Can the protein costs of bacterial resistance be offset by altered feeding behaviour?

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Running headline: Protein costs of bacterial infection

Summary

1. Mounting an immune response is likely to be costly in terms of energy and nutrients, and so it is predicted that dietary intake should change in response to infection to offset these costs. The present study focuses on the interactions between a specialist grass-feeding caterpillar species, the African armyworm *Spodoptera exempta*, and an opportunist bacterium, *Bacillus subtilis*.
2. The main aims of the study were (i) to establish the macronutrient costs to the insect host of surviving a systemic bacterial infection, (ii) to determine the relative importance of dietary protein and carbohydrate to immune system functions, and (iii) to determine whether there is an adaptive change in the host's normal feeding behaviour in response to bacterial challenge, such that the nutritional costs of resisting infection are offset.
3. We show that the survival of bacterially-infected larvae increased with increasing dietary protein-to-carbohydrate (P:C) ratio, suggesting a protein cost associated with bacterial resistance. As dietary protein levels increased, there was an increase in antibacterial activity, phenoloxidase (PO) activity and protein levels in the haemolymph, providing a potential source for this protein cost. However, there was also evidence for a physiological trade-off between antibacterial activity and phenoloxidase activity, as larvae whose antibacterial activity levels were elevated in response to immune activation had reduced PO activity.
4. When given a choice between two diets varying in their P:C ratios, larvae injected with a sub-lethal dose of bacteria increased their protein intake relative to control larvae whilst maintaining similar carbohydrate intake levels. These results are consistent with the notion that *S. exempta* larvae alter their

1 feeding behaviour in response to bacterial infection in a manner that is likely
2 to enhance the levels of protein available for producing the immune system
3 components required to resist bacterial infections (‘self-medication’).

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6 **Keywords:** nutrition**,** parasite, immunity, life-history, insect

Introduction

It has long been assumed that immune function is costly to the host and so must be traded-off against other fitness-related traits (Sheldon & Verhulst 1996). Recent studies have assumed that there are significant resource costs involved in both the deployment and maintenance of the immune system (Lochmiller & Deerenberg 2000; Schmid-Hempel 2003). Evidence for these resource costs comes from studies showing that starvation disproportionately reduces fitness in animals that are immune-challenged. For example, mealworm beetles, *Tenebrio molitor*, exposed to short-term nutrient deprivation exhibited a down-regulation of immune system function (Siva-Jothy & Thompson 2002), and bumble bees, *Bombus terrestris*, denied access to food showed reduced survival compared to controls when their immune systems were activated (Moret & Schmid-Hempel 2000) and their parasites showed increased virulence in starved hosts (Brown *et al.* 2000).

However, starvation is a fairly blunt tool for measuring the specific nutritional costs of mounting an immune response. Many studies have shown that animals regulate their macronutrient intake through dietary self-selection, thereby optimising growth and performance (Raubenheimer & Simpson 1997; Waldbauer & Friedman 1999). Experimental work on dietary self-selection in insects has mainly focused on investigating the regulation of two macronutrients, protein and carbohydrate (e.g. Chambers *et al.* 1995; Lee *at al.* 2003, 2004; Thompson *et al.* 2005). At any given time, the animal will require a specific quantity and mixture of nutrients, termed the intake target (Simpson & Raubenheimer 1995). However, the nutritional requirements for growth, reproduction and survival of healthy individuals, may be very different from those in individuals that are immune-challenged.

The insect immune system comprises both cellular and humoral components, which often interact to combat infections (Lavine & Strand 2002). Cellular processes are mediated by an array of highly differentiated haemocytes that are responsible for a number for immune reactions, including cellular encapsulation, nodulation and phagocytosis (Gupta 1991; Eslin & Prevost 1996; Chapman 1998). The humoral responses largely involve soluble proteins, such as inducible antimicrobial peptides and polypeptides. These molecules are produced in the haemocytes, fat body and epithelial tissues and secreted into the haemocoel, where they affect the parasite either directly or indirectly by influencing the behaviour of haemocytes. Phenoloxidase (PO) is a key enzyme in the insect immune system, involved in melanisation of the cuticle, wound repair, cytotoxin production, and the encapsulation of larger pathogens (Sugumaran 2002; Gupta 1991), and is often used as a measure of constitutive immune function in insects as its expression is both repeatable and heritable (Cotter & Wilson 2002; Cotter *et al.* 2008).

Due to the costs involved in maintaining and activating the immune system, it would be expected that macronutrient intake requirements would change in the face of an immune insult. If resources are limiting or inadequate, especially those required to fight off infections, then immune function could be compromised. Furthermore, it is possible that different immune function traits could be traded-off against each another. For example, phenoloxidase activity in bumble bees injected with an immune elicitor was negatively phenotypically correlated with growth-inhibition antibacterial activity (Moret & Schmid-Hempel 2001). Similarly, in the Egyptian cotton leafworm, *Spodoptera littoralis*, haemocyte density was positively genetically correlated with phenoloxidase activity but negatively genetically correlated with lysozyme-like antibacterial activity (Cotter *et al.* 2004b). Trade-offs within the immune system may

become more apparent when resources are limiting, due to a general shortage of resources for all necessary immune responses (van Noordwijk & de Jong 1986).

Numerous studies have focused on how survival of the host changes when specific metabolites are included in their diet (e.g. DeWitt *et al.* 1999; Buentello & Gatlin 2001; Lee *et al.* 2005). However, a question that remains largely unanswered is whether animals have the ability to actively self-select diets that will improve their resistance to parasite infection. In the field, herbivorous insects may balance their intake of nutrients by consuming different plant species, different individual plants of the same species, or different parts of the same plant (Raubenheimer & Bernays 1993; Mody *et al.* 2007). More recently, it has been reported that even predatory invertebrates are capable of balancing their intake of multiple nutrients (Mayntz *et al.* 2005). In the laboratory, artificial diet is often used to study nutritional regulation because levels of specific nutrients can be accurately manipulated, whilst maintaining constant other aspects of the food quality and quantity. It has been shown that parasitism can affect the relative intake of protein and carbohydrate (Thompson & Redak 2005). It would be expected that if specific nutrients, or nutrient ratios, are beneficial to the host in mounting an immune response against a parasite, infected hosts would actively select diets with high levels of that particular nutrient. This approach was adopted by Lee *et al*. (2005) with virally-infected *S. littoralis*. Uninfected larvae performed best on diets that had an even P:C ratio, but virally-infected larvae that successfully resisted the infection selected a significantly more protein-biased diet than that selected by lethally-infected and control larvae. However, it remains to be established how ubiquitous this response is and whether it is dependent on the optimal diet of the uninfected host, or the nature of the parasitic challenge.

The present study examines the impact of dietary macronutrients on the interaction between the African armyworm, *Spodoptera exempta*, and an opportunistic bacterium, *Bacillus subtilis*. *S. exempta* is a specialist grass feeder which performs best on moderately carbohydrate-biased diets when uninfected (Lee *et al.* 2004), contrasting with the larvae of most other lepidopteran species, which tend to perform best on protein-biased diets (see references in Lee *et al.* 2004). The main aims of this study were to address the following questions: (1) How does bacterial resistance vary in relation to the macronutrient content (protein-to-carbohydrate ratio) of the host's diet? (2) What is the impact of dietary macronutrients on insect immune function, specifically antibacterial activity and phenoloxidase activity? (3) If the optimal diet for immune system function and bacterial resistance differs from that normally chosen, do infected insects alter their diet-choice decisions to reflect this difference?

