

14/11/2014

Povey et al. Protein costs of bacterial infection

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

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19 Running headline: Protein costs of bacterial infection

1 **Summary**

- 2 1. Mounting an immune response is likely to be costly in terms of energy and
3 nutrients, and so it is predicted that dietary intake should change in response to
4 infection to offset these costs. The present study focuses on the interactions
5 between a specialist grass-feeding caterpillar species, the African armyworm
6 *Spodoptera exempta*, and an opportunist bacterium, *Bacillus subtilis*.
- 7 2. The main aims of the study were (i) to establish the macronutrient costs to the
8 insect host of surviving a systemic bacterial infection, (ii) to determine the
9 relative importance of dietary protein and carbohydrate to immune system
10 functions, and (iii) to determine whether there is an adaptive change in the
11 host's normal feeding behaviour in response to bacterial challenge, such that
12 the nutritional costs of resisting infection are offset.
- 13 3. We show that the survival of bacterially-infected larvae increased with
14 increasing dietary protein-to-carbohydrate (P:C) ratio, suggesting a protein
15 cost associated with bacterial resistance. As dietary protein levels increased,
16 there was an increase in antibacterial activity, phenoloxidase (PO) activity and
17 protein levels in the haemolymph, providing a potential source for this protein
18 cost. However, there was also evidence for a physiological trade-off between
19 antibacterial activity and phenoloxidase activity, as larvae whose antibacterial
20 activity levels were elevated in response to immune activation had reduced PO
21 activity.
- 22 4. When given a choice between two diets varying in their P:C ratios, larvae
23 injected with a sub-lethal dose of bacteria increased their protein intake
24 relative to control larvae whilst maintaining similar carbohydrate intake levels.
25 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').

4

5

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

1 **Introduction**

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

14 However, starvation is a fairly blunt tool for measuring the specific nutritional
15 costs of mounting an immune response. Many studies have shown that animals
16 regulate their macronutrient intake through dietary self-selection, thereby optimising
17 growth and performance (Raubenheimer & Simpson 1997; Waldbauer & Friedman
18 1999). Experimental work on dietary self-selection in insects has mainly focused on
19 investigating the regulation of two macronutrients, protein and carbohydrate (e.g.
20 Chambers *et al.* 1995; Lee *at al.* 2003, 2004; Thompson *et al.* 2005). At any given
21 time, the animal will require a specific quantity and mixture of nutrients, termed the
22 intake target (Simpson & Raubenheimer 1995). However, the nutritional requirements
23 for growth, reproduction and survival of healthy individuals, may be very different
24 from those in individuals that are immune-challenged.

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

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

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

3 Numerous studies have focused on how survival of the host changes when
4 specific metabolites are included in their diet (e.g. DeWitt *et al.* 1999; Buentello &
5 Gatlin 2001; Lee *et al.* 2005). However, a question that remains largely unanswered is
6 whether animals have the ability to actively self-select diets that will improve their
7 resistance to parasite infection. In the field, herbivorous insects may balance their
8 intake of nutrients by consuming different plant species, different individual plants of
9 the same species, or different parts of the same plant (Raubenheimer & Bernays 1993;
10 Mody *et al.* 2007). More recently, it has been reported that even predatory
11 invertebrates are capable of balancing their intake of multiple nutrients (Mayntz *et al.*
12 2005). In the laboratory, artificial diet is often used to study nutritional regulation
13 because levels of specific nutrients can be accurately manipulated, whilst maintaining
14 constant other aspects of the food quality and quantity. It has been shown that
15 parasitism can affect the relative intake of protein and carbohydrate (Thompson &
16 Redak 2005). It would be expected that if specific nutrients, or nutrient ratios, are
17 beneficial to the host in mounting an immune response against a parasite, infected
18 hosts would actively select diets with high levels of that particular nutrient. This
19 approach was adopted by Lee *et al.* (2005) with virally-infected *S. littoralis*.
20 Uninfected larvae performed best on diets that had an even P:C ratio, but virally-
21 infected larvae that successfully resisted the infection selected a significantly more
22 protein-biased diet than that selected by lethally-infected and control larvae. However,
23 it remains to be established how ubiquitous this response is and whether it is
24 dependent on the optimal diet of the uninfected host, or the nature of the parasitic
25 challenge.

