

Commissioned Review Article

Body condition constrains immune function in field populations of female Australian plague locust *Chortoicetes terminifera*R. I. GRAHAM,^{1,2,3} J. M. DEACUTIS,^{1,2} S. J. SIMPSON^{1,2} & K. WILSON³¹School of Biological Sciences, University of Sydney, Sydney, NSW, Australia, ²Charles Perkins Centre, University of Sydney, Sydney, NSW, Australia, ³Lancaster Environment Centre, Lancaster University, Lancaster, UK

SUMMARY

The insect innate immune system comprises both humoral and cellular defence responses. In the laboratory, the insect immune system is well characterized. In the field, however, little is known about the role of constitutive insect immune function and how it varies within and between populations. Laboratory studies suggest that host nutrition has significant impact upon insect immune function. Thus, the rationale for this study was to sample natural populations of the Australian Plague Locust *Chortoicetes terminifera* to establish whether locust body condition (as determined by protein and lipid content) impacted their constitutive immune system and, as a result, has the potential to impact on their capacity to respond to a pathogenic challenge. We found that body condition varied greatly between individual female locusts within sites and that haemolymph protein levels, but not body lipid content, varied between sites. Moreover, our measures of immune function were correlated with the haemolymph levels of protein (in the case of haemocyte density), lipid (prophenoloxidase activity) or both (lysozyme-like antimicrobial activity). We discuss the implications of these findings for the role of biological pesticides in the control of locust populations.

Keywords Australian plague locust, *Chortoicetes terminifera*, ecological immunology, immune function, nutrition ecology

INTRODUCTION

The ability of organisms to tolerate or resist infection is largely dependent upon their capacity to maintain an

appropriate immune response during the course of their development. The insect immune system is highly responsive (1–4) and capable of reacting very effectively against parasites and pathogens. The insect innate immune system comprises both humoral and cellular defence mechanisms (5). 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 include responses such as nodulation, phagocytosis and encapsulation that are mediated by insect haemocytes (5). Immune defence can be both constitutive (always expressed) and induced (activated upon exposure to a disease challenge); indeed, some immune responses are both constitutively expressed and up-regulated as a consequence of immune challenge (5).

A wide spectrum of parasites have been shown to infect insects, including viruses, bacteria, fungi, protozoa, nematodes, helminths, mites and parasitoids (reviewed in Ref. (6)). Host investment in constitutive immune defences will be advantageous in parasite-rich environments. However, in the absence of parasites, such a costly investment may be disadvantageous in the longer term and may negatively impact other life-history traits e.g. (7). As a consequence, immune function is often condition dependent (8–10), with food limitation and other stressors such as humidity, light and temperature constraining immune function, which in turn may facilitate disease epizootics in stressed insect populations (11). Poor nutrition is considered an important stressor upon insect populations. In particular, migratory insects are often deficient in key nutritional resources, and their marching and swarming behaviour is often precipitated by a lack of specific nutrients (12, 13). Previous studies have demonstrated that host nutritional stress (i.e. starvation or imbalanced nutritional intake) can exacerbate the costs involved in immune activation (14) and can lead to reduced immune responsiveness (9, 15).

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Animals in nutrient-poor habitats may redirect physiological resources from other life-history traits (e.g. reproduction) to those that may increase survival. Lipid is often a critical nutrient required to survive periods of starvation and to fuel locomotion (16, 17). Meanwhile, dietary protein appears to be essential for constitutive immune function, suggesting higher protein costs of mounting an immune response than carbohydrate or overall energy costs (18). Numerous studies have quantified insect nutrition and immunity in the laboratory [e.g. (19–24)], but much less is known about immune function in natural populations (25), especially those that migrate (26).

In the field, heterogeneous nutritional environments encountered by animals have a major impact upon their ecology and evolution, including traits such as mating success (7) and migratory potential (12). For example, studies of marching bands of Mormon crickets and locusts (12, 27) discovered swarm movement to be driven by the requirement of salt and protein. In turn, protein deficiency in crickets had a major impact on immune function (28). One likely cost of band formation is increased disease transmission within the crowded populations, and the limited immune function of protein-starved individuals could exacerbate this. The role of nutrition in modulating host immune traits has clear implications for future use of integrated pest management strategies, especially at a time when the public are becoming increasingly aware of the need to reduce chemical pesticides and invest in biological control programmes (29). A previous laboratory study highlighting the variability in biopesticide efficacy on locusts fed different diets (18) highlighted the importance of considering pest nutrition when designing biological control programmes.

