

Heavy metal pollution disturbs immune response in wild ant populations

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Heavy metal pollution affects immune function in ants.

Abstract

Concern about the effects of environmental contaminants on immune function in both humans and wildlife is growing and practically nothing is known about this impact on terrestrial invertebrates, even though they are known to easily accumulate pollutants. We studied the effect of industrial heavy metal contamination on immune defense of a free-living wood ant (*Formica aquilonia*). To find out whether ants show an adapted immune function in a polluted environment, we compared encapsulation responses between local and translocated colonies. Local colonies showed higher heavy metal levels than the translocated ones but the encapsulation response was similar between the two groups, indicating that the immune system of local ants has not adapted to high contamination level. The encapsulation response was elevated in moderate whereas suppressed in high heavy metal levels suggesting higher risk for infections in heavily polluted areas.

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1. Introduction

There is growing concern about the effects of environmental contaminants on the immune function of both humans and wildlife. Some of the contaminants may act as immunotoxins causing increased susceptibility to infectious diseases and parasites (reviewed in Galloway and Depledge, 2001). In insects, factors causing stress—such as chemical poisoning—are known to have an effect on immune function in several laboratory experiments (reviewed in Brey, 1994). Heavy metals have been shown to accumulate easily in insects (Rabitsch, 1995), but the effect on immune function in wild insect populations is still largely unknown.

Compared to vertebrates, the immune system in insects is simple because they lack an antibody-based immune system (Rolff and Siva-Jothy, 2003). Insects are, however, capable of responding very effectively to foreign invaders such as parasites and pathogens by cellular encapsulation. Encapsulation is an unspecific, constitutive, cellular response through which insects defend themselves against multicellular pathogens such as nematodes, fungi and parasitoids (Gillespie et al., 1997), but it also plays a role in defense against viruses (Washburn et al., 1996). Encapsulation of large foreign bodies is a common phenomenon of cellular immune response in insects (Salt, 1970). During the encapsulation process, specialized hemocytes recognize invading particles as non-self and cause other hemocytes to aggregate and form a capsule. A cascade of biochemical reactions leads to a deposition of melanin and hardening of the capsule (Gillespie et al., 1997). The enclosed intruder dies from suffocation or from

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the release of necrotizing compounds (Nappi et al., 1995). The magnitude of encapsulation response has been found to vary with age, body size, condition, density, genotype, sex, state of health, developmental stability and activity etc. (see e.g. Köning and Schmid-Hempel, 1995; Boete et al., 2002; Doums et al., 2002; Wilson et al., 2002; Rantala et al., 2003a,b, 2004; Cotter et al., 2004; Vainio et al., 2004; Rantala and Roff, 2005). Although insects are known to respond effectively to foreign invaders by the encapsulation response, the association between environmental pollution and immune function is poorly understood.

Social Hymenoptera, such as wasps, bees and ants, live in colonies and exploit local resources. Among them, ants are excellent study organisms for exploring the effects of environmental pollution, because their colonies are perennial and due to wingless workers they use resources on a very local scale. Owing to their perennial colonies, ants might adapt to heavy metal pollution during a long-term exposure. This adaptation should express itself as smaller effect of heavy metal exposure on encapsulation response in long time exposed populations compared to newly exposed ones. However, evidence on such adaptations in arthropods is very scarce (Donker, 1992; Donker et al., 1993). Reproductive state may affect immune response (Galloway and Depledge, 2001) and cause additional variation in immune response measures when free-living study animals are used. Ant workers, however, represent a quite homogeneous sample in relation to their reproductive state. In this respect, and considering the knowledge on the accumulation of heavy metals (Rabitsch, 1995; Eeva et al., 2004), ants are exceptionally good study organisms in studies concerning effects of atmospheric pollution on immune functions of free-living animal populations.

A previous study showed that heavy metals accumulate in a wood ant (*Formica aquilonia*) at higher rate around Harjavalta copper smelter compared to populations further away from the smelter (Eeva et al., 2004). Having a prior knowledge about heavy metal accumulation in ants in this area, we asked:

- (i) whether aerial heavy metal pollution affects encapsulation rates of wild populations of *F. aquilonia*;
- (ii) whether encapsulation rate is related to nutritional status (fat content) and size of the ants; and
- (iii) whether local ants are adapted to long-term heavy metal exposure by having weaker association between encapsulation response and heavy metals compared to newly exposed population.

