Juvenile immune system activation induces a costly upregulation of adult immunity in field crickets *Gryllus campestris*

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Inducible immune defence may allow organisms a state-dependent upregulation of costly immunity in order to minimize the risk of anticipated future parasitism. The basic costs of elevated immune activity might involve a reduction in other fitness-related traits as well as an increased risk of immunopathology. In male field crickets *Gryllus campestris* we experimentally investigated the condition-dependent effects of immune system activation in nymphs on immunity and physiological condition during adulthood. Following a nymphal injection of bacterial lipopolysaccharides, adult males showed significantly elevated levels of two major immune parameters, i.e. haemolymph antibacterial activity and the concentration of prophenoloxidase (proPO). By contrast, the active enzyme, phenoloxidase (PO), did not increase, suggesting a strategic long-term upregulation of the inactive proenzyme proPO only. This may help avoid the cytotoxic effects associated with high standing levels of the active enzyme. The nymphal immune insult further caused a reduction in adult haemolymph protein load, suggesting a long-term decline in overall metabolic condition. Nymphal food availability positively affected adult lysozyme activity, while PO and proPO concentrations were not affected. Our data thus suggest the long-term upregulation of immunity in response to antigenic cues as an adaptive, yet costly, invertebrate strategy to improve resistance to future parasitism.

Keywords: *Gryllus campestris*; induced immunity; lipopolysaccharide; lysozyme; prophenoloxidase protein load

1. INTRODUCTION

Variation in immunity is a key issue for understanding the life history (Sheldon & Verhulst 1996; Zuk & Stoehr 2002), sexual selection process (Hamilton & Zuk 1982; Folstad & Karter 1992; Westneat & Birkhead 1998) and population dynamics (Lochmiller 1996) of animals. In the face of omnipresent selection by parasites and pathogens, the immune system evolved as the major host defence, restraining the detrimental effects of parasitism (Hoffmann *et al.* 1996; Roitt *et al.* 1996; Wakelin 1996; Goater & Holmes 1997). However, in spite of the obvious immediate benefits of protection against disease, maximum immune defence might not always be optimal, because of its associated cost on a life-history scale (Sheldon & Verhulst 1996).

Life-history theory predicts that optimal immunity requires a plastic, inducible regulation to maximize fitness over a wide range of individual states and parasite pressures. The optimal investment thus depends on the benefits of enhanced parasite resistance and the costs associated with the reduced contribution to fitness of other costly

life-history traits (Sheldon & Verhulst 1996). Such allocation costs of immunity might involve both the expenses for development and maintenance of the immune system and the deployment costs arising when the immune system is actually used to fight an infection (Schmid-Hempel 2003). Therefore, the immune system requires two levels of regulation: a long-term, life-history decision about the investment to immune defence, and a short-term, direct response in the case of infection. As far as we are aware, no attempts have yet been made to separate such long- and short-term immunological decisions. Insects, which metamorphose from clearly separated larval to adult life-history stages, offer an opportunity to do so.

Although invertebrates have recently been shown to be capable of some form of specific immune memory (Kurtz & Franz 2003), their primary mode of enhancing future resistance is probably through systemic upregulation of relatively unspecific immune components (Rolff & Siva-Jothy 2003). Invertebrates show elevated immunity after an infection has been cleared (Iwanaga *et al.* 1995; Söderhäll & Cerenius 1998). Their immune system consists of constitutive and inducible defence mechanisms (Hoffmann *et al.* 1996; Gillespie *et al.* 1997; Frost 1999). Important components are antimicrobial peptides and proteins, such as lysozyme, which are inducible above constitutive levels for many days after bacterial infection (Morishima *et al.* 1995; da Silva *et al.* 2000; Adamo 2004).

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An induced upregulation of antibacterial activity is further associated with an increased resistance to later infection (Faulhaber & Karp 1992; Moret & Siva-Jothy 2003). Another important pathway is the prophenoloxidase (proPO) cascade, which is activated by a variety of pathogens and involved in the melanization process and encapsulation (Sugumaran & Kanost 1993; Söderhäll & Cerenius 1998; Sugumaran 1998; da Silva *et al.* 2000). The key catalysing enzyme phenoloxidase (PO) is stored mainly in haemocytes as the inactive proenzyme proPO, which is rapidly activated upon infection. The accumulation of proPO instead of PO might reduce the risk of oxidative damage to host tissues by quinones and other reactive oxygen metabolites that are produced by the active enzyme (Söderhäll & Cerenius 1998; Kumar *et al.* 2003). Despite the obvious relevance of a distinction between inactive proPO and active PO, previous studies in the field of ecological immunology assessed only the activity of the active enzyme, PO (Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Moret & Siva-Jothy 2003).

