface and the interior of their nests (do Nascimento et al., 1996; Hölldobler and Engel-Siegel, 1985 (“1984”); Maschwitz, 1974; Maschwitz et al., 1970). In *Camponotus* ants, these glands are partially or even completely atrophied (Brown, 1968; Hölldobler and Engel-Siegel, 1985 (“1984”)). However, these ants do not remain unprotected since the secretions of other glands show microbicidal action, e.g. the formic acid from poison gland (Revis and Waller, 2004).

Besides these preventive defences, ants possess an elaborate immune system, shared with all insects and other arthropods. The cuticle itself constitutes an excellent barrier against parasite invasion (Schmid-Hempel and Ebert, 2003), but once it is crossed the immune system is activated by a specific antigen recognition system able to detect different components of microorganisms: lipopolysaccharides from the outer membrane of Gram-negative bacteria, peptidoglycan (PGN) unique to Gram-positive cell walls, as well as β-1,3-glycans and β-1,3-mannans from fungal cell walls (Gillespie et al., 1997). The main immune responses of insects and other arthropods include opsonization, phagocytosis, melanization, encapsulation, coagulation and production of antibacterial peptides (Gillespie et al., 1997), a large range of defences that have been very efficient throughout millions of years of evolution of these organisms.

Sociality is based on a trade-off between costs and benefits. For example, trophallaxis — the exchange of food among colony members-may favour the transmission of pathogens in colonies of fire ants (Jouvenaz, 1986). Grooming behaviour can remove parasites from a nestmate, but it also may infect termite workers engaged in grooming (Kramm et al., 1982). This seems valid in

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respect to both invertebrates and vertebrates: grooming represents an important way of nematode transmission among groups of mice (Heitman et al., 2003).

Immunity is costly in several ways. As shown by Moret and Schmid-Hempel (2000), bumblebee workers mounting an immune response to lipopolysaccharides or to microlatex beads survived for shorter time periods in conditions of starvation than control individuals. In *Apis mellifera*, the associative learning can be negatively affected by immune response (Mallon et al., 2003).

Research devoted to the impact of immune responses on insect social behaviour is still at its beginning, especially in the case of ants. If we assume that both social relations and immune responses are costly, we will have to assume as well that each individual has to choose where to invest its energy. To throw more light on that question, we studied the relations between immune responses and social behaviour in the ant *Camponotus fellah*, a species known to engage in intense social interactions (Boulay et al., 1999). First, we examined the temporal dynamics of the antibacterial immune response induced by the administration of PGN from *Saccharomyces cerevisiae*. PGNs are the major component of the cell wall of Gram-positive organisms that confer to the cell wall great mechanical strength, eliciting immune reactions in the infected organisms (Stewarttull, 1980). For that purpose, we performed a test of bacterial inhibition and measured the encapsulation response to estimate the immune responses arising as a consequence of the PGN treatment. Social costs associated with the immune response were estimated by observations of the behaviour displayed by workers subjected to PGN treatment. We hypothesized that these "infected workers" might change their behaviour as a consequence of a trade-off between immune responses and social behaviour, and, particularly, that they would show decreased level of social interactions.

2. Material and methods

2.1. Ants

The colonies of *C. fellah* were obtained by rearing newly mated queens collected in Tel Aviv (Israel) in March 2003. They were maintained in a temperature-controlled room (28 ± 2 °C) under 12:12 h light–dark conditions. Colonies were installed in artificial plaster nests allowing direct observations of intranidal activities. Each nest was connected with a foraging area. The ants were fed with diet consisting of dead insects (mealworms, flies and moths) and sugar solution supplied twice a week. We used 10 colonies containing each one queen, at least 200 workers and abundant brood. We used as subjects media workers present in the foraging area.

2.2. Immune treatments

Peptidoglycans were extracted from *S. cerevisiae* (Sigma-Aldrich, No. 72789). The ants received one of the following three treatments: 'Naive' (N), 'Ringer' (R) and PGN. Workers in the naive group were chilled on ice prior to the experiment. Workers in the Ringer group received an injection of 0.5 μl of Ringer solution after being chilled on ice. Workers in the PGN group received 0.5 μl of a 0.5 mg/ml solution of PGN dissolved in Ringer solution. We injected the ants into the haemocoel through the pleural membrane between the second and the third tergite, using a sterilized fine glass capillary.

