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Pathogen and immune dynamics during maturation are explained by Bateman's Principle

CHARLOTTE V. L. MILLER[1,2] and SHEENA C. COTTER[2] [1]School of Biological Sciences, Medical Biology Centre, Queen's University Belfast, Belfast, U.K. and [2]School of Life Sciences, Joseph Banks Laboratories, University of Lincoln, Lincoln, U.K.

Abstract. 1. Bateman's principle, stating that female fitness is maximised by increasing longevity, suggests females are likely to exhibit a stronger immune response than males. Applying these ideas to maturation, it is predicted that immature beetles will also show enhanced immunity. This study looks at how life-history strategies interplay with infection responses in a well-established immunological model, the burying beetle, *Nicrophorus vespilloides* (Herbst (Silphidae)), and a pathogenic bacteria, *Photorhabdus luminescens* (Thomas and Poinar (Enterobacteriaceae)).

2. To test this, the immune enzyme phenoloxidase (PO) and bacterial loads in sexually mature and immature beetles were tested over time after injection with *P. luminescens*, and survival was monitored. Breeding after infection was also tested.

3. Bacterial loads were lower in females than males, and clearance seems more prolonged in immature than mature beetles, and both these groups showed increased survival, supporting the application of Bateman's principle to the effects of maturation and sex on immunity.

4. Mature beetles were found to undergo a shorter period of PO suppression after injection with bacteria. However, while high PO was beneficial up to 20 h post-infection, it became detrimental after that. This temporal factor has rarely been investigated but is shown here to be influential on the interpretation of PO activity, which is generally perceived as beneficial after infection.

5. A trade-off between reproduction and immunity was also found, revealed only by a highly pathogenic infection. This contrasts with the effects found in studies using non-pathogenic bacteria, suggesting an enforced, resource-based trade-off is the driving force behind the change, rather than an adaptive strategy.

Key words. Ecological immunology, insect, *Nicrophorus vespilloides*, pathogen dynamics, phenoloxidase, *Photorhabdus luminescens*.

Introduction

Variation in life-history creates differing selection pressures on individuals. For example, females (due to larger gamete size) usually have more to lose if a single reproductive event fails, resulting in sex-specific selection pressures on parental investment (Parker *et al.*, 1972). Bateman's principle suggests that as female reproduction is limited by time, whereas male reproduction is limited by mating opportunities, females should invest more in longevity than males (Rolff, 2002). Correspondingly, there is evidence of increased investment in immunity in females across a range of species (Rolff, 2001; Cotter *et al.*, 2008; Steiger *et al.*, 2011).

As organisms of different ages also differ in reproductive capacities, they are similarly subject to different selective pressures. Organisms need to survive to reproductive maturity to achieve more than zero fitness unless kin selection results in social breeding efforts that provide inclusive fitness. To ensure they reach maturity, organisms must invest in defence against parasites, which inflict a perpetual pressure on all organisms (Haldane, 1992). According to Bateman's principle, immature organisms can be expected to invest more heavily in immunity, to ensure future reproductive opportunities, whereas mature organisms may invest more in current reproduction to the detriment of immune function (Rolff, 2001). This investment may be based on limited resources such as time or nutrition, and

Correspondence: Charlotte V. L. Miller, School of Life Sciences, University of Lincoln, Joseph Banks Laboratories, Green Lane, Lincoln, U.K. LN6 7DL. E-mail: chmiller@lincoln.ac.uk

so result in a trade-off between reproductive and immune traits (Reavey *et al.*, 2014a). For example, there was found to be a relationship between wing-spot melanisation (a sexually-selected trait) and activity levels of the immune enzyme phenoloxidase (PO) in the damselfly, *Calopteryx splendens* (Harris (Calopterygidae)), indicating that the process the two traits rely on (melanisation) is limited by a restricted substrate (Siva-Jothy, 2000).

However, other explanations for the decrease in immune function seen at maturity have been suggested, such as the immunocompetence handicap hypothesis. This states that the suppression of immune function by sex hormones, such as juvenile hormone in insects (Rolff & Siva-Jothy, 2002; Rantala *et al.*, 2003), makes secondary sex signals, such as pheromone production in male *Tenebrio molitor* (Linnaeus (Tenebrionidae)), an 'honest' reflection of individual condition (Rantala *et al.*, 2003). However, evidence of 'cheating' in *Tenebrio molitor* pheromone signalling in response to an immune challenge suggests that perceived longevity, as well as condition, is influential on how this trade-off is managed (Sadd *et al.*, 2006).

The costs of having an immune system are the result of the energetic and nutritional demands of both maintaining immune responsivity and reacting to a pathogen threat (Siva-Jothy & Thompson, 2002). The costs of immunity also mean that the timing and duration of the immune response are important as costs can be minimised by reducing the duration of the upregulation. Faster reaction times to infection may allow hosts to more easily clear infection before the parasite population increases. However, reducing the duration of immune mechanisms may result in the survival of more resistant phenotypes within the infecting population, and so persistent, resistant infection. The temporal interaction of different immune parameters, for example delaying some responses, may prevent this (Haine *et al.*, 2008). Therefore, the timing and duration of the immune responses are subjected to selective pressures, but a widely ignored aspect of immunity.