We investigated the condition-dependent effects of immune system activation in nymphs on immunity and physiological condition during adulthood in male field crickets *Gryllus campestris*. A long time-span of 24 days, on average, separated nymphal challenge from adult immune measurements. To manipulate condition we subjected penultimate instar nymphs to one of two feeding regimes and subsequently induced an immune response with bacterial lipopolysaccharide (LPS) in half of the individuals of each food treatment. LPS is an established immune elicitor that activates the humoral immune response without direct pathogenic effects (da Silva *et al.* 2000; Imler *et al.* 2000) and is usually cleared within a few hours (Mathison & Ulevitch 1979). The immune parameters we studied in adults included the haemolymph concentration of the antibacterial protein lysozyme and of the enzymes PO and proPO. To examine the long-term costs in terms of physiological state we measured the total protein load (i.e. concentration) in the haemolymph of the adult individuals. Adult immune parameters were measured twice, first early (3 days) after adult eclosion, then again later (9 days post-eclosion), after an additional adult challenge with LPS 24 h previously. On this later occasion, animals were also implanted with a nylon filament to acquire an additional measure of immunity, the encapsulation response towards foreign material.

2. MATERIAL AND METHODS

(a) Rearing conditions

Field crickets used in the present study were male offspring of mated females that had been caught on an uncut meadow near Hinterkappelen (Switzerland) in late June 2002. Females were transferred to a climate chamber at 26 °C, 65% relative humidity, and 14 L:10 D. For egg laying, females were kept singly in small containers containing a humid sandy peat substrate. Groups of 30 newly hatched nymphs were kept in plastic containers (1 × w × h = 38 × 20 × 25 cm³) in the same climate chamber. Rearing containers contained a layer of sand, egg cardboards for shelter, a vial with water, and a plastic tray with food *ad libitum* (i.e. fish chow, a 1:1 mixture of JBL Novovort and JBL Novogrand, JBL GmbH/Neuhausen, Germany). Approximately 90 days post-hatching, a total of 152 fourth instar male nymphs

were housed individually in plastic jars (1 × w × h = 13 × 9 × 18 cm³) inside a second climate chamber under similar climate and light conditions as experienced during the initial period of juvenile development. The jars contained sand bedding, a cardboard shelter and two plastic cups for water and food.

(b) Experimental design

The condition-dependent effects of immune system activation during the nymphal stage on adult immunity and condition were investigated using a two-by-two experimental design with food treatment and immune treatment as the main factors, each with two levels. The assignment of the nymphs to the experimental treatments was at random, controlling for variation in initial body mass across treatment groups (food treatment: $p = 0.94$; immune treatment: $p = 0.87$; interaction term: $p = 0.41$). Nymphs were subjected to one of two different feeding regimes, which started immediately upon individual housing and lasted until adult eclosion. The daily mean food ratio of nymphs in the high-food and the low-food group was, on average, 0.200 and 0.015 g, respectively. The food was refreshed every second or third day. These food ratios reflect an *ad libitum* and restricted feeding regime, respectively (Scheuber *et al.* 2003). One week after the start of the feeding regime we induced an immune response in half of the nymphs of each food treatment group, by injecting 10 µl of a Grace's insect medium-based 0.1% LPS solution. The remaining control nymphs received 10 µl of Grace's insect medium only. The LPS was derived from the Gram-negative bacterium *Serratia marcescens* (Sigma, L6136). LPS is an established non-pathogenic immune elicitor that induces several pathways of the immune response (Hoffmann *et al.* 1996; Söderhäll & Cerenius 1998). LPS has been shown to activate vertebrate immune functions via binding to toll-like receptor 4 (TLR 4). For arthropods, there are numerous studies using LPS to activate essential all-important parts of the immune system. Not only the two main pathways of the humoral immune response, the Toll and the Imd pathway (Hoffmann 2003), but also the proPO cascade are highly sensitive to LPS (Söderhäll & Cerenius 1998). Further immune traits such as phagocytosis and superoxide production are also enhanced in the presence of LPS (Wittwer *et al.* 1997). Prior to the injection we physically immobilized the nymphs and cleaned the sternites with 70% EtOH. Injections were applied ventrally between the third and fourth sternites using a 10 µl Hamilton syringe. After injection, males were immediately returned to their home cage. Mortality and moult into adulthood were checked at least every other day. The mean nymphal development period from the start of the experiment until adult eclosion was 23.9 ± 1.1 days, and was not affected by the food treatment and immune treatment. After adult eclosion, food was available *ad libitum* for all individuals, thus restricting the manipulation of nutritional condition to the nymphal stage only. Body mass was recorded immediately after adult eclosion and again 3 days later. On day 3 we obtained a haemolymph sample to assess adult measures of immunity.

