

Research article

Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinator* and the effect of infection

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Summary. Parasites represent significant challenges to social insects. The high density, interaction rate and relatedness of individuals within colonies are all predicted to make social insect colonies particularly vulnerable to parasites. To cope with this pressure, social insects have evolved a number of defence mechanisms. These include the immune response, which, aside from in bumblebees, has been relatively little studied in social insects. Here we compare the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinator* and examine the effect upon immunocompetence of prior exposure to a virulent parasite. Males have a far lower immune response than workers, suggesting either haploid susceptibility or reduced investment in immunity by the short-lived males. There was also significantly less variation in the immune response of males than of workers, which may be due to leaf-cutting ant workers being more variable in age or more genetically diverse within colonies. When exposed to the entomopathogenic fungus *Metarhizium*, workers expressed a substantially reduced immune response 96 h after infection, suggesting that the immune system was either depleted by having to respond to the *Metarhizium* infection or was depressed by the parasite. The results suggest that the immune response is a costly and limited process, but further experiments are needed to distinguish between the alternative explanations for the effects observed.

Key words: Encapsulation, immunocompetence, *Metarhizium*, *Acromyrmex*, haploid susceptibility hypothesis.

Introduction

Social insect colonies are characterised by a dense aggregation of individuals that are generally highly related to one another. These features facilitate the transmission of disease and make social insect colonies particularly vulnerable to parasites (Alexander, 1974; Schmid-Hempel, 1998; Boomsma et al., in press). Consequently, social insects have evolved a number of specialised mechanisms to defend their colonies against parasites, including grooming, antibiotic secretions and hygienic behaviour (e.g. Kermarrec et al., 1986; Rosengaus et al., 1998, 2000, 2004; Schmid-Hempel, 1998; Christie et al., 2002; Hart and Ratnieks, 2001, 2002; Hughes et al., 2002; Poulsen et al., 2002a,b; Turillazzi et al., 2004; Boomsma et al., in press). The effectiveness of these mechanisms is such that rather than the group-living lifestyle being associated with increased susceptibility to disease, as is expected, it may instead result in decreased susceptibility (Rosengaus et al., 1998; Hughes et al., 2002; Shimizu and Yamaji, 2003). In addition to these specialised defence mechanisms, social insects also have the individual immune systems that are found in most insects and a number of antibacterial peptides have been identified from them (Casteels et al., 1990, 1993; Casteelsjossion et al., 1993; Mackintosh et al., 1998; Taguchi et al., 1998; Lamberty et al., 2001). The action of the immune system has been intensively studied in the bumblebee *Bombus terrestris* (reviewed in Schmid-Hempel, 2001), but there have been relatively few investigations of the immune system in other social insects (Rosengaus et al., 1999; Traniello et al., 2002; Vainio et al., 2004). This is in spite of its probable importance to the survival of individual social insects and thus of their colonies.

In this study, we examine the immune response of *Acromyrmex* leaf-cutting ants (Hymenoptera: Formicidae: Attini) by implanting a nylon filament into the haemolymph of the ants and then measuring the resulting encapsulation response.

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Leaf-cutting ants have large long-lived colonies (Hölldobler and Wilson, 1990), and exist in habitats that can contain a high diversity of particular parasites (Hughes et al., 2004a). Although much is known about their other defence mechanisms against disease (Kermarrec et al., 1986; Jaccoud et al., 1999; Bot et al., 2001, 2002; Hart and Ratnieks, 2001, 2002; Hughes et al., 2002; Poulsen et al., 2002a,b), their immune system has not previously been investigated. We set-out to determine two basic features of the immune response: 1) whether males and workers differ in their encapsulation response, and 2) whether the encapsulation response is either increased or decreased by prior exposure to a parasite.

Lower immunocompetence of males compared to females has been found in two other social insect species (*Bombus terrestris*: Gerloff et al., 2003; B. Baer and P. Schmid-Hempel, unpubl.; *Formica exsecta*: Vainio et al., 2004;), and can be expected for two reasons. In the Hymenoptera, males arise from unfertilised eggs and are therefore haploid, whereas females arise from fertilised eggs and are diploid. Heterozygosity is often considered to improve an individual's resistance to disease, and it has been suggested that haploid individuals, such as ant and bee males, will be less immunocompetent than diploid females (the 'haploid susceptibility hypothesis'; O'Donnell and Beshers, 2004). A non-mutually exclusive alternative explanation derives from life-history differences rather than ploidy. In leaf-cutting ants and most social Hymenoptera, the males spend the vast majority of their life within their natal colony and leave it only for a short mating flight after which they die (Baer, 2003). Within their natal colony males will be protected from parasites by the colony's workers, while the very short duration of the mating flight means that any diseases that males contact there will not impact upon their fitness. Female workers, in contrast, spend a significant part of their lives foraging outside the colony and therefore have a much higher lifetime exposure to parasites than do males. Workers are likely to be selected to be resistant to parasites both to maximise their individual value to their colony and to reduce the risk of them transmitting diseases to nestmates. The immune system of insects is costly (Kraaijeveld and Godfray, 1997; Fellowes et al., 1998; Kraaijeveld et al., 2002; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; 2005), and the much lower importance of diseases to males compared to workers means that males may benefit by diverting resources from immunity to mating-related traits (Rolff, 2002; Schmid-Hempel, 2005).