Costs also result from the risk of the immune system causing damage to the host as well as the pathogen, as a result of recognition failures or non-specific immune actors being part of the immune defences. For example, insects rely heavily on the PO enzyme cascade, which provides an extremely reactive resistance to infection (González-Santoyo & Córdoba-Aguilar, 2012). It is activated by the molecular patterns common to a range of pathogens such as lipopolysaccharide or peptidoglycan, and its activation causes the release of reactive oxygen species (Cerenius *et al.*, 2008). These are indiscriminately cytotoxic, and have been shown to be measurably costly to the host when activated (Sadd & Siva-Jothy, 2006). However, the comprehensive action of PO makes it highly effective as an immune response, and it is also important in other immune defences, such as the cellular immune defences. When a particularly large pathogen, such as a clump of bacteria or a parasitoid egg is recognised, haemocytes surround it and melanise through PO action (the processes of nodulation or encapsulation), cutting the pathogen off from host tissues and resources, and subjecting it to cytotoxic reactive oxygen species (Lavine & Strand, 2002). In cases of smaller invaders such as single bacterial cells, some haemocytes can carry out phagocytosis, engulfing the bacteria entirely (Lavine & Strand, 2002). PO is also involved in the melanisation of the primary defence against pathogens, the cuticle which has been demonstrated to confer functional survival effects in the face of infection (Barnes & Siva-Jothy, 2000).

In the present study, a model host–parasite system is used to explore how sex and reproductive maturity affect the response to infection in terms of investment in immunity versus reproduction. The burying beetle, *Nicrophorus vespilloides*, spends the first 2 weeks of its adult life in a pre-reproductive state. Once mature, it can only reproduce if a breeding resource, in the form of a small vertebrate carcass, is available (Pukowski, 1933). Upon locating a carcass, the juvenile hormone is upregulated rapidly, instigating egg maturation and inducing the parental behaviour necessary for breeding (Trumbo, 1997). These parental behaviours include preparation of the carcass by burying it, stripping it of fur or feathers and coating it with oral and anal secretions. These secretions contain lysozyme (Rozen *et al.*, 2008; Cotter *et al.*, 2010; Arce *et al.*, 2012; Palmer *et al.*, 2016), antimicrobial peptides (Jacobs *et al.*, 2016), and other antimicrobial molecules (Degenkolb *et al.*, 2011), which protect the breeding resource, and so the offspring feeding on it, from microbial competition (Hall *et al.*, 2011). Due to the protection provided to offspring, this is classified as an immune social effort (Cotter & Kilner, 2010), but it is down-regulated in response to wounding (Cotter *et al.*, 2013), signalling a trade-off between personal immunity and social immunity.

If carcasses (and so breeding resources) are very rare, the fitness benefits of surviving to the detriment of the current brood may be less than those of raising one brood now and dying. Indeed, reproduction has been shown to be traded off with the immune system in *N. vespilloides*, as PO activity is downregulated during a breeding bout (Reavey *et al.*, 2014a). However, if the individual experienced a non-pathogenic immune challenge early in the breeding bout, the immune response was upregulated to the detriment of breeding success (defined by larval number) (Reavey *et al.*, 2014a), suggesting that beetles prioritise their health over that of the current brood.

The parasite used, *Photorhabdus luminescens,* is a bacterial symbiont of an entomopathogenic soil nematode (*Heterorhabditis bacteriophora* (Poinar (Heterorhabditidae)), which seeks out and invades insects in the soil, carrying the bacteria in its gut (Ciche & Ensign, 2003). The nematode can invade through the mouth, anus, trachea or by breaking open the cuticle, and then regurgitate the bacteria carried in its intestine (Ciche & Ensign, 2003). The bacteria then aggregates in the midgut of the host insect (Silva *et al.*, 2002) and produces a cocktail of insecticidal toxins which result in apoptosis in the midgut and haemocytes (Daborn *et al.*, 2002), and the suppression of phagocytosis (Silva *et al.*, 2002), PO activity in the haemolymph, and related nodulation activity (Eleftherianos *et al.*, 2007). The suppression of PO was shown to be the result of a molecule in the bacterial cell wall, and when transferred to a different bacterium, it conferred pathogenicity to the previously un-pathogenic *Escherichia coli* (Migula (Enterobacteriaceae)) in *Manduca sexta* (Linnaeus (Sphingidae)) larvae (Eleftherianos *et al.*, 2009). PO is a key defence against this pathogen; a *P. luminescens* strain that is unable to suppress PO activity showed drastically reduced virulence against *Manduca sexta* larvae, with

host survival extended by around 100 h (Eleftherianos *et al.*, 2007). *Photorhabdus luminescens* is highly pathogenic, and has been shown to have an LD50 at 72 h of less than 40 cells in several insect species (Khan & Brooks, 1977; Milstead, 1979), however, in *N. vespilloides*, the LD50 over 8 days lies around 300 000 cells (C. Miller, unpublished data), suggesting that the beetles employ a robust immune defence when infected.

Using the *Nicrophorus vespilloides*/ *Photorhabdus luminescens* system, the timing of infection and immune responses in pre-reproductive and mature beetles and how these affect reproduction were investigated. Specifically, it is asked:

1 Do pre-reproductive beetles show a more robust immune response to infection than mature beetles, leading to lower bacterial loads and higher phenoloxidase activity in immature beetles?
2 Do females show a more robust immune response than males, leading to lower bacterial loads and higher phenoloxidase activity in females?
3 Do infected beetles increase their investment in reproduction in response to infection, and so their likelihood of survival?

Materials and methods

Nicrophorus vespilloides colony

A population of *N. vespilloides* was established using individuals sourced from woods near Lincoln, England and maintained in the lab for five generations prior to experiments. Each beetle was given a unique number and kept individually in $12 \times 8 \times 2$ cm boxes under an LD 16:8 h cycle at 21 °C. The population was maintained by breeding non-siblings. These non-sibling pairs were each provided with a mouse carcass of a recorded weight (12.89 *g* +/− SD 1.99) and placed in a larger box ($17 \times 12 \times 6$ cm) two-thirds filled with damp soil and placed in darkness to replicate the natural breeding conditions of the species.

Around 8 days after the pairs were set up, the resulting larvae disperse from the carcass into the surrounding soil. At this point, the parents were removed, and the larvae weighed and counted. Each larva was placed into a cell of a 25-cell Petri dish (discarding excess individuals) to pupate and covered with damp soil, with a different Petri dish for each family. Approximately 20 days later, the offspring eclose, and around 15 days after that they would reach sexual maturity.

Adult beetles were fed with beef mince twice a week to allow *ad libitum* feeding. Experimental beetles and their offspring were not used in breeding for the colony to avoid possible confounding effects on later experiments due to immune priming. As the beetles reach sexual maturity at around 15 days old, experiments were carried out using virgin 19- to 22-day-old beetles, ensuring all individuals were mature and of a similar breeding status.

Experiment 1: Reproductive maturation and infection dynamics

Immature (7–10 days post eclosure) and mature (21 days post eclosure) virgin beetles were injected with approximately 260 000 *P. luminescens* cells in 1 μl of nutrient broth. Beetles were then placed in their standard housing with dampened paper in place of soil for the duration of the experiment to reduce the likelihood of environmental contaminants entering their wounds. Haemolymph samples were taken from 30 previously unsampled individuals at 0, 15, 30, 45, 60 and 75 h after infection (15 per age group, approximately 50 : 50 male : female in each age group), using new individuals for each time point. The haemolymph samples were then used to quantify the number of *P. luminescens* cells infecting the organism (see below for details).

Experiment 2: Immune response to bacterial proliferation

To test the immune response of the host to *P. luminescens* infection, 7 or 21 days after eclosion, beetles were injected with 100 000 bacterial cells in 1 μl of nutrient broth then housed as described above. At 0, 5, 8, 15, 25, 40, 50 and 65 h after injections haemolymph samples were taken from 15 previously unsampled individuals (approximately equal numbers of males and females in each age group of 15, with no resampling). Haemolymph was also taken once from 20 individuals (10 males, 10 females) of the two ages used in the experiment (10 mature, 10 immature) that were not immune treated. Samples were frozen for later assays, which tested the PO enzyme activity of each of these samples (see below for details). Survival of the experimental beetles was subsequently monitored. Previous studies have shown that survival in uninfected beetles is not impacted by haemolymph sampling (Cotter *et al.*, 2013; Reavey *et al.*, 2014a). Adult beetles typically live well over 3 months in the lab under the conditions of the experiment (clean boxes with dampened paper only as a substrate) and survival monitoring ended well before this time so survival data were not collected for the control beetles. However, we have included average survival data of uninfected beetles from the lab colony which were kept under the same conditions. These data were collected independently as part of another study at another time and so are included for visual comparison only.

Experiment 3: Infection, social immunity and subsequent breeding

Fifty-five, 21-day-old, adult, virgin female *N. vespilloides* beetles were injected with one of three immune treatments, two consisting of injections of 1 μl of nutrient broth, the other consisting of handling for an equivalent period (20 females). The two injection treatments were either sterile nutrient broth (15 females) or nutrient broth containing 3 700 000 *P. luminescens* cells (20 females). The females were then immediately set up with a mouse carcass (weighing 12.89 *g* +/− SD 1.99) and a male, and allowed to breed. Their exudate was collected 3 days later and tested for lysozyme content (see below for details). The resulting breeding success was recorded by measuring the number of larvae, brood mass, and the average mass of one larva in the offspring produced.

Injection challenge

Beetles were injected with 1 μl of sterile nutrient broth. In bacterial treatments, the nutrient broth was mixed to the appropriate cell density with an overnight culture (incubated at 30 °C and 2.24 × *g*) of *Photorhabdus luminescens* (strain TT01).

Doses were estimated from the overnight culture using spectrophotometry and dilution to the appropriate dose. Accurate counts of live cells take 24 h and so are only available after treatment. The discrepancy between the estimated dose and the actual dose is a combination of the precision of the density estimate, which is expected to contain some error and the fact that the density estimate will include inviable cells, which are not counted using the plate technique. Treatments were administered by injection with a Hamilton syringe through the pleural membrane between the third and fourth abdominal sternites on the right dorsal side of the beetle. The beetle was wiped with ethanol before and after injection to prevent contamination during the process and to reduce haemolymph loss.

To control for the effect of the injection treatment, some beetles were handled for an equivalent amount of time to induce the stress this may cause. Others were injected but with a sham treatment, consisting of sterile nutrient broth.

Haemolymph collection

Haemolymph was collected from beetles by holding the beetle firmly and piercing the pleural membrane beneath the pronotum, between the thorax and abdomen with a sterile needle. The internal pressure of the beetle forced haemolymph to extrude. The droplet which formed could then be pipetted off. Due to the high levels of melanin, the haemolymph of *N. vespilloides* beetles contains, the sample must be either used quickly (where bacterial quantification was carried out) or mixed with an equal amount of PBS EDTA buffer and frozen at −20 °C to prevent coagulation (to carry out PO assays).

Bacterial quantification in beetle haemolymph

One micro liter of fresh haemolymph was pipetted onto one of four sections of a 0.005 *g* per 100 ml Rifampicin 1% agar plate. Including rifampicin in plates reduced the risk of contamination of the sample by a different bacterial species present constitutively or coincidentally in the beetle's haemolymph. Another 1 μl of fresh haemolymph was also 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. 10 μl of this was then removed and added to another 90 μl of sterile nutrient broth, creating a run of three serial dilutions.

These were then gently inverted, and 10 μl of each was pipetted onto the remaining three sections of the rifampicin agar plates and spread. The plates were then incubated overnight at 30 °C, and the colonies in the section of the plate containing between 20–200 colonies were counted. The dilution of that section was then used to calculate the original concentration of the bacteria in 1 μl haemolymph.

PO quantification

To quantify PO enzyme levels in haemolymph, the frozen blood sample (mixed 50 : 50 with phosphate-buffered saline EDTA at the time of collection) was vortexed until melted, then 3 μl of it was added to 500 μl of chilled sodium cacodylate buffer and vortexed again. One hundred microliters of this was added to multiple wells of a 96 well plate on ice. One hundred microliters 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 activity and therefore the quantity of the PO enzyme.

