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Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*

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A primary infection by a parasite may indicate a higher risk of being reinfected in the near future (since infection may indicate that enemies are becoming more abundant). Acquired immunity does not exist in invertebrates despite the fact that they also face increased risks of reinfection following primary exposure. However, when subjected to immune insult, insects can produce immune responses that persist for long enough to provide prophylaxis. Because these immune responses are costly, persistence must be maintained through a selective advantage. We tested for the possibility that these long-lasting immune responses provided increased resistance to later infections by experimentally mimicking a primary immune insult (pre-challenge) in larvae of the mealworm beetle, *Tenebrio molitor*, with lipopolysaccharides (LPS) prior to early or late exposure to spores of the entomopathogenic fungus *Metarhizium anisopliae*. We found that pre-challenged larvae produced a long-lasting antimicrobial response, which provided a survival benefit when the larvae were exposed to fungal infection. These results suggest that the observed response is functionally 'adaptive'.

Keywords: ecological immunology; long-lasting immunity; antimicrobial peptides; phenoloxidase

1. INTRODUCTION

Encounters with pathogens or parasites are often temporally unpredictable (Combes 1995; Schmid-Hempel 1998). However, once a naive organism has received an immune insult it might subsequently be exposed to a higher probability of being reinfected because pathogens that are now present in the environment are likely to increase in frequency because of their reliance on hosts for transmission (e.g. Lui 2000). In short, experience of an infection may predict a higher risk of becoming infected in the near future. This is likely to have been part of the selection behind the evolution of the acquired immune response in vertebrates (Rinkevich 1999; Klimovich 2000). Acquired immunity, based on immunoglobulins, improves the efficiency of the host's immune response during the second encounter by being directed at a specific, repeated, insult and provides long-lasting 'responsive-mode prophylaxis' by maintaining effector systems on standby after the initial insult has been neutralized. Acquired immunity does not exist in invertebrates (Hoffmann *et al.* 1996), but both vertebrates and invertebrates possess innate immunity. Because the vertebrate acquired immune system is adaptive, flexible and specific, we tend to view the invertebrates' reliance on an innate system as a poor alternative in terms of the breadth of response options. However, recent inclusion of ecological considerations in the understanding of immune system function is changing the entrenched view that invertebrate innate systems are limited simply by the number of available effector systems. Arthropods show some fairly sophisticated integration of their defence physiology to produce some very effective functional outcomes. For example, when the risk of parasitism increases, even in the absence of parasites, insects show prophylactic investment in immune defence (Reeson *et al.* 1998; Huang & Song 1999; Barnes & Siva-Jothy 2000; Moret & Schmid-Hempel 2001; Wilson *et al.* 2001; Little *et al.* 2003). Arthropods subjected to immune insult also appear to produce immune responses that persist after the pathogen is neutralized (Söderhäll 1982; Ratcliffe *et al.* 1985; Kato *et al.* 1994; Lemaître *et al.* 1996). So far, no explanation has been provided for the occurrence of such long-lasting immune responses. Because immune responses impose fitness costs (Moret & Schmid-Hempel 2000, 2001) there must be some selective advantage for maintaining these long-lasting immune responses. We propose that such long-lasting immune responses are functionally adaptive. They can be described as providing 'responsive-mode prophylaxis' to later parasite attacks, which are now ecologically more likely. Under this hypothesis the first insult received by a naive individual indicates an elevated likelihood of parasite insult.