2.3. Dynamics of antibacterial immune response

We collected 0.5 μl of haemolymph per individual from 32 naive workers to evaluate the normal conditions of workers from 10 studied colonies. The haemolymph of two workers was pooled in a 0.5 ml Eppendorf tube containing 1.0 μl of cold cacodylate/$CaCl_2$ buffer (0.01 M Na-Cac, 0.005 M $CaCl_2$). We repeated this procedure for the injected workers (Ringer and PGN), determining antibacterial activity at 24, 48, 72, 96, and 120 h post-injection. Antibacterial test plates (diameter 9 cm) were prepared by adding 0.05 ml of live *Arthrobacter globiformis* bacteria suspension (10e7 cells/ml) to 7 ml of sterile broth medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, 1000 ml of distilled water, pH 7.5), with 1% of bacto-agar at 45 °C. Twelve holes per plate were made in the agar, and 2 μl of the haemolymph solution extracted from the workers was added per hole for the test. The plates were then incubated at 28 °C overnight. The antibacterial substances present in the haemolymph inhibit development of bacterial colonies on the plate, leading to a circular, clear zone around each hole with a diameter proportional to the concentration of antibacterial substances. We measured the diameter of each zone of inhibition.

2.4. Encapsulation rate assay

To measure encapsulation rate, we inserted a 2 mm long piece of sterile nylon monofilament (0.12 mm diameter) in the pleural membrane between the second and third tergite. This procedure was carried out in the case of 36 workers immediately after the injection of PGN solution ($n = 18$) or Ringer solution ($n = 18$). After 24 h after, the implants were removed from the haemocoel and placed on a slide to be mounted into Clarion™ mounting medium. The removed monofilament was examined under a light microscope and photographed using a digital camera. The mean grey value of the whole implant was measured using the ImageJ 1.37v software. We assumed that the darkest grey received the highest encapsulation rate (total black). The background grey value was used to correct the values of the implants.

2.5. Behavioural assays

We performed 108 encounters of dyads of workers sampled in almost the same proportions from each of ten tested colonies. They were divided into three groups receiving the following treatments: (1) dyads consisting of two naive workers just chilled in ice (N/N); (2) one chilled worker and another worker treated with PGN (N/Ip); (3) one naive and one injected with Ringer (N/Ir); (4) both ants treated with PGN (Ip/Ip); and (5) two workers injected with Ringer (Ir/Ir). Prior to the dyadic tests and just after the treatment, we isolated the workers for 24 or 48 h in test tubes ($18 \times 180\,mm^2$) where they had *ad libitum* access to water and food. Bioassays consisted of dyadic encounters between individuals that were previously subjected to the same isolation period. We placed two ants in the same test tube and started the observation 3–5 min afterwards. Each test was continued during 30 min with observations taking place every minute according to the method of the scan sampling. For each encounter, we observed behaviours on 30 sample occasions, and noted the occurrence of three behaviour patterns (trophallaxis, self-grooming, and allogrooming). Frequency of each behaviour was quantified as the percentage of the 30 sample points.

2.6. Statistical analyses

We used a factorial ANOVA with two independent variables (time and type of treatment) to analyse the dynamics of antibacterial immune response. The dependent variable was the diameter of each inhibition zone. We compared the encapsulation rate, expressed by the grey mean value of nylon beads, between

