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The presence of conifer resin decreases the use of the immune system in wood ants

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Abstract. 1. Wood ants (*Formica paralugubris*) incorporate large amounts of solidified conifer resin into their nest, which reduces the density of many bacteria and fungi and protects the ants against some detrimental micro-organisms. By inducing an environment unfavourable to pathogens, the presence of resin may allow workers to reduce the use of their immune system.

2. The present study tested the hypothesis that the presence of resin decreases the immune activity of wood ants. Specifically, three components of the humoral immune defences of workers kept in resin-rich and resin-free experimental nests (antibacterial, lytic, and prophenoloxidase activities) were compared.

3. The presence of resin was associated with reduced bacterial and fungal densities in nest material and with a small decrease in worker antibacterial and lytic activities. The prophenoloxidase activity was very low in all workers and was not affected by the presence of resin.

4. These results suggest that collective medication with resin reduces pathogen pressure, which in turn decreases the use of the inducible part of the immune system. More generally, the use of plant secondary compounds might be an efficient and economical way to fight pathogens.

Key words. Antibacterial activity, *Formica paralugubris*, immunity, lytic activity, medication, plant secondary metabolites, prophenoloxidase.

Introduction

Parasites are widespread and almost all organisms face their attack. As parasites reduce the fitness of their host, hosts have evolved behavioural and immune defence mechanisms to counteract these negative effects. When resource limited, hosts often have to divert some resources from other fitness-related traits in order to re-allocate them to defence mechanisms (Williams, 1966; Coustau *et al.*, 2000).

To better resist parasites, hosts have evolved behavioural strategies (Hart, 1990), such as parasite avoidance (Christe *et al.*, 1994), grooming (Mooring *et al.*, 2004), hygienic behaviours (Arathi *et al.*, 2006), and self-medication (Lozano, 1998; Christe *et al.*, 2003; Chapuisat *et al.*, 2007). These behaviours are likely to be associated with two types of costs. First, the behaviour may be energetically costly (Giorgi *et al.*, 2001) and, second, the time spent performing the behaviour may be lost for other tasks such as feeding, resting, or reproducing (Christe *et al.*, 1996; Eckstein & Hart, 2000; Hawlena *et al.*, 2007).

The second line of defence is the immune system. Immunity, which relies on both cellular and humoral mechanisms to recognise and respond to non-self entities, is mainly innate in insects (Gillespie *et al.*, 1997). A common factor that links all components of immune function is that they are supposed to be costly (Sheldon & Verhulst, 1996; Schmid-Hempel, 2005). Physiological costs are indeed likely to be associated with both the maintenance and the activation of the immune system (Rolff & Siva-Jothy, 2003). Whereas the costs of maintenance are intrinsically difficult to quantify (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2003), activation costs are readily measured and have been demonstrated (reviewed in Siva-Jothy *et al.*, 2005). In bumblebees, for instance, immune system activation reduces the workers' survival under starvation (Moret & Schmid-Hempel, 2000) and the reproductive success of the colony (Moret & Schmid-Hempel, 2004).

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Anti-parasite behaviours and immunity may be used alternatively and investment in one type of defence may permit reduced investment in another type. More specifically, as the expression of immune defences is phenotypically plastic and is thus likely to be modulated according to environmental factors (Siva-Jothy *et al.*, 2005), a behaviour that decreases exposure to parasites should result in lower use of the immune system, either by permitting a reduced investment in constitutive defences or by lessening activation of inducible responses.

The medication behaviour of wood ants (*Formica paralugubris*) provides an interesting model to test for such a trade-off between behavioural defences and immunity. Workers of this species actively collect pieces of solidified conifer resin from their habitat and incorporate them into their mounds (Castella *et al.*, 2008). Resin, produced by conifer trees when attacked by insects or mechanically wounded (Martin *et al.*, 2002), contains a complex mixture of mono- and sesqui-terpenes showing antibacterial and antifungal properties (Cowan, 1999; Phillips & Croteau, 1999). Recent studies have demonstrated that this medication behaviour provides benefits in terms of defence against pathogens. The resin reduces the densities of bacteria and fungi in ant nest material and inhibits *in vitro* the growth of some potential ant pathogens (Christe *et al.*, 2003). It also increases the survival of experimentally infected ants (Chapuisat *et al.*, 2007). Resin, by inducing an unfavourable environment to pathogens in the nest, reduces the probability and the potential costs of parasitic infections. To collect resin may thus allow workers to invest fewer resources in immunity.