To investigate the effect of juvenile immune insult on adult induced immunity, all males were challenged on day 8 after eclosion with LPS. Measures of immunity and protein load were assessed 24 h later, by taking a haemolymph sample. Following procedures described by König & Schmid-Hempel (1995) and Ryder & Siva-Jothy (2000), all males were subsequently implanted with a nylon monofilament between the third and fourth dorsal sternites, for a period of 24 h and transferred to a freezer at -20 °C. Nylon monofilaments activate an encapsulation response in insects and are supposed to be non-pathogenic. Crickets were dissected at a later stage to recover the encapsulated

implant. Using image analysis software (NIH Image 1.61, National Institutes of Health, USA) we measured mean grey value and size of the nylon filament. Implant size did not vary among treatment groups (food treatment: $F_{1,60} = 2.11$, $p = 0.15$; immune treatment: $F_{1,60} = 1.06$, $p = 0.31$; interaction term: $F_{1,58} < 0.001$, $p = 0.98$) and had no effect on encapsulation rate ($F_{1,57} = 1.11$, $p = 0.30$). Pronotum size, calculated as the product of length and width, was obtained as an index of structural body size. Body condition was determined as the residuals from the regression of body mass on pronotum size.

(c) Haemolymph collection

Prior to collection of haemolymph samples, individuals were cooled on ice for *ca.* 20 min until torpid and then punctured with a sterile injection needle (BD Microlance, 25G^{5/8}) under the dorsal pronotum plate. Aliquots of 5 μ l of outflowing haemolymph were obtained with a 5 μ l microcapillary (Corning Pyrex) and immediately drained into 45 μ l of ice-cold Grace's medium. Samples were immediately frozen in liquid nitrogen at -196°C and then stored at -80°C .

(d) Lysozyme assay

To obtain a measure of general antibacterial activity we assessed lysozyme-like activity, with a 'lytic zone assay' modified from Mohrig & Messner (1968) and Wiesner *et al.* (1998). Agar plates containing 5 ml of 1% agar L11 (Oxoid) with 5 mg ml⁻¹ freeze-dried *Micrococcus luteus* were prepared following Kurtz *et al.* (2000). Eight holes with a diameter of 3 mm were punched in the agar layers and 3 μ l of thawed haemolymph solution were added to each hole. After incubation of the plates at 30°C for 24 h lytic zones were analysed using a digital imaging system and the public domain NIH Image program. Standard curves were obtained by dilution series of lysozyme from hen egg white (Merck 5282, 100 000 units mg⁻¹). Based on the logarithmic relation to lysozyme concentration, diameters of lytic zones obtained from the haemolymph samples were converted to lysozyme-like activities, i.e. ng ml⁻¹ equivalents of hen egg white lysozyme. Within-individual repeatability of this measurement was obtained by analysing two samples per individual and was highly significant ($r = 0.74$, $F_{102,103} = 6.68$, $p < 0.01$).

(e) PO and proPO assay

To assess PO and proPO activity we followed a modified protocol from Kopáček *et al.* (1995). From each individual, the haemolymph concentrations of active PO and the proenzyme proPO were measured. PO was quantified without further activation, while the proenzyme proPO was assayed after its activation with chymotrypsin to the active PO. For PO measurements, reaction mixtures contained 2 μ l of haemolymph and 50 μ l of 0.1 M Bis-Tris-HCl buffer, pH 6.5, and 98 μ l of distilled water. For proPO measurements, 5 μ l of chymotrypsin (5 mg ml⁻¹ in H₂O) were added to the mixture (containing only 93 μ l of distilled water in this case) and incubated for 5 min at room temperature. As a substrate, 50 μ l of DL-DOPA (4 mg ml⁻¹ in 0.1 M Bis-Tris-HCl buffer, pH 6.5; prepared freshly and protected from light) was added, comprising a final volume of 200 μ l. The reaction was allowed to proceed for 60 min and readings were taken every minute at 490 nm on a BIO-TEK PowerWave X Select microplate scanning spectrophotometer (BIO-TEK Instruments, Inc., Winooski, Vermont, USA), using the BIO-TEK KC4 (2000) software. The activity was measured as the slope of product formation during 20 min of the linear phase starting after 20 min. Negative controls, containing either no haemolymph or traces of phenylthiourea (PTU), an inhibitor of PO, showed only minimal activity. Repeatabilities, calculated on the

