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Evidence for a cost of immunity when the crustacean *Daphnia magna* is exposed to the bacterial pathogen *Pasteuria ramosa*

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Summary

1. The deployment of the immune system has the obvious potential to ameliorate infection outcomes, but immune responses can also harm hosts by either damaging host tissues or monopolizing resources, leading to enhanced mortality. To gain insight into such a 'cost of immunity' when the crustacean *Daphnia magna* is challenged with the bacterium *Pasteuria ramosa*, we measured survivorship among hosts that resisted infection following exposure to various strains and doses of the parasite.
2. In the first of two experiments, these exposures were: single exposures with relatively non-aggressive strains, double exposures with non-aggressive strains, and exposure to aggressive strains. Mortality increased across this gradient of exposure. In a second experiment, we varied the dose of the most aggressive *P. ramosa* strain and found that resisting infection when a large dose was applied resulted in greater mortality than when a medium or low dose was applied.
3. Assuming that resistance is accomplished with an immune response, and that more aggressive parasites and/or larger doses of parasites are more immunostimulatory, these data are compatible with a cost of immunity. Indeed, in terms of survival, resisting parasites can be more harmful than infection.

Key-words: acquired immunity, cost of resistance, immunopathology, innate immunity, parasite, specificity.

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Introduction

Parasites are ubiquitous and frequently cause considerable harm to their hosts. However, harm during the infection process may have more than one source. First, parasites directly cause harm through their own activities that damage host tissue or alter host behaviour. Secondly, parasitism indirectly harms because immune responses may self-harm. Such a cost of immunity may be due to the immune response damaging host tissue (typically called immunopathology), or because immune systems monopolize resources that could have been used for other important functions. Depending on the magnitude of their fitness effects, costs of immunity may play a crucial part in the evolution of virulence or how host–parasite coevolution impacts population genetic structure or breeding systems (e.g. Haldane 1949; Lively 1987; Hamilton, Axelrod & Tanese 1990; Jokela, Schmid-Hempel & Rigby 2000).

It is important to distinguish the two main types of costs that are commonly discussed in the coevolutionary literature. The first cost is associated with genetic differences among hosts: some genotypes invest heavily in defence systems at the expense of other functions. Genetic-based costs are a form of pleiotropy and are evident as reduced performance of resistant compared with susceptible genotypes in the absence of parasitism. The second cost is the cost of launching an immune response, which is simply the loss in performance suffered due to the energetic requirements or immunopathological effects of immune responses. This type of cost is detected by comparing the fitness of hosts that have deployed their immune systems to those that have not.

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The present study concerns this second cost of immunity – the cost of launching an immune response. A significant challenge when trying to detect such a cost is to stimulate an immune response and measure the consequences of that response while not measuring direct costs of parasitism. To circumvent this problem, studies have, for example, stimulated immune responses by exposing hosts to compounds that mimic parasites (Moret & Schmid-Hempel 2001) or used tissue grafts to investigate self-reactivity (Sadd & Siva-Jothy 2006). An alternate approach, pioneered by Hasu, Valtonen & Jokela (2006) and that the present study adopts, is to study live parasite strains, but include noninfective host–parasite combinations. The major assumption here is that resistance is due to a host immune response, and if true, reductions in host fitness will be due to the costliness of that immune response. Although making this assumption in the absence of explicit measurement of an immune reaction is not faultless, this approach has advantages over the use of pathogen mimics or dead pathogens because these may not qualitatively or quantitatively stimulate immune responses to the extent a natural invasion route would.

We studied host mortality when the crustacean *Daphnia magna* was exposed to one of four parasite strains of the bacterium *Pasteuria ramosa*. Two of these strains are not aggressive, being essentially noninfective on the host clone studied, while the other two strains are highly infective. We assumed that the cases where an infection failed to establish (essentially always with the noninfective strains, occasionally with the infective ones) were attributable to an immune response. Our experimental designs tested this assumption. In particular we predicted that immune responses, and thus costs of immunity, should intensify as treatments moved from single exposures with relatively non-aggressive strains, to double exposures with non-aggressive strains. Resisting aggressive strains should see the cost of immunity rise still higher, with higher doses of aggressive strains inducing greater costs than lower doses. These predictions were met and, further, hosts that resisted infection showed higher mortality than those that succumb to infection.

