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RESEARCH ARTICLE

Weaving resistance: silk and disease resistance in the weaver ant *Polyrhachis dives*

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Abstract Social insects are at risk from a diverse range of parasites. The antibiotic-producing metapleural gland is an ancestral trait in ants which is thought to be one of their primary mechanisms of resistance. However, the metapleural gland has been lost secondarily in three ant genera, which include weaver ants that are characterised by the remarkable construction of their nests using larval silk. Silken nests may have allowed reduced investment in costly disease resistance mechanisms like the metapleural gland if the silk has antimicrobial properties, as in other insects, or is a hygienic substrate. Here we examine this hypothesis in the weaver ant *Polyrhachis dives*. We found no evidence of a beneficial effect of silk. The presence of silk did not improve the already high resistance of ants to the entomopathogenic fungus *Metarhizium*, the ants only rarely interacted with the silk regardless of whether they were exposed to *Metarhizium* or not, and silk also did not inhibit the in vitro germination or growth of *Metarhizium*. Furthermore, silk was found in vitro to be heavily contaminated with the facultative entomopathogenic fungus *Aspergillus flavus*, and many more ants sporulated with this fungus when kept with silk in vivo than when they were kept without silk. Further work is needed to examine the effects of silk on other parasites and of silk from other weaver ants. However, the results in combination suggest that silk in *P. dives* is unlikely to provide protection against parasites and that it is also not a hygienic substrate. Alternative explanations may therefore be needed for the loss of the metapleural gland in weaver ants.

Keywords Parasites · Social insects · *Metarhizium* · *Aspergillus* · Pathogens · Metapleural gland

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Introduction

Disease poses one of the greatest threats to social insects. The high density, interaction rate and relatedness of individuals within colonies will all facilitate the transmission and adaptation of parasites (Schmid-Hempel, 1998; Boomsma et al., 2005). While this may be offset to some extent by a reduced rate of between-colony transmission (Wilson et al., 2003), parasites nevertheless represent a serious threat to social insect colonies. Social insects have consequently evolved a sophisticated suite of defence mechanisms to protect themselves against parasites (Boomsma et al., 2005; Wilson-Rich et al., 2009). These include the recognition and avoidance of parasites, first-line individual defences of self-grooming and prophylactic production of antimicrobial secretions, and the second-line individual defence provided by the physiological immune response. In addition, social insects also have group defences, including allogrooming, the transfer of antimicrobial secretions and physiological immune products, and the incorporation of antimicrobial compounds from the environment. These group defences, termed 'social immunity' in its broad sense, are adaptive and proactive, and can result in the social lifestyle producing a net benefit to social insect hosts in terms of the within-colony interactions with parasites (Hughes et al., 2002; Morelos-Juárez et al., 2010; Walker and Hughes, 2009; Rosengaus et al., 1998; Ugelvig and Cremer, 2007; Cremer et al., 2007).

A key component of disease resistance in ants has long been considered to be the metapleural gland (Hölldobler and Engel-Siegel, 1984; Schmid-Hempel, 1998; Boomsma et al., 2005; Hölldobler and Wilson, 1990). This gland is an ancestral characteristic of ants which produces a wide range of antimicrobial compounds (Schlüns and Crozier, 2009; Bot et al., 2002; Beattie et al., 1986; Veal et al., 1992; Mackintosh et al., 1995). The gland has been shown to be extremely costly, using as much as 20% of the energetic expenditure in leaf-cutting ants, and to be essential for ants to resist parasites (Poulsen et al., 2002). The size of the gland in other ants suggests that it is similarly costly and important in them as well. In accord with this, the gland is reduced in socially parasitic ants which rely on their host workers for protection against disease, and is not present in male ants which are similarly protected for all but a short portion of their lives by their nestmate workers (Brown, 1968; Sumner et al., 2003; Hölldobler and Wilson, 1990).

However, a small number of ant species lack the metapleural gland, with it having been lost secondarily on at least two occasions in the subfamily Formicinae, once in the genus *Oecophylla* and once in a clade containing the *Polyrhachis* and *Camponotus* genera (Johnson et al., 2003). This is perplexing. If the metapleural gland is as essential to disease resistance as it appears, how are *Polyrhachis*, *Camponotus* and *Oecophylla* able to survive without it? Possibly they may have evolved to rely on alternative defence mechanisms. A non-mutually exclusive alternative is that they may be less exposed to parasites and thus have less need for the costly metapleural gland. It is notable that many species of *Polyrhachis*, *Camponotus* and *Oecophylla* have evolved the otherwise unusual habit of weaving their nests out of larval silk (Robson and Kohout, 2005; Hölldobler and Wilson, 1990). It would seem logical that silk may provide a more hygienic substrate to form a nest than the soil used by most ants, which contains numerous transmission stages of a diversity of parasites, particularly fungi (Hughes et al., 2004b; Keller et al., 2003; Meyling and Eilenberg, 2006; Boucias and Pendland, 1998). In addition, silk has been shown to have antimicrobial properties in some other insects and so may itself provide a defence against disease (Li et al., 2007; Korayem et al., 2007). However, whether the silk used by weaver ants is indeed hygienic or has antimicrobial properties is unknown.

Here we examine the role of silk in the south-east Asian weaver ant *Polyrhachis dives*. This is an arboreal nesting species, distributed from Japan to Australia, which constructs its nests out of thin sheets of silk (Robson and Kohout, 2005). In order to determine whether silk may protect ants against disease, we exposed them experimentally to a fungal parasite and recorded ant survival, parasite success, and the interaction between ants and silk. We also investigate in vitro whether silk reduces the germination or growth of the fungal parasite. In both experiments we record the occurrence of contaminant fungi to confirm if silk is a hygienic substrate.

Materials and methods

The study used workers from three colonies of *P. dives*, with colonies being maintained at 80 ± 5% relative humidity and 26 ± 2°C on a diet of *Tenebrio molitor* larvae and 10% sucrose solution, with water provided ad libitum. As an experimental parasite, we used strain KVL02-73 of the entomopathogenic fungus *Metarhizium anisopliae* (Bischoff et al., 2009), which is highly virulent in many ant species, including *P. dives* (P. Graystock and W.O.H. Hughes, unpubl. data; (Hughes et al., 2004b; Hughes et al., 2004a). *M. anisopliae* var. *anisopliae* is a globally distributed, generalist parasite, found in the region where *P. dives* occurs, and known to infect numerous ant species as well as many other insects (Schmid-Hempel, 1998; Freed et al., 2011; Boucias and Pendland, 1998; Dong et al., 2009; Sun and Liu, 2008; Hughes et al., 2004a). It is thus highly likely to represent a natural threat to *P. dives*, but one which will not have coevolved to exploit any particular host species.

Experiment 1: disease resistance and silk

The effect of silk presence on disease resistance was examined in a factorial design, with ants being exposed to either the *Metarhizium* parasite or a control solution, and provided either with silk or not. The experiment used 120 workers from three parent colonies. A suspension of *Metarhizium* conidia was made-up from a sporulated plate and diluted to concentration of 1×10^7 conidia/ml. A 0.5 µl dose of either the *Metarhizium* suspension or a 0.05% Triton-X control solution was applied to the dorsal thorax of each ant with a micropipette. Viability of the *Metarhizium* conidia used was checked after 24 h by plating on to Sabouraud dextrose agar media and found to be 75%, so the dose applied equated to approximately 3,750 viable conidia per ant. Following treatment, the ants were immediately placed in groups of three nestmates, all exposed to the same treatment, in containers (8 cm height, 5 cm diameter). Half of these containers had already been given a 2 cm^2 piece of silk from their parent colony, and all had cotton wool balls soaked in water and 10% sucrose water. There were thus 10 replicate groups for each combination of *Metarhizium* or control solution, with or without silk. The numbers of ants in each group that engaged in self-grooming, allogrooming or grooming involving the silk (either manipulating the silk or grooming on the silk) were recorded during 30-s observation periods at 1, 2, 3, 4, 5, 10, 15, 30 and 60 min after application. The survival of the ants was recorded daily for 14 days. Dead

ants were removed, surface sterilised (Lacey, 1997), and placed in a Petri dish on a piece of moistened filter paper to maintain a humid environment. The cadavers were checked daily for fungal sporulation, with both the *Metarhizium* experimental parasite and the common *Aspergillus flavus* facultative parasite being identified by their characteristic conidia and conidiophores.

Experiment 2: antimicrobial effect of silk in vitro

Six 90 mm Petri dishes of media (65 g Sabouraud dextrose agar, 1 ml 10% streptomycin sulphate and 1 ml 5% chloramphenicol per litre of water) were each divided into six equal sections. Silk extracts were made by placing three 0.5 cm^2 pieces of silk in 1.5 ml pentane (99+%, Sigma) for 3 h. On each agar dish, a 0.5 mm^2 piece of silk was placed in two sections, 100 µl of silk extract was placed in two other sections and 100 µl of pentane placed in the final two sections. The media dishes were left for 30 min for any antimicrobial compounds to absorb into the media, and 1 ml of a 1×10^7 conidia/ml suspension of *Metarhizium anisopliae* was then spread evenly over the plate. The media dishes were stored at 27°C and 80% RH for 4 days, after which the presence or absence of *Metarhizium* growth in each section was assessed.

Statistical analysis

We used generalized linear models with poisson distributions and log link functions to examine the effects of treatment (*Metarhizium* or control), silk presence and colony on the numbers of cadavers which sporulated with *Metarhizium* or *Aspergillus*, and on the total amount of grooming observed. The survival of ants over the 14-day observation period, and the grooming of ants over the 60-min observation period, contained repeated measures and were thus analysed using generalized estimating equations with time modelled as a within-subjects variable. The minimum adequate models were obtained in all cases. All analyses were carried out in SPSS 15.0.

Results

Experiment 1: disease resistance and silk

There was no significant effect of treatment (*Metarhizium* exposure or control; Wald $\chi^2 = 1.24$, $df = 1$, $P = 0.266$) or silk (Wald $\chi^2 = 0.04$, $df = 1$, $P = 0.842$) on the survival of ants over the 14-day experimental period. Survival, however, did differ significantly between the three source colonies used (Wald $\chi^2 = 20.5$, $df = 2$, $P < 0.001$). Survival was generally high, with 50–60% of ants surviving in

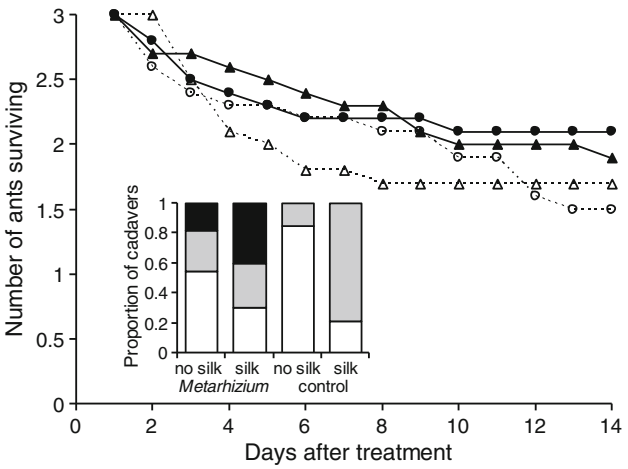

Fig. 1 Mean number of weaver ants surviving after treatment with either a suspension of the *Metarhizium* parasite (*black symbols* and *solid lines*) or control solution (*open symbols* and *dashed lines*), and maintained either with (*circles*) or without (*triangles*) silk. Ants were maintained in groups of three. *Error bars* excluded for clarity. *Inset* are the proportion of cadavers that showed no fungal sporulation (*white*), or which sporulated with either the facultative parasite *Aspergillus flavus* (*grey*) or *Metarhizium* (*black*)

all treatment/silk combinations (Fig. 1). There was no effect of silk (Wald $\chi^2 = 0.641$, $df = 1$, $P = 0.423$) or colony (Wald $\chi^2 = 1.28$, $df = 2$, $P = 0.257$) on the number of cadavers sporulating with *Metarhizium*, all of which were in the *Metarhizium* treatment (Fig. 1, inset). There was a significant effect of silk on the number of cadavers sporulating with *A. flavus* (Wald $\chi^2 = 4.52$, $df = 1$, $P = 0.033$), but no effect on this of treatment (Wald $\chi^2 = 0.789$, $df = 1$, $P = 0.374$) or colony (Wald $\chi^2 = 3.301$, $df = 2$, $P = 0.192$). A large proportion of the control ants with silk which died sporulated with *A. flavus*, whereas very few of the control ants without silk which died produced this parasite (Fig. 1, inset).

There was a significant interaction between the effects of treatment and silk on the total amount of grooming over the course of the 60-min observation period (Wald $\chi^2 = 7.13$, $df = 1$, $P = 0.008$), while the effect of colony was non-significant (Wald $\chi^2 = 3.84$, $df = 2$, $P = 0.147$). Grooming peaked at 2 min after treatment in all treatment/silk combinations, but was otherwise broadly constant (Fig. 2). The most grooming took place in the *Metarhizium*-treated groups without silk, while the least grooming was in the control-treated groups without silk. The groups with silk and treated with *Metarhizium* or control solution were intermediate and similar. There was no effect of treatment on the overall amount of grooming involving silk in the 60-min observation period in those groups with silk (Wald $\chi^2 = 0.053$, $df = 1$, $P = 0.819$), or, marginally, on self-grooming (Wald $\chi^2 = 3.29$, $df = 1$, $P = 0.07$; Fig. 2, inset). However, there were significant effects of treatment (Wald $\chi^2 = 5.72$, $df = 1$, $P = 0.017$) and silk (Wald

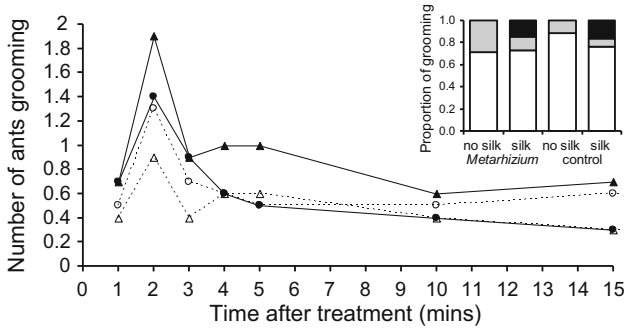

Fig. 2 Mean number of individuals grooming per group of three weaver ants during the first 15 min after treatment with either a *Metarhizium* suspension (*black symbols* and *solid lines*) or control solution (*open symbols* and *dashed lines*), and maintained either with (*circles*) or without (*triangles*) silk. *Error bars* excluded for clarity. *Inset* are the mean proportions of these grooming events which were self-grooming (*white*), allogrooming (*grey*) or grooming involving silk (*black*)

$\chi^2 = 7.19$, $df = 1$, $P = 0.007$) on the total amount of allogrooming, with more allogrooming taking place in the groups exposed to *Metarhizium* and without silk (Fig. 2, inset). There were no significant differences between colonies in the total amounts of either grooming involving silk (Wald $\chi^2 = 1.77$, $df = 1$, $P = 0.413$) or allogrooming (Wald $\chi^2 = 1.53$, $df = 1$, $P = 0.465$).

Experiment 2: antimicrobial effect of silk in vitro

Metarhizium grew in all of the replicate sectors and there was therefore no effect of either whole silk or silk extract on its growth. *Aspergillus flavus* developed in 10 of the 12 replicate sectors which had whole silk, but in no other sectors (Fisher's Exact Test $P < 0.001$).