The aim of this study is to test whether the conifer resin present in wood ant nests permits a reduction in worker's immune activity. Resin-rich and resin-free experimental nests were created and the immune activity of workers kept in these two types of nests was compared. More precisely, three components of their humoral activity were tested, namely the antibacterial, lytic, and prophenoloxidase activities. As resin decreases the densities of fungi and bacteria in wood ant nest material, lower activity in all three immune components is predicted.

Methods

Sampling

In October 2004, 20 litres of nest material and workers were collected from 10 randomly chosen nests of the study population located near the Chalet à Roch in the Swiss Jura Mountains. Nest material was sampled from within the upper part of the mounds, and consisted of twigs and spruce needles. Nest material and workers from all colonies were mixed together, taking advantage of the lack of aggression between individuals of this population (Holzer *et al.*, 2006). All pieces of resin were removed with soft forceps and kept apart.

Experimental nests

In order to create 30 experimental nests, the homogeneous stock of workers and nest material was split into 30 parts. Each part, consisting of approximately 0.6 litres of nest material and 200 workers, was deposited in a plastic container ($21 \times 37 \times 18$ cm) side-coated with Fluon to prevent ants from escaping. One tube filled with liquid food (distilled water with 8% honey) and one tube filled with distilled water were deposited in each container. Half of the nests were allocated to the resin-rich group and the other half to the resin-free group. In each of the nests of the former group, 6 g of resin (corresponding to $\sim$ 300 pieces) were deposited on the surface of the nest material. All experimental nests were kept for 35 days at 25 °C with a LD 12:12 h cycle and with regular vaporisation of sterile water to maintain high humidity.

In order to verify that the presence of resin effectively reduced bacterial and fungal densities in the experiment, the concentration of micro-organisms in the nest material of each experimental nest after 35 days was assessed, as described in Christe *et al.* (2003). A significantly lower number of bacterial colony-forming units (CFU) was measured in the resin-rich experimental nests as compared to the resin-free ones (mean $\pm$ SD of CFU per 10^{-9} g: 2.81 ± 0.19 and 3.67 ± 0.19 in nests with resin and without resin respectively; $t_{28} = 3.05$, $P = 0.005$). The density of fungi was also significantly lower in resin-rich nests as compared to resin-free ones (square-root transformed mean $\pm$ SD of CFU per 10^{-4} g: 1.11 ± 0.31 and 1.40 ± 0.27 in nests with resin and without resin respectively; $t_{27.6} = 2.69$, $P = 0.01$). The mean number of CFU formed by gram-negative bacteria in the resin-rich nests was however not significantly different from the one found in resin-free nests (Mean $\pm$ SD of CFU per 10^{-9} g: 3.48 ± 0.29 and 3.58 ± 0.29 in nests with resin and without resin respectively; $t_{28} = 0.24$, $P = 0.81$). These results confirm that the resin treatment significantly reduced the microbial load in nest material (Christe *et al.*, 2003).

For all workers tested for immune activity, body size was estimated from the measure of head size with a stereomicroscope Nikon Profile V-12 (Schwander *et al.*, 2005). There was no difference in the mean head size between resin-rich and resin-free nests, both after 15 and 35 days (mean head size per nest $\pm$ SE in millimetres; day 15: 1.48 ± 0.02 for the resin-rich group and 1.51 ± 0.02 for the resin-free group, $t_{27.1} = 1.32$, $P = 0.20$; day 35: 1.47 ± 0.02 for the resin-rich group and 1.48 ± 0.01 for resin-free group, $t_{25.3} = 0.69$, $P = 0.50$).

