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The double-edged sword of immune defence and damage control: do food availability and immune challenge alter the balance?

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Summary

1. Animal immune systems must adaptively balance aggressive immune resistance (ability to destroy pathogens) with infection tolerance (ability to withstand the negative effects of infection; e.g. immunopathology or damage due to pathogen metabolism).

2. Insects offer unique insight into this balancing act because phenoloxidase (PO)-mediated melanization is a key feature of immune resistance, but PO activation obligates the production of nonspecific reactive species that can cause self-damage. The antioxidant glutathione (GSH) can provide protection against such reactive molecules, but high levels of GSH can impair melanization. In support of the hypothesis that GSH can protect insects (e.g. crickets) from self-damage during an immune response, we found that bacterially infected crickets showed a significant positive relationship between GSH haemolymph concentration and fecundity after controlling for bacterial growth rate. That is, GSH may be a mechanism of infection tolerance because it correlated with fecundity despite bacterial proliferation.

3. Next, we factorially manipulated food availability and immune activation in female crickets to examine whether the relative balance between a component of immune resistance (i.e. PO) and protection from self-damage (i.e. GSH) was plastic and sensitive to environmental conditions.

4. Glutathione and PO were positively correlated, and the PO:GSH ratio was robust and not affected by food availability or immune activation. Thus, increased investment in a mechanism of immune resistance may obligate a concomitant increase in GSH to reduce self-damage (i.e. increase infection tolerance). Chronic immune activation led to greater tolerance of oxidative stress suggesting that repeated immune activation upregulates infection tolerance mechanisms. Food limitation led to reduced PO activity, but not GSH concentration. This result suggests that mechanisms of immune resistance may be more sensitive to resource scarcity than mechanisms of infection tolerance.

5. We demonstrate that some mechanisms of immune resistance and infection tolerance can be correlated and that they can be affected by food availability or immune activation.

Key-words: ecological immunology, glutathione, *Gryllus texensis*, oxidative stress, phenoloxidase, self-damage

Introduction

Organisms use two types of defence to protect themselves from infection (reviewed in Schneider & Ayres 2008; Ayres & Schneider 2012). First, organisms can employ a suite of resistance mechanisms (e.g. phagocytic cells or melanin production) designed to attack pathogens or otherwise limit their proliferation. However, some resistance mechanisms may cause self-damage or immunopathology (e.g. reactive oxygen species) (Sadd & Siva-Jothy 2006; Lambeth 2007; Lambeth, Kawahara & Diebold 2007). Therefore, organisms use a second type of defence (infection tolerance) to reduce the impact of a pathogen's presence. Tolerance mechanisms have been categorized into several classes and include such processes as detoxification of pathogen-produced molecules, host repair mechanisms and energy redistribution (Ayres & Schneider 2012). One class of infection tolerance mechanisms mitigates the damage

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produced by the immune system to the host (i.e. the 'bystander effect'). For example, oxidative stress resistance pathways, such as the production of antioxidants and molecular chaperones, are tasked with preventing or repairing damage to macromolecules (categorized as 'damage prevention' and 'repair' components, respectively: Ayres & Schneider 2012).

Immune resistance and infection tolerance may be related to one another or co-regulated (Raberg, Sim & Read 2007; Ayres & Schneider 2008; Schneider & Ayres 2008). Yet, the relationship between specific mechanisms underlying resistance and tolerance is unclear, and insect innate immunity offers key insight into this link or co-regulation. Pathogen defence in insects relies strongly upon melanization, an immune response that includes cuticular wound healing and encapsulation (Siva-Jothy, Moret & Rolff 2005; Christensen *et al.* 2005). Melanization is facilitated by the activity of phenoloxidase (PO; Siva-Jothy, Moret & Rolff 2005; Kanost & Gorman 2008; Gonzalez-Santoyo & Cordoba-Aguilar 2012), and PO has been shown to be a mechanism of immune resistance as it is associated with pathogen clearing and/or disease resistance (e.g. reviewed in Cerenius, Lee & Soderhall 2008; Srygley & Jaronski 2011). Yet, PO-mediated melanization obligates the production of reactive oxygen species (Christensen *et al.* 2005). Although these reactive intermediates likely enhance pathogen killing (Cerenius, Lee & Soderhall 2008), they can also damage the internal organs of the host insect (Sadd & Siva-Jothy 2006). Thus, insects must balance melanization-inducing PO activity with mechanisms of infection tolerance, such as those that reduce immunopathology. For example, glutathione (GSH), an important insect antioxidant, inhibits melanization *in vitro* (Matsuki *et al.* 2008) and *in vivo* (Clark, Lu & Strand 2010). Therefore, some mechanisms of tolerance may impede resistance.