Materials and methods

HOSTS AND PARASITES

Daphnia magna is a cyclically parthenogenetic crustacean found in temperate freshwater ponds. The *D. magna* clone used in this study was collected from a pond near Gaarzerfeld, northern Germany in 1997. *Pasteuria ramosa* is a naturally occurring bacterial endoparasite of *D. magna*. Infection occurs when spores are released from dead hosts and then ingested through filter feeding. *Pasteuria ramosa* infection causes a severe reduction in host fecundity. Four *P. ramosa* strains were used in this study, and these were isolated from the same pond at the host clone in 1997. These strains, designated Sp1, Sp7, Sp8 and Sp13, have been studied extensively since their original description (Carius, Little & Ebert 2001) and they vary substantially in their infectivity when exposed to this particular host clone. Strains Sp8 and Sp13 are essentially incapable of establishing infection (0–3% of hosts become infected when exposed to this strain), Sp1 is highly infective (80–100% infection) and Sp7 is of intermediate infectivity (20–60% infection). Prior to the experiments, solutions containing 50 000 transmission spores per mL were made up for each parasite strain and stored at –20 °C until required.

EXPERIMENT I: PATHOGENIC AND NON PATHOGENIC STRAINS

The aim of this experiment was to study host mortality when resisting relatively non-aggressive parasites, when resisting double exposure to these non-aggressive parasites, and when resisting pathogenic parasites. Prior to experiments, *Daphnia* were kept for three generations in 20 jars containing 1·5 L of *Daphnia* medium (Aachener Daphnien Medium; Klüttgen *et al*. 1994) at 20 °C in a light : dark (LD) 12 : 12 h cycle, with 100 *Daphnia* per jar. The animals were fed $3{\cdot}5 \times 10^6$ algal cells (*Scenedesmus* sp.) per *Daphnia* per day and water was changed every other day. The experiment was a 'split-jar' design (analogous to a split-brood design), and thus on the first day of the experiment 42 second to fourth brood female neonates (< 24 h old) were collected from each of the 201·5-L jars and split into seven groups. These seven groups, each a jar containing six *Daphnia*, were: exposure to no spores (NS), single exposure to Sp8, Sp13, Sp1 or Sp7 or dual exposure to SP13 or Sp8 (Fig. 1).

For hosts in the dual exposure treatments parasite spores were added on both day 1 and day 6, while in the single exposure treatments, parasite spores were added only on day 6. All hosts remained in the jars with parasite spores until day 10. For this 10-day period, hosts were kept in 60 mL jars containing *Daphnia* medium, a small quantity of sand and 1 mL of the appropriate spore solution (NS Controls received 1 mL of *Daphnia* water). They were maintained at 20 °C in a LD 12 : 12 h cycle, and were fed 5×10^5 algal cells per *Daphnia* per day. Jars were randomly allocated to trays (24 jars per tray) and both jars and trays were rotated daily to minimize positional effects. For the post-exposure phase, beginning on day 11, *Daphnia* were moved to 200 mL jars (12 jars per tray) and feeding was

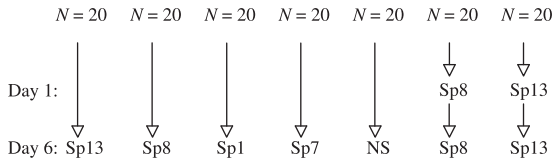

Fig. 1. Schematic of the experimental design to test for a cost of immunity.

increased to $3{\cdot}5 \times 10^6$ algal cells per Daphnia per day. The lower food during the infection period encourages browsing on the jar bottom, which increases contact with parasite spores. During the post-exposure phase, medium was changed every other day and, again, jars and trays were rotated daily to minimize positional effects. The post-exposure phase lasted until day 31, and during this time we recorded mortality and daily offspring production in each jar. At the end of the experiment the number of infected and uninfected females was recorded. Hosts that died before the age of 12 (the day on which infections with parasite–host combinations from this population are easily detectable) were not included.