Immune measures

The immune defences were measured for five and ten workers per experimental nest sampled 15 and 35 days after the start of the experiment respectively. Immune defences were measured individually on worker homogenates. Workers were put in 0.5 ml Eppendorf tubes and chilled on ice. The three last segments of the abdomen and the head were removed. The thorax and the remaining part of the abdomen were put in a tube containing 30 µl of sodium cacodylate (Na-Cac: 0.01 m; $CaCl_2$: 0.005 m) and squashed with a pellet pestle. The sample was then vortexed for 1 min and centrifuged (6600 rpm) for 15 min at 4 °C. Twenty microlitres of the supernatant were collected and immediately frozen at –80 °C.

The antibacterial and lytic activities were measured with the inhibition zone (Moret & Schmid-Hempel, 2000) and clearing

zone (Y. Moret, pers. comm.) assays respectively. These two assays consist of depositing a drop of homogenate solution on a culture medium inseminated with live bacteria for the inhibition zone and with bacterial cell walls extract for the clearing zone assays. The antibacterial and lytic activities of the sample are proportional to the surface of the zone where bacterial growth has been inhibited and to the surface of the zone where cell walls have been lysed respectively.

For the inhibition zone assay, 2 μl of the homogenate solution were placed on the surface of a thin layer of agar (6 ml in Petri dishes, diameter 9 cm; agar recipe: 10 g bactotryptone, 10 g bacto-agar, 10 g NaCl, 5 g yeast extract, 1000 ml distilled water, pH 7.5) mixed with the test bacteria *Arthrobacter globiformis* (Institut Pasteur Paris, nr. 81.84 T) adjusted to 10^5 cells ml^{-1}. After overnight incubation at 30 °C, the minimum and maximum diameters (in mm) of each zone of inhibition were measured and their mean was used to calculate the surface of the corresponding area [with the formula: π(mean diameter/2)^2].

For the clearing zone assay, 2 μl of the homogenate solution were placed on the surface of a thin layer of agar (5 ml in lids of cell culture plates; agar recipe: 1 g bacto-agar, 10 mg streptomycin sulphate, Triton X-100 2%, 100 ml distilled water) mixed with *Micrococcus luteus* cell walls extract (Sigma, 5 mg ml^{-1}). After overnight incubation at 30 °C, the minimum and maximum diameters (in mm) of each zone of lysis were measured and their mean was used to calculate the surface of the corresponding area.

Prophenoloxidase activity was measured as described by Moret and Siva-Jothy (2003), with slight modifications. The total amount of enzymes is proportional to the increase in optical density induced by the transformation of L-DOPA to dopachrome by phenoloxidase (Soderhall & Cerenius, 1998). Samples were tested in a 96-well microplate reader. Ten microlitres of homogenate solution were mixed with 10 μl of phosphate-buffered saline (PBS) and 50 μl of trypsin (0.25%) in a microplate well and left for 5 min at room temperature. Trypsin proteolytically activates prophenoloxidase (the inactive form of the enzyme) into phenoloxidase (the active form) and thus allows measurement of the total enzymatic activity of the sample. Ten microlitres of L-DOPA (4 mg ml^{-1}) were then added to the mix. The mixture absorbance at 492 nm was measured at 30 °C every 10 s for 50 min. The prophenoloxidase activity was measured as the slope of the reaction curve during the linear phase of the reaction (V_{max}, i.e. the rate of change in optical density $\times 10^2$ per s).

To evaluate the immune activity of workers before the start of the experiment, five workers were sampled per nest immediately after the split of the common stock of workers and they were tested for all three immune measures. As expected, no difference was found between the new experimental nests destined to be allocated to the two treatment groups (mean inhibition surface $\pm$ SE in mm^2: 15.97 $\pm$ 3.21 in nests destined to be resin-rich and 18.33 $\pm$ 2.69 in nests destined to be resin-free; $t_{27.2} = 0.56$, $P = 0.58$; log transformed mean lysis surface $\pm$ SE in mm^2: 2.05 $\pm$ 0.11 in nests destined to be resin-rich and 2.05 $\pm$ 0.13 in nests destined to be resin-free; $t_{27.4} = 0.01$, $P = 0.99$; median prophenoloxidase activity: 0.064 in nests destined to be resin-rich and 0.108 in nests destined to be resin-free; Wilcoxon rank sum test, $Z = 0.604$, $n = 30$, $P = 0.55$).

