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

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Running Title:

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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, we predict 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*, and a pathogenic bacteria, *Photorhabdus luminescens*.

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 show 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, whilst high PO was beneficial up to 20 hours post-infection, it became detrimental thereafter. 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.

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 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*, 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*, 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 is 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 are 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).

Here we use a model host-parasite system to ask 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 two 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, 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 a social immune 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 own health over that of the current brood.

The parasite we use, *Photorhabdus luminescens,* is a bacterial symbiont of an entomopathogenic soil nematode (*Heterorhabditis bacteriophora*), 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 *E. coli* in *Manduca sexta* 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 hours (Eleftherianos *et al.*, 2007). *P. luminescens* is highly pathogenic, and has been shown to have an LD50 at 72 hours of less than forty cells in several insect species (Khan & Brooks, 1977; Milstead, 1979), however, in *N. vespilloides*, the LD50 over eight 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, we investigate the timing of infection and immune responses in pre-reproductive and mature beetles and how these affect reproduction. Specifically we ask:

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 *Nicrophorus 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 12x8x2 cm boxes under a 16:8 light: dark 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 (17x12x6 cm) two-thirds filled with damp soil and placed in darkness to replicate the natural breeding conditions of the species.

Around eight 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 fifteen 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 19-22 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 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 hours 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 hours 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 re-sampling). 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 was not collected for the control beetles. However, we have included average survival data of uninfected beetles from the lab colony who 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

55, 21 day old, adult, virgin female *N. vespilloides* beetles were injected with one of three immune treatments, two consisting of injections of 1 µl nutrient broth, the other consisting of handling for an equivalent period of time (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 three 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 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 200rpm) 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 hours and so are only being 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

1 µl of fresh haemolymph was pipetted onto one of four sections of a 0.005g per 100ml 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. 100 µl of this was added to multiple wells of a 96 well plate on ice. 100 µl of 50mM 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 480nm was then read every ten seconds for ten minutes, maintained at a temperature of 27°C. The Vmax of the slope was then used as an indicator of the activity and therefore 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 13,000 rpm for three minutes to remove any dirt included in the exudate. 1 µl of the resulting supernatant was pipetted into 1mm diameter wells in agar containing lyophilized *Micrococcus leuteus*. 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, except when modelling the PO activity, when

a generalised linear model was used due to non-normal variance. 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: $F_{1, 158} = 4.897$, $p = 0.028$; Figure 1Figure 1a, b) and bacterial loads appear to start increasing again in mature beetles after 60 hours (in agreement with data from Miller and Cotter, under review) whilst 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 Figure 1a and 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 *Photorhabdus luminescens* was assessed by measuring how much PO enzyme activity there was in the blood of infected beetles over the 65 hours 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 hours in the mature beetles, and 40 hours in immature beetles (see Figure 2Figure 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 hours after infection, high PO prolonged survival, but from 20 to 55 hours, higher PO was detrimental to survival (Figure 2Figure 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, Figure 2Figure 2c). Immature beetles survived longer than mature beetles (age: $F_{1, 203}$ = 5.082, p = 0.025), 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, Figure 2d).

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, Figure 3Figure 3a). If the female was infected with *P. luminescens*, 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 Figure 3Figure 3b). The offspring of females treated with a bacterial injection took significantly longer to disperse from the carcass than those which were treated with either a sterile injection (t = 2.343, p = 0.023) or just handling (t = 2.308, p =

