

## Mixed infections and insect–pathogen interactions

Matthew B. Thomas<sup>1,2\*</sup>, Emma L. Watson<sup>2</sup> and Pablo Valverde-García<sup>2,3</sup>

<sup>1</sup>Department of Agricultural Sciences, Imperial College, Wye, Kent TN25 5AH, UK

<sup>2</sup>Centre for Population Biology, Imperial College, Silwood Park, Ascot, Berks SL5 7PY, UK

<sup>3</sup>Entomología Agrícola, Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, Universidad de Córdoba, Apartado 3048, Avda. Menéndez Pidal s/n, 14080 Córdoba, Spain

\*Correspondence: E-mail: m.thomas@ic.ac.uk

## Abstract

Most studies of insect–pathogen interactions consider the direct interaction between one disease agent and one species of host. However, given that hosts are subject to challenge from many pathogen/parasite species, mixed infections are probably common. In this study, using the desert locust and two species of fungal entomopathogen, we show how mixed infection with a largely avirulent pathogen can alter the virulence and reproduction of a second, highly virulent pathogen. We find that two strains of the avirulent pathogen vary in their interaction with the virulent pathogen, depending on the order of infection and environmental conditions. We propose that avirulent pathogens, which have largely been overlooked to date, could play a significant role in host–pathogen dynamics, with implications for biological control and evolution of virulence.

## Keywords

Biocontrol, disease dynamics, fungal entomopathogens, insect–pathogen interactions, locusts and grasshoppers, mixed infection, super infection, virulence.

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## INTRODUCTION

Over the years there have been many studies of insect–pathogen/parasite interactions, with emphases ranging from fundamental research into host and/or pathogen dynamics, through to investigations into use of microbial agents for biological pest control. Whether empirical or theoretical in nature, the great majority of these studies consider the direct interaction between one disease agent or parasite, and one host. However, evidence from a range of host–pathogen systems indicates that ‘concomitant’ or ‘mixed’ infections involving two or more parasite species or genotypes, are common, and may even be the rule (Cox 2001; Read & Taylor 2001). For example (and this list is by no means exhaustive), mixed infections and their effects have been detailed in numerous mammal (e.g. Nilssen *et al.* 1998; Behnke *et al.* 2001; Cox 2001), bird (e.g. Forbes *et al.* 1999), reptile (Schall & Bromwich 1994; Lainson 2002) and fish (Sousa *et al.* 1996; Barker *et al.* 2002) host systems. Given that insects are subject to challenge from a diversity of parasites and pathogens [e.g. see Tanada & Kaya (1993) and references therein], with potential for simultaneous exposure to multiple pathogen species or strains (e.g. Lecuona *et al.* 1996; Malakar *et al.* 1999; Ishii *et al.* 2002), mixed infections are also likely to be common in insect hosts. Indeed, there are a number of studies which investigate interactions between pathogens and/or parasites in inverte-

brates (e.g. Ritter & Tanada 1978; Barbercheck & Kaya 1990; Chandler *et al.* 1993; Inglis *et al.* 1997, 1999; Koppenhöfer & Kaya 1997; Korenberg *et al.* 1999; Malakar *et al.* 1999; Ishii *et al.* 2002). These examples notwithstanding, amongst the vast body of research which exists in insect–parasite/pathogen interactions, studies of mixed infections remain rare.

In mixed infections, complex interactions between pathogens and host may arise such that the burden of one or both of the infectious agents may be increased, one or both may be suppressed, or one may be increased and the other suppressed (Cox 2001). As such, mixed infection has the potential to dramatically alter population dynamics of a particular insect–pathogen interaction, something which has received very little attention to date.

In this study, we examined mixed infection in the desert locust, *Schistocerca gregaria* (Forskål). The primary pathogen was a strain of the mitosporic fungus, *Metarhizium anisopliae* var. *acridum* (Driver, Milner & Trueman.); a virulent pathogen utilized in biocontrol of locusts and grasshoppers in Africa, and which is at present being tested for locust control in Spain (Lomer *et al.* 2001). The co-infecting pathogens were two strains of another mitosporic fungus, *Beauveria bassiana* (Bals.), isolated from locust hosts in Spain. Our aim was to examine how mixed infection with these indigenous pathogens affect performance of *M. anisopliae*.

