



# Locusts increase carbohydrate consumption to protect against a fungal biopesticide



Robert I. Graham<sup>a,b,c,\*</sup>, Juliane M. Deacutis<sup>a,b,c</sup>, Tamara Pulpitel<sup>a,b</sup>, Fleur Ponton<sup>a,b</sup>, Stephen J. Simpson<sup>a,b</sup>, Kenneth Wilson<sup>c</sup>

<sup>a</sup> School of Biological Sciences, University of Sydney, NSW 2006, Australia

<sup>b</sup> Charles Perkins Centre, University of Sydney, NSW 2006, Australia

<sup>c</sup> Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK

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## ABSTRACT

There is growing evidence to suggest that hosts can alter their dietary intake to recoup the specific resources involved in mounting effective resistance against parasites and pathogens. We examined macronutrient ingestion and disease-resistance in the Australian plague locust (*Chortoicetes terminifera*), challenged with a fungal pathogen (*Metarhizium acridum*) under dietary regimes varying in their relative amounts of protein and digestible carbohydrate. Dietary protein influenced constitutive immune function to a greater extent than did carbohydrate, indicating higher protein costs of mounting an immune defence than carbohydrate or overall energy costs. However, it appears that increased immune function, as a result of greater protein ingestion, was not sufficient to protect locusts from fungal disease. We found that locusts restricted to diets high in protein (P) and low in carbohydrate (C) were more likely to die of a fungal infection than those restricted to diets with a low P:C ratio. We hypothesise that the fungus is more efficient at exploiting protein in the insect's haemolymph than the host is at producing immune effectors, tipping the balance in favour of the pathogen on high-protein diets. When allowed free-choice, survivors of a fungus-challenge chose a less-protein-rich diet than those succumbing to infection and those not challenged with fungus locusts. These results are contrary to previous studies on caterpillars in the genus *Spodoptera* challenged with bacterial and baculoviral pathogens, indicating that nutrient ingestion and pathogen resistance may be a complex interaction specific to different host species and disease agents.

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## 1. Introduction

Parasites and pathogens (broadly defined to include viruses, bacteria, protozoans, helminths and arthropods) pose major threats to their hosts, and theory predicts that only those hosts that can effectively protect themselves will be evolutionarily selected for. Disease and infection are known to impose significant fitness costs, such as reducing host survival and host reproductive output (Hurd, 2001). Intricately connected to these direct impacts, pathogen infections are also thought to impose resource-costs associated with the maintenance and activation of resistance mechanisms (Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2005). In turn, these costs impact the ability of the host to fight and withstand infection, all of which may be dependent on host nutritional

state (Lochmiller and Deerenberg, 2000; Coop and Kyriazakis, 2001). Evidence from insect herbivores (Lee et al., 2002, 2008; Simpson et al., 2004), and more recently from predatory invertebrates (Wilder et al., 2013), has shown that animals balance their intake of multiple essential nutrients (notably protein and non-protein energy, such as carbohydrate), and it is known that this may have a large impact on their ability to fight infection (Lee et al., 2006; Ponton et al., 2011a,b).

Previous studies have demonstrated that host nutritional-stress (i.e. starvation) can increase parasite virulence (Brown et al., 2000), can exacerbate the costs involved in immune activation (Moret and Schmid-Hempel, 2000), and can lead to reduced immune responsiveness (Siva-Jothy and Thompson, 2002; Rolff et al., 2004). A key principle in the evolutionary ecology of disease is the degree to which hosts invest in defence against parasites and pathogens, including the concept that increased risk of disease leads to selection on the host to minimize the potential costs of disease (Sheldon and Verhulst, 1996). It has been reported that host survival under

\* Corresponding author at: Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK. Tel.: +44 1524593406.

E-mail address: [r.graham@lancaster.ac.uk](mailto:r.graham@lancaster.ac.uk) (R.I. Graham).

parasite challenge can improve as a result of selecting foods containing anti-parasitic chemicals (Bernays and Singer, 2005; Singer et al., 2009; Smilanich et al., 2011), known as “therapeutic medication” (de Roode and Lefevre, 2012). Therapeutic self-medication does not necessarily rely on the consumption of chemical substances, but may also be facilitated through the interaction between nutrition and physiological immune responses (Raubenheimer and Simpson, 2009; Ponton et al., 2011a,b; Ponton et al., 2013): insects can select particular nutrients or nutrient ratios to increase their resistance or tolerance to pathogen challenge (Lee et al., 2006; Povey et al., 2009, 2013). In particular, protein is an important macronutrient for the immune system, and several studies have found that increased protein intake increases host resistance to disease challenge, possibly by providing the limiting resources to produce or replace infected cells or immune effectors such as haemocytes, antimicrobial peptides and enzyme cascades (Lee et al., 2006; Povey et al., 2009, 2013). To date, experiments to determine whether insects alter their intake of macronutrients to offset the costs of microparasite infection have only been undertaken using two types of pathogen, baculoviruses (Lee et al., 2006; Povey et al., 2013) and bacteria (Povey et al., 2009); and notably, only on closely-related host caterpillars, noctuid moths from the genus *Spodoptera*. These questions have not been tested in other insect hosts or other microparasite–pathogen systems; however, a similar study has been undertaken in a macroparasite system, *Tenebrio molitor* and its tapeworm *Hymenolepis diminuta* (Ponton et al., 2011a,b).

