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Resistance and tolerance: The role of nutrients on pathogen dynamics and infection outcomes in an insect host

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Abstract

1. Tolerance and resistance are the two ways in which hosts can lessen the effects of infection. Tolerance aims to minimize the fitness effects resulting from incumbent pathogen populations, whereas resistance aims to reduce the pathogen population size within the host. While environmental impacts on resistance have been extensively, recorded their impacts on variation in tolerance are virtually unexplored.
2. Here, we ask how the environment, namely the host diet, influences the capacity of an organism to tolerate and resist infection, using a model host–parasite system, the burying beetle, *Nicrophorus vespilloides* and the entomopathogenic bacteria, *Photorhabdus luminescens*.
3. We first considered dose–responses and pathogen dynamics within the host, and compared our findings to responses known from other host species. We then investigated how investment in tolerance and resistance changed under different nutritional regimes. Beetles were maintained on one of five diets that varied in their ratio of protein to fat for 48 hr and then injected with *P. luminescens*. Survival was monitored and the phenoloxidase (PO) response and bacterial load at 24-hr postinfection were ascertained.
4. The dose required to kill 50% of individuals in this species was several magnitudes higher than in other species and the bacteria were shown to display massive decreases in population size, in contrast to patterns of proliferation found in other host species. Diet strongly modified host survival after infection, with those on the high fat/low protein diet showing 30% survival at 8 days, vs. almost 0% survival on the low-fat/high-protein diet. However, this was independent of bacterial load or variation in PO, providing evidence for diet-mediated tolerance mechanisms rather than immune-driven resistance.
5. Evolutionary ecology has long focussed on immune resistance when investigating how organisms avoid succumbing to infection. Tolerance of infection has recently become a much more prominent concept and is suggested to be influential in disease dynamics. This is one of the first studies to find diet-mediated tolerance.

KEYWORDS
immunity, insect, lipophorin, *N. vespilloides*, nutrition, *P. luminescens*, pathogen dynamics, phenoloxidase, tolerance

1 | INTRODUCTION

All organisms suffer from limited access to resources and so must provision resources appropriately to maximize fitness. Immune responses are a large sink of resources. This is because immune responses are costly in several ways. Firstly, they are directly energetically costly, as the construction of responses such as immune proteins requires the use of nutritional inputs (Rahnamaeian et al., 2015; Siva-Jothy & Thompson, 2002). This cost applies both constantly, in the maintenance of the body's capacity to respond to infection, and during infection, when the immune defences are upregulated in response to pathogen presence. Insect immune defences consist broadly of two branches—cellular and humoral immunity. Cellular immunity is affected by circulating cells called haemocytes, which are able to isolate a range of types of pathogen (from single bacteria to parasitoid eggs) through the processes of phagocytosis (similar to the process in vertebrates), nodulation and encapsulation. Encapsulation and nodulation involve the formation of a haemocyte layer surrounding the pathogen, which undergoes apoptosis and melanization, creating a barrier between the pathogen and host tissues. Melanization is carried out through the phenoloxidase (PO) enzyme cascade and is part of the humoral immune defences. When activated, PO catalyses the oxidation of phenols to form quinones and then melanin (Cerenius & Söderhäll, 2004). In the process, reactive oxygen species are also produced, which are highly damaging to both host and pathogen tissues in the immediate locality (Sadd & Siva-Jothy, 2006). This leads to the second and significant cost of immune activation: auto-immune damage. Auto-immune damage costs the host through both loss of function in the affected tissues, and the energy costs of rectifying this damage. Other mechanisms of the humoral immune response include the release of antimicrobial peptides; a functionally additive group of peptides active against particular classifications of pathogen, such as Gram-positive bacteria (Rahnamaeian et al., 2015).

Conversely, recent studies suggest that the immunity of an organism to disease is determined by more than just the capacity of it to demonstrate physiological resistance. Along with the complicating factors of behavioural immune defences, it has been suggested that an entirely different option is available to hosts, other than attempting to eliminate the infectious agent. By negating the effects a pathogen directly inflicts on the host, and so tolerating the presence of the parasite, the host can avoid becoming ill in spite of infection (Schneider & Ayres, 2008). Tolerance may be used in conjunction with resistance, and contributes to overall immunity. For example, the resources removed from the host circulatory system by pathogens can be compensated for by a reduction in movement by the host, conserving energy or by foraging for specific nutrients (Lee, Cory, Wilson, Raubenheimer, & Simpson, 2006; Povey, Cotter, Simpson, Lee, & Wilson, 2009).

However, tolerance is limited by host resources, as it is costly through loss of function in damaged tissues and the associated healing (Medzhitov, Schneider, & Soares, 2012) and through the nutritional demands of the pathogen population (Cressler, Nelson, Day, & Mccauley, 2014). As such, the adaptive value of tolerance would vary depending on the level of damage the pathogen causes. Variation in investment in resistance mechanisms has been found to be affected by a variety of factors (reviewed Schmid-Hempel, 2003), but variation in tolerance has been predominantly investigated from a genetic stand-point (Clough, Prykhodko, & Raberg, 2016). To our knowledge, only two studies have so far shown environmental factors affecting tolerance, with low-protein diets increasing intestine wall permeability (a sign of tissue damage due to infection) for a given load of the nematode pathogen in mice (Clough et al., 2016), and low-sugar diets improving survival after bacterial infection in *Drosophila melanogaster* (Howick & Lazarro, 2014).