We analyzed a colony level heavy metal and fat contents in workers, and measured their individual body masses and encapsulation rates. We assayed encapsulation rate by measuring the magnitude of encapsulation response to a sterile nylon monofilament. To examine the effect of possible adaptation of ants to long-term heavy metal pollution, we moved ant colonies from a distant unpolluted site to the pollution gradient and compared immune responses between newly translocated and long time exposed local colonies.

2. Materials and methods

2.1. Study area

The study material was collected in the surroundings of a copper smelter in the town Harjavalta (61°20' N, 22°10' E), SW Finland in summer 2003. Sulphuric oxides and heavy metals (especially Cu, Ni, As, Pb and Zn) are common pollutants in the area (Kiikkilä, 2003). Heavy metals accumulate in wood ants and the highest enrichment factors (polluted/unpolluted) are found for As (4.1), Ni (2.4), Cu (2.1) and Pb (1.8) (Eeva et al., 2004). Elevated heavy metal concentrations occur in the polluted area due to current and long-term (since the 1940s) deposition, and metal contents decrease exponentially with increasing distance to the smelter approaching background levels at sites further than 5 km from the smelter (Jussila et al., 1991; Eeva and Lehtikoinen, 1996). There are no major sources of pollutants in the background area. Study plots of similar forest type, i.e. relatively barren Scots pine (*Pinus sylvestris* L.) dominated forests typical to our study area were selected for the study.

2.2. Study populations

In June 2001, all local colonies of *Formica aquilonia* were searched at 14 study plots (mean area $7.9 \pm \text{SE } 1.22$ ha) locating 0.5–12 km from the pollution source. For this study, 24 of the colonies (minimum separation always >100 m), representing all of the study plots were sampled (the same colonies as in an earlier study; Eeva et al., 2004). In addition, 24 ant nests were moved from an uncontaminated area (60°44' N, 21°58' E) 64 km from the smelter to the pollution gradient (0.5–12 km) around Harjavalta copper smelter in May 2002. Nest material (50 L) containing workers, queens and offspring were excavated and ants were put into plastic buckets and transferred immediately into the study area by pick-up car. Colonies were immediately released into field in selected locations along the pollution gradient, into similar habitat than local colonies (over 150 m from the nearest local colony).

2.3. Heavy metal analyses

Heavy metal contamination from 41 out of 48 studied colonies ($n = 22$ local + 19 translocated nests) were measured. Workers at the top of the mounds were collected in June 2002 (local ants) and September 2003 (translocated ants) for heavy metal analyses. Heavy metal concentrations may increase in spring when ants start to tend aphids more intensively (Stary and Kubiznáková, 1987; Rabitsch, 1995). However, we consider it unlikely that there would be major changes during the period between June and September, because intensive tending of aphids continues from mid summer to autumn. Samples (ca. 80 workers each) were dried at 50 °C for 48 h and analyzed for contents of As, Cd, Cu, Ni and Pb. The concentrations of these metals in *F. aquilonia* have previously shown to correlate negatively with the distance to the Harjavalta copper smelter (Eeva et al., 2004). The samples were accurately weighed in the range of 0.15–0.20 g. Two milliliters of supra-pure HNO₃ and 0.5 ml of H₂O₂ were added to the samples into Teflon bombs for digestion with a microwave system (Milestone High Performance Microwave Digestion Unit MLS 1200 mega). After the digestion, the samples were diluted to 50 ml with de-ionized water (Elgastat Maxima). The determination of concentration of the analytic elements was done with inductively coupled plasma mass spectrometer (ICP-MS) Elan 6100 DRC+ from PerkinElmer-Sciex (Montaser, 1998). The detection limits for most of the elements are around ppt (ng L⁻¹) level and below. The calibration of the instrument has been done with certified solution (Claritas PPT, Multi element solution 2A) from Spex Certiprep.

