Title: Trade-offs Between Personal Immunity and Reproduction in the Burying Beetle, *N. vespilloides*

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Running Title:

Trade-offs between reproduction and immunity

Summary/Abstract

We know that parental investment and immune investment are costly processes, but it is unclear which trait will be prioritised when both may be required. Here we address this question using the burying beetle *Nicrophorus vespilloides*, carrion breeders that exhibit biparental care of young. Our results show that

immunosuppression occurs during provision of parental care. We measured Phenoloxidase (PO) on Day 1-8 of the breeding bout and results show a clear decrease in PO immediately from presentation of the breeding resource onwards. Having established baseline immune investment during breeding we then manipulated immune investment at different times by applying a wounding challenge. Beetles were wounded prior to and during the parental care period and reproductive investment quantified. Different effects on reproductive output occur depending on the timing of wounding. Challenging the immune system with wounding prior to breeding does not affect reproductive output and subsequent Lifetime Reproductive Success (LRS). LRS is also unaffected by applying an immune elicitor prior to breeding, though different arms of the immune system are up/downregulated, perhaps indicating a trade-off between cellular and humoral immunity. In contrast, wounding during breeding reduces reproductive output and to the greatest extent if the challenge is applied early in the breeding bout. Despite being immunosuppressed, breeding beetles can still respond to wounding by increasing PO, albeit not to pre-breeding levels. This upregulation of PO during breeding may affect parental investment, resulting in a reduction in reproductive output. The potential role of juvenile hormone in controlling this trade-off is discussed.

Key Words

Immunity, Reproduction, Parental Care, Trade-off, Juvenile Hormone, *Nicrophorus*, Phenoloxidase, Ecological Immunology, Insect, Wounding

Introduction

Accounting for the costs and benefits of a particular trait is central to evolutionary biology. The diversity of organisms in nature is a testament to the many

strategies by which organisms have optimised investment in the face of costs and constraints. These optimisation 'decisions' result in trade-offs, which are often observed as negative associations between traits. Classic life history trade-offs include: age and size at maturity, and aspects of reproductive effort such as clutch size and offspring size (Roff 1992).

Competitive allocation of resources to growth, maintenance, and reproduction requires a system of resource allocation. Control may be genetic, in the form of pleiotropy or linkage, or environmental, for example resource acquisition (energy/nutrients), predation and time-based conflicts. Whilst resource acquisition is a central issue surrounding trade-offs, the system of control of these resources is the crucial factor. In this light, hormonal studies are becoming more important; relatively little is known about the endocrine mechanisms underlying trade-offs but an involvement of hormones is supported to date (Zera & Harshman 2001). Hormones regulate many life history components, e.g. egg production, growth, metabolism, and so provide a means for mediating trade-offs (Stearns 1989; Zera & Harshman 2001).

Classical immunology considers the physiological mechanisms behind the function of the immune system, both in a state of disease and at times of health (Schulenburg et al. 2009). It has only been in recent decades (Schmid-Hempel 2003) that immune function has been given due attention by evolutionary ecologists. Ecological immunology makes the transition from the study of biochemical pathways and the molecular mechanisms involved, to an integrated study of these components in an ecological context under the process of evolution (Rolff & Siva-Jothy 2003).

A range of empirical studies across taxa have provided evidence for immune costs. These can be broken down into (1) Evolutionary costs of defence (Kraaijeveld & Godfray 2001; Cotter et al. 2004a; Simmons & Roberts 2005; McKean et al. 2008),

(2) Usage costs of defence due to either maintenance (Valtonen et al. 2010) or activation of the immune system (Ilmonen et al. 2000; Hasselquist et al. 2001; Siva-Jothy & Thompson 2002; Cotter et al. 2004b, Reaney & Knell 2010), and (3) Auto-reactivity/auto-immunity costs (Sadd & Siva-Jothy 2006).

Invertebrates have provided excellent, productive model systems to address many of the key questions within the field of ecological immunology. This is mainly due to the ease with which many species can be kept (in large numbers), bred, and their immune systems studied. The invertebrate immune system, whilst complex and efficient, is nonetheless simpler than that of vertebrates. Immune responses can be loosely categorised into two main arms: cellular immunity and humoral immunity. The cellular response is largely constitutive, i.e. it is present at a basal level. It involves haemocytes and is the primary defence to invasion, acting in a generalised manner. The mechanisms include phagocytosis of microparasites, nodulation of clumps of microparasites and encapsulation of macroparasites, which combat pathogens in a fairly generic approach (Gillespie et al. 1997). A central feature of the constitutive response is the activation of the prophenoloxidase (proPO) cascade (Gillespie et al. 1997). As well as having a role to play in non-self recognition (Söderhäll & Cerenius 1998), activation of the proPO cascade leads to the production of melanin (Götz 1986), a substance used in the encapsulation response. Phenoloxidase (PO) plays a key role in the coordination of the cellular response (Gillespie et al. 1997) and it is also involved in cuticular hardening (Sugumaran et al. 2000). Whilst PO activity is constitutive, it can be further activated and upregulated by a wide range of parasitic challenges (Gillespie et al. 1997).

