

1 **Ageing in personal and social immunity: do immune traits**
2 **senesce at the same rate?**

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18 **Running title**

19 Changes in personal and social immunity with age

20

21 **Summary**

- 22 1) How much should an individual invest in immunity as it grows older? Immunity
23 is costly and its value is likely to change across an organism's lifespan. A limited
24 number of studies have focused on how personal immune investment changes
25 with age in insects, but we do not know how social immunity, immune responses
26 that protect kin, changes across lifespan, or how resources are divided between
27 these two arms of the immune response.
- 28 2) In this study both personal and social immune function are considered in the
29 burying beetle, *Nicrophorus vespilloides*. We show that personal immune
30 function declines (phenoloxidase levels) or is maintained (defensin expression)
31 across lifespan in non-breeding beetles but is maintained (phenoloxidase levels) or
32 even upregulated (defensin expression) in breeding individuals. In contrast, social
33 immunity increases in breeding burying beetles up to middle age, before
34 decreasing in old age. Social immunity is not affected by a wounding challenge
35 across lifespan, whereas personal immunity, through PO, is upregulated following
36 wounding to a similar extent across lifespan.
- 37 3) Personal immune function may be prioritised in younger individuals in order to
38 ensure survival until reproductive maturity. If not breeding, this may then drop
39 off in later life as state declines. As burying beetles are ephemeral breeders,
40 breeding opportunities in later life may be rare. When allowed to breed beetles
41 may therefore invest heavily in 'staying alive' in order to complete what could
42 potentially be their final reproductive opportunity. As parental care is important
43 for the survival and growth of offspring in this genus, staying alive to provide care
44 behaviours will clearly have fitness payoffs.
- 45 4) This study shows that all immune traits do not senesce at the same rate. In fact,
46 the patterns observed depend upon the immune traits measured and the breeding
47 status of the individual.

48 **Key-words**

49 Ageing, ecological immunology, insect, lifespan, lysozyme, *Nicrophorus*, parental
50 care, phenoloxidase, wounding, defensin

51

52 **Introduction**

53 Senescence is the change in physiological processes and tissue function with
54 age, exhibited in nearly all organisms (Stanley 2012). It results in a gradual loss of
55 function at the level of the cells, tissue and whole organism, culminating in some
56 degree of irreversible decline with age. The rate of senescence shows a broad
57 phylogenetic distribution. For example, *Drosophila melanogaster* live to 52 days,
58 whereas Japanese women live to 102 years (5% of the population surviving) (Jones et
59 al. 2013). Such a broad range of senescence rates across taxa indicates highly varied
60 cellular and physiological processes, as well as widely different investment strategies.

61 Essentially, it is cumulative damage by biological processes that causes
62 senescence (e.g. Kaszubowska 2008; Oliveira et al. 2010). Mechanisms such as
63 antagonistic pleiotropy (Hughes & Reynolds 2005), adverse gene actions at older ages
64 (Kirkwood & Austad 2000) and damage by reactive oxygen species (ROS)
65 (Hoffmann 1995) can all contribute to senescence. Even with a very low rate of
66 senescence, an organism will not live indefinitely due to environmental pressures.
67 Therefore, the optimal investment strategy for all traits with respect to time will
68 evolve in conjunction with externally imposed schedules of survival and reproduction
69 (Kirkwood & Austad 2000); senescence is a key life-history trait.

70 A central question in evolutionary biology is that of the proximate and
71 ultimate reasons for changing investment patterns with age. The costs and benefits of
72 a particular trait are likely to change across the lifespan of an individual. Investing in
73 the immune system is one such trait that should vary substantially, either because of a
74 decline in state and the changing risk of external mortality or because of changes in
75 environment or behaviour throughout the lifespan of a species (Rigby & Jokela 2000;
76 Wilson et al. 2002; Lawniczak & Begun 2004). Furthermore, the value of investment
77 in the immune system is likely to change as the individual ages. It is crucial that
78 juvenile organisms maintain an efficient immune system in order that they reach
79 adulthood and reproduce. If they do not, their direct fitness will be zero. We know
80 that immune function is costly (Lenski 1988; Kraaijeveld & Godfray 1997; Koella &
81 Boëte 2002; Hanssen et al. 2005; Lee et al. 2006; Sadd & Siva-Jothy 2006; Valtonen
82 et al. 2010; Simmons 2011), which raises the question of determining the optimal
83 pattern of investment with age.