Logistic regression (SAS procedure GENMOD with the scale = dev option invoked to account for some minor overdispersion of the data) was used to relate mortality (the proportion of individuals dying in each jar) to a single explanatory variable with nine levels: NS, Sp8 (single exposure), Sp13 (single exposure), Sp8 (double exposure), Sp13 (double exposure), Sp1 (uninfected), Sp7 (uninfected), Sp1 (infected) and Sp7 (infected). We invoked the 'contrast' statement within GENMOD to compare factor levels. Because life-history theory predicts a trade-off between longevity and reproduction, we repeated the analysis with reproduction (the mean number of offspring produced per host) included as a covariate. Host reproduction (the mean number of (square-root + 0·5 transformed) offspring produced per host) was also separately analysed as a response variable using the explanatory variables described above.

EXPERIMENT 2: MULTIPLE SPORE DOSES OF INFECTIVE PATHOGENS

The goal of this experiment was to compare the consequences of being exposed to different doses of spores of the highly pathogenic strain Sp1. Replicates of a single *Daphnia* clone were maintained for three generations under controlled conditions in 200-mL jars before the experiment to equilibrate environmental variation. In this experiment we kept only one *Daphnia* per jar at all times before and during the experiments. There were 120 replicates, and on the first day of experiment three 3rd clutch newborn (< 24 h old) were taken and allocated to the treatments: high spore dose (50 000 spores), medium spore dose (10 000 spores) and low spore dose (1000 spores). Parasite spores were added on day 1 and the exposure period lasted 5 days. All *Daphnia* were exposed to parasites in this experiment (i.e. there were no unexposed controls). During the exposure period *Daphnia* were kept in 60-mL jars with a small quantity of sand on the bottom and were given 1×10^6 algae cells per day. Following the exposure period, hosts were moved into 200-mL jars and fed $3{\cdot}5 \times 10^6$ algae cells per day. For this post-exposure period, water was changed every other day and survivorship was monitored for 65 days, at which point the experiment was ended. Hosts that died before the age of 12 were not included.

As this experiment housed *Daphnia* singly, it was straightforward to monitor the fate of individuals and thus perform formal survival analysis. Our response variable was therefore time to death (in days) and individuals that survived to day 65 were censored. We used proportional hazards to compare survivorship. Focusing only on hosts that resisted infection, we compared survivorship between those that resisted a low, medium or high dose of parasites. We did not study reproduction as a covariate in this experiment because so many hosts did not reproduce before death.

Results

EXPERIMENT 1: PATHOGENIC AND NONPATHOGENIC STRAINS

Survivorship differed between the treatment groups, with infected *Daphnia* showing the least mortality, and those fending off the most aggressive strain (Sp1) showing the most (Fig. 2; $\chi^2 = 17{\cdot}4$, $df_N = 8$, $df_D = 119$, $P = 0{\cdot}026$). This significance was not solely due to the very high levels of mortality in the class that fended off Sp1, as pair-wise comparisons (using the 'contrast statement' in GENMOD) also showed that the following pairs of treatments differed: Sp13 (two exposures) vs. NS ($P = 0{\cdot}036$), Sp13 (two exposures) vs. infected with Sp1 ($P = 0{\cdot}002$), Sp13 (two exposures) vs. infected with Sp7 ($P = 0{\cdot}021$), and Sp8 (two exposures) vs. infected with Sp1 ($P = 0{\cdot}022$). On average, 40% of host exposed to Sp7 showed signs of infection, while 90% exposed to Sp1 became infected in this experiment. We detected an

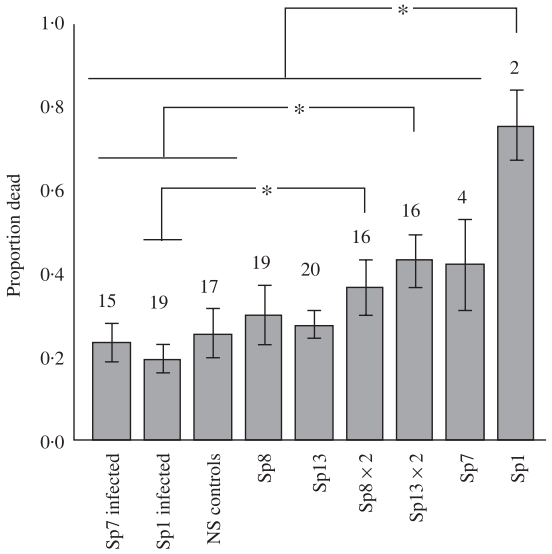

Fig. 2. The proportion of *Daphnia* hosts that died following exposure to *Pasteuria ramosa.* Stippled lines with a indicate factor levels that differed at the $P < 0{\cdot}05$ level. Numbers above each bar are sample sizes (which, in this experiment, are the number of jars in each treatment. Each jar initially contained five hosts).

infection in only one jar of host exposed to a noninfective parasite strain. This was a treatment twice exposed to Sp8, and in it just one of the six *Daphnia* became infected (data excluded).

