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Pathogen- and diet-dependent foraging, nutritional and immune ecology in mealworms

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ABSTRACT

Background: Feeding habits and dietary nutritional content may play a key role in pathogen-dependent foraging ecology, because mounting an effective immune response is costly for the host.

Hypothesis: Since immune defence is the final line of protection against infective aggression, an adequate provision of dietary macromolecules – through a selective foraging behaviour – is required to maintain immunocompetence in infected hosts.

Goal: We studied dietary switching and its consequences on immune response performance after an immune challenge using mealworms (*Tenebrio molitor*) as a model host.

Methods: We evaluated diet selection and body mass balance (proxy of fitness) of larvae following a lipopolysaccharide challenge under three experimental nutritional treatments: an isocaloric low-protein/high-carbohydrate or high-protein/low-carbohydrate diet offered either independently (no-choice experiment) or simultaneously (dual-choice experiment). Furthermore, we studied the effect of diet composition on three immune traits: antibacterial activity, phenoloxidase activity, and total haemocyte count.

Results: Immune-challenged larvae ate almost five times more than did control larvae in the dual-choice experiment. In addition, 50.7% of total food intake by immune-challenged larvae corresponded to the high-protein/low-carbohydrate diet, significantly higher than challenged or unchallenged control larvae (3.7% and 2.3% respectively). However, no significant differences in body mass change were observed. In contrast, in the no-choice diet condition, immune-challenged larvae lost body mass compared with naïve mealworms. Furthermore, we found that dietary protein had a positive effect on antibacterial activity and total haemocyte count but not phenoloxidase activity, and that mealworms feeding on a balanced diet did not have a better immune performance.

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Conclusions: The immune response activation triggers a compensatory shift in host foraging behaviour that is not necessarily associated with the prevailing physiological state, but can have considerable influence on Darwinian fitness.

Keywords: antibacterial activity, dietary nutrients, ecoimmunology, feeding, haemocytes, phenoloxidase activity, *Tenebrio molitor*.

INTRODUCTION

Animals feed selectively and, to a large extent, what an animal eats defines its ecological and evolutionary success (Bozinovic and Martinez del Río, 1996; Pirolli, 2007; Stephens *et al.*, 2007). Indeed, dietary habits are associated with specific structural, physiological, and behavioural traits, and even life-history ecology (Stephens *et al.*, 2007). As a corollary, foraging ecology and animal diets are central to ecological theory and also important drivers for evolution (Keller, 1999; Roff, 2002; Crutchfield and Schuster, 2003). On the other hand, ecological physiologists posit that not only food type but also physiological constraints and design of consumers are important determinants of diet selection (Karasov, 2011). For instance, to maintain the energy/matter budget when consuming different prey, the predicted foraging behaviour is a change in the rate of food intake; however, a critical point may eventually be reached as a consequence of digestive constraints (e.g. food assimilation, digesta retention time, enzyme activity, nutrient absorption) for utilization of different food qualities (Hughes, 1993; Bozinovic, 1995; Bozinovic *et al.*, 1997; López-Calleja *et al.*, 1997; Sabat *et al.*, 1998; Karasov *et al.*, 2011).

Recent seminal studies have demonstrated that dietary chemical composition can affect immune function (Schulenburg *et al.*, 2009). Theoretically, when given a choice between food types with different nutritional values, pathogen-infected consumers may select those diets that improve their immune system and hence increase survival or fitness. This idea suggests that dietary nutritional content may play a role in pathogen-dependent foraging ecology, both at the population and community level (Seppälä and Jokela, 2010). Thus, since triggering the immune system is costly (Moret and Schmid-Hempel, 2000; Armitage *et al.*, 2003; Siva-Jothy *et al.*, 2005; Rolff and Reynolds, 2009), feeding on low-quality diets and/or deficient nutrition may impair immune function and increase the susceptibility of animals to diseases with negative impact on Darwinian fitness (Chandra, 1996). In this sense, allocation decisions between reproductive effort, development time, and immune defence are hypothesized to be targets of optimizing selection, favouring individuals that allocate their resources in a manner that maximizes their lifetime reproductive success (Sheldon and Verhulst, 1996; Rantala and Roff, 2005). A trade-off between traits associated with growth and development and immune function may be expected because substantial nutritional and energetic demands are associated with immune activation and the maintenance of an efficient immune system (Lochmiller and Deerenberg, 2000).