2.4. Encapsulation rate assay

In arthropods, one of the most informative ways to assay the strength of immune defense is to measure the magnitude of the encapsulation response to a novel and standardized antigen such as a nylon monofilament (e.g. Köning and Schmid-Hempel, 1995; Rantala and Kortet, 2003, 2004; Ahtiainen et al., 2004, 2005; Vainio et al., 2004). Many studies indicate that parasites and their

hosts are locally co-adapted in their encapsulation response (see Bouletreau, 1986). Thus, general encapsulation response is best measured by the defense reaction against a novel, “passive” and standardized antigen, rather than by the reaction against a coevolved, interactive, actively evading and variable parasitic organism. Furthermore, the ability to encapsulate synthetic substrate is shown to be strongly positively related to ability to encapsulate parasites (Paskewitz and Riehle, 1994; Gorman et al., 1996).

The ants used in encapsulation rate assays were simultaneously (within 3 days) collected from both local and translocated nests in early June 2003. Due to the short life span of *Formica* red wood ant workers (approx. <1 year; Rosengren et al., 1985, 1986) the most of the translocated ants were grown up in the new habitat in the previous growing season. To measure encapsulation rate, we prepared 1 mm long pieces of nylon monofilament (0.18 mm diameter) that was rubbed with sandpaper before cut, knotted at one end and stored in 95% ethanol to keep them sterile. The nylon monofilament was inserted through a puncture made with a sterile needle in the first gastral tergite. The immune system of ants was allowed to react to this object for 3 h, while they were kept individually in small plastic jars (diameter 3 cm, height 5 cm) at constant temperature (28 ± 1 °C). The knot allowed to non-destructively removing the monofilament after 3 h. The removed monofilament was photographed using a digital video recorder attached to a light microscope. The pictures were then analyzed using an image analysis program (Image J). The degree of encapsulation was analyzed as the gray value of reflected light from the implants. The scale was calibrated to indicate that the darkest gray received the highest encapsulation rate (total black). Previously it has been shown that the repeatability for this measurement is very high ($r = 0.997$; Rantala et al., 2002). The encapsulation process is associated with a visible melanization of the formed capsule (reviewed in Ratcliffe et al., 1985). Thus, the implant's darkness reflects a combination of cellular encapsulation and degree of melanization, with higher values indicating a stronger encapsulation response. Encapsulation rate is known to correlate with hemocyte count and phenoloxidase activity, two other measures of immune function (Rantala et al., 2000, 2002). The encapsulation rate was measured individually from 1502 worker ants from 24 local and 24 translocated nests (24–33 workers per nest, mean $31.3 \pm \text{SE } 0.2$).

2.5. Body mass and fat content

Body mass and fat content were measured from the same workers that had been used in encapsulation rate assays. The fat extraction method was modified after Sundström (1995). Ants were dried (48 h at 55 °C) and scaled with Mettler Toledo MX5 scale (accuracy 0.001 mg) for dry body mass (d.w.) first individually and then pooled as a nest sample and extracted in 90 ml of petrol spirit (boiling point 40–60 °C) 24 h in a water bath of 40 °C. New petrol spirit was added when needed during the extraction. After extraction the ants were dried (24 h at 55 °C) and scaled again. The weight difference represents the amount of fat in a colony sample. In the analyses we used percentage of fat from dry body mass before extraction. Fat extraction was done for 48 nests, one of which was excluded from the analysis due to a failure in the process.

2.6. Statistical methods

To handle five intercorrelated heavy metal concentrations, principal component analysis was used to produce one variable describing the heavy metal exposure. First principal component (PC1) explained 69% of variation. The eigenvectors in PC1 were as follows: Cu (0.52), As (0.51), Ni (0.47), Pb (0.43), and Cd (0.26). The same heavy metals have earlier shown to be the most accumulated in *F. aquilonia* in the study area (Eeva et al., 2004). PC1 was used as an independent factor in the following analyses. Mixed model ANOVAs (GLMM) were used to analyze the association between heavy metal levels and nest mound volume with encapsulation rate, and association between worker size and origin (local vs. translocated) of ant workers by using nest of origin (nested within translocation treatment) as a clustering factor (a repeated subject in GLMM). Pearson correlations were used to explore the association between heavy metal levels and distance to the copper smelter, body mass and encapsulation rate, fat content and encapsulation rate, and fat

content and heavy metal level. Analyses were made with statistical software SAS version 8.2 (SAS institute, Cary, NC, USA) using procedures Corr, Mixed and Princomp.