Figure 1. Adult antibacterial activity (mean + s.e.m.) 3 days post-eclosion in relation to nymphal food and immune treatment. The LPS challenge and the food treatment had a positive effect on lysozyme-like activity (immune treatment: $F_{1,101} = 8.29$, $p < 0.01$; food treatment: $F_{1,101} = 5.33$, $p = 0.02$; interaction term: $F_{1,100} = 0.57$, $p = 0.45$).

basis of two samples per individual, were highly significant for both measurements (PO: $r = 0.76$, $F_{109,110} = 7.35$, $p < 0.01$; proPO: $r = 0.83$, $F_{102,103} = 10.79$, $p < 0.01$).

(f) Haemolymph protein load

To obtain a measure of physiological condition we assessed the total protein load of the haemolymph (Ozbay & Riley 2002; Nakamatsu & Tanaka 2003). The concentration of total protein was determined photometrically in 5 μ l of diluted haemolymph, using the test solution Roti Nanoquant (K 880.1, Carl Roth GmbH & Co., Karlsruhe, Germany), following the protocol of the manufacturer. Measurements of OD₅₉₀/OD₄₅₀ were taken in 96-well plates on a BIO-TEK PowerWave X Select microplate scanning spectrophotometer (BIO-TEK Instruments, Inc., Winooski, Vermont, USA), using the BIO-TEK KC4 (2000) software. Standard curves were obtained from serial dilutions of bovine serum albumin (SIGMA, Taufkirchen, A 3912) and included on each 96-well plate. By analysing two samples for each individual we found a significant repeatability for this measurement ($r = 0.64$, $F_{87,88} = 4.48$, $p < 0.01$).

(g) Data analysis

Data analyses were performed with the STATA 8.0 statistical package (StataCorp LP, USA). To describe the variation in immune parameters we used multivariate general linear models with the immune parameters as variates, and the food and the immune treatments with their mutual interaction as predictor variables. Body condition and protein load were analysed univariate. Time until adult eclosion was further included as a covariate to control for developmental variation in adult immune parameters and protein load. The effect of the adult immune insult was analysed in a repeated-measures ANOVA with immune parameters and protein load on day 3 and day 9 as repeats.

Figure 2. The effects of nymphal food treatment and immune treatment on proPO activity (a) and PO activity (b) in male adult field crickets 3 days post-eclosion. LPS males showed higher proPO activity ($F_{1,101} = 4.17$, $p = 0.04$) independent of food treatment ($F_{1,101} = 0.83$, $p = 0.36$; interaction term: $F_{1,100} = 0.16$, $p = 0.69$), while there was no effect of the immune treatment on PO activity (immune treatment: $F_{1,102} = 1.23$, $p = 0.27$; food treatment: $F_{1,102} = 0.48$, $p = 0.49$; interaction term: $F_{1,100} = 3.16$, $p = 0.08$).

3. RESULTS

(a) Adult immune parameters

Adult antibacterial activity was significantly affected by the immune treatment. In addition, there was a significant effect of nymphal nutrition, which was independent of nymphal immunity. Adult antibacterial activity was higher in LPS males than in control males; given the effect of the nymphal immune treatment, nymphal food restriction reduced lysozyme-like activity (figure 1). Lysozyme-like activity was not explained by variation in nymphal development time ($F_{1,101} = 0.06$, $p = 0.81$).

The haemolymph concentration of proPO was higher in immune-treated individuals, whereas the variation was independent of the food treatment (figure 2a), and independent of nymphal development time ($F_{1,101} = 0.49$, $p = 0.48$). PO activity showed no significant relationship with the food or immune treatments (figure 2b). Nymphal development time ($F_{1,100} = 1.49$, $p = 0.22$) did not affect PO activity.

Experimental treatments did not affect adult body condition (food treatment: $F_{1,98} = 1.69$, $p = 0.20$; immune treatment: $F_{1,98} = 3.34$, $p = 0.07$; interaction term: $F_{1,96} = 2.93$, $p = 0.09$). Total haemolymph protein load was significantly affected by the immune treatment, while there was no additional effect of the nymphal food treatment or nymphal development time ($F_{1,100} = 1.55$, $p = 0.22$). Control males had a higher haemolymph protein load than LPS males (figure 3).

(b) Induced adult immunity

Measures of induced adult immunity on day 9 were stronger than measures of immunity on day 3 post-eclosion (repeated measures ANOVA; PO: $F_{1,92} = 33.75$, $p < 0.01$; proPO: $F_{1,95} = 74.47$, $p < 0.01$; lysozyme-like activity: $F_{1,95} = 26.86$, $p < 0.01$; table 1). While lysozyme- and proPO activity increased by ca. 22–23%, PO activity increased by 60%. Neither the absolute value, nor the increase in lysozyme activity and in PO or proPO activity was related to nymphal immune or food treatment (table 1). Furthermore, the level of proPO activity on day 3 was a predictor for the level of PO activity on day 9 ($F_{1,93} = 16.44$, $p < 0.01$). Encapsulation of the nylon implant was independent of the food treatment ($F_{1,86} = 0.33$, $p = 0.57$) and immune treatment ($F_{1,86} = 1.55$, $p = 0.22$; for interaction term: $F_{1,84} = 0.27$, $p = 0.60$).

Males in the different experimental groups did not differ in body condition on day 9 (food treatment: $F_{1,92} = 0.66$, $p = 0.42$; immune treatment: $F_{1,92} = 1.39$, $p = 0.24$; interaction term: $F_{1,90} = 0.05$, $p = 0.83$). The change in physiological state, from day 3 to day 9 was not related to the experimental treatments (table 1).

4. DISCUSSION

This study revealed that adult male field crickets show significantly elevated levels of two key parameters of immunity, i.e. antibacterial activity and the haemolymph concentration of the enzyme proPO, in response to an antigenic challenge several weeks earlier during the juvenile stage. Interestingly, under *ad libitum* food conditions, the inactive precursor enzyme proPO was elevated upon juvenile immune challenge, while the level of the active enzyme PO was not above the values found in the corresponding controls.

Conceivably, the animals may thereby avoid the immunopathological costs of the enzymes' activity. PO can produce highly toxic intermediates, such as quinones, peroxides and free oxygen radicals, which can cause apoptosis at the site of release (Söderhäll & Cerenius 1998). This process has been suggested as a functional part of the immune response in cases of parasitic infection (Söderhäll & Cerenius 1998), but can also lead to oxidative stress and immunopathological effects (Söderhäll & Cerenius 1998; Sugumaran *et al.* 2000) that may eventually reduce lifespan (Sohal 1988). By contrast, the precursor proPO can be safely stored in high quantities and rapidly activated upon infection (Söderhäll & Cerenius 1998). The strong upregulation of lysozyme in the haemolymph after a juvenile immune challenge may have a function as a safeguard against future reinfection or to control latent infections (Stanley-Samuelson *et al.* 1991; Yu & Kanost 2003). Antimicrobial peptides are relatively slowly synthesized and activate a non-oxidative antimicrobial pathway without

Figure 3. Total adult protein load (mean $\pm$ s.e.m.) in relation to nymphal food treatment and immune treatment. LPS males had a lower haemolymph protein load than control males ($F_{1,101} = 6.64$, $p = 0.01$) independent of food treatment ($F_{1,100} = 0.01$, $p = 0.91$; interaction term: $F_{1,99} = 1.84$, $p = 0.15$).

cytotoxic effects on non-target cells (Hoffmann 1995). LPS-challenged animals may thereby enhance future parasite resistance without risk of oxidative damage to self-tissues. However this long-term upregulation of general immunity depletes resources and comes with a metabolic cost, seen as a reduced adult haemolymph protein load in immune-challenged individuals. Protein load of the haemolymph has been used as an adequate indicator of physiological metabolic condition (Ozbay & Riley 2002; Nakamatsu & Tanaka 2003) and disease resistance (Adamo 2004) in invertebrates.

Nymphal nutritional condition positively affected lysozyme activity, while there was no effect on the proPO cascade. This discrepancy in the conditional effects on immune parameters suggests that nutritional constraints and/or limitations during juvenile development act more strongly on antimicrobial peptide production than on proPO-related immunity. As previously mentioned, bacterial infections are often latent, i.e. cannot be fully cleared by the host. Here, a lasting upregulation of specific antimicrobial peptides may give optimal long-term protection. While there are a few studies demonstrating the short-term nutritional effects on immunity (Feder *et al.* 1997; Siva-Jothy & Thompson 2002; Rantala *et al.* 2003), to our knowledge this is the first study demonstrating the lasting effects of juvenile food availability on adult immunity.

The immediate effect of an adult immune stimulation markedly differed from the long-term effect of the juvenile treatment. Here, all individuals increased proPO, PO and lysozyme activity, irrespective of the experimental treatment in the juvenile phase. In addition, encapsulation rate of an implanted monofilament was independent of juvenile food availability and juvenile immune status. The absence of any difference between the treatment groups might indicate that all individuals now show a maximal response to immune stimulation in order to resist an ongoing infection. The fitness benefits of an induced immune system upregulation are

Table 1. Measures of induced adult immunity and protein load in relation to food and immune treatment as nymphs. (All Δ values are differences (mean $\pm$ s.e.m.) between day 3 and day 9 post adult eclosion. The absolute initial values for immune parameters and protein load are given in figures 1–3. Statistics are based on repeated-measurement ANOVAs with absolute values and indicate within-individual effects. Encapsulation was measured as the mean grey value ($\pm$ s.e.m.) of the implanted nylon filament.)

	ad libitum food				food-restricted				time $\times$			
	sham-injected		LPS		sham-injected		LPS		time	food	challenge	food $\times$ challenge
Δ PO	0.30 $\pm$ 0.08		0.12 $\pm$ 0.06		0.33 $\pm$ 0.10		0.27 $\pm$ 0.11		$p < 0.01$	$p = 0.35$	$p = 0.12$	$p = 0.40$
Δ proPO	3.50 $\pm$ 0.64		1.93 $\pm$ 0.78		3.96 $\pm$ 0.66		3.28 $\pm$ 0.78		$p < 0.01$	$p = 0.19$	$p = 0.28$	$p = 0.46$
Δ lysozyme	15.21 $\pm$ 5.23		4.10 $\pm$ 9.33		10.41 $\pm$ 3.60		18.68 $\pm$ 7.89		$p < 0.01$	$p = 0.92$	$p = 0.19$	$p = 0.14$
Δ protein load	2.60 $\pm$ 3.24		1.70 $\pm$ 4.35		-2.57 $\pm$ 5.21		4.51 $\pm$ 4.04		$p = 0.34$	$p = 0.57$	$p = 0.86$	$p = 0.34$
encapsulation	66.28 $\pm$ 3.29		73.46 $\pm$ 4.30		65.88 $\pm$ 4.07		68.66 $\pm$ 5.38			$p = 0.57$	$p = 0.22$	$p = 0.60$

not necessarily linked only to the magnitude of the immune response but also to the short- and long-term costs of the host response. In addition, the adaptive benefits of enhanced immunity might only be detectable under real parasitism with its complex host–parasite interactions, whereas a challenge with a non-pathogenic antigen will mainly give information about the short-term host response.

In conclusion, our study revealed that juvenile field crickets respond to an antigenic challenge with a long-term up-regulation of constitutive immune function that extends into adulthood. Such induced responses presumably reflect the main invertebrate strategy to deal with variation in future parasitism. Immunopathological costs can be reduced via storage of inactive precursors. However, the upregulation of immunity comes with a lasting reduction in metabolic state that may eventually restrain host fitness (Jacot *et al.* 2004). Consequently, this underlying trade-off plays a key role in the evolution of adaptive invertebrate immunity.

