Elevated haemocyte number is associated with infection and low fitness potential in wild Daphnia magna

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Summary
1. Immune activity may be a cause of resistance to parasites, but it can also be a consequence of infection. Thus, the adaptive significance of an immune response is more accurately assessed when it is measured alongside both host fitness and infection status.
2. We sought to determine the significance of immune responses in a naturally coevolving host–parasite system in the wild, with support from laboratory experiments. We measured haemocyte numbers in Daphnia magna in relation to an infection that has a clear fitness consequence: infection with the bacterium Pasteuria ramosa causes sterilization.
3. Haemocyte number was consistently elevated in infected Daphnia in the field and in parasite exposed or infected hosts in the laboratory. Thus, elevated haemocyte numbers were essentially a symptom of infection, and we found no evidence that haemocytes help hosts exclude the parasite.
4. Consequently, these results provide an especially clear example where increased immune activity does not mean increased immunity or fitness: hosts with the highest haemocyte counts have extremely low health and low fitness potential.

Key-words: ecoimmunology, host–parasite interactions, immunocompetence, Pasteuria ramosa, resistance

Introduction
Parasites reduce the fitness of the hosts they infect, and their ubiquity makes them an important selective agent for many organisms. Host immune systems, by preventing parasite establishment and/or proliferation, may consequently play an important role in preserving fitness of infected organisms. But what exactly is the relationship between immune response magnitude and fitness? In some cases, the intuitive scenario will apply: a stronger immune response will be helpful and will lead to greater host fitness by reducing the probability of becoming infected or the harm caused by infection. However, strong immune responses have drawbacks as well, as they can drain energy reserves or cause immunopathology, thus reducing host fitness (Graham, Allen & Read 2005). Moreover, greater immune activity could equally reflect a greater current parasite burden (and hence susceptibility) or past exposure (and hence successful defence) (Stasewski et al. 2007). In other words, an immune response can be either a cause or a consequence of parasite burden (Osnas & Lively 2006; Graham et al. 2011).

Thus, the relationship between immune response magnitude and fitness may be complex, and more immune responsiveness will not always mean more fitness (Graham, Allen & Read 2005; Day, Graham & Read 2007; Graham et al. 2011).

These complications suggest that drawing conclusions about the role of host immunity in parasite-mediated selection requires the simultaneous measurement of three parameters: immune activity, host fitness and infection status (Graham, Allen & Read 2005; Viney, Riley & Buchanan 2005; Bradley & Jackson 2008; Graham et al. 2011), preferably across genetic and environmental variation (Lazzaro & Little 2009) and/or in the field with natural parasites (Stasewski et al. 2007). Much pure immunological work is poorly poised to achieve this, as the careful work required to elucidate immunological mechanism requires tightly controlled conditions, inbred strains and often the use of artificial stimulants of the immune response. Ecological immunology (ecoinmunology) has sought to fill this gap, but not all studies in this field measure all three parameters of immune activity, infection status and host fitness. Thus, while the assumption that more immune activity means greater fitness has long been criticized (Behnke, Barnard & Wakelin 1992;
many still assume the magnitude of the immune response will be positively associated with host fitness. The current study is an empirical example of how heightened immune activity can be associated with low, not high, host fitness.

Using the crustacean *Daphnia magna* and its naturally co-evolving parasite, *Pasteuria ramosa* as a model (Fig. 1), we measured infection status and host immune activity in the field. Because *P. ramosa* sterilizes its host, infection status simultaneously provides a measure of host fitness — *Pasteuria* infection has the same effect on host fitness as death. Infection status provides an accurate measure of fitness, because much of the variation in reproductive fitness in this system is attributable to being infected or not, and there is less variation attributable to reproductive differences amongst infected hosts. In *Daphnia*, we counted circulating host haemocytes as a measure of immune activity, because haemocytes generally play an important anti-parasite role in invertebrates (Ataev & Coustau 1999; Elrod-Erickson, Mishra & Schneider 2000; Kraaijeveld, Limentani & Godfray 2001; Canesi et al. 2002; Cotter, Kruuk & Wilson 2004). A very early study showed *Daphnia* maintain a population of circulating amoeboid haemocytes (Metchnikoff 1884), and our more recent work has shown that some genotypes of *Daphnia* mount a cellular response shortly after exposure to *P. ramosa* (Auld, Scholefield & Little 2010).