As in other arthropods, immune defence in insects relies on both constitutive and inducible mechanisms that provide non-specific and fairly specific immunity, respectively (Hoffmann *et al.* 1996; Gillespie *et al.* 1997). Infection activates multiple systemic responses, including phagocytosis and encapsulation by haemocyte blood cells (Ratcliffe *et al.* 1985; Hoffmann *et al.* 1996), and accompanying melanization reactions (Söderhäll & Cerenius 1998). These latter reactions are based on the pro-phenoloxidase (proPO) cascade, which is a common and generalized response to immune insult. It can be experimentally triggered by non-pathogenic elicitors such as lipopolysaccharides (LPS), important antigens that characterize the surface of some micro-organisms (Söderhäll & Cerenius 1998). The proPO system involves numerous enzymes that are constitutively synthesized and located both in the haemolymph and in circulating haemocytes (Gillespie *et al.* 1997; Söderhäll & Cerenius 1998). In addition, the recognition of micro-organism cell walls induces the production of antifungal or antibacterial

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peptides by the fat body. These peptides are secreted into the haemolymph (Hoffmann *et al.* 1996). These antimicrobial pathways are induced within the hour following the challenge and the synthesis of antimicrobial peptides can persist for many days post-challenge (Söderhäll 1982; Ratcliffe *et al.* 1985; Kato *et al.* 1994; Lemaître *et al.* 1996). Moreover, some of these insect antimicrobial responses are induced in the absence of microbial antigens by a trauma alone, for example during wounding (Lemaître *et al.* 1997), presumably as a prophylactic response.

In this study, we mimicked a primary immune insult by experimentally challenging larvae of the mealworm beetle, *Tenebrio molitor*, with LPS. The larvae were then exposed to early or late infection with spores of the entomopathogenic fungus *Metarhizium anisopliae* to test for the survival costs and benefits of producing a long-lasting prophylactic immune response. If the primary insult provides long-lasting protection, we expected to observe a survival benefit to the fungal infection in previously challenged larvae. By contrast, because mounting an immune response is costly (Moret & Schmid-Hempel 2000) we expected to see a survival cost in previously challenged larvae that were not exposed to fungal infection.

2. MATERIAL AND METHODS

(a) *Mealworm cultures*

The beetle larvae used in this study were taken at random from a stock culture maintained at the University of Sheffield. Nine cultures, each containing 75 to 80 larvae at the same developmental stage (10 to 15 mm in length), were set up for the experiment. Cultures were fed *ad libitum* and kept at 28 ± 1 °C for the duration of the experiment.

(b) *Immune treatments*

Experimental cultures were assigned to one of the three treatments: 'naive', 'Ringer' and 'LPS'. Larvae in the 'naive' group were chilled on ice prior to inclusion in the experiment. Larvae in the 'Ringer' group received a single injection of 5 µl of saline solution (Ringer solution) after being chilled on ice. Larvae in the 'LPS' group received a 0.5 mg ml^{-1} dose of LPS (Sigma: L8274) in 5 µl Ringer solution after being chilled on ice. LPS is the non-pathogenic, non-living surface molecule derived from *E. coli*. It is highly immunogenic (Söderhäll 1982; Ratcliffe *et al.* 1985; Jomori *et al.* 1990) and elicits the production of antimicrobial peptides over many hours (Söderhäll 1982; Ratcliffe *et al.* 1985; Kato *et al.* 1994; Lemaître *et al.* 1996). All injections were made through the pleural membrane between the second and the third abdominal segments, using sterilized glass capillaries that had been pulled out to a fine point.

(c) *Fungal infection*

Larvae of each immune treatment were then assigned to one of the three infection treatments with the entomopathogenic fungus *M. anisopliae*: 'control' (larvae were not exposed to the fungi), 'early' infection (larvae were exposed to the fungi 4 days post-challenge) and 'late' infection (larvae were exposed to the fungi 7 days post-challenge). The antimicrobial pathway induced by the recognition of LPS in insects is different from the one that would be induced through the recognition of fungi (Gottar *et al.* 2002; Tzou *et al.* 2002). However, the resulting antimicrobial peptides that are produced work efficiently against a broad range of microbial agents (Gillespie *et al.* 1997). We therefore expected the antimicrobial response to LPS challenge to be efficient against *M. anisopliae*.