Exudate collection

Exudate was collected from adult beetles using a capillary tube. Gently tapping the ventral side of the tip of the beetle's abdomen usually causes the beetle to extrude a small amount of the exudate, which can then be picked up in the capillary tube. The sample can then be gently blown into an Eppendorf and stored at −20 °C until analysis for lytic activity.

Lysozyme quantification

Exudate samples were defrosted, then centrifuged at 11337 × *g* for 3 min to remove any dirt included in the exudate. One microliter of the resulting supernatant was pipetted into 1 mm diameter wells in agar containing lyophilized *Micrococcus luteus*. This was incubated overnight at 34 °C, and the diameter of the resulting zone of clearance was measured in two directions, taking the average as the final measurement. By comparing this to the measurements of a series of standard concentrations of pure lysozyme, the lysozyme activity of the exudate could be calculated.

Statistical analysis

PO activity data and bacterial count data were logged. Data were analysed using general linear models and stepwise removal of variables. All analyses were carried out in the statistical software R (version 2.15.3).

Results

Experiment 1: Reproductive maturation and infection dynamics

Bacterial proliferation assays were carried out in beetles of two different ages (7–10 days old and 21 days old). Bacterial load decreased over time (time: $F_{1, 158} = 33.256$, $P < 0.001$, time^2: $F_{1, 158} = 14.777$, $P < 0.001$), but this was more rapid in immature compared to mature beetles (age: $F_{1, 158} = 0.007$, $P = 0.932$; time^2*age: $F_{1, 158} = 4.897$, $P = 0.028$; Fig. 1a,b) and bacterial loads appear to start increasing again in mature

beetles after 60 h while still decreasing in immature beetles. Females had lower bacterial loads than males (sex: $F_{1, 158} = 7.245$, $P = 0.008$) and this was unaffected by time since injection (time*sex: $F_{1, 158} = 0.423$, $P = 0.321$) or age (age*sex: $F_{1, 158} = 0.059$, $P = 0.711$). The three-way interaction term did not significantly affect the model fit (age*sex*time: $F_{1, 152} = 1.297$, $P = 0.257$). While differences in bacterial load between males and females appear small in Fig. 1a,b, it must be noted that this is merely the bacterial load in a single microlitre of blood, displayed on a log scale, and each beetle contains around 20 μl of blood, making small differences in this measurement highly significant.

Experiment 2: Personal immune response to injection with Photorhabdus luminescens

The internal immune response of adult *N. vespilloides* beetles to injection with *P. luminescens* was assessed by measuring how much PO enzyme activity there was in the blood of infected beetles over the 65 h after treatment. PO activity decreased over time (time: $F_{1, 210} = 27.221$, $P < 0.001$; time^2: $F_{1, 210} = 26.932$, $P < 0.001$) until around 30 h in the mature beetles, and 40 h in immature beetles (see Fig. 2a). PO activity then increased, resulting in a quicker recovery to constitutive levels in mature beetles (age: $F_{1, 210} = 4.537$, $P = 0.034$; time*age: $F_{1, 210} = 10.748$, $P = 0.001$). There was no difference in the PO response between the sexes (sex: $F_{1, 210} = 0.003$, $P = 0.955$).

Survival was found to be affected by the organisms' PO level depending on the time the haemolymph was sampled (PO: $F_{1, 203} = 5.951$, $P = 0.015$; PO*time: $F_{1, 203} = 23.374$, $P < 0.001$; PO*time^2: $F_{1, 203} = 18.731$, $P < 0.001$). In the initial 20 h after infection, high PO prolonged survival, but from 20 to 55 h, higher PO was detrimental to survival (Fig. 2b). Females survived considerably longer than males (sex: $F_{1, 203} = 4.714$, $P = 0.031$) but female survival varied less than male survival depending how long after infection the sample was taken (sex*time: $F_{1, 203} = 8.976$, $P = 0.003$; sex*time^2: $F_{1, 203} = 12.341$, $P < 0.001$, Fig. 2c). Immature beetles survived longer than mature beetles (age: $F_{1, 203} = 5.082$, $p = 0.025$, Fig. 2d), but this was more pronounced in the groups whose blood was sampled later (age*time: $F_{1, 199} = 7.959$, $P = 0.005$; age*time^2: $F_{1, 199} = 11.295$, $P < 0.001$, Fig. 2e).

Experiment 3: Infection, social immunity and subsequent breeding

Treatment with *P. luminescens* significantly affected the lysozyme content of a female's exudate produced while breeding (treatment: $F_{2, 46} = 15.075$, $P < 0.001$, Fig. 3a). If the female was infected with *P. luminescens*, the exudate lysozyme concentration was significantly lower than the exudate lysozyme concentration of females either receiving a sterile injection ($t = 3.834$, $P < 0.001$) or handling ($t = 5.142$, $P < 0.001$). The mass of the mouse carcass used for breeding had no effect on the female's exudate content (carcass mass: $F_{1, 45} = 2.685$, $P = 0.108$).

The immune treatment the female received was found to significantly increase the time taken for the breeding bout to be completed (treatment: $F_{2, 46} = 3.721$, $P = 0.032$), defined as the number of days between the exposure of the parents to the carcass and the dispersal of the offspring into the soil surrounding the carcass (see Fig. 3b). The offspring of females treated with a bacterial injection took significantly longer to disperse from the carcass than those who were treated with either a sterile injection ($t = 2.343$, $P = 0.023$) or just handling ($t = 2.308$, $P = 0.026$). The carcass mass did not affect the number of days taken to breed (carcass mass, $F_{1, 45} = 1.741$, $P = 0.194$).