The humoral arm is most often induced in response to infection and is more specific (Casteels et al. 1994; Lemaitre et al. 1997). It includes lysozyme and other

smaller antimicrobial peptides (AMPs) (Hoffmann 1995). Some AMPs are also induced in the absence of microbial antigens, for example, during wounding, most likely as a preventative measure against potential microorganisms entering through the wound (Lemaitre et al. 1997). Whilst the PO cascade is predominantly associated with cell based immunity, it also has a humoral function - its intermediate products (quinones) have been shown to have antimicrobial/toxic activity in the haemolymph (Nappi & Ottaviani 2000).

Here we focus on the life history constraints of immune function, in the burying beetle *N. vespilloides* (Figure 1). Burying beetles provide a highly tractable system for studying the trade-off between immune investment and reproduction. The nature of the constitutive immune system in insects makes it possible to measure investment in immune function without actually stimulating their immune system. The central feature associated with the life cycle of burying beetles is the availability of a small vertebrate carcass; reproduction is completely reliant on the presence of this resource. Extended biparental care is rare in insects and burying beetles use this strategy to great effect. Once a carcass has been located, the parents will cooperate to bury it underground and prepare it for consumption by their offspring by removing hair/feathers and shaping it into a ball (Pukowski 1933; Scott 1998). The beetles coat the carcass with antimicrobial anal exudates to delay decomposition (Cotter & Kilner 2010a). As this primarily benefits the offspring it is a form of social immunity (Cotter & Kilner 2010b). Eggs are laid in the soil, near the carcass (Trumbo et al. 1995; Scott 1998). A further component of parental care is the capacity for brood reduction against insurance larvae. If more larvae arrive at the carcass than it can adequately sustain, the parents can carry out ovicide and/or larvicide (Trumbo 1990; Trumbo & Fernandez 1995). Two-three days after egg laying in *N. vespilloides*, the

larvae crawl to the carcass where parents will provision them with pre-digested food and protect them from predators and competitors (Eggert & Müller 1997, Scott 1998). Around 6 days after hatching, the larvae disperse to pupate in the soil (Eggert & Müller 1997, Scott 1998). Parental care improves offspring growth and survival in *N. vespilloides* (Eggert et al. 1998), we therefore use number and mass of larvae produced as a proxy for parental investment.

Research on immunity in this species is still at the early stage. Whilst there are interesting findings with regards to social immunity (Cotter & Kilner 2010a; Cotter et al. 2010b; Steiger et al. 2011; Arce et al. 2012; Cotter et al. 2013), there is little information regarding the personal immune response. Three papers have addressed this. The first identified a range of immune-inducible genes (Vogel et al. 2011), and two studies by Steiger et al. considered changes in immunity in a congeneric species whilst breeding (Steiger et al. 2011) and the effect of dominance status on immunity (Steiger et al. 2012).

A key aspect of the burying beetle breeding cycle is the surge in Juvenile Hormone (JH) that occurs upon discovery of a carcass (Trumbo et al. 1995; Trumbo 1997). This is further upregulated when larvae appear (Trumbo 1997) and then falls off during the remainder of the breeding bout. JH has been shown to be immunosuppressive in other species (Hiruma & Riddiford 1988; Khafagi & Hegazi 2001; Rolff & Siva-Jothy 2002; Rolff 2002; Rantala et al. 2003; Amdam et al. 2004; Franssens et al. 2006, Flatt et al. 2008), which suggests that the immune response might be downregulated in this species during breeding. However, one could hypothesise that immune function should be *upregulated* during breeding, as disease risk may also be higher at this time due to high loads of soil and/or carrion-associated

143 microbes. However, this would be a substantial investment in two traits that often
144 experience trade-offs in other taxa (Richner et al. 1995; Hanssen et al. 2005).
145 Here we address this question by measuring constitutive investment in
146 immunity during breeding to look for evidence of immunosuppression. We then
147 consider the effect of challenging the immune system both prior to and during
148 breeding to assess the effects of immune upregulation on reproductive investment.
149

