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Interplay between thermal and immune ecology: Effect of environmental temperature on insect immune response and energetic costs after an immune challenge

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ABSTRACT

Although the study of thermoregulation in insects has shown that infected animals tend to prefer higher temperatures than healthy individuals, the immune response and energetic consequences of this preference remain unknown. We examined the effect of environmental temperature and the energetic costs associated to the activation of the immune response of *Tenebrio molitor* larvae following a lipopolysaccharide (LPS) challenge. We measured the effect of temperature on immune parameters including phenoloxidase (PO) activity and antibacterial responses. Further as proximal and distal costs of the immune response we determined the standard metabolic rate (SMR) and the loss of body mass (m_b), respectively. Immune response was stronger at 30 °C than was at 10 or 20 °C. While SMR at 10 and 20 °C did not differ between immune treatments, at 30 °C SMR of LPS-treated larvae was almost 25–60% higher than SMR of PBS-treated and naïve larvae. In addition, the loss in m_b was 1.9 and 4.2 times higher in LPS-treated larvae than in PBS-treated and naïve controls. The immune responses exhibited a positive correlation with temperature and both, SMR and m_b change, were sensitive to environmental temperature. These data suggest a significant effect of environmental temperature on the immune response and on the energetic costs of immunity.

1. Introduction

The study of thermal and immune ecology has the potential to provide a mechanistic bridge between the physiology of immunity and thermoregulation during pathogen–host encounter and the resulting life histories in an ecological and evolutionary context (Blanford and Thomas, 1999). Because environmental temperature varies in time and space, organisms are continuously challenged to maintain homeostasis (Johnston and Bennett, 1996) and thermal constraints are not only the result of ambient conditions, but also those achieved through thermoregulation. Such a constraint may increase when individuals are exposed to infectious agents, including bacteria.

Environmental temperature can significantly influence the components of host–pathogen biological interaction by affecting both: pathogen virulence and growth rate (Inglis et al., 1996; Ouedraogo et al., 1997; Arthurs and Thomas, 2001), as well as the host capacity to fight infections (Blanford and Thomas, 1999; Thomas and Blanford, 2003). However, the mechanisms responsible for the modulation of the host–pathogen interaction by environmental temperature remain unknown (Ouedraogo et al., 2003). In fact, there is only scarce empirical evidence in relation to the influence of environmental temperature on the energetic costs of the immune response and its consequences on host fitness. Changes in body temperature induced by fluctuations in ambient temperature can affect several traits of ectotherm energetics (Wiener, 1992; Angilletta, 2009). It has been proposed that the adaptive significance of preferred body temperatures correlates with temperature values that optimise physiological performance and consequently maximise Darwinian fitness (Huey and Bennett, 1987; Angilletta et al., 2004). Consistent with this notion is the behavioural fever phenomenon, whereby an infection is accompanied by a change in the host preference for higher environmental temperatures, which may enhance or improve the immune response (Kluger et al., 1998) with a consequent increase in energy expenditure (Boorstein and Ewald, 1987; Chown and Nicolson, 2004).

In insects, the immune system consists of cellular and humoral responses acting synergistically to control the spread of an infection.

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Cellular responses are mainly mediated by hemocytes and include encapsulation, nodulation and phagocytosis (Gillespie et al., 1997; Lavine and Strand, 2002). On the other hand, humoral defense includes the production of reactive species derived from oxygen and nitrogen and of antibacterial peptides. Perhaps one of the most important constitutive effectors of immune response in insects is the tyrosinase phenoloxidase (PO), which works as an effector of the innate immune response in invertebrates. Such a response is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004; Siva-Jothy et al., 2005) and leads to the production of sticky proteins, which immobilise and render the pathogen susceptible to other host defence effector mechanisms, including phagocytosis and encapsulation (Siva-Jothy et al., 2005).

Early studies have shown that warmer temperatures increase insect immune performance through the increase of the enzymatic activity of phenoloxidase and lysozyme-like enzymes (Adamo and Lovett, 2011; Fuller et al., 2011), as well as the maintenance of hemocytes numbers and haemolymph protein levels (Ouedraogo et al., 2003). At the molecular level, thermal as well as physical stress may elicit changes in the expression of antimicrobial peptides, which contribute to the initiation and maintenance of the immune response (Mowlds et al., 2008). Nevertheless, in theory, high immunocompetence is costly and difficult to sustain over extended periods of times (Moret and Schmid-Hempel, 2000; Haine et al., 2008a). Thus, an optimal immunocompetence may not only require a specific environment temperature, but also an energetic supply that is defined by the environmental conditions.