The optimum balance between mechanisms of resistance and tolerance is likely to vary as the environment fluctuates (Adamo 2014). The abundance and composition of food varies in nature, and both of these dietary factors influence immune resistance and/or infection tolerance (Vale *et al.* 2011; reviewed in Chambers & Schneider 2012; Sternberg *et al.* 2012; Ponton *et al.* 2013; Cornet *et al.* 2014; Howick & Lazzaro 2014). For example, Ayres & Schneider (2009) elegantly demonstrated the effects of food limitation on resistance and tolerance in *Drosophila* using host survival and pathogen growth rate as proxies. Alternatively, one can use a nonlethal immune insult (e.g. nylon monofilament) to uncouple the adverse effects of pathogen proliferation from those of self-damage (Sadd & Siva-Jothy 2006). For example, repeated exposure to heat-killed bacteria chronically activates an insect's immune response, thereby 'priming' it for subsequent exposure (reviewed in Chambers & Schneider 2012). Although immune resistance and overall disease susceptibility are affected by previous immune activation, little is known about how tolerance mechanisms are influenced. Further, chronic immune activation and diet simultaneously vary in nature, and they may exert independent and/or interactive effects on immune resistance and infection tolerance (Vale *et al.* 2011).

In this study, we measured the GSH concentration of haemolymph in female Texas field crickets (*Gryllus texensis*) in relation to females' abilities to maintain reproductive output during a live bacterial challenge, to provide evidence that GSH is a mechanism of infection tolerance. We then used factorial manipulations of food availability and immune activation in *G. texensis* to address two hypotheses. First, we hypothesized that some mechanisms of immune resistance and infection tolerance would be correlated with one another. Specifically, we predicted that individuals strongly investing in PO activity as a mechanism of resistance will also exhibit higher concentrations of the antioxidant GSH in the haemolymph, given the self-damage obligated by PO-induced melanization. Secondly, we hypothesized that PO activity and GSH concentration will be variably influenced by food availability and immune activation. Based on previous work, we predicted that total PO activity will be lower in nutrient-limited females (Cotter *et al.* 2011). If food limitation decreases PO, it should also result in a reduction in the concomitant demand for GSH. Next, we predicted that chronic immune activation would increase demand for GSH concentration (to mitigate self-damage) and lead to enhanced oxidative stress resistance [survival to a paraquat (PQ) challenge, see below]. We made no prediction on the effects of chronic immune activation on PO activity because available information indicates conflicting results on the topic – some researchers report a transient increase in PO activity due to acute immunogen exposure (e.g. Sung, Yang & Song 1996; Thanardkit *et al.* 2002), while others demonstrate no change in the expression of PO-related genes due to chronic exposure to a range of immunogens (Hauton, Brockton & Smith 2006). Together, this design allowed us to examine the effects of food availability and immune activation on mechanisms of immune resistance (total PO activity) and infection tolerance (PQ survival and GSH concentration), as well as relationships between PO and GSH.

Materials and methods

STUDY ANIMALS

We used long-winged adult *G. texensis* that were part of a long-term colony, which has been described previously (Adamo & Lovett 2011). We supplied crickets with water *ad libitum* and housed crickets in a room maintained at 26 ± 1 °C and a 12-h light:12-h dark cycle. All studies were approved by the Animal Care Committee of Dalhousie University (#I9-026) and are in accordance with the Canadian Council on Animal Care.

EVIDENCE THAT GSH IS A MECHANISM OF INFECTION TOLERANCE

Phenoloxidase is an important mechanism of immune resistance in arthropods (reviewed in Cerenius, Lee & Soderhall 2008). Yet, it is unclear whether GSH is a mechanism for infection tolerance – that