For this study, we used the economically important Australian plague locust, *Chortoicetes terminifera*, as a system to investigate body condition and immune function in the field. Based on our previous laboratory studies (18), we hypothesized that locust immune function in the field would reflect their nutritional status, with protein in particular being an important constraint on constitutive immunity. Specifically, we (i) quantified female locust body condition in different field populations, (ii) examined immune traits in different populations and (iii) determined the relationships between locust macronutrient content and immune function under field conditions.

METHODS

Field collection of locusts

Chortoicetes terminifera were sampled at three geographically separated sites (Site A [Yatina, S 33 00-98, E 138

41-76], Site B [Yongala, S 33 01-17, E 138 42-76] and Site C [Mt. Bryan, S 33 29-76, E 138 51-56]) in the Jamestown area of South Australia during November 2012. Sites A and B were approximately 1.4 km apart and Site C was a further 55 km away. While all three sites were geographically isolated from each other, we cannot be sure that they are not part of a single large population. Site A was close to a creek bed, and so the vegetation was relatively lush, comprising both graminoid and forb species, whereas the vegetation at sites B and C contained dried graminoid species only. All three sites comprised 4th instar nymphs to prereproductive adult life stages. Density was estimated using a sweep net (total number of locusts per sweep) along five 10-metre transects per site. All body condition and immune function analyses were undertaken on females, as only this gender was of a suitable size to extract the required volume of haemolymph for the subsequent experiments. Fifth instar females were retained and taken back to our field laboratory for further analysis.

Within 1 h of capture, approximately 20 µL of haemolymph was collected from the base of the hindleg of each fifth instar female. The haemolymph was immediately placed on dry ice for use in assays to assess prophenoloxidase activity, total haemolymph protein, lysozyme activity and haemocyte density. The locusts were then sacrificed and the carcasses stored on dry ice for subsequent lipid analysis assay. The samples were transported back to University of Sydney on dry ice where the laboratory assays were undertaken.

Haemolymph protein and lipid extraction assays

Haemolymph protein levels

Following haemolymph collection, samples were diluted (1 : 100) in PBS (149.6 mM NaCl; 10 mM Na₂ HPO₄; pH 6.5) and frozen at –80°C until needed. Haemolymph protein levels were determined using a standard curve created using a BSA standard (Bio-Rad, Hercules, CA, USA); 10 µL of the haemolymph sample (1 : 100 dilution) was added to wells in a microtitre plate containing 200 µL of the dye reagent, and the resulting colour was measured at 600 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Three technical replicates were undertaken per sample.

Lipid extraction

Locust carcasses were dried to constant mass at 40°C and weighed to within 0.1 mg. Dried locusts were placed individually into 5-mL glass vials and covered with chloroform. After 12 h of soaking, the chloroform was removed with a suction pipette and replaced with fresh chloroform. This soak-extraction process was repeated for a total of

three washes. Locusts were then dried at 40°C and weighed again. Lipid content was calculated as prechloroform dry mass minus post-chloroform dry mass.

Constitutive immune traits assays

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 infection (5). In addition, haemocytes contain antimicrobial proteins such as *defensin* and *gallerimycin*, which are involved in antimicrobial activity (30). Immediately after collection, haemolymph was stored at -80°C until needed. Haemocyte counts were performed by diluting the sample 10-fold and pipetting 10 µL of the sample onto each side of an improved Neubauer haemocytometer. Haemocytes were counted in five nonadjacent squares on each side of the haemocytometer to give an estimate of the haemocyte density for each locust.

Prophenoloxidase activity

The prophenoloxidase cascade generates highly cytotoxic quinones that can inactivate a range of insect pathogens (31). Haemolymph samples were diluted (1 : 10) in PBS (149.6 mM NaCl; 10 mM Na₂ HPO₄; pH 6.5) and frozen at -80°C until needed. Prophenoloxidase (pro-PO) activity was measured after activation using chymotrypsin. Reaction mixtures contained 8 µL of haemolymph supernatant, 5 µL of chymotrypsin (5 mg/mL in distilled water; Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia), 8 µL of PBS and 51 µL of distilled water. The mixture was incubated in a 96-well plate for 10 min at room temperature before the addition of 8 µL of L-DOPA as substrate (4 mg/mL in distilled water) (32). Readings were taken every 12 s for 60 min on a VERSAmax microplate reader (Molecular Devices) at 492 nm. Enzyme activity is measured as V_{max} (the slope of the reaction curve during the linear phase), which directly correlates with the concentration of Pro-PO in the sample (33).