3. Results

Heavy metal levels (PC1) correlated strongly with distance to the copper smelter both in local and translocated colonies (local: Pearson $r = -0.92$, $n = 22$, $p < 0.0001$; translocated: Pearson $r = -0.94$, $n = 19$, $p < 0.0001$). For example, arsenic, that showed the highest enrichment factor (polluted/unpolluted) in our previous study (Eeva et al., 2004) correlated strongly with the distance to the smelter both in the local and translocated populations (local: Pearson $r = -0.811$, $n = 21$, $p < 0.0001$; translocated: Pearson $r = -0.931$, $n = 19$, $p < 0.0001$; Fig. 1). The heavy metal levels (PC1) were higher in local ants than in translocated colonies (GLMM: $F_{1,39} = 6.24$, $p = 0.017$). Nest mound volume (a proxy for colony size; nested within translocation treatment) did not affect significantly encapsulation rates (GLMM: $F_{2,1186} = 2.01$, $p = 0.13$) and was thus removed from the final model. Encapsulation rates of workers did not differ between translocated and local ants (GLMM: $F_{1,1218} = 1.50$, $p = 0.22$). There were no linear responses between encapsulation rate and heavy metal PC1 (GLMM: $F_{1,1218} = 0.05$, $p = 0.81$), but similar quadratic association between encapsulation rate and PC1 was found in both groups ($\text{PC1} \times \text{PC1}$; GLMM: $F_{1,1218} = 12.7$, $p = 0.0004$): encapsulation rate first increased along an increasing heavy metal concentrations (PC1) but started to decrease at higher heavy metal concentrations (Fig. 2).

Translocated workers were larger than local workers (model based means of body mass \pm SE, translocated: 2.33 ± 0.02 mg,

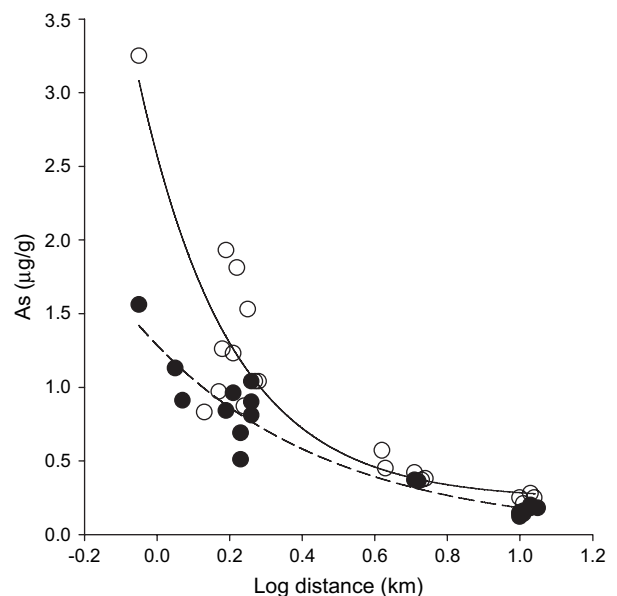


Fig. 1. Relationships between arsenic (As) concentrations (d.w. µg/g) in ant workers and distance to the copper smelter in local and translocated colonies. Filled circles are translocated colonies ($n = 19$) and open circles are local colonies ($n = 22$). Third order exponential curves are fitted in the data.

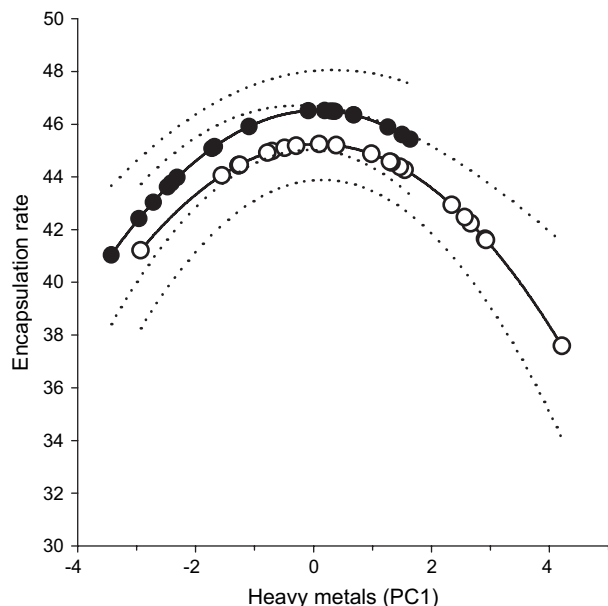


Fig. 2. Encapsulation rates (artificial unit) as a function of heavy metal levels (principal component, PC1) in ants. The encapsulation rate was measured as relative darkness of nylon implants inserted into ants. Increasing PC1 means increase in As, Cd, Cu, Ni and Pb. Model based predicted values (filled circles: translocated colonies, $n = 19$; open circles: local colonies, $n = 22$) and 95% confidence intervals are shown.