Methods.

HOST AND PATHOGEN CULTURES

A continuous culture of *S. exempta*, originally from Tanzania, has been maintained at Lancaster University for 4 years. Experimental larvae were reared in isolation in 25ml plastic pots from the third instar onwards. In this pre-experimental phase, the larvae were fed on a wheatgerm-based semi-artificial diet containing approximately 33% protein and 29% carbohydrate (Hoffman *et al.* 1996) and kept at a constant temperature of 25ºC under a 12h:12h light:dark regime. All experiments were performed on newly-moulted final (6^{th}) instar larvae.

The bacterial culture used in the following experiments was the gram-positive bacterium, *Bacillus subtilis*, (Blades Biological Ltd, Kent.), chosen because it is an

effective pathogen against *S. exempta*. *B. subtilis* enters the insect through the cuticle (St. Leger 1991) rather then being ingested, the usual route for baculovirus infection (Volkman 1997). Bacteria were supplied on agar slopes, and experimental bacteria were grown in nutrient broth (10g bacto-tryptone, 5g yeast extract, 10 NaCl, 1000 ml distilled water, pH. 7.50) (Lacey 1997). The bacterial concentration was determined using a haemocytometer, at x40 phase-contrast magnification.

ARTIFICIAL DIETS

Upon reaching the final instar, experimental larvae were transferred to experimental diets that varied in their macronutrient content. These diets were based on those of Simpson & Abisgold (1985) and have been used previously in studies using *S. exempta* (Lee *et al.* 2004). These diets varied in their protein and digestible carbohydrate content. The protein portion of the diet consisted of a 3:1:1 ratio of casein, peptone and albumen, and the carbohydrate content consisted of a 1:1 mixture of sucrose and dextrin. Other constituents of the diets were Wesson's salts (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mixture (0.2%). The remaining portion of the diets was made up of cellulose, a non-nutritive bulking agent. The dry ingredients of the diet were mixed in a 1% agar solution in a 6:1 agar solution:dry diet ratio. Five diets were used in total: 35% protein with 7% carbohydrate (P:C = 35:7), 28:14, 21:21, 14:28 and 7:35. In each case the protein and carbohydrate portion made up 42% of the final diet, which meant that all diets were near isocaloric.

INFECTIONS

All bacterial infections were performed on final-instar larvae using an electronic micro-injector (Narishign, IM-200 microinjector, New York) attached to an air supply at a pressure of 248.19 Pka +/- 0.24 S.E. Larvae were chilled on ice, and then injected by inserting an Eppendorf Femtotip needle (Eppendorf UK Ltd, Cambridge), with an inner opening diameter of 0.5 µm, between the pro-legs of the caterpillar, taking care not to rupture the gut.

EXPERIMENT 1: EFFECTS OF DIETARY P:C RATIO ON LARVAL SURVIVAL

Larvae were injected with 5µl of *B. subtilis* suspended in nutrient broth at the LD_{50} concentration of $1x10^7$ cells/ml (S. Povey, unpublished data). Three control groups were used: those injected with insect Ringer solution (NaCl 8.0g, $CaCl_2$ 0.25g, KCl 0.25g, $NaHCO_3$ 0.25g, distilled water 1000ml) (Lacey 1997), to control for the effects of the injection process; those injected with nutrient broth, to control for any mortality caused by the nutrient broth rather then the bacteria; and a group of naïve larvae, which were handled but not injected. The results indicate no difference between the mortality pattern of the injected controls (see Results) and so in future experiments just the nutrient broth control treatment was used as an injection control. In total, there were 32 larvae in each control group and 32 *B. subtilis*-injected larvae on each diet (a total of 640 larvae, across two blocks).

Following injection, larvae were placed in a 25ml plastic pot containing one of the five test diets varying in their protein-to-carbohydrate ratio (P:C): 7:35, 14:28, 21:21, 28:14 or 35:7. At the same time, naïve larvae were also switched on to their test diet. Each day, until all larvae had either died or pupated, the larvae were provided with fresh test diet and any deaths were recorded. Larvae dying due to systemic bacterial

infection exhibit a characteristic darkening of the cuticle and flaccid posture, and the haemolymph becomes packed with bacterial cells. However, bacteria may contribute indirectly to larval mortality without the insect showing any outward signs of infection. Therefore, in the mortality analyses we did not attempt to distinguish between those larvae that died of obvious bacterial infection and those apparently dying of other causes. However, it is worth noting that the majority of larvae that died in the bacteria-injected treatment group showed clear signs of systemic bacterial infection.

EXPERIMENT 2: EFFECT OF DIETARY P:C RATIO ON LARVAL IMMUNE FUNCTION

A total of 576 larvae were used in this experiment, across three experimental blocks. For each of the five test diets there were three treatment groups: naïve larvae, those injected with 5µl of insect Ringer solution, and those injected with 5µl of a 50:50 solution of 0.5 mg/ml lipopolysaccharide (LPS) and 1.0 mg/ml peptidoglycan (PEP), (Morishima *et al.* 1997; Korner & Schmid-Hempel 2004). LPS and PEP are products from gram-negative and gram-positive bacterial cell walls, so when injected into the larvae should elicit antibacterial immune responses without causing mortality. Following injection, all larvae were immediately assigned to one of the five diets varying in P:C ratio, except for a further set of control larvae that had haemolymph taken at the same time as test larvae were injected. Larvae allocated to the five test diets had haemolymph collected 24h after injection, as this was shown in a preliminary study to be the peak in the antibacterial response (S. Povey, unpublished data). Haemolymph was extracted from the insect by piercing the larval cuticle between the prolegs with a fine gauge needle and allowing the haemolymph to drip

into an Eppendorf tube on ice. All of the samples were then frozen at -80^{o}C until they were to be measured. The exact amount of haemolymph collected varied between larvae, but 15 µl was sufficiently to conduct all of the immune function assays reported.

For the antibacterial assay, we modified the methods described in Korner and Schmid-Hempel (2004). Briefly, test plates were made up the day before haemolymph collection, using an agar overlay technique (Rahalison *et al.* 1991) and the bacterium *Micrococcus luteus*. Directly after collection, two 2µl replicates of each haemolymph sample were pipetted straight into the labelled holes on the agar plates. Each plate also contained two control holes: one left blank and one containing insect Ringer. The plates were incubated for 24h at 37ºC and antibacterial activity was measured as the radius of the clear zone of bacterial inhibition around the holes in the plate. Measurements were made using Image Pro Plus software 4.1 (Media Cybernetics, USA).