Statistical analyses

For all three immune measures, a nest mean was calculated for each sampling (i.e. on five workers per nest after 15 days and on 10 workers per nest after 35 days) and used as data point. Groups were compared using Student's *t*-tests (allowing unequal variance) when the data followed a normal distribution and Wilcoxon rank sum tests otherwise. To test for an overall effect of the treatment, a *Z*-transform test was used (Whitlock, 2005). Statistical analyses were carried out using JMP 6.0 (SAS Institute Inc., Cary, North Carolina).

Results

The antibacterial activity of workers from resin-rich and resin-free nests did not differ significantly after 15 days (one-tailed *t*-test: $t_{27.4} = 0.24$, $P = 0.403$; Fig. 1). However, after 35 days, workers kept in resin-rich nests had a significantly lower level of antibacterial activity than workers kept in resin-free nests (one-tailed *t*-test: $t_{20.7} = 2.02$, $P = 0.028$; Fig. 1). Workers kept in resin-rich nests had a lower lytic activity than workers kept in resin-free nests after 15 days but this difference was no longer significant after 35 days (one-tailed *t*-tests: after 15 days, $t_{24.8} = 1.80$, $P = 0.042$; after 35 days, $t_{21.5} = 1.31$, $P = 0.102$; Fig. 2). Workers kept in resin-rich and resin-free nests did not differ significantly in their prophenoloxidase activity (Wilcoxon rank sum tests: after 15 days, $Z = -0.069$, $n = 30$, $P = 0.464$; after 35 days, $Z = 0.251$, $n = 30$, $P = 0.408$; Fig. 3). When combining *P*-values of the comparisons on days 15 and 35 for the three immune components, the presence of resin was associated with a lower immune activity in workers (*Z*-transform test, $P = 0.013$).

Discussion

When pieces of solidified conifer resin are mixed into their nest material, wood ant workers show a small but significant decrease in their antibacterial and lytic activities. In contrast, prophenoloxidase activity does not seem affected by the presence of resin.

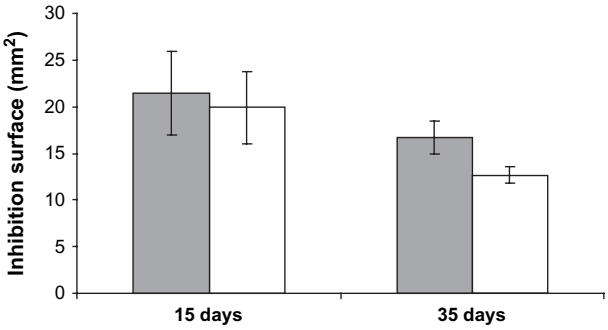

Fig. 1. Mean antibacterial activity expressed as inhibition zone surface $\pm$SE in mm^2 for workers kept in resin-free (grey bars) and in resin-rich (white bars) nests, 15 days and 35 days after the beginning of the experiment. Nest means are used as data points.

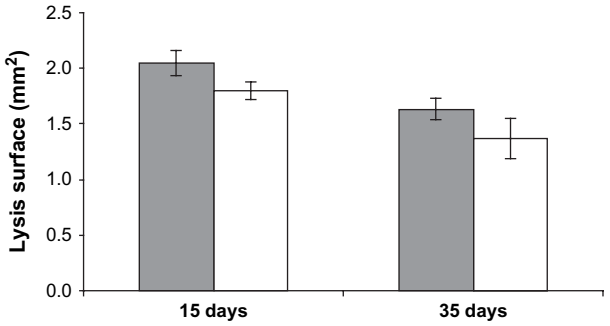

Fig. 2. Mean lytic activity expressed as lysis zone surface ±SE in mm^2 (log_e transformed) for workers kept in resin-free (grey bars) and in resin-rich (white bars) nests, 15 days and 35 days after the beginning of the experiment. Nest means are used as data points.