The immune treatment also affected the efficiency of the use of the breeding resource (treatment: $F_{2, 46} = 21.533$, $P < 0.001$, Fig. 3c). Bacterially treated females produced a lower mass of larvae per gramme of carcass than handled mothers ($t = 6.553$, $P < 0.001$). Sterile injected mothers also produced a lower mass of larvae than handled mothers ($t = 3.892$, $P < 0.001$). Bacterial treated mothers produced broods with the lowest conversion rate. Carcass mass had no effect on the rate of conversion of mass into larval mass (carcass mass, $F_{1, 45} = 0.008$, $P = 0.929$).

The immune treatments had an impact on the number of offspring in the brood produced immediately after treatment (treatment: $F_{2, 45} = 13.659$, $P < 0.001$). Females which were handled produced on average 27 larvae per brood, whereas sterile-injected mothers produced 22 larvae, and bacterially treated mothers only 13 larvae. There was a significant difference between the handled and bacteria treated groups ($t = 5.020$, $P < 0.001$), and the sterile-injected groups also produced higher numbers of larvae than bacterially treated females ($t = 3.456$, $P = 0.001$). The number of larvae produced per brood was positively correlated with carcass mass (carcass mass: $F_{1, 45} = 4.470$, $P = 0.040$).

The average weight of a single larva in each brood was unaffected by the immune treatment the mother received (treatment: $F_{2, 46} = 0.363$, $P = 0.698$). Individual larval weight was also unaffected by the mass of the carcass they were raised on (carcass mass: $F_{1, 45} = 0.295$, $P = 0.589$). Infection reduced the total mass of the broods produced (treatment: $F_{2, 45} = 24.800$, $P < 0.001$, Fig. 3d), with broods produced by bacteria-treated mothers being lighter than handled mothers ($t = 6.938$, $P < 0.001$) and lighter than those produced by sterile-injected females ($t = 4.051$, $P < 0.001$). Brood mass was also affected by the mass of the carcass the brood was raised on (carcass mass: $F_{1, 45} = 15.197$, $P < 0.001$), with bigger broods being produced on larger carcasses, regardless of treatment the mother received.

Discussion

The response to infection showed different patterns in immature and mature beetles, and in males and females, although not all aspects of the immune response changed in the same way across these groups. Bacterial loads were lower in females, and immature beetles seemed to show more prolonged clearance, and both had better survival. This conforms to our predictions based on Bateman's principle. Both mature and immature

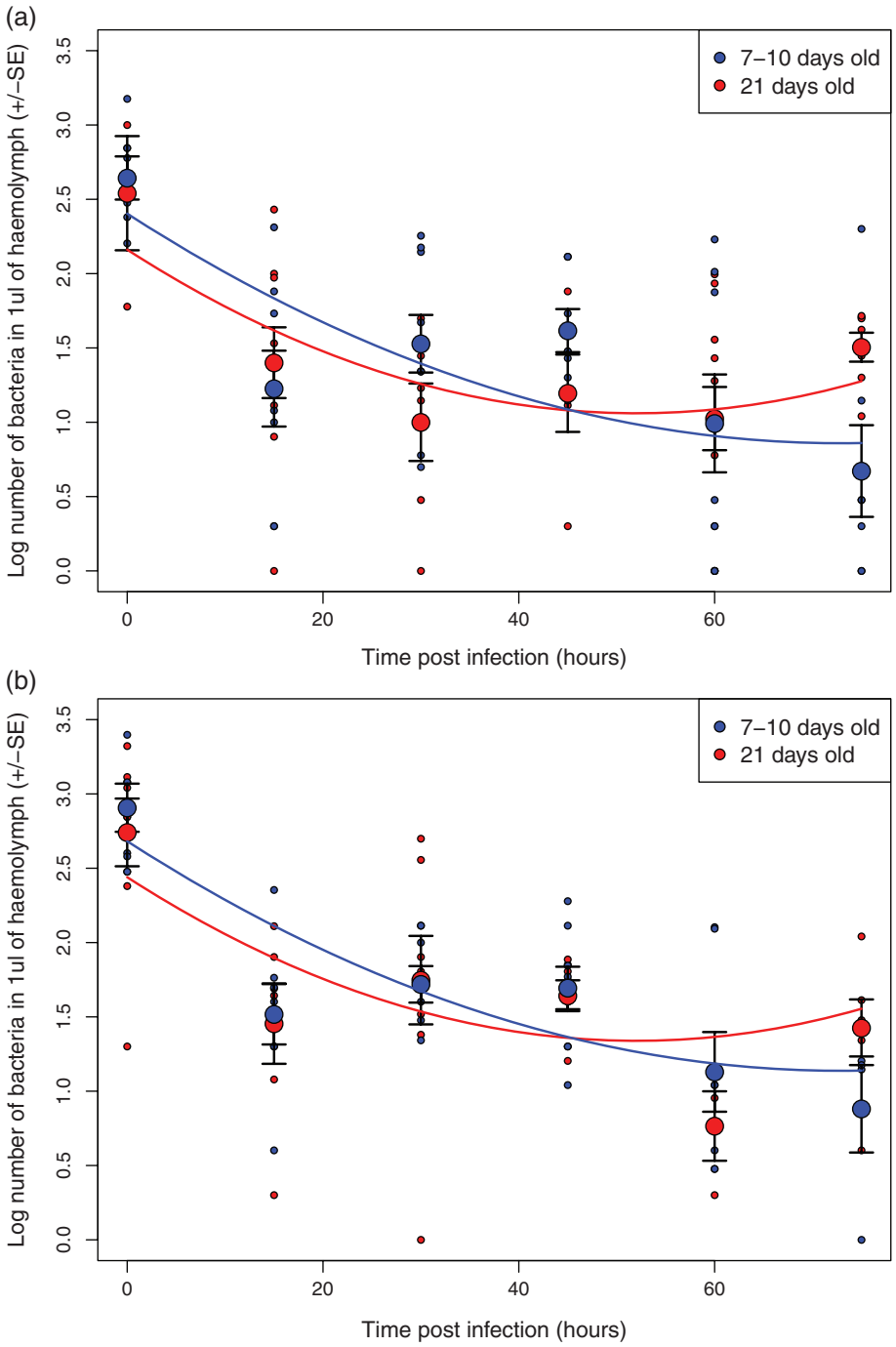

Fig. 1. The dynamics of *Photorhabdus luminescens* infection in the haemolymph of immature and mature (a) females, and (b) males over 75 h post infection.