The costly nature of immunity in insects also makes it likely that there will be limitations on the number and level of challenges that an individual can deal with at any one time. In addition, many parasites can directly affect the immune response by producing immunodepressant compounds (Gillespie et al., 2000a; Schmid-Hempel, 2005). However, evidence for an impact of parasites upon the immune response is limited and conflicting. The encapsulation response of bumblebees has been found to be reduced (Doums and Schmid-Hempel, 2000), and unaffected (Allander and Schmid-Hempel, 2000) by infection with the gut parasite *Crithidia*, while the immune response of termites can be increased by prior exposure to a parasite (Rosengaus et al., 1999).

Materials and methods

Acromyrmex echinator colonies were collected in Gamboa, Panama and maintained in the laboratory at 25°C, 70% RH on a diet of bramble leaves and rice. Adult males and large workers (head width $2.07 \text{ mm} \pm 0.015 \text{ mm}$) were sampled from six colonies (Ae33, Ae48, Ae109, Ae153, Ae162 and Ae177). They were maintained in a container with an *ad libitum* supply of water and 10% sucrose water. We quantified immunocompetence by measuring the encapsulation response. This cellular response is a commonly used measure of immunity and involves haemocytes attaching to a foreign particle, melanising and eventually forming a capsule around it. We modified the protocol used previously with bumblebees (Allander and Schmid-Hempel, 2000; Doums and Schmid-Hempel, 2000; Baer and Schmid-Hempel, 2003a; Gerloff et al., 2003), and utilised equipment designed for the artificial insemination of bees (Baer and Schmid-Hempel, 2000). Ants were anaesthetized with CO₂ and held in place in a plastic holder. Two injection needles with bent tips were used as hooks to stretch the individual's sternites allowing access to the intersegmental membranes. A needle was used to pierce the intersegmental membrane between the second and the third sternite and a nylon filament ($0.13 \times 0.5 \text{ mm}$) was implanted into the animal's haemolymph. Ants were allowed to recover and were then frozen 24 h later. Mortality during this procedure was low (<2%). The implants were subsequently dissected out, mounted on microscope slides with Eukitt, and photographed with a digital camera (Canon EOS D30) connected to a Leica dissecting microscope. The degree of encapsulation was measured using imaging software (NIH Image Program) by subtracting the mean grey value of the background from the mean grey value of the melanised implant.

To examine the effect of prior exposure to a parasite upon the immune response, we sampled large workers from each of ten colonies of *A. echinator* (Ae33, Ae48, Ae109, Ae132, Ae143, Ae153, Ae154, Ae155, Ae168 and Ae177) and maintained them as above. As the experimental parasite, we used strain KVL02-73 of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*. This strain was isolated from the same site in Gamboa, Panama, as that from which the ant colonies were collected (Hughes et al., 2004a), and it has previously been shown to be highly pathogenic to *A. echinator* (Hughes et al., 2002; 2004b). We harvested spores from a freshly sporulating plate of the fungus and used these to make a suspension of 1×10^7 spores per ml in 0.05% Triton-X. Spore viability was checked by plating the suspension on to agar plates (Lacey and Brooks, 1997), and was confirmed to be >95%. We treated half of the ants from each colony with 0.5 µl of the spore suspension of the parasite, and the other half of the ants with 0.5 µl of a 0.05% Triton-X control solution. The ants were then maintained in individual pots with access to water and 10% sucrose water. For each treatment and each colony, the encapsulation responses of a third of the ants were measured immediately, and of the remaining thirds at 48 h and 96 h after treatment. Implants were inserted at these times, and the ants were then maintained for 24 h before being frozen. The encapsulation response was quantified as described above.