Preparation of *M. anisopliae* spores and assays was performed as described by Goettel & Inglis (1997). Fungus was cultured on potato dextrose agar plates and the dose was prepared by scraping fungal spores into 0.05% Triton X-100 solution. The spore concentration was determined by counting spores using a haemocytometer with a compound microscope (Leitz Diaplan) and was then adjusted to 2×10^6 spores ml^{-1} of Triton X-100 solution for inoculation. Inoculates were never older than 24 h and were stored at 4 °C when not used.

Tenebrio molitor larvae were exposed to the fungus by dipping them in 1 ml of spore solution for 5 s. Control larvae were dipped in 0.05% Triton X-100 solution without fungal spores. Larvae were then allowed to crawl over tissue paper to remove excess moisture and placed back in their box. Mortality was scored over 50 days after fungal inoculation.

(d) *Immune parameters*

Six larvae were sampled at random from each immune treatment at day 4 and day 7 after the pre-challenge but prior to the treatment exposure to fungal spores to test for the antibacterial and the phenoloxidase (PO) activity in their haemolymph. Each larva was chilled on ice for 10 min and the pleural membrane between the second and the third abdominal segments was punctured with a sterile hypodermic needle. The droplet of haemolymph that came out of the wound was collected into a sterile, pre-chilled glass capillary. For each insect, 5 µl of haemolymph were collected and flushed into a 1.5 ml microcentrifuge tube containing 50 µl of cold sodium cacodilate/$CaCl_2$ buffer (0.01 M Na-Cac, 0.005 M $CaCl_2$, pH 6.5). A 15 µl subsample was kept in a 0.5 ml microcentrifuge tube and stored at –80 °C until later examination for antibacterial activity using a zone-of-inhibition test. The methods for this test were as described in Moret & Schmid-Hempel (2000) except that the assay was performed using haemolymph diluted 12 times with sodium cacodilate/$CaCl_2$ buffer. The remaining haemolymph solution was diluted with 30 µl of cold sodium cacodilate/$CaCl_2$ buffer and immediately stored at –80 °C for later measurement of PO activity. PO activity was assayed by thawing samples of frozen haemolymph solution (dilution 1 in 20; haemolymph/sodium cacodilate/$CaCl_2$ buffer) on ice and then adding 20 µl to a microplate well containing 140 µl of distilled water, 20 µl of phosphate buffer saline (PBS: 8.74 g NaCl; 1.78 g Na_2HPO_4, $2H_2O$; 1000 ml distilled water; pH. 6.5) and 20 µl of L-DOPA solution (4 mg ml^{-1} of distilled water). The reaction was allowed to proceed at 30 °C in a microplate reader (Versamax, Molecular Devices) for 40 min. Readings were taken every 10 s at 490 nm and analysed using SoftMax Pro 4.0 software (Molecular Devices, Sunnyvale, CA). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction (Barnes & Siva-Jothy 2000; Plaistow *et al.* 2003).

(e) *Statistics*

Antibacterial and PO activities were analysed using a multivariate analysis of variance (MANOVA) with pre-challenges and time post-injection as factors. Antibacterial activity was log transformed to homogenize the variance.

We used a Cox regression with a time-dependent covariate to analyse the differences in survival rates with respect to fungal and pre-challenge treatments. This analysis allows the