Several studies have suggested the occurrence of large immunity costs through trade-offs between immunocompetence and life history traits (Barnes and Siva-Jothy, 2000; Armitage et al., 2003; Fedorka et al., 2004; Gwynn et al., 2005; Rantala and Roff, 2005; Springate and Thomas, 2005). However, a direct cost of the immune response on metabolic rate has been difficult to demonstrate (for invertebrates see Freitak et al., 2003 and for vertebrates Malvin and Kluger, 1979; Demas et al., 1997; Svensson et al., 1998; Lochmiller and Deerenberg, 2000; Moreno et al., 2001). The prediction of the outcome of the pathogen–host interaction under various natural and human-induced environmental conditions would require the measurement of immunocompetence and costs of immune traits. In this study we have assessed the question as to whether the environmental temperature can modulate the immune response in insects. Further, we explored a possible dependence for energetic cost on both, the immune response and the environmental temperature. We hypothesised that warmer temperatures increase the immune activation, in parallel with an increase in metabolic rate and loss of body mass.

An immune response was induced in *Tenebrio molitor* larvae by the challenge with *Escherichia coli* lipopolysaccharide (LPS) and two parameters of the innate immune response were measured: phenoloxidase (PO) and antibacterial responses. These traits have been largely used in insects as indicators of resistance to pathogens and parasites (Moret and Siva-Jothy, 2003). Further, to determine the costs of the immune response under different environmental temperature conditions we measured metabolic rate as a proximal cost and body mass (m_b) change as an ultimate cost, two well known parameters for Darwinian fitness (Angilletta et al., 2004).

2. Materials and methods

2.1. Mealworm cultures

As a study model we employed the insect *T. molitor* L. (Coleoptera: Tenebrionidae) because unlike vertebrates, insects are able to elicit only innate immune responses and do not exhibit specific adaptive immune responses. Therefore, *T. molitor* enables to estimate the cost associated with innate immunity without the potentially confounding effect of adaptive immunity. Additionally, short term energetic costs would be expected due to the previous demonstration of life-history trade-offs associated to immune costs in *T. molitor* (Armitage et al., 2003; Krams et al., 2011). We used the larval stage because, at this stage, sex and reproduction are not particularly relevant; moreover, animals are gaining body mass as their main physiological output. Hence, any alteration of energy allocation can be easily determined by measuring body mass and standard metabolic rate.

Larvae of *T. molitor* were randomly selected from a stock culture maintained since year 2000 under laboratory conditions (23 ± 2 °C and 12:12 photoperiod) and supplied with food consisting of apple peelings and a mixture of flour (60%), oats (20%), yeast (10%) and bran (10%) provided *ad libitum*. Insects were kept in plastic chambers carefully cleaned and autoclaved; mixed dry food was heated at 70 °C for 72 h to eliminate pathogens and parasites. Larvae with similar body mass at the fifth instar were employed and different sets of animals were used for each measurement to avoid any effect of previous manipulations on our results. Animals used showed no signs of moulting, such as colour change or immobility; furthermore, they were observed for 3 days under laboratory conditions (23 ± 2 °C and LD = 12:12 photoperiod) after SMR and m_b measurements, to ensure that they were not near moulting at the time in which measurements were performed. Any observable sign of moulting during this period led to the exclusion of animals from all analyses.

2.2. Immune response

To induce an innate immune response, animals were challenged with 4 μl (0.5 mg/ml) of *E. coli* lipopolysaccharide (LPS, Sigma 8274). Because this immune stimulus is not pathogenic or replicative, it allows measuring of the costs of deploying the immune system in invertebrates without the influence of pathological costs associated to the parasites themselves (Fellowes et al., 2005). As a control procedure, mealworms were injected with 4 μl of Phosphate Buffered Saline solution (PBS pH 6.4), and untreated (naïve) larvae were included as controls in each experiment. These control animals allowed determining the constitutive level of immune activity. Injections were made through the pleural membrane between the second and the third abdominal segments, using a sterile Hamilton syringe.

Two haemolymph immune variables were measured for each individual larva: haemolymph phenoloxidase (PO) activity and antibacterial activity. Haemolymph (10 μl per animal) was collected in pre-chilled glass capillaries by puncturing the pleural membrane. Each animal was bled only once. Immune traits were measured at 24 and 72 h after the challenge (Haine et al., 2008a y *b*). Animals (n = 10) were maintained at three different temperatures (10, 20 and 30 °C) during the post-injection period. Thus, we used a factorial design with environmental temperature and immune challenge treatment as fixed factors.

2.2.1. Antibacterial activity

Haemolymph (μl) was diluted with 24 μl PBS and 1 μl of an overnight culture (approximately 3×10^7 CFU ml^{-1}) of streptomycin-resistant *Micrococcus luteus* was added to the solution. The mixture was incubated at 30 °C with agitation at 150 rpm for 1 h. Then, the mixture was diluted 100 times and plated on LB agar containing 5 μg ml^{-1} streptomycin (Sigma S6501). The mixture (50 and 100 μl) was spread onto two plates per each animal. Plates were incubated at 30 °C for 48 h. The number of colonies was quantified on each plate and the mean for the two plates number of colony-forming units (CFUs) per μl of mixture spread was used

to calculate the antibacterial activity as the percentage of CFUs relative to control plates without larval haemolymph (modified from Cotter et al., 2004; Haine et al., 2008a; Ahmed et al., 2002).