150 Materials and Methods

151 *Nicrophorus vespilloides*

152 The colony was established in February 2011 from an outbred colony
153 maintained in the Zoology department at the University of Cambridge. Non-breeding
154 adult beetles were housed in individual boxes (measuring 12×8×2 cm) at 20°C under
155 a 16:8 light:dark cycle, and fed twice weekly *ad libitum* with minced beef. During
156 breeding, each pair was placed together in a breeding container (17×12×6 cm), 1/3
157 filled with moist soil and provided with a newly defrosted mouse carcass of
158 approximately 20-25g in weight. For experimental beetles mouse weight was 24.93g
159 +/- 0.28 (means are presented +/- 1 Standard Error throughout the manuscript). At
160 this time the beetles were placed in a compartmentalised cupboard so that conditions
161 were similar to those underground.
162 Larvae were removed from the breeding container as soon as they began
163 dispersing from the carcass, typically 8 days after the parents were paired, and were
164 placed individually in compartments of 25 cell petri dishes, with different petri dishes
165 used for each family. The containers were topped up with moist soil and the larvae
166 left to pupate. Eclosion occurs around 20 days following dispersal, after which the

beetles were again set up in their individual containers and were either used as colony beetles or used in later experiments. Life history data including pronotum width, date of dispersal, date of eclosion, and reproductive success was recorded for all beetles, both colony and experimental.

Experiment 1: Constitutive Immunity During Breeding

PO is present constitutively and we can exploit this as a proxy for investment into immune function during non-challenged conditions. Due to its multi-faceted role, it seems a realistic proxy and empirical studies support its role as an indicator of parasitic resistance (Wilson et al. 2001; Zhao et al. 2007). Haemolymph could only be sampled from each beetle once, as wounding alone will trigger an immune response (as illustrated for this species by Experiment 2). Therefore separate individuals were required for each day of the bout. To achieve this, three-week old, unrelated males and females were assigned to one of 11 treatment groups. 8 treatment groups were set up so that haemolymph could be collected from a discrete group of beetles on every day of the breeding bout (1-8). Day 1 corresponds to the day the beetles were presented with the carcass.

In addition, there were 3 control groups (C0, C1, and C4) that were housed in the same conditions but were not provided with a carcass. C0 consisted of virgin beetles and therefore represented standing levels of PO prior to reproduction. C1 & C4 consisted of mated but non-breeding beetles from which haemolymph samples were obtained on days 1 and 4 of the breeding bout respectively. Due to constraints on beetle numbers, controls could not be provided for all days, 1-8. Therefore in addition to day 1, we selected day 4 as it corresponded to the time when breeding beetles would be dealing/about to deal with a brood of small, very demanding larvae, which should be very taxing on resources. Control beetles were fed mince *ad libitum*.

In total, 24 males and 24 females were used in each treatment group and paired accordingly in the breeding treatments. Haemolymph samples were obtained from both sexes from 1.30pm on the appropriate day. From the 528 beetles used in the experiment, haemolymph samples were obtained from 483.

Experiment 2: The Effect of Immune Challenge Before Breeding on Immunity and Reproduction

a) Stimulating the immune response

In order to measure potential costs of immune deployment, the immune system must be activated, but the condition of the organism must not be compromised. We therefore use immune elicitors, which have been used constructively across many taxa, illustrating usage costs of the immune system (Moret & Schmid-Hempel 2000; Mallon et al. 2003; Riddell & Mallon 2006).

In insects the Toll signalling pathway controls the defence against fungal or Gram-positive bacterial molecules whilst the Immune Deficiency Pathway (IMD) targets Gram-negative bacteria. We decided to activate Toll by using peptidoglycan (PEP) from *Bacillus subtilis*, a Gram-positive bacterium, and IMD by using lipopolysaccharides (LPS). The idea was that by triggering both pathways, we would apply a greater challenge to the immune system. Data from a pilot experiment were used to determine the dose of LPS/PEP to use (C.Reavey, unpublished data).

Non-breeding, two-week old, virgin *N. vespilloides* were assigned to one of three treatment groups, (1) handled, (2) injected with autoclaved insect ringer's solution (referred to as wounded in the text) and (3) injected with elicitor dissolved in autoclaved insect ringer's solution. All injections occurred on the cuticle behind the pronotum. For group 3, 1mg of LPS and 2.5mg of PEP were suspended in 1ml of

sterile insect ringer's solution and 1ul of this solution injected into each beetle using a Hamilton syringe. Beetles in the wounded treatment were injected with sterile insect ringer's solution only, whilst controls were handled but not injected. Haemolymph samples were obtained for 227 beetles across the three treatment groups 24 hours after immune exposure. PO activity was measured in accordance with the protocol below. RNA was extracted from 12 virgin, 2-week old, female beetles (n = 4 per treatment group) and defensin upregulation was measured in accordance with the protocol below.