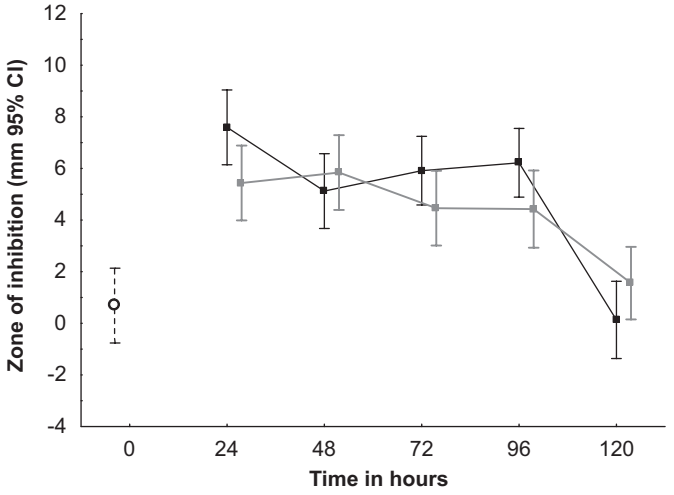

Fig. 1. Temporal dynamics of antibacterial activity after an injection of sterile Ringer solution only (in grey) and an injection of PGN solution (0.5 mg/ml in Ringer Solution) (in black). Each dot is the mean of 14–16 measured pairs of individuals taken from 10 colonies of *Camponotus fellah*. The two lines are not significantly different by factorial ANOVA, $p > 0.05$, for the interaction time/treatment. The circle in the time O represents the baseline obtained from naive workers ($n = 16$).

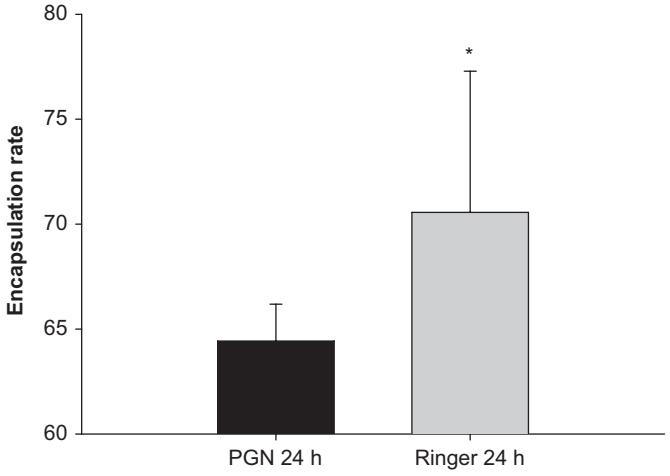

Fig. 2. Mean encapsulation rate (artificial units ± SD) of media workers injected with peptidoglycans (PGN) or Ringer, after 24 h. Sample size values are $N = 18$, for the two bars. Means are significantly different (t-test, $p < 0.001$).

Ringer- and PGN-injected ants with Student's t-test. For behavioural essays, we normalized the data by arcsine transformation, compared the groups by ANOVA, and performed a Fisher LSD test for post-hoc comparisons when necessary.

3. Results

3.1. Dynamics of antibacterial immune response

The haemolymph of naive workers did not contain significant antibacterial activity (Fig. 1). PGN- and Ringer-treated workers displayed a similar antibacterial immune response during the period 1–4 days after injection ($F_{(4,155)} = 2.41$, $p = 0.052$, Fig. 1). No interaction between time and treatment was observed. The variation in antibacterial response was statistically significant throughout time ($F_{(4,155)} = 17.77$, $p < 0.00001$), but no variation was detected between treatment and control ($F_{(1,155)} = 1.99$, $p = 0.16$).This implies that a simple trauma caused by the injection allowed mounting of an immune response. This activity was maintained for at least 96 h and ceased after 120 h.

3.2. Encapsulation rate assay

Ants subjected to injections of Ringer solution showed a significantly higher encapsulation rate than ants treated with PGN ($P < 0.001$, t-test, Fig. 2).

3.3. Social interactions

Self-grooming and allogrooming were observed with low frequency (less than 10%) and did not show significant differences between the tested groups in either of two post-injection periods used (ANOVA: $P > 0.05$). Trophallaxis was observed more frequently. At 24 h post-injection, all treated workers (PGN or Ringer solution) devoted more time to trophallaxis than dyads consisting exclusively of naive workers ($F_{(4,80)} = 2.7288$, $p = 0.04$, Fig. 3a). In the ants tested 48 h post-injection, the level of trophallaxis increased in all groups, including naive workers. However, we did not find significant differences among them ($F_{(4,76)} = 2.32$, $p = 0.07$, Fig. 3b).

4. Discussion

The injection of either PGN or Ringer solution into *C. fellah* ants produced strong and closely similar antibacterial immune responses lasting 4 days after the trauma. The dynamics of the elicited antibacterial response show that the immune response had a shorter duration than that observed in bumblebees (Korner and Schmid-Hempel, 2004) and in *Tenebrio molitor* (Moret and Siva-Jothy, 2003), whatever the type of challenge. Therefore, we can hypothesize that the antibacterial response in *Camponotus* ants restrains microbial infection and avoids another infection of the individual by maintaining a high level of antibacterial activity for at least 4 days.