2.2.2. Haemolymph PO activity

Haemolymph (5 μl) was added to 200 μl of ice-cold PBS at pH 6.4 in an Eppendorf tube and vortexed. Samples were frozen at −80 °C until use. PO activity was assayed spectrophotometrically with L-Dopa as a substrate (Wilson et al., 2001; Cotter et al., 2004). L-Dopa (Sigma D9628, 100 μl, 20 mM) was added to 100 μl buffered haemolymph and the OD (492 nm) of the mixture was determined at 25 °C with a microplate reader (Packard Bioscience model AS 10001) at 10 min intervals during 90 min. Enzyme activity, expressed as PO units (in min^{-1}) per μl haemolymph, was obtained by: (i) adjusting the kinetic data for each sample to the sigmoid Eq. (1); (ii) calculating ΔOD from Eq. (2), and (iii) dividing ΔOD by haemolymph volume and 0.001 (Lee et al., 2006)

$$OD_t = \frac{OD_{max}}{1 + e^{-\left(\frac{t - t_{max}}{V_{max}}\right)}} \quad (1)$$

$$\Delta OD = \frac{OD_{max} - OD_{control}}{2t_{max}} \quad (2)$$

2.3. Immune response costs

2.3.1. Metabolism

Standard metabolic rate (SMR) was measured as carbon dioxide production (V_{CO_2}) within a closed system as previously described (Lighton, 2008). At 24 and 72 h after injections and temperature acclimation, naïve, PBS- and LPS-treated insects were individually placed inside a glass syringe (2 ml) fitted with three way valves and completely sealed for 4 h under controlled environmental temperature at 10, 20 or 30 °C, respectively. Then, air from the syringe (1 ml) was slowly injected into a short Tygon tube connected to a glass tube (10 cm long) to avoid any loss before CO_2 readings (Lighton, 2008). The air pumped from the ambient was passed through a Drierite column to scrub the CO_2 before the injection. Samples passed directly to the CO_2 analyzer (Fox-Box, Sable system) with a 350 ml min^{-1} flow. Data were transformed from percentage to volume per h and the total CO_2 production per animal was calculated from the integral of the curve obtained with the program EXPEDATA (Sable Systems). All animals ($n = 15$) were weighed in an analytical balance (±0.0001 g; JK-180, Chyo, Kyoto) before and after each measurement.

2.3.2. Body mass loss

Body mass (m_b) loss was determined as the percentage of m_b loss of each larva after 24 and 72 h. Larvae ($n = 15$) were weighed and randomly assigned to immune challenge treatments (naïve, PBS and LPS) and to environmental temperature of 10, 20 and 30 °C. Animals were fasted for the experimental time and weighed again in an analytical balance (±0.0001 g; JK-180, Chyo, Kyoto).

2.4. Statistical analyses

To determine the effects of environmental temperature and immune challenges on the immune response traits and weight loss, a factorial ANOVA test with fixed factors (temperature: 10, 20 and 30 °C and immune treatments: naïve, PBS and LPS) was performed. In all cases a correlation between m_b and immune and cost traits was assessed. Differences in SMR were analysed with an ANCOVA using body mass as covariate. All data were tested for normality and homoscedasticity with Kolmogorov–Smirnov and Cochran C tests. When necessary, data were transformed to meet statistical assumptions. When differences were significant at $P < 0.05$ after the general linear model tests, *a posteriori* Newman Keuls test for multiple comparisons were used. All statistical analyses were conducted using Statistica 6.0 software (StatSoft Inc., 2001, Tulsa, OK).

3. Results

3.1. Immune response

Antibacterial activity differed significantly between immune challenge factors (naïve, PBS and LPS treatments) at 24 and 72 h after challenge, but the effect of environmental temperature was only significant at 24 h (Fig. 1; Table 1), when CFU counts were minimal. As expected, LPS-treated larvae exhibited lower CFUs percentages than did PBS-treated or naïve larvae at both time points tested (Newman Keuls test: naïve-LPS, $P < 0.0001$; PBS–LPS, $P < 0.005$ at 24 and 72 h). Although PBS-treated larvae showed less bacterial growth than did naïve larvae (Newman Keuls test: naïve-PBS, $P = 0.006$ at 24 and $P = 0.005$ at 72 h), differences were smaller than between naïve and LPS-treated animals (Newman Keuls test: naïve-LPS, $P < 0.001$ at 24 and 72 h) (Fig. 1). Antibacterial response increased with environmental temperature at 24 h after challenge (Fig. 1a), but at 72 h any co-relation with environmental temperature was no longer observed (Table 1 and Fig. 1b). Thus, plate-assays showed that the antibacterial activity in the haemolymph of LPS-treated animals

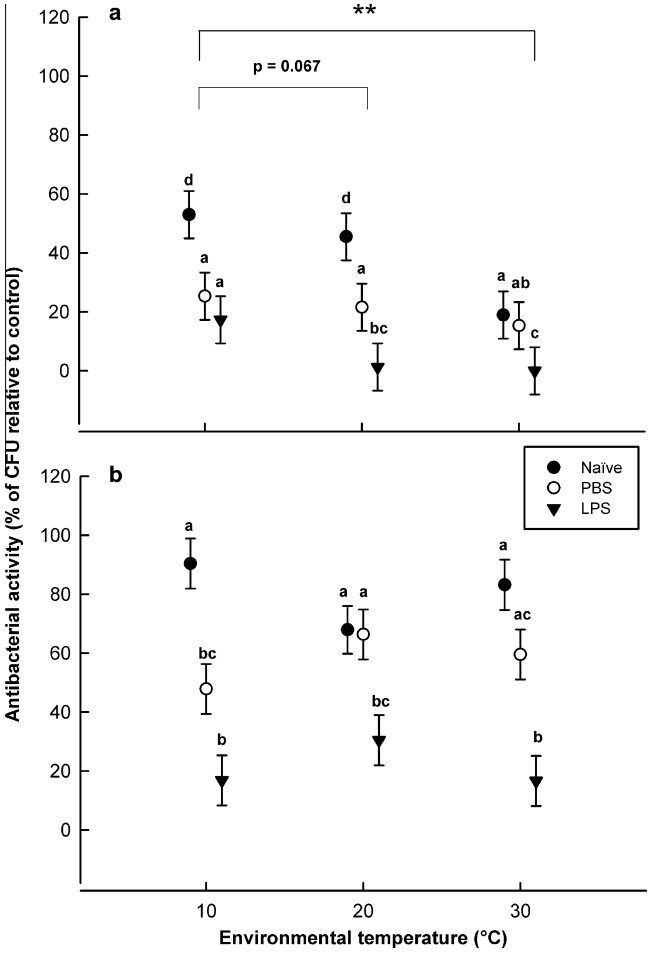

Fig. 1. Antibacterial activity (±SE) in the haemolyph of naïve, PBS- (Phosphate Buffered Saline solution) and LPS- (*E. coli* lipopolysaccharide) treated *T. molitor* larvae tested at 10, 20 and 30 °C at (a) 24 h and (b) 72 h after challenge. Different letters show significant differences among treatments. Asterisks indicate statistical differences between temperatures (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Table 1
Results of factorial two-way ANOVA testing for the effects of environmental temperature (fixed factor) and immune challenge treatment (fixed factor) on antibacterial activity at 24 and 72 h.

Factor	Sum of squares	Df	Mean square	*F*	*P*-value
24 h Antibacterial activity (% of CFU relative to control)					
Environmental temperature (°C)	1.323	2	0.662	5.642	0.005**
Immune challenge treatment	4.040	2	2.020	17.224	<0.001***
Temperature × treatment	0.608	4	0.152	1.296	0.278
Error	9.499	81	0.117		
72 h Antibacterial activity (% of CFU relative to control)					
Environmental temperature (°C)	0.013	2	0.006	0.042	0.958
Immune challenge treatment	9.631	2	4.816	32.226	<0.001***
Temperature × treatment	0.912	4	0.228	1.526	0.202
Error	12.104	81	0.149		

** $P < 0.01$.
*** $P < 0.001$.

Table 2
Results of factorial two-way ANOVA testing for the effects of environmental temperature (fixed factor) and immune challenge treatment (fixed factor) on PO activity at 24 and 72 h.

Factor	Sum of squares	Df	Mean squares	*F*	*P*-value
24 h PO activity (log PO units μl^{-1})					
Environmental temperature (°C)	0.567	2	0.284	13.927	<0.001***
Immune challenge treatment	0.592	2	0.296	14.534	<0.001***
Temperature × treatment	0.086	4	0.022	1.059	0.3822
Error	1.650	81	0.020		
72 h PO activity (log PO units μl^{-1})					
Environmental temperature (°C)	0.382	2	0.191	8.390	<0.001***
Immune challenge treatment	0.003	2	0.002	0.072	0.9310
Temperature x treatment	0.021	4	0.005	0.231	0.9201
Error	1.845	81	0.023		

*** $P < 0.001$.

led to CFU percentages equal to 1.28% and 0.01% at 20 and 30 °C, at 24 h, and 30.46% and 16.65% at 72 h, respectively.

Consequently, this response appeared to be more temperature-sensitive in naïve rather than in procedure control (PBS) or in immune challenged (LPS) larvae at 24 h (Fig. 1); this pattern was lost at 72 h, when antibacterial activity decayed considerably and CFU percentage was between 16.65% (for LPS treatment) and 90.38% (naïve treatment), independent of the temperature (Table 1 and Fig. 1b).

PO activity differed significantly between environmental temperatures at which animals were kept during 24 or 72 h after challenge (Table 2; Fig. 2). However, the effect of challenge treatment was significant only at 24 h (Fig. 2a). At 24 h post-challenge, LPS-treated larvae showed a significantly higher PO activity than did naïve animals (Newman Keuls: LPS-Naïve, $P = 0.007$), but did not than PBS injected larvae (LPS–PBS, $P = 0.1044$; Naïve-PBS, $P = 0.5749$) (Fig. 2a). Similar to antibacterial activity, higher PO activity was observed at 24 (3.47 ± 1.08, 4.00 ± 1.08 and 5.39 ± 1.05 units μl^{-1} at 10, 20 and 30 °C, respectively) than at 72 h (2.82 ± 1.08, 3.62 ± 1.05 and 4.03 ± 1.06 units μl^{-1} at 10, 20 and 30 °C, respectively) (Fig. 2). Finally, a higher PO activity was observed at 30 °C than at 10 and 20 °C, at each time point evaluated (24 and 72 h, Fig. 2).

3.2. Immune response costs

As expected, SMR was significantly affected by environmental temperature. At 24 h, the SMR increased as a direct function of the temperature (ANCOVA: $F_{(2,125)} = 151.81$; $P < 0.0001$), with no significant effect of immune insult ($F_{(2,125)} = 0.88$; $P = 0.417$). However, it was striking to observe that an immune challenge significantly affect SMR at 72 h after the immune insult (Table 3). Thus, animals treated with LPS, 72 h after the challenge showed significantly higher SMR than did naïve larvae and marginally higher SMR than did PBS-treated larvae (Newman Keuls test: LPS-naïve, $P < 0.0001$; LPS–PBS, $P = 0.053$) (Fig. 3). Such a marginal difference between LPS- and PBS-treated larvae can be explained by the finding of significant differences only between treatments at 30 °C but not at 10 and 20 °C. No significant differences were found between SMR of naïve and control procedure (PBS-treated) animals (Newman Keuls test: $P = 0.104$).

In addition, the interaction between factors was statistically significant only at 72 h after the insult (Table 3). At 10 and 20 °C no differences were observed between immune treatments, but at 30 °C the SMR of LPS-treated larvae was almost 60% and 25% higher than SMR of naïve and PBS-treated larvae, respectively (Fig. 3).

Similar to SMR, a clear effect of immune treatment and environmental temperature on body mass loss was observed only at 72 h (Table 3). At 24 h, both factors did not show an effect on body mass loss (ANOVA: Temperature: $F_{(2,126)} = 0.002$; $P = 0.998$; Treatment: $F_{(2,126)} = 0.004$; $P = 0.999$). On the contrary, at extreme temperatures, 10 and 30 °C, larvae lost 1.7 and 2.6 times more body mass than did at 20 °C (Newman Keuls test: 10–20 °C, $P = 0.008$; 20–30 °C, $P = 0.013$). Furthermore, LPS-treated larvae lost 4.2 and 1.9 times more body mass than did naïve and PBS-treated larvae, respectively. However, the effect of immune treatments was not significant at all tested temperatures. Whereas at 20 °C there were no differences between controls (naïve and PBS-treated) and LPS-treated larvae (Newman Keuls test: Naïve-LPS, $P = 0.397$; PBS–LPS, $P = 0.887$), at 10 and 30 °C, LPS-treated larvae lost 3.1 and 7.0 times more body mass than did naïve larvae (Newman Keuls test: Naïve-LPS, $P = 0.034$ at 10 °C and $P < 0.001$ at 30 °C) and 2.8 and 1.9 times more than did PBS-treated larvae, at the respective temperatures (Newman Keuls test: Naïve-LPS, $P = 0.032$ at 10 °C and $P = 0.006$ at 30 °C) (Fig. 4).