In order to test for potential toxicity of the elicitor, we injected a treatment group with 1ul of 1mg/ml of LPS and 2.5mg/ml PEP and a control group with 1ul of insect ringer's solution and measured their subsequent longevity (n=15 per treatment group).

b) What effect does immune upregulation have on lifetime reproductive success?

Deploying the immune system is a costly process (Schulenburg et al. 2009). Recognising/pin-pointing where these costs are 'paid' is sometimes difficult. Lifetime reproductive success provides an excellent proxy for fitness, enabling us to potentially unravel previously hidden costs of deployment.

Three treatment groups were established as above, (1) handled, (2) injected with autoclaved insect ringer's solution (referred to as wounded in the text), and (3) injected with elicitor dissolved in autoclaved insect ringer's solution (1µl of 1mg/ml of LPS & 2.5mg/ml PEP) with 48 breeding females/treatment group. The treatment was applied to two-week old females in the morning and the pairs set up in the afternoon on the same day. The male was removed on day 2 of the breeding bout to minimise his contribution to parental investment. Reproductive output was recorded and the female then repeatedly bred with young virgin males (male age: 16.68 days

+/- 0.27) until death. Eleven females never produced a brood (3/handled, 4/ringer's, 4/elicitor), and two escaped mid-experiment. These samples were excluded from the analysis.

Experiment 3: The Effect of Immune Challenge During Breeding on Immunity and Reproduction

a) Is the immune system still upregulated when wounded during breeding?

The results of the above experiments showed that PO was suppressed during breeding and upregulated following wounding in non-breeding beetles. However, we do not know the effect of wounding on PO in breeding beetles. To determine this, 80 pairs were set up and immune function was measured on and off the carcass in both immune challenged (wounded with a sterile needle on the cuticle behind the pronotum) and control individuals (aged three-weeks). Those beetles off the carcass were mated pairs and they were fed *ad libitum*. Haemolymph samples were taken 24 hours after treatment application and processed to determine PO levels.

b) What effect does wounding whilst breeding have on reproductive output?

Three-week old, unrelated males and females were paired and placed in a breeding container and presented with a newly defrosted mouse carcass. Nine treatment groups were established; each group corresponding to a different day of the breeding bout (1-8), as well as a non-wounded control. The beetles were wounded with a sterile needle on the cuticle behind the pronotum at various stages throughout the reproductive bout (day 1-day 8). In total, 24 pairs were used in each treatment group in the experiment, alongside 48 control pairs (a total of 240 pairs). 210 pairs bred successfully (breeding success = 87.5%). Those that did not breed were omitted

from the analysis. The number of larvae produced, the total mass of the brood and the mean mass of larvae were considered separately in the analysis. Wounding with a sterile needle and wounding by injection of autoclaved insect ringer's solution result in similar net haemolymph loss.

Haemolymph Sampling

Haemolymph was obtained from *N. vespilloides* by piercing the cuticle behind the pronotum with a sterile needle and then collecting the haemolymph as it was released with a pipette. The haemolymph was then diluted with an equal quantity of anticoagulant buffer (EDTA anticoagulant in PBS – pH 7.4) and then stored in a freezer (-20°C) prior to analysis. Whilst in some taxa costs of haemolymph loss have been observed (Ardia et al. 2012), in *N. vespilloides* haemolymph extraction had no effect on survival (Cotter et al. 2010a) and so we expect costs to be due to immune activation rather than costs due to state.

Phenoloxidase (PO) Assay

Pilot experiments established the kinetics of the phenoloxidase (PO) reaction for this species in order that an appropriate level of dopamine was used, such that it was not limiting as a substrate, nor was it inhibiting the PO itself. The concentration of dopamine most appropriate for the levels of PO observed in this species was 10mM for a 2µl haemolymph per ml PBS concentration. Following defrosting of the haemolymph samples, 2 µl of haemolymph/anticoagulant buffer solution was added to 500 µl of PBS (pH 7.4). 100 µl of this solution was placed in a well of a 96-well microplate with 100 µl of 10mM dopamine as a substrate. For this species, the PO rate is only linear during the first few minutes of the reaction (C.Reavey, unpublished data), therefore readings were taken every 10 seconds for three minutes at 490nm and

25°C on a Thermo Scientific Multiscan Spectrum spectrophotometer. The maximum rate of reaction was then used as an approximation of PO level.