Lysozyme-like antimicrobial activity

Lytic activity against *Micrococcus luteus* was determined using a lytic zone assay, as is the standard procedure for determining insect antimicrobial activity (34) and previously used for determining locust immune function (35). Agar plates containing 10 mL of 1% agar with 5 mg per mL freeze-dried *M. luteus* (Sigma-Aldrich Pty. Ltd.) were prepared. For each plate, 20 holes with a diameter of 2 mm were punched in the agar. A total of 1 µL PTU (phenylthiourea saturated in 70% ethanol, to inhibit melanization) and 1 µL haemolymph were placed in each

well, three replicates per sample. The plates were incubated at 33°C for 48 h and then photographed using a digital camera, and the area of the clear zones was calculated using IMAGEJ software (36). Standard curves were obtained using a serial dilution of hen egg white lysozyme, and concentration of antimicrobial activity was then calculated.

Statistical analysis

All analyses were conducted using the R statistical software package (version 3.0.2) (37). For the immune parameters, analysis was by linear models (LM) after prior transformation to normalize the data, where appropriate (square-root transformation: haemolymph protein and haemocyte density; log₁₀ transformation: lysozyme activity). All interaction terms were tested for significance but, except where stated, these were nonsignificant. Model simplification (combining factor levels and testing the change in explained variance) was used to establish whether differences between sites were statistically significant. The effects of body condition as estimated by haemolymph protein levels (P) and body lipid content (L) on each of the immune traits were analysed using linear mixed effects models (LME), using the *lme* function in R, including the site from which the locusts originated as a random effect. Fixed terms in the models included the linear main effects (P and L), both squared terms (P² and L²), and the interaction between protein and lipids (P*L) (38). Minimal models were established via a backwards stepwise deletion process (39). The shape of the response surface for each trait was then visualized using nonparametric thin-plate splines using the *fields* package in R (40). However, it should be noted that these are simply an aid to visualizing the surfaces and are not a direct output from the statistical models.

RESULTS

Body condition

Population density did not differ significantly across the three sites' samples (LM: $F_{2,12} = 0.33$, $P = 0.72$), averaging 21.93 + 8.40 (mean + SD) locusts per 10 m sweep. There was also no significant difference in the dry masses of locusts in the three sites ($F_{2,120} = 0.63$, $P = 0.54$; Figure 1a). However, levels of protein in the haemolymph did differ between the three populations ($F_{2,120} = 5.79$, $P = 0.0040$; Figure 1b) and model simplification revealed that this was because haemolymph protein levels were lower in population C than in the other two populations, which did not differ significantly from each other and were

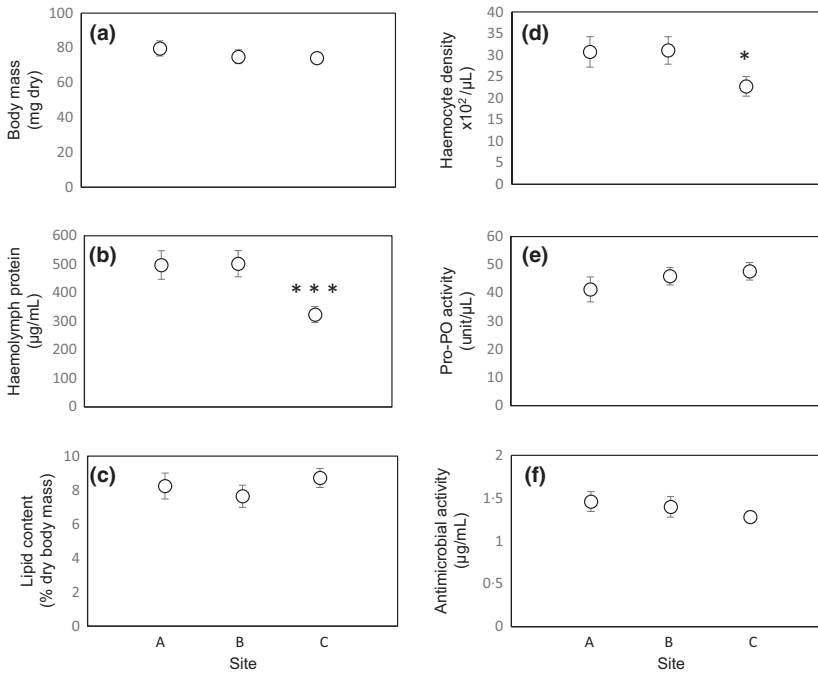


Figure 1 Body condition and immune traits at the three populations. (a) Mean body mass (\pm SE), (b) mean haemolymph protein content (\pm SE), (c) mean body lipid content (\pm SE), (d) mean total haemocyte density (\pm SE), (e) mean prophenoloxidase activity (\pm SE) and (f) mean lysozyme-like antimicrobial activity (\pm SE). Asterisks indicate significant differences.

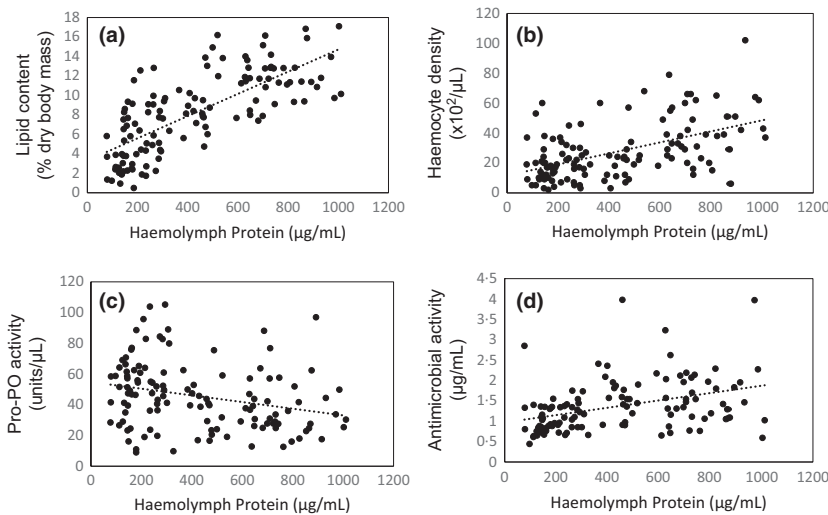


Figure 2 Interaction between haemolymph protein and immune traits in field-collected locusts. (a) Lipid content, (b) total haemocyte density, (c) prophenoloxidase activity and (d) lysozyme-like antimicrobial activity.

therefore combined ($F_{1,121} = 11.57$, $P = 0.0009$). In contrast, the lipid content of the locusts did not differ across the three populations ($F_{2,120} = 0.93$, $P = 0.40$, Figure 1c), but was strongly positively correlated with haemolymph protein levels ($F_{1,121} = 159.09$, $P < 0.0001$, $r^2 = 0.564$; Figure 2a). After taking this relationship into account, there was a significant difference between the three populations in their *relative* body lipid contents ($F_{2,119} = 22.98$, $P < 0.0001$), with locusts in population C having lower protein:lipid (P:L) ratios ($F_{1,120} = 45.47$, $P < 0.0001$).

Immune function

Haemocyte density differed between the three populations ($F_{2,118} = 3.07$, $P = 0.049$; Figure 1d), and again this was because values were significantly lower in population C ($F_{1,119} = 6.17$, $P = 0.014$). However, haemocyte density was significantly positively correlated with haemolymph protein levels ($F_{1,119} = 40.53$, $P < 0.0001$, $r^2 = 0.248$; Figure 2b) and when this was taken into account, the population differences in haemocyte density disappeared

($F_{1,118} = 0.87$, $P = 0.35$), suggesting that population differences were probably driven by the differences in their relative haemolymph protein pools.

Conversely, haemolymph prophenoloxidase activity did not differ between populations ($F_{2,118} = 0.84$, $P = 0.43$; Figure 1e) and was significantly negatively correlated with the amount of protein in the haemolymph ($F_{1,119} = 8.21$, $P = 0.0049$, $r^2 = 0.057$; Figure 2c). Even after taking protein levels into account, there was still no difference between the three populations in their relative prophenoloxidase activity levels ($F_{2,117} = 0.45$, $P = 0.64$).