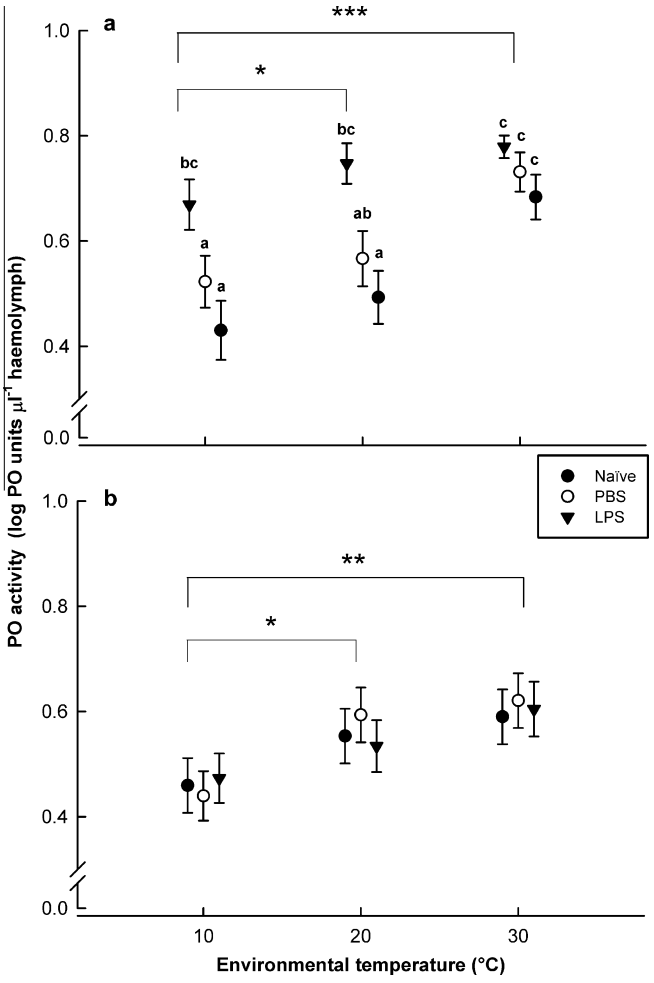

Fig. 2. PO activity in the haemolymph (±SE) of naïve, PBS- (Phosphate Buffered Saline solution) and LPS- (*E. coli* lipopolysaccharide) treated *T. molitor* larvae tested at 10, 20 and 30 °C at (a) 24 h and (b) 72 h after challenge. Different letters show significant differences among treatments. Asterisks indicate statistical differences between temperatures (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

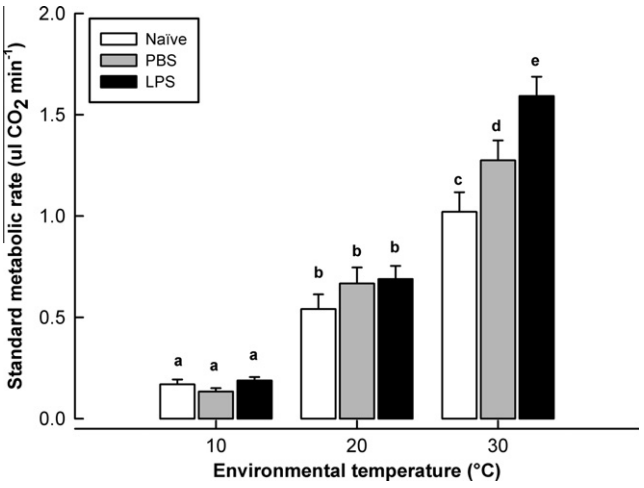

Fig. 3. Standard metabolic rate (±SE) of naïve, PBS- (Phosphate Buffered Saline solution) and LPS- (*E. coli* lipopolysaccharide) treated *T. molitor* larvae tested at 10, 20 and 30 °C. Different letters show significant differences among treatments.

4. Discussion

Ecological immunology proposes that immune defense varies according to biotic and abiotic habitat conditions and successfully establishes an evolutionary physiological ecology framework around immunology. Here we have evaluated the effect of temperature on immune responses and the proximal (metabolic) and ultimate (body mass loss) costs of this response in larvae of the insect *T. molitor*. Albeit the direct effect of temperature on pathogens and on its ability to infect the host has been largely investigated (Inglis et al., 1996; Blanford and Thomas, 1999; Elliot et al., 2002), few studies have approached the question as to how temperature influences host immune response and its consequences on the host energy allocation (but see Adamo and Lovett, 2011). Here we have explored for the first time the effect of temperature on immune responses and their associated costs.

Our results support the notion that at higher temperatures, all biochemical processes are accelerated, including protein synthesis, cellular migration and enzyme activity, as well as higher duration of responses (Haynie, 2008), with a positive effect on the immune response. These observations agree with previous data on *Locusta migratoria* (Ouedraogo et al., 2003) and *Eurygaster integriceps* (Zibaee et al., 2009). In both cases, thermoregulation above normothermy prevents the decrease in the hemocytes number and promotes the nodulation and the engulfment of silica beads during a fungal infection. These cellular responses, accompanied by antimicrobial peptides synthesis (Rolff and Reynolds, 2009) and the increase of the activity of lysozyme-like enzymes (Adamo and Lovett, 2011) are consistent with the positive effect of higher temperatures on antibacterial and PO activity observed on larvae 24 h after an LPS challenge. It is known that the production of the components required for an antibacterial response takes at least 1–3 h (Lavine et al., 2005) and 12–48 h to reach peak levels (Haine et al.,

Table 3
Results of factorial two-way ANOVA and ANCOVA testing for the effects of environmental temperature (fixed factor) and immune challenge treatment (fixed factor) on SMR and body mass change.