AMP Upregulation

To measure potential changes in expression of the immune related gene defensin, we extracted RNA 24 hours after treatment application and used qRT-PCR to determine any changes in defensin expression following treatment. Total RNA was isolated from each beetle using Trizol® Reagent (Invitrogen, Life Technologies) in accordance with the manufacturer's instructions. Contaminating DNA was removed by treating total RNA with TURBO™ DNase (Invitrogen, Life Technologies) and converted to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies). Primers were designed for defensin and the housekeeping gene Beta Tubulin from ESTs known for *N. vespilloides* (Vogel et al. 2011). For each PCR reaction 10µl of SYBR, 0.4µl FWD primer, 0.4µl REV primer, 7.2µl of water and 2µl of 25ng/µl of cDNA was used. Real time PCR was carried out using a Biorad Thermo Cycler with the following conditions; 95°C for 3 mins, and 50x (95°C for 10 seconds, 52°C for 10 seconds and 72°C for 20 seconds) with a melt analysis from 65°C to 95°C ramping at 0.5°C. RNA was extracted from 12 beetles (handled, injected with insect ringer's solution and injected with elicitor, n = 4/treatment group) with a corresponding negative control for every experiment.

Statistical Analyses

Data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.v811c. Analyses were carried out using either linear mixed effects REML models in Genstat 15 (VSN International, Hemel Hempstead, UK) or general linear models in R 2.15.1 (Development Core Team, 2013). Genstat

produces both Wald statistics, and if the design and sample size permit it, F statistics. F statistics are more reliable than Wald statistics, and so are the statistical outputs that we quote in our results (A Guide to REML in GenStat® 15th Edition). The assumptions of the models were tested by visual inspection of the diagnostic plots produced by either program. When multiple measurements were taken from the same individuals, beetle ID was included as a random effect; this was the case in Experiment 2 when repeat breeding to measure LRS. Box was included as a random effect in Experiment 1 and when measuring PO in breeding beetles in response to wounding in Experiment 3 to account for any similarities between males and females breeding on the same carcass. Two-way interactions were included in the models for each experiment, but three-way interactions were not considered. PO and defensin data were normalised by log transformation. The statistics presented are estimations from the minimum adequate model following stepwise deletion of non-significant variables.

In Experiment 1 we used a REML model to analyse for any potential changes in PO throughout the breeding bout. We considered a factor with 9 levels; 8 levels accounting for each day of the bout and 1 level accounting for all control groups pooled. Whilst ideally we would have had a control on each day of the breeding bout, this was not logistically possible. Due to this constraint we could not consider day of bout*presence of carcass interaction. We therefore pooled those beetles that were not in the presence of a carcass into one control group for the analysis. Variables included in the initial model were day of bout, sex, carcass weight and whether the beetle bred or not, alongside two-way interactions. As there was no significant effect of box we considered any changes between the control groups with a linear model. We used a 3-levelled factor to include C0, C1 & C4.

In Experiment 2a when considering upregulation of PO we used a General Linear Model with 3 levels for treatment and 2 levels for sex. A One-Way ANOVA, not assuming equal variances, was used for the defensin data. Only females were used in this experiment. A REML model was used in Experiment 2b when considering potential changes to reproductive output. Treatment, brood and carcass weight were included in the initial model, alongside two-way interactions.

A REML model was used in Experiment 3a. Treatment (wounded?), presence of carcass and sex were included in the initial model, alongside two-way interactions. General Linear Models were used in Experiment 3b to consider potential changes to reproductive output following wounding at different times during the bout, including carcass weight in the model. Time of wounding within bout was treated as a continuous variable in Experiment 3b, in contrast to day being treated as a factor in Experiment 1. We believe day can be treated as either a factor or linear effect depending on its biological effects. For experiment 1, we hypothesised that PO would decline when we expected JH to peak, and we knew from the congener that JH does not change linearly over the breeding bout. As we expected, initial data exploration suggested a non-linear fit for day in this experiment. For experiment 3 we did not have an a priori expectation of the effect of day of wounding on reproductive output, and data exploration suggested a linear fit. As the response variables (PO and reproductive output) are very different, it is reasonable to assume that the effects of time on those variables are also different.

Results

Experiment 1: Constitutive Immunity During Breeding

PO was suppressed during breeding, with levels changing throughout the bout ($F_{8,242} = 7.25$, $p<0.001$; Figure 2). PO declined up to the third day of breeding, one day prior to larvae appearing on the carcass, but thereafter started to recover to pre-breeding levels (Figure 2). However, mating in the absence of a carcass (C1) caused PO to increase above control individuals (C0) ($F_{2,136} = 7.65$, $p<0.001$; Figure 2). PO levels dropped in C4 to a level similar to that of C0 individuals, most likely due to repeat mating for 4 days without a carcass being an unnatural situation. PO levels were not affected by sex (sex: $F_{1,260} = 0.16$, $p = 0.689$; treatment*sex: $F_{8,238} = 1.80$, $p = 0.078$), carcass weight ($F_{1,178} = 0.34$, $p = 0.562$), or whether a beetle bred successfully or not ($F_{1,177} = 0.46$, $p = 0.498$).