84 In insects, there are two main arms to the immune system; cellular immunity
85 and humoral immunity. The standing immune response is the cellular system. A
86 range of haemocytes act as the initial, generalised defence to invaders, using
87 mechanisms such as phagocytosis of microparasites, nodulation of clumps of
88 microparasites and encapsulation of macroparasites (Gillespie et al. 1997). A main
89 component of the constitutive response is the activation of the prophenoloxidase (pro-
90 PO) cascade (Gillespie et al. 1997). The end product of this cascade is melanin (Götz
91 1986), which is used for encapsulation. Further roles of phenoloxidase (PO) include:
92 involvement in non-self recognition (Söderhäll & Cerenius 1998), coordination of the
93 cellular response (Gillespie et al. 1997) and cuticular hardening (Sugumaran et al.
94 2000). Although present at a basal level, PO can be further activated and upregulated
95 by a wide range of parasitic challenges (Gillespie et al. 1997). The PO cascade is also
96 associated with humoral immunity; the intermediate quinones produced exhibit
97 antimicrobial activity in the haemolymph (Nappi & Ottaviani 2000). Invasion also
98 prompts a further induction of the humoral immune response, which is relatively
99 specific in comparison to the generalised cellular response (Casteels et al. 1994;
100 Lemaitre et al. 1997). The molecules involved include lysozyme and other small
101 antimicrobial peptides (AMPs) (Hoffmann 1995). There are a huge range of AMPs,
102 e.g. cecropins, attacins and defensins, to name a few (Hoffmann 1995). Defensin is a
103 ubiquitous AMP found across the animal kingdom, and forms part of the human
104 immune response (Ganz 2003).

105 Various components of the personal immune system in insects have shown
106 changes with age in the literature. PO has been observed to decrease with age in
107 crickets, *Gryllus texensis* (Adamo et al. 2001), bumblebees, *Bombus terrestris* and
108 *Bombus muscorum* (Moret & Schmid-Hempel 2009; Whitehorn et al. 2011) and
109 honeybees, *Apis mellifera* (Roberts & Hughes 2014). In contrast, in a study on the
110 leaf-cutting ant, *Acromyrmex octospinosus*, PO increases in older workers (Armitage
111 & Boomsma 2010). Research by Li *et al.* (1992) showed a decrease in both PO
112 associated with haemocytes and haemolymph PO with age. The encapsulation
113 response has also been observed to decline in older age classes in *Bombus terrestris*
114 (Doums et al. 2002). Older mosquitoes, *Aedes aegypti*, showed an age-associated
115 mortality in response to challenge and this corresponded to a decrease in haemocyte
116 numbers (Hillyer et al. 2005). Haemocyte density has also been observed to decrease
117 with age in *Bombus terrestris* (Moret & Schmid-Hempel 2009). A decline in the

118 nodulation response with age has been observed in several species; in honeybees, *Apis*
119 *mellifera*, the number of nodules produced in response to freeze-dried bacterial
120 challenge declines with age (Bedick et al. 2001). In male crickets, *Gryllus assimilis*,
121 declining numbers of nodules were formed in a graded response to lipopolysaccharide
122 (LPS) injections with age (Park et al. 2011). Both haemocyte density and phagocytic
123 ability have been shown to decline in *Drosophila melanogaster* (Mackenzie et al.
124 2011). *Drosophila* have yielded great insight into the genes involved in
125 immunosenescence. Interestingly, the most obvious change in genome-wide
126 expression with age seems to be for the genes involved in the immune response
127 (Sarup et al. 2011; Iliadi et al. 2012). Across studies, the increase in transcripts of
128 immune response genes in *Drosophila melanogaster* with age has been observed
129 (Pletcher et al. 2002; Zerofsky et al. 2005). These studies hypothesise that this
130 upregulation may be due to a lifetime of exposure to pathogens, or it may result from
131 a decline in the function of transcripts (Iliadi et al. 2012).

132 The above studies all consider changes in personal immune responses, but
133 there are a suite of immune responses, called social immune responses (*sensu* Cotter
134 & Kilner 2010b), that have been selected to increase the fitness of the challenged
135 individual and one or more recipients (Cotter & Kilner 2010a). These responses
136 occur across the animal kingdom. For example, the beewolf, *Philanthus triangulum*,
137 provisions brood cells with bees upon which their offspring feed. The bee corpses are
138 embalmed using a cocktail of hydrocarbons that create an environment unsuitable for
139 fungal growth (Herzner & Strohm 2007; Herzner et al. 2007). Eggs of the three-
140 spined stickleback, *Gasterosteus aculeatus*, are protected from microbes by an
141 antimicrobial mucus that glues the nest together (Little et al. 2008). Indeed, the
142 provisioning of antibodies in mammalian milk also falls within the definition of a
143 social immune response (Cotter & Kilner 2010a). Despite their ubiquity, we do not
144 know how social immune responses change with age, or indeed how the balance of
145 investment in personal and social immunity changes over an organism's lifetime.