Factor	Sum of squares	Df	Mean squares	*F*	*P*-value
Standard metabolic rate (µl CO_2/min)					
m_b initial (mg)	0.433	1	0.433	6.015	0.016*
Environmental temperature (°C)	29.219	2	14.610	203.150	<0.001***
Immune challenge treatment	1.347	2	0.673	9.365	<0.001**
Temperature x treatment	1.143	4	0.286	3.975	0.005**
Error	8.990	125	0.072		
Body mass change (% loss)					
Environmental temperature (°C)	0.090	2	0.045	4.991	0.008**
Immune challenge treatment	0.320	2	0.160	17.809	<0.001***
Temperature × treatment	0.087	4	0.022	2.417	0.052
Error	1.133	126	0.009		

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

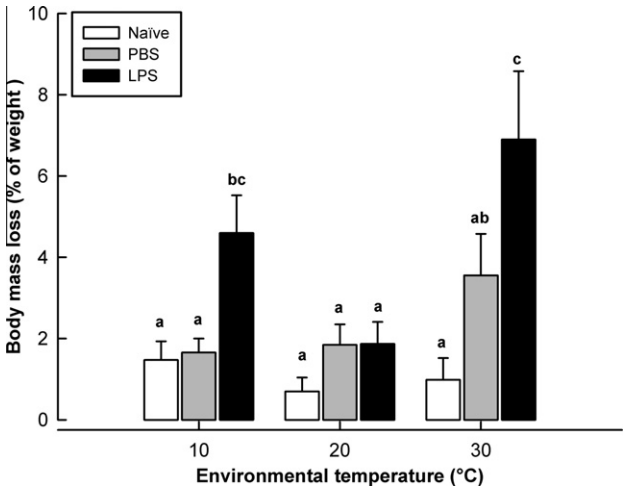

Fig. 4. Body mass loss (±SE) of naïve, PBS- (Phosphate Buffered Saline solution) and LPS (*E. coli* lipopolysaccharide) treated *T. molitor* larvae tested at 10, 20 and 30 °C. Different letters show significant differences among treatments.

2008b). Further, induced responses can persist even for weeks in a variety of insects (Hoffmann, 1980; Bulet et al., 1992; Jarosz, 1993; Korner and Schmid-Hempel, 2004), as is the case for the mealworm beetle *T. molitor*, in which they persist for at least 14 days (Haine et al., 2008b). Consistently with previous studies, we observed that antibacterial activity reached maximal values at 24 h after LPS challenge in animals that were kept at 20 and 30 °C. However, this response fails to persist in time and decays about 17–30% at 72 h, probably due to the high energetic cost associated with a higher immune performance resulting from higher environmental temperatures (Moret and Schmid-Hempel, 2000). At 10 °C, antibacterial activity was higher at 72 than at 24 h, suggesting that the maximum can be displaced in time at lower temperatures. Then, environmental temperature can affect not only the maximal antibacterial response but also the kinetics of this response. However additional time points need to be included in order to conclusively define this issue.

PO activity showed a pattern similar to the antibacterial response. LPS-treated mealworms showed at 24 h a significant increase in PO units as compared to naïve larvae but no changes were observed relative to PBS-treated larvae. Our results are consistent with previous studies showing that injury due to injection of PBS or LPS, can by itself promote an immune response triggering (Dushay, 2009). However, we observed that there is almost a 30% of PO activity increase in LPS-treated larvae relative to PBS controls at 20 and 30 °C, suggesting that PO activity increased in response to LPS challenge in *T. molitor* (Armitage and Siva-Jothy, 2005) and in environmental temperature context, similar to antibacterial activity. Nevertheless, further studies are required to define whether the effect of immune challenge was either directly on PO activity or on PO precursor (proPO) level, or on haemocytes number (responsible of transporting proPO) (Ashida and Brey, 1995; Zibaee et al., 2009).

Our findings on the effects of temperature on PO activity are consistent with previous field studies showing that environmental temperature was positively correlated with PO activity (Fuller et al., 2011). This is similar to reports on *E. integriceps* in which activation of PO activity was affected by temperature, being higher and more persistent in time at 40 °C (at least 72 h) than at 20 or 30 °C (Zibaee et al., 2009). However, our results showed that the increase of PO was not sustained over time and decayed at 72 h, albeit maintaining the temperature pattern observed at 24 h, i.e., PO activity at 30° was higher than at 10 or 20 °C.

It is noteworthy that both immune traits (antibacterial and PO activity) are highly sensitive to environmental temperature for naïve larvae, being higher at higher temperatures. In fact, naïve larvae at 30 °C seem able to confront a bacterial infection more effectively, without the investment in immune components required at the moment of pathogenic interaction and having a prophylactic protection, but with a higher maintenance cost (Chown and Nicolson, 2004).