Results

Comparison of the immune responses of males and workers

Encapsulation data were collected for a total of 131 large workers and 102 males. The encapsulation response of males (5.89 ± 0.53) was significantly lower than that of the workers (19.9 ± 1.55 ; ANOVA, $F_{1,221} = 68.5$, $p < 0.001$). This difference was present in all colonies, but the magnitude of it varied significantly between colonies (Fig. 1; $F_{5,221} = 4.10$, $p = 0.001$), and colonies differed overall in their encapsulation response ($F_{5,221} = 3.43$, $p = 0.005$). The variation in the encapsulation response was significantly greater between

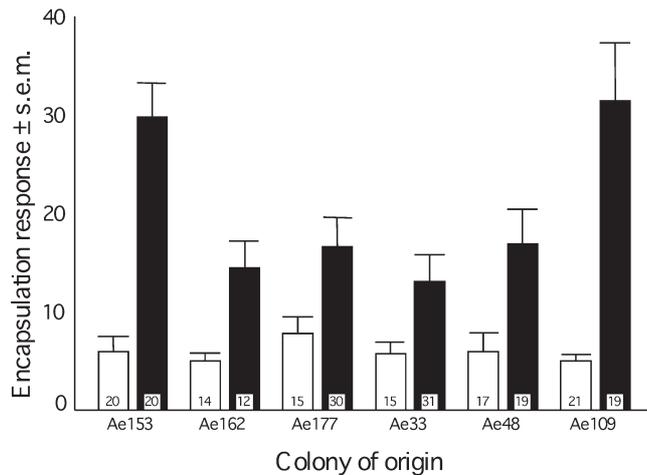


Figure 1. The mean (\pm s.e.) encapsulation response to a nylon filament implanted in the haemolymph of males (clear bars) and workers (shaded bars) from six colonies of *A. echinator*. Sample sizes are presented as numbers within bars

large workers than between males (ANOVA using ranks of standard deviations in encapsulation response as a dependent variable, $F_{1,12} = 36.0$, $p = 0.002$), but did not differ between colonies ($F_{5,12} = 1.33$, $p = 0.380$).

Effect of parasitic infection on immune response

The encapsulation response of large workers treated with *Metarhizium* spores and those treated with the control solution did not differ immediately after treatment or 48 h later (Fig. 2). However at 96 h after treatment ants exposed to the *Metarhizium* parasite had a significantly lower encapsulation response than those treated with the control solution (ANO-

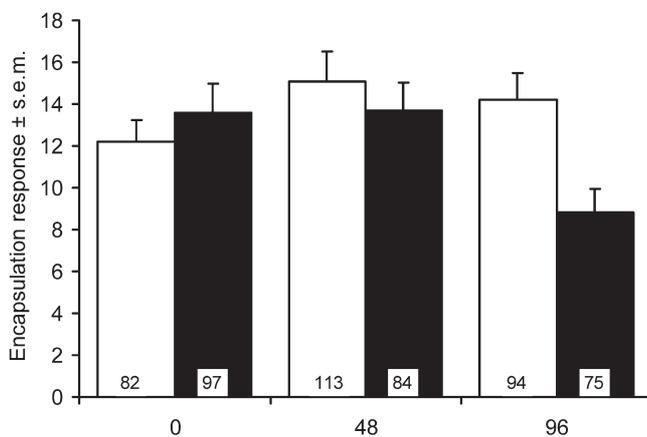


Figure 2. The mean (\pm s.e.) encapsulation response to a nylon filament implanted in the haemolymph of *A. echinator* workers treated with the *Metarhizium* parasite (shaded bars) or a control solution (clear bars). The encapsulation response of workers was examined either immediately, 48 h or 96 h after treatment. Sample sizes are presented as numbers within bars

VA, $F_{2,487} = 4.55$, $p = 0.011$). There was also significant variation between colonies in the overall level of encapsulation response ($F_{9,487} = 2.25$, $p = 0.018$), but not in the way the encapsulation response was affected by the two treatments ($F_{9,487} = 0.58$, $p = 0.82$).

Discussion

The leaf-cutting ant *A. echinator* was found to have a clear difference in immune response between males and workers, with the former exhibiting a much lower encapsulation response than the latter. Males have similarly been found to have lower immunocompetence than females in both of the other social insect species previously examined (Gerloff et al., 2003; Vainio et al., 2004; B. Baer and P. Schmid-Hempel, unpubl.), as well as in some other insects (Adamo et al., 2001; Rolff, 2001, 2002; Siva-Jothy et al., 2001; Schmid-Hempel, 2005). Although the data in our study do not allow us to directly distinguish between haploid susceptibility and life-history as explanations for low male immunocompetence, there are two points that may shed light on the issue. The first is that all male ants lack metapleural glands (Hölldobler and Wilson, 1990). These glands are known to be important in disease defence (Bot et al., 2002; Hughes et al., 2002; Poulsen et al., 2002a), and are large and costly (Poulsen et al., 2002a). Male ants have thus eliminated investment in this costly disease defence mechanism, presumably because disease resistance is less important to them than traits that increase the number of matings they obtain. The reduced immunocompetence of males might therefore be for the same reason. Secondly, the differences in immunocompetence and life-history between the males and workers of *A. echinator* and of the bumblebee *Bombus terrestris* exhibit an intriguing pattern. Male leaf-cutting ants leave the protection of their natal colony only for a brief nuptial flight after which they die. There is therefore very little risk of diseases impacting upon the fitness of male leaf-cutting ants and little that they can gain by investing in immune defences. In contrast, male bumblebees patrol mating territories or other colonies for several weeks (Goulson, 2003). During this time the males will be exposed to parasites and if infected may have reduced lifespan and fitness. The benefit of being able to resist parasites is therefore similar for the males and workers of *B. terrestris*, whereas in *A. echinator* the benefit is much lower for males than for workers. In *A. echinator*, the encapsulation response of males was approximately four times lower than that of workers, with the response of males being minimal. In *Bombus terrestris*, the difference between males and workers is much less with both having considerable immune responses (Gerloff et al., 2003; B. Baer and P. Schmid-Hempel, unpubl.). The lack of metapleural glands in male leaf-cutting ants and the relative male-worker differences in the encapsulation response of *A. echinator* and *B. terrestris*, both suggest that the low immunocompetence of male leaf-cutting ants may be due to reduced investment in immunity by males rather than haploid susceptibility. However, data from more species are obviously needed to obtain a clearer comparative picture.