Finally, lysozyme activity levels also did not differ significantly between populations ($F_{2,113} = 0.54$, $P = 0.59$; Figure 1f) but were significantly positively correlated with haemolymph protein levels ($F_{1,114} = 25.90$, $P < 0.0001$, $r^2 = 0.178$; Figure 2d). Inclusion of protein in the analysis revealed that there was a significant relationship between haemolymph protein levels and lysozyme activity, which differed across the three populations (interaction term – protein*population: $F_{2,110} = 5.47$, $P = 0.0054$). Model simplification resulted in populations B and C again being combined and indicated that the interaction was because haemolymph protein and lysozyme activity levels were significantly positively correlated in populations B and C ($t_{88} = 6.35$, $P < 0.0001$, $r^2 = 0.307$), but not in population A ($t_{23} = -0.453$, $P = 0.65$, $r^2 = 0.008$; final model interaction term – protein*population: $F_{1,112} = 9.93$, $P = 0.0021$).

Relationship between body condition and immune function

To tease apart the interaction between body condition and immune function further, we followed Cotter *et al.* (20) and used LME in which the three immune function parameters were analysed in relation to the haemolymph protein (P) and body lipid (L) contents of the locusts, as main effects, squared terms and their linear interaction (i.e. $y = P + L + P^2 + L^2 + P*L$), with the site that the locusts were collected included as a random effect. We then used thin-plate splines to visualize these relationships. Haemocyte density was not correlated with body lipid content but was strongly, linearly and positively related to the amount of protein in the haemolymph (Table 1; parameter estimate + SE, P: $0.1369 + 0.0215$). In contrast, prophenoloxidase activity was not correlated with haemolymph protein levels but was strongly, linearly and negatively related to the locust's lipid content (Table 1; parameter estimate, L: $-1.699 + 0.4507$). Lysozyme-like antimicrobial activity was related to both the protein and lipid content of the insect, with most variation being explained by haemolymph protein levels (Figure 4;

Table 1 The relationships between locust body composition and immune function

Fixed term	Haemocyte density	Pro-PO activity	Lysozyme activity
P	$F_{1,117} = \mathbf{40.53}$ $P < \mathbf{0.0001}$	$F_{1,116} = 0.02$ $P = 0.90$	$F_{1,110} = \mathbf{8.92}$ $P = \mathbf{0.0035}$
L	$F_{1,116} = 0.086$ $P = 0.77$	$F_{1,117} = \mathbf{14.22}$ $P < \mathbf{0.0001}$	$F_{1,110} = 0.07$ $P = 0.79$
P ²	$F_{1,115} = 3.40$ $P = 0.068$	$F_{1,115} = 0.84$ $P = 0.36$	$F_{1,109} = 1.24$ $P = 0.27$
L ²	$F_{1,114} = 0.16$ $P = 0.69$	$F_{1,114} = 0.80$ $P = 0.37$	$F_{1,110} = \mathbf{5.13}$ $P = \mathbf{0.025}$
P:L	$F_{1,113} = 1.85$ $P = 0.18$	$F_{1,113} = 0.94$ $P = 0.33$	$F_{1,108} = 0.03$ $P = 0.86$

Results from the linear mixed models examining the relationships between haemolymph protein (P) and tissue lipid (L) on haemocyte density, prophenoloxidase activity and lysozyme-like antimicrobial activity. Site was included as a random effect. Significant terms are highlighted in bold type. Significance of the nonsignificant terms is determined at the position that they dropped out of the model.

Table 1: parameter estimates, P: $0.0109 + 0.0036$; L: $0.0341 + 0.0155$; L²: $-0.0019 + 0.0009$.

DISCUSSION

The principal aim of this study was to assess the body condition of field-collected female locusts and investigate how this impacted their immune system. We found that haemolymph protein levels differed between sites, as did protein:lipid ratios, but lipid levels did not. Our measurement of female locust immune traits demonstrated that constitutive levels of immune function, in the form of lysozyme-like antimicrobial activity, prophenoloxidase activity and haemocyte density, were highly variable between individual female locusts within field populations of *C. terminifera*. This suggests that certain individuals would be much more susceptible to infection than others, even when in close proximity to one another. Haemocyte density was the only immune trait to differ significantly between populations, but all three immune traits were highly correlated with the protein pool in the haemolymph: haemocyte density and lysozyme-like antimicrobial activity were positively correlated whereas prophenoloxidase was negatively correlated, suggesting that two of the three tested immune responses were constrained by protein reserves. It is known that protein is a major substrate for producing immunological components used for resisting parasite and pathogen infection (41).

This study complements previous work undertaken on female *C. terminifera* in our laboratory, where locust immune function was found to be highly correlated with



Figure 3 Locusts in the field. (a) Adult locusts basking, (b) a 5th instar female sourcing protein through cannibalism of an injured locust and (c) a band of locusts on the march across a road.

dietary protein intake and the corresponding haemolymph protein pool (18). 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 (18). Studies on other insect groups (i.e. lepidopterans) have also found that immune traits (encapsulation response, lysozyme-like antimicrobial activity and PO activity) were all significantly higher in insects fed high-protein diets, as would be expected if protein is needed for the production of these immunological effectors (19,21). However, it has been found that immune traits differentially respond to variation in the ratios of macronutrient, and peak in different regions of macronutrient space, suggesting that nutrient-immune trait interactions may be more complex than initially anticipated (20). While the current experiment did not allow for the investigation of induced immunity in our samples (due to the inherent logistical difficulties of undertaking field research in remote areas), investigating induced immunity in field populations of locusts would be an exciting avenue to explore.

Our results suggest that some locusts are experiencing nutritional stress in the field. This phenomenon could reduce immune responsiveness in individuals possessing a lower haemolymph protein pool, thereby increasing susceptibility to disease and parasites. As previously stated, knowledge of nutrition upon immune defence is particularly lacking in field populations (25), and in migratory species (26) such as locusts and other orthopterans (42). Of the few studies undertaken, a manipulative study on *C. terminifera* field populations found haemocyte density

to fluctuate with host density, but body condition was not determined (42). In a related species, field populations of the Mormon cricket (*Anabrus simplex*) were generally protein deficient, and this deficiency induced their marching behaviour (12). Access to a proteinaceous diet subsequently resulted in increased immune function (as measured by phenoloxidase activity), suggesting that migrating Mormon crickets had low phenoloxidase activity as a result of their nutritional deficiency (13). Further, Srygley *et al.* (13) suggest that such limited immune activity of protein-starved individuals was likely to enhance disease transmission.

A benefit of associating with a high-density band of conspecifics is the reduced risk of predation (43), but as a result, the risk of disease and cannibalism by protein-deficient band members increases (12). Cannibalistic acts may carry a cost in terms of disease transmission via ingestion, but they most likely also provide nutritional benefits to enhance immune function. An interesting observation during our current field study was the high prevalence of cannibalism seen in field populations of *C. terminifera* (Figure 3b), suggesting that individuals were protein deficient. In particular, cannibalism of injured conspecifics was observed in populations A and B (>50 acts of cannibalism were recorded, whereas none were observed at site C). It is therefore perhaps not surprising that populations A and B had higher mean-protein haemolymph pools than population C, as cannibalism is a rich source of protein (12).

From an applied perspective, knowledge of pest population immunology could be incredibly important when determining the use of an effective biological control agent