Experiment 2: The Effect of Immune Challenge Before Breeding on Immunity and Reproduction

a) Stimulating the immune response

Wounding increased PO levels, whereas the elicitor treatment decreased PO levels, relative to the non-challenged control group ($F_{2,224} = 18.18$, $p<0.001$; Figure 3a). Sex did not affect PO levels ($F_{1,223} = 0.52$, $p = 0.472$).

Wounding increased defensin expression relative to control beetles, and injection with elicitor increased defensin expression above the level of both the wounded group and the control group ($F_{2,4} = 296.76$, $p<0.001$; Figure 3b). The elicitor did not affect the beetles' subsequent longevity ($t = -0.48$, $df = 27$, $p = 0.634$).

b) What effect does immune challenge have on lifetime reproductive success?

Immune challenge did not affect the beetles' LRS in terms of larval number ($F_{1,411} = 0.07$, $p = 0.790$), mean larval weight ($F_{1,381} = 0.63$, $p = 0.428$) or total larval weight ($F_{1,374} = 0.01$, $p = 0.922$). After accounting for the effect of carcass weight on all three reproductive proxies (number of larvae: $F_{1,406} = 11.55$, $p<0.001$; mean larval weight: $F_{1,383} = 12.72$, $p<0.001$; total larval weight: $F_{1,381} = 4.06$, $p = 0.045$), reproductive output declined in the later broods (number of larvae: $F_{4,329} = 14.57$, $p<0.001$; mean larval weight: $F_{3,297} = 6.46$, $p<0.001$; total larval weight: $F_{3,291} = 17.26$, $p<0.001$). Interactions were not significant for any of the reproductive components (larval number: $F<1.71$, $p>0.146$, mean larval weight: $F<1.16$, $p>0.325$, total larval weight: $F<2.47$, $p>0.061$).

The number of broods per female ($F_{1,124} = 4.45$, $p = 0.037$) and the successful broods per female were predicted by average carcass weight ($F_{1,124} = 12.07$, $p<0.001$). Larger carcasses resulted in more broods and more successful broods. The number of broods per female and number of successful broods per female are shown in Table 1.

Experiment 3: The Effect of Immune Challenge During Breeding on Immunity and Reproduction

a) Is the immune system still upregulated when wounded during breeding?

Immune upregulation following wounding occurs prior to carcass acquisition, as shown previously. This experiment showed that beetles can also upregulate PO following wounding whilst breeding ($F_{1,75} = 5.07$, $p = 0.027$; Figure 4). Immunosuppression was observed on the carcass, as previously shown in the first experiment ($F_{1,76} = 44.38$, $p<0.001$). Sex had no effect on PO ($F_{1,77} = 1.52$, $p = 0.221$). No interaction terms were significant ($F<1.07$, $p>0.304$).

b) What effect does wounding whilst breeding have on reproductive output?

When the parent was wounded early in the bout, fewer larvae were produced ($F_{1,143} = 7.35$, p=0.008; Figure 5) and a lower total weight of larvae ($F_{1,142} = 7.24$, p = 0.008), however mean larval weight was not affected ($F_{1,142} = 0.71$, p = 0.402). This effect was observed after accounting for carcass weight where required (mean larval weight: $F_{1,143} = 76.56$, p<0.001; total larval weight: $F_{1,142} = 6.24$, p = 0.014; number of larvae: $F_{1,142} = 3.29$, p = 0.071).

For all three reproductive proxies, there was no interaction between day of wounding and carcass weight (F<3.08, p>0.081). Data from day 8 was omitted from all analyses as some larvae had already dispersed by then.

Discussion

Trade-offs between reproduction and immune function are supported in the literature (Sheldon & Verhulst 1996). To date, immune research in this genus (*Nicrophorus*) has been mostly directed towards quantifying social immunity. Knowledge of personal immune strategies will yield a fuller understanding of how organisms associated with microbe-rich environments cope with both surviving and providing costly parental care.

The study we present provides evidence of immunosuppression during breeding. However, the beetles can still upregulate their immune system during breeding if presented with a challenge, albeit not to pre-breeding levels. We show that timing of immune challenge is important; a trade-off is present if challenged during the reproductive bout, but if applied prior to breeding, no trade-off with reproduction is observed.