The role of nutrition in resistance to pathogen-challenge has clear implications for future use of biological control agents, especially at a time when policy makers are becoming increasingly aware of the need to reduce chemical pesticide use and invest in biological control programs (Wilson et al., 2013). For this study we used the Australian plague locust, *Chortoicetes terminifera*, and the commercially available fungal biopesticide Greenguard<sup>®</sup>, *Metarhizium acridum* ARSEF 324, as a model system to further study the role of nutrition in disease resistance. Locusts are highly phenotypically plastic, and are known to undertake a number of behavioural responses to pathogen challenge, including a phenomenon known as “behavioural fever”, whereby locusts move towards heat sources so as to increase their body temperature to cure themselves of an infection (Blanford and Thomas, 2000). Fungal pathogens are used extensively as biopesticides. *M. acridum* is especially useful as a specialist biopesticide, due to its host specificity to only certain locusts and grasshoppers (Driver et al., 2000). Host-to-host transmission can occur mainly horizontally via environmental contamination by the infectious form of the fungus, the spore. The spore attaches to the insect’s external cuticle. It germinates after 24 h and then penetrates, using enzymes and mechanical force, through the cuticle to the inside of the body cavity. Here it circulates and reproduces within the insect haemolymph, progressively consuming host nutrients and digesting the body cavity. They are highly virulent pathogens, with infections usually resulting in the death of the host within one week.

The insect innate immune system comprises both humoral and cellular defence responses (Lavine and Strand, 2002). Humoral responses include antimicrobial peptides, the cascades that regulate coagulation and melanization of haemolymph, and the production of reactive intermediates of oxygen and nitrogen. Cellular defences refer to haemocyte-mediated responses like nodulation, phagocytosis and encapsulation. Immune responses can be both constitutive (always expressed) and induced (activated upon exposure to a disease-challenge); some immune responses are both constitutively expressed and up-regulated in response to an immune challenge. The locust’s defensive responses to *Metarhizium* include encapsulation of infected cells via haemocytes, activation of the prophenoloxidase (pro-PO) enzyme cascade, and general

antimicrobial defences via the lysozyme enzyme (Wilson et al., 2001; Lavine and Strand, 2002; Royet, 2004; Strand, 2008; Wang et al., 2013).

Our study focuses on the effects of two key macronutrients, protein and digestible carbohydrate, after an initial pathogen challenge. Intake of both these nutrients is known to be regulated by locusts, with carbohydrate providing the major source of energy and protein being used for growth (Simpson and Abisgold, 1985). We monitored the performance consequences of pathogen challenge in fungal-infected locusts that were confined to feeding on one of three diets varying only in their protein to carbohydrate (P:C) ratios (Experiment 1). We then examined whether diet-related differences in resistance to fungal infection correlated with cellular and humoral immune responses (Experiment 2). Finally, we investigated whether fungal-infected and non-infected (control) locusts regulated their nutritional intake when given a diet-choice (Experiment 3), an action that may increase their ability to resist pathogen challenge.

## 2. Methods

### 2.1. Locust and fungal cultures

The locust *C. terminifera* rearing colony was established in 2006 from approximately 25,000 field-collected locusts. The colony has since been maintained in gregarious cultures retaining most of the genetic variation found in the field (Berthier et al., 2010). The fungal isolate *M. acridum* (ARSEF 324) was used in all experiments. Working cultures were grown for 14 days at 27 °C on Potato Dextrose Agar (BD, North Ryde, NSW, Australia) petri dishes. Conidial suspensions were prepared in sterile water plus 0.07% (v/v) Triton X-100 (Sigma–Aldrich Pty. Ltd., Sydney, Australia). Locust inoculation was undertaken by applying the fungal spores topically, 1 µl under the pronotum (Prior et al., 1995).

### 2.2. Locust artificial diets

Three dry, granular synthetic diets differing in the percentage of protein and carbohydrate (%P:C) (35:7, 21:21 and 7:35) were used as previously described (Simpson and Abisgold, 1985), with protein being a 3:1:1 mixture of casein, bacteriological peptone and egg albumen, and carbohydrate a 1:1 mixture of sucrose and dextrin. All diets contained 4% micronutrients (salts, vitamins and sterols) and 54% indigestible  $\alpha$ -cellulose (C8002, Sigma–Aldrich Pty. Ltd., Sydney, Australia) and were ground to a fine powder. *C. terminifera* develop well on 21:21 diet, with their optimal diet previously found to be slightly carbohydrate-rich (Clissold et al., 2009). The use of 35:7 and 7:35 allowed extreme P:C ratios to be examined.