From the current study is clear that the metabolic and body mass costs of the immune response not only depend on the immune challenge itself, but also on its interaction with the immediate abiotic environment, in this case environmental temperature. Although the costs of immune response have been studied largely indirectly, direct evidence for such costs is very scarce. In white cabbage butterfly pupae, standard metabolic rate increased nearly 8% after an immune insult (Freitak et al., 2003), well below 25–60% of SMR increase that we observed at 30 °C for mealworms injected with PBS and LPS, respectively. In our study, only animals maintained at 30 °C after immune challenge tended to increase their SMR with PBS and LPS injection. Therefore, at 10 and 20 °C biochemical processes may not be as augmented as at 30 °C. In fact, even PBS-treated larvae showed a higher SMR than naïve larvae at 30 °C. Which is not wear, given the considerable amount of peptides that insect can synthesized in response to an injury (Fehlbaum et al., 1994; Dushay, 2009).

The initial induction of several immune peptides (Lavine et al., 2005) and the late production and mobilisation of hemocytes (Mowlds et al., 2008) can explain the expenditure of extra energy leading to SMR and body mass loss for LPS- and PBS-treated larvae. But, considering that immune performance at 30 °C was higher at 24 h and not at 72 h like costs traits, it is likely that oxidative stress accompanying melanotic encapsulation, the generation of quinones by activated phenoloxidase and free radicals species derived from the immune response and wound repair (Nappi and Vass, 1993; Fehlbaum et al., 1994; Söderhäll and Cerenius, 1998; Dushay, 2009) are responsible to create a toxic environment in the host, which promotes detoxification processes with the consequent increase in SMR impacting deleteriously body mass balance. It is thus possible that the increase of SMR could be at least partly due to energy demanding repair functions and the clearance of damaged tissues. This notion is consistent with the increase in SMR and body mass loss at 72 h and not at 24 h when immune activities were maximised for PBS- and LPS-treated larvae.

The costs of immune response seem to be asymmetrical between temperatures. At 10 °C LPS-treated larvae did not present an increase in SMR, but body mass loss was significantly bigger as compared to controls (PBS and naïve groups); furthermore, at 20 °C there were no differences in SMR and body mass change between different groups. Probably, this pattern is the result of a conjunction of different physiological processes associated to stress and immunity (Diehl-Jones et al., 1996; Linder et al., 2008; Mowlds et al., 2008).

At 30 °C, differences in SMR and body mass loss between naïve and LPS-treated larvae are easily explained by the high immune performance observed in PO and antibacterial activities. However, differences in body mass loss at 10 °C without change in SMR may be rather related to a stress process. During acute stress, insects release octopamine to haemolymph, a neurohormone that induces a number of physiological changes in insects, including the liberation of energy (e.g. lipids) from the body fat that is needed to fuel fight or flight behaviours (Woodring et al., 1989; Adamo, 2010). Hence, body mass loss without change in SMR and low immune activity at 10 °C can be the result of both the hyperlipaemia caused by thermal stress and by the immune challenge (Mullen et al., 2004; Adamo, 2010). And due to illness-induced anorexia is a common phenomenon whereby insects immune-challenged tend to decrease feeding

(Dunn et al., 1994; Adamo et al., 2010), we fasted larvae during our 'body mass balance'' experiment in order to avoid possible mass (or energy) compensations in used treatments. Consequently a putative effect of anorexia on body mass loss was discarded.

In conclusion, it is possible that at high temperatures, the immune response could quickly reach the maximum but at such high energetic cost that it does not allow individuals to have a long-lasting response, contrary to what would happen at low temperatures. When ectotherm animals are exposed to low temperatures the immune response can be poor and late, with a consequent low energy cost, but with high fitness consequences. It is well known that the immune response acts as a ''double edged sword'' i.e., immunity activation is fundamental to resist a pathogen, but can be detrimental if the response is maintained in the long term (Moret and Schmid-Hempel, 2000; Haine et al., 2008b), and the energetic costs to fight off an infection can become too high to be sustainable. Hence, a maximal response might not always be an optimal response.

Finally, further research is needed to conclusively define the consequences of the observed immune responses on energy allocation under different environmental temperature conditions as well as under a diversity of infecting agents and different physiological states. Indeed, questions as to how the persistence in time and the effectiveness of the immune response could be affected by environmental factors, such as temperature or developmental stage, requires further investigation. Future studies should attempt to explain how immunological traits are affected by high levels of temperature variability encountered over different habitats and conditions in a global change scenario (IPCC, 2001). Clearly, the impact of rising temperatures will depend in part on the physiological vulnerability of organisms and their capabilities to deal with infections as well as on the diversity of pathogens.

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