As both tolerance and resistance mechanisms are often highly costly, they compete with other traits for resources, and distribution of resources between traits is subject to selection, resulting in an optimum allocation strategy (Moret, 2006). Resource ecology is the result of both the use of and uptake of nutrients, with different diets being more beneficial under different circumstances (Povey, Cotter, Simpson, & Wilson, 2014). This suggests that maximizing intake of nutrition would be optimal in all circumstances, to minimize conflicts in allocation. For example, reducing nutrient intake caused significant immune suppression in the mealworm beetle, *Tenebrio molitor*, and allowing access to food allowed immunity to recover (Siva-Jothy & Thompson, 2002). However, infection-induced anorexia (the reduced intake of nutrients in response to infection) is commonly found in insects and has been shown to enhance immune traits, possibly due to the prophylactic stress-related induction of immune defences (Ayres & Schneider, 2009). In contrast, in many studies, immune traits have been maximized on restricted diets but this has failed to translate into increased survival after infection (Ayres & Schneider, 2009). Theoretically, diet restriction could help either the host or pathogen, depending on the pathogen and hosts' relative abilities to monopolize nutrients in the body (Cressler et al., 2014).

Considering nutritional intake from the perspective of one measure (such as total calories) may be deceptively simple, considering an organism's need for multiple types of nutrient in varying quantities. The consideration of these varying nutrient ratios is known as nutritional ecology (Raubenheimer, Simpson, & Mayntz, 2009), and is revealing a previously hidden complexity, affecting both immune traits and life-history traits (Cotter, Simpson, Raubenheimer, & Wilson, 2011). Applying the techniques of nutritional geometry has supported the complexity of nutritional effects, with immune traits in the plague locust (*Chortoicetes terminifera*) for example, being maximized on a high-protein diet, but survival highest on a low-protein diet (Graham et al., 2014). This effect of protein was disconnected from total carbohydrate intake, suggesting that macronutrient effects should be investigated rather than total intakes. Insect hosts were also found to self-medicate by choosing the diet on which survival after infection was most enhanced, increasing protein intake in all cases (Graham et al., 2014; Povey et al., 2009, 2014). Evidence for modification of the allocation of resources after an immune challenge was also found in *Spodoptera littoralis*, rather than diet modification (Cotter et al., 2011).

In this study, the burying beetle species *Nicrophorus vespilloides* and its interactions with the entomopathogenic bacteria *Photorhabdus luminescens* were investigated. The burying beetle has a rather unusual ecology. Not only does it feed on carrion but it also breeds on carrion. When an adult burying beetle finds a suitable carcass (somewhere between 7 g and 75 g, usually small mammals or birds) it will prepare it for breeding, by removing fur or feathers and shaping it into a ball and burying it (Pukowski, 1933). Once larvae hatch they feed on the carcass, both fed and protected by the parents, for around 8 days, at which point they disperse into the soil and pupate. The carcass is vital for the beetles to successfully reproduce, but they have to compete with microbial competitors to prevent it being quickly degraded. Living in an environment which is so heavily infested with microbes requires these beetles to have an extremely effective immune system to survive, at both the larval and adult stages. The secretion of lysozyme in the anal exudate of both larvae (Arce, Smiseth, & Rozen, 2013; Reavey, Beare, & Cotter, 2014) and adults (Arce, Johnston, Smiseth, & Rozen, 2012; Cotter & Kilner, 2010) is an evolved response reducing the microbial degradation of the carcass and reducing pathogen density, and so reducing competition and infection risk associated with the carcass.

Photorhabdus luminescens is an entomopathogenic bacteria, symbiotic with a species of entomopathogenic nematode. The nematode acts as a vector for the bacteria, carrying it through the soil until a suitable host is located, invading the host and releasing the bacteria into the host haemocoel by regurgitation (Ciche & Ensign, 2003). The bacteria and nematodes both then begin to multiply within the host, feeding off the nutrient-rich haemolymph. *P. luminescens* also begins to release multiple toxins, which quickly cause apoptosis in both the haemocytes, and the midgut epithelial cells (Daborn, Waterfield, Silva, Au, & Sharma, 2002). As the midgut is important in water regulation and retention the insect quickly loses turgidity and dies. Once the resources of the host carcass begin to run out, the nematodes begin to reproduce asexually and the "infective juveniles" produced take up bacteria and begin to leave the carcass. These bacteria are obligate killers, as without killing, they cannot be transmitted and the host becomes a dead end. As a result, they are highly pathogenic, and LD50 doses have been recorded to be extremely low (see Table 1) and typically all hosts are dead within 48 hr. However, the insect immune enzyme PO has been shown to be highly influential on *P. luminescens* infection, with a *P. luminescens* strain with no ability to suppress PO allowing prolonged host survival (Eleftherianos et al., 2007).

As such, this paper seeks to investigate what immune mechanisms are utilized in the host, and how they are affected by the resources available to the host by answering the following questions:

1. Is *N. vespilloides* more tolerant/resistant to *P. luminescens* infection than other insect hosts?
2. Can diet mediate resistance/tolerance to infection?

2 | MATERIALS AND METHODS

2.1 | *Nicrophorus vespilloides* colony

A population of *N. vespilloides* was established using individuals from a pedigreed colony kept at Cambridge University. Each beetle was given an individual number and kept in a 12 × 8 × 2 cm box under a 16:8 light:dark cycle. Adult beetles were fed beef mince twice a week to allow ad libitum feeding. The population was maintained by breeding non-sibling pairs, which were each provided with a mouse carcass of a suitable size and placed in a larger box (17 × 12 × 6 cm) two-thirds filled with damp soil. They were then placed in darkness to replicate the natural breeding conditions of the species.

Around 8 days after the pairs were set up, the resulting larvae dispersed from the carcass into the surrounding soil. The parents and offspring were then removed, and the larvae were each put into a cell of a 25-cell Petri dish to pupate and covered with damp soil, with a different Petri dish for each family. Approximately 20 days later, the offspring would eclose, and around 15 days after that they reached sexual maturity. Beetles were used in experiments 19–21 days after eclosure.

2.2 | Diet modification

Five diets were used in this study, each varying in the ratio of protein to fat (20:80, 35:65, 50:50, 65:35, 80:20) (see Table 2). Ingredients were warmed slightly and mixed thoroughly to ensure even distribution of components.

2.3 | Injection challenge

Beetles were injected with 1 μl of nutrient broth through the pleural membrane between the third and fourth abdominal sternites on the right side of the beetle. The nutrient broth was mixed to the appropriate strength (see below) with an overnight culture of *P. luminescens* (strain TT01, inoculated from primary form colonies, remaining in primary form in vitro), incubated at 30°C and 200 r.p.m. The concentration of bacterial cells was initially estimated by measuring the optical density of the culture. This number was then more accurately determined using serial dilutions to carry out viable colony counts, but these more accurate counts could only be calculated after 24 hr, and

TABLE 1 LD50 dose of *Photorhabdus luminescens* recorded in previous studies

Host	Pathogen (strain)	LD50 (hr)	Reference
Tenebrio molitor	*P. luminescens* (TT01)	50 cells (46)	Blackburn et al. (2016)
Manduca sexta	*P. luminescens* (TT01)	100 cells (48)	Eleftherianos et al. (2007)
Galleria mellonella	*P. luminescens* (TT01)	50 cells (39)	Blackburn et al. (2016)
Drosophila melanogaster	*P. luminescens* (TT01)	50 cells (36)	Aymeric et al. (2010)

TABLE 2 Content of artificial diets used in experiments 3–5 (in grams)

Fat:protein ratio	Lard	Casein	Peptone	Albumen	Vitamin mix	Wesson's salts	Sugar
20:80	7.68	1.2	0.4	0.4	0.018	0.25	0.1
35:65	6.24	2.0	0.7	0.7	0.018	0.25	0.1
50:50	4.80	2.9	1.0	1.0	0.018	0.25	0.1
65:35	3.36	3.7	1.2	1.2	0.018	0.25	0.1
80:20	1.92	4.6	1.5	1.5	0.018	0.25	0.1

TABLE 3 Dose and number of samples used in each time point in the four replicates of experiment 2

Replicate	Dose (cells)	Time points (number of samples per time point)
1	7,500,000	0 (15), 1 (13), 5 (13), 7 (13), 9 (9), 24 (12)
2	7,500,000	0 (12), 5 (11), 10 (9), 15 (14), 20 (13), 25 (10), 28 (9)
3	250,000	0 (15), 15 (15), 30 (13), 40 (14), 45 (14), 50 (12), 55 (12)
4	257,000	96 (9), 120 (8), 144 (10), 168 (10; 8f, 2 m)

so after the treatment had been carried out. The beetle was wiped with ethanol before and after injection to prevent contamination during the process and to reduce haemolymph loss.

2.4 | Experiment 1: Dose–response curve to *Photorhabdus luminescens* infection

Adult *N. vespilloides* were injected with 1 μl of nutrient broth containing 1 of 15 doses of *P. luminescens* cells (0, 10, 100, 1,000, 10,000, 47,000, 100,000, 234,000, 257,000, 469,000, 478,000, 651,000, 937,000, 2,027,000, 5,881,000, 8,433,000 cells). The concentration of *P. luminescens* cells was initially estimated by testing the optical density of the liquid culture. The exact concentration of cells was then determined by plating up a serial dilution of the injected culture and incubating overnight (see above). A ratio of 1 male: 1 female was used in treatment groups where possible (n = 13–20). Survival was monitored daily over 8 days subsequent to inoculation, and beetles were cared for as described for the colony.

2.5 | Experiment 2: Bacterial proliferation dynamics

Beetles were injected with 1 μl of nutrient broth containing *P. luminescens* cells. Exact doses were established after inoculation as described above. Haemolymph samples were taken from beetles at regular time points after infection (see Table 3), and the bacterial loads of each sample were quantified using plate counts as described below. As wounding has been shown to activate the immune defences in this species, each beetle could only be sampled once, to avoid sampling affecting later bacterial proliferation. This experiment was repeated four times, using varying concentrations of *P. luminescens* cells (run 1 = 7,500,000; 2 = 7,500,000; 3 = 250,000; 4 = 257,000 cells), but with each replicate extending the time-scale of the experiment.

2.6 | Experiment 3: Diet effect on survival of *Photorhabdus luminescens*

Adult beetles of two ages (2 or 5 weeks posteclosion) were fed on one of five chemically defined artificial diets varying in the ratio of protein to fat (see above) for 2 days. After 2 days the beetles were injected with 1 μl of nutrient broth, either sterile or containing 2,480,000 *P. luminescens* cells, which corresponds to an LD70 dose on standard diet. Their survival was then monitored until every individual was dead. Beetles of the same ages were also fed on the five diets for 2 days (19 of 20 individuals per diet, 96 total, *c.* 50:50 male: female and 50:50 3:5 weeks old), and were then either injected with 1 μl sterile nutrient broth (9 of 10 per diet) or handled for an equivalent time (9 of 10 per diet), and their survival was monitored for 22 days.

2.7 | Experiment 4: Diet effect on bacterial proliferation in the haemolymph

Adult *N. vespilloides* beetles were fed on one of five diets (either 2 or 5 weeks posteclosion), varying in the ratio of protein to fat (as in experiment 3) for 2 days. After 2 days, the beetles were injected with 1 μl of nutrient broth containing 2,250,000 *P. luminescens* cells. Twenty-four hours after the treatment, haemolymph was sampled and spread on agar to quantify *P. luminescens* cells. The survival of each individual was monitored for 25 days after infection.

2.8 | Experiment 5: Diet effect on phenoloxidase activity in the haemolymph

Nicrophorus vespilloides beetles (2 or 5 weeks after eclosion) were fed on one of five diets varying in the ratio of protein to fat (as in experiment 3) for 2 days. After this the beetles were injected with 1 μl of nutrient broth containing 3,375,000 *P. luminescens* cells. Twenty-four hours later, the blood of each individual was sampled and later assayed for PO activity. This experiment was carried out separately to experiment 4 despite the similarity in the protocols due to the difficulty in consistently extracting enough haemolymph for PO and bacterial load measurements. Constitutive (i.e. untreated) levels of PO enzyme activity were measured in beetles of the same ages and both sexes, but with no time component. Age and sex did not affect PO activity in this untreated group and so they were pooled, and the average is added to Figure 5 for comparison.