### 2.3. Experiment 1: the effects of P:C ratio on survival in locusts challenged with fungi

The aim of this experiment was to assess the impact of dietary protein-to-carbohydrate (P:C) ratio on locust survival. 130 locusts per diet treatment were used in this experiment, 30 control locusts and 100 challenged with an LD<sub>50</sub> dose of *M. acridum* (1000 spores per locust; as evaluated from a preliminary dose–response experiment; R.I. Graham unpublished data), split over 3 blocks (total:  $n = 390$ ). From egg-hatch to fourth instar, all locusts were reared under normal culture conditions (see Section 2.1), feeding upon wheat-grass and dry wheat-germ. Upon reaching the fifth instar, female locusts were inoculated either with 1 µl sterile water (controls) or with the LD<sub>50</sub> dose of *M. acridum*, and randomly placed on one of three diets varying in P:C ratio from extremely

protein-biased to extremely carbohydrate-biased: 35:7, 21:21 or 7:35. Fresh diet was provided each day post-inoculation (p.i.), and deaths monitored daily until the end of the experiment at day 14 p.i. To confirm mortality from fungal-challenge, locust cadavers were plated in humid Petri dishes, and *Metarhizium* confirmed by the presence of sporulation. Development rate was recorded as the number of days to complete the fifth instar.

#### 2.4. Experiment 2: the effects of P:C ratio on locust immune function in insects challenged with fungi

The aim of this experiment was to assess the impact of P:C ratio on insect immune function. Forty locusts per diet treatment were used in this experiment, 20 control locusts and 20 challenged with an LD<sub>50</sub> dose of *M. acridum* (1000 spores per locust) (total:  $n = 120$ ). All locusts were inoculated at the start of the fifth instar, and randomly placed on one of three diets varying in P:C ratio from extremely protein-biased to extremely carbohydrate-biased: 35:7, 21:21 or 7:35. Locusts were sacrificed 48 h p.i. and haemolymph collected from the base of the hindleg (Mullen and Goldsworthy, 2006).

##### 2.4.1. Haemolymph protein levels

Following haemolymph collection, samples were diluted (1:100) in PBS (149.6 mM NaCl; 10 mM Na<sub>2</sub> HPO<sub>4</sub>; pH 6.5) and frozen at  $-80^{\circ}\text{C}$  until needed. Haemolymph protein levels were determined using a standard curve created using a BSA standard (BioRad, Hercules, CA, USA); 10  $\mu\text{l}$  of the haemolymph sample (1:100 dilution) was added to wells in a microtitre plate containing 200  $\mu\text{l}$  of the dye reagent and the resulting colour measured at 600 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Three technical replicates were undertaken per sample.

##### 2.4.2. Haemocyte density

Haemocytes are the immune cells of insects and are important effectors against parasites and pathogens, activating phagocytosis, nodule formation, and encapsulation to clear and/or restrict fungal cells (Lavine and Strand, 2002). In addition, haemocytes contain antimicrobial proteins such as *defensin* and *gallerimycin*, which are involved in anti-fungal activity (Bulet and Stocklin, 2005). Immediately after collection, haemolymph was stored at  $-80^{\circ}\text{C}$  until needed. Haemocyte counts were performed by diluting the sample 10-fold, and pipetting 10  $\mu\text{l}$  of the sample onto each side of an Improved Neubauer Haemocytometer. Haemocytes were counted in five non-adjacent squares on each side of the haemocytometer to give an estimate of the haemocyte density for each locust.

##### 2.4.3. Lysozyme-like antimicrobial activity

Lytic activity against *Micrococcus luteus* was determined using a lytic zone assay, as is standard procedure for determining insect antimicrobial activity (Cotter et al., 2004) and previously used for determining locust immune function (Wilson et al., 2002). Agar plates containing 10 ml of 1% agar with 5 mg per ml freeze-dried *M. luteus* (Sigma–Aldrich Pty. Ltd., Sydney, Australia) were prepared. For each plate, 20 holes with a diameter of 2 mm were punched in the agar. 1  $\mu\text{l}$  PTU (phenylthiourea saturated in 70% ethanol, to inhibit melanisation) and 1  $\mu\text{l}$  of haemolymph and was placed in each well, three replicates per sample. The plates were incubated at  $33^{\circ}\text{C}$  for 48 h then photographed using a digital camera and the area of the clear zones calculated using Image-J software (Schneider et al., 2012).

##### 2.4.4. Prophenoloxidase activity

The prophenoloxidase cascade generates highly cytotoxic quinones that can inactivate a range of insect pathogens, including fungi (Bidochka and Hajek, 1998). Following haemolymph

collection, samples were diluted (1:10) in PBS (149.6 mM NaCl; 10 mM Na<sub>2</sub> HPO<sub>4</sub>; pH 6.5) and frozen at  $-80^{\circ}\text{C}$  until needed. Prophenoloxidase (pro-PO) activity was measured after activation using chymotrypsin. Reaction mixtures contained 8  $\mu\text{l}$  of haemolymph supernatant, 5  $\mu\text{l}$  of chymotrypsin (5 mg/ml in distilled water, Sigma–Aldrich Pty. Ltd., Sydney, Australia), 8  $\mu\text{l}$  PBS and 51  $\mu\text{l}$  distilled water. The mixture was incubated in a 96-well plate for 10 min at room temperature before the addition of 8  $\mu\text{l}$  of L-DOPA as substrate (4 mg/ml in distilled water) (Haine et al., 2008). Readings were taken every 12 s for 60 min on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 492 nm. Enzyme activity is measured as  $V_{\text{max}}$  (the slope of the reaction curve during the linear phase), which directly correlates with the concentration of Pro-PO in the sample (Thompson, 2002).