146 In this study we look at patterns of immunity across lifespan in the burying
147 beetle, *Nicrophorus vespilloides* (Figure 1). This species is a carrion breeder, and
148 exhibits biparental care of young (Pukowski 1933). The parents cooperate to bury a
149 small vertebrate carcass, and prepare it for their offspring by removing hair or
150 feathers and shaping it into a ball (Pukowski 1933; Scott 1998). Antimicrobial anal
151 exudates are used to delay decomposition of the carcass (Cotter & Kilner 2010a),

152 which is a form of social immunity (Cotter & Kilner 2010b). The antimicrobial
153 exudates improve offspring survival; larvae do not develop as well on carcasses in an
154 advanced state of decay (Rozen et al. 2008). Breeding success in this species drops
155 off significantly as the beetles age (Cotter et al. 2010a). We know that the production
156 of antimicrobial exudates i.e. social immunity, is costly (Cotter et al. 2010b) and that
157 maintaining personal immunity is also costly (Reavey et al. 2014). Burying beetles
158 therefore provide us with a system that allows us to easily consider both personal and
159 social immune investment across lifespan. Is a change in the balance of these traits
160 with age selected for?

161 We hypothesise that personal immune function will decline with age and that
162 it will be suppressed in breeding beetles (Reavey et al. 2014), but perhaps this
163 suppression will be exacerbated with old age when residual reproductive value
164 declines. If social immunity follows a pattern of parental investment, we might
165 expect an initial increase, but lower levels of lytic activity in later life. Currently
166 there are little or no studies on social immunity across lifespan or changes in the
167 balance of personal and social immunity with age. Therefore a central aim of this
168 study is to further understand variation in personal and social immune function.
169 Study of social immunity is very much in its early stages; the external social immune
170 response is still part of the immune response but much less studied in terms of costs
171 (Otti et al. 2014). Furthermore, many of the organisms in which social immunity has
172 been studied are typically eusocial species in which the majority of individuals do not
173 reproduce. Therefore, considering the balance of personal and social immunity in
174 reproductive individuals is especially interesting, as both survival and reproduction
175 are the central components contributing to fitness.

176 **Materials and methods**

177 *Nicrophorus vespilloides*

178 The lab population was maintained as described previously (Reavey et al.
179 2014). In brief, non-breeding adult beetles were housed in individual boxes
180 containing moist soil at 20°C under a 16:8 light:dark cycle and fed twice weekly *ad*
181 *libitum* with minced beef. Pairs were placed together in a breeding container, 1/3
182 filled with moist soil and provided with a mouse carcass, and placed in a dark
183 cupboard to mimic underground conditions. Larvae were removed from the breeding

184 container as soon as they began dispersing from the carcass, typically 8-10 days after
185 the parents were paired, placed individually in compartments of 25 cell petri dishes,
186 covered with moist soil and left to pupate. Eclosion occurs around 20 days following
187 dispersal, after which the beetles were set up in their individual containers for either
188 lab population beetles or for use in later experiments.

189 The mean lifespan of beetles from our pedigree data, was 51.92 days +/- 0.23,
190 with mortality rising sharply thereafter. Adult age is measured from the point of
191 eclosion, rather than since the hatching from the egg. Therefore, age classes for the
192 experiments were selected from 0-8 weeks old. Discrete groups of beetles were used
193 in each experimental set-up. Different age classes were used across experiments; for
194 non-breeding beetles PO was measured from 0-8 weeks. Due to the time required for
195 reproductive maturation and a decline in breeding with age potentially providing less
196 data, age classes from 2-7 weeks were used in experiments carried out on breeding
197 beetles. When measuring antimicrobial activity (AMP – defensin), the age classes
198 selected were 2, 5 and 8 weeks. This range was selected to cover as much of the
199 lifespan of the beetles as possible in uniform intervals, but due to logistical
200 constraints, more age classes could not be included.

201 **Experiment 1: Changes in personal and social immunity across lifespan**

202 **a) Changes in personal immunity (PO) across lifespan in breeding and non-breeding** 203 **beetles**

204 Constitutive PO levels were measured in this part of the study. Firstly, PO
205 activity in non-breeding beetles across lifespan was measured. Standing levels of PO
206 in non-breeding beetles were measured on a weekly basis from 0 to 8 weeks of age,
207 with week 0 being 2 days following eclosion. Haemolymph could only be sampled
208 from each beetle once, as wounding alone will trigger an immune response (Reavey et
209 al. 2014). Therefore, separate individuals in discrete groups (n=18) were used for
210 each age class (total sample size = 162 beetles). Due to death in the later stages of
211 this experiment, some individuals did not provide samples. 130 samples were
212 obtained in total. Individuals were fed mince *ad libitum* on the day prior to sampling,
213 and sampling took place at the same time of day.

214 PO in breeding beetles across lifespan was then measured. Six age classes
215 were used, beetles aged 2 weeks to 7 weeks at weekly intervals, with each age class

216 consisting of a discrete group of female beetles. This experiment focused on females
217 only in order that any effect of age class could be considered for each individual in
218 isolation, without potentially confounding effects from a partner. Females can raise
219 offspring without the assistance of a male (Scott 1998). 10 beetles were allocated to
220 each age class and paired at the appropriate time for breeding. Beetles were mated
221 (males were aged 2 weeks for all experