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Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants

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Within-host competition is an important factor in host–parasite relationships, yet most studies consider interactions involving only single parasite species. We investigated the interaction between a virulent obligate entomopathogenic fungus, *Metarhizium anisopliae* var. *anisopliae*, and a normally avirulent, opportunistic fungal pathogen, *Aspergillus flavus*, in their leaf-cutting ant host, *Acromyrmex echinatior*. Surprisingly, the latter normally out-competed the former in mixed infections and had enhanced fitness relative to when infecting in isolation. The result is most probably due to *Metarhizium* inhibiting the host's immune defences, which would otherwise normally prevent infections by *Aspergillus*. With the host defences negated by the virulent parasite, the avirulent parasite was then able to out-compete its competitor. This result is strikingly similar to that seen in immunocompromised vertebrate hosts and indicates that avirulent parasites may play a more important role in host life histories than is generally realized.

Keywords: virulence; competition; entomopathogen

1. INTRODUCTION

Although host–parasite relationships are restricted in most studies to involving only a single species of parasite, it is becoming increasingly clear that infections with multiple parasite species are commonplace in nature (Cox 2001; Read & Taylor 2001). Hosts represent limited resources, so the co-occurrence of different parasites within a single host makes within-host competition between the parasites inevitable. Interactions between different parasites are often considered to result in increased virulence, but may also lead to decreased virulence depending on the dynamics (May & Nowak 1995; Frank 1996; Cox 2001; Thomas *et al.* 2003). The nature of the within-host competition has been described as a continuum between two extremes: superinfections, which exhibit contest competition with the most virulent parasites and eliminate those less virulent, and coinfections, where the parasites differ little in virulence and resources end up being shared among individuals via scramble competition (Nowak & May 1994; May & Nowak 1995).

We examine the interaction between the fungal parasites *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) (Deuteromycotina: Hyphomycetes) and *Aspergillus flavus* Link. (Ascomycota: Eurotiomycetes) in the leaf-cutting ant host *Acromyrmex echinatior* Forel (Hymenoptera: Formicidae: Attini). *Metarhizium anisopliae* var. *anisopliae* is an obligate entomopathogenic fungus that is a generalist, infecting a wide range of insects, including leaf-cutting ants (Schmid-Hempel 1998; Jaccoud *et al.* 1999; Hughes *et al.* 2002). *Aspergillus flavus* is a facultative pathogen of many plants and animals (Boucias & Pendland 1998; St Leger *et al.* 2000) and appears to be an occasional parasite of leaf-cutting ants, including *A. echinatior* (Schmid-Hempel 1998; A. N. M. Bot, unpublished data; W. O. H. Hughes, personal observation). Both parasites are semelparous 'obligate killers' (Ebert & Weisser 1997): they grow inside their host, eventually killing it, and produce transmission stages only after host death.

2. MATERIAL AND METHODS

Four colonies of *A. echinatior*, originally collected from Gamboa, Panama, were used as sources of experimental ants. The isolate of *M. anisopliae* var. *anisopliae* used (KVL02-73) had been isolated from soil at the same field site as that from which the ants were collected, while the strain of *A. flavus* used had been isolated from an *A. echinatio*r worker.

Spore (conidia) suspensions were made and the concentration of spores quantified with a haemocytometer. The viability of the spore suspensions was checked (Lacey & Brooks 1997) and was greater than 95% in all cases. The suspension of *A. flavus* was serially diluted to give suspensions of 1×10^7, 1×10^6, 1×10^5 and 1×10^4 spores ml^{-1}. A 1×10^5 spores ml^{-1} suspension of the *M. anisopliae* var. *anisopliae* isolate was also made. We tested a range of doses of *A. flavus* because, unlike *M. anisopliae* var. *anisopliae*, the dynamics of this parasite has not previously been investigated in leaf-cutting ants. Finally, a mixed suspension of *M. anisopliae* var. *anisopliae* and *A. flavus* was made that contained 1×10^5 spores ml^{-1} in total, with an equal proportion originating from each species.

Large workers (head width of 2.1–2.4 mm) were removed from each of the colonies and placed individually in plastic pots (diameter of 2.5 cm, height of 4 cm), where they were maintained at 24 °C with an *ad libitum* supply of water and 10% sucrose water. The ants were treated by applying 0.5 µl of a spore suspension to their thorax using a micropipette. Control ants had 0.5 µl of a 0.05% Triton-X solution applied in the same way. Eight ants from each of the four colonies were treated with each of the four dilutions of *A. flavus* spores alone, with the suspension of *M. anisopliae* var. *anisopliae* spores alone, with the suspension containing both *A. flavus* and *M. anisopliae* var. *anisopliae* spores, or with a control solution of 0.05% Triton-X. Following application, ant mortality was assessed daily for 16 days. The survival of ants over this period was analysed using a Cox regression analysis, which allows for the inclusion of surviving individuals as censored cases (Cox 1972; SPSS 1999). Ants that died during the 16-day period were surface sterilized (Lacey & Brooks 1997) and placed in a Petri dish lined with damp filter paper. Ten days after the end of the experimental period, the cadavers of these ants were scored for the presence of either *A. flavus* or *M. anisopliae* var. *anisopliae*, both of which produce characteristic external conidia and conidiaphores (Boucias & Pendland 1998). The proportions of ants sporulating were compared using *G*-tests for heterogeneity.

3. RESULTS

The survival distributions of the ants differed significantly between the treatments (Wald = 24.2, d.f. = 6, $p < 0.001$; figure 1*a*) (see electronic Appendix A: available on The Royal Society's Publications Web site). Ant survival at the three lowest doses of *A. flavus* did not differ significantly from that in the control treatment, but ants treated with either the highest dose of *A. flavus*, the *M. anisopliae* var. *anisopliae* suspension or the mixed suspension suffered significantly greater mortality. The proportion of

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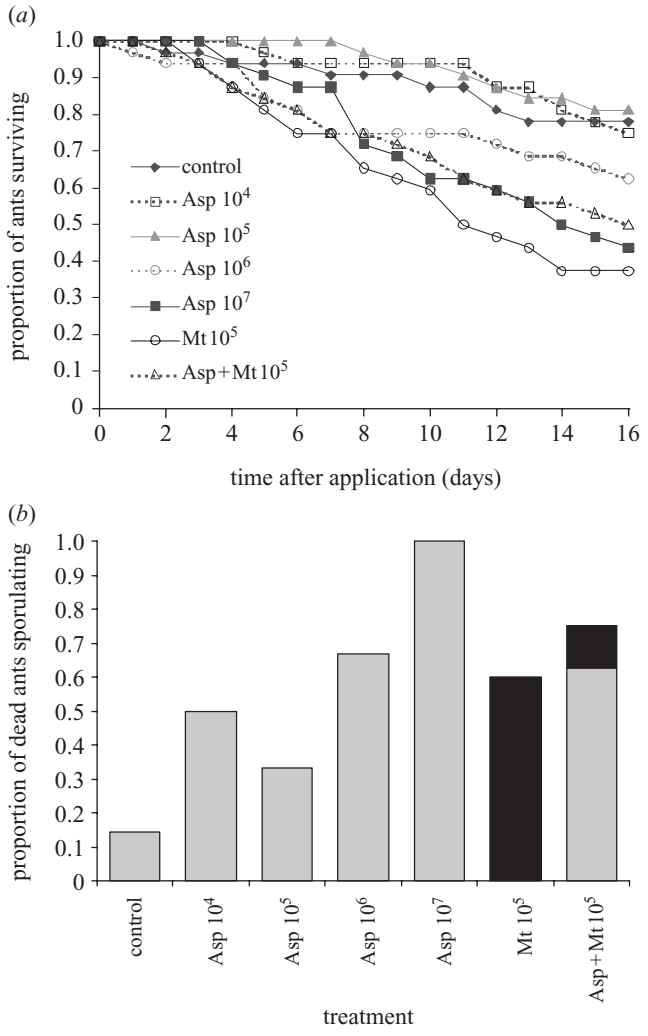

Figure 1. (*a*) Survival of ants treated with either a range of doses of *Aspergillus flavus* (Asp), a 1×10^5 spores ml^{-1} dose of *Metarhizium anisopliae* var. *anisopliae* (Mt), a mixed 1×10^5 spores ml^{-1} dose of *A. flavus* and *M. anisopliae* var. *anisopliae* (Asp + Mt), or a control solution. (*b*) Proportion of cadavers of ants exposed to the above treatments that sporulated with either *M. anisopliae* var. *anisopliae* (dark shading) or *A. flavus* (light shading).

dead ants sporulating also differed significantly between the treatments ($G_{\mathrm{Het}} = 27.4$, d.f. = 6, $p < 0.01$), with a high sporulation rate of *M. anisopliae* var. *anisopliae* from the cadavers of ants that were treated with *M. anisopliae* var. *anisopliae* alone and with more cadavers sporulating with *A. flavus* when higher doses of *A. flavus* were applied (figure 1*b*). A single control ant also produced *A. flavus* spores, indicating the presence of the latent *A. flavus* enzootic that appears to often be present in colonies of *Acromyrmex* (A. N. M. Bot, unpublished data; W. O. H. Hughes, personal observation). Out of the three treatments involving a dose of 1×10^5 spores ml^{-1}, significantly more ants sporulated when treated with either *M. anisopliae* var. *anisopliae* alone or with the mixture of *M. anisopliae* var. *anisopliae* and *A. flavus* than when treated with *A. flavus* alone ($G_{\mathrm{Het}} = 12.5$, d.f. = 2, $p < 0.01$). In the mixed infection treatment, the sporulation rate consisted predominantly of *A. flavus* with very few of the cadavers producing *M. anisopliae* var. *anisopliae* spores.