#### 2.5. Experiment 3: the effects fungal challenge on locust diet choice

This experiment was to determine whether, when given a choice, locusts infected with fungi would actively select a diet that improved their survival chances. 100 fifth instar female locusts were inoculated with either an LD<sub>25</sub> dose (500 spores) of fungi ( $n = 60$ ) or with distilled water ( $n = 40$ ). After inoculation, locusts were individually housed and given a choice between the two most extreme diets (P:C 35:7 vs. 7:35). Handling deaths were discarded from the experiment. Diet was replaced each day and monitored from day 2 p.i. until day 5 p.i. Diet monitoring was not continued beyond 5 days p.i. because the locusts stopped feeding in preparation for ecdysis into adulthood. Any uneaten food was dried to a constant mass in a desiccating oven (having first removed any contaminating frass). Consumption was calculated as the difference between the initial and final dry weight of each diet. From the dry mass of food eaten, the amount of protein and carbohydrate consumed on each day was estimated. The diet regime was maintained, and deaths monitored daily until day 14 p.i. To confirm mortality from fungal-challenge, locust cadavers were plated in humid petri-dishes, and *Metarhizium* confirmed by the presence of sporulation.

#### 2.6. Statistical analysis

All analyses were conducted using the R statistical package (version 3.0.1) (R Development Core Team 2012). Mortality data were analysed using generalized linear models (GLMs) with binomial errors and logit link functions. Survival analysis was conducted by fitting a parametric survival regression model (using the *survreg* function in R), assuming a Weibull survival function. Diet-choice data were analysed using linear mixed-effects models with the proportion of protein in the chosen diet as the dependent variable and locust identity included as a random effect. All other analyses were conducted using linear models after transforming the data where appropriate to normalise, as indicated below. During analysis, all main effects and interaction terms were tested for inclusion in the model and removed if they failed to explain significant levels of variation. Subsequently, model simplification was conducted by comparing models where factor levels were combined, and these were retained where they explained similar levels of variation to the original factor (Crawley, 2007). Parameter estimates were compared using model summary tables in R.

### 3. Results

#### 3.1. Experiment 1: the effects of P:C ratio on survival in locusts challenged with fungi

The aim of this experiment was to quantify the effects of dietary P:C ratio on the survival and development of *C. terminifera* fifth

instar nymph when challenged with *M. acridum* fungus. Regardless of which of the three diets they were fed on, all of the control insects survived to adulthood. In contrast, survival of the fungus-challenged locusts was diet-dependent (GLM with binomial errors: Diet:  $\chi^2_2 = 8.40$ ,  $P = 0.015$ ) and increased with the relative amount of carbohydrate in the diet: 25%, 38% and 45%, respectively, on the protein-rich (P:C = 35:7), balanced (21:21) and carbohydrate-rich (7:35) diets (Fig. 1a). Examination of the parameter estimates revealed that locusts fed the protein-rich diet suffered significantly higher mortality than those in the carbohydrate-rich group ( $z = 2.83$ ,  $P = 0.0047$ ), whereas mortality on the balanced diet was only marginally higher ( $z = 1.813$ ,  $P = 0.069$ ).

Survival analysis takes account of not just the level of mortality but also the timing of deaths. This revealed that the mortality rate in fungus-challenged insects was also a function of diet (parametric regression model: Diet:  $\chi^2_2 = 12.51$ ,  $P = 0.0019$ ; Fig. 1a), with locusts on the 35:7 diet dying at a significantly faster rate than those on the 21:21 diet ( $z = 2.27$ ,  $P = 0.023$ ) and the 7:35 diet ( $z = 3.45$ ,  $P = 0.00056$ ), but there was no difference in the mortality rates of those on the two latter diets ( $z = 1.25$ ,  $P = 0.21$ ). Model simplification indicated that fungus-challenged individuals on the protein-rich diet suffered significantly higher mortality than those on the balanced or carbohydrate-rich diet (Diet:  $\chi^2_1 = 10.93$ ,  $P = 0.00094$ ). Development rate during the fifth instar (1/instar duration) was not significantly affected by whether or not the locust received a fungal-challenge (linear model: Challenge:  $F_{1,181} = 0.69$ ,  $P = 0.41$ ; Diet \* Challenge interaction:  $F_{2,181} = 0.14$ ,  $P = 0.87$ ). However, development rate was affected by diet (Diet:  $F_{2,184} = 83.8$ ,  $P < 0.0001$ ; Fig. 1b), and model simplification revealed that there was no significant difference in the development rate of insects on the balanced and protein-rich diets ( $t_{114} = 0.088$ ,  $P = 0.93$ ), and hence individuals on the

carbohydrate-rich diet developed at a significantly slower rate than those on the other two diets (Diet:  $F_{1,185} = 168.5$ ,  $P < 0.0001$ ).