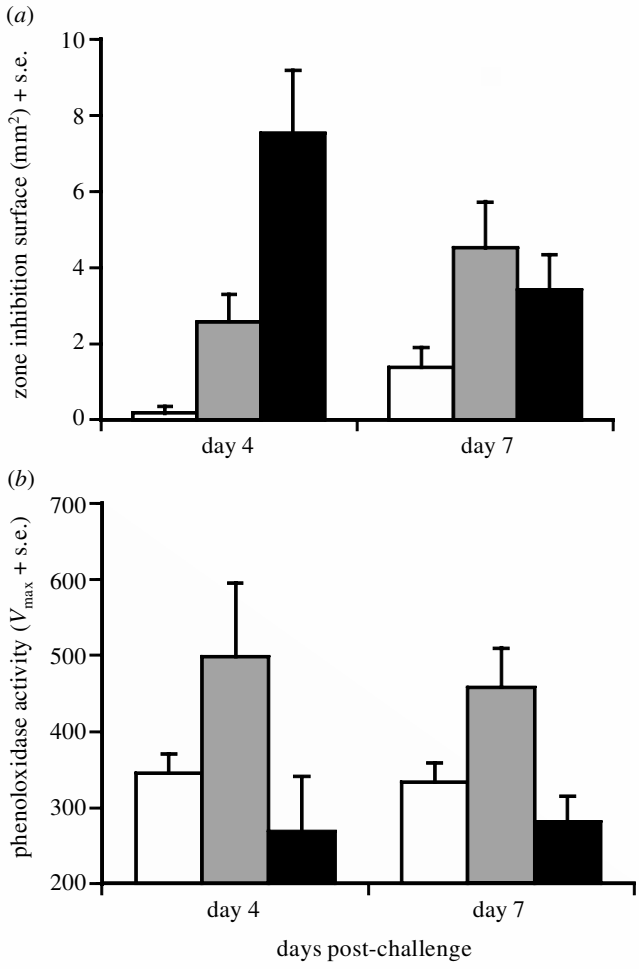

Figure 1. (*a*) Antibacterial and (*b*) PO activities in the haemolymph of *Tenebrio molitor* larvae from naive (open bars), Ringer (grey bars) and LPS (black bars) pre-challenges tested at days 4 and 7 post-injection.

simultaneous comparison of entire survival curves against an assigned reference survival function (baseline function) for different variables (Norusis & Inc. 1989). Our statistical model used a stepwise procedure and the reference survival function was generated from the control data derived from the pre-challenge and fungal treatments (e.g. naive and no fungal infection). The pre-challenges were coded as categorical variables (0, 1, 2) for naive, Ringer and LPS, respectively, and with the fungal treatments (0, 1, 2) for control, early (at day 4 post-injection) or late inoculation (at day 7 post-injection), respectively. Time (in days) was incremented as covariate in the model because hazard ratios of the survival functions were not constant across time. All data were analysed using SPSS 10 for Macintosh.

3. RESULTS

(a) *Antibacterial and PO activities*

MANOVA showed a significant effect of the pre-challenges on the measured immune parameters (these response variables are zone of inhibition and PO activity (figure 1; Pillai's trace: $F_{4,60} = 9.14$, $p < 0.001$)). By contrast, neither time post-injection nor its interaction with pre-challenge treatments affected the measured immune parameters (time post-injection: $F_{2,29} = 0.34$, $p = 0.712$; pre-challenges × time post-injection: $F_{4,60} = 1.55$, $p = 0.185$). As expected from the pre-challenge treatments, the haemolymph of LPS-treated larvae had a higher antibacterial activity than that of Ringer-treated or naive larvae (figure 1*a*; ANOVA for zone of inhibition: $F_{2,30} = 12.27$, $p < 0.001$). We conclude that LPS-treated larvae had activated their antibacterial immune pathway for at least 7 days post-insult. Ringer-treated larvae also had higher antibacterial activity compared with naive larvae but the level of their antibacterial response did not reach that of LPS-challenged larvae (figure 1*a*). Antibacterial activity levels of each pre-challenge treatment (Ringer and LPS pre-challenges) seemed to converge towards a similar value at day 7 (figure 1*a*), but these changes across time were only marginally significant (pre-challenges × time post-injection: $F_{2,30} = 3.23$, $p = 0.054$). PO activity did not significantly differ between LPS-treated and naive larvae (figure 1*b*), but the PO activity of Ringer-treated larvae was enhanced (figure 1*b*; $F_{2,30} = 6.58$, $p = 0.004$). Time post-injection did not affect PO activity (figure 1*b*; $F_{2,30} = 0.77$, $p = 0.783$) and the effect of the immune challenges on the enzyme activity remained the same across time (figure 1*b*; $F_{2,30} = 0.109$, $p = 0.897$).