## METHODS

We conducted two bioassays to explore the effects of mixed infection and how environmental factors may mediate the interactions between host and pathogens. The test locusts were acquired as fifth instar nymphs from Blades Biological (Edenbridge, Kent, UK), and held in standard aluminium locust cages with mesh climbing frames and light bulbs until use in the bioassays as 3–5-day-old adults (Elliot *et al.* 2002). Locusts were of mixed sex with 1 : 1 sex ratio. The isolate of *M. anisopliae* var. *acridum* (IMI 330189) was the standard isolate used in locust biocontrol in Africa (Lomer *et al.* 2001) and was obtained from the mycological culture collection held by CABI Bioscience, UK. The *B. bassiana* isolates were two of 20–30 strains which we isolated from locust hosts in La Serena, Spain, during surveys in June 2000.

In both assays, locusts were infected following a standard technique in which individual adult locusts were inoculated with a 2- $\mu$ L drop of spore suspension, placed at the base of the pronotal shield (see Prior *et al.* 1995; Blanford & Thomas 1999; Arthurs & Thomas 2001; Elliot *et al.* 2002). Single pathogen treatments used  $10^3$  spores per insect. Combination treatments used  $10^3$  spores of each isolate per insect. Controls received blank peanut oil formulation. Locusts were fed freshly cut wheat seedlings and frass was removed daily. Time to death for each insect was recorded. Dead locusts were removed from the cages and placed on filter paper in aerated Petri dishes for 2 days at 20 °C. The filter paper was subsequently moistened with sterile distilled water and the cadavers observed and incubated under humid conditions for a further 5–7 days to encourage sporulation (Arthurs & Thomas 2001; Elliot *et al.* 2002). Cumulative mortality responses of locusts in both bioassays were analysed using Kaplan–Meier Survival Analysis (SPSS vs. 10.0 for Windows), with differences between treatment mean survival times compared by the log-rank test. Proportion data for sporulation were analysed using ANOVA following logit transformation (using R v.1.4.1).

In the first bioassay, we examined each of the fungal isolates separately, together with combinations of *M. anisopliae* and one or other of the *B. bassiana* isolates, either as simultaneous co-infections, or as sequential infections with *B. bassiana* applied 4 days before *M. anisopliae* (these prior treatments of *B. bassiana* reflecting the biocontrol scenario where *M. anisopliae* is introduced into a locust population carrying indigenous pathogens). Three replicates of 12 locusts each were used per treatment. In this initial assay, insects were maintained at 30 °C to facilitate pathogen growth (Blanford & Thomas 1999; Inglis *et al.* 1999; Elliot *et al.* 2002).

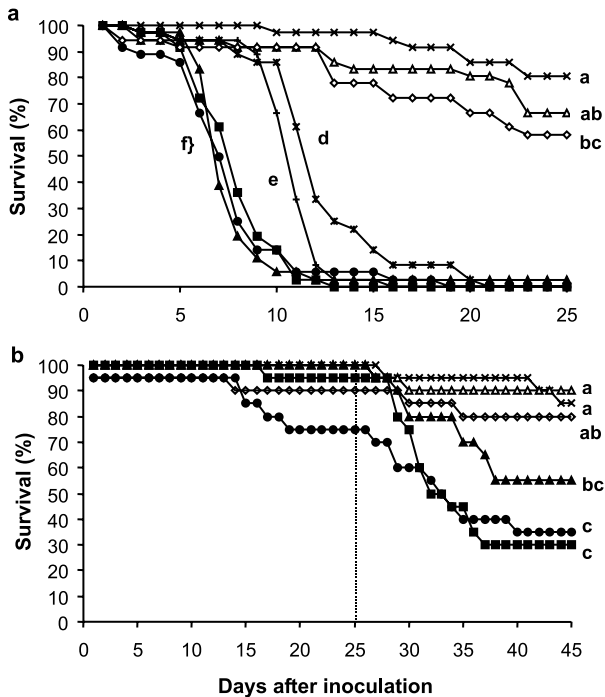
Locusts are active behavioural thermoregulators with capacity to mount fever responses upon infection (Blanford & Thomas 1999; Elliot *et al.* 2002). These behaviours have