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

13

14 **Methods.**

15 HOST AND PATHOGEN CULTURES

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

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

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

7

8 ARTIFICIAL DIETS

9 Upon reaching the final instar, experimental larvae were transferred to
10 experimental diets that varied in their macronutrient content. These diets were based
11 on those of Simpson & Abisgold (1985) and have been used previously in studies
12 using *S. exempta* (Lee *et al.* 2004). These diets varied in their protein and digestible
13 carbohydrate content. The protein portion of the diet consisted of a 3:1:1 ratio of
14 casein, peptone and albumen, and the carbohydrate content consisted of a 1:1 mixture
15 of sucrose and dextrin. Other constituents of the diets were Wesson's salts (2.4%),
16 cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mixture
17 (0.2%). The remaining portion of the diets was made up of cellulose, a non-nutritive
18 bulking agent. The dry ingredients of the diet were mixed in a 1% agar solution in a
19 6:1 agar solution:dry diet ratio. Five diets were used in total: 35% protein with 7%
20 carbohydrate (P:C = 35:7), 28:14, 21:21, 14:28 and 7:35. In each case the protein and
21 carbohydrate portion made up 42% of the final diet, which meant that all diets were
22 near isocaloric.

23

1 INFECTIONS

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

8

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

10 Larvae were injected with 5 μl of *B. subtilis* suspended in nutrient broth at the LD₅₀
11 concentration of 1x10⁷ cells/ml (S. Povey, unpublished data). Three control groups
12 were used: those injected with insect Ringer solution (NaCl 8.0g, CaCl₂ 0.25g, KCl
13 0.25g, NaHCO₃ 0.25g, distilled water 1000ml) (Lacey 1997), to control for the effects
14 of the injection process; those injected with nutrient broth, to control for any mortality
15 caused by the nutrient broth rather than the bacteria; and a group of naïve larvae,
16 which were handled but not injected. The results indicate no difference between the
17 mortality pattern of the injected controls (see Results) and so in future experiments
18 just the nutrient broth control treatment was used as an injection control. In total, there
19 were 32 larvae in each control group and 32 *B. subtilis*-injected larvae on each diet (a
20 total of 640 larvae, across two blocks).

21 Following injection, larvae were placed in a 25ml plastic pot containing one of the
22 five test diets varying in their protein-to-carbohydrate ratio (P:C): 7:35, 14:28, 21:21,
23 28:14 or 35:7. At the same time, naïve larvae were also switched on to their test diet.
24 Each day, until all larvae had either died or pupated, the larvae were provided with
25 fresh test diet and any deaths were recorded. Larvae dying due to systemic bacterial

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

9

10 EXPERIMENT 2: EFFECT OF DIETARY P:C RATIO ON LARVAL IMMUNE 11 FUNCTION

12 A total of 576 larvae were used in this experiment, across three experimental
13 blocks. For each of the five test diets there were three treatment groups: naïve larvae,
14 those injected with 5µl of insect Ringer solution, and those injected with 5µl of a
15 50:50 solution of 0.5 mg/ml lipopolysaccharide (LPS) and 1.0 mg/ml peptidoglycan
16 (PEP), (Morishima *et al.* 1997; Korner & Schmid-Hempel 2004). LPS and PEP are
17 products from gram-negative and gram-positive bacterial cell walls, so when injected
18 into the larvae should elicit antibacterial immune responses without causing mortality.
19 Following injection, all larvae were immediately assigned to one of the five diets
20 varying in P:C ratio, except for a further set of control larvae that had haemolymph
21 taken at the same time as test larvae were injected. Larvae allocated to the five test
22 diets had haemolymph collected 24h after injection, as this was shown in a
23 preliminary study to be the peak in the antibacterial response (S. Povey, unpublished
24 data). Haemolymph was extracted from the insect by piercing the larval cuticle
25 between the prolegs with a fine gauge needle and allowing the haemolymph to drip

1 into an Eppendorf tube on ice. All of the samples were then frozen at -80°C until they
2 were to be measured. The exact amount of haemolymph collected varied between
3 larvae, but $15\ \mu\text{l}$ was sufficiently to conduct all of the immune function assays
4 reported.