Thus, we hypothesize that since immune defence is the final line of protection against parasites, adequate provision of dietary macromolecules is needed to maintain immunocompetence and/or lifetime reproductive success in infected animals, and that dietary shift and diet selection – and its nutritional impacts – are immune-dependent in different ecological scenarios (Lee *et al.*, 2006). Here, we evaluate the effect of experimental changes in diet composition (differences in protein content) on food selection behaviour, body mass balance, and immune performance in the mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae). As far as we are aware, few studies have considered how the interaction between diet chemistry and pathogens influences foraging ecology (but see Lee *et al.*, 2006; Cotter

et al., 2010). Hence, we tested: (1) the effect of immune challenge on diet selection, i.e. preferences for high or low dietary protein content; (2) the effect of two diets (low-protein/high-carbohydrate and high-protein/low-carbohydrate) in no-choice and dual-choice scenarios on mass balance – as a proxy of fitness – in immune-challenged animals; and (3) the effect of diets differing in protein and carbohydrate content on the immune response.

Insects are attractive model organisms because, unlike vertebrates, they posses only an innate immune system consisting of cellular and humoral responses acting together to control the spread of an infection. We used tyrosinase phenoloxidase activity, antibacterial activity, and haemocyte count as indicators of an insect's immune defence because: phenoloxidase is considered the key enzyme for encapsulation, melanization, and wound repair (Söderhäll and Cerenius, 1998; Sugumaran, 2002); antibacterial activity represents the action of humoral defence, including lysozymes actions that are critical for the degradation of bacterial cell walls (Schneider, 1985); and haemocytes mediate cellular responses such as encapsulation, nodulation, and phagocytosis (Gillespie *et al.*, 1997; Lavine and Strand, 2002). Thus, these systems provide an efficient defence against pathogens and parasites (Siva-Jothy *et al.*, 2005). Furthermore, larvae were used because at this stage, animals are extremely sensitive to environmental conditions (Min *et al.*, 2006; Morales-Ramos *et al.*, 2009). In addition, changes in diet composition would be expected due to the previous demonstration of life-history trade-offs associated with immune costs in *T. molitor* (Armitage *et al.*, 2003; Krams *et al.*, 2011).

Thus, given the potential fitness benefits of diet selection under an infection condition, we predicted that larvae of *T. molitor* would regulate intake of both nutrients (carbohydrates and proteins) according to their physiological state (i.e. immune challenge) and surrounding ecological conditions (i.e. nutrient availability); such regulation will show a positive effect not only on immune traits, but also on body mass balance, given that carbohydrates provide the major source of energy and proteins are fundamental as structural components for growth (Lee *et al.*, 2002).

METHODS

Animals and experimental design

Larvae of *T. molitor* were randomly selected from a stock culture maintained since the year 2000 under laboratory conditions (23 ± 2°C and light/dark = 12/12 h), and supplied with food consisting of apple peelings and a mixture of flour (60%), oats (20%), yeast (10%), and bran (10%) provided *ad libitum*. Larvae with similar body mass (*c.* 0.1015 ± 0.02 g) were used and different sets of animals ($n = 15$ per set) were used for each measurement to avoid any effect of previous manipulations on the results. Animals showed no signs of moulting. Isocaloric pathogen-free diets (Table 1) were custom-made by MP Biomedicals™, LLC (Solon, OH, USA). Differences in energy content were less than 4% (18.1 ± 0.07, 18.9 ± 0.14, and 18.5 ± 0.48 kJ $\cdot$ g^{-1} for the low-protein/high-carbohydrate, high-protein/low-carbohydrate, and balanced diet, respectively). To induce an immune response, animals were challenged with 4 μL (0.5 mg $\cdot$ mL^{-1}) of *Escherichia coli* lipopolysaccharide (LPS, Sigma 8274) (Wilson, 2005). As controls for the constitutive level of immune activity, some mealworms were injected with 4 μL of phosphate buffered saline solution (PBS; pH 6.4); the other mealworms were not challenged, whether with LPS or with PBS. Animals were injected through the pleural membrane, between the second and the third abdominal segments, using a sterile Hamilton syringe.

Table 1. Composition of experimentally purified pathogen-free low-, high-, and balanced protein diets

	Low-protein		Balanced		High-protein	
Ingredients	grams	%	grams	%	grams	%
Soy protein	25	5.0	137.5	27.5	250	50.0
Corn starch	250	50.0	137.5	27.5	25	5.0
α-Cellulose	189	37.8	189	37.8	189	37.8
Vegetable oil	35	7.0	35	7.0	35	7.0
Vitamin mix	1	0.2	1	0.2	1	0.2
Total	500	100	500	100	500	100

Note: Weights are expressed in grams and on an air-dry basis.

Diet selection and body mass balance

The food preference experiments were conducted in 5-cm diameter Petri dishes with two diets (low-protein/high-carbohydrate and high-protein/low-carbohydrate) offered simultaneously. The powdered dry mass of each diet was suspended in a 1:1 ratio agar solution (15 $mg \cdot L^{-1}$). The behavioural tests were conducted at 20°C and a light/dark cycle of 12:12 h for 72 h. Water was provided with small pieces of cotton wool and controls were performed according to the same protocol described above. Fifteen replicates and the disposition of dietary items inside the dishes were randomly assigned (Raffa *et al.*, 2002). Food intake was measured gravimetrically after correction for evaporative mass loss. Food preferences were determined as the ratio between individual diets ingested and total mass ingested. Larvae and food were weighed in an analytical balance (±0.0001 g; JK-180, Chyo, Kyoto) before and after each experiment.

The nutritional effect of dietary composition in immune-challenged and control animals was assessed through changes in body mass. Mass changes were determined as the percentage of weight gained by each larva feeding on both diets simultaneously (dual-choice experiment) and on either the low-protein/high-carbohydrate or high-protein/low-carbohydrate diet (no-choice diet experiment). Larvae were weighed and randomly assigned to immune challenge treatments (naïve, PBS, and LPS) and to diet treatments (no-choice and dual-choice). Animals were weighed again after 72 h in an analytical balance (±0.0001 g; JK-180, Chyo, Kyoto).

Diet composition and immune response

To test for the effect of experimental diets on immune response, we measured three haemolymph immune variables in individual larvae, namely: antibacterial activity, haemolymph phenoloxidase (PO) activity, and total haemocyte count (THC). Haemolymph (10 mL per animal) was collected in pre-chilled glass capillaries by puncturing the pleural membrane. Each animal was bled only once. Animals were maintained with a low protein/high carbohydrate, balanced, or high protein/low carbohydrate diet for 72 h. Injections were made 48 h after diets were offered and immune traits were measured 24 h after injections (Haine *et al.*, 2008a, 2008b). To measure antibacterial activity, haemolymph (5 μL) was diluted

with 24 mL PBS, and 1 mL of an overnight culture (approximately 3×10^7 CFU $\cdot$ mL^{-1}) of streptomycin-resistant *Micrococcus luteus* was added to the solution. The mixture was incubated for 1 h at 30°C with agitation at 150 rpm. Then, the mixture was diluted 100 times and plated on LB agar containing 5 μg $\cdot$ mL^{-1} streptomycin (Sigma S6501). The mixture (50 and 100 μL) was spread onto two plates for each animal. Plates were incubated at 30°C for 48 h and the number of colony-forming units (CFU) per millilitre of mixture spread determined. Mean values for both plates were used to determine antibacterial activity as the percentage of CFU over control plates without larval haemolymph (modified from Ahmed *et al.*, 2002; Cotter *et al.*, 2004; Haine *et al.*, 2008a).

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

$$\mathrm{OD}_t = \frac{\mathrm{OD}_{\max}}{1 + e^{-\left(\frac{t - t_{\max}}{V_{\max}}\right)}} \tag{1}$$

$$\Delta\mathrm{OD} = \frac{\mathrm{OD}_{\max} - \mathrm{OD}_{\text{control}}}{2t_{\max}} \tag{2}$$

Finally, the number of haemocytes per millilitre of haemolymph was determined using a Neubauer haemocytometer. Haemolymph (2 mL) was diluted 10 times in PBS (pH 6.4) and trypan blue (0.4%) was used to stain cells (1:1) and determine their viability.

Statistical analyses

To determine the effects of diets and immune challenges on feeding behaviour, the immune response traits, and change in body mass, repeated-measures and factorial analyses of variance (ANOVA) were performed. All data were tested for normality and homoscedasticity using the 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 tests for multiple comparisons were used. All statistical analyses were conducted using Statistica v.6.0 software (Statsoft Inc., Tulsa, OK).

RESULTS

In the dual-choice diet experiment, immune treatment affected both total food intake (ANOVA: $F_{2,27} = 129.8$, $P < 0.0001$) and uptake of individual diets (repeated-measures ANOVA: diet/treatment, $F_{2,27} = 115.2$, $P < 0.0001$) (Fig. 1A). Indeed, naïve and PBS-treated animals exhibited similar total food intake (13.69 ± 0.60 and 11.03 ± 1.10 mg $\cdot$ day^{-1},

respectively) and preferred mainly the low-protein/high-carbohydrate diet (96.3% and 97.7%, respectively; Fig. 1A). In contrast, challenged (LPS-treated) larvae consumed a higher amount of both diets (62.89 ± 4.21 mg · day^{-1}) with a marked preference for the high-protein diet (50.7%) compared with controls (3.7% for naïve and 2.3% for PBS; Fig. 1A). Animals with access to a single diet only (no-choice diet experiment) exhibited a markedly higher consumption of the low-protein/high-carbohydrate diet compared with the high-protein/low-carbohydrate diet, independently of the immune treatment (factorial ANOVA: diet, $F_{1,54} = 47.33$, $P < 0.0001$; treatment, $F_{2,54} = 1.76$, $P = 0.182$; Fig. 1B).

Body mass change was affected by the interaction of the immune challenge and diet treatments (factorial ANOVA: diet × treatment, $F_{4,126} = 2.68$, $P = 0.034$), as well as by both factors independently (factorial ANOVA: diet, $F_{2,126} = 5.86$, $P = 0.004$; treatment, $F_{2,126} = 10.34$, $P < 0.0001$) (see Fig. 2). Naïve animals maintained with either a low-protein/high-carbohydrate or high-protein/low-carbohydrate diet exhibited a significantly higher increase in body mass compared with PBS- and LPS-treated animals, which showed a similar increase in body mass (Fig. 2). Nevertheless, when given a choice between diets, animals in the two conditions exhibited a similar change in body mass with no statistically difference between them (Fig. 2). Furthermore, in the dual-choice diet experiment, larvae gained more body mass than in the no-choice diet experiment (*a posteriori* Newman-Keuls test: both diets vs. low-protein/high-carbohydrate diet: $P = 0.011$; both diets vs. high-protein/low-carbohydrate diet: $P = 0.003$; and low-protein/high-carbohydrate diet vs. high-protein/low-carbohydrate diet: $P = 0.473$).

Regarding the effect of diet composition on the immune response, antibacterial activity was significantly affected by the interaction of diet and immune challenge (factorial ANOVA: $F_{4,81} = 2.54$, $P = 0.046$) as well as by both factors independently (factorial ANOVA: diet, $F_{2,81} = 4.55$, $P = 0.013$; treatment, $F_{2,81} = 25.96$, $P < 0.0001$). Antibacterial activity was significantly higher in animals consuming the high-protein/low-carbohydrate diet than animals consuming either the low-protein/high-carbohydrate (*a posteriori* Newman-Keuls test: $P = 0.023$) or balanced diet (*a posteriori* Newman-Keuls test: $P = 0.014$) (Fig. 3A). On a low-protein/high-carbohydrate diet, PBS- and LPS-treated animals exhibited similar antibacterial activity but higher activity than naïve animals. In contrast, on a high-protein/low-carbohydrate diet, naïve and PBS-treated animals showed similar antibacterial activity and both showed significantly lower activity than LPS-treated animals. On a balanced diet, there were no statistical differences between immune treatments (Fig 3A).