The remaining haemolymph samples were frozen at -80ºC for assaying phenoloxidase activity. Before freezing, 8 µl of haemolymph was diluted in 8 µl of a 50:50 solution of phosphate-buffered saline (PBS): glycerol solution. PO activity and the amount of protein per haemolymph sample were measured using a modified version of the procedure described by Cotter *et al.* (2004a). Briefly, 8µl of each diluted haemolymph sample was mixed with 200µl of PBS, 90µl of the resulting solution was pipetted in duplicate into a microtitre plate. 4 mM dopamine was used as a substrate for the PO reaction, with 90µl added to each sample. The absorbance of the resulting mixture was measured at 492nm for 10 min on a VERSAmax microplate reader (Molecular Devices Corp., USA) at a constant temperature of 25ºC, which was during the linear phase of the reaction. Haemolymph protein levels were determined

using a standard curve created using BSA standards ranging from 0 – 1 mg BSA/ml distilled water, at intervals of 0.1 mg/ml. 5µl of the haemolymph sample in PBS/glycerol was pipetted into a microtitre plate with 200µl of BioRad dye reagent and the absorbance of the mixture measured at 600nm.

EXPERIMENT 3: DIETARY SELF-SELECTION IN RESPONSE TO AN IMMUNE CHALLLENGE

This experiment used a total of 108 larvae, across two blocks of 54. The experiment included three treatments. Test larvae were injected with 5µl of *B. subtilis* suspended in nutrient broth at a concentration of $1x10^4$ cells/ml. This concentration was chosen as pilot studies showed that it is low enough to cause minimal mortality (<10%) yet high enough to elicit a significant antibacterial response in the larvae. There were two control groups; those injected with nutrient broth, again to test the effects of the suspension liquid and the injection process, and a group of naïve larvae that were handled but not injected. Following injection, larvae were transferred to Petri dishes containing the experimental diets. Each insect was given a choice between two blocks of diet: one that was carbohydrate-biased (P:C = 14:28) and one that was either slightly protein-biased (28:14) or strongly protein-biased (35:7). These diets were chosen as they allow the larvae the option of consuming their predicted optimal diet when uninfected (14:28) (Lee *et al*. 2004) or to deviate from this to a protein-biased diet. Two protein-biased diets were chosen as they would allow a comparison between a slightly and strongly protein-biased diet, and therefore show the degree to which the larvae were able to deviate from their optimal diet when bacterially-infected.

Diet blocks, each weighing 2-3g, were replaced every two days; any uneaten food was dried to a constant mass in a desiccating oven. Diet blocks were replaced until the larvae had ceased feeding at the pre-moult stage. Consumption was calculated as the difference between the initial and final dry weight of each diet block. The initial dry weight of the blocks was estimated by regression analysis, using control blocks of each diet type that were wet-weighed then dried to constant mass and reweighed (Lee *et al*. 2005).

STATISTICAL ANALYSIS

All analyses were carried out using S-Plus 6.2 (Insightful Corp., Washington) statistical package. All the statistical models were run with *Block* included as a factor; however the qualitative results were the same as when *Block* was excluded, therefore *Block* is not expanded on in the results.

Experiment 1: The survival analysis was performed using accelerated failure time (AFT) models. These describe the relationship between the hazard function, or the risk of death, and a set of covariates or factors (Cox 1972). Death rate was set as the response variable and *Treatment* (*B. subtilis*-injected, broth-injected, Ringer-injected or naïve), *Diet* (percentage protein in the diet), and all interactions, were considered as explanatory terms.

Experiment 2: Immune function data were analysed using general linear models (GLM). The PO and protein data were square-root transformed to obtain normally-distributed data. PO activity, protein (mg), and antibacterial activity were response variables in the model, and *Diet* (percent protein), and *Treatment* (naïve, Ringer-injected or LPS/PEP-injected) were treated as explanatory terms.

Experiment 3: The dietary self-selection data were also analysed using GLMs. The daily P:C ratio selected every two days, the final P:C ratio selected, final stadium duration, and pupal weight were set as response variables in the model. The two diet-choices was analysed separately. The diet-choice data were log-transformed to conform to the normality assumptions of the linear regression model. *Treatment* (*B. subtilis*-injected, Ringer-injected or naïve), *Larval weight*, *Diet choice*, and *Stadium duration*, plus all potential interactions, were treated as explanatory terms.

In all cases, maximal models were considered, and using a stepwise deletion process, non-significant terms were removed from the model one by one until only significant terms remained and no removed terms explained significant variation in the data (Crawley 2002).

Results

EXPERIMENT 1: EFFECT OF DIETARY P:C RATIO ON LARVAL SURVIVAL

The aim of this experiment was to identify any differences in survival of *S. exempta* larvae when provided with diets differing in their P:C ratio. Larvae started dying 24h post-injection and deaths continued until 144h, at which point all larvae had either died or pupated. There were significant effects of *Treatment* and *Diet* on larval survival (AFT model: *Treatment*: $\chi^2_2 = 95.96$, $p < 0.001$, *Diet*: $\chi^2_1 = 4.08$, $p = 0.043$), but there was no significant interaction between these two main effects (*Diet* x *Treatment* interaction*:* $\chi^2_2 = 2.67$, $p = 0.26$). Although the interaction term was non-significant, it is worth noting that when the three treatment groups were analysed separately, the effect of dietary protein content on mortality rate was strongest for the

bacterially-infected larvae (*Diet*: bacteria-injected: $\chi^2_1 = 4.22$, $p = 0.039$, injected-controls: $\chi^2_1 = 1.98$, $p = 0.160$; naïve: $\chi^2_1 = 0.69$, $p = 0.406$). After controlling for dietary protein content, there were significant differences between all three treatment groups in both their predicted mean time to death (hours $\pm$ s.d.) and their overall mortality rates: bacteria-injected larvae = 138.3 $\pm$ 13.5 h (65% mortality); injected-controls = 237.7 $\pm$ 23.2 h (34% mortality); naïve larvae = 403.9 $\pm$ 39.6 h (16% mortality) (Fig. 1a).

There were significant effects of *Diet* and *Treatment* on the time taken for the surviving larvae to pupate (GLM: *Treatment*: $F_{2,394} = 19.93$, $p < 0.001$; *Diet:* $F_{1,394} = 10.46$, $p = 0.001$), but there was no significant interaction between these two main effects (*Diet* x *Treatment*: $F_{,2,392} = 0.41$, $p = 0.667$). Across all treatment groups, time to pupation increased as the protein content of the diet declined (Fig. 1b). Further analyses indicated that *B. subtilis*-injected larvae took significantly longer to pupate than larvae in the control groups (*Treatment*: *B. subtilis*-injected (mean $\pm$ s.d. = 5.56 $\pm$ 0.10 d) vs. controls (5.03 $\pm$ 0.10 d): $F_{1,395} = 30.15$, $p < 0.001$), and that surviving larvae in the injected-control group (5.11 $\pm$ 0.10 d) took significantly longer to pupate than survivors from the naïve group (4.91 $\pm$ 0.10 d) ($F_{1,341} = 8.83$, $p = 0.003$).

EXPERIMENT 2: EFFECT OF DIETARY P:C RATIO ON LARVAL IMMUNE FUNCTION

The aim of this experiment was to determine the effect of dietary P:C ratio on haemolymph protein levels and the relationship between protein levels and larval immune function. Test larvae were injected with LPS/PEP, while control larvae were either injected with insect Ringer or were naïve.

Haemolymph protein levels

Twenty-four hours after larvae were switched on to one of the five chemically-defined diets, larval haemolymph protein levels reflected the P:C ratio of the new diet (*Diet*: $F_{1,432} = 31.37$, $p < 0.001$), suggesting a rapid incorporation of dietary protein into the haemolymph protein pool (Fig. 2a). However, the injection treatment received did not significantly affect haemolymph protein levels (*Treatment*: $F_{2,430} = 0.39$, $p = 0.677$, Fig. 2a).