4. DISCUSSION

These results provide an intriguing contrast to the dynamics that are normally assumed to occur during interspecific within-host competition between parasites. That *A. flavus* is pathogenic to *Acromyrmex* ants was demonstrated by the increased mortality of ants treated with increasing doses of *A. flavus* spores, thus confirming previous findings (Schmid-Hempel 1998, A. N. M. Bot, unpublished data; W. O. H. Hughes, personal observation). The mortality of ants treated with the 1×10^5 spores ml^{-1} suspension of *A. flavus* was significantly lower than of those ants treated with the same dose of *M. anisopliae* var. *anisopliae* spores, confirming that *A. flavus* is less virulent than *M. anisopliae* var. *anisopliae*. In an interaction between a virulent and an avirulent parasite, the former would be expected to dominate. The fact that the virulent parasite used was also an obligate entomopathogen, whereas the avirulent parasite was only an opportunistic parasite, further supports this prediction. However, when ants were exposed to a mixed application of *M. anisopliae* var. *anisopliae* and *A. flavus*, it was the latter that appeared to dominate. Cadavers only ever produced spores of one or the other parasite, and never both. Almost all of the cadavers that sporulated produced only *A. flavus* spores. The dynamics was that of a superinfection (Nowak & May 1994), except that it was the less virulent parasite that was the superior competitor. Furthermore, *A. flavus* actually gained from superinfection with *M. anisopliae* var. *anisopliae* because it sporulated from more ants during mixed infections than when it was applied at the same dose in isolation.

The results seem most likely to derive from the interactions between the two parasites and the host's immune system. Invertebrates have an innate immune system that includes both cellular and humoral components (Rolff & Siva-Jothy 2003). Natural *A. flavus* infections of leafcutting ants (A. N. M. Bot, unpublished data; W. O. H. Hughes, personal observation) and other animals (Boucias & Pendland 1998; St Leger *et al.* 2000) are rare, and this appears to be because the immune system is normally able to prevent successful infections. Although *A. flavus* can release immunodepressant toxins and is able to produce hyphae in insects in spite of the host's immune response, it is relatively poor at doing this and proliferates through the haemocoel only very slowly (Boucias & Pendland 1998; St Leger *et al.* 2000). By contrast, the hyphae of *M. anisopliae* var. *anisopliae* are well known to produce toxins that are extremely effective at inhibiting the immune responses of many insects (Huxham *et al.* 1989; Boucias & Pendland 1998). The germination of *Aspergillus niger* spores has been shown to increase as a result of the inhibition of the encapsulation response by the toxins produced by *Metarhizium* (Vey *et al.* 2002). A similar effect has been reported for *Candida albicans* yeast cells injected shortly after infection by *Beauveria bassiana* (Hung *et al.* 1993), another specialized entomopathogenic fungus that produces immunodepressant toxins. The most likely explanation for the increased success of *A. flavus* during mixed infections with *M. anisopliae* var. *anisopliae* is that the toxins produced by *M. anisopliae* var. *anisopliae* hyphae degraded the immune response of the host to the point that it was ineffective at preventing the spread of *A. flavus*. With the immune response depressed, *A. flavus* was

then able to out-compete its otherwise more virulent competitor.

The effect documented here is strikingly similar to that seen in immunocompromised vertebrates, where otherwise avirulent micro-organisms can become virulent pathogens. Indeed, infections of *Aspergillus* in humans are almost entirely limited to immunocompromised patients because of the effectiveness of the immune response in healthy individuals (Ampel 1996; Roilides & Meletiadis 2003). There are many described cases in vertebrates of parasites showing increased virulence during concomitant infection with other parasites that depress the host immune response (Cox 2001). In invertebrates the data are more limited, but certain parasitoid wasps are known to rely on poly-DNA viruses to suppress the immune responses of hosts (Godfray 1994), and it is well established that stressed or unhealthy hosts are more susceptible to infection because of depressed immune systems (Rolff & Siva-Jothy 2003). Given that concomitant infections of multiple parasites are commonplace (Cox 2001; Read & Taylor 2001) and that many parasites will have some impact upon the host's immune system, understanding the nature of this impact will be critical to understanding the dynamics of the interaction. Our results demonstrate that the outcome of within-host competition between parasites may not necessarily be as would be predicted from the virulence of parasites infecting in isolation, and that avirulent parasites may play a far greater role than is generally recognized.

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