

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

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13 **Running Title:**

14 Trade-offs between reproduction and immunity

15 **Summary/Abstract**

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

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

38 **Key Words**

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

41 **Introduction**

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

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

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

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

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

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

73 Invertebrates have provided excellent, productive model systems to address
74 many of the key questions within the field of ecological immunology. This is mainly
75 due to the ease with which many species can be kept (in large numbers), bred, and
76 their immune systems studied. The invertebrate immune system, whilst complex and
77 efficient, is nonetheless simpler than that of vertebrates. Immune responses can be
78 loosely categorised into two main arms: cellular immunity and humoral immunity.
79 The cellular response is largely constitutive, i.e. it is present at a basal level. It
80 involves haemocytes and is the primary defence to invasion, acting in a generalised
81 manner. The mechanisms include phagocytosis of microparasites, nodulation of
82 clumps of microparasites and encapsulation of macroparasites, which combat
83 pathogens in a fairly generic approach (Gillespie et al. 1997). A central feature of the
84 constitutive response is the activation of the prophenoloxidase (proPO) cascade
85 (Gillespie et al. 1997). As well as having a role to play in non-self recognition
86 (Söderhäll & Cerenius 1998), activation of the proPO cascade leads to the production
87 of melanin (Götz 1986), a substance used in the encapsulation response.
88 Phenoloxidase (PO) plays a key role in the coordination of the cellular response
89 (Gillespie et al. 1997) and it is also involved in cuticular hardening (Sugumaran et al.
90 2000). Whilst PO activity is constitutive, it can be further activated and upregulated
91 by a wide range of parasitic challenges (Gillespie et al. 1997).

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

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

101 Here we focus on the life history constraints of immune function, in the
102 burying beetle *N. vespilloides* (Figure 1). Burying beetles provide a highly tractable
103 system for studying the trade-off between immune investment and reproduction. The
104 nature of the constitutive immune system in insects makes it possible to measure
105 investment in immune function without actually stimulating their immune system.
106 The central feature associated with the life cycle of burying beetles is the availability
107 of a small vertebrate carcass; reproduction is completely reliant on the presence of
108 this resource. Extended biparental care is rare in insects and burying beetles use this
109 strategy to great effect. Once a carcass has been located, the parents will cooperate to
110 bury it underground and prepare it for consumption by their offspring by removing
111 hair/feathers and shaping it into a ball (Pukowski 1933; Scott 1998). The beetles coat
112 the carcass with antimicrobial anal exudates to delay decomposition (Cotter & Kilner
113 2010a). As this primarily benefits the offspring it is a form of social immunity
114 (Cotter & Kilner 2010b). Eggs are laid in the soil, near the carcass (Trumbo et al.
115 1995; Scott 1998). A further component of parental care is the capacity for brood
116 reduction against insurance larvae. If more larvae arrive at the carcass than it can
117 adequately sustain, the parents can carry out ovicide and/or larvicide (Trumbo 1990;
118 Trumbo & Fernandez 1995). Two-three days after egg laying in *N. vespilloides*, the

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

125 Research on immunity in this species is still at the early stage. Whilst there
126 are interesting findings with regards to social immunity (Cotter & Kilner 2010a;
127 Cotter et al. 2010b; Steiger et al. 2011; Arce et al. 2012; Cotter et al. 2013), there is
128 little information regarding the personal immune response. Three papers have
129 addressed this. The first identified a range of immune-inducible genes (Vogel et al.
130 2011), and two studies by Steiger et al. considered changes in immunity in a
131 congeneric species whilst breeding (Steiger et al. 2011) and the effect of dominance
132 status on immunity (Steiger et al. 2012).

133 A key aspect of the burying beetle breeding cycle is the surge in Juvenile
134 Hormone (JH) that occurs upon discovery of a carcass (Trumbo et al. 1995; Trumbo
135 1997). This is further upregulated when larvae appear (Trumbo 1997) and then falls
136 off during the remainder of the breeding bout. JH has been shown to be
137 immunosuppressive in other species (Hiruma & Riddiford 1988; Khafagi & Hegazi
138 2001; Rolff & Siva-Jothy 2002; Rolff 2002; Rantala et al. 2003; Amdam et al. 2004;
139 Franssens et al. 2006, Flatt et al. 2008), which suggests that the immune response
140 might be downregulated in this species during breeding. However, one could
141 hypothesise that immune function should be *upregulated* during breeding, as disease
142 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

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

171 **Experiment 1: Constitutive Immunity During Breeding**

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

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

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

196 **Experiment 2: The Effect of Immune Challenge Before Breeding on** 197 **Immunity and Reproduction**

198 **a) Stimulating the immune response**

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

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

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

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

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

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

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

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

241 +/- 0.27) until death. Eleven females never produced a brood (3/handled, 4/ringer's,
242 4/elicitor), and two escaped mid-experiment. These samples were excluded from the
243 analysis.