(b) *Survival to pre-challenges and fungal infections*

Insect survival was monitored for 50 days after exposure to fungal spores (figure 2). While exposure to fungal infection increased insect death rate by a factor of 1.5–2 (see odds ratio for infection (4) and infection (7) in table 1), pre-challenge with Ringer or LPS provided a survival benefit for the duration of the experiment by reducing the death rate by 33% and 58%, respectively (table 1; figure 2). However, this survival benefit decreased across time (table 1: T × Ringer, and T × LPS). The negative survival effect of the fungal infections remained constant except for the early fungal exposure that induced less mortality across time (table 1: T × infection (4) and T × infection (7)).

The survival benefit of pre-challenge was dependent on the fungal treatment to which the beetle larvae were exposed. There was no survival benefit from the Ringer challenge when the larvae were exposed to fungal infections, regardless of time after injection (see interaction terms: infection (4) × Ringer and infection (7) × Ringer in table 1). By contrast, the LPS challenge provided a survival benefit when the larvae were exposed to early or late fungal infections (figure 2*b*,*c*; table 1: infection (4) × LPS and infection (7) × LPS). However, this benefit was not constant over time as survival decreased for larvae exposed to early fungal infection (table 1: T × infection (4) × LPS).

4. DISCUSSION

We demonstrated that the immune response following pre-challenge provides resistance against subsequent pathogen insults. LPS-treated *T. molitor* larvae showed better survival to fungal infection 4 or 7 days after pre-challenge (table 1; figure 2*b*,*c*). This enhanced resistance to infection was correlated with elevated antimicrobial activity in the haemolymph of LPS-treated larvae for the period of the fungal inoculations (figure 1*a*). By contrast, PO activity of LPS-treated larvae was lowest when com-