beetles showed some suppression of PO activity in response to injection of bacteria (Fig. 2a), but certainly not the complete suppression was seen in *Manduca sexta* larvae (Eleftherianos *et al.*, 2007). Indeed, injection of an inactive immune elicitor has also been shown to suppress PO activity in this species of beetle partially, potentially as a result of a trade-off with concomitantly upregulated AMP activity, so this suppression is unsurprising (Reavey *et al.*, 2014a). PO activity was shown to recover more quickly in mature beetles, contradicting our prediction that immature beetles would have heightened immune responses. However, the effect of PO on survival in this study brings its perception as beneficial during infection into question.

The difference in pathogen loads between male and female beetles, with both immature and mature males carrying

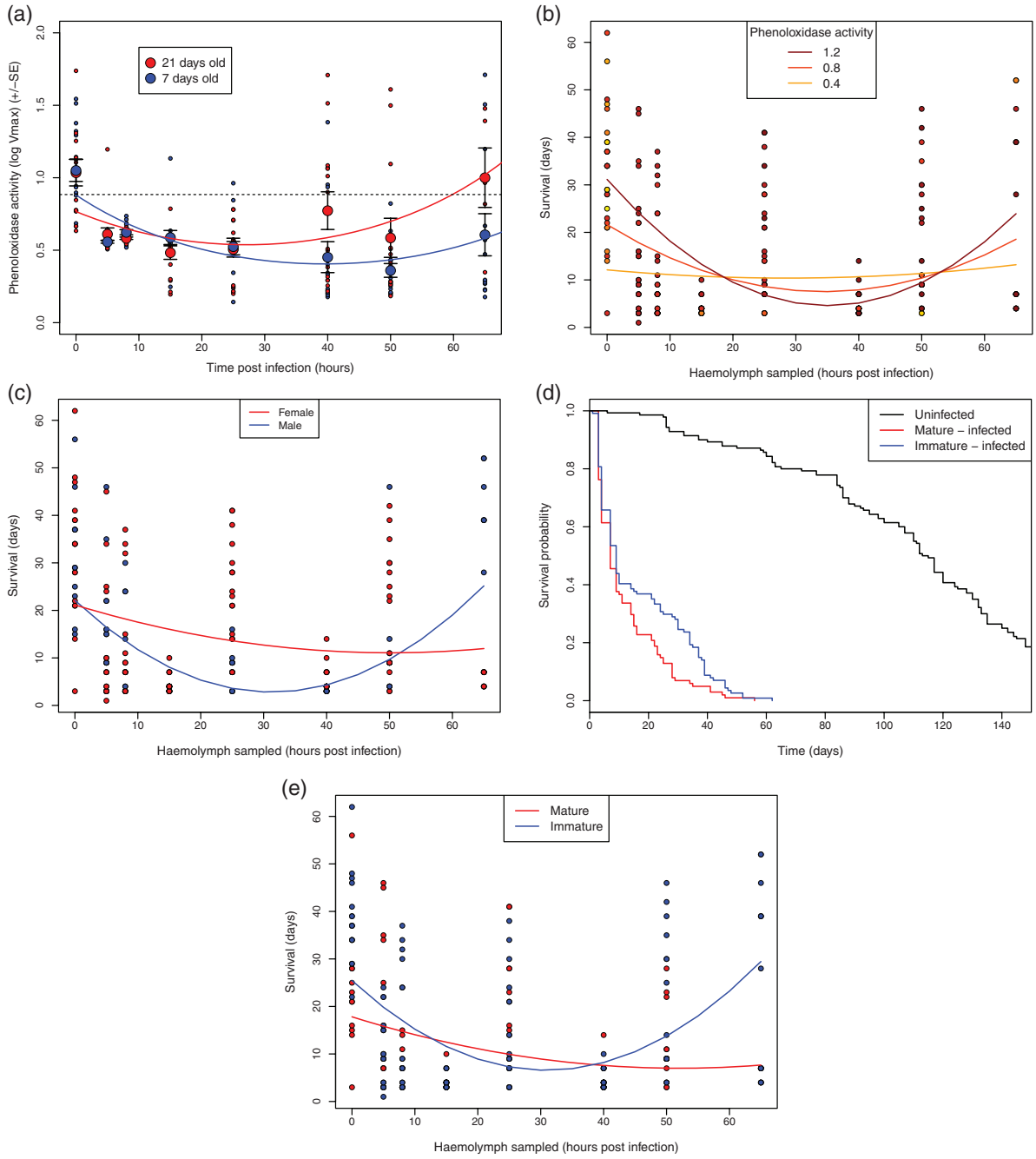

Fig. 2. The effects of infection on phenoloxidase (PO) activity and survival post infection. (a) PO activity in immature and mature beetles over 65 h post infection, (b) the effects of PO activity on survival, (c) survival differences between females and males, and (d) survival effects of age and (e) age with time sampled.

significantly higher numbers of bacteria than females, was accompanied by males succumbing more rapidly to infection. There were also differences in bacterial load between immature and mature beetles, with bacterial load decreasing up to 75 h post infection in immature beetles, wherea bacterial numbers decreased initially in mature beetles but levelled off at 40 h and seemed to begin to increase again at 50 h post infection. The rapid bacterial clearance in both age groups suggests that burying beetles have highly effective cellular responses to combat *P. luminescens* infection, unlike in *M. sexta,* where clearance is not apparent, and bacteria proliferate within the haemocoel until death (Eleftherianos *et al.*, 2007). The continued clearance in immature beetles suggests a more robust cellular response than in mature beetles, and this is supported by the increased