The field survey recorded both haemocyte number and *P. ramosa* prevalence over a 9-month period, during which several epidemics were documented. We complemented these observations with two laboratory experiments subjecting *Daphnia* from this same population to controlled exposure to *P. ramosa* spores (also collected from the same local population). First, we tested for an early cellular response in parasite-exposed hosts (5 h post-exposure) by comparing haemocyte numbers between parasite-exposed and unexposed (control) *Daphnia*; second, we tested whether well-established infection (21 days post-exposure) was associated with longer-term differences in haemocyte number. The present study adds to our earlier work (Auld, Scholefield & Little 2010), but is novel in that it examines the relationship between host immune activity and fitness *in the wild* and then corroborates this relationship under controlled laboratory conditions.

**Materials and methods**

**HOST AND PARASITE ORGANISMS**

*Daphnia magna* is a cyclically parthenogenetic freshwater crustacean that lives in shallow eutrophic ponds. They are host to the obligate microparasite, *Pasteuria ramosa*: a spore-forming bacterium that is transmitted horizontally from the corpses of previously infected hosts (Ebert et al. 1996). Infection occurs when *Daphnia* filter-feed, taking in the transmission spores along with their food. Once in the host, the *P. ramosa* spores go through a 10–20 day developmental process, resulting in many millions of transmission spores that are released on the death of the host. Infection nearly always results in the complete sterilization of the host.

**FIELD COLLECTIONS AND HAEMOCYTE COUNTS**

*Daphnia magna* were sampled from a pond at Kaims Farm, Leitholm, Scottish Borders (2°20′43″W, 55°42′15″N) twice per month between April and October 2009 and once in November and December. Adult *Daphnia* were categorized according to infection status (healthy or infected; infection can be easily diagnosed by eye, as the symptoms include an absence of eggs in the brood pouch or a lack of enlarged ovaries, redness and obvious bacterial growth in the haemolymph). Hosts from each categorization were placed in groups of five in a cell extraction chamber with 40 μL of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: Lavine, Chen & Strand 2005), and their hearts were pierced using a 25-gauge needle (BD Microlance, Drogheda, Ireland), causing haemolymph to pool into the buffer. This haemolymph-buffer solution was then transferred into a 0.5 mL Eppendorf tube and placed on ice for the hour-long journey back to the laboratory. In the laboratory, each of the samples was mixed thoroughly using a pipette, 2 μL was placed in a fertility counting chamber [0.001 mm² × 0.100 mm (depth)] (Hawksley, Lancing, Sussex, UK), and the number of haemocytes was counted. These counts were converted to number of cells per microlitre of haemolymph-buffer solution.

We also measured a set of variables within the pond at each sampling date: water temperature was measured using a digital field thermometer (HANNA instruments HI93510), and the population density of *Daphnia* was estimated by sweeping a 0.063 m² net through 1 m of pond water from three fixed locations around the pond. These live collections were taken back to the laboratory, and counts of infected adults, healthy adults and juveniles were determined. The population density (in *Daphnia* per litre) of each life stage was then just this count divided by the volume of water the net was passed through [water volume = 0.063 m² (net area) × 1 m (the sweep distance)]. *Pasteuria ramosa* infection was assessed in the adult portion of all subsamples: this was usually performed by eye, but in the few ambiguous cases, individuals were crushed under a glass coverslip on

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**Fig. 1.** Two genetically identical (clones) of *Daphnia magna*. The female on the left is healthy and carrying a clutch of offspring; the female on the right has been sterilized by an infection with *Pasteuria ramosa* (note the empty brood pouch).
a microscope slide and then examined under a transmission microscope for the presence of *P. ramosa* spores.

**EXPERIMENTAL SET-UP**

First, we tested whether exposure to *P. ramosa* resulted in a rapid increase in haemocyte count in *Daphnia*, as seen previously in a laboratory study of a different population of *D. magna* (Auld, Scholefield & Little 2010). This first experiment is referred to below as the early cellular response experiment. Second, we exposed hosts to parasites but then waited (21 days) for infections to become established and then tested whether *Daphnia* with established infections had greater haemocyte counts than their exposed but uninfected counterparts. This scenario more closely resembles the hosts we collect from the wild, which have established infections. This second experiment is referred to below as the infection experiment. In both experiments, *Daphnia* were exposed to a mixture of *P. ramosa* isolates, as opposed to a single isolate, to more accurately mimic *Daphnia-* *P.asterias* interactions in the field (wild *Daphnia* will likely encounter spores from different genotypes).

Methods were similar to Auld, Scholefield & Little (2010). For both experiments, independent replicates of four *Daphnia* genotypes (KA25, KA30, KA71 and KA93) were kept in the laboratory in a state of clonal reproduction for three generations, to minimize variation in condition. Hosts were kept in groups of five in jars containing 200 mL of artificial medium (Kluttgen et al. 1994) and fed 50 ABS of chemostat-grown *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 60 nm white light by the *C. vulgaris* culture). Medium was changed three times per week, and jars were incubated at 20 °C on a 12L : 12D light cycle. Second clutch neonates formed the experimental replicates in each of the two experiments.

The parasite spores used here were from a solution containing a mix of *P. ramosa* isolates from the Kaimes pond (Allen & Little in press). This spore solution was prepared by homogenizing multiple *P. ramosa*-infected hosts with ddH₂O, yielding a solution containing a mixture of spore isolates.

**EARLY CELLULAR RESPONSE EXPERIMENT**

Replicates were allocated to one of two parasite treatments: parasite-exposed or non-exposed (control). There were 48 replicates in total: six replicates per genotype per treatment, and each replicate consisted of five *Daphnia*. Experimental replicates were kept in the same conditions as maternal generations until at least three of the five *Daphnia* deposited eggs in their brood pouch, at which point they were ready for parasite exposure. Parasite treatments were carried out as follows: for each replicate, the five adult *Daphnia* were placed in the well of a 24-well plate (Costar; Corning Inc., Corning, NY, USA). The parasite spore solution was thawed, thoroughly mixed with a pipette, and the number of spores was determined using a Neubauer (Improved) counting chamber (0.0025 mm² × 0.1 mm depth). Replicates assigned to the parasite-exposed treatment then received 100 000 *P. ramosa* spores, and control replicates received an identical volume of homogenized healthy *Daphnia* as a placebo. This dose was chosen because, in previous studies, it has led to infection levels similar to those observed in the field (e.g. Duncan, Mitchell & Little 2006).

Treatment exposure lasted for 5 h, after which the *Daphnia* were removed from the cell plate and washed in artificial medium. Hosts from each replicate were placed in a cell extraction chamber, their hearts were then pierced, and their haemolymph pooled in 4 µL of ice-cold anticoagulant buffer. Haemocytes were then counted using methodology described earlier. These *Daphnia* were exposed to the parasites as adults to ensure we obtained enough haemolymph from which haemocyte numbers could be estimated reliably.

**INFECTION EXPERIMENT**

Methods were similar to the early cellular response experiment outlined above. Here, there were a total of 72 replicates: there were 12 parasite-exposed replicates and six control replicates per genotype. Once again, a replicate consisted of five *Daphnia*. Parasite exposures were carried out as follows: for each replicate, the five female neonates (<24 h old) were placed in a jar with 200 mL of artificial medium and 5 g of sterile sand. The parasite spore solution was thawed, mixed, and the spores were counted as before. Parasite-exposed replicates received a dose of 100 000 *P. ramosa* spores; control replicates received the same volume of placebo. Jars were stirred daily and fed low amounts (1.5 ABS per day) of *C. vulgaris* throughout the infection period, which lasted 7 days. The low food levels forced the *Daphnia* to filter-feed the sand at the bottom of the jars. This procedure was meant to mimic a natural infection process, where *Daphnia* ingest spores from the sediment.

On day 8, the replicates were changed into clean jars with 200 mL of fresh artificial medium, and they were then fed 5.0 ABS of *C. vulgaris* per day and their medium was refreshed three times per week. On day 21, hosts from each replicate were grouped according to infection status and placed in the cell extraction chamber with 0.8 µL of ice-cold anticoagulant buffer per host. The hosts' haemolymph was extracted, and their haemocytes were counted using the methodology described earlier. Some replicates contained both infected and uninfected hosts, thus leading to haemocyte counts for the two infection categories (exposed-infected and exposed-uninfected) from the same replicate.

**ANALYSIS**

All data were analysed using general linear models in R (Ihaka & Gentleman 1996, R, 2005). The heterogeneity of variance was assessed for all models, and the assumptions for the tests were fulfilled. The model fitting process was repeated until a minimum adequate model was achieved.

**Field data**

We aimed to test whether haemocyte numbers in the field could be explained by infection status (infected or not), host density, parasite prevalence or temperature. However, we considered that the haemocyte numbers could reflect current conditions or might more closely reflect events occurring in the past. Haemocyte numbers might also influence traits measured in the future. We, therefore, examined how current haemocyte counts were influenced by current infection status, parasite prevalence (current, past or future), host density (current, past or future) and temperature (current and past). All models included sample date (when haemocyte counts were made) as a random effect, to control for any temporal autocorrelation. The fixed effects in each model were as follows:

\[ H_1 = I_t + \text{Prevalence,} + \text{Density,} + \text{Temperature,} + \epsilon \]

\[ H_2 = I_t + \text{Prevalence,} - 2 + \text{Density,} - 2 + \text{Temperature,} - 2 + \epsilon \]

\[ H_t = I_t + \text{Prevalence,} + 2 + \text{Density,} + 2 + \epsilon \]

where $H$ is the log$_{10}$-transformed haemocyte count, $I$ is the infection status (infected or healthy), and $\varepsilon$ is the error. The subscript $t$ denotes the lag (in weeks) between when haemocyte counts were made and the other variables were recorded.

As a final analysis of the field data, we hypothesized that high parasite prevalence might be followed by a reduction in host population density. This hypothesis was tested by examining whether there was a correlation between current (arc sine square root transformed) parasite prevalence and host density 2 and 4 weeks in the future.

**Experimental data**

Data from the cellular response experiment were used to test whether exposure to *P. ramosa* led to an increase in the number of circulating haemocytes, and whether any response differs between the four host genotypes (i.e. if there was a *P. ramosa*-exposure-by-genotype interaction for haemocyte number). This was performed by using a two-way ANOVA with host genotype and parasite exposure included as fixed effects. Haemocyte counts were also log$_{10}$-transformed to achieve normality. As some jars contained both infected hosts and hosts that were exposed but were uninfected, jar was fitted as a random effect to control for partial non-independence of the data. Haemocyte counts were exposed but were uninfected, jar was fitted as a random effect to achieve normality. Some jars contained both infected hosts and hosts that were exposed but were uninfected, jar was fitted as a random effect to control for partial non-independence of the data. Haemocyte counts were also log$_{10}$-transformed to achieve normality. Finaly, we examined whether host genotype affected the proportion of parasite-exposed hosts that became infected with *P. ramosa*, using a generalized linear model (GLM) with quasibinomial error structure.

**Results**

**HAEMOCYTE COUNTS AND PARASITEMISM IN THE FIELD**

*Pasteuria ramosa*-infected *Daphnia* first appeared in early June and prevalence peaked three times over the season; in early June, late August and late September, achieving a maximum prevalence of 32%, which is likely to be an underestimate as individuals with low level infections can be overlooked. There were also three peaks in the number of circulating haemocytes in the hosts, which occurred at approximately the same times as parasite prevalence peaks (Fig. 2).

Of the ecological variables tested, only infection status explained a significant amount of the variation in haemocyte counts: healthy wild *Daphnia* had a mean of 1261±52 ± 115±24 haemocytes, whereas their parasite-infected counterparts had 5609±71 ± 814±37 circulating haemocytes, and this pattern was clearly consistent on nearly all sampling dates (Fig. 2). No other variable, from either the past or future, significantly determined haemocyte counts (Table 1). Finally, there was a significant negative correlation between current *P. ramosa* prevalence and *Daphnia* population density 2 weeks in the future ($r_s = -0.51$, $P < 0.05$), and this pattern was even stronger 4 weeks into the future ($r_s = -0.74$, $P < 0.01$) (Fig. 3).