Antibacterial activity

The amount of protein in the diet had a significant effect on antibacterial activity (*Diet*: $F_{1,423} = 37.67$, $p < 0.001$). Within each of the three treatment groups, as the relative protein content of the diet increased, so too did the level of antibacterial activity (*Diet*: LPS/PEP-injected larvae, $F_{1,137} = 9.77$, $p = 0.002$; Ringer-injected larvae, $F_{1,132} = 16.45$, $p < 0.001$; naïve larvae, $F_{1,148} = 12.15$, $p < 0.001$, Fig. 2b). In addition to a *Diet* effect, there was an independent positive effect of haemolymph protein levels on antibacterial activity (*Haemolymph protein*: $F_{1,423} = 21.05$, $p < 0.001$, Fig. 3a).

The antibacterial activity levels of naïve larvae at 24 hours were not significantly different from those of the control larvae whose antibacterial activity levels were measured at the start of the experiment (*Treatment*: $F_{1,211} = 2.62$, $p = 0.107$). In contrast, there was a significant increase in the antibacterial activity of Ringer-injected larvae ($F_{1,201} = 119.64$, $p < 0.001$) and LPS/PEP-injected larvae ($F_{1,206} = 344.86$, $p < 0.001$), relative to these controls, suggesting that injection resulted in a significant up-regulation of antibacterial activity.

There was a significant effect of injection-treatment on antibacterial activity (*Treatment*: $F_{2,423} = 498.18$, $p < 0.001$): larvae that received an injection of Ringer

solution had significantly higher antibacterial activity than naïve larvae (*Treatment*: $F_{1,339} = 360.02$, $p < 0.001$), while larvae injected with LPS/PEP had significantly higher antibacterial activity than those injected with Ringer solution (*Treatment*: $F_{1,269} = 158.64$, $p < 0.001$). Although larvae in all three treatment groups had similar levels of protein in their haemolymph (Fig 2a), larvae injected with LPS/PEP appear to allocate more of their haemolymph protein pool to antibacterial activity than do Ringer-injected or naïve larvae (*Treatment*: $F_{1,428} = 360.46$, $p < 0.001$, Fig. 3a)

Phenoloxidase activity

Not all insects bled enough haemolymph to allow for measurement of both antibacterial activity and phenoloxidase activity, therefore slightly fewer insects were used in the PO assay ($n = 470$). There was no significant difference between the PO activity levels of control larvae at the start of the experiment and naïve larvae that had been feeding on chemically-defined diet for 24 h (*Treatment*: $F_{1,186} = 0.57$, $p = 0.450$). However, there was a significant decline in PO activity in Ringer-injected larvae ($F_{1,168} = 46.92$, $p < 0.001$) and LPS/PEP injected larvae ($F_{1,172} = 38.37$, $p < 0.001$), compared to control larvae at the start of the experiment, suggesting that PO activity was down-regulated only in challenged insects.

PO activity was significantly influenced by the amount of protein in the diet (*Diet*: $F_{1,431} = 17.30$, $p < 0.0001$), with an increase in dietary protein generally resulting in an increase in PO activity (Fig. 2c). When each treatment group was analysed separately, it appeared that the effect of dietary protein on PO activity was strongest for naïve larvae ($F_{1,152} = 10.74$, $p < 0.0001$), intermediate for larvae injected with Ringer solution ($F_{1,134} = 4.97$, $p = 0.027$), and lowest for those larvae injected with LPS/PEP ($F_{1,137} = 3.13$, $p = 0.079$). However, the statistical interaction between

Diet and *Treatment* was non-significant (*Diet* x *Treatment*: $F_{2,429} = 1.64$, $p = 0.20$). In addition to a *Diet* effect, there was also an independent positive effect of haemolymph protein levels on haemolymph PO activity (*Haemolymph protein*: $F_{1,431} = 50.09$, $p < 0.0001$, Fig. 3b).

There was a significant difference in larval haemolymph PO activity associated with injection-treatment (*Treatment*: $F_{1,431} = 105.23$, $p < 0.0001$), with the highest PO activity being observed in naïve larvae that had been handled but not injected (naïve vs. injected larvae, $F_{1,432} = 206.40$, $p < 0.0001$, Fig. 2c). Naive larvae invested more of their haemolymph protein pool into PO activity than the injected larvae (Fig. 3b), but there was no significant difference between the PO activity levels of LPS/PEP-injected and Ringer-injected larvae ($F_{1,275} = 3.98$, $p = 0.086$, Fig. 2c).

Immune function trade-offs

Both antibacterial activity and phenoloxidase activity are positively correlated with dietary protein levels (Fig. 2b,c) and haemolymph protein levels (Fig. 3a,b) and, as a consequence, we might expect to see a positive correlation between antibacterial activity and phenoloxidase activity within each of the three injection-treatment groups. Whilst this was true for *Naïve* larvae (Pearson's $r_{153} = 0.242$, $p = 0.0024$), there was no correlation between antibacterial and phenoloxidase activity in the two injection treatment groups (*Ringer*: $r_{135} = 0.100$, $p = 0.24$; *LPS/PEP*: $r_{141} = -0.152$, $p = 0.069$). Across the three treatment groups, there was a strong negative correlation between these two traits ($r_{466} = -0.356$, $p < 0.0001$), which became stronger after accounting of differences between larvae in their haemolymph protein levels ($r_{466} = -0.421$, $p < 0.0001$; Fig. 3c); correlations within treatment groups were either non-significant (*Naïve*: $r_{153} = 0.132$, $p = 0.10$; *Ringer*: $r_{135} = -0.026$, $p = 0.77$) or significantly negative (*LPS/PEP*: $r_{141} = -0.196$, $p = 0.019$).

EXPERIMENT 3: DIETARY SELF-SELECTION IN RESPONSE TO AN IMMUNE CHALLENGE

The aim of this experiment was to establish whether the larvae could offset the costs associated with mounting an antibacterial immune response through self-selecting a diet that would allow them to gain deficient macronutrients. The larvae were offered one of two choices: a low-protein diet-choice (14:28 paired with 28:14) or a high-protein diet-choice (14:28 paired with 35:7). In this experiment, just nine of the 108 larvae died or failed to pupate: five from the bacterially-injected treatment group, and four from the broth-injected treatment group, apparently due to the injection process, rather than from a bacterial infection. These larvae were omitted from the analysis. Although a few of the larvae were still feeding on day 7 post-challenge, 37% of larvae had reached the pre-pupal stage by day 6 and so were no longer feeding. Therefore, data analyses were focussed on the diet-choice decisions made over the first 4 days post-challenge when all larvae were still feeding.

Diet consumption

The total amount of diet consumed over the first four days was affected by the diet-choice offered (*Diet-choice*: $F_{1,190} = 18.03$, $p < 0.001$), with larvae on the high-protein diet-choice consuming more diet than larvae on the low-protein diet-choice. However, there was no effect of injection treatment on the total amount of food eaten (*Treatment*: $F_{1,94} = 1.06$, $p = 0.35$), and the interaction between diet-choice and injection treatment was marginally non-significant (*Diet-choice* x *Treatment*: $F_{1,92} = 2.93$, $p = 0.056$). The difference in food consumption between the two diet-choices was mainly due to differences in protein consumption ($F_{1,96} = 77.61$, $p < 0.0001$), with

larvae on the high-protein diet-choice consuming more protein than those on the low-protein choice across all three treatment groups (Fig. 4a). In contrast, the amount of carbohydrate consumed was not affected by the diet-choice offered ($F_{1,96} = 0.44$, $p = 0.51$, Fig. 4a).