Phenoloxidase activity was significantly different between immune-challenge treatments (factorial ANOVA: $F_{2,81} = 3.79$, $P = 0.027$), but not between diet treatments (factorial ANOVA: $F_{2,81} = 0.72$, $P = 0.492$) (Fig. 3B). LPS-treated larvae showed higher PO activity than naïve larvae (*a posteriori* Newman-Keuls test: $P = 0.021$), but not PBS-treated larvae (*a posteriori* Newman-Keuls test: $P = 0.099$). Finally, THC was not significantly different between treatments on a low-protein/high-carbohydrate diet (Fig. 3C). Nevertheless, on a high-protein/low-carbohydrate diet, LPS-treated animals responded with a significant increase in haemocyte number compared with the PBS-treated (*a posteriori* Newman-Keuls test: $P = 0.049$) and naïve animals (*a posteriori* Newman-Keuls test: $P = 0.014$) (Fig. 3C).

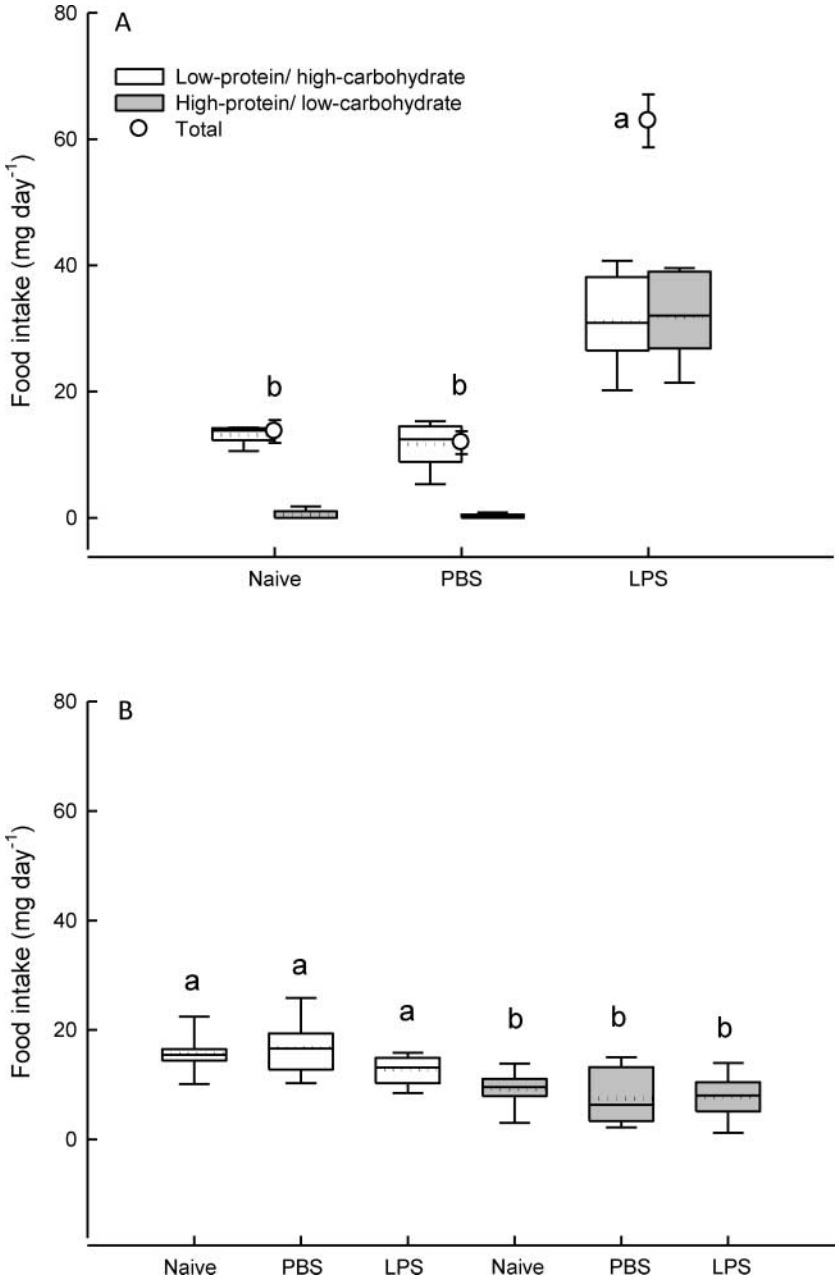

Fig. 1. Relationships between immune treatment and food intake in the dual-choice diet experiment (A) and the no-choice diet experiment (B). In (A), boxes show partial food intake of different diets and open circles represent total food intake. In (B), boxes show differences in food intake between larvae maintained on the