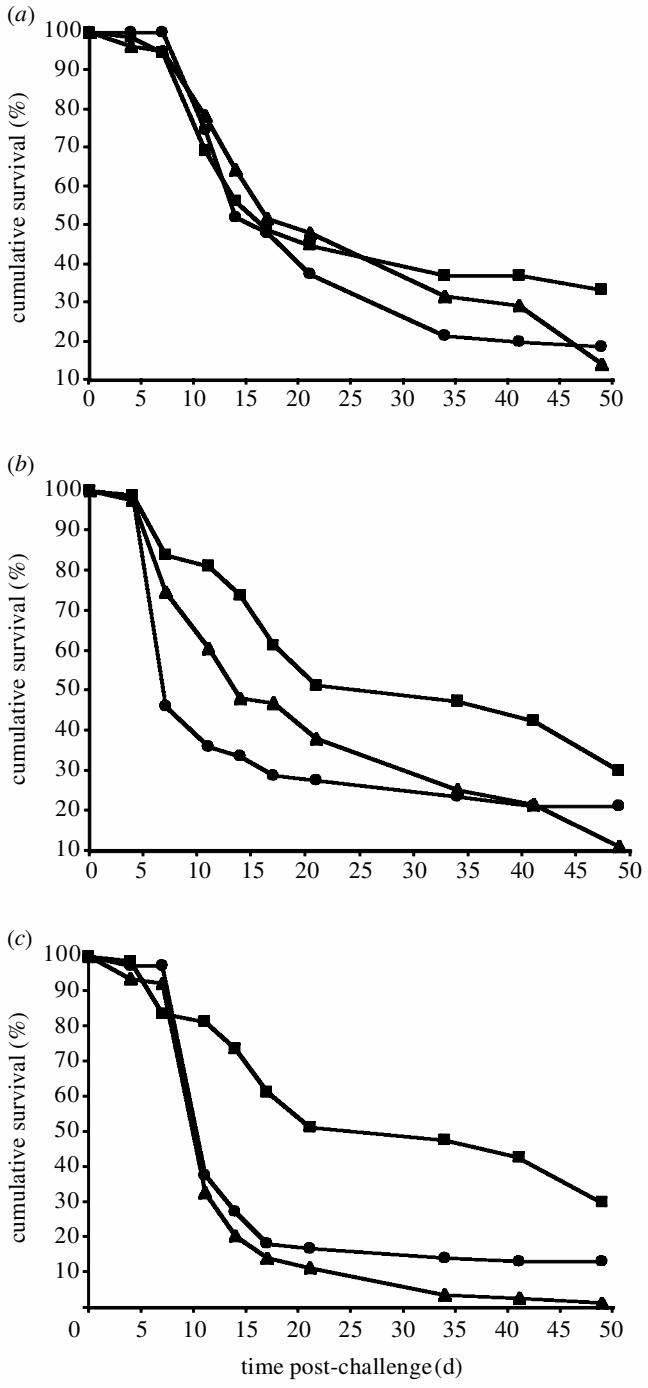

Figure 2. Survival curves of the mealworm beetle *Tenebrio molitor* by immune pre-challenge when exposed to (*a*) no infection; (*b*) to fungal infection at day 4 after pre-challenge and (*c*) to fungal infection at day 7 after pre-challenge. (Circles, naive; triangles, Ringer; squares, LPS.)

pared with the other treatments, especially when compared with the Ringer pre-challenge where the larvae had an increased enzyme activity (figure 1*b*). This latter result is in accordance with a study in bumble-bees showing a negative correlation between antibacterial and PO activities in response to LPS treatment (Moret & Schmid-Hempel 2001) and could indicate a trade-off between the two immune pathways. The elevated PO activity found in Ringer-treated larvae did not correlate with a better protection against fungal infections. This suggests that the PO system might not be involved in long-lasting responsive-mode prophylaxis, which seems only to be provided by the antimicrobial response. Increased PO activity on wounding in the absence of specific 'pathogen associated molecular patterns' (the 'Ringer' treatment) may prevent some low-level opportunistic infections (i.e. as an induced non-specific response to wounding) and/or may be the result of the mobilization of the wound-healing apparatus, which requires the production of new exocuticle and melanin (Ashida & Brey 1995; Sugumaran *et al.* 2000).

Another important result from our study is that the survival benefit of the LPS pre-challenge is greater for infected larvae, as is shown by the significant interaction terms in table 1: infection (4) or (7) × pre-challenge. The analysis shows that the pre-challenge offers increased survival over the period during which the fungal pathogen has its negative effects, but bears a slight but significant cost (T × pre-challenges; table 1) once that advantage has passed. By contrast, naive (no pre-challenge) animals show high initial mortality owing to their lack of resistance to the infection, which then stabilizes (T × infections at day 4; table 1) in the absence of a cost of the prophylaxis. In our experiment, such a cost, albeit small, was detectable despite ideal conditions in terms of food and temperature that are not commonly found in natural conditions. The actual mechanism that maintains high levels of antimicrobial activity during long-lasting immune responses in insects is as yet not known. Such a persistent antimicrobial activity may either be due to a continuous synthesis of antimicrobial peptides in the haemolymph or to a slow turnover of these peptides when produced. In terms of the cost of the immune response, the production mode of these antimicrobial peptides may have different impact, because the first might be more costly than the second. However, this is as yet not known. Because mounting an immune response is costly (Moret & Schmid-Hempel 2000), a long-lasting immune response is not expected to be adaptive unless the advantage of producing it over-compensates for the cost. Variable probabilities of reinfection after a primary infection may balance the cost–benefit ratio of such adaptive prophylactic investment in immune defence.