Discussion

We found no evidence of a beneficial effect of silk on the resistance of *P. dives* weaver ants to the *Metarhizium* fungal parasite. Survival of ants was high whether they were with or without silk, although the sporulation of *Metarhizium* from some cadavers showed that infection of ants did take place. The experiment therefore had limited power to detect any beneficial effect of silk on survival. However, the numbers of ant cadavers sporulating with *Metarhizium* were similar with or without silk (4/10 cadavers when silk was present, compared with 2/11 when silk was not present). In addition, the ants, whether exposed to *Metarhizium* or the control solution, interacted very rarely with the silk during the observation period, providing no behavioural evidence for the ants utilising silk to resist parasites. Finally, neither silk nor silk extracts caused any inhibition of *Metarhizium* germination and growth in vitro. Silk in other insects has been shown to have antimicrobial properties (Li et al., 2007; Korayem et al., 2007), and it remains possible that the silk of *P. dives* may be active against other parasites or that the ants use their silk to provide a more general, colony-level resistance than tested here. Wood ants, for example, use tree resin both to enhance individual-level resistance and to provide colony-level protection against parasites (Castella et al., 2008; Chapuisat et al., 2007; Christe et al., 2003). However, the results in combination suggest that *P. dives* do not use, or gain individual-level benefits from, silk, at least when resisting the parasite and dose tested experimentally here.

Even if silk does not provide any direct antimicrobial benefit, it may still reduce exposure to disease if it is a particularly hygienic substrate from which to build a nest. However, this also does not appear to be the case in *P. dives*. Most of the silk plated in vitro produced growth of the facultative entomopathogen *A. flavus* and a significantly greater number of the control ants which died sporulated with *A. flavus* when they had been kept with silk than without. This together suggests that the silk is heavily contaminated with the fungus. *Aspergillus* has been recorded parasitizing and killing many ant species, as well as many other insects, either by infecting in isolation or by exploiting hosts stressed by other parasites (St Leger et al., 2000; St Leger et al., 1993; Hughes and Boomsma, 2004; Poulsen et al., 2006; Schmid-Hempel, 1998). The high rate of *Aspergillus* sporulation in our experiment from the surface-sterilised cadavers of *P. dives* supports it being a facultative parasite of this species as well. The use of silk to weave nests may be of some indirect benefit as a direct barrier to particular parasites or by allowing nests to be built in the less parasite-rich arboreal habitat (Boomsma et al., 2005). However, our results show that weaver ant silk harbours a significant threat from *Aspergillus* and therefore does not appear to be a sufficiently hygienic substrate to have allowed the evolutionary loss of the metapleural gland.

In spite of the lack of benefit from silk, *P. dives* workers appeared to be highly resistant to *Metarhizium*, at least compared to *Acromyrmex* leaf-cutting ants which show greater susceptibility to the strain and dose of parasite tested here (Hughes et al., 2004a). Some infection with *Metarhizium* did occur, as evidenced by cadavers sporulating with the characteristic *Metarhizium* conidia/conidiophores, but the ants were able for the most part to resist the dose used, even though this was relatively high. Although *P. dives* ants lack metapleural glands, they evidently have other defence mechanisms, such as self-grooming and other antimicrobial secretions (P.G. Graystock and W.O.H. Hughes, unpubl. data), which are quite effective against this parasite. *Polyrhachis* have been described as intermediate weaver ants, with less derived weaving behaviour than the

advanced weavers *Oecophylla* (Hölldobler and Wilson, 1990). Further work is therefore warranted to determine whether the silk of *Oecophylla* also lacks antimicrobial properties and harbours potential parasites, as well as to confirm whether the silk of *P. dives* is similarly inactive against other parasites. However, the results in combination suggest that at least in *Polyrhachis*, mechanisms other than silken nests may be needed to explain their evolutionary loss of the metapleural gland.

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