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

15 The remaining haemolymph samples were frozen at -80°C for assaying
16 phenoloxidase activity. Before freezing, $8\ \mu\text{l}$ of haemolymph was diluted in $8\ \mu\text{l}$ of a
17 50:50 solution of phosphate-buffered saline (PBS): glycerol solution. PO activity and
18 the amount of protein per haemolymph sample were measured using a modified
19 version of the procedure described by Cotter *et al.* (2004a). Briefly, $8\ \mu\text{l}$ of each
20 diluted haemolymph sample was mixed with $200\ \mu\text{l}$ of PBS, $90\ \mu\text{l}$ of the resulting
21 solution was pipetted in duplicate into a microtitre plate. $4\ \text{mM}$ dopamine was used as
22 a substrate for the PO reaction, with $90\ \mu\text{l}$ added to each sample. The absorbance of
23 the resulting mixture was measured at 492nm for 10 min on a VERSAmax microplate
24 reader (Molecular Devices Corp., USA) at a constant temperature of 25°C , which was
25 during the linear phase of the reaction. Haemolymph protein levels were determined

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

5

6 EXPERIMENT 3: DIETARY SELF-SELECTION IN RESPONSE TO AN IMMUNE 7 CHALLENGE

8 This experiment used a total of 108 larvae, across two blocks of 54. The
9 experiment included three treatments. Test larvae were injected with 5µl of *B. subtilis*
10 suspended in nutrient broth at a concentration of 1×10^4 cells/ml. This concentration
11 was chosen as pilot studies showed that it is low enough to cause minimal mortality
12 (<10%) yet high enough to elicit a significant antibacterial response in the larvae.
13 There were two control groups; those injected with nutrient broth, again to test the
14 effects of the suspension liquid and the injection process, and a group of naïve larvae
15 that were handled but not injected. Following injection, larvae were transferred to
16 Petri dishes containing the experimental diets. Each insect was given a choice
17 between two blocks of diet: one that was carbohydrate-biased (P:C = 14:28) and one
18 that was either slightly protein-biased (28:14) or strongly protein-biased (35:7). These
19 diets were chosen as they allow the larvae the option of consuming their predicted
20 optimal diet when uninfected (14:28) (Lee *et al.* 2004) or to deviate from this to a
21 protein-biased diet. Two protein-biased diets were chosen as they would allow a
22 comparison between a slightly and strongly protein-biased diet, and therefore show
23 the degree to which the larvae were able to deviate from their optimal diet when
24 bacterially-infected.

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

8

9 STATISTICAL ANALYSIS

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

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

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

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

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

12

13

14 **Results**

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

16 The aim of this experiment was to identify any differences in survival of *S.*
17 *exempta* larvae when provided with diets differing in their P:C ratio. Larvae started
18 dying 24h post-injection and deaths continued until 144h, at which point all larvae
19 had either died or pupated. There were significant effects of *Treatment* and *Diet* on
20 larval survival (AFT model: *Treatment*: $\chi^2_2 = 95.96$, $p < 0.001$, *Diet*: $\chi^2_1 = 4.08$, $p =$
21 0.043), but there was no significant interaction between these two main effects (*Diet* x
22 *Treatment* interaction: $\chi^2_2 = 2.67$, $p = 0.26$). Although the interaction term was non-
23 significant, it is worth noting that when the three treatment groups were analysed
24 separately, the effect of dietary protein content on mortality rate was strongest for the

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

8 There were significant effects of *Diet* and *Treatment* on the time taken for the
9 surviving larvae to pupate (GLM: *Treatment*: $F_{2,394} = 19.93$, $p < 0.001$; *Diet*: $F_{1,394} =$
10 10.46 , $p = 0.001$), but there was no significant interaction between these two main
11 effects (*Diet* x *Treatment*: $F_{2,392} = 0.41$, $p = 0.667$). Across all treatment groups, time
12 to pupation increased as the protein content of the diet declined (Fig. 1b). Further
13 analyses indicated that *B. subtilis*-injected larvae took significantly longer to pupate
14 than larvae in the control groups (*Treatment*: *B. subtilis*-injected (mean \pm s.d. = $5.56 \pm$
15 0.10 d) vs. controls (5.03 ± 0.10 d): $F_{1,395} = 30.15$, $p < 0.001$), and that surviving
16 larvae in the injected-control group (5.11 ± 0.10 d) took significantly longer to pupate
17 than survivors from the naïve group (4.91 ± 0.10 d) ($F_{1,341} = 8.83$, $p = 0.003$).