Homologies exist between vertebrate and invertebrate innate immunity (Medzhitov & Janeway 1998), but the vertebrate acquired immune system has no direct equivalent in invertebrates (Hughes 1998). For this reason, it is generally held that invertebrates are not able to mount any kind of adaptive immune response (Arala-Chaves & Sequeira 2000). Clearly the definition of 'adaptive' is important in this argument: we argue that if the ultimate function of vertebrate acquired immunity is to provide better protection against repeated parasite infections then our data show a similar functional outcome in an invertebrate. By looking at the invertebrate immune system from an ecological perspective (i.e. the response to the probability of reinfection), we suggest that the prophylactic benefits of prolonged expression of innate pathways may represent the functional equivalent of the acquired immune response, and we argue that that kind of regulation is an adaptive response. Clearly the resolution and memory of the invertebrate system are not comparable with that of vertebrates in these mechanistically disparate systems, but both provide prophylactic cover in response to the onset of a parasitic insult. The chasm between

Table 1. Results of the time-dependent Cox regression model. The table contains the relevant terms identified by a backward stepwise procedure.
(The full model is: survival, $S(t) = S_0(t)^p$, where $p = \exp[b(\text{pre-challenges}) + b(\text{infections}) + b(\text{infections} \times \text{pre-challenges}) + (t/1)(b(\text{T} \times \text{pre-challenges}) + b(\text{T} \times \text{infections}) + b(\text{T} \times \text{infections} \times \text{pre-challenges})]$.)

factors[a]	b[b]	s.e.[c]	Wald[d]	d.f.	p[e]	odds[f]
pre-challenges			26.25	2	< **0.001**	
Ringer	−0.39	0.162	5.91	1	**0.015**	0.67
LPS	−0.86	0.169	26.23	1	< **0.001**	0.42
infections			18.15	2	< **0.001**	
infection (4)	0.40	0.173	5.43	1	**0.02**	1.50
infection (7)	0.71	0.168	18.14	1	< **0.001**	2.04
infections × pre-challenges			17.50	4	**0.002**	
infection (4) × Ringer	−0.79	0.409	3.72	1	0.054	0.45
infection (7) × Ringer	0.27	0.400	0.46	1	0.496	1.31
infection (4) × LPS	−1.43	0.422	11.44	1	**0.001**	0.24
infection (7) × LPS	−0.76	0.406	3.54	1	**0.06**	0.47
T × pre-challenges			12.20	2	**0.002**	
T × Ringer	0.01	0.007	5.38	1	**0.02**	1.01
T × LPS	0.02	0.006	11.72	1	**0.001**	1.02
T × infections			5.02	2	0.081	
T × infection (4)	−0.01	0.006	4.42	1	**0.036**	0.99
T × infection (7)	−0.01	0.007	2.53	1	0.111	0.99
T × infections × pre-challenges			10.09	4	**0.039**	
T × infection (4) × Ringer	0.03	0.014	4.19	1	**0.041**	1.03
T × infection (7) × Ringer	0.01	0.017	0.37	1	0.542	1.01
T × infection (4) × LPS	0.04	0.014	9.01	1	**0.003**	1.04

[a] 'Ringer': (5 µl); 'LPS': (5 µl, 0.5 mg ml^{-1}); 'infection (4)': exposure to fungal inoculums (2.10^6) at day 4 post immune challenge; 'infection (7)': exposure to fungal inoculums (2.10^6) at day 7 post immune challenge; 'T': time covariate (in days).
[b] b, regression coefficient of overall survival function for variable.
[c] Standard error of regression coefficient.
[d] Wald statistic for variable. Values $p \leqslant 0.05$ are given in bold.
[e] Significance level for Wald statistic.
[f] Odds ratio of survival for variable relative to control (= $\exp(b)$).

invertebrate and vertebrate immune mechanisms may be less obvious at this functional level: one system provides highly specific, responsive-mode prophylaxis in generally long-lived complex organisms whereas the other provides generic, responsive-mode prophylaxis in generally short-lived organisms. Life-history differences between these taxa may be better reflected in the manner in which the available immune responses are coordinated, rather than the differences in specificity and memory.

Our study demonstrates that invertebrates show long-lasting (in the context of a semelparous, short-lived organism) prophylaxis against secondary parasite insults after a 'pre-challenge'. We conclude that such a response is functionally 'adaptive' and is not unique to vertebrates. This suggestion is based on the notion that vertebrates and invertebrates are subjected to similar parasite-imposed selection, which has provided a similar ultimate (functional) response, albeit based on different mechanisms.

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