is, whether it limits the damage caused by immune responses, such as PO (Sadd & Siva-Jothy 2006) and pathogen metabolism. If GSH is a mechanism of infection tolerance, then GSH haemolymph concentration should be related to the ability of individuals to maintain reproductive output during an infection. Thus, we examined the relationship between GSH concentration and fecundity (as a proxy for fitness or condition) after accounting for the effects of pathogen proliferation. Over the span of one month, we isolated three cohorts of female *G. texensis* (10–15 days post-adult moult) from the colony ($n = 29$). For 3 day, we kept each female in clear containers (17 × 15 × 9·5 cm) and provided water and food *ad libitum*. We then injected crickets through the pronotal membrane with a median lethal dose (LD_{50}) of the Gram-negative bacterium, *Serratia marcescens* (approx. 1×10^4 cells/2 μL in culture media: Adamo *et al.* 2010). *Serratia marcescens* is found world-wide in both soil and water, and it has been recovered from the bodies of orthopterans in the field (Steinhaus 1959). After 2 day, we counted the number of eggs each female laid in a cotton-plugged water bottle as a measure of fecundity during infection. We also removed 10 μL of haemolymph from each cricket's pronotal membrane 2 day after *S. marcescens* injections. We used half of this haemolymph to determine GSH concentration and the other half to determine bacterial count (detailed below). We excluded from analyses the two females that died due to infection by *S. marcescens* prior to haemolymph and egg sampling.

We isolated a separate group of females ($n = 27$), and we kept them in clear containers and mated them as described above. After mating, we collected eggs for 6 days. At the end of this period, we collected 8 μL of haemolymph, added it to 42 μL of phosphate-buffered saline and measured GSH concentration. For further details, see Assays for total circulating protein, phenoloxidase activity, and glutathione concentration and Bacterial count (below).

EFFECTS OF FOOD AVAILABILITY AND IMMUNE ACTIVATION ON RESISTANCE AND TOLERANCE

We next used a 2 × 3 design to manipulate food availability and immune activation in a factorial fashion throughout adulthood. Over the span of seven months, we isolated 23 cohorts of newly moulted (≤1 day post-adult moult) females and housed them individually in transparent 2000-mL plastic containers. As described previously (Stahlschmidt *et al.* 2013), we manipulated each female's access to food: '*ad lib.* food' (which reflects a regime where females have an abundance of resources available for physiological processes) or 'food limited' (access to food for 3 h every 3 day, which results in *G. texensis* with similar levels of body fat as those found in the field: Adamo *et al.* 2012). From ≤1 day to 17 days post-adult moult, we manipulated the level of females' immune activation: 'bacterial challenge' treatment (abdominal injection of heat-killed bacteria every 3 day), 'wounded' treatment (sham injection every 3 day) or 'control' treatment (not handled). We induced a chronic systemic immune challenge by injecting females with heat-killed *S. marcescens*. We used the LD_{50} dosage of *S. marcescens* for *G. texensis* (Adamo *et al.* 2010), which we then heat-killed for this study. Using this concentration of heat-killed *S. marcescens*, we chronically activated crickets' immune systems without pathogenesis (Adamo 2004). As a control for wounding and handling stress, we pierced females in the abdomen with a sterile dissection pin every 3 day. Wounding activates a truncated version of the insect immune response (Gillespie & Khachatourians 1992; Wigby *et al.* 2008; Ardia *et al.* 2012). These dietary and immune treatment groups independently and interactively influence the number and/or quality of eggs and hatchlings (e.g. protein content of eggs and body size, respectively), as well as female body condition – for further details, see Stahlschmidt *et al.* (2013).

On the evening of 11 day post-adult moult, we mixed females with sexually mature males at a ratio of one female per 1–2 males (depending on the availability of age-matched males) to elicit overnight mating, and we excluded from analyses females that did not mate (the number of nonmated females did not vary across treatment groups). Mating status was determined by the presence of spermatophore-filled spermatheca post-mortem. At 17 day post-adult moult, females were assigned to one of two experimental groups. We injected some females ($n = 117$) with a lethal dose (LD) of paraquat (PQ; LD range: 54–86%), an herbicide that induces oxidative stress in a range of animal taxa (reviewed in Rzezniczak *et al.* 2011). For details, see Paraquat challenge (below). We checked each female every day for 5 day to determine whether it survived this challenge (a proxy for resistance to oxidative stress).

Also at 17 day post-adult moult, we removed ≤10 μL of haemolymph from the pronotal membrane of the other females ($n = 133$) to determine haemolymph values for total PO activity and GSH concentration. We also determined the total circulating protein of the haemolymph because it was affected by food availability (Fig. S1, Supporting information) and can reflect condition (Adamo 2004). Further, we determined the ratio between PO and GSH to examine whether the balance between these mechanisms of immune resistance and infection tolerance shifted due to food availability and/or immune activation. For further details, see Assays for total circulating protein, phenoloxidase activity and glutathione concentration.