0.026). 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, Figure 3Figure 3c). Bacterially treated females produced a lower mass of larvae per gram 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, while 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 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, Figure 3Figure 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, though 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 beetles showed some suppression of PO activity in response to injection of bacteria (Figure 2a), but certainly not the complete suppression seen in *Manduca sexta* larvae (Eleftherianos *et al.*, 2007). Indeed, injection of an inactive immune elicitor has also been shown to partially suppress PO activity in this species of beetle, potentially due to 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 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 hours post infection in immature beetles, whilst bacterial numbers decreased initially in mature beetles but levelled off at 40 hours and seemed to begin to increase again at 50 hours 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 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 hours in mature beetles is in agreement with other data (Miller and Cotter, under review), 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 possibly 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 and colleagues (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 hours after infection, and beyond 55 hours 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 to 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 hours, 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 hours after infection, which corresponds to the point at which PO activity has the strongest effect of infection. Beyond 25 hours 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, though there are some documented complications. Burying beetle parents have been shown to recoup some or all of the costs of reproduction subsequent to 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 hours 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 *Nicrophorus 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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Contribution of Authors

SCC & CVLM conceived the idea, analysed the data and wrote the paper. CVLM carried out the experiments.

References

Adamo, S.A. (2004) Estimating disease resistance in insects: Phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*, **50**, 209–216.

Adamo, S.A., Jensen, M. & Younger, M. (2001) Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Animal Behaviour*, **62**, 417–425.

Arce, A.N., Johnston, P.R., Smiseth, P.T. & Rozen, D.E. (2012) Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology*, **25**, 930–7.

Barnes, A.I. & Siva-Jothy, M.T. (2000) Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): Cuticular melanization is an indicator of

investment in immunity. *Proceedings of the Royal Society B: Biological Sciences*, **267**, 177–182.

Boncoraglio, G. & Kilner, R.M. (2012) Female burying beetles benefit from male desertion: sexual conflict and counter-adaptation over parental investment. *PloS ONE*, **7**, e31713.

Cerenius, L., Lee, B.L. & Söderhäll, K. (2008) The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, **29**, 263–71.

Ciche, T. a. & Ensign, J.C. (2003) For the Insect Pathogen *Photorhabdus luminescens*, Which End of a Nematode Is Out? *Applied and Environmental Microbiology*, **69**, 1890–1897.

Cotter, S.C. & Kilner, R.M. (2010) Personal immunity versus social immunity. *Behavioral Ecology*, **21**, 663–668.

Cotter, S.C., Littlefair, J.E., Grantham, P.J. & Kilner, R.M. (2013) A direct physiological trade-off between personal and social immunity. *The Journal of Animal Ecology*, **82**, 846–853.

Cotter, S.C., Myatt, J.P., Benskin, C.M.H. & Wilson, K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology*, **21**, 1744–54.

Cotter, S.C., Topham, E., Price, a J.P. & Kilner, R.M. (2010) Fitness costs associated with mounting a social immune response. *Ecology Letters*, **13**, 1114–23.

Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P.Y. & Sharma, S. (2002) A single *Photorhabdus* gene, makes caterpillars floppy (mcf), allows *Escherichia coli* to persist within and kill insects. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 10742–10747.

Degenkolb, T., Düring, R.A. & Vilcinskas, A. (2011) Secondary Metabolites Released by The Burying Beetle Nicrophorus vespilloides: Chemical Analyses and Possible Ecological Functions. *Journal of Chemical Ecology*, **37**, 724-735.

Eleftherianos, I., Boundy, S., Joyce, S. a, Aslam, S., Marshall, J.W., Cox, R.J., *et al.* (2007) An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 2419–2424.

Eleftherianos, I., Waterfield, N.R., Bone, P., Boundy, S., Ffrench-Constant, R.H. & Reynolds, S.E. (2009) A single locus from the entomopathogenic bacterium *Photorhabdus luminescens* inhibits activated *Manduca sexta* phenoloxidase. *FEMS Microbiology Letters*, **293**, 170–176.

Freitak, D., Wheat, C.W., Heckel, D.G. & Vogel, H. (2007) Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. *BMC Biology*, **5**, 56.

González-Santoyo, I. & Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata*, **142**, 1–16.

Haine, E., Moret, Y., Siva-Jothy, M. & Rolff, J. (2008) Antimicrobial defense and persistent infection in insects. *Science*, **322**, 1257–1259.