local: 2.11 ± 0.02 mg; GLMM: $F_{1,1479} = 45.01$, $p < 0.0001$), but the encapsulation rate did not correlate with body mass (Colony means, Pearson $r = 0.031$, $n = 47$, $p = 0.84$). Colony mean encapsulation rate did not correlate with fat content of workers (Pearson $r = 0.105$, $n = 47$, $p = 0.48$). Similarly, the fat content of workers did not correlate with the principal component (PC1) of heavy metals (Pearson $r = -0.178$, $n = 40$, $p = 0.27$).

4. Discussion

As a result of increase in heavy metal levels the immune response of ants first elevated, but was suppressed at higher heavy metal levels. Our data suggest that moderate levels of heavy metals enhance immune response, but higher levels start to suppress immune system when the dose exceeds a certain critical point. Parallel to our results, some previous studies in vertebrates and marine invertebrates have shown that immune responses at elevated heavy metal levels may be enhanced after a short-term exposure, whereas longer exposure may inhibit the same responses (reviewed in Galloway and Depledge, 2001). Contrary to the local adaptation hypothesis, the association between heavy metals and encapsulation rate was similar in local ants and translocated ants. This suggests that immune system of local ants in the polluted area has not adapted to long-term heavy metal contamination. This result is not biased by the fact that the translocated ants were slightly larger than local ants in the study area, because the body size was not associated with encapsulation rate. Furthermore, in an earlier study we did not find an association

between body mass and distance to pollution source (Eeva et al., 2004).

It has been found that insects' immune defense is condition-dependent, being downregulated by nutritional stress (e.g. Suwanchaichinda and Paskewitz, 1998; Siva-Jothy and Thompson, 2002; Rantala et al., 2003a). Fat is the major energy reserve in insects, and in some studies fat reserves are known to be correlated with encapsulation rate (e.g. Koskimäki et al., 2004). Thus, the effects of heavy metal pollution could be indirectly mediated through poor environmental conditions such as food resource levels. This, however, was controlled by measuring fat content of workers as an indicator of food resource levels. Fat content was similar along the heavy metal gradient and the encapsulation rate was not associated with body fat content of ants. Thus, the response found in encapsulation rate is more likely to have been caused by direct toxic effects rather than by the indirect effects of the nutritional status.

Aerial pollution can have implications for welfare of populations. In laboratory mice chronic exposure to lead prior to antigenic challenge caused enhanced immune responses, whereas host resistance was reduced with acute exposure at the time of infection (Laschi-Loquerie et al., 1987). Wide spectrum of pathogens such as viruses, bacteria and other prokaryotes, fungi, protozoa, nematodes, helminths, mites, and parasitic insects have been shown to parasitize social insects such as ants (Schmid-Hempel, 1998). Disturbed immune functions can lower parasite resistance and may have further implications in fitness of ant colonies in polluted areas. In fact, mortality may explain why the nest mound sizes were smaller in the polluted environments than in less polluted ones, as documented in the same study area (Eeva et al., 2004). Pollution-related immunomodulation and lack of adaptation to heavy metals are possible reasons for declining ant populations, a phenomenon observed in several locations where forests are affected by atmospheric pollution (Katayev et al., 1983; Sary and Kubiznáková, 1987; Koricheva et al., 1995).

To conclude, our study along with the study of van Ooik et al. (in press) is one of the first to show that heavy metal pollution has toxic effects on immune responses in free-living insect populations, and further, that ants have not adapted to increased heavy metal levels during a long-term exposure. Corrupted immune functions may cause potential risk for infections in ants as well as other organisms exposed to heavy metal pollution. Perennial social insects seem to be good bio-monitors of the impacts of heavy metal pollution.

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