We began by considering constitutive immunity i.e. the natural baseline of immunity during breeding, with no manipulation. As we might expect from the JH profile, PO levels were suppressed during breeding. Suppression was at its greatest on Day 3. Larvae arrive on the carcass at Day 4, and this suppression may occur in anticipation of this resource intensive period. We know that both reproduction and investment into immunity are costly (Lochmiller & Deerenberg 2000; Davies et al. 2012) and this finding supports a trade-off between these traits. However, mating increased PO levels. This may be adaptive in order to deal with the increased risk of invading microbes during mating as well as being unconstrained by additional components of costly breeding processes. With mating increasing PO, the immunosuppressive effect of breeding relative to individual virgin beetles is even greater given that the beetles are also mating at this time.

Conversely, a similar experiment on *Nicrophorus orbicollis* (Steiger et al. 2011) showed no change in PO during the breeding bout in the species, alongside an upregulation of encapsulation. However, this experiment did not include any unchallenged control beetles. Therefore whilst PO activity in breeding beetles was similar to non-breeding beetles, this shows an equal level of response to challenge, but no clear picture as to the baseline levels. We find that *N. vespilloides* can still upregulate PO whilst on a carcass following wounding, and as encapsulation presents a much larger challenge, the expected increase would be substantially greater. This response to immune insult could therefore mask any immunosuppression present.

Deciphering the proximate basis of trade-offs is important. JH is increasingly being invoked as the potential mechanism for this trade-off and this study lends support to it acting in an antagonistic manner on reproduction and immune investment. However, as we do not currently have the JH breeding profile for this

452 species, it would be just as valid to consider that the trade-off may arise from
453 physiological constraints, resource based trade-offs not coupled to JH control or
454 autoimmunity.
455 We found that whether a beetle successfully bred or not did not affect PO
456 levels. Immunosuppression may be implicated in anticipation of events or in many
457 cases the efforts utilised even in failed broods may still merit immunosuppression in
458 order to occur.
459 No difference was observed between the sexes in their standing immune
460 function. Cotter and Kilner (2010b) predicted that male investment in personal
461 immunity would be greater as they have a greater residual reproductive value (Ward
462 et al. 2009), and so more to gain from a longer life. In contrast, Steiger et al. found
463 higher PO activity in the haemolymph of females (Steiger et al. 2011). These
464 congenerics may have different investment strategies. In *N. vespilloides*, males and
465 females seem to balance the costs of their reproduction related activities with immune
466 investment in a similar fashion.
467 Burying beetles do seem to have the capacity to treat immune investment as a
468 plastic trait, in a manner already observed for their reproductive strategy, and can alter
469 their standing immunity at a time when they are investing heavily in reproduction.
470 PO suppression during breeding provides good evidence for a trade-off
471 between reproduction and immune investment. Whilst manipulative experiments
472 often show the best evidence of trade-offs, there is also great scope in exploiting times
473 of natural resource pressure such as reproduction, especially in species exhibiting
474 parental care.
475

After establishing baseline immunity during breeding, we considered what would happen if we perturbed the system at different times, both prior to and during breeding. First we considered effects of wounding and an immune elicitor on PO and defensin levels. The expectation that PO would increase with wounding was confirmed in this study. However, PO was suppressed upon elicitor injection, both relative to the control and wounding treatments. Conversely, defensin was upregulated in the elicitor treatment relative to wounding. It is possible that this shows an internal immune trade-off between PO and upregulation of the humoral system, as has been shown in other systems (Cotter et al. 2004a; Moret & Schmid-Hempel 2009; Povey et al. 2009; Rao et al. 2010). However, whether it is a resource-based trade-off or not is unclear. It could also arise due to physiological constraints, autoimmunity or perhaps an effect of PO on AMP action.

We then went on to consider if this immune upregulation prior to breeding had an effect on LRS. Although our experiments show no effect of elicitor treatment on LRS, this does not contradict the costly nature of immunity. This finding is similar to the study that used dead bacteria as a treatment, where LRS was also unaffected (Cotter et al. 2013). We successfully triggered the immune system so the burying beetles must have been able to recoup the associated costs elsewhere. A common way to recoup costs is simply to eat more (Lee et al. 2006; Povey et al. 2009) and our burying beetles were not studied under any form of nutrient limitation, indeed they could also eat from the breeding resource. Furthermore, humoral immunity may not actually be that costly resulting in potential effects being too small to detect or else being easily compensated for. As a laboratory system, some costly processes are bypassed; for example, there are no competitors and energy does not have to be utilised in carcass location. Whilst they have no difference in the lifetime number and