ASSAYS FOR TOTAL CIRCULATING PROTEIN, PHENOLOXIDASE ACTIVITY AND GLUTATHIONE CONCENTRATION

We mixed 10 μL of fresh haemolymph with 50 μL double-distilled H_2O (ddH_2O). After brief vortexing, we removed 17 μL and stored it at −80 °C for assays of total PO and circulating protein. The remaining blood was deproteinated. We added 40 μL of 1·25 M meta-phosphoric acid (Sigma-Aldrich, St. Louis, MO, USA) to the remainder of the haemolymph–ddH_2O solution. We spun this solution at 2500 *g* for 3 min after 5 min of incubation at room temperature. We then removed 70 μL of the supernatant and stored it at −80 °C for GSH assays.

We thawed the stored samples (17 μL of haemolymph–ddH_2O solution) on ice before adding 34 μL of ddH_2O. We removed 20 μL from this solution for Bradford assays of total circulating protein in triplicate and kept the remaining solution for total PO assays (see below). We centrifuged this solution in a microcentrifuge tube for 10 min at 10 000 ***g***. We removed 4 μL of the supernatant and added it to 180 μL of Bradford reagent (Sigma-Aldrich) in a 96-well microtitre plate. After a 10-min incubation period at room temperature, we measured absorbance at 590 nm using a spectrophotometer (Molecular Devices, Thermomax microplate reader, Sunnyvale, CA, USA). We compared absorbance values of samples with those from a standard calibration curve that was calculated using bovine serum albumin (Sigma-Aldrich).

We used a kinetic spectrophotometric assay modified from that of Bidochka, Gillespie & Khachatourians (1989) to measure the total PO activity of haemolymph. To the remaining solution, we added 30 μL of a solution containing bovine pancreas α-chymotrypsin (2 mg mL^{-1} PBS) and 420 μL of ddH_2O. We incubated this mixture for 20 min at room temperature, including 5 min of centrifugation at 10 000 ***g***. We then added 15 μL of the supernatant to 180 μL of a solution containing l-DOPA (0·02 mol L^{-1}) in a 96-well microtiter plate. We measured the change in absorbance for 20 min at 490 nm using a spectrophotometer (Molecular Devices, Thermomax microplate reader). We compared absorbance values of samples with those from a standard calibration curve that was calculated using tyrosinase (Sigma-Aldrich). We

measured both standards and samples in triplicate. We report absolute PO activity (μg tyrosinase equivalent μL^{-1}). However, because total circulating protein can correlate with increased disease resistance (Adamo 2004), we included total circulating protein as a covariate in our mixed model for PO activity (see below). This procedure helped us to disentangle total circulating protein effects from PO activity.

We performed GSH assays using a kit from Cayman Chemicals (#703002), following the manufacturer's directions, with minor modifications. We deproteinated haemolymph samples using metaphosphoric acid (0·5 g/5 mL ddH_2O). We then vortexed an equal volume of MPA and sample and allowed this solution to stand at room temperature for 5 min. We next centrifuged at 5200 ***g*** for 3 min before storing the supernatant at −80 °C. We analysed samples by first thawing them on ice and then adding 50 μL of a 4 M solution of triethanolamine per 1 mL of sample. We used GSH MES buffer [2-(N-morpholino)ethanesulphonic acid, phosphate, EDTA, pH 6·0] to dilute both the samples and GSSG (glutathione disulphide dimer) standards. All GSSG was reduced to GSH by glutathione reductase to ensure measurement of both GSSG and GSH. We added glucose-6-phosphate dehydrogenase with co-factors $NADP^+$ and glucose-6-phosphate to the samples and standards. Addition of 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) results in a reaction with the sulfhydryl group of GSH to produce 5-thio-2-nitrobenzoic acid (TNB) and mixed disulphide GSTNB (GSH and TNB). Glutathione reductase reduces GSTNB to GSH and TNB. This results in the enzymatic recycling of GSH. The rate of TNB production through the enzymatic recycling is directly proportional to GSH concentration. We measured the change in absorbance of the yellow-coloured TNB for 30 min at 405 nm using a spectrophotometer (Molecular Devices, Thermomax microplate reader). We compared the absorbance values of samples with those from a standard calibration curve to calculate GSH concentration.

BACTERIAL COUNTS

We transferred 5 μL of freshly sampled haemolymph into 50 μL of sterile phosphate-buffered saline using a sterile pipette tip and vortexed briefly. We then pipetted the haemolymph–saline mixture onto a nutrient agar plate and spread using a sterile spreader (i.e. spread-plating method). We placed plates in a biosafety cabinet at room temperature and incubated for 3 days. After which, we counted the number of bacterial colonies on each plate.