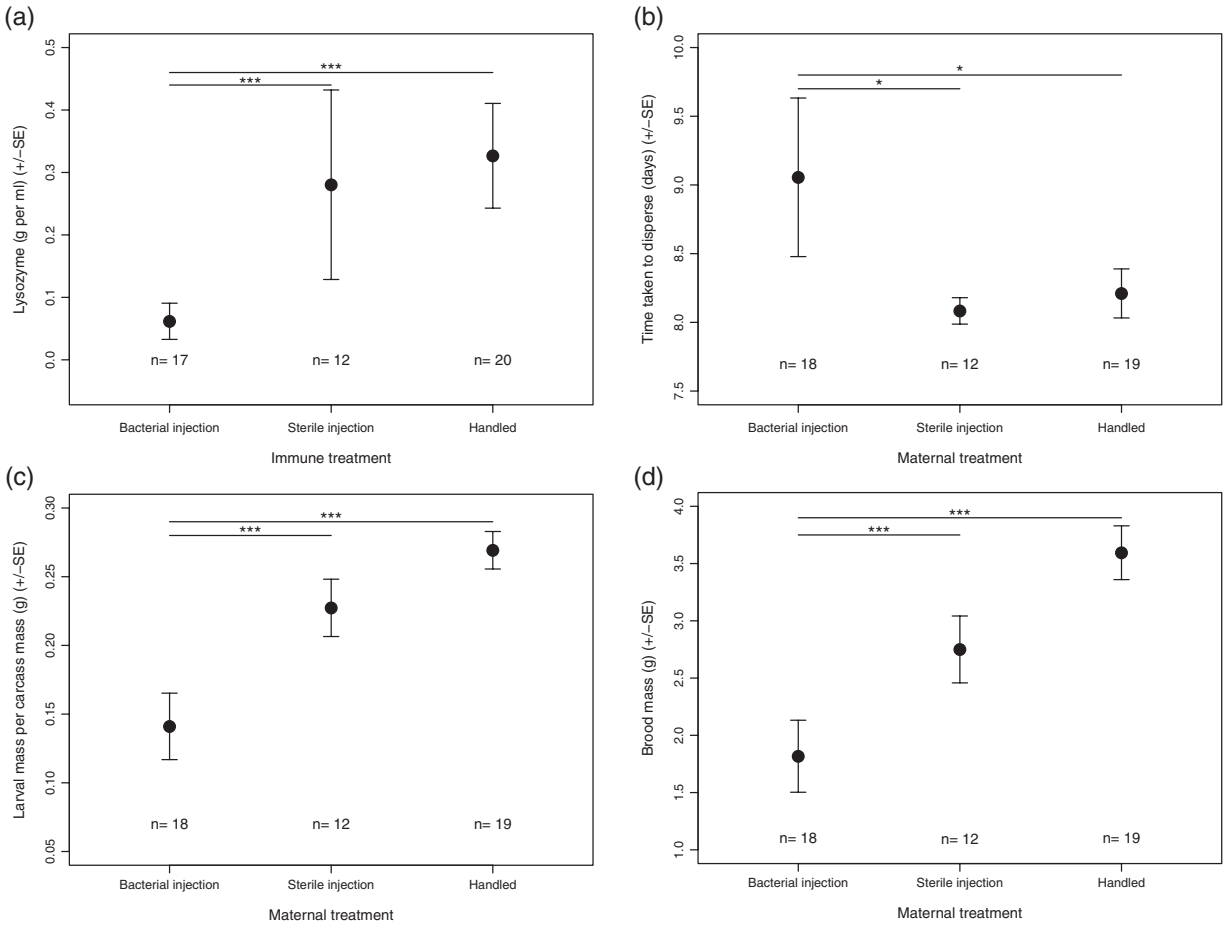

Fig. 3. The effects of *Photorhabdus luminescens* infection on (a) the social immune effort of breeding female *Nicrophorus vespilloides*, in terms of lysozyme activity of the anal exudate, (b) time taken for larvae to disperse from the carcass (c) the efficiency of conversion of carcass to offspring and (d) the total weight of broods produced from females from each treatment group.

survival of immature beetles. While there has been shown to be a decrease in cellular immunity in *N. vespilloides* between the pupal and adult stage (Urbański *et al.*, 2013), this has not been investigated within the adult life stage. The increase in bacterial load seen after 60 h in mature beetles is in agreement with other data (C. Miller, unpublished data), and this increase may be absent or delayed in immature beetles. This enhanced immunity in females has been found in multiple species (Adamo *et al.*, 2001; Rolff, 2001), and has been explained by several contrasting theories, which also attempt to explain changes in immunity between mature and immature organisms, as were also found in this study.

The immuno-competence handicap hypothesis predicts that sex hormones such as juvenile hormone have an immune-suppressing effect, causing the down-regulation of immunity after maturity (Rantala *et al.*, 2003). However, as insects lack sex-specific hormones, this would be expected to affect both males and females (Rolff, 2002). Therefore, while juvenile hormone levels could account for changes in PO activity levels, they are not likely to be affecting bacterial clearance from the haemocoel, or survival, both of which are higher in females. Resource-mediated trade-offs have also been suggested to be responsible for changes in immunity before and after maturation (Sheldon & Verhulst, 1996). However, as no breeding was allowed to take place in the first two experiments, only gamete development could be inflicting costs on the individuals used, and so costs would apply more to females than males, predicting a down-regulation of female immune competence, which, again, is not what was observed. The last explanation is made by applying Bateman's principle to the differing life-history strategies of males and females, to assess how selection pressure differs between the sexes. This proposes that female reproductive opportunities are predominantly restricted by time, whereas male reproduction is restricted by mating opportunities. As such, females can be expected to invest more in immunity than males to prolong their longevity and so maximise their reproduction, resulting in enhanced immunity in females (Rolff, 2002). This should be evident in both mature and immature organisms. Female bacterial loads were lower, and survival was higher, in both mature and immature beetles, in agreement with Bateman's principle. Further to this, by applying Bateman's principle to maturation, we predicted that immunity would be higher in

immature beetles, which was confirmed by faster, more prolonged clearance rates, and longer survival in immature beetles.