**EARLY CELLULAR RESPONSE EXPERIMENT**

Haemocyte counts were obtained from 240 *Daphnia* from 48 replicate jars. We found that *Daphnia* mounted a cellular response to *P. ramosa* exposure: there were 358 ± 37 haemocytes per *Daphnia* in parasite-exposed hosts and 128 ± 14 haemocytes per *Daphnia* in control (unexposed) hosts ($F_{1,40} = 45.43$, $P < 0.0001$). The number of circulating haemocytes also depended on the identity of the host genotype ($F_{3,40} = 2.93$, $P = 0.05$; Fig. 4), but there was no host genotype-by-parasite exposure interaction ($F_{3,40} = 1.46$, $P = 0.24$; Fig. 4).
INFECTION EXPERIMENT – LONG-TERM CELLULAR RESPONSE

Haemocyte counts were obtained from 474 Daphnia from 96 jars. There was no natural host mortality during the course of the experiment. Pasteuria ramosa-infected Daphnia had considerably more haemocytes circulating in their haemolymph: 8458 ± 3 ± 1190 for infected and 3164 ± 2 ± 249 for healthy hosts (Fig. 5, \( F_{1,63} = 29.64, P < 0.0001 \)), and haemocyte counts were also affected by host genotype (\( F_{3,63} = 3.62, P < 0.05 \)). Jar identity accounted for a very small proportion of the variance in the data (3.54 ± 10⁻⁵). These haemocyte counts were consistent with those from wild Daphnia. There were no significant differences between haemocyte counts from control replicates and uninfected Pasteuria ramosa-exposed replicates (\( F_{1,67} = 0.01, P = 0.93 \)), but the differences between host genotypes remained (\( F_{3,67} = 4.40, P < 0.01 \)). Finally, none of the Daphnia from control replicates became infected, and the likelihood of infection in exposed replicates was dependent on the genotype of the host (\( F_{3,67} = 6.60, P < 0.001 \)).

Discussion

The number of circulating haemocytes in wild Daphnia magna was far higher in infected than in uninfected hosts. Thus, this putative immune trait is a reporter for infection with the sterilizing bacterium, Pasteuria ramosa (Fig. 2). Greater immune activity is sometimes associated with strong defence capability: either a high potential to resist parasites or as evidence of a successful defence in the past. However, as observed here, immune activity can simply be indicative of high-current parasite burden, and hence a low fitness potential (Viney, Riley & Buchanan 2005; Lindsey & Altizer 2009; Auld, Scholefield & Little 2010). Drawing this conclusion required
simultaneously diagnosing infection and its fitness consequences whilst measuring the immune response (Graham et al. 2011). With comprehensive measurement, it is possible to draw robust conclusions about what high immune activity means for host fitness, and in the present case, a large haemocyte response can be equated with being (genetically) dead because *P. ramosa* sterilizes its hosts. Indeed, the consequences of this sterilization may have been evident at the population level, as high parasite prevalence predicted lower host population density in the future (Fig. 3), consistent with earlier studies (Little & Ebert 1999; Decaestecker et al. 2005; Duncan, Mitchell & Little 2006).

The current results are compatible with a previously proposed heuristic model of defence in *Daphnia*: for successful infection to occur, *P. ramosa* spores need to (i) pass from the *Daphnia*'s gut into its haemocoel and then (ii) avoid haemolymph-based host immune effectors (Auld, Scholefield & Little 2010; Duneau et al. 2011). Strong resistance in *Daphnia* appears to be based upon parasites not passing the gut wall, and this could be based on specific recognition factors that either do not allow penetration or suppress the parasite at a very early stage. In susceptible hosts, which lack the specific recognition factors that lead to resistance, parasites gain entry and haemocyte numbers rapidly increase in response. Thus, an increase in haemocyte numbers is not causally linked to susceptibility/resistance. Haemocyte numbers report infection.