18

19 EXPERIMENT 2: EFFECT OF DIETARY P:C RATIO ON LARVAL IMMUNE 20 FUNCTION

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

1 *Haemolymph protein levels*

2 Twenty-four hours after larvae were switched on to one of the five chemically-
3 defined diets, larval haemolymph protein levels reflected the P:C ratio of the new diet
4 (*Diet*: $F_{1,432} = 31.37$, $p < 0.001$), suggesting a rapid incorporation of dietary protein
5 into the haemolymph protein pool (Fig. 2a). However, the injection treatment received
6 did not significantly affect haemolymph protein levels (*Treatment*: $F_{2,430} = 0.39$, $p =$
7 0.677 , Fig. 2a).

8 *Antibacterial activity*

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

17 The antibacterial activity levels of naïve larvae at 24 hours were not
18 significantly different from those of the control larvae whose antibacterial activity
19 levels were measured at the start of the experiment (*Treatment*: $F_{1,211} = 2.62$, $p =$
20 0.107). In contrast, there was a significant increase in the antibacterial activity of
21 Ringer-injected larvae ($F_{1,201} = 119.64$, $p < 0.001$) and LPS/PEP-injected larvae ($F_{1,206}$
22 $= 344.86$, $p < 0.001$), relative to these controls, suggesting that injection resulted in a
23 significant up-regulation of antibacterial activity.

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

1 solution had significantly higher antibacterial activity than naïve larvae (*Treatment:*
2 $F_{1,339} = 360.02$, $p < 0.001$), while larvae injected with LPS/PEP had significantly
3 higher antibacterial activity than those injected with Ringer solution (*Treatment:* $F_{1,269}$
4 $= 158.64$, $p < 0.001$). Although larvae in all three treatment groups had similar levels
5 of protein in their haemolymph (Fig 2a), larvae injected with LPS/PEP appear to
6 allocate more of their haemolymph protein pool to antibacterial activity than do
7 Ringer-injected or naïve larvae (*Treatment:* $F_{1,428} = 360.46$, $p < 0.001$, Fig. 3a)

8

9 *Phenoloxidase activity*

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

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

1 *Diet* and *Treatment* was non-significant (*Diet* x *Treatment*: $F_{2,429} = 1.64$, $p = 0.20$). In
2 addition to a *Diet* effect, there was also an independent positive effect of haemolymph
3 protein levels on haemolymph PO activity (*Haemolymph protein*: $F_{1,431} = 50.09$, $p <$
4 0.0001 , Fig. 3b).

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

12 *Immune function trade-offs*

13 Both antibacterial activity and phenoloxidase activity are positively correlated
14 with dietary protein levels (Fig. 2b,c) and haemolymph protein levels (Fig. 3a,b) and,
15 as a consequence, we might expect to see a positive correlation between antibacterial
16 activity and phenoloxidase activity within each of the three injection-treatment
17 groups. Whilst this was true for *Naïve* larvae (Pearson's $r_{153} = 0.242$, $p = 0.0024$),
18 there was no correlation between antibacterial and phenoloxidase activity in the two
19 injection treatment groups (*Ringer*: $r_{135} = 0.100$, $p = 0.24$; *LPS/PEP*: $r_{141} = -0.152$, $p =$
20 0.069). Across the three treatment groups, there was a strong negative correlation
21 between these two traits ($r_{466} = -0.356$, $p < 0.0001$), which became stronger after
22 accounting of differences between larvae in their haemolymph protein levels ($r_{466} = -$
23 0.421 , $p < 0.0001$; Fig. 3c); correlations within treatment groups were either non-
24 significant (*Naïve*: $r_{153} = 0.132$, $p = 0.10$; *Ringer*: $r_{135} = -0.026$, $p = 0.77$) or
25 significantly negative (*LPS/PEP*: $r_{141} = -0.196$, $p = 0.019$).

1

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

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

16 *Diet consumption*

17 The total amount of diet consumed over the first four days was affected by the
18 diet-choice offered (*Diet-choice*: $F_{1,190} = 18.03$, $p < 0.001$), with larvae on the high-
19 protein diet-choice consuming more diet than larvae on the low-protein diet-choice.
20 However, there was no effect of injection treatment on the total amount of food eaten
21 (*Treatment*: $F_{1,94} = 1.06$, $p = 0.35$), and the interaction between diet-choice and
22 injection treatment was marginally non-significant (*Diet-choice* x *Treatment*: $F_{1,92} =$
23 2.93 , $p = 0.056$). The difference in food consumption between the two diet-choices
24 was mainly due to differences in protein consumption ($F_{1,96} = 77.61$, $p < 0.0001$), with