### 3.2. Experiment 2: the effects of P:C ratio on locust immune function in insects challenged with fungi

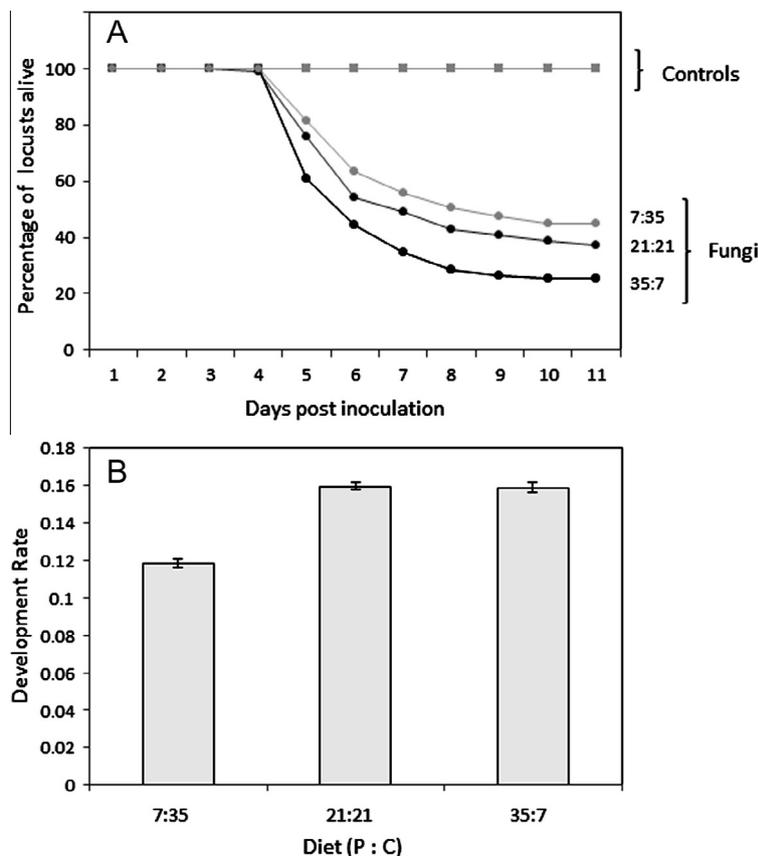
The aim of this experiment was to determine the effect of dietary P:C ratio on haemolymph protein levels and locust constitutive (basal) and induced immune function (after fungal challenge), to establish whether the higher mortality of fungus-challenged locusts on the protein-rich diet was due to it compromising immune function.

#### 3.2.1. Haemolymph protein levels

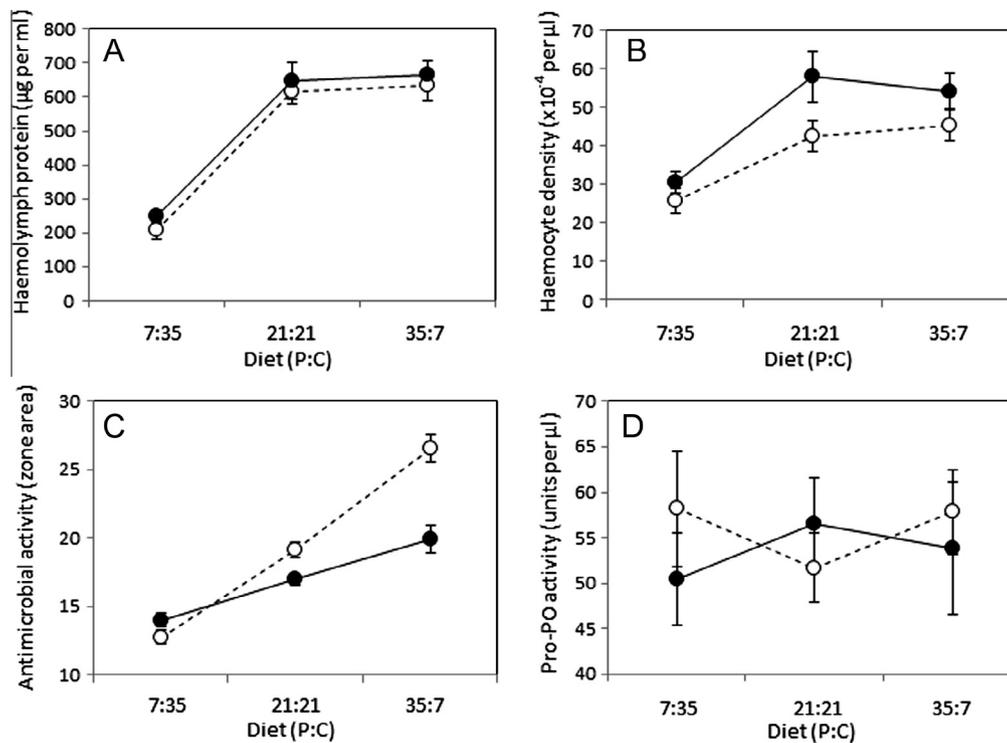
The levels of protein in the haemolymph varied with diet but not with fungal challenge-status (linear model: Diet:  $F_{2,113} = 79.38$ ,  $P < 0.0001$ ; Challenge:  $F_{1,110} = 0.93$ ,  $P = 0.34$ ; Diet \* Challenge:  $F_{2,110} = 0.01$ ,  $P = 0.98$ ). Model simplification revealed that haemolymph protein levels were significantly lower in locusts fed a carbohydrate-rich diet (P:C = 7:35) than in those fed either a balanced diet (21:21) or a protein-rich diet (35:7) (Diet:  $F_{1,114} = 159.64$ ,  $P < 0.0001$ , Fig. 2a).