Nonetheless, haemocyte activity may still be beneficial, perhaps by delaying sterilization in susceptible hosts. In the *Daphnia–Pasteuria* system, however, most fitness variation is explained by being infected or not, as opposed to differences amongst infected hosts. Thus, an effect of delayed sterilization in infected hosts would be small compared to the benefits of excluding the parasite entirely. For example, during a typical 30-day experiment, healthy hosts produce 70–100 offspring, while infected hosts usually achieve between 0 and 20 (Ebert et al. 2004; Vale, Stjernman & Little 2008). The variation in offspring numbers observed in infected hosts, which is the variation that could potentially be altered by haemocyte activity, is thus relatively small alongside the large fitness consequences of becoming infected in the first place. Additionally, as has been observed in Mosquito–*Plasmodium* interactions (Cirimotich et al. 2011), it may be that *Daphnia* haemocytes are not responding to *Pasteuria* at all, but are instead a response to opportunistic bacteria of the gut that enter the haemocoel as *Pasteuria* crosses the gut barrier. By preventing the establishment of opportunistic bacteria, the cellular response may keep host alive long enough to achieve at least some reproduction. Ultimately assessing any benefit of haemocyte activity will be greatly aided by functional readouts, for example, measurement of nitric oxide production.

One of the aims of this study was to determine whether the cellular response patterns observed in the laboratory were mirrored in noisy natural environments, where environmental conditions, and thus host condition, should be very different from those in the laboratory (Harvell et al. 2001; Klemola et al. 2007). For example, to what degree do the intricate mechanisms of immunity, which are typically studied under tightly controlled conditions and in a limited range of genetic backgrounds, manifest under more stressful conditions or in the wild? In this study, what was true for *Daphnia* in the field was also true for those that had been experimentally exposed to *P. ramosa* and reared under controlled laboratory conditions. Moreover, although *Daphnia* haemocyte counts fluctuated over the course of the field season (Fig. 2), they remained a robust marker for parasitism: variation in parasite prevalence host density or pond temperature did not reduce the strength of the signal, even when lags were included in the analyses.

Still, variation in environmental conditions is known to substantially affect infection outcome in many host–parasite systems (see Lazzaro & Little, 2009, Vale, Stjernman & Little 2008). For example, based on past research on temperature (Mitchell et al. 2005; Vale, Stjernman & Little 2008; Vale & Little 2009), we expected, but did not observe, a relationship between temperature and infection levels (that is, beyond the superficial observation that epidemics tend to occur in the summer months: Duncan, Mitchell & Little 2006). Additionally, because elevated temperature also favours an increased rate of development and reproduction in healthy *Daphnia*, we also expected, but did not observe, some linkage between temperature and population growth. An extensive study in the *Daphnia dentifera–Metschnikowia bicuspidata* system has also failed to detect an effect of temperature on disease phenomena in the wild (Duffy et al. 2009). In both instances, effects may be hard to detect because of joint increases in host and parasite metabolism with increasing temperature.

Genetic differences for the cellular response to *P. ramosa* were evident in the experimental studies presented here, but each of the host clones showed a significant cellular response (and some infection), and difference between clones was one of the magnitude. In a previous study on a different population (Auld, Scholefield & Little 2010), some host genotypes showed no response at all, and these were the ones that also showed complete resistance (there were no successful infections in these genotypes). This contrast between populations confirms that a strong immune response is associated with, though does not cause, susceptibility. In particular, the hosts used in the previous study show very dichotomous patterns of variation for susceptibility (and hence all-or nothing cellular responses, at least to the single parasite strain used in that study). In the current population, although there is only subtle variation in how readily different host clones succumb to infection, all hosts are ultimately susceptible to the mixed parasite spore solution used (and thus all were expected to show a cellular response, as observed).

In conclusion, studies linking immune activity with infection status, and host and parasite fitness, are essential for our understanding of host–parasite coevolution (Graham et al. 2011), but we acknowledge that is not always possible for a study to be comprehensive. It is simply important to bear in mind that studies using a restricted suite of measurements should be cautious when making assumptions about the functional significance of immune activity. Specifically, studies
that measure immune activity and infection (but not host fitness) will often overlook the possible role of immunopathology; studies that measure immune function and host fitness (but do not assess parasite burden) may overlook the possible role of infection in causing variation in host immune capabilities.

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