low-protein/high-carbohydrate and high-protein/low-carbohydrate diets (letters indicate statistical differences between total food intakes). Boundaries of boxes show the 25th and 75th percentile, respectively; solid line within the box marks the median, dotted line indicates the mean, and error bars indicate the 90th and 10th percentiles.

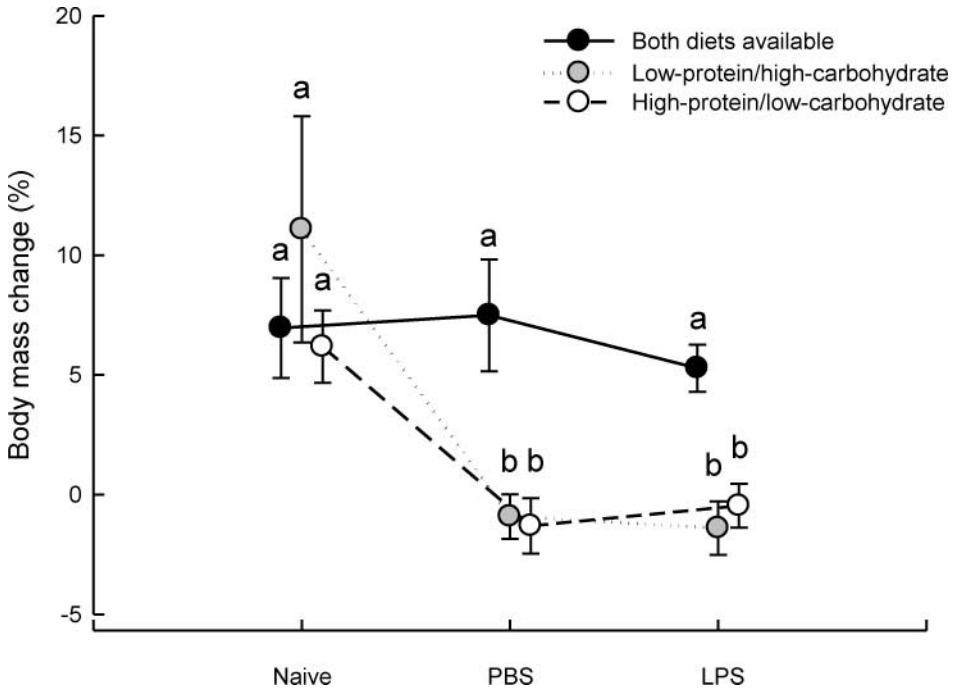

Fig. 2. Relationship between immune treatments and changes in body mass in the different dietary treatments. We observed no differences between immune treatments under dual-choice conditions (black circles). Under no-choice conditions (white and grey circles), challenged (LPS) and control procedure (PBS) animals lost body mass compared with naïve animals. Data were transformed to meet statistical assumptions (letters indicate statistical differences).

DISCUSSION

Provenza and Cincotta (1993) proposed that the palatability of a particular diet stems in part from its chemical nutritional composition. In addition, Pirolli (2007) and Stephens *et al.* (2007) addressed the question of how ecological interactions and adaptive interaction with environmental information can influence the dynamics of foraging.