Control injections using Ringer solution induced an immune response not discernible from the one caused by a PGN injection. Thanks to subsequent observations of naive workers we verified that haemolymph of normal, non-challenged ant workers does not contain bactericidal peptides, or at least does not contain them at detectable levels. The question whether insects do respond to a bacterial infection by the coordinated synthesis of a specific array of antibacterial proteins remains open. We find conflicting results in the literature, with insects developing specific and adaptive humoral response to foreign substances (Karp and Rheins, 1980; Rheins et al., 1980) or unspecific response (Mohrig and Messner, 1968). In *Drosophila melanogaster*, it was demonstrated that septic injury activates two signalling pathways involved in humoral immune response: the Toll pathway that controls resistance to fungal and Gram-positive bacterial infections, and the Imd pathway that is responsible for defence against Gram-negative bacteria (Lemaitre et al., 1997), although these responses were lower than those provoked by the injury infected with Gram-negative and Gram-positive bacteria. The unpredictability of antibacterial immune response could be related to the differential lifestyle of studied species. After unravelling of the complete genome sequence of honeybee (Honey Bee Genome Sequencing Consortium, 2006), the authors verified that this social insect presents fewer proteins implicated in insect immune pathways when compared to other insect genomes, like that of *Drosophila*. The smaller diversity of immune-related proteins could be compensated by unspecific but efficient action of