244 **Experiment 3: The Effect of Immune Challenge During Breeding on** 245 **Immunity and Reproduction**

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

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

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

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

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

268 **Haemolymph Sampling**

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

277 **Phenoloxidase (PO) Assay**

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

288 25°C on a Thermo Scientific Multiscan Spectrum spectrophotometer. The maximum
289 rate of reaction was then used as an approximation of PO level.

290 **AMP Upregulation**

291 To measure potential changes in expression of the immune related gene
292 defensin, we extracted RNA 24 hours after treatment application and used qRT-PCR
293 to determine any changes in defensin expression following treatment. Total RNA was
294 isolated from each beetle using Trizol® Reagent (Invitrogen, Life Technologies) in
295 accordance with the manufacturer's instructions. Contaminating DNA was removed
296 by treating total RNA with TURBO™ DNase (Invitrogen, Life Technologies) and
297 converted to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems,
298 Life Technologies). Primers were designed for defensin and the housekeeping gene
299 Beta Tubulin from ESTs known for *N. vespilloides* (Vogel et al. 2011). For each PCR
300 reaction 10µl of SYBR, 0.4µl FWD primer, 0.4µl REV primer, 7.2µl of water and 2µl
301 of 25ng/µl of cDNA was used. Real time PCR was carried out using a Biorad
302 Thermo Cycler with the following conditions; 95°C for 3 mins, and 50x (95°C for 10
303 seconds, 52°C for 10 seconds and 72°C for 20 seconds) with a melt analysis from
304 65°C to 95°C ramping at 0.5°C. RNA was extracted from 12 beetles (handled,
305 injected with insect ringer's solution and injected with elicitor, n = 4/treatment group)
306 with a corresponding negative control for every experiment.

307 **Statistical Analyses**

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

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

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

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

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

358 **Results**

359 **Experiment 1: Constitutive Immunity During Breeding**

360 PO was suppressed during breeding, with levels changing throughout the bout
361 ($F_{8,242} = 7.25$, $p < 0.001$; Figure 2). PO declined up to the third day of breeding, one
362 day prior to larvae appearing on the carcass, but thereafter started to recover to pre-
363 breeding levels (Figure 2). However, mating in the absence of a carcass (C1) caused
364 PO to increase above control individuals (C0) ($F_{2,136} = 7.65$, $p < 0.001$; Figure 2). PO
365 levels dropped in C4 to a level similar to that of C0 individuals, most likely due to
366 repeat mating for 4 days without a carcass being an unnatural situation. PO levels
367 were not affected by sex (sex: $F_{1,260} = 0.16$, $p = 0.689$; treatment*sex: $F_{8,238} = 1.80$, p
368 $= 0.078$), carcass weight ($F_{1,178} = 0.34$, $p = 0.562$), or whether a beetle bred
369 successfully or not ($F_{1,177} = 0.46$, $p = 0.498$).

370 **Experiment 2: The Effect of Immune Challenge Before Breeding on** 371 **Immunity and Reproduction**

372 **a) Stimulating the immune response**

373 Wounding increased PO levels, whereas the elicitor treatment decreased PO
374 levels, relative to the non-challenged control group ($F_{2,224} = 18.18$, $p < 0.001$; Figure
375 3a). Sex did not affect PO levels ($F_{1,223} = 0.52$, $p = 0.472$).

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

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

381 Immune challenge did not affect the beetles' LRS in terms of larval number
382 ($F_{1,411} = 0.07$, $p = 0.790$), mean larval weight ($F_{1,381} = 0.63$, $p = 0.428$) or total larval
383 weight ($F_{1,374} = 0.01$, $p = 0.922$). After accounting for the effect of carcass weight on
384 all three reproductive proxies (number of larvae: $F_{1,406} = 11.55$, $p < 0.001$; mean larval
385 weight: $F_{1,383} = 12.72$, $p < 0.001$; total larval weight: $F_{1,381} = 4.06$, $p = 0.045$),
386 reproductive output declined in the later broods (number of larvae: $F_{4,329} = 14.57$,
387 $p < 0.001$; mean larval weight: $F_{3,297} = 6.46$, $p < 0.001$; total larval weight: $F_{3,291} =$
388 17.26 , $p < 0.001$). Interactions were not significant for any of the reproductive
389 components (larval number: $F < 1.71$, $p > 0.146$, mean larval weight: $F < 1.16$, $p > 0.325$,
390 total larval weight: $F < 2.47$, $p > 0.061$).