PARAQUAT CHALLENGE

Because immune activation results in the production of reactive oxygen species (ROS) and oxidative stress (OS) (Sadd & Siva-Jothy 2006; Molina-Cruz *et al.* 2008), we used the ability of crickets to withstand OS as a proxy for infection tolerance. Paraquat (PQ, 1,1′-dimethyl-4,4′-bipyridinium dichloride) is commonly used to induce OS (reviewed in Rzezniczak *et al.* 2011). Paraquat undergoes an *in vivo*, NADPH-dependent reduction, which results in a stable PQ radical that reacts with oxygen to generate ROS (Bus & Gibson 1984). The accumulation of ROS results in OS, which entails ROS-induced damage to lipids, proteins and DNA.

We determined a variably lethal dose of PQ for a cricket of average mass (0·54 g) to be approximately 2 μL of 12·5 mM PQ in ddH_2O. We used the same concentration (12·5 mM) for all injections, but we adjusted the volume of injectate for each cricket based on its body mass (BM) where volume of injectate (μL) = $(4{\cdot}7\ BM)^{0{\cdot}75}$. We returned crickets to their individual containers after they were injected at 17 day post-adult moult. We checked these containers every day for 5 day to determine mortality, the vast majority of which occurred 2–3 days postinjection.

STATISTICAL ANALYSES

We performed analyses with SPSS (version 21, IBM Corp., Armonk, NY, USA). We determined two-tailed significance at $\alpha < 0{\cdot}05$ and display all values as mean ± SEM. All data met the assumptions of parametric statistics or were transformed as necessary. To confirm the deleterious effect of bacterial growth on our proxy of fitness/condition for our validation study, we ran a simple correlation between bacterial count and the number of eggs females laid. To examine relationships among GSH concentration, egg laying and bacterial count (e.g. if GSH haemolymph concentration was related to the ability of individuals to maintain reproductive output during an infection), we performed a partial correlation analyses among these three variables.

We used linear mixed models to determine the main and interactive effects of food availability (two levels: *ad lib.* or limited) and immune activation (three levels: bacteria, wound and control) on each dependent variable (total PO activity, GSH concentration, PO:GSH, total circulating protein) in separate models. Each of our initial models included cohort as a random effect, and treatments as fixed effects. For these models, we also initially included total circulating protein (for all models other than the total circulating protein model) and femur length as covariate(s). In addition, we included total PO activity as a covariate in our initial GSH model and *vice versa*. From each initial mixed model, we removed nonsignificant effects that did not affect the Akaike's Information Criterion (AIC). Thus, we report the final models with the fewest number of independent variables that conserved the AIC. When a covariate was significant (e.g. covariation of PO and GSH), we examined its regression coefficient to determine the sign of the correlation. For PQ survival, we used a binary logistic generalized linear model. Our model included treatments as fixed effects and femur length (a proxy for body size) as a covariate.

Results

Bacterial concentration in the haemolymph exhibited a significant negative relationship with the number of eggs females laid (Pearson's correlation, $n = 27$, $R = -0{\cdot}42$, $P = 0{\cdot}030$). Partial correlation analyses revealed that egg laying was positively related to GSH concentration after controlling for bacterial count (Table 1; Fig. 1). In

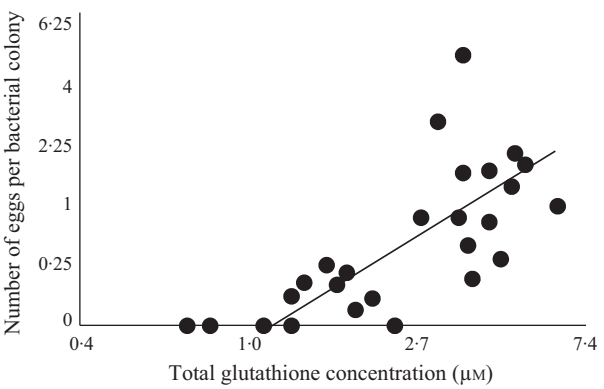

Fig. 1. Relationship between glutathione concentration and infection tolerance – specifically, the number of eggs laid by *Gryllus texensis* per colony of bacteria, *Serratia marcescens*. The *x*- and *y*-axes are formatted to reflect the manner in which data were transformed for statistic analyses (i.e. via natural logarithm and square root, respectively).