The three treatment groups showed some differences in their pattern of protein and carbohydrate consumption within the two diet-choices. On the high-protein diet-choice, larvae in the two treatment groups that had been injected (with or without bacteria) consumed relatively less carbohydrate than naïve larvae offered the same diet-choice (*Treatment*: $F_{1,48} = 7.31$, $p = 0.009$); while on the low-protein diet-choice, bacterially-injected larvae consumed more protein than the two control treatment groups ($F_{1,45} = 7.34$, $p = 0.009$, Fig. 4a).

P:C ratio chosen

During the first 4 days post-challenge, P:C diet-choice decisions made by larvae were highly repeatable (Pearson's correlation between P:C chosen by larvae on day 2 vs day 4: $r_{97} = 0.403$, $p < 0.0001$) and the P:C ratio chosen by larvae did not differ significantly between day 2 and day 4 (GLM: *Day*: $F_{1,192} = 2.72$, $P = 0.10$). In order to account for multiple samples from larvae across the four days, subsequent analyses were therefore conducted on the average P:C ratio chosen over the first four days. The mean P:C ratio selected by larvae during this period was affected by the injection-treatment they received and the diet-choice they were offered (*Treatment*: $F_{2,94} = 11.70$, $p < 0.001$, *Diet-choice*: $F_{1,94} = 172.37$, $p < 0.0001$), but there was no significant interaction between these two main effects (*Treatment* x *Diet-choice*: $F_{2,92} = 0.366$, $p = 0.69$). Larvae offered the high-protein diet-choice selected a higher P:C ratio than larvae offered the low-protein choice (Fig. 4a,b). However, on both diet-choices, bacterially-injected larvae selected a significantly higher P:C ratio than that selected

by larvae in the two control treatment groups (low-protein choice: $F_{1,45} = 9.94$, $p = 0.0029$; high-protein choice: $F_{1,48} = 10.11$, $p = 0.0025$), and the two control groups did not differ in their P:C ratio choices (low-protein choice: $F_{1,30} = 2.05$, $p = 0.16$; high-protein choice: $F_{1,31} = 0.62$, $p = 0.44$).

Discussion

Bacterially-challenged larvae had highest survival on the most protein-rich diets, with predicted survival rates declining from nearly 50% on the most protein-rich diet to less than 25% on the most protein-poor diet (Fig. 1a). However, no such obvious diet-related effect was observed for naïve larvae, for which survival was generally high (>80%) and independent of dietary P:C ratio. This result is similar to that found by Lee *et al.* (2005) for *S. littoralis* larvae infected with an NPV, as although naïve larvae had high survival on all diets, the virally-infected larvae had highest survival on the most protein-rich diet (35:7). The higher survival rates seen on the higher-protein diets could reflect the protein cost involved in surviving a bacterial infection and investing in protein-dependent immune responses or replenishing lost reserves. However, the results from the immune assays indicate that the answer may be more complicated than this.

As expected, the level of protein in the haemolymph increased as the amount of dietary protein available to the larvae increased (Fig. 2a). As the protein-content of the diet (and haemolymph) increased, there was a proportional increase in both antibacterial activity and PO activity, suggesting that the larvae were allocating a relatively constant proportion of their dietary protein intake to haemolymph immune function. This result is consistent with those of Lee *et al.* (2005), who found that the encapsulation response, lysozyme-like antimicrobial activity and PO activity were all

significantly higher in *S. littoralis* larvae fed high-protein diets, as would be expected if protein is needed for the production of immunological effectors.

In the present study, however, whereas an increase in dietary protein resulted in an increase in PO and antibacterial activity, the injection treatment affected these two immune responses differently. As expected, the greater the specific bacterial immune insult, the higher the antibacterial activity. Conversely, naïve larvae had higher PO levels than those that had received an immune insult (Fig. 2c). This negative correlation between the two immune functions (PO activity and antibacterial activity), suggests a possible physiological trade-off within the immune system; larvae injected with a bacterial immune elicitor appear to be investing more of their available protein into antibacterial peptide production, at the expense of PO activity. It appears that unchallenged *S. exempta* larvae maintain a fairly high level of constitutive PO activity in the haemolymph; whereas, antibacterial peptides are maintained at relatively low levels. Yet, if immunity is costly to maintain, as predicted by life-history theory (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000), it might be expected that both PO and antibacterial peptides would be maintained at low levels when insects are unchallenged. A possible explanation for the high PO activity levels in unchallenged larvae could be that phenoloxidase is involved in functions other than immunity. For example, PO is a key enzyme in the production of the pigment melanin, which has a role in darkening and strengthening of the cuticle through sclerotization (Sugumaran 2002). In the face of a bacterial challenge, however, protein resources that are used to maintain high PO levels, could be used for the production of antibacterial peptides, generating in the apparent trade-off (Fig. 3c).

Other studies have also identified potential trade-offs within the immune system of insects. In unchallenged *S. littoralis* larvae, there is evidence for both

phenotypic and genetic trade-offs between PO activity and lysozyme-like antibacterial activity (Cotter *et al.* 2004a,b; Cotter *et al.* 2008), and similar correlations have been found in both mealworm beetles (Moret & Siva-Jothy 2003) and bumble bees (Moret & Schmid-Hempel 2001) that have been LPS-challenged. Trade-offs have also been suggested in field crickets, where encapsulation ability is negatively associated with antibacterial activity (Rantala & Kortet 2003; Rantala & Roff 2005).

The mechanisms underpinning putative trade-offs within the immune system are not fully understood, however it has been suggested that they could be mediated hormonally, for example by juvenile hormone (JH) (Cotter *et al.* 2004b; Wilson & Cotter 2008). Recent studies suggest a role for JH in mediating mating-induced immune suppression in the mealworm beetle (Rolff & Siva-Jothy 2002; Rantala *et al.* 2004) and modulating the cellular encapsulation response in *S. littoralis* (Khafagi & Hegazi 2001). There is also evidence that adipokinetic hormone (AKH) may play a regulatory role in the insect immune system (Adamo *et al.* 2007) and may modulate PO activity (Goldsworthy *et al.* 2005).

The bacterial bioassay and immune function assays suggest that there is a protein cost associated with fighting bacterial infections in *S. exempta*. With more protein in the diet, caterpillars are more likely to survive a bacterial infection, and there is an indication that protein is allocated to the production of antibacterial activity at the expense of PO activity, especially on the higher protein diets. The diet-choice experiments should therefore reveal whether bacterially-infected *S. exempta* larvae demonstrate an adaptive response to infection by actively selecting a diet rich in protein to compensate for these protein costs.