Haldane, J.B.S. (1992) Disease and Evolution (Reprinted from La Ricerca Scientifica Supplemento, Vol 19, Pg 1-11, 1949). *Current Science*, **63**, 599–604.

Hall, C.L., Wadsworth, N.K., Howard, D.R., Jennings, E.M., Farrell, L.D., Magnuson, T.S., *et al.* (2011) Inhibition of microorganisms on a carrion breeding resource: the antimicrobial peptide activity of burying beetle (Coleoptera: Silphidae) oral and anal secretions. *Environmental Entomology*, **40**, 669–78.

Jacobs, C.G.C., Steiger, S., Heckel, D.G., Wielsch, N., Vilcinskas, A. & Vogel, H. (2016) Sex, offspring and carcass determine antimicrobial peptide expression in the burying beetle. *Scientific Reports*, **6**, 25409.

Khan, A. & Brooks, W.M. (1977) A Chromogenic Bioluminescent Bacterium Associated with the Entomophilic Nematode *Chromonema heliothidis*. *Journal of Invertebrate Pathology*, **29**,

253–261.

Lavine, M.D. & Strand, M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, **32**, 1295–309.

Milstead, J.E. (1979) *Heterorhabditis bacteriophora* as a vector for introducing its associated bacterium into the hemocoel of *Galleria mellonella* larvae. *Journal of Invertebrate Pathology*, **33**, 324–327.

Palmer, W.J., Duarte, A., Schrader, M., Day, J.P., Kilner, R. & Jiggins, F.M. (2016) A gene associated with social immunity in the burying beetle *Nicrophorus vespilloides*. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20152733.

Parker, G.A., Baker, R.R. & Smith, V.G.F. (1972) The origin and evolution of gamete dimorphism and the male-female phenomenon. *Journal of Theoretical Biology*, **36**, 529–553.

Pukowski, E. (1933) Okologische Untersuchungen and Necrophorus. *F. Z. Morph Okol Tiere*, **27**, 518–586.

Rantala, M.J., Vainikka, A. & Kortet, R. (2003) The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 2257–2261.

Reavey, C.E., Beare, L. & Cotter, S.C. (2014b) Parental care influences social immunity in burying beetle larvae. *Ecological Entomology*, **39**, 395–398.

Reavey, C.E., Silva, F.W.S. & Cotter, S.C. (2015) Bacterial infection increases reproductive investment in burying beetles. *Insects*, **6**, 926–942.

Reavey, C.E., Warnock, N., Vogel, H. & Cotter, S.C. (2014a) Trade-offs between personal immunity and reproduction in the burying beetle, *Nicrophorus vespilloides*. *Behavioral Ecology*, **25**, 415–423.

Rolff, J. (2001) Effects of age and gender on immune function of dragonflies (Odonata,

Lestidae) from a wild population. *Canadian Journal of Zoology*, **79**, 2176–2180.

Rolff, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society B: Biological Sciences*, **269**, 867–872.

Rolff, J. & Siva-Jothy, M.T. (2002) Copulation corrupts immunity: a mechanism for a cost of mating in insects. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 9916–8.

Rozen, D.E., Engelmoer, D.J.P. & Smiseth, P.T. (2008) Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 17890–5.

Sadd, B., Holman, L., Armitage, H., Lock, F., Marland, R. & Siva-Jothy, M.T. (2006) Modulation of sexual signalling by immune challenged male mealworm beetles (*Tenebrio molitor*, L.): evidence for terminal investment and dishonesty. *Journal of Evolutionary Biology*, **19**, 321–5.

Sadd, B.M. & Siva-Jothy, M.T. (2006) Self-harm caused by an insect's innate immunity. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 2571–4.

Sheldon, B.C. & Verhulst, S. (1996) Ecological immunology - costly parasite defenses and trade- offs in evolutionary ecology. *Trends in Ecology and Evolution*, **11**, 317–321.

Silva, C.P., Waterfield, N.R., Daborn, P.J., Dean, P., Chilver, T., Au, C.P.Y., *et al.* (2002) Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cellular Microbiology*, **4**, 329–339.