Adding reproduction as a covariate to the analysis did not change conclusions regarding the relationship between treatment and mortality ($\chi^2 = 18{\cdot}1$, $df_N = 8$, $df_D = 119$, $P = 0{\cdot}020$), but it was clear that reproduction was related to mortality ($\chi^2 = 17{\cdot}3$, $df_N = 1$, $df_D = 110$, $P = 0{\cdot}007$). A simple correlation relating offspring production to mortality (the proportion surviving in each jar) in this experiment gave a positive relationship ($r^2 = 0{\cdot}07$, $P = 0{\cdot}003$), suggesting that more reproduction resulted in more mortality.

Studying reproductive output as a response variable showed that reproduction differed between treatments ($F_{8,119} = 54{\cdot}4$, $P < 0{\cdot}0001$), but this was entirely due to the inclusion of jars with infected hosts [*P. ramosa* pathology severely compromises reproduction, as evidenced by the observation that mean reproduction per female ranged from 3·37 offspring (in jars with six infected hosts) to 22·44 offspring (in jars with no infected hosts)]. Excluding the two treatment levels that included infected hosts [Sp1 (infected) and Sp7 (infected)], treatment did not explain differences in reproduction between jars containing only uninfected *Daphnia* ($F_{6,87} = 1{\cdot}57$, $P = 0{\cdot}17$), which indicates that infections were not overlooked.

EXPERIMENT 2: MULTIPLE SPORE DOSES OF INFECTIVE PATHOGENS

The high, medium and low spore doses resulted in 93, 89 and 63% of hosts becoming infected, respectively. Among hosts that resisted infection, mortality was higher (Fig. 3, left) and clearly linked to the dose of parasites they resisted (Fig. 3, right; likelihood ratio $\chi^2 = 15{\cdot}6$, $df_N = 2$, $df_D = 54$, $P < 0{\cdot}0003$) with resisting higher doses leading to greater mortality.

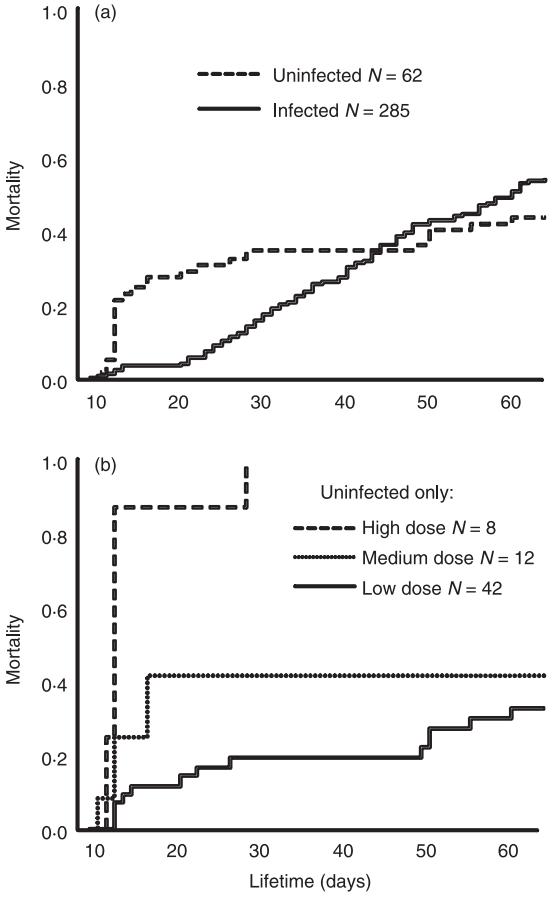

Fig. 3. (a) Mortality of *Daphnia* that resisted infection (i.e. remained 'uninfected') following exposure to *P. ramosa* compared with those that succumb to infection. (b) Mortality of uninfected *Daphnia* at each of the three doses of transmission spores they were exposed to. Numbers are sample sizes (which, in this experiment, are the number of individual hosts in each treatment).