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

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

12 *P:C ratio chosen*

13 During the first 4 days post-challenge, P:C diet-choice decisions made by larvae
14 were highly repeatable (Pearson's correlation between P:C chosen by larvae on day 2
15 vs day 4: $r_{97} = 0.403$, $p < 0.0001$) and the P:C ratio chosen by larvae did not differ
16 significantly between day 2 and day 4 (GLM: *Day*: $F_{1,192} = 2.72$, $P = 0.10$). In order to
17 account for multiple samples from larvae across the four days, subsequent analyses
18 were therefore conducted on the average P:C ratio chosen over the first four days. The
19 mean P:C ratio selected by larvae during this period was affected by the injection-
20 treatment they received and the diet-choice they were offered (*Treatment*: $F_{2,94} =$
21 11.70 , $p < 0.001$, *Diet-choice*: $F_{1,94} = 172.37$, $p < 0.0001$), but there was no significant
22 interaction between these two main effects (*Treatment* x *Diet-choice*: $F_{2,92} = 0.366$, p
23 $= 0.69$). Larvae offered the high-protein diet-choice selected a higher P:C ratio than
24 larvae offered the low-protein choice (Fig. 4a,b). However, on both diet-choices,
25 bacterially-injected larvae selected a significantly higher P:C ratio than that selected

1 by larvae in the two control treatment groups (low-protein choice: $F_{1,45} = 9.94$, $p =$
2 0.0029; high-protein choice: $F_{1,48} = 10.11$, $p = 0.0025$), and the two control groups did
3 not differ in their P:C ratio choices (low-protein choice: $F_{1,30} = 2.05$, $p = 0.16$; high-
4 protein choice: $F_{1,31} = 0.62$, $p = 0.44$).

6 Discussion

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

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

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

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

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

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

7 The mechanisms underpinning putative trade-offs within the immune system
8 are not fully understood, however it has been suggested that they could be mediated
9 hormonally, for example by juvenile hormone (JH) (Cotter *et al.* 2004b; Wilson &
10 Cotter 2008). Recent studies suggest a role for JH in mediating mating-induced
11 immune suppression in the mealworm beetle (Rolff & Siva-Jothy 2002; Rantala *et al.*
12 2004) and modulating the cellular encapsulation response in *S. littoralis* (Khafagi &
13 Hegazi 2001). There is also evidence that adipokinetic hormone (AKH) may play a
14 regulatory role in the insect immune system (Adamo *et al.* 2007) and may modulate
15 PO activity (Goldsworthy *et al.* 2005).

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

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

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

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

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

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

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

- 1 by altering their normal feeding behaviour to ingest relatively more dietary protein,
- 2 consistent with the notion of 'self medication'.
- 3

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1 **Figure Legends**

2

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

9

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

15

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

24

25

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

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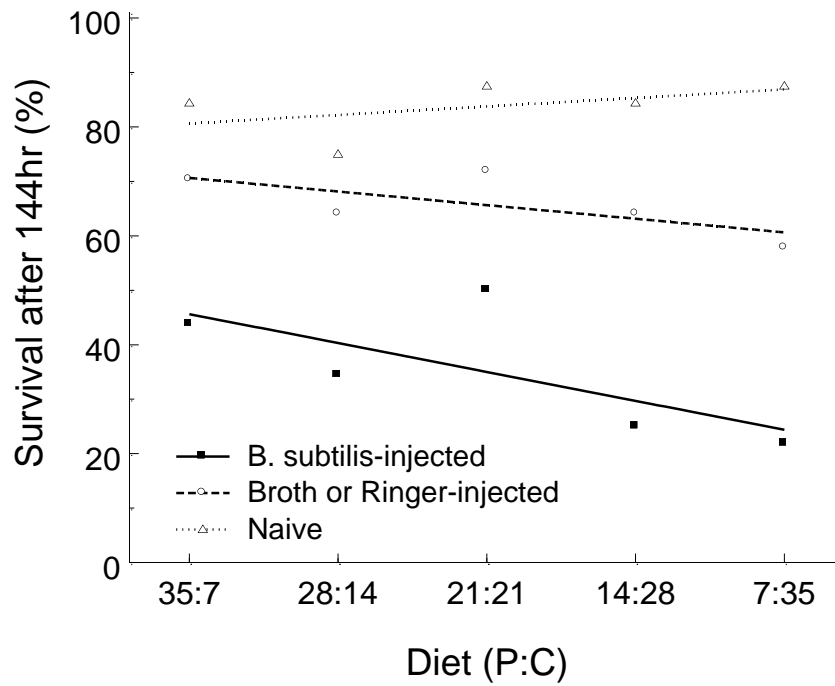
14