Antibacterial peptides and lysozyme, which are responsible for antibacterial and lytic activities respectively, are mainly produced by the insect fat body in response to bacterial or fungal infection (Gillespie *et al.*, 1997). These two immune pathways are likely to be activated if the nest material contains high microbial densities. Resin inhibits the growth of bacteria and fungi (Christe *et al.* 2003; this study) and may thus reduce the activation of these inducible immune defences.

On the other hand, prophenoloxidase activity, considered a constitutive defence (Schmid-Hempel, 2005), was not impacted by the presence of resin in the nest. Prophenoloxidase is a zymogen that, once activated into phenoloxidase by proteolysis, leads to melanisation (Gillespie *et al.*, 1997). Both in the presence and absence of resin, wood ant workers exhibit very low prophenoloxidase activity. Indeed only 13% of all the workers had detectable prophenoloxidase activity, whereas 69% and 58% of them showed antibacterial and lytic activities respectively. These proportions were not affected by the resin treatment. Wood ant workers therefore seem to invest little in the phenoloxidase cascade regardless of the presence of resin.

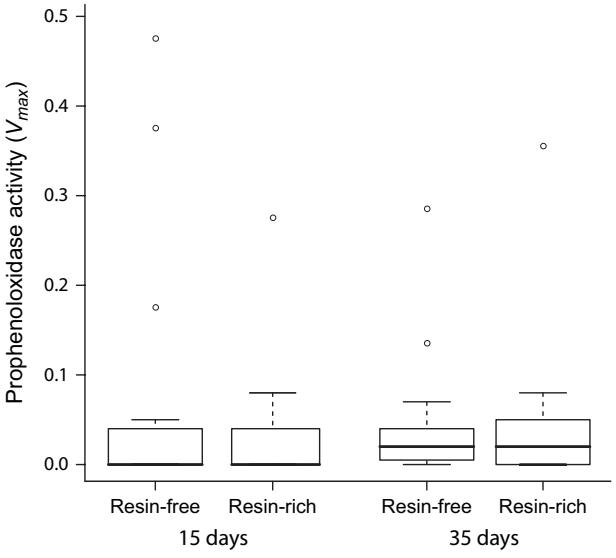

Fig. 3. Prophenoloxidase activity expressed as V_{max} for workers kept in resin-free and in resin-rich nests, 15 days and 35 days after the beginning of the experiment. Nest means are used as data points. Boxes correspond to the interquartile range and solid lines to the median for each group. Whiskers contain all points that are not further from the box than 1.5 times the interquartile range.

These results show that resin collection, by inducing unfavourable conditions for pathogens, results in a slightly lower activation of workers' inducible immune defences. In addition, the presence of resin reduced microbial densities in nest material (Christe *et al.*, 2003) and increased the survival of infected ants (Chapuisat *et al.*, 2007). As immune activation is likely to be associated with energetic costs, the resources spared might then be re-allocated to other fitness-related tasks (Siva-Jothy *et al.*, 2005). Several studies in mammals and birds demonstrated that individuals in germ-free environments increased their productivity and growth (reviewed in Lochmiller & Deerenberg, 2000).

Interestingly, plants may also reduce their level of defences in a reverse situation where plants use ants for their own protection. In myrmecophyte associations, plants provide food and shelter to ants that protect them against herbivorous insects (Heil & McKey, 2003). It has been suggested that plants with active ant colonies have a lower level of chemical defences than plants that are not defended by ants (Dyer *et al.*, 2001).

Many plant species produce secondary metabolites that are biologically active. Animals can recycle these complex substances and use them to fight pathogens (Hart, 2005) or defend themselves against predators (Eisner *et al.*, 1974; Narberhaus *et al.*, 2005). Sociality offers a new level of interactions and permits the collective use of the plant resources, along with many other social mechanisms that have evolved to counter the threat of parasites (Cremer *et al.*, 2007). These various forms of *social immunity* may reduce the physiological costs of immune activation, and might in the long term even lead to simpler genetic immune systems, as indicated by the recent finding that honeybees lack several of the immune genes of non-social insects (Evans *et al.*, 2006).

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