The results showed that bacterially-challenged larvae selected a higher P:C diet relative to uninfected larvae, especially when they were offered a higher protein

diet-choice (Fig. 4). This result is consistent with that found for virally-infected *S. littoralis* larvae, where infected larvae selected diets with higher levels of protein relative to naïve larvae from day 2 post-challenge (Lee *et al.* 2005). However, the current study provides even stronger support for pathogen-induced protein compensation since *S. exempta* normally prefers a carbohydrate-biased diet. The overall P:C chosen by larvae on the higher protein diet-choice was significantly elevated in the bacterially-injected larvae relative to the two control treatments. Again, this strongly suggests that *S. exempta* larvae actively modulate their food choice in response to bacterial infection.

Clayton & Wolfe (1993) describe "self-medication" as behaviour that increases the defence against parasites by one species by using substances that have been produced by another species. They state that self-medication can be classified into one of four categories: ingestion, absorption, topical application or proximity and that these categories can be tested using three predictions: (1) the mediator actively seeks contact with the medicinal substance; (2) the substance causes negative effects on the parasite; and (3) host fitness is increased due to the detrimental effects on the parasite. Circumstantial evidence for self-medication through ingestion comes from several studies of vertebrates, with the most well-known examples coming from chimpanzees using plant-derived medicinal substances when infected with protozoa or helminths (Huffman & Seifu 1989; Fowler *et al.* 2007). Medicinal chemicals have also been shown to be utilised in several insect species (e.g. Christe *et al.* 2003; Castella *et al.* 2008). For example, tobacco hornworm infected with *Bacillus thuringiensis* had improved fitness and decreased the bacterial colony growth and toxicity when they ingested the nicotine in tobacco leaf (Krischik *et al.* 1988). When attacked by endoparasitoids, some generalist Arctiid caterpillars are reported to

increase their gustatory responsiveness to specific plant toxins, pyrrolizidine alkaloids and iridoid glycosides, the consumption of which may improve survival (Bernays & Singer 2005).

The present study provides evidence consistent with self-medication in *S. exempta*, as all three of the stipulated conditions for self-medication are satisfied. The infected larvae actively seek the higher-protein diets; by ingesting more protein, the larvae are able to increase their immune function, such as antibacterial activity, which would have negative effects on the bacterial infection; and, finally, larvae that were provided with high-protein diets had increased survival and hence fitness. All larvae, with the exception of naïve larvae on the high-protein diet-choice, maintained a relatively constant absolute intake of carbohydrate, but varied the amount of protein eaten as a function of immune challenge. Therefore, protein appears to take precedence over carbohydrate when *S. exempta* are bacterially-infected, possibly so that larvae can increase the production of the immune system components, such as antibacterial peptides, needed to fight the infection. Given the apparent immuno-protective effects of protein, the increase in protein intake by the immune-challenged insects can therefore be seen as a form of self-medication, though it is yet to be established whether the ingested protein is metabolised to produce immune effectors or if it simply replaces resources lost fighting the infection.

In conclusion, the present study suggests that resistance to bacterial pathogens is positively related to levels of dietary protein. High levels of protein in the diet enhance protein levels in the haemolymph, which can be directed towards resistance mechanisms, including antibacterial peptides, providing a potential mechanism for this cost of resistance. Moreover, infected individuals may offset these protein costs

by altering their normal feeding behaviour to ingest relatively more dietary protein, consistent with the notion of ‘self medication’.

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Figure Legends

Figure 1. The effect of a bacterial infection and dietary P:C ratio on (a) survival, and (b) stadium duration of larvae at 144hr post injection. Means and standard errors are shown for naïve larvae, injected controls (broth- and Ringer-injected combined) and bacterially-injected larvae. The three lines represent (a) logistic regression lines through the raw survival data and (b) linear regression lines through the raw stadium duration data.

Figure 2. The relationship between dietary P:C ratio and (a) mean haemolymph protein levels (+/- S.E.), (b) mean antibacterial activity (+/- S.E.) and (c) mean phenoloxidase levels (+/- S.E.) for LPS/PEP-injected, Ringer-injected and naïve larvae. The three lines on each graph represent the linear regression lines through the raw data.

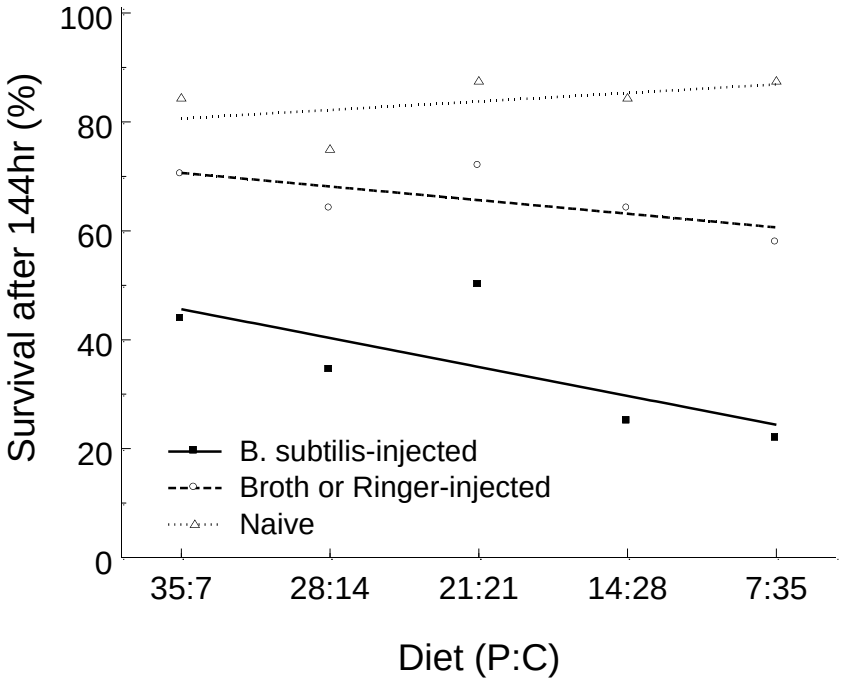
Figure 3. The relationships between haemolymph protein levels, antibacterial activity and phenoloxidase activity, for naïve larvae, larvae injected with Ringer solution and larvae injected with bacterial LPS/PEP to stimulate an antibacterial immune response. In (a) and (b), the correlation between haemolymph protein levels and haemolymph antibacterial and PO activity is shown, respectively, and in (c) the relationship between residual antibacterial activity and residual phenoloxidase activity is illustrated, after controlling for haemolymph protein levels. The symbols represent the raw data and the lines are linear regressions fitted to the raw data.

Figure 4. The relationship between injection treatment (naïve, broth-injected and bacteria-injected) and (a) mean protein and carbohydrate consumption over four days post-injection, and (b) mean P:C selected during the same period for larvae offered the choice between a carbohydrate-rich diet block (P:C = 14:28) and a protein rich diet block (P:C = 28:14, small symbols; or 35:7, large symbols). Symbols represent means (+/- S.E.). In (a), the grey lines represent different P:C ratios. In both (a) and (b), the thick dashed line shows the P:C ratio 1:1, which is the ratio that larvae in the low-protein diet-choice would have chosen if they had fed indiscriminately on the two diet blocks; the thin dotted line shows the ratio 1.4:1, which is the ratio that larvae in the high-protein diet-choice would have chosen if they had fed indiscriminately.