#### 3.2.2. Haemocyte density

The density of haemocytes in the haemolymph (square-root transformed) varied with both diet and challenge-status, but the interaction between the two was non-significant (linear model: Diet:  $F_{2,112} = 19.04$ ,  $P < 0.0001$ ; Challenge:  $F_{1,112} = 7.21$ ,  $P = 0.0083$ ; Diet \* Challenge:  $F_{2,110} = 0.33$ ,  $P = 0.72$ ). Model simplification indicated that haemocyte density was significantly lower in locusts fed a carbohydrate-rich diet than in those fed either a balanced



**Fig. 1.** (A) Survival curves for locusts restricted to one of three diets varying in their protein and carbohydrate ratios (P:C). Locusts were either inoculated with an LD<sub>50</sub> dose of *M. acridum* or with water (controls); (B) development rate of locusts in the no-choice diet experiment (Experiment 1) fed upon the three different diets. These mean values ( $\pm$ SE) are calculated as 1/no. days in fifth instar.



**Fig. 2.** Dietary influence on constitutive immune function in locusts restricted to one of three diets (Experiment 2): (A) mean haemolymph-protein levels ( $\pm$ SE); (B) mean total haemocyte density ( $\pm$ SE); (C) mean lysozyme-like antimicrobial activity ( $\pm$ SE) and; (D) mean prophenoloxidase activity ( $\pm$ SE), for *M. acridum*-challenged and control locusts. Solid markers = *M. acridum*-challenged locusts; open markers = control locusts.

or a protein-rich diet, as well as being higher in challenged insects, indicating that haemocyte production or mobilization was induced following infection (Diet:  $F_{1,113} = 38.40$ ,  $P < 0.0001$ ; Challenge:  $F_{1,113} = 7.28$ ,  $P = 0.0080$ ; Fig. 2b).

### 3.2.3. Antimicrobial activity

The area of the lytic zone on the plates was used as a measure of lysozyme-like activity. Antimicrobial activity was determined by the interaction between diet and challenge-status (linear model: Diet:  $F_{2,110} = 89.98$ ,  $P < 0.0001$ ; Challenge:  $F_{1,110} = 18.97$ ,  $P < 0.0001$ ; Diet \* Challenge:  $F_{2,110} = 15.14$ ,  $P < 0.0001$ ; Fig. 2c). Analysis of the parameter estimates indicated that all of the differences between the two treatment-group combinations (Challenge and Diet) were significant except that between the challenge and control groups feeding on a carbohydrate-rich diet ( $t_{36} = 1.205$ ,  $P = 0.23$ ). In other words, lytic activity was induced following fungal infection, except when the protein-content of the diet was low (P:C = 7:35).

### 3.2.4. Pro-phenoloxidase activity

Prophenoloxidase activity did not vary with either locust diet or challenge-status, or the interaction between the two (linear model: Diet:  $F_{2,113} = 0.059$ ,  $P = 0.94$ ; Challenge:  $F_{1,114} = 0.23$ ,  $P = 0.63$ ; Diet \* Challenge:  $F_{2,110} = 0.69$ ,  $P = 0.50$ ; Fig. 2d).

### 3.3. Experiment 3: the effects of fungal challenge on locust diet choice

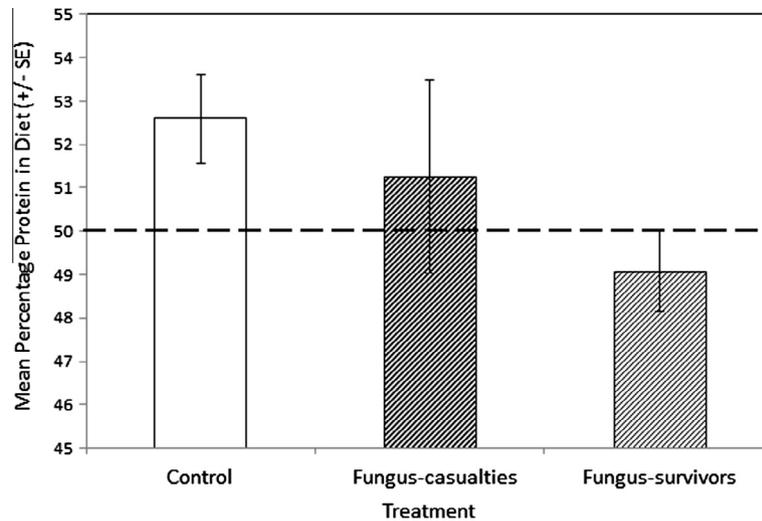
Macronutrient ingestion was compared between non-challenged controls and fungus-challenged locusts (both those surviving and those succumbing to infection) in order to establish whether individuals changed their diet choice in response to infection. Overall, fungus-challenged locusts chose a diet that was significantly less protein-biased than that of non-challenged control insects (mean percentage of protein in diet  $\pm$  s.e. =  $49.6 \pm 0.9\%$  vs.

$52.6 \pm 1.0\%$ , respectively; mixed-effects model: Diet:  $\chi^2_1 = 4.78$ ,  $P = 0.029$ ). There was no significant variation in diet choice across days and the interaction between diet and time was also non-significant (Day:  $\chi^2_3 = 3.60$ ,  $P = 0.31$ ; Diet \* Day:  $\chi^2_6 = 6.05$ ,  $P = 0.42$ ).

A comparison of the diets chosen by locusts that survived fungal-challenge with those that either succumbed to infection or were in the control group revealed that there was a significant difference in the relative amounts of protein chosen by locusts in the three groups (mixed-effects model: Diet:  $\chi^2_2 = 6.12$ ,  $P = 0.047$ ; Fig. 3); again, there was no significant variation in diet choice across days and the interaction term was non-significant (Day:  $\chi^2_3 = 3.61$ ,  $P = 0.31$ ; Diet \* Day:  $\chi^2_6 = 7.60$ ,  $P = 0.58$ ). Examination of the parameter estimates indicated that whilst the locusts that died of fungal infection did not differ from the non-challenged control insects in the relative amount of protein chosen ( $t_{53} = -0.71$ ,  $P = 0.48$ ), those that survived infection chose a diet that was significantly less protein-biased than that of the controls, consistent with adaptive diet choice ( $t_{76} = -2.46$ ,  $P = 0.016$ ).

## 4. Discussion

Our measurement of locust immune responses demonstrated that even constitutive (basal) levels of immune function, in the form of lysozyme-like antimicrobial activity and haemocyte density, were compromised in locusts that fed on a low-protein/high-carbohydrate diet (P:C = 7:35); only pro-PO activity was unaffected by diet. These differences were correlated with a significant decline in the haemolymph protein pool with decreasing dietary P:C ratio, suggesting that these constitutive immune responses were constrained by protein reserves. Protein is a major substrate for producing immunological components used for resisting pathogen infection (Strand, 2008). The results presented here are consistent with those of Lee et al. (2006), who found that immune function (encapsulation response, lysozyme-like antimicrobial



**Fig. 3.** The relationship between fungus-challenge and mean ( $\pm$ SE) percentage protein in the diet selected by locusts offered the choice between a protein-rich diet (P:C = 35:7) and a carbohydrate-rich diet (P:C = 7:35). Locusts were inoculated with either an LD<sub>25</sub> dose of fungi (*M. acridum*) or with water (controls). Data are taken from Experiment 3, self-selecting treatment. The dashed horizontal line at 50% protein is the null expectation if the locusts fed randomly on the two diets.

activity and PO activity) were all significantly higher in *Spodoptera littoralis* caterpillars fed high-protein diets, as would be expected if protein is needed for the production of these immunological effectors. If these immune responses are involved in resistance to fungal infection, which we presume to be the case, then these data suggest that locusts feeding on protein-rich diets should be more resistant to *Metarhizium* fungal challenge than those fed on low-protein diets.

However, locusts challenged with *Metarhizium* fungus had the highest survival (45%) on a low-protein/high-carbohydrate diet, with survival rates declining to 25% on the most protein-rich diet. These results are in stark contrast to those previously found in similar experiments with lepidopteran hosts. As previously stated, this study builds on three investigations into macronutrient-impact on insect pathogen resistance and dietary choice when faced with a pathogen challenge. Lee et al. (2006) and Povey et al. (2013) found that for *S. littoralis* and *Spodoptera exempta*, respectively, NPV-infected larvae had highest survival on the most protein-rich diet (35:7). Povey et al. (2009) also found higher survival rates on the higher protein diets when insects were challenged with a bacterial pathogen, suggesting that it could reflect the protein cost involved in surviving an infection and investing in protein-dependent immune responses.

Both the fungal bioassay (Experiment 1) and immune function assay (Experiment 2) suggest that there is a nutritional cost associated with fighting fungal infections in locusts. With more protein (and less carbohydrate) in the diet, locusts are more likely to succumb to a fungal infection, despite enhancing their immune responses. To build on this, we undertook the diet-choice experiment (Experiment 3) which we hoped would reveal whether locusts demonstrate an adaptive response to infection by actively selecting a diet that would enhance their chances of survival.

Our results showed that both challenged and non-challenged insects tended to choose a slightly P-rich diet (Fig. 3). This is perhaps not unexpected, given the much slower development rate of locusts when given a highly carbohydrate-rich diet (see Fig. 1b). However, given the reduced survival of fungus-challenged insects on protein-rich diets (Fig. 1a), we predicted that when given a choice between low- and high-P diets, the locusts would choose a diet with a lower protein content when challenged with fungus. As predicted, those that were challenged with fungus chose a significantly less protein-rich diet (although they still chose a diet

with a P:C ratio greater than one). Importantly, only locusts that switched their feeding behaviour to include relatively less protein and more carbohydrate (P:C < 1) survived the fungal challenge; those that succumbed to fungal infection chose a diet that had a similar protein content to that of the control insects (Fig. 3). Again, this finding is contrary to the results of the previous three studies (Lee et al., 2006; Povey et al., 2009, 2013), which found that pathogen-challenged Lepidoptera increased the amount of protein in their diet to combat infection. However, it demonstrates an adaptive switch in feeding behaviour that improves survival in the face of pathogen challenge in locusts. Indeed, fungal challenge appears to induce a response more like a macroparasite infection: beetles infected with a helminth tapeworm also increased their carbohydrate intake (Ponton et al., 2011a,b).