Here, we predicted that pathogen-infected consumers may select those dietary nutrients that allow them to pay the cost of eliciting an immune response against an infection, and thus proposed that pathogens may play an important role in foraging ecology. We observed that when mealworms were maintained either on a low-protein/high-carbohydrate or a high-protein/low-carbohydrate diet, intake of the low-protein/high-carbohydrate diet was higher than that of the high-protein/low-carbohydrate diet even when larvae were challenged with LPS to induce an immune response. However, when animals were allowed to choose their diet, LPS-treated larvae presented a higher total food intake than naïve and PBS-challenged larvae. Two interesting conclusions emerge from these results.

First, mealworms seem to compensate the energy depletion of immune activation (Siva-Jothy *et al.*, 2005) by increasing food intake, which is an unusual response compared with illness-induced anorexia (Adamo *et al.*, 2010). This suggests that compensation and/or allocation

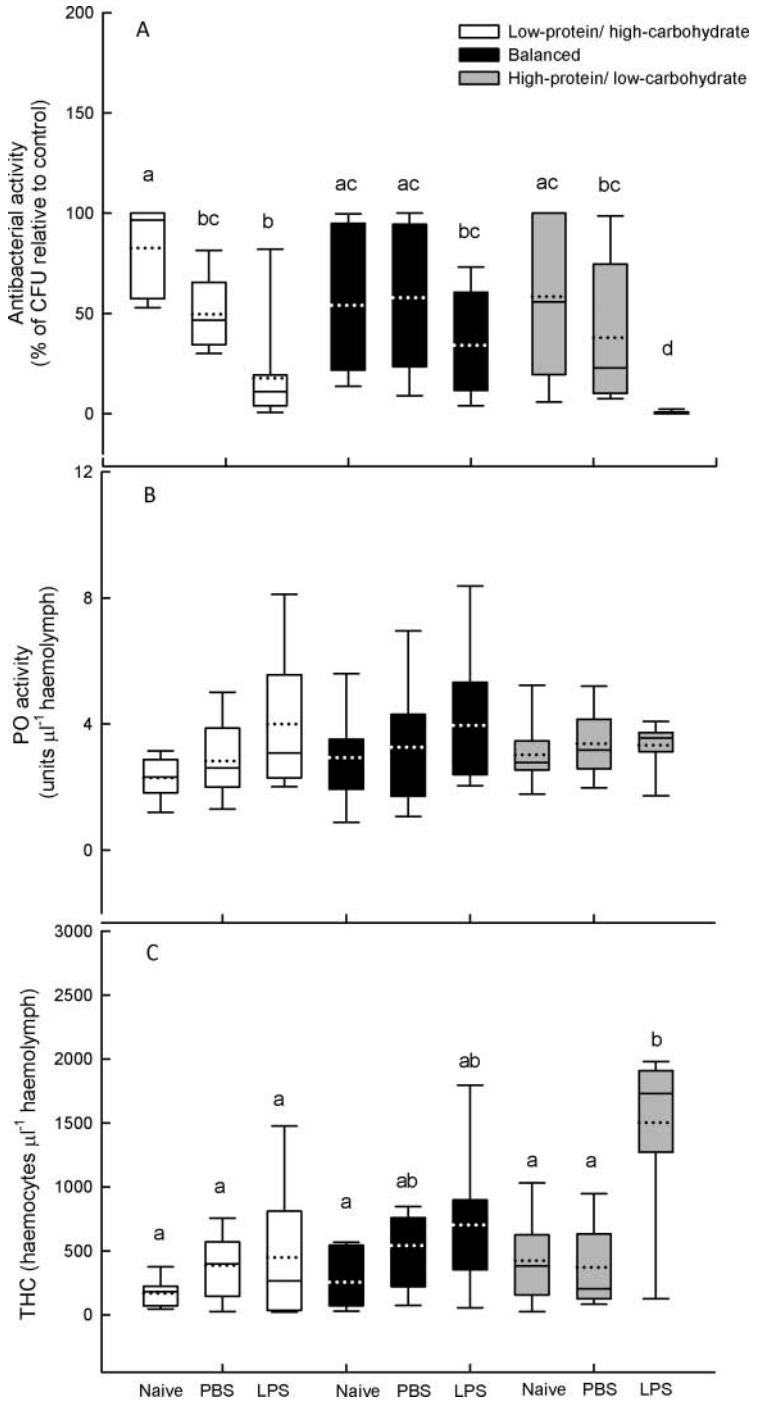

Fig. 3. Influence of diet composition on immune traits: (A) antibacterial activity, (B) phenoloxidase (PO) activity, and (C) total haemocyte count (THC). Letters indicate statistically significant differences between treatments.