However, our test of PO as an immune parameter produced more contradictory results. PO has been shown to slow *P. luminescens* proliferation but not completely suppress it; using a mutant strain of *P. luminescens* that cannot produce the ST toxin responsible for PO suppression, Eleftherianos *et al.* (2007) showed that bacteria reached fatal levels in *M. sexta* larvae 8 days post infection, rather than the 2–3 days it takes the wild type to kill its host. In this study, it was found that higher PO levels are only beneficial in the first 20 h after infection, and beyond 55 h after infection. Between these points, higher PO activity decreases survival. This may be due to the damaging nature of the PO immune response (Sadd & Siva-Jothy, 2006), making temporally limited responses more valuable. While extremely effective due to its responsivity and wide-ranging action, long-term PO activation may cause more damage than a pathogen, making a shorter-lived or less vigorous response more adaptive, explaining the beneficial effect of high PO early after infection, but apparently detrimental effect later on. The PO response is commonly interpreted (at any one time point) as being positively correlated with resistance, without integrating the effects of response persistence and survival effects. However, other recent studies (Adamo, 2004; Freitak *et al.*, 2007) suggest a more complex interpretation, and the effect of time after infection on the adaptive value of PO activity in this study may provide a more comprehensive explanation.

While differences in PO were found here between mature and immature beetles, with mature beetles showing higher PO than younger beetles after around 40 h, there were no sex differences in PO activity. Both these results were contrary to our predictions based on Bateman's principle. There were minimal differences in PO between mature and immature beetles in the first 25 h after infection, which corresponds to the point at which PO activity has the strongest effect of infection. Beyond 25 h PO activity begins to increase faster in mature beetles, diverging from immature beetles. This, however, was not accompanied by increased survival. As PO is constitutively necessary for functions such as wound healing and melanisation of the cuticle, it may be that selection on PO levels is not reduced in maturity as is suggested by Bateman's principle. As wounding is, in fact, common among adult *N. vespilloides* during intra-specific competition (Trumbo, 2007), it may be that PO is subject to different selection pressures to those affecting immune mechanisms, and so does not conform to predictions based on Bateman's principles, in cases of either sex or maturation.

Females which were infected with *P. luminescens* immediately before breeding were shown to invest less in reproduction. This came in the form of lower lysozyme content in the exudate of infected females which has also previously been found in response to sterile wounding and non-pathogenic bacterial infection (Cotter *et al.*, 2013). As this exudate functions to exclude microbes which can function both as competitors or pathogens of the larval offspring (Hall *et al.*, 2011), reduced production of exudate by the female indicates significantly reduced investment in reproductive effort. Larvae have been shown to decrease their exudate's lysozyme content in response to the removal of a parent (Reavey *et al.*, 2014b), so the compounded effects of these decreased contributions to carcass preservation would be expected to significantly reduce the quality of the breeding resource, in comparison to that of a normally functioning family.

If females contributed less to protecting the carcass from microbes when infected with *P. luminescens*, offspring may be able to utilise less of the nutritional value of the carcass, resulting in slower weight gain and so the decreased larval mass, slower development, and less efficient utilisation of the carcass seen in this study. As the offspring do not eat during the pupal stage, larval body mass is highly influential on adult body size, which is in turn highly influential on the outcome of intraspecific competition for carcasses and so on fitness (Steiger, 2013). However, the lack of a change in average larval mass indicates that the difference in brood mass and carcass utilisation efficiency is a result of bacterially treated females producing smaller clutches (through fewer eggs or infanticide). In contrast, both Reavey *et al.* (2015) and Cotter *et al.* (2013) found that infection of *N. vespilloides* prior to breeding increased the number of larvae in the brood immediately produced. This was suggested to be the result of a switch to a high reproductive output strategy, investing in reproduction as much as possible in the face of a negative change in the environment which may reduce future reproductive opportunities (Reavey *et al.*, 2015).

Evolutionary theory indicates that reproduction must be costly, otherwise fitness could be increased infinitely by reproduction, and maximising reproductive output would always be the most adaptive strategy. That reproduction is costly has widely proven to be true, although there are some documented complications. Burying beetle parents have been shown to recoup some or all of the costs of reproduction after larval dispersal and to provide parental care to an extent determined by the likelihood of this recuperation, based on the size of the carcass which is being utilised for the breeding bout (Boncoraglio & Kilner, 2012). Still, this recuperation of the costs of breeding is different to breeding itself being beneficial. As the first 24 h of the breeding bout are highly costly (Boncoraglio & Kilner, 2012), there must still be a trade-off during this time between reproduction and immunity. The downregulation of PO enzyme activity which has been shown to occur during breeding bouts in *N. vespilloides* females (Reavey *et al.*, 2014a) is evidence of a trade-off between breeding and immunity. However, this was shown to be short-term, just like the costs of breeding, with PO activity returning to constitutive levels by the end of the breeding bout. The decrease in reproductive investment found here may, therefore, be evidence of a resource based trade-off which was not evident in previous studies due to the use of a non-pathogenic bacteria.

In conclusion, this study provides support for the idea that maturation and sex effects on immunity are adaptive, responding to varying selection pressures on certain life stages, rather than the result of restrictions on resources or immunosuppressive sex hormones. It shows the importance of integrating the timing of immune responses into evolutionary ecological experiments, as a binary classification of immune defence as inactive or upregulated poorly characterises it. It also suggests a key role of

tolerance in the immune defences of adult *N. vespilloides* which should be further investigated to complement the species' role as a model system in ecological investigations.

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