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REFERENCES

- Adamo, S. A. 2004 Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J. Insect Physiol.* **50**, 209–216.
- Barnes, A. I. & Siva-Jothy, M. T. 2000 Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proc. R. Soc. Lond. B* **267**, 177–182. (doi:10.1098/rspb.2000.0984.)
- da Silva, C., Dunphy, G. B. & Rau, M. E. 2000 Interaction of *Xenorhabdus nematophilus* (Enterobacteriaceae) with the antimicrobial defenses of the house cricket, *Acheta domesticus*. *J. Invertebr. Pathol.* **76**, 285–292.
- Faulhaber, L. M. & Karp, R. D. 1992 A diphasic immune response against bacteria in the American cockroach. *Immunology* **75**, 378–381.
- Feder, D., Mello, C. B., Garcia, E. S. & Azambuja, P. 1997 Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdyson. *J. Insect Physiol.* **43**, 513–519.
- Folstad, I. & Karter, A. J. 1992 Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**, 603–622.
- Frost, S. D. W. 1999 The immune system as an inducible defense. In *The ecology and evolution of inducible defenses* (ed. R. Tollrian & C. D. Harvell), pp. 104–126. Princeton, NJ: Princeton University Press.
- Gillespie, J. P., Kanost, M. R. & Trenczek, T. 1997 Biological mediators of insect immunity. *A. Rev. Entomol.* **42**, 611–643.
- Goater, C. P. & Holmes, J. C. 1997 Parasite-mediated natural selection. In *Host-parasite evolution. General principles and avian models* (ed. D. H. Clayton & J. Moore), pp. 9–29. Oxford University Press.
- Hamilton, W. D. & Zuk, M. 1982 Heritable true fitness and bright birds: a role for parasites? *Science* **218**, 384–387.
- Hoffmann, J. A. 1995 Innate immunity of insects. *Curr. Opin. Immunol.* **7**, 4–10.
- Hoffmann, J. A. 2003 The immune response of *Drosophila*. *Nature* **426**, 33–38.
- Hoffmann, J. A., Reichhart, J. M. & Hetru, C. 1996 Innate immunity in higher insects. *Curr. Opin. Immunol.* **8**, 8–13.
- Imler, J. L., Tauszig, S., Jouanguy, E., Forestier, C. & Hoffmann, J. A. 2000 LPS-induced immune response in *Drosophila*. *J. Endotoxin Res.* **6**, 459–462.
- Iwanaga, S., Söderhäll, K. & Vasta, G. 1995 *Invertebrate immunology*. Fair Haven, NJ: SOS Publications.
- Jacot, A., Scheuber, H. & Brinkhof, M. W. G. 2004 Costs of an induced immune response on sexual display and longevity in field crickets. *Evolution*. **58**, 2280–2286.
- König, C. & Schmidt-Hempel, P. 1995 Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. *Proc. R. Soc. B* **260**, 225–227.
- Kopáček, P., Weise, C. & Götz, P. 1995 The prophenoloxidase from the wax moth *Galleria mellonella*: purification and characterization of the proenzyme. *Insect Biochem. Mol. Biol.* **25**, 1081–1091.
- Kumar, S., Christophides, G. K., Cantera, R., Charles, B., Han, Y. S., Meister, S., Dimopoulos, G., Kafatos, F. C. & Barillas-Mury, C. 2003 The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA* **100**, 14 139–14 144.
- Kurtz, J. & Franz, K. 2003 Evidence for memory in invertebrate immunity. *Nature* **425**, 37–38.
- Kurtz, J., Wiesner, A., Götz, P. & Sauer, K. P. 2000 Gender differences and individual variation in the insect immune system of the scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). *Devl Comp. Immunol.* **24**, 1–12.
- Lochmiller, R. L. 1996 Immunocompetence and animal population regulation. *Oikos* **76**, 594–602.
- Mathison, J. C. & Ulevitch, R. J. 1979 Clearance, tissue distribution, a cellular-localization of intravenously injected lipopolysaccharide in rabbits. *J. Immunol.* **125**, 2133–2143.
- Mohrig, W. & Messner, B. 1968 Immunreaktionen bei Insekten. Lysozym als grundlegender antibakterieller Faktor im humoralen Abwehrsystem der Insekten. *Biol. Zbl.* **4**, 439–470.
- Moret, Y. & Siva-Jothy, M. T. 2003 Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. *Proc. R. Soc. B* **270**, 2475–2480.
- Morishima, I., Horiba, T., Iketani, M., Nishioka, E. & Yamano, Y. 1995 Parallel induction of cecropin and lysozyme in larvae of the silkworm, *Bombyx mori*. *Devl Comp. Immunol.* **19**, 357–363.
- Nakamatsu, Y. & Tanaka, T. 2003 Venom of ectoparasitoid, *Euplectrus* sp. near *plathypenae* (Hymenoptera: Eulophidae) regulates the physiological state of *Pseudaletia separata* (Lepidoptera: Noctuidae) host as a food resource. *J. Insect Physiol.* **49**, 149–159.
- Ozbay, G. & Riley, J. G. 2002 An analysis of refractometry as a method of determining blood total protein concentration in the American lobster *Homarus americanus* (Milne Edwards). *Aquacult. Res.* **33**, 557–562.
- Rantala, M., Kortet, R., Kotiaho, J. S., Vainikka, A. & Suhonen, J. 2003 Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Funct. Ecol.* **17**, 534–540.
- Roitt, I., Brostoff, J. & Male, D. 1996 *Immunology*. London: Mosby.
- Rolff, J. & Siva-Jothy, M. T. 2003 Invertebrate ecological immunology. *Science* **301**, 472–475.
- Ryder, J. J. & Siva-Jothy, M. T. 2000 Male calling song provides a reliable signal of immune function in a cricket. *Proc. R. Soc. B* **267**, 1171–1175. (doi:10.1098/rspb.2000.1125.)
- Scheuber, H., Jacot, A. & Brinkhof, M. W. G. 2003 The effect of past condition on a multicomponent sexual signal. *Proc. R. Soc. B* **270**, 1779–1784. (doi:10.1098/rspb.2003.2449.)
- Schmid-Hempel, P. 2003 Variation in immune defence as a question of evolutionary ecology. *Proc. R. Soc. B* **270**, 357–366. (doi:10.1098/rspb.2002.2265)
- Sheldon, B. C. & Verhulst, S. 1996 Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317–321.
- Siva-Jothy, M. T. & Thompson, J. J. W. 2002 Short-term nutrient deprivation affects immune function. *Physiol. Entomol.* **27**, 206–212.