Table 1. Partial correlations among egg laying, bacterial counts and glutathione (GSH) concentration in *Gryllus texensis* challenged with the bacterium, *Serratia marcescens*. Correlation coefficients are displayed, and all d.f. = 24

	GSH concentration (μM)	Egg laying (number of eggs)
Egg laying (number of eggs)	0·74*	
Bacterial count (number of colonies)	−0·28	−0·058

The only significant relationship was the one between egg laying and GSH concentration after controlling for bacterial count.
*$P < 0{\cdot}001$.

uninfected females, egg number was uncorrelated with GSH concentration in the haemolymph (Pearson's correlation, $R = 0{\cdot}12$, $P = 0{\cdot}56$, $n = 27$).

Total PO activity of haemolymph was affected by food availability and was positively correlated with concentrations of GSH concentration and protein in the haemolymph, (Table 2a; Fig. 2a). GSH concentration was negatively correlated with total circulating protein and positively correlated with total PO activity of the haemolymph (Table 2b; Fig. 2b). The PO:GSH ratio was nearly correlated with total circulating protein (Table 2c), but it was not significantly affected by treatment – that is, food availability, immune activation and the food × immune interaction were not significant and removed from the final model. Total circulating protein was positively correlated with food availability (Table 2d; Fig. S1, Supporting information).

Table 2. Final linear mixed models describing the effects on (a) total phenoloxidase (PO) activity of haemolymph, (b) total glutathione (GSH) concentration, (c) ratio between PO and GSH (PO:GSH) and (d) total circulating protein

	d.f.	*F*	*P*
(a) Total PO activity			
Food availability	1,63	4·2	0·045
Circulating protein	1,63	4·3	0·041
GSH	1,63	25	<0·001
Femur length	5,63	0·18	0·68
(b) GSH concentration			
Circulating protein	1,64	1·0	0·31
PO	1,64	20	<0·001
Femur length	1,64	0·72	0·41
Cohort*	5,64	3·5	0·008
(c) PO:GSH			
Circulating protein	1,65	3·5	0·067
Femur length	1,65	0·56	0·46
Cohort*	5,65	1·5	0·20
(d) Circulating protein			
Food availability	1,75	5·3	0·045
Femur length	1,75	0·89	0·35
Cohort*	5,75	4·7	0·001

*Random effect.

Survival to paraquat treatment was significantly affected by immune activation (immune-challenged crickets were more likely to survive: Wald $\chi^2 = 11$, d.f. = 2, $P = 0{\cdot}005$) and an interaction between food availability and immune activation (Wald $\chi^2 = 6{\cdot}6$, d.f. = 2, $P = 0{\cdot}037$), but it was not independently influenced by food availability (Wald $\chi^2 = 0{\cdot}43$, d.f. = 1, $P = 0{\cdot}51$) or femur length (Wald $\chi^2 = 2{\cdot}3$, d.f. = 1, $P = 0{\cdot}13$) (Fig. 2c).

Discussion

After controlling for bacterial count, crickets infected with *S. marcescens* laid more eggs if they had a higher GSH haemolymph concentration. In other words, given the same number of proliferating bacteria, females with more GSH in their haemolymph were better able to maintain egg output. This result suggests that haemolymph GSH could be a mechanism of infection tolerance. More definitive evidence that GSH is a mechanism of infection tolerance would require manipulating GSH concentrations. Nevertheless, this report is one of the few that provides evidence of a plausible mechanism of infection tolerance that also influences immune resistance (Matsuki *et al.* 2008; Clark, Lu & Strand 2010).

Phenoloxidase (a component of immune resistance) and GSH (a likely component of infection tolerance) exhibited a strong positive relationship to one another – that is, individuals with high PO activity (i.e. heavy investment into a resistance mechanism that generates damaging reactive molecules) also had high levels of GSH. One explanation for this result is that increased investment in melanization capacity (and, thus, PO activity) obligates a concomitant increase in potential components of infection tolerance (e.g. GSH concentration) to reduce self-damage. Yet, the literature has examples of both positive and negative relationships between immune resistance and infection tolerance at the organismal level (e.g. Raberg, Sim & Read 2007; Howick & Lazzaro 2014). Predicting how immune resistance and infection tolerance should relate to one another (e.g. trade-off vs. positively correlated) is complicated by the multiplicity of mechanisms underlying these two traits. Although researchers have called for a better description of these mechanisms (e.g. Howick & Lazzaro 2014), our results demonstrate that biologists' understanding of these concepts requires further development. The potential mechanisms underlying infection tolerance (see Ayres & Schneider 2012) have multiple functions. For example, GSH is upregulated during both bacterial and insecticidal challenge (Huang, Wu & Ye 2011) meaning GSH is also be a key molecule in the detoxification of noxious chemicals. Similarly, the protein apolipophorin III is best known as an insect lipid transport molecule (Weers & Ryan 2006), but it is also involved in both immune resistance (e.g. Whitten *et al.* 2004) and infection tolerance (e.g. as an antioxidant: Seo, Park & Cho 2008). In fact, unlike mechanisms of immune resistance that often appear to be dedicated to pathogen eradication (e.g. attacin), all

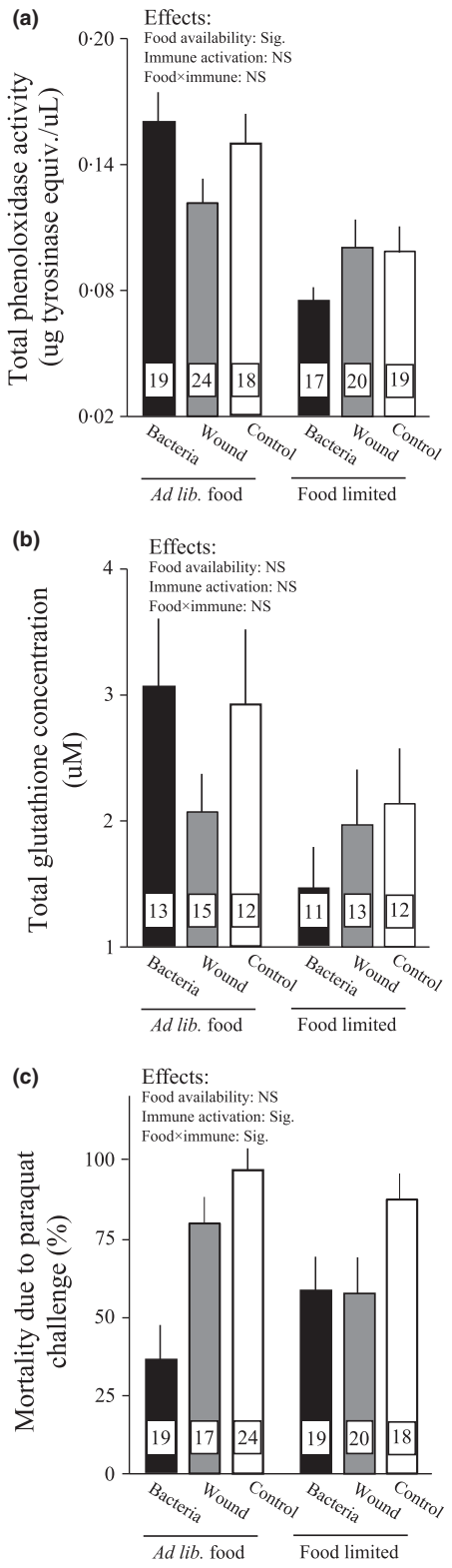

Fig. 2. Effects of treatment on a type of immune resistance [phenoloxidase (PO) activity], a type of tolerance mechanism [glutathione (GSH) concentration] and a proxy for infection tolerance (mortality due to an oxidative stressor). (a) Total PO activity of haemolymph, (b) total GSH concentration of haemolymph and (c) mortality due to paraquat (an oxidative stressor) exposure of *Gryllus texensis* that experienced differential access to food (*ad libitum* or limited) and were exposed to a bacterial challenge, a wound (sham injection) or no immune challenge (unhandled control). Sample sizes of treatment groups are represented on each bar. Values are displayed as mean ± SEM. Note: The results of full statistical models (e.g. including covariates and test statistics) for PO and GSH are reported in Table 2.

hypothesized mechanisms of infection tolerance across taxa are also necessary for other functions. For example, GSH is important for a wide range of metabolic processes (Meister & Anderson 1983). The enzyme alkaline phosphatase is another suggested mechanism of infection tolerance (Ayres & Schneider 2012). It removes phosphate groups from many types of molecules and plays a role in a variety of physiological functions in addition to infection tolerance (e.g. during reproduction: Lei *et al.* 2013). Similarly, Bessede *et al.* (2014) found that an aryl hydrocarbon receptor is important for infection tolerance. This same receptor also plays a role in normal development (Bessede *et al.* 2014). Thus, mechanisms of infection tolerance are likely to be more pleiotropic than those underlying immune resistance. This difference between immune resistance and infection tolerance will have ramifications for the evolution of both sets of mechanisms, including dynamics of co-regulation or co-evolution.

Not unexpectedly, haemolymph protein concentration was greater when food was readily available (Fig. S1, Supporting information). However, greater haemolymph protein concentration correlated with *lower* total GSH concentration. Unfortunately, this result is difficult to interpret when the identity of the proteins that are increasing in concentration is unknown. For example, it is possible that when proteins that can also function as antioxidants [e.g. vitellogenin (Havukainen *et al.* 2013) or apolipophorin III (Seo, Park & Cho 2008)] increase in concentration, less GSH is synthesized. Food availability also influenced total PO activity (Fig. 2a), but its effect was not strong enough to shift the highly significant correlation between PO and GSH. This result is similar to recent research demonstrating a positive relationship between immune resistance and infection tolerance, although the strength of that relationship was sensitive to nutrition (Howick & Lazzaro 2014). Clearly further work is required to better understand the robustness of the PO:GSH ratio. For example, it would be interesting to explore whether the ratio between the two molecules is actively maintained.

As predicted, total PO (a measure of resistance) was affected by food availability – total PO activity was lower in food-limited females (Fig. 2). This supports a growing body of literature demonstrating the strong role of nutrition in immune function ('nutritional immunology', reviewed in Ponton *et al.* 2013). However, contrary to our prediction, GSH was not reduced by our food limitation paradigm, even though this level of food limitation dramatically reduces egg production (Stahlschmidt *et al.* 2013). The lack of effect of food limitation on GSH differs from work in vertebrates that indicates a link between GSH concentration and fat content (reviewed in Grim

et al. 2011), and it also contrasts with studies demonstrating that host nutrition influences tolerance when measured at the organismal level in other invertebrates (Ayres & Schneider 2009; Vale *et al*. 2011; Howick & Lazzaro 2014). However, resistance to PQ was also maintained during food limitation. This result suggests that, when resources were short, resistance to oxidative stress was prioritized more than PO-dependent immune defences. Further studies should continue to investigate this effect under a range of dietary regimes. The quantity and composition (e.g. protein:carbohydrate ratio) of food influences immunocompetence, immune resistance and infection tolerance in a complex manner in other systems. For example, depriving *Drosophila* of food increases their infection tolerance to one pathogen (*Salmonella typhimurium*) while reducing their immune resistance to another pathogen (*Listeria monocytogenes*) (Ayres & Schneider 2009).

Chronic immune activation did not affect PO activity, but it did increase oxidative stress (PQ) resistance, a predicted mechanism of infection tolerance (Ayres & Schneider 2012) (Fig. 2a,c). Our result for PO was not unexpected because the effect of chronic immune challenge on PO activity is variable across insects (Gonzalez-Santoyo & Cordoba-Aguilar 2012). However, to our knowledge, we are the first to demonstrate that immune-activated individuals can also be 'primed' for future oxidative stressors unrelated to the immune response. Upregulation of GSH contributes to insect resistance to organophosphate insecticides (reviewed in Ketterman, Saisawang & Wongsantichon 2011). Thus, GSH concentration would be expected to be higher in crickets that were chronically immune challenged because these crickets were more likely to survive a PQ challenge – yet, we did not find this to be the case in *G. texensis* (Fig. 2b). Either other, nonmeasured antioxidants (e.g. superoxide dismutase or catalase) or repair mechanisms (e.g. heat-shock proteins) were constitutively upregulated. Clearly, future work is required to fully understand the link between chronic immune activation and oxidative stress resistance.

To conclude, we provide evidence that GSH may be a mechanism of infection tolerance in crickets, and we bring new insight into the balancing act that individuals make with regard to investment into immune resistance and infection tolerance mechanisms. We found that PO and GSH are positively related in crickets. Further, we show that although food availability and chronic immune activation variably influence both PO activity and GSH levels, neither treatment affects the positive relationship between these two measures. At least within the parameters tested, these two components of immune resistance and infection tolerance remain in balance. We advocate further research into the mechanisms underlying this relationship, as well as on the effects of an immune phenotype (e.g. an individual with high PO and GSH vs. an individual with low PO and low GSH) on other life-history traits, such as behaviour (Niemela *et al*. 2012) or reproduction (Roff & Fairbairn 2013).

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Data accessibility

Data for this study are accessible at the Dryad Digital Repository (doi: 10·5061/dryad.h0d12) (Stahlschmidt *et al*. 2015).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. Effects of treatment on total circulating protein of *Gryllus texensis* that experienced differential access to food (*ad libitum* or limited) and were exposed to a bacterial challenge, a wound (sham injection) or no immune challenge (unhandled control).