been shown to affect pathogen virulence and locust fitness (Elliot *et al.* 2002). Therefore, we conducted a second bioassay under a more realistic fluctuating temperature regime with locusts maintained at 20 °C for 10 h during the night, followed by a gradual increase in temperature to a peak of 42 °C for 4 h during the day. These conditions simulate the diurnal fluctuation in average body temperatures of locusts infected with *M. anisopliae*, with active behavioural thermoregulation and fever during the day, and simple thermoconforming to track ambient temperatures during the night (Blanford & Thomas 1999; 2000; Elliot *et al.* 2002). Locusts were maintained under these conditions for 25 days following infection, at which time temperatures were returned to a constant 30 °C to facilitate expression of any surviving pathogens (and which in the field would be consistent with a change in thermoregulatory conditions through, for example, increased cloud cover). Insects were inoculated as before, although for technical reasons, we were not able to include sequential infection treatments.

In addition, to investigate the host immune response further, an additional batch of locusts were infected with the *Metarhizium* and *Beauveria* isolates, to determine the effects of infection on preferred body temperatures and identify any behavioural fever responses. Preferred body temperatures were estimated using an aluminium thermal gradient providing a temperature range from 25 to 50 °C. Locusts were introduced into the middle of the gradient and were left for 30 min to acclimate. Body temperatures were then recorded every 15 min for 1 h before returning the locusts to their cages which were maintained at 30 °C. Body temperature measurements were taken for each of the single pathogen treatments together with controls ( $n = c. 50$  per treatment) on days 4 and 5 after inoculation, and again on day 13 for the *Beauveria* and control treatments only. Pairwise comparisons of the preferred temperature distributions for each treatment were conducted using the Mann–Whitney test (SPSS). Further details of the methodology can be found in Blanford & Thomas (1999) and Wilson *et al.* (2002).

## RESULTS

In line with previous studies (e.g. Prior *et al.* 1995; Blanford & Thomas 1999; Elliot *et al.* 2002), under the constant temperature conditions of the first bioassay, *M. anisopliae* was highly virulent with mean survival time of infected locusts around 8 days (Fig. 1a) and 75% of cadavers sporulating to produce new fungal conidia. In contrast, the *B. bassiana* isolates showed very low virulence with only one of them (isolate 2) affecting survival time relative to controls, and just one cadaver showing any sporulation. The simultaneous mixed-infection treatments showed no effects beyond those consistent with the activity of *M. anisopliae* alone (i.e.



**Figure 1** Cumulative survival of desert locusts following single and co-infection with three strains of fungal pathogen. (a) The results of a bioassay at 30°C; (b) results of a second bioassay in which locusts experienced fever temperatures during the day for the first 25 days and were then maintained at constant 30°C. Treatments are denoted by: control (×); *M. anisopliae* (■); *B. bassiana* isolate 1 (△); *B. bassiana* isolate 2 (◇); simultaneous co-infection of *M. anisopliae* and *B. bassiana* isolate 1 (▲); simultaneous co-infection of *M. anisopliae* and *B. bassiana* isolate 2 (●); infection with *B. bassiana* isolate 1 before *M. anisopliae* (\*); infection with *B. bassiana* isolate 2 before *M. anisopliae* (+). Different lower case letters indicate significant differences in mean survival time between treatments at the 5% level.

equivalent survival times and percentage sporulation). However, pre-inoculation with *B. bassiana* produced an apparent interaction between pathogens whereby *Beauveria*-isolate 2 caused a 1-day reduction in locust survival time (when accounting for the lag in infection with *M. anisopliae*) and both caused a significant decrease ( $P < 0.001$ ) in sporulation of *M. anisopliae* to  $<20\%$ .

The simulated fever regime substantially reduced pathogen virulence with little or no mortality in any of the single-pathogen treatments during the first 25 days (Fig. 1b). However, *M. anisopliae* performed better in this first period under mixed infection with *Beauveria*-isolate 2, causing a significant reduction in locust survival compared with all other treatments, with 80% of the resulting cadavers sporulating.