2.9 | Survival monitoring

Each beetle was checked for survival at the same time each day for the duration of survival.

2.10 | Haemolymph collection

Haemolymph was collected from beetles between the thorax and abdomen, by holding the beetle firmly and piercing the pleural membrane beneath the pronotum. Internal pressure forces a droplet to form which can then be pipetted off. The sample was then either quickly assayed (for bacterial quantification) or mixed with an equal amount of phosphate-buffered saline with EDTA and frozen to prevent coagulation (to carry out PO assays).

2.11 | Bacterial quantification in beetle haemolymph

A total of 0.005 g per 100 ml rifampicin 1% agar plates were divided into four sections. Rifampicin inclusion ensured only the target bacterial species was included in the assay. One microlitre of fresh haemolymph was pipetted onto one of the four sections. Another 1 μl of fresh haemolymph was used in a serial dilution to allow quantification of larger numbers, and so was added to 99 μl of sterile nutrient broth. This was mixed, and then 10 μl of it was removed, and added to 90 μl of sterile nutrient broth. Ten microlitres of this was then removed and added to another 90 μl of sterile nutrient broth, creating a run of three dilutions.

These were then gently inverted and 10 μl of each was pipetted onto the remaining three sections of the rifampicin agar plates. The plates were then incubated overnight at 30°C, and the colonies in the section of the plate containing between 50 and 300 colonies were counted. The dilution of that section was then used to calculate the original concentration of the bacteria in the haemolymph sample.

2.12 | Phenoloxidase quantification

To measure PO enzyme quantities in haemolymph, each frozen haemolymph sample (collected as above) was vortexed for 20 s, then 3 μl of the sample was added to 500 μl of chilled sodium cacodylate buffer and vortexed again. 100 μl of this diluted sample was added to multiple wells of a 96 well plate on ice. One hundred microlitres of 50 mM dopamine hydrochloride was then added to the wells and the plate was put immediately into a plate reader. The optical density of the plate at 480 nm was then read every 10 s for 10 min, maintained at a temperature of 27°C. The Vmax of the slope was then used as an indicator of the quantity of the PO enzyme.

2.13 | Statistical analysis

All analyses were carried out using the statistical software R (version 2.15.3). For all experiments, examination of the model residuals confirmed that the data conformed to assumptions of normality. For experiment 1, the DRC package was used to apply a dose–response model to proportions of individuals survived to 8 days at different doses in experiment one, obtaining an LD50.

For experiment 2, due to the approximate dichotomy in doses (see Table 3) used (lower doses were necessary to extend the time-scale of the experiment), the experiment was analysed as two separate models (high and low dose). The first two (high dose) runs covered the first 28 hr of infection at around an LD80. The second two runs covered 0–168 hr after infection, using a dose of around LD30. Both doses were modelled using general linear models, and non-significant predictors were removed using stepwise deletion and type II sums of squares tests. One data point at time 0 was removed from the analysis due to a bacterial count of 0, suggesting the injection process was not successful.

Cox proportional hazards were applied to experiment 3 data using the SURVIVAL package in R. The significance of predictors was tested through stepwise deletion of non-significant variables and log-likelihood ratio tests.

For experiment 4, analyses of the effects of dietary protein: fat ratios, age and sex, on PO activity and bacterial load at 24 hr, were carried out using general linear models constructed through stepwise deletion and log-likelihood ratio tests. PO activity required Box–Cox transformation to fulfil assumptions of normality. In addition, the data from experiment 4 was used to assess the effects of sex, age, bacterial load and diet on survival (again, Box–Cox transformed), verifying analysis of experiment 3, and further testing how this related to bacterial load. Non-significant factors were removed from the model, remaining predictors were assessed using type II sums of squares.

3 | RESULTS

3.1 | Dose—response curve

At very low doses of *P. luminescens*, from zero to 10,000 cells, mortality at 8 days was minimal (see Figure 1). Above 10,000 cells, percentage survival decreased rapidly, dropping to around 20% at doses of 10,000,000 cells. An LD50 dose was found to consist of 305,000 cells.

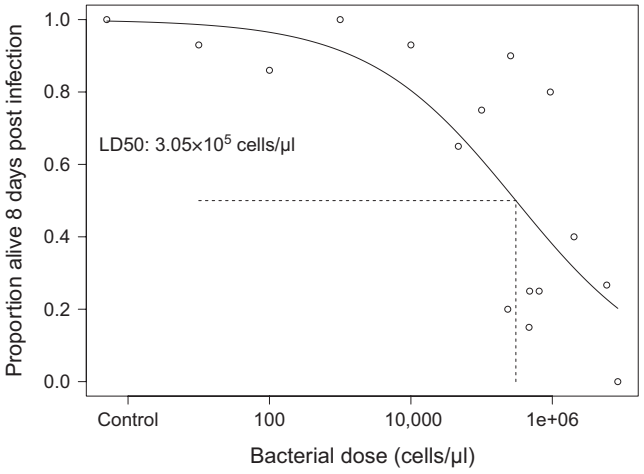

FIGURE 1 Dose–response to *Photorhabdus luminescens* infection in *Nicrophorus vespilloides* measured in percentage survival of infected individuals over 8 days. Line indicates model fit (survival ~ dose)

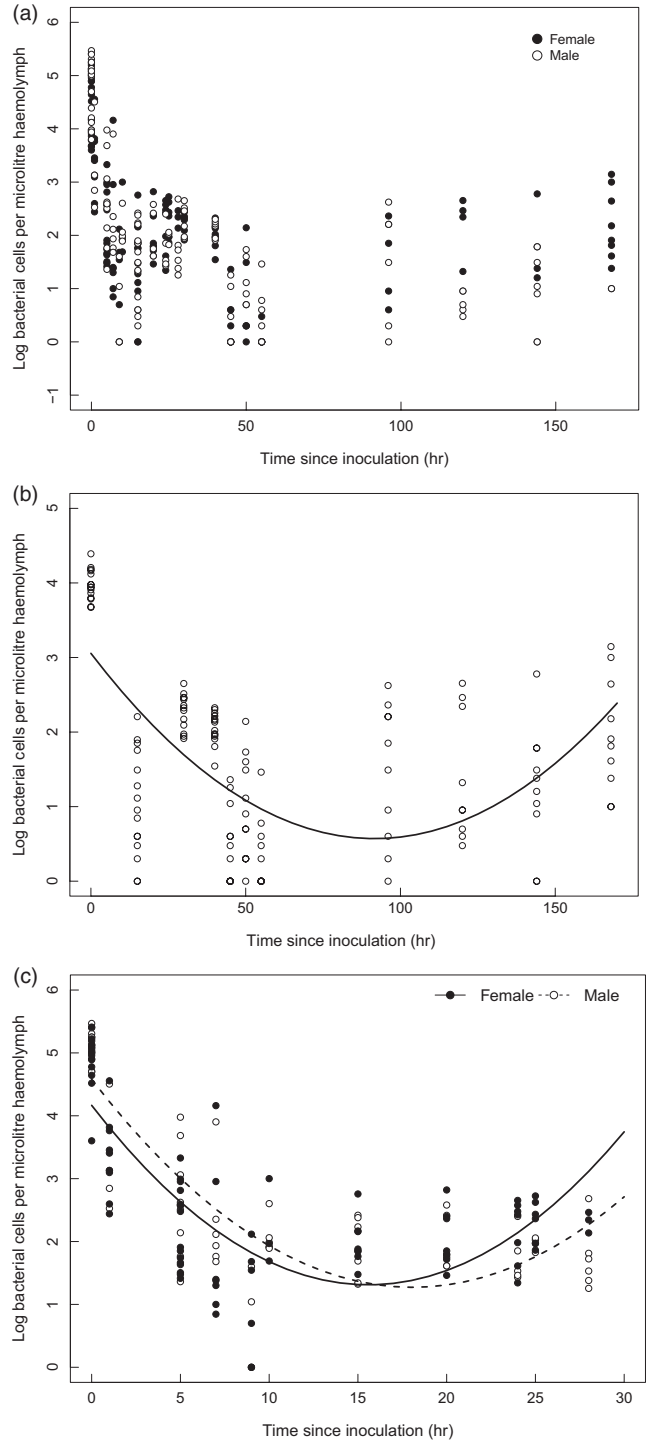

FIGURE 2 (a) Bacterial load in beetle haemolymph significantly varied over time at both (b) low and (c) high doses. At high doses bacterial proliferation was different in males and females. Lines indicate model fits in b) (bacterial load ~ time + time^2) and c) (bacterial load ~ time + time^2 + sex + time^2:sex)

3.2 | Bacterial proliferation

Bacterial population size within the beetle host was determined by counting bacteria in a 1-μl haemolymph sample taken from infected beetles at multiple time points postinoculation. At both high and low doses, the bacterial numbers dropped significantly (Figure 2a). In the low doses, bacterial load in haemolymph dropped from 10,000 to 10/μl (Table 4a; Figure 2b). Just before 100 hr, bacterial loads began to increase again, leading to a significant curvature (Table 4a). At the low dose, no other predictors or interactions were found to be significant.

At the high doses, a similar pattern was found but over a shorter time-scale, with significant decreases in bacterial load from 0 to 15 hr (Table 4b, Figure 2c) from around 100,000 to 100/μl of haemolymph. This was again, followed by an increase in bacterial load (Table 4b), and the resulting curve was affected by the sex of the individual (Table 4b), with females having lower bacterial loads initially, but males increasing sooner leading to higher bacterial loads in males subsequently.

3.3 | Diet effect on survival after infection

Adults on lower protein diets survived significantly longer than those on high-protein diets (Table 5, Figure 3a, see Figure S1 for full monitored period). Females survived significantly longer than males (Table 5, Figure 3b). Age did not significantly affect the likelihood of survival (Table 5) and there were no significant interactions between the above variables.

3.4 | Diet effect on bacterial proliferation in the haemolymph

Although there was a general trend for higher dietary protein to result in a lower bacterial load (Figure 4a) in the haemolymph at 24 hr, this was marginally non-significant (Table 6a). There was also no significant effect of either age (Table 6a), or the individuals' sex (Table 6a) on the bacterial load at 24 hr.

Survival in the 25 days after infection (and blood sampling) was negatively correlated with dietary protein content (Table 6b) with females surviving longer than males (Table 6b), and this difference being exaggerated by higher protein diets (Table 6b, Figure 4b). It was also affected by the individual's bacterial load at 24 hr (Table 6b), with higher bacterial loads being associated with shorter survival (Figure 4c).

3.5 | Diet effect on phenoloxidase activity in the haemolymph

Dietary protein content was found to significantly affect the levels of PO activity in blood samples (Table 7), with those on higher protein diets exhibiting lower PO activity (Figure 5). However, neither age nor sex affected PO activity (Table 7).

4 | DISCUSSION

This paper shows that the burying beetle species *N. vespilloides* is highly immune to the virulent bacteria *P. luminescens* compared to other investigated insect hosts, and that this immunity appears to be driven by a combination of tolerance and resistance mechanisms.

TABLE 4 General linear models fitted to (a) Low-dose bacterial proliferation data and (b) High-dose bacterial proliferation data. Terms retained in the minimum adequate model are highlighted in bold

	Sum of squares	*df*	Residual *df*	*F* value	*p*
(a)					
Time	70.967	1	131	77.212	**<.001**
Time^2	62.152	1	131	67.621	**<.001**
(b)					
Time	124.451	1	147	197.295	**<.001**
Sex	0.227	1	147	0.3593	**.550**
Time^2	79.129	1	147	125.4455	**<.001**
Sex:time^2	6.307	1	147	9.998	**.002**

TABLE 5 Cox proportional hazards model applied to diet effect on survival. Terms retained in the minimum adequate model are highlighted in bold

	Log-likelihood	Chi-square	*df*	*p*
Diet:age:sex	−810.31	0.063	1	.802
Diet:age	−810.31	0.278	1	.598
Diet:sex	−810.96	1.018	1	.313
Age:sex	−810.96	3.109	1	.079
Age	−813.6	2.173	1	.140
Sex	−813.6	74.737	1	**<.001**
Diet	−813.6	17.679	1	**<.001**

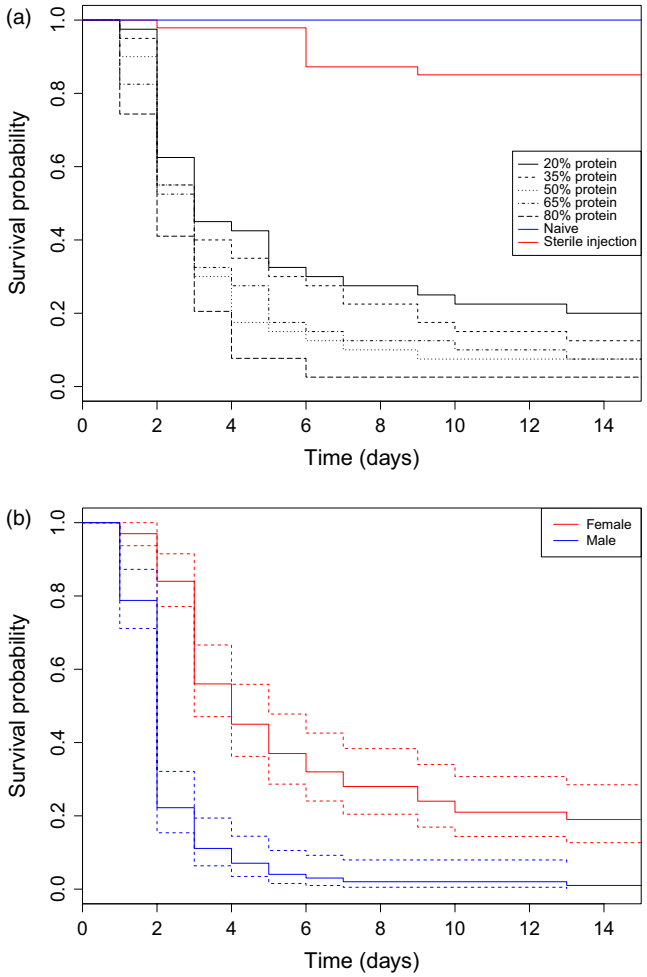

FIGURE 3 Dietary protein: fat ratio and sex significantly affected survival of *Photorhabdus luminescens* infection in *Nicrophorus vespilloides*, with (a) lower protein resulting in increased survival and (b) females surviving significantly longer than males (with confidence intervals)

These mechanisms, conveying higher survival, are favoured by a diet high in fat and low in protein, and tolerance is higher in females than males.

The LD50 at 8 days postinfection in *N. vespilloides* was found to occur at 305,000 injected *P. luminescens* cells. This is in contrast to previous studies, in which the LD50 has been found in other species to be around 50 cells at just 2 days postinfection (Table 1), suggesting that *N. vespilloides* is either extremely resistant to or tolerant of infection by *P. luminescens*, an otherwise highly virulent pathogen.

However, the proliferation of the bacteria within the haemolymph of *N. vespilloides* appears to be completely different to that seen in *Manduca sexta*, in which *P. luminescens* proliferates steadily after injection in the haemolymph, fat body and midgut, with no decrease shown in the 72 hr measured (Silva et al., 2002). This suggests that *N. vespilloides* produces more effective immune resistance than *M. sexta*, at least in the early stages of infection. As *P. luminescens* has been shown to secrete immune-suppressing factors (Eleftherianos et al., 2009), the resurgence in bacterial numbers could be due to the build-up of toxins such as these, reducing host immune upregulation to a point at which the bacteria begin to proliferate again. This suppression may also be the result of temporally controlled shifts in host immune strategy, which serve to limit costs to the host resulting from immune upregulation (Korner & Schmid-Hempel, 2004).

The pattern of bacterial proliferation we see in *N. vespilloides* is closer to that seen in the proliferation of *P. luminescens* in *D. melanogaster* (Aymeric, Givaudan, & Duvic, 2010) and *S. littoralis* (Mouammine et al., 2017), which mirrors the initial drop in bacterial numbers and subsequent resurgence. However, in both cases the resurgence occurred after 6 hr postinfection, and growth was exponential thereafter. The resurgence we see is much slower with no evidence of exponential growth, even up to 150 hr postinfection.

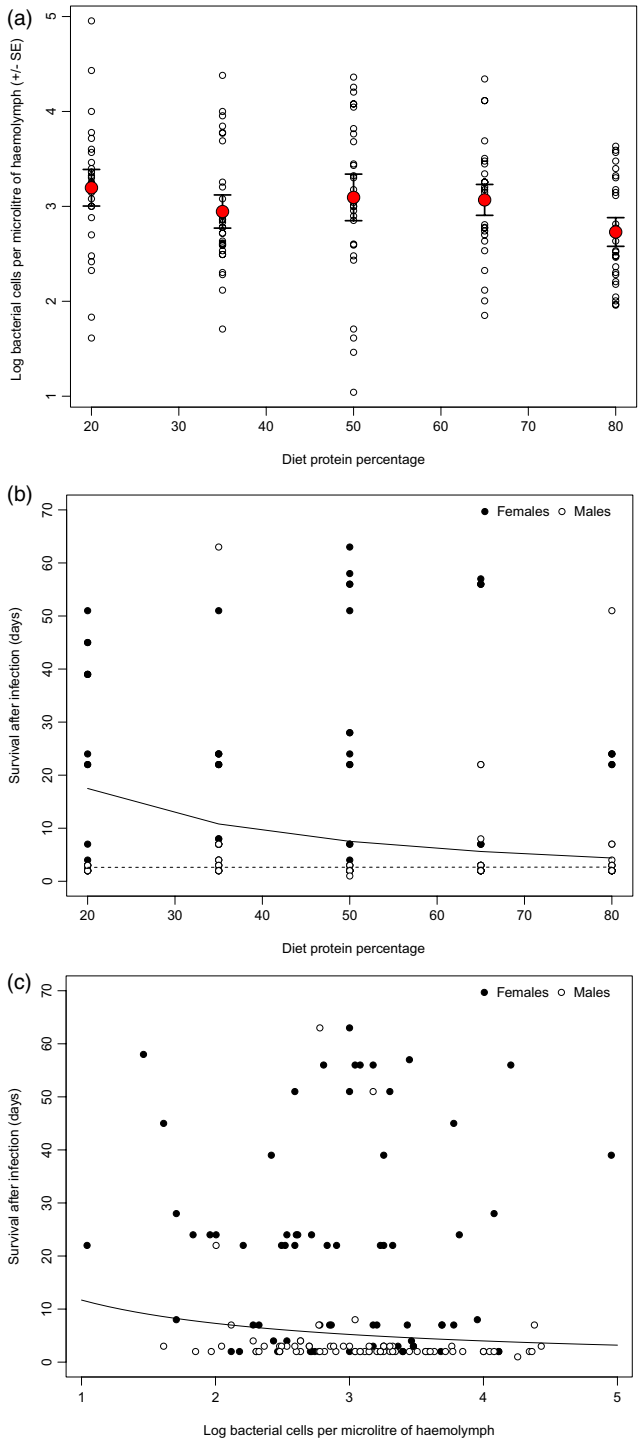

FIGURE 4 (a) Dietary protein:fat ratio did not significantly affect the bacterial load in the haemolymph 24 hr after infection. Line indicates model fit (bacterial load ~ diet). (b) Survival was higher in females than males on high-fat diets, but this effect was diminished on higher protein diets. (c) Survival was higher in beetles with a lower pathogen load regardless of diet. Lines indicate model fit (survival ~ diet + sex + bacterial load + diet:sex)

In *S. littoralis*, the resurgence in bacterial numbers was due to the retention of a highly resistant strain in the host (Mouammine et al., 2017). Further experiments would be required to test if a resistant strain is responsible for the resurgence in *Nicrophorus*, though, if a resistant strain is responsible for the resurgence, its effects on host mortality are clearly different. In *S. littoralis*, growth of the resistant strain resulted in 90% mortality within 48 hr (Mouammine et al., 2017), unlike in our study, where 90% mortality occurred around 9 days postinfection. The ability to restrict the exponential growth of the remaining bacteria suggests this species is highly resistant to *P. luminescens* infection.

Beetles on a lower protein to fat ratio were shown to have higher survival and PO activity. PO is very important in the response to infection to this pathogen, the bacteria can inhibit PO activity completely in *M. sexta*, and a mutant that lacks this ability takes much longer to kill the host (Eleftherianos et al., 2007). We have found that PO inhibition in *N. vespilloides* does occur, but that it is much weaker than the effect seen in *M. sexta* (Miller & Cotter, 2017a). Therefore, the higher PO levels on the high-fat diet could be playing a role in the increased survival by helping to facilitate phagocytosis and nodulation of bacterial cells. This contrasts surprisingly with previous studies which documented beneficial effects of protein on both PO and antimicrobial peptides (Graham et al., 2014; Povey et al., 2009, 2014). The exception to this is a study by (Cotter et al., 2011), which found differing complex effects of protein: carbohydrate ratios on PO and lysozyme activity, resulting in a trade-off between the two traits. One study found benefits to a carbohydrate-rich diet in ants, in which social immunity against fungal infection, facilitated by social grooming with secretions from the metapleural gland, was maximized on a high-carbohydrate diet (Kay et al., 2014). However, diet modification was carried out in all these studies by varying protein: carbohydrate ratios rather than protein: fat ratios, as was done in this study.

A relationship between fat intake and immune function has been shown in a study by Adamo, Roberts, Easy, and Ross (2008). Lipid transport in response to increased energy output and immune responsivity were found to be traded-off in crickets, due to competition between the two functions of apolipophorin III. Apolipophorin III is not only a lipoprotein used to transport lipids within the body but it also stimulates the immune system (Halwani & Dunphy, 1999; Wiesner, Losen, Ek, Weise, & Gotz, 1997), and the two functions appear to be closely structurally related (Niere, Dettloff, Maier, Ziegler, & Wiesner, 2001). This results in upregulation or enhancement of both phagocytosis (Wiesner et al., 1997) and lysozyme activity (Halwani & Dunphy, 1999) in the blood, possibly by making changes to the structure of the bacterial membrane, making recognition more likely.

However, the effect of diet on bacterial load at 24 hr was marginally non-significant, and in fact high-fat, low-protein diets (with the highest survival) tended to have the highest bacterial load. This could be because 24 hr was not a suitable time point at which to measure bacterial load, perhaps there is too little variation at this point to see differences across diets, and indeed, very few beetles died at 24 hr. However, we did find a strong effect of bacterial load at 24 hr on subsequent survival; beetles with high bacterial loads at this point died more rapidly than those with low bacterial loads, suggesting that our measures of bacterial load are meaningful, but they do not explain the diet- or sex-mediated differences in survival. This suggests

TABLE 6 General linear models applied to (a) bacterial load in the haemolymph at 24 hr and (b) survival. Terms retained in the minimum adequate model are highlighted in bold

	Residual *df*	*df*	Sum of squares	*F*	*p*
(a)					
Diet:sex:age	136	1	0.498	1.076	.301
Diet:sex	138	−1	−0.033	0.071	.791
Sex:age	139	−1	−0.045	0.098	.755
Diet:age	139	1	0.357	0.782	.378
Age	140	1	0.451	0.989	.322
Sex	141	1	1.214	2.661	.105
Diet	143	−1	−1.770	3.839	.052
(b)					
Diet:age:sex:bacteria	128	1	0.029	0.911	.342
Age:sex:bacteria	130	−1	−0.001	0.047	.829
Diet:bacteria:age	131	−1	−0.001	0.010	.920
Diet:bacteria:sex	131	1	0.004	0.141	.707
Sex:age:diet	132	1	0.014	0.455	.501
Age:bacteria	133	1	0.005	0.162	.688
Diet:bacteria	134	1	0.014	0.442	.507
Diet:age	136	−1	−0.053	1.712	.193
Sex:bacteria	136	1	0.007	0.222	.638
Age:sex	138	−1	−0.100	3.215	.075
Age	138	1	0.003	0.088	.767
Diet:sex	139	1	0.222	7.070	**.009**
Sex	139	1	2.226	70.766	**<.001**
Bacteria	139	1	0.192	6.103	**.015**
Diet	139	1	0.194	6.164	**.015**

that it is tolerance, rather than resistance that explains these gross differences across treatment groups. So how might this tolerance be mediated?

Phenoloxidase has been shown to be suppressed in insect hosts by the secretion of the *P. luminescens* toxin ST (Eleftherianos et al., 2007). The resistance to PO suppression seen in the beetles on higher fat diets suggests that this toxin (and possibly others) is being inactivated in some way in these individuals. High-fat diets may result in increased production of lipoproteins, such as lipophorins, which are responsible for transporting lipids around the insect body (Chino & Downer, 1982). Lipophorins are also released from haemocytes in response to infection (Andrejko, Mizerska-Dudka, & Jakubowicz, 2008) and have been shown to form complexes with toxins such as lipopolysaccharides and lipoteichoic acids (Freudenberg, Bog-Hansen, Back, & Galanos, 1980; Halwani, Niven, & Dunphy, 2000). This binding was shown to reduce the damaging behaviour of the toxins in vitro (Halwani et al., 2000; Kato et al., 1994), and may be the mechanism allowing a high-fat diet to favour survival after infection, with lipoproteins being more prevalent in individuals with a higher fat intake to facilitate lipid transport.

The effects seen in this study, of both sex and dietary makeup on survival, which were not explained by changes in bacterial load, provide strong evidence for an important role for tolerance mechanisms in immune defence against *P. luminescens*. It seems that while females show better survival, this is not due to increased clearance of the bacteria, and is enhanced by a lower protein: fat ratio. This

TABLE 7 General linear models applied to phenoloxidase activity in the haemolymph 24 hr postinfection. Terms retained in the minimum adequate model are highlighted in bold

	Residual *df*	*df*	Sum of squares	*F*	*p*
Diet:sex:age	180	−1	−0.102	1.307	.255
Diet:sex	180	1	0.007	0.096	.757
Age:sex	182	−1	−0.010	0.126	.723
Diet:age	182	1	0.121	1.571	.212
Age	183	1	0.004	0.051	.821
Sex	185	−1	−0.042	0.544	.462
Diet	185	1	1.257	15.070	**<.001**

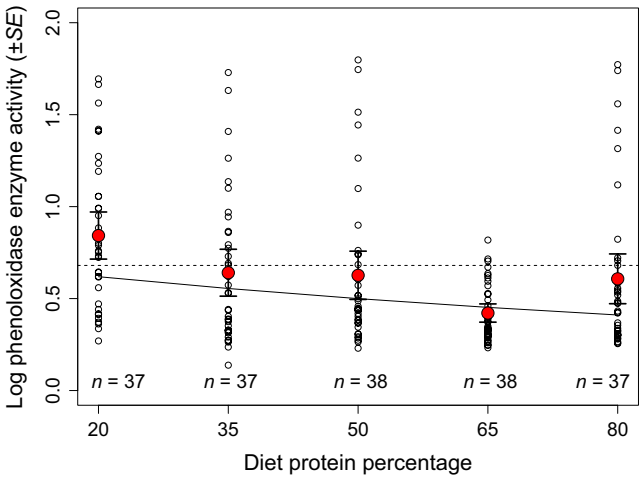

FIGURE 5 Dietary protein:fat ratio was shown to significantly affect the phenoloxidase response to *Photorhabdus luminescens* infection. Dotted line indicates constitutive levels of phenoloxidase. Solid line indicates model fit (PO ~ diet)

shows effects of the environment on tolerance—a very novel area of research (Clough et al., 2016). The much higher LD50 seen in this species also indicates strong immunity to *P. luminescens* in this species, with immune tolerance as a significant contributor to this immunity, especially on a high-fat, low-protein diet. In a species which is heavily exposed to microbial competitors during the utilization of carcasses for feeding and breeding, physiological tolerance of infection provides significant additive benefits to overall immunity in tandem with resistance.

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AUTHORS' CONTRIBUTIONS

C.M. and S.C. conceived the ideas, designed methodology, analysed the data, and wrote the manuscript; C.M. collected the data.

DATA ACCESSIBILITY

Data is publically available on Figshare https://doi.org/10.6084/m9.figshare.5372737 (Miller & Cotter, 2017b).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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