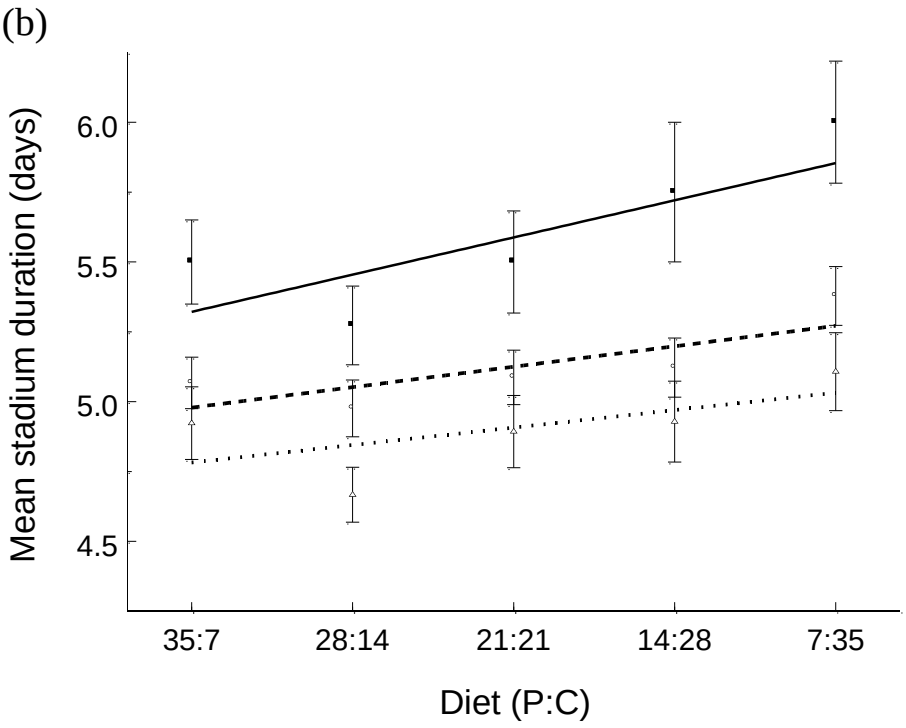
Figures

Fig. 1

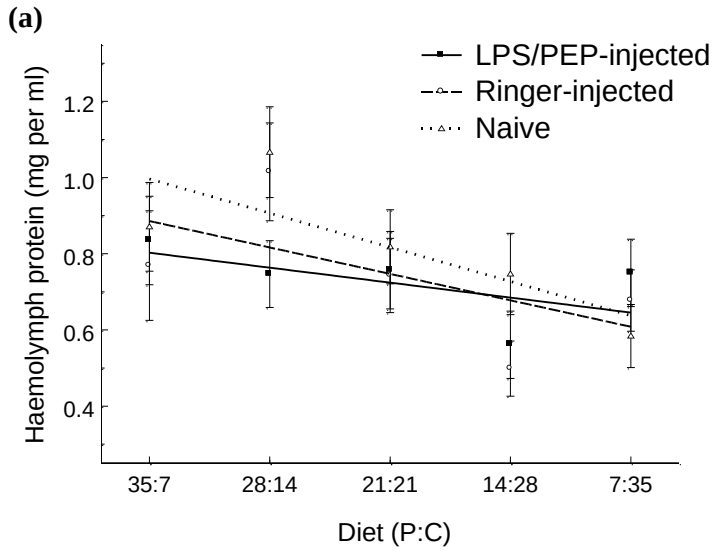
(a)



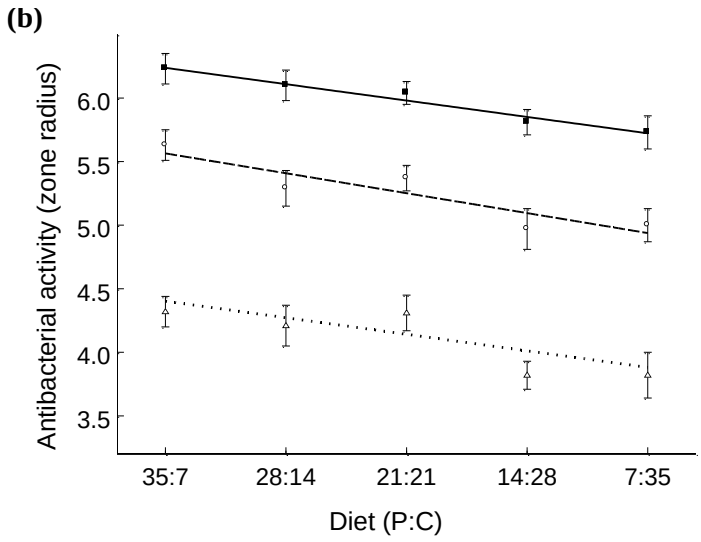
(b)



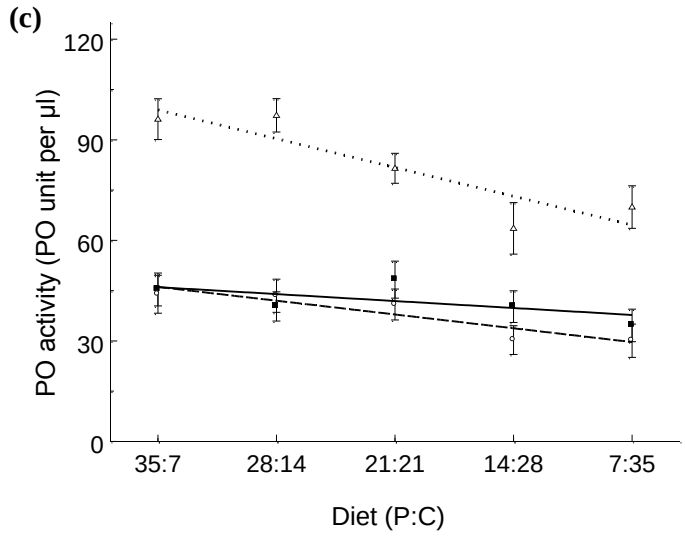
1 **Fig. 2**


(a)
LPS/PEP-injected
Ringer-injected
Naive
Haemolymph protein (mg per ml)
1.2
1.0
0.8
0.6
0.4
35:7
28:14
21:21
14:28
7:35
Diet (P:C)

2
3

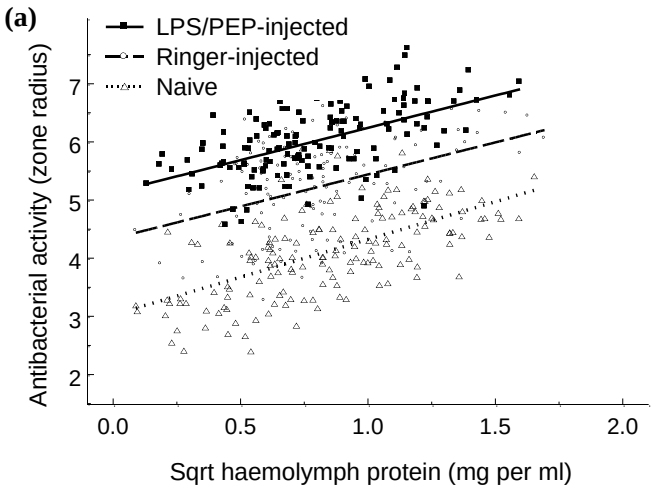

(b)
Antibacterial activity (zone radius)
6.0
5.5
5.0
4.5
4.0
3.5
35:7
28:14
21:21
14:28
7:35
Diet (P:C)