1 **Figures**

2 **Fig. 1**

3

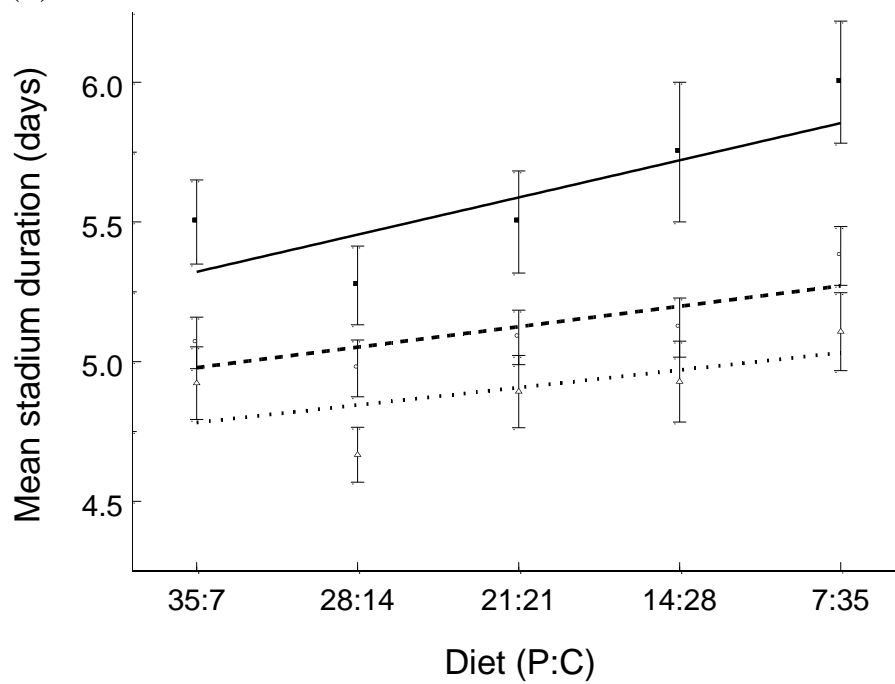
4 (a)



5

6

(b)

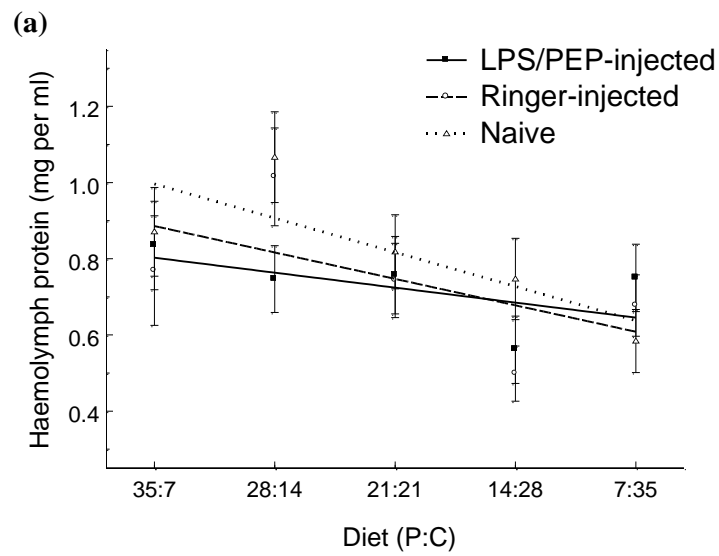


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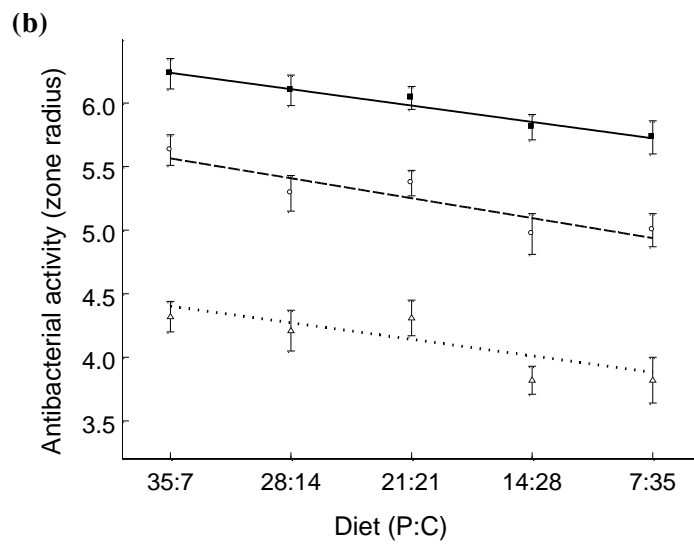
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9