Siva-Jothy, M.T. (2000) A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society B: Biological Sciences*, **267**, 2523–2527.

Siva-Jothy, M.T. & Thompson, J.J.W. (2002) Short-term nutrient deprivation affects immune function. *Physiological Entomology*, **27**, 206–212.

Steiger, S. (2013) Bigger mothers are better mothers: disentangling size-related prenatal and postnatal maternal effects. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20131225.

Steiger, S., Gershman, S.N., Pettinger, A.M., Eggert, A.-K. & Sakaluk, S.K. (2011) Sex differences in immunity and rapid upregulation of immune defence during parental care in the burying beetle, *Nicrophorus orbicollis. Functional Ecology*, **25**, 1368–1378.

Trumbo, S.T. (1997) Juvenile Hormone–Mediated Reproduction in Burying Beetles: From Behavior to Physiology. *Archives of Insect Biochemistry and Physiology*, **35**, 227–235.

Trumbo, S.T. (2007) Defending young biparentally: female-risk taking with and without a male in the burying beetle, *Nicrophorus pustulatus*. *Behavioral Ecology and Sociobiology,* **61**, 1717-1723.

Urbański, A., Czarniewska, E., Baraniak, E. & Rosiński, G. (2013) Developmental changes in cellular and humoral responses of the burying beetle *Nicrophorus vespilloides* (Coleoptera, Silphidae). *Journal of Insect Physiology*, **60**, 98–103.

Figure legends

Figure 1. The dynamics of *P. luminescens* infection in the haemolymph of immature and mature a) females, and b) males over 65 hours post infection.

Figure 2. The effects of infection on PO activity and survival post-infection. a) PO activity in immature and mature beetles over 65 hours 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) sex.

Figure 3. The effects of *P. luminescens* infection on a) the social immune effort of breeding female *N. 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.

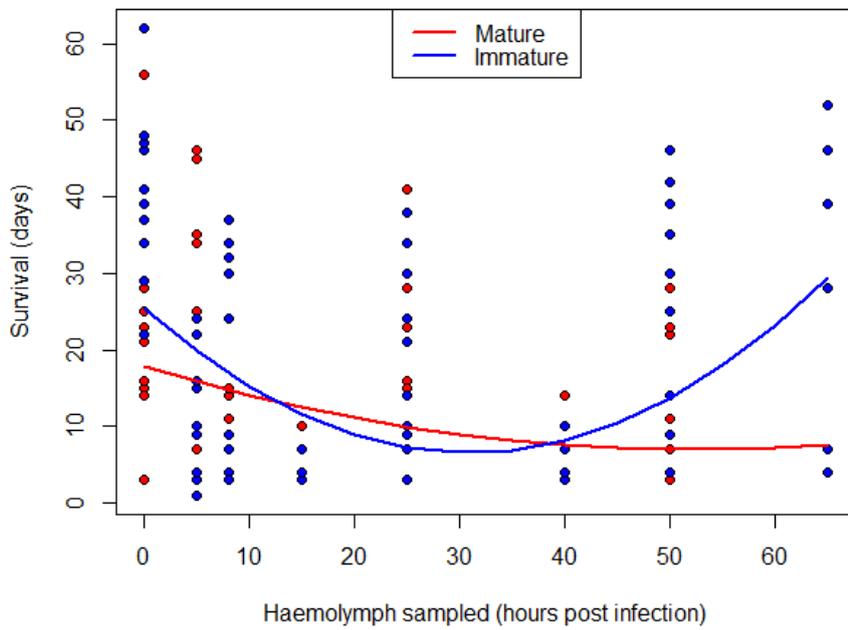# Figures

Figure 1

a) Females

b) Males

Figure 2

a)

b)

c)

Haemolymph sampled (hours post infection)

d)

Haemolymph sampled (hours post infection)

e)

Figure 3

a)

b)

c)

d)