Discussion

When comparing hosts expected to have activated their immune systems to differing degrees, we found that mortality was highest among hosts expected to have launched a stronger response. This pattern might be attributable to direct damage caused by parasites that tried but failed to establish infection, but then infected hosts would be expected to suffer the most parasite-mediated damage, and thus the most mortality. Infected hosts, however, showed relatively low mortality. Our observations are compatible with the hypothesis that fighting off infection compromises longevity because deployment of the immune response comes with costs. This cost could be due to immune-mediated damage to host tissues (immunopathology), but it could equally be due to the energetic cost of launching an immune response, i.e. energy devoted to fighting parasites is unavailable for growth or maintenance. This cost of immunity is largely evident in the short term (30 or fewer days); over longer time frames, infection also compromises longevity (Fig. 3) (Jensen, Little, Skorping & Ebert 2006).

We studied costs of immunity with respect to mortality because we reasoned that patterns of mortality would be relatively easy to interpret. Reproduction, for example, is confounded because the principal effect of *P. ramosa* infection is fecundity reduction, while at the same time hosts may show fecundity compensation during the pre-patent period (Ebert, Carius, Little & Decaestecker 2004). The present study was not designed to disentangle these nuances. In addition, reproduction itself can compromise longevity, and our work provided evidence that short-term levels of mortality experienced by uninfected hosts is partly attributable to reproductive effort: having more offspring increased mortality. This relationship between reproduction and longevity is surprising because longer lived hosts have more opportunity to reproduce, and thus our estimate of how reproduction compromises longevity is likely conservative.

The cost of immunity described here – the cost of deploying an immune response – should not be confused with other types of costs, such as those associated with evolving greater resistance. For example, lines of *Drosophila melanogaster* artificially selected for greater parasitoid resistance also have reduced levels of larval competitive ability (Kraaijeveld & Godfray 1997; Fellowes, Kraaijeveld & Godfray 1998; Fellowes, Kraaijeveld & Godfray 1999; Kraaijeveld, Limentani & Godfray 2001), while artificial selection for phenoloxidase activity in dung flies revealed a trade-off with survivorship (Schwarzenbach & Ward 2006). There is no evidence for such a genetic-based cost of resistance in *D. magna*. In particular, a comparison of a range of susceptible and resistant genotypes in terms of mortality, life-history traits and competitive ability in the absence of parasites found no evidence for standing costs of resistance (Little, Carius, Sakwinska & Ebert 2002). It remains conceivable, however, that different *Daphnia* genotypes would show quantitatively or qualitatively different immune responses upon exposure to parasites, and this could be a focus of future experiments. A further focus of future experiments could be studying costs (pleiotropic or costs of launching the immune response) under a range of environmental and host conditions, because these have clearly been shown to effect the capacity of hosts to resist infection (Krist, Jokela, Wiehn & Lively 2004; Mitchell, Rogers, Little & Read 2005) and they ought to similarly impact immunopathology or energy use by the immune system.

Studies of fitness variation (which, requiring the study of a substantial number of individuals, may not always feasibly include detailed immunological assays) are required to determine how immune responses mediate host and parasite evolution. Nevertheless, studies of the fitness costs of immunity in *Daphnia* would be enhanced by immune assays that can verify that immune responsiveness mediates mortality. This is a challenging task with the *Daphnia–Pasteuria* system, which is practical for the efficient measurement of fitness parameters, but for which knowledge of immune mechanisms is lacking. The simple immune measures commonly used in other systems (e.g. Kraaijeveld *et al*. 2001; Moret & Siva-Jothy 2003) might be adapted to the *Daphnia* system, or, alternatively, the recent sequencing of a *Daphnia* genome may help advance knowledge of the immune-related genome to the stage where a large range of immune molecules can be assayed. Overall, knowledge of the mechanistic basis of either costs of immunity or costs of evolving resistance in invertebrates is scarce (reviewed in Kraaijeveld, Ferrari & Godfray 2002; Rolff & Siva-Jothy 2003), compared with, for example, the related field of costs of resistance to insecticides where extremely detailed knowledge is available (e.g. Labbé, Lenormand & Raymond 2005). This situation seems likely to change given the increasing interest in invertebrate immune systems (Kurtz 2004; Little, Hultmark & Read 2005).

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