On return to 30 °C, mortality of locusts infected with *M. anisopliae* (either singly or in combination) increased

greatly, with  $>60\%$  sporulation. At this time, however, the positive interaction with *Beauveria*-isolate 2 was no longer apparent and instead, there was a negative effect of *Beauveria*-isolate 1 reducing mortality and sporulation of *M. anisopliae* by 30–40% (Fig. 1b). This is in spite of the fact that this *Beauveria* isolate appeared to have no significant effect on the host itself.

The thermal gradient observations revealed a significant difference ( $P < 0.05$ ) in preferred body temperatures between treatments, with *Metarhizium*-infected locusts and those infected with *Beauveria*-isolate 1, selecting significantly higher temperatures (means of 40 and 38.2 °C, respectively) compared with locusts treated with *Beauveria*-isolate 2 and the controls (both  $c. 36$  °C) at days 4 and 5 after inoculation. Thirteen days after inoculation, locusts treated with *Beauveria*-isolate 1 showed a similar significant increase in preferred temperature compared with *Beauveria*-isolate 2 and control treatments.

## DISCUSSION

These results indicate that the performance (i.e. speed of kill as a measure of virulence and sporulation as a fitness correlate) of a virulent insect pathogen may be altered by the presence of another, largely avirulent pathogen. Moreover, the co-infecting pathogens could act independently, synergistically or antagonistically depending on environmental conditions and order of infection.

The mechanisms for these diverse effects remain unclear. In vertebrate systems, the possibility for enhanced performance of individual parasite clones or species may occur through suppressive effects on host immune responses. Competitive interactions between parasites (including exploitation, interference and apparent competition) may also occur, affecting both the within-host dynamics of individual parasites and host health (see Cox 2001 and Read & Taylor 2001 for review and discussion). For invertebrates, the types of interactions and possible mechanisms appear similar. The increased virulence of *M. anisopliae* under co-infection with *Beauveria*-isolate 2 in the simulated fever regime, is consistent with a number of studies which report additive (Fuxa 1979; Koppenhöfer & Kaya 1997) or synergistic effects (Fuxa 1979; Barbercheck & Kaya 1990; Koppenhöfer & Kaya 1997; Malakar *et al.* 1999) following co-inoculation of insect hosts with entomopathogens. This synergy might result from an interspecific interaction, such as the production of toxic metabolites by *B. bassiana* (Hung *et al.* 1993), acting to reduce host cellular defences and in so doing, indirectly enhancing the performance of *M. anisopliae*. A similar host-mediated effect could result if the high temperatures lead to increased host recognition of *B. bassiana* hyphal bodies [e.g. through a change in cell wall structure, see Hajek & St Leger (1994) and Gillespie *et al.* (1997) and

references therein], promoting an active immune response and causing an effective depletion of host haemocytes. There may also be a dose–response effect, with the higher number of spores from the combined treatment acting to saturate the host immune response (importantly, this dose effect is a real consequence of mixed infection because the simultaneous or sequential challenge by more than one pathogen is additive). Interestingly, the positive interaction was most apparent under conditions which were suboptimal for the pathogens. In a similar way, Inglis *et al.* (1997) and Malakar *et al.* (1999) report synergistic effects of mixed infections becoming apparent at higher temperatures.

Considering the negative effects of mixed infection, for at least one of the *Beauveria* isolates, the observed fever response indicates host recognition and the possibility of some level of heterologous immunity (Clark 2001). The immune response of locusts involves cellular encapsulation and phagocytosis of foreign bodies such as fungal blastospores (Bidochka *et al.* 1997) and it is possible that the level of haemocyte activity, together with other non-specific elements of the immune response (see Gillespie *et al.* 1997; Wilson *et al.* 2001), are enhanced under multiple disease challenge. Interestingly, prior activation of the immune system, as occurs in sequential infection, may result in stronger heterologous effects than simultaneous activation by two or more parasites (see Lowenberger *et al.* 1999); a response which is consistent with the results in the first bioassay.

There is also the possibility that direct antagonistic interactions between pathogens could lead to reduced virulence and reproduction of *M. anisopliae*. For example, production of antifungal metabolites has been reported in some isolates of *B. bassiana* (Gemma *et al.* 1984), although mixed *in vitro* culture of our study isolates indicated no obvious zones of inhibition between colonies (M.B. Thomas, E.L. Watson, P. Valverde-Garcia, unpublished data). In addition, competition for nutrients within the host haemocoel is likely to occur. Studies on mixed virus infections in lepidoptera indicate that direct competition for common host resources may limit pathogen replication (Ishii *et al.* 2002). An effective reduction in pathogen replication or reproduction may also occur where mortality is enhanced through mixed infection (Malakar *et al.* 1999). That said, one of the most striking results in the current study was the reduction in sporulation of *M. anisopliae* under certain conditions of mixed infection, without necessarily strong effects on mortality rate. *Metarhizium anisopliae* is a relatively weak saprophyte and it seems that, in general, the process of cadaver colonization is quite sensitive to interactions with other pathogens (see also Ritter & Tanada 1978; Barbercheck & Kaya 1990; Chandler *et al.* 1993; Inglis *et al.* 1999; Elliot *et al.* 2002; Ishii *et al.* 2002).

One additional insight to emerge from this study is that the magnitude of behavioural fever appears to vary depending on which pathogen is infecting. On days 4 and 5 following infection, *Metarhizium* infection induced a fever of *c.* 4 °C, which is in agreement with previous studies (e.g. Blanford & Thomas 1999). *Beauveria*-isolate 1, on the other hand, induced a fever of just 2 °C and isolate 2 appeared to induce no fever at all. On day 13, a fever response was again detected with *Beauveria*-isolate 1, whereas locusts infected with isolate 2 still showed no response. Of note here is that of these two *Beauveria* isolates, isolate 2 appears marginally more virulent, with isolate 1 apparently having little direct effect. This indicates that measurement of pathogen-induced effects in terms of just host mortality, may miss important consequences of infection on, for example, host physiology and behaviour.

In a recent theoretical study, Gardner & Thomas (2002) suggested that the behavioural fever response should carry costs with, for example, high temperature causing physiological or cellular damage, and extended basking reducing feeding and increasing susceptibility to predators. An associated prediction is that infected hosts should exhibit a variable fever response with extent and magnitude of fever scaled in line with the thermal sensitivity and virulence of the disease (Gardner & Thomas 2002). The results of the current study provide some support for this prediction and suggest that costs of defence may be balanced by tailoring investment in induced defence to the different pathogens.

As indicated in the introduction, there is a growing recognition that mixed infections are likely to be common. A key result of the current study is that through mixed infection, avirulent pathogens, which hitherto have been given little attention and may go largely undetected in the field (and may not even result in infection proper but simply elicit an immune response from the host), could play a significant role in mediating the outcome of coupled host–pathogen interactions. Pathogen virulence and productivity (or variants thereof) are identified as key parameters in the vast majority of host–pathogen models. From a population dynamic perspective, our results suggest that mixed infections with avirulent pathogens could contribute unexpected variability in host–pathogen dynamics over time and/or space. In terms of biocontrol, this could translate to variable efficacy of an agent depending on the nature of the resident microbial community. With respect to our study system, for example, the background prevalence of *B. bassiana* in locust populations in Spain has been observed to range from <2 to >50% in different sites and years (Hernandez-Crespo & Santiago-Alvarez 1997; M.B. Thomas, E.L. Watson, P. Valverde-Garcia, unpublished data). Accordingly, we would anticipate considerable variation in prevalence of mixed infections following biopesticide spray treatments of *M. anisopliae* and through this, different patterns of mortality

and pathogen spread. As an extension of this suggestion, there is at present considerable global interest in invasive species and emergent infectious diseases. In this context, our results suggest potential for avirulent, non-lethal indigenous diseases to mediate the fitness and establishment potential of virulent, exotic pathogens, providing mechanisms whereby microbial community diversity may influence invasion resistance (cf. Levine & D'Antonio 1999).

Finally, one area where mixed infection has attracted considerable attention in recent years is in the study of the evolution of virulence (e.g. van Baalen & Sabelis 1995; Ebert & Mangin 1997; Gandon *et al.* 2001; Brown *et al.* 2002). In this literature, models and data are presented which indicate that mixed infection can lead to the evolution of either increased or decreased virulence; the outcome depends critically on specific aspects of parasite activity or behaviour, and host response to (super)infection. In this context, the strong condition-dependency of mixed infection identified in the current study adds complexity, and points to the need for further detailed studies of the mechanisms of mixed infection.

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