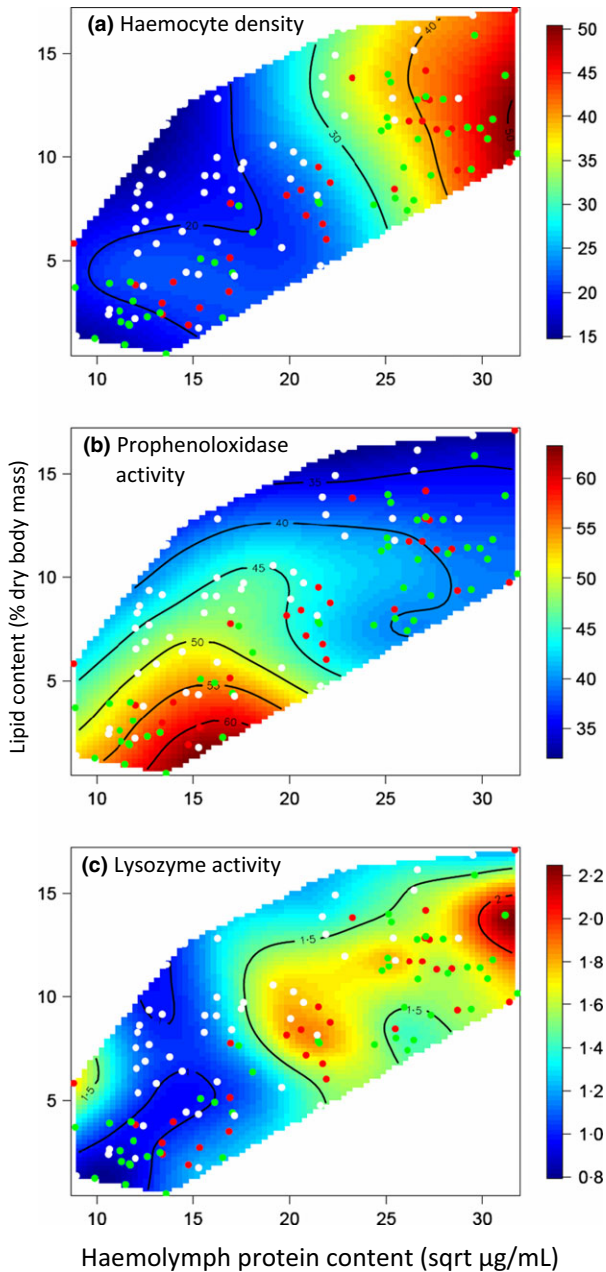


Figure 4 Thin-plate spline displaying relationships between the lipid (*y*-axis) and protein (*x*-axis) content of locust bodies with immune traits. (a) Total haemocyte density ($\times 10^2$ per μL), (b) prophenoloxidase activity (units per μL) and (c) lysozyme-like antimicrobial activity (μg per mL). Thin-plate splines were fitted to the data using the *fields* package in R (version 3.0.2). The data points represent the individual sampled locusts: (red) population A, (green) population B and (white) population C.

as part of a sustainable pest management programme. If certain populations were found to have high levels of protein and strong immune responses, it suggests that those

populations would be more resistant or tolerant to a biological pesticide than more nutritionally stressed populations with lower immune function. However, the nutrition–immunity picture is possibly further complicated, as revealed by the previous findings in our laboratory (18), which suggested that protein ingestion potentially increases an individual’s risk of mortality due to fungal–pathogen infection, possibly because fungal–pathogens are better able to utilize the host protein pool for growth. It should be noted that from an ecological and evolutionary point of view, this specific fungal–pathogen association with locusts is probably relatively rare in natural field populations (44), and that other parasites and predators are probably a greater survival threat. However, from a pest management perspective, this is an important finding with regard to the efficacy potential of Australia’s primary registered biocontrol product against the Australian plague locust, the *Metarhizium acridum* fungi commercially known as Greenguard®.

We have largely concentrated on nutritional aspects and body condition in this paper; however, there is an array of other factors that have been found to impact immune function (45). Although we found no significant differences in locust density between the three populations sampled in our study, population density has been found to be an important factor in determining immune trait function in *C. terminifera* populations (42), as well as other species (35, 46). The density-dependent prophylaxis hypothesis predicts that organisms living in high population densities will invest more in immune defence (46) due to an increased risk of disease when living in crowded conditions. However, this prediction does not hold for all species; indeed, Miller and Simpson (42) found that solitary *C. terminifera* had higher haemocyte densities than crowded locusts, thereby suggesting that solitary locusts would be more resistant to infection than crowded locusts: increased haemocyte densities clearly promote host defence (41).

In conclusion, our study suggests that body condition does impact locust immune function in field populations, namely haemocyte density, lysozyme-like antimicrobial activity and prophenoloxidase activity. Further investigation of immune parameters in field populations will provide a broader understanding of the role of pathogens in host dynamics and the relationship between host immunocompetence, body condition and population density. These results suggest that host body condition should be carefully considered as part of an integrated pest management strategy. However, field manipulation experiments will be required to disentangle how much of the variation in immune function we see is adaptive and how much is truly driven by nutritional constraints.

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