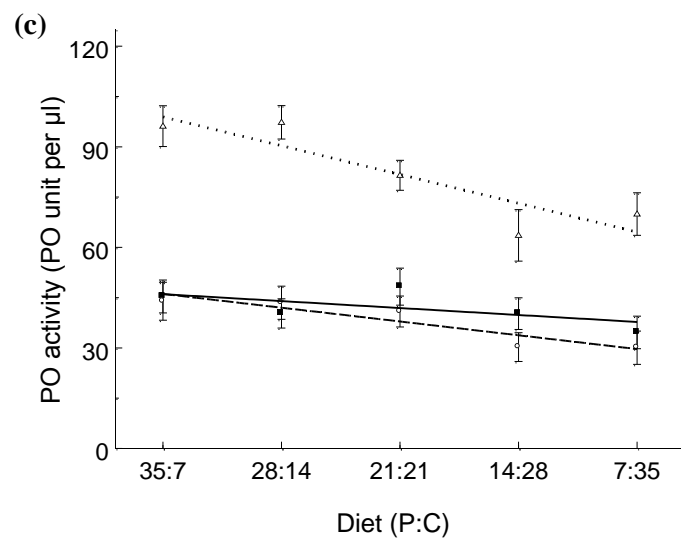
1 **Fig. 2**



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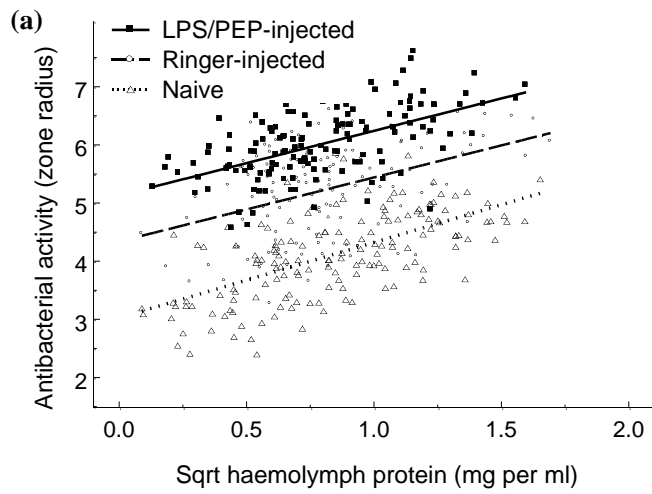
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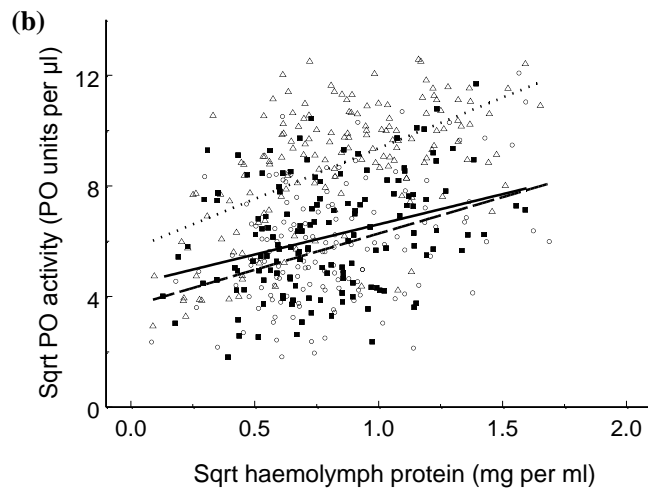
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1 **Fig. 3**