mass of larvae produced, there may still be effects downstream with regards differences in the quality of the offspring. The ability to turn traits on and off minimises cost utilisation, and we did not study the duration of PO/AMP upregulation. However, we did find that as AMPs were upregulated, PO was downregulated so maybe the costs are paid in reducing PO activity, in order that LRS is unaffected. If so, this would be similar to the finding that upregulating personal immunity through wounding in *N. vespilloides* downregulates social immunity in order to defend LRS (Cotter et al. 2013). An immune elicitor is clearly much less costly than an actively replicating pathogen, therefore future studies will investigate the effects of live parasites on immune-reproduction trade-offs. A point of note must be made regarding the difference in effect of an LPS/PEP elicitor and the utilisation of dead bacteria as in previous studies (Cotter et al. 2010a; Cotter et al. 2013). In these studies dead bacteria triggered an increase in reproductive output – a lifting of reproductive restraint. Perhaps there is a difference in the recognition and effector systems, with the dead bacteria representing a more natural scenario.

Challenging the immune system prior to breeding does so at a time when the immune system is not suppressed. Therefore the challenge could be 'dealt with' before the breeding bout commenced. We therefore wanted to consider what would happen if the beetle was called upon to invest in immunity and reproduction at the same time. Timing with regards development has been shown to be important in the regulation of this trade-off; in crickets an immune challenge in early adulthood results in a decline in reproductive output (Stahlschmidt et al. 2013), whereas in middle age this trade-off does not occur (Shoemaker & Adamo 2007). Our experiment looked at the timing with regards a specific event; before and after location of a breeding resource. The results of our study showed that wounding during breeding still

upregulated PO but at the detriment to reproductive output. The ability to upregulate PO is important due to the microbe-rich nature of the environment and also the likelihood of injuries from fights for carcasses (Scott 1998, Steiger 2012). The timing of wounding is important; wounding later in the bout does not have as large an effect. As the average larval weight is not affected by time of wounding, it would suggest that the mechanism for lowered reproductive output is not lack of care, but brood reduction (Trumbo 1990; Trumbo & Fernandez 1995) through ovicide and larvicide, with the same amount of care provided for the remaining offspring. A further possibility is that wounding during egg laying caused females to lay fewer eggs overall, resulting in a reduced brood size at dispersal. Larval mass at dispersal is the key measure of offspring quality due to its high correlation with adult body size (Bartlett & Ashworth 1988), which in turn is a central factor affecting the outcome of competitive interactions in burying beetles (Bartlett & Ashworth 1988; Muller et al. 1990). In this situation the optimal strategy for the beetles may be fewer, high quality, offspring. The changing value of the brood may also affect how resources are distributed. PO is suppressed the most in the early stages. If this is strategic, it follows that forcing them to upregulate PO early in the bout will have the largest effect on larval output.

To conclude, we have demonstrated that immunosuppression occurs during breeding. However, immune upregulation only affected reproductive output if it occurred once breeding had commenced. In both cases the burying beetle seems to act to optimise fitness in accordance with theory; they can ameliorate costs of immune investment incurred prior to breeding and the beetles can still respond to a wounding challenge when on the carcass, although at the detriment to reproduction.

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738

Figure Legends

Figure 1

A *Nicrophorus vespilloides* burying beetle providing care for her brood. Photo courtesy of O. Kruger.

Figure 2

The change in phenoloxidase levels across the breeding bout. The graph shows means and SE of log transformed raw data. Beetles are paired on day 1 of the bout and larvae begin dispersing around day 8. Breeding beetles are shown in black and control beetles (C0, C1 & C4) are shown in white.

Figure 3

The change in a) phenoloxidase levels and b) defensin expression following wounding and elicitor treatment. The graphs show means and SE of log transformed raw data. Means with different subscripted letters are significantly different from each other ($P<0.05$).

Figure 4

The effect of wounding on phenoloxidase levels, both on and off the carcass. Means and SEs are predicted values from a REML model controlling for box. White circles represent non-wounded beetles, with black circles representing wounded beetles.

Figure 5

The number of larvae produced when wounded on each day of the breeding bout. Day 8 is omitted as some larvae had already dispersed. The graph shows means and SE of log transformed raw data alongside a line showing the predicted model of the effect of day controlling for carcass weight.

Tables

Table 1: Means and SE for number of broods per female and number of successful broods per female for Experiment 2.

	Mean number of broods per female +/-SE	**Mean number of successful broods per female +/-SE**
Control	4.17+/-0.19	3.39+/-0.19
Wounded	3.72+/-0.17	3.03+/-0.16
Elicitor	4.07+/-0.20	3.30+/-0.19