of energy and nutrients not only depends on physiological state, but also on ecological-nutrient availability (Simpson *et al.*, 2004; Cotter *et al.*, 2010). Second, a mixed diet leads to less intake of food to cope with different physiological states (see Bozinovic and Muñoz-Pedreros, 1995a, 1995b). Larvae exposed to individual diets ate more than larvae exposed to both diets simultaneously, except LPS-treated larvae. Despite the above, PBS- and LPS-treated larvae on individual diets lost a significant percentage of body mass compared with naïve animals, probably as a consequence of the nutritional imbalance, which implies a metabolic cost of transforming excess protein to carbohydrate to supplement energy deficiencies (Thompson and Redak, 2000) or accessing protein from reserve tissue to build immunological components (Washburn *et al.*, 1996; Trudeau *et al.*, 2001). In contrast, larvae on the dual-choice experiment gained body mass independently of the immune treatment. This result is at variance with the observation that virus-challenged caterpillars, *Spodoptera littoralis*, exhibited decreased performance relative to control animals, owing to the protein content of the diet (Lee *et al.*, 2006). Similar results have been reported for bacteria-challenged *Spodoptera exempta* (Povey *et al.*, 2009). In both studies, a protein-rich diet conferred a higher fitness to immune-challenged caterpillars than low-protein diets.

The immune response was not improved by a balanced diet, as expected from the dual-choice diet experiment. Only LPS-treated larvae showed increases in antibacterial activity and THC when feeding on the high-protein diet, and PO activity did not differ between diet treatments. These data are consistent with observations made on caterpillars. For instance, *S. littoralis and S. exempta* infected with live pathogens showed a higher antimicrobial activity when feeding on a high-protein diet, even at baseline levels (Lee *et al.*, 2006, 2008; Povey *et al.*, 2009; Cotter *et al.*, 2010). In this sense, it would be expected that a high-protein diet might enhance antibacterial activity through production of lysozymes and/or maintenance of haemolymph levels. Lysozymes are constitutively expressed in most insect species, are upregulated upon recognition of microbial cell wall components, such as LPS (Briese, 1981), and contribute to infection control. Nevertheless, a dietary shift to improve an immune trait such as antibacterial activity can cause a trade-off with other immune traits, such as PO activity (Moret and Schmid-Hempel, 2001; Cotter *et al.*, 2004). In fact, reports of the effects of diet on PO activity are contradictory: Lee *et al.* (2006) and Povey *et al.* (2009) found that PO activity in caterpillars was higher when feeding on diets rich in protein, whereas Cotter *et al.* (2010) observed the highest activity to occur when feeding on a carbohydrate-based diet. In line with this notion is that the melanization response of the mosquito *Anopheles stephensi* increases with food sugar concentration after a blood meal (Koella and Sorensen, 2002). These data add further support to the notion that the PO response requires carbohydrates, perhaps in greater amounts than antibacterial activity given the need for haemocytes to burst open to release PO into the haemolymph (Ashida and Brey, 1998) with subsequent haematopoiesis. In addition, it is important to consider that PO is involved in diverse physiological functions, including melanization after moulting (Hiruma and Riddiford, 1988), in addition to its role in immune response.

Published data support our predictions for pathogen- and diet-dependent foraging ecology. That is, in a pathogen–host ecological interaction, infected host dynamic plasticity allows a partial increase of the immune response when feeding on a high-protein diet. In fact, since triggering the immune system is costly (Freitak *et al.*, 2003; Siva-Jothy *et al.*, 2005) a selective feeding behaviour by pathogen-infected consumers apparently minimizes a decrease in fitness, as evidenced by our data showing differential changes in body mass. However, no diet can optimize all components of the immune response and the allocation of nutrients

to different tasks will be subject to physiological state as well as the particular ecological scenario in space and time.

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