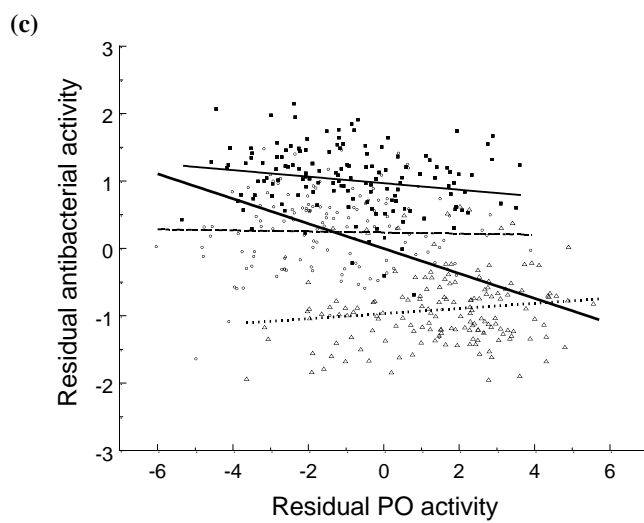
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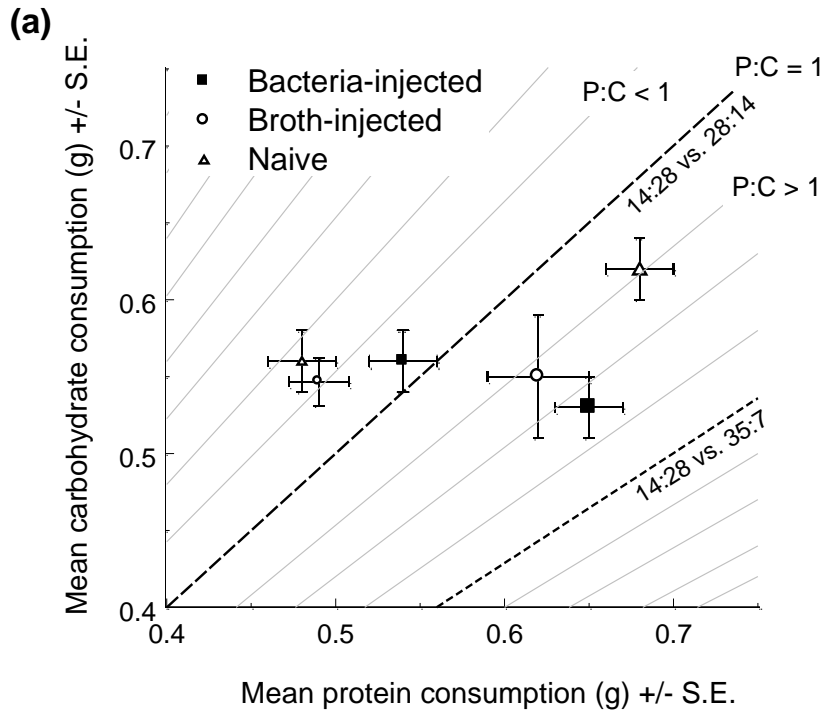
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8

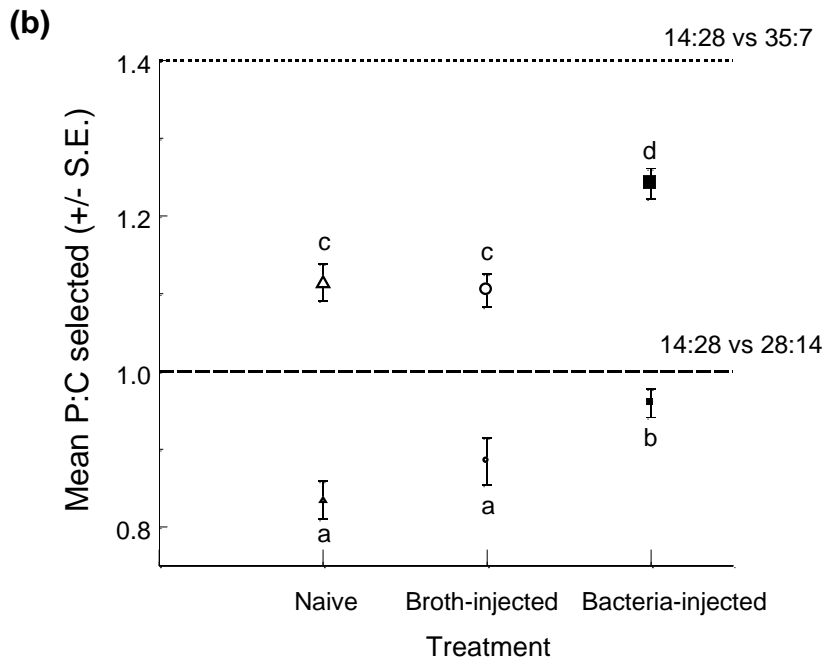
1 **Fig. 4**

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