The phenomenon observed in this experiment can be classed as therapeutic medication under the current definitions (de Roode and Lefevre, 2012); which includes responses in which infected individuals actively modulate their nutritional intake to combat infection (Raubenheimer and Simpson, 2009; Ponton et al., 2011a,b). This behavioural and physiological response may be directed at the infected individual itself, at its offspring, or at other relatives (de Roode and Lefevre, 2012). The major distinction with prophylactic medication, which is displayed by both infected and uninfected individuals, is that therapeutic medication is used solely by infected individuals. Thus, prophylactic medication is a fixed response, while therapeutic medication is a plastic response, used only when individuals are infected. In addition to the nutritional responses seen in this current study and already described (Lee et al. 2006; Povey et al., 2009, 2013), nutritional therapeutic medication has also been described in fruit flies, in which infected individuals appeared to be able to preferentially consume yeast species that increased their ability to encapsulate parasitoid eggs (Anagnostou et al., 2010).

This current study demonstrates the importance of testing different host systems, and suggests that the mode of therapeutic self-medication may be highly specific to the particular host-pathogen interaction. It may not be possible to generalise about a pathogen or parasite causing a particular host nutritional-behavioural response, but different pathogens and parasites most likely induce different therapeutic responses. Typically, this may be very difficult to unravel in the field where several different species of pathogens are likely to be present, though the response

observed here may generalise to other fungal pathogens. We propose, for example, that the enhanced mortality of challenged locusts fed a protein-rich diet is because the fungus is more efficient at exploiting protein in the insect's haemolymph than the host is at producing appropriate immune effectors, tipping the balance in favour of the pathogen on high-protein diets. High nitrogen resource levels are necessary for the closely-related fungus *Metarhizium anisopliae*, particularly for mycelial growth and toxin production (Barnes et al., 1975; Campbell et al., 1983), and substrates with high protein content (but low starch, carbohydrate content) have been shown to produce most rapid mycelial growth (Rachappa et al., 2005). As previously stated, this observation is contrary to that seen for viral and bacterial challenges (Lee et al., 2006; Povey et al., 2009, 2013), but this is likely a consequence of the different modes of action and nutritional requirements of these different organisms. In order to make predictions about the outcome of host–pathogen interactions, future studies need to tease apart the separate nutritional requirements of the host resistance mechanisms and the pathogen's population growth rate. Considering the host–pathogen interaction as a resource competition problem is likely to be a fruitful area going forward (Cressler et al., 2014). It is also worth noting that as both digestion and metabolism are temperature-dependent processes, the relationship between diet and infection outcome is also likely to vary with temperature, especially for insects such as locusts that practice behavioural fever (Miller et al., 2009).

Knowledge of nutritional effects on immune defence is particularly lacking in field populations (Boggs, 2009), and even more so in migrating animals such as locusts (Weber and Stilianakis, 2007). In a closely related species, field populations of the Mormon cricket (*Anabrus simplex*) were found to be generally protein-deficient, and it is this deficiency that induced the marching behaviour in these species (Simpson et al., 2006). Access to a proteinaceous diet subsequently resulted in increased immune function (as measured by phenoloxidase activity), suggesting that migrating Mormon crickets have low phenoloxidase activity as a result of their nutritional deficiency (Srygley et al., 2009). Further to this, Srygley et al. (2009), suggest that such limited immune activity of protein-starved individuals is likely to enhance disease transmission. A benefit of associating with a high-density band of conspecifics is the reduced risk of predation (Sword et al., 2005), but as a result, the risk of cannibalism by protein-deficient band members increases (Simpson et al., 2006). Cannibalistic acts have a cost in disease transmission via ingestion, but they most likely also provide nutritional benefits to enhance immune function. This picture is now possibly further complicated by our findings in the laboratory, which suggests that protein ingestion potentially increases an individual's risk of mortality due to fungal infection. However, it should be noted that from an ecological and evolutionary point of view, *Metarhizium* association with locusts is believed to be relatively rare in natural field populations (Farrow, 1982), and that other predators and parasites (including other fungi) are probably a greater survival threat. However, from a biological control perspective, this is an important finding for the efficacy-potential of Australia's primary registered biocontrol product against the Australian plague locust.

In conclusion, this study suggests that despite stronger immune responses when fed on protein-rich diets, enhanced immune function was not sufficient to protect locusts against the fungal pathogen, *Metarhizium acridum*. Indeed, although insects fed a low-protein/high-carbohydrate diet (7:35) had very poor immune function (as measured here), they had the highest survival when challenged with the fungus. Contrary to previous studies investigating macronutrient intake and pathogen challenge, highest mortality was observed in insects feeding on a protein-rich diet (35:7). To explore this host–nutrient–fungus interaction further, future

studies should determine the optimal nutrient-ratio favoured by the fungal pathogen. We propose that *M. acridum* may grow most rapidly on protein (nitrogen) rich substrates, and it is therefore utilising the high protein content of the locust haemolymph, resulting in high host mortality on protein-rich diets. We also found that survivors of fungal challenge chose a less protein-rich diet than those succumbing to infection. It is already well established that locusts undertake a number of behavioural and physiological changes to avoid infection, including a thermal “behavioural-fever” response (Blanford and Thomas, 2000; Thomas and Blanford, 2003; Klass et al., 2007) – nutritional therapeutic medication may now be added to this list.

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