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

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

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

398 Immune upregulation following wounding occurs prior to carcass acquisition,
399 as shown previously. This experiment showed that beetles can also upregulate PO
400 following wounding whilst breeding ($F_{1,75} = 5.07$, $p = 0.027$; Figure 4).
401 Immunosuppression was observed on the carcass, as previously shown in the first
402 experiment ($F_{1,76} = 44.38$, $p < 0.001$). Sex had no effect on PO ($F_{1,77} = 1.52$, $p =$
403 0.221). No interaction terms were significant ($F < 1.07$, $p > 0.304$).

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

405 When the parent was wounded early in the bout, fewer larvae were produced
406 ($F_{1,143} = 7.35$, $p=0.008$; Figure 5) and a lower total weight of larvae ($F_{1,142} = 7.24$, $p =$
407 0.008), however mean larval weight was not affected ($F_{1,142} = 0.71$, $p = 0.402$). This
408 effect was observed after accounting for carcass weight where required (mean larval
409 weight: $F_{1,143} = 76.56$, $p<0.001$; total larval weight: $F_{1,142} = 6.24$, $p = 0.014$; number of
410 larvae: $F_{1,142} = 3.29$, $p = 0.071$).

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

414 **Discussion**

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

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

427 We began by considering constitutive immunity i.e. the natural baseline of
428 immunity during breeding, with no manipulation. As we might expect from the JH
429 profile, PO levels were suppressed during breeding. Suppression was at its greatest
430 on Day 3. Larvae arrive on the carcass at Day 4, and this suppression may occur in
431 anticipation of this resource intensive period. We know that both reproduction and
432 investment into immunity are costly (Lochmiller & Deerenberg 2000; Davies et al.
433 2012) and this finding supports a trade-off between these traits. However, mating
434 increased PO levels. This may be adaptive in order to deal with the increased risk of
435 invading microbes during mating as well as being unconstrained by additional
436 components of costly breeding processes. With mating increasing PO, the
437 immunosuppressive effect of breeding relative to individual virgin beetles is even
438 greater given that the beetles are also mating at this time.

439 Conversely, a similar experiment on *Nicrophorus orbicollis* (Steiger et al.
440 2011) showed no change in PO during the breeding bout in the species, alongside an
441 upregulation of encapsulation. However, this experiment did not include any
442 unchallenged control beetles. Therefore whilst PO activity in breeding beetles was
443 similar to non-breeding beetles, this shows an equal level of response to challenge,
444 but no clear picture as to the baseline levels. We find that *N. vespilloides* can still
445 upregulate PO whilst on a carcass following wounding, and as encapsulation presents
446 a much larger challenge, the expected increase would be substantially greater. This
447 response to immune insult could therefore mask any immunosuppression present.

448 Deciphering the proximate basis of trade-offs is important. JH is increasingly
449 being invoked as the potential mechanism for this trade-off and this study lends
450 support to it acting in an antagonistic manner on reproduction and immune
451 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 congeners 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

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

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

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

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

526 upregulated PO but at the detriment to reproductive output. The ability to upregulate
527 PO is important due to the microbe-rich nature of the environment and also the
528 likelihood of injuries from fights for carcasses (Scott 1998, Steiger 2012). The timing
529 of wounding is important; wounding later in the bout does not have as large an effect.
530 As the average larval weight is not affected by time of wounding, it would suggest
531 that the mechanism for lowered reproductive output is not lack of care, but brood
532 reduction (Trumbo 1990; Trumbo & Fernandez 1995) through ovicide and larvicide,
533 with the same amount of care provided for the remaining offspring. A further
534 possibility is that wounding during egg laying caused females to lay fewer eggs
535 overall, resulting in a reduced brood size at dispersal. Larval mass at dispersal is the
536 key measure of offspring quality due to its high correlation with adult body size
537 (Bartlett & Ashworth 1988), which in turn is a central factor affecting the outcome of
538 competitive interactions in burying beetles (Bartlett & Ashworth 1988; Muller et al.
539 1990). In this situation the optimal strategy for the beetles may be fewer, high
540 quality, offspring. The changing value of the brood may also affect how resources are
541 distributed. PO is suppressed the most in the early stages. If this is strategic, it
542 follows that forcing them to upregulate PO early in the bout will have the largest
543 effect on larval output.

544

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

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560

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738

739

740 **Figure Legends**

741 **Figure 1**

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

744 **Figure 2**

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

749 **Figure 3**

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

754 **Figure 4**

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

758

759

760 **Figure 5**

761 The number of larvae produced when wounded on each day of the breeding bout.

762 Day 8 is omitted as some larvae had already dispersed. The graph shows means and

763 SE of log transformed raw data alongside a line showing the predicted model of the

764 effect of day controlling for carcass weight.

765

766 **Tables**

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

769

	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

770