The comparison of male-worker immunocompetence also demonstrated that workers were more variable in their encapsulation response than males. An individual's age can influence its encapsulation response (Adamo et al., 2001; Doums et al., 2002), and the males were probably of a narrower age-range than the workers. The greater variation in worker immunocompetence might therefore be because workers varied more in age than males. An alternative explanation is that workers were more genetically variable than males, and that genotypes vary in their immunocompetence. Given the well-established costs of immunity in insects and the consequent trade-offs between the immune response and other life history traits (Kraaijeveld and Godfray, 1997; Fellowes et al., 1998; Kraaijeveld et al., 2002; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; 2005), genotypic differences in immunocompetence seem likely. Genetic variation for resistance has been found in several social insect species (Baer and Schmid-Hempel, 2003b; Palmer and Oldroyd, 2003; Tarry, 2003; Hughes and Boomsma, 2004a), although these results could relate to other defences as well as the immune system. Variation in the encapsulation response did not differ between males and females in either of the previous studies that compared the immunocompetence of social insect males and females (Gerloff et al., 2003; Vainio et al., 2004). However, queens in these two study species are either obligately monoandrous, or have an effective number of patrines per colony that is close to one (Schmid-Hempel and Schmid-Hempel, 2000; Sundström et al., 2003). *A. echinatio* queens mate with approximately ten different males (Sumner et al., 2004), so the number of worker genotypes is high, and far greater than that of the males which only carry maternal genes. The disease resistance of leaf-cutting ant workers (Hughes and Boomsma, 2004a) is known to vary between patrines, and it may be that this relates to the observed variation in the immune response. Further experiments with individuals of known genotype and age are needed to establish whether there is indeed a direct link between genotype and immunocompetence in leaf-cutting ants.

In the second experiment, we found no effect of infection with the *Metarhizium* parasite at 48 h after application, but that the encapsulation response of infected workers was substantially reduced 96 h after treatment. *Metarhizium* will take 24–48 h to germinate and penetrate into the host insect, and the switch from growth as protoplasts to the more antigenic hyphal bodies occurs 48–96 h after infection (Gillespie et al., 2000a). Mortality of infected leaf-cutting ant workers is normally seen 6–10 days after treatment with the strain and dose used here (Hughes et al., 2004b). After 48 h there had therefore probably been relatively little parasite growth inside the workers, whereas after 96 h parasite growth was likely to be quite substantial. The hyphal bodies of *Metarhizium* do stimulate an encapsulation response in insects (Gillespie et al., 2000a), and so the reduced response seen in workers 96 h after infection could be due to the immune response being depleted from responding to the multiple hyphal bodies that were probably circulating within the ants by this time. However, *Metarhizium* also produces immunodepressant toxins (Boucias and Pendland, 1998; Gillespie et al., 2000a,b; Vey

et al., 2002), and the increased growth of an avirulent parasite when coinfecting with *Metarhizium* has been attributed to the immunodepressant activity of *Metarhizium* toxins (Hughes and Boomsma, 2004b). The toxins are produced by the hyphal bodies of *Metarhizium* and so the quantity of toxins produced will be positively correlated with parasite growth. The reduced encapsulation response of workers 96 h after infection could therefore be due to depletion or to toxin-mediated immunodepression and further work is needed to distinguish these possibilities.

The immune response is a costly trait (Kraaijeveld and Godfray, 1997; Fellowes et al., 1998; Kraaijeveld et al., 2002; Rolff, 2002; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; 2005; Boomsma et al., 2005) and it is therefore to be expected that there are trade-offs involved in the allocation of resources to it. Investment will depend upon life-history and the importance of being resistant to diseases, while the response itself will be finite and may be depleted by multiple challenges. The results of this study are suggestive of such trade-offs occurring.

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