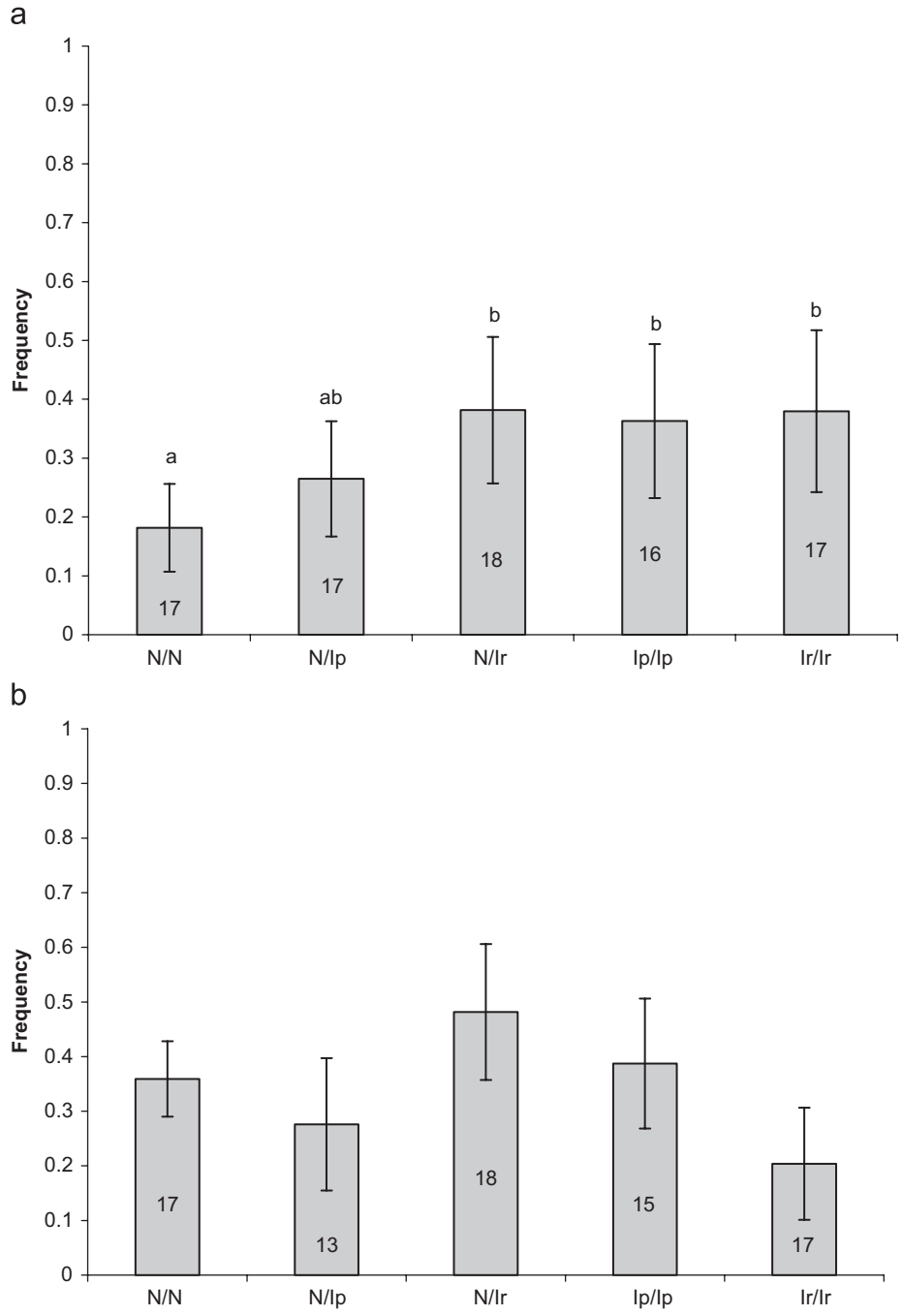

Fig. 3. Mean frequency ($\pm$SD) of trophallaxis between naive workers (N), workers injected with peptidoglycan (Ip), and workers injected with Ringer solution (Ir) observed during dyadic encounters after (a) 24 h of isolation. Different letters denote significant differences (Fisher's LSD, $p < 0.05$). (b) 48 h of isolation. The mean frequencies are not significantly different (ANOVA, $F_{(4,76)} = 2.32$, $p = 0.07$).

antibacterial peptides associated with other immune defence responses and the hygienic behaviour typical of social insects. Further studies devoted to insect immune responses will undoubtedly discover yet other evolutionary strategies of antibacterial defence.

Determination of encapsulation rates showed that PGN injections trigger a different immune response than Ringer solution injections. Interestingly, workers injected with PGN subsequently displayed a lower encapsulation rate than workers injected with Ringer. Studies of the interrelationship of cellular and humoral responses are scant but we may hypothesize that PGN injection depleted components of the encapsulation process (enzymes and cells).

In spite of mounting an immune reaction, *C. fellah* workers did not decrease the level of their social interactions with their nestmates: self-grooming and allogrooming were maintained at the same levels in the control and treated groups, at both 24 and 48 h after the injection. Interestingly, the presence of at least one worker previously subjected to an injection caused a significant intensification of trophallactic behaviour when tested 24 h after the injection, but not when tested 48 h post-injection. We cannot explain precisely the reasons for this effect but we should consider the effects of social isolation. As previously shown by other authors (Boulay et al., 2000; Cybulska et al., 2000), social isolation induces an increase of frequency and/or duration of trophallaxis in ants of the genus *Camponotus*. Forty-eight hours of

isolation is a sufficiently long time to cause an increase in trophallaxis rate, even in naive ants.

In contrast to our hypothesis, costs associated with the immune challenges did not cause a significant decrease in energy allocated to social behaviour. On the contrary, the induction of an immune response was associated with increased trophallaxis. An increased level of trophallaxis could be interpreted as an alert signalling the infected status of a worker to other colony members. If this is the case, other workers could be able either to help the infected nestmate to overcome the infection or to change their behaviour and to attempt to avoid the possible contamination of the entire colony. Our study supports the first of these two hypotheses.

We do not know precisely how trophallaxis may be involved in parasite transmission in ants, but we already know that viruses, nematodes and other parasites could be transmitted via trophallaxis in social insects (Schmid-Hempel, 1998). Considering this possibility, trophallactic behaviour should be inhibited in cases of infection. A search for the presence of antibacterial peptides in the trophallatic liquid of social insects might provide an interesting problem for future research. Studies conducted on *Drosophila* have shown that antibacterial peptides can be induced on surface epithelium (Tzou et al., 2000). For example, the tissue of adult's salivary glands synthesizes an important amount of drosomycin. It will be interesting to analyse the composition of trophallactic fluid to know its possible role in disease prevention in ant colonies.

Our work showed that infection did not induce an immediate reduction of social interactions of workers. On the contrary, trophallactic behaviour was significantly increased. No avoidance behaviour of infected workers was verified so far. It remains thus an open question whether in ants social interactions and physiological mechanisms do indeed operate mutually to minimize the effects of infections on individual worker.

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