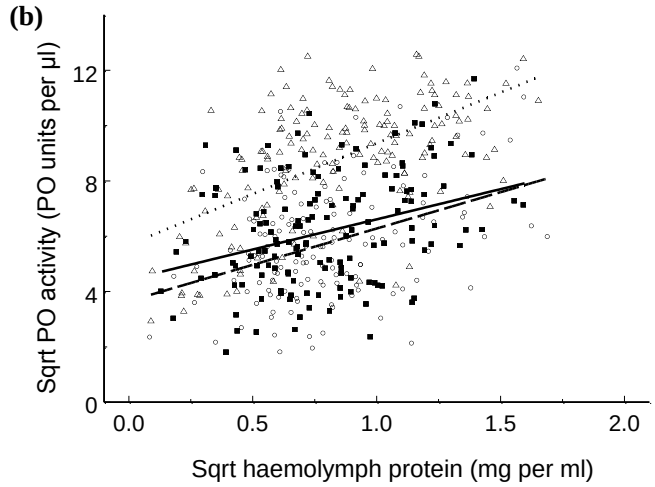
4


(c)
PO activity (PO unit per µl)
120
90
60
30
0
35:7
28:14
21:21
14:28
7:35
Diet (P:C)

5

Fig. 3


(a)
LPS/PEP-injected
Ringer-injected
Naive
Antibacterial activity (zone radius)
Sqrt haemolymph protein (mg per ml)


(b)
Sqrt PO activity (PO units per µl)
Sqrt haemolymph protein (mg per ml)

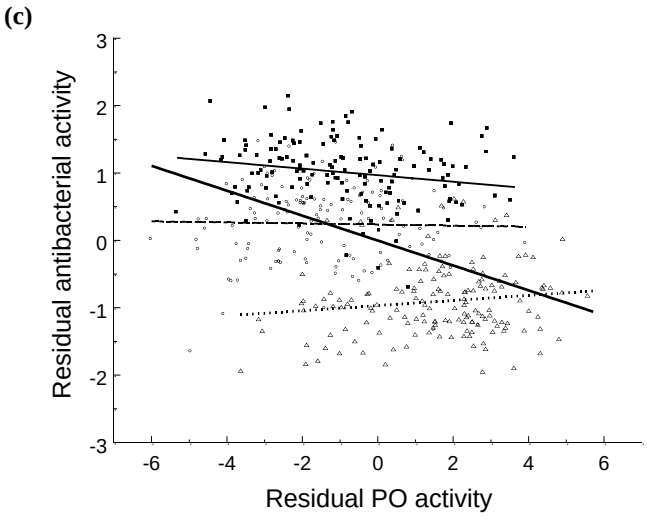
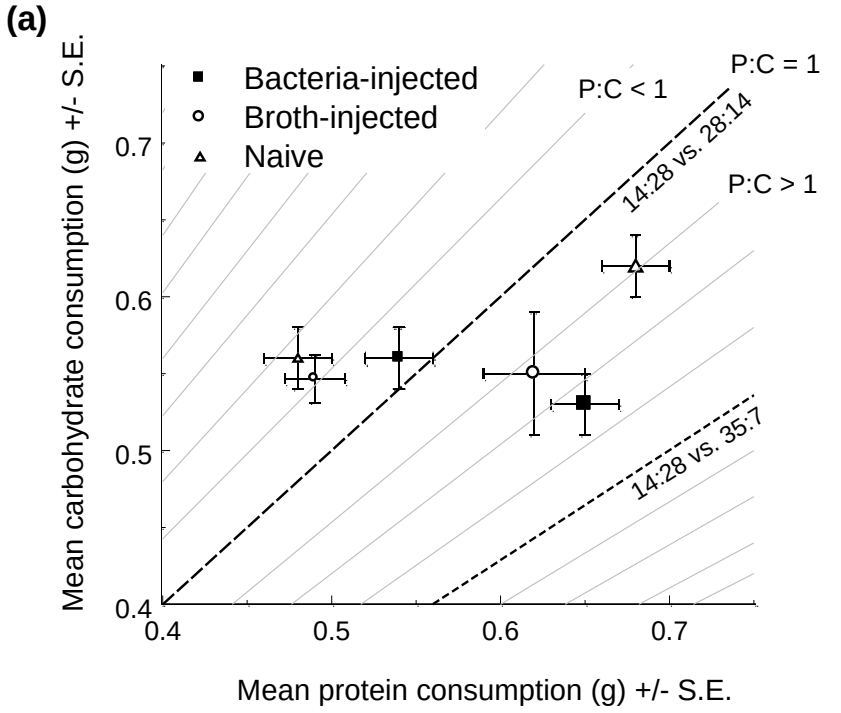
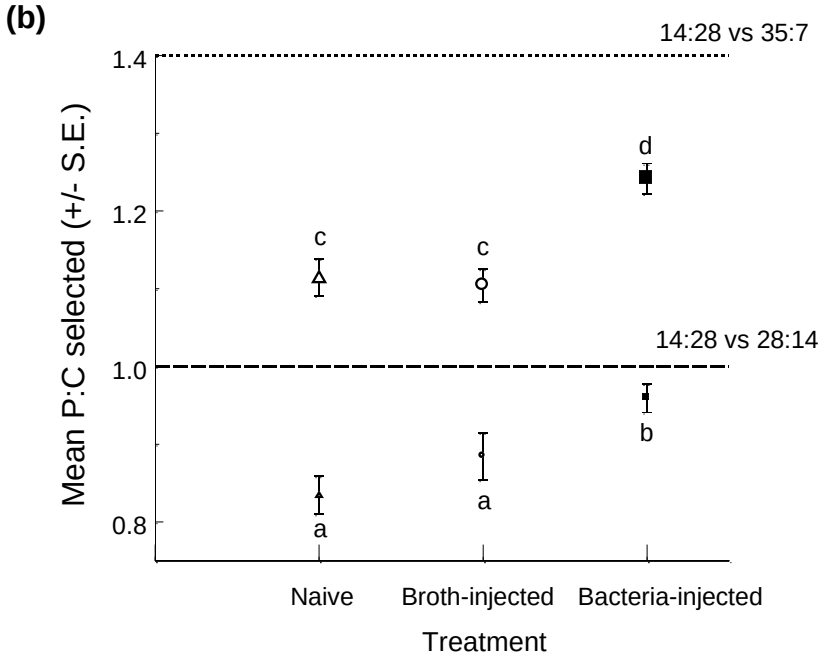

(c)
Residual antibacterial activity
Residual PO activity

Fig. 4


(a)
Bacteria-injected
Broth-injected
Naive
P:C = 1
P:C < 1
P:C > 1
14:28 vs. 28:14
14:28 vs. 35:7
Mean carbohydrate consumption (g) +/- S.E.
Mean protein consumption (g) +/- S.E.
0.4
0.5
0.6
0.7


(b)
14:28 vs 35:7
14:28 vs 28:14
Mean P:C selected (+/- S.E.)
1.4
1.2
1.0
0.8
a
a
b
c
c
d
Naive
Broth-injected
Bacteria-injected
Treatment