- Söderhäll, K. & Cerenius, L. 1998 Role of the prophenol-oxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **10**, 23–28.
- Sohal, R. S. 1988 Effect of hydrogen peroxide administration on life span, superoxide dismutase, catalase, and glutathione in the adult housefly, *Musca domestica*. *Exp. Gerontol.* **23**, 211–216.
- Stanley-Samuelson, D. W., Jensen, E., Nickerson, K. W., Tiebel, K., Ogg, C. L. & Howard, R. W. 1991 Insect immune response to bacterial infection is mediated by eicosanoids. *Proc. Natl Acad. Sci. USA* **88**, 1064–1068.
- Sugumaran, M. 1998 Characterization of phenoloxidase complexes. In *Techniques in insect immunology* (ed. A. Wiesner, G. B. Dunphy, V. J. Marmaras, M. Sugarman & M. Yamakawa), pp. 205–215. Fair Haven, NJ: SOS Publications.
- Sugumaran, M. & Kanost, M. R. 1993 Regulation of insect hemolymph phenoloxidase. In *Parasites and pathogens of insects* vol. 1 (ed. N. E. Beckage, S. N. Thompson & B. A. Federici), pp. 317–342. San Diego, CA: Academic Press.
- Sugumaran, M., Nellaiappan, K. & Valivittan, K. 2000 A new mechanism for the control of phenoloxidase activity: inhibition and complex formation with quinone isomerase. *Arch. Biochem. Biophys.* **379**, 252–260.
- Wakelin, D. 1996 *Immunity to parasites*. Cambridge University Press.
- Westneat, D. F. & Birkhead, T. R. 1998 Alternative hypotheses linking the immune system and mate choice for good genes. *Proc. R. Soc. B* **265**, 1065–1073. (doi:10.1098/rspb.1998.0400)
- Wiesner, A., Rohloff, L.-H., Wittwer, D., Pohl, U., Van Sambeek, J., Kurtz, J. & Götz, P. 1998 Phagocytosis by insect hemocytes in vitro. In *Techniques in insect immunology* (ed. A. Wiesner, G. B. Dunphy, V. J. Marmaras, M. Sugumaran & M. Yamakawa), pp. 11–20. Fair Haven, NY: SOS Publications.
- Wilson, K., Cotter, S. C., Reeson, A. F. & Pell, J. K. 2001 Melanism and disease resistance in insects. *Ecol. Lett.* **4**, 637–649.
- Wittwer, D., Weise, C., Gotz, P. & Wiesner, A. 1997 LPS (lipopolysaccharide)-activated immune responses in a hemocyte cell line from *Estigmene acrea* (Lepidoptera). *Devl Comp. Immunol.* **21**, 323–336.
- Yu, X.-Q. & Kanost, M. R. 2003 *Manduca sexta* lipopolysaccharide-specific immuelectin-2 protects larvae from bacterial infection. *Devl Comp. Immunol.* **27**, 189–196.
- Zuk, M. & Stoehr, A. M. 2002 Immune defense and host life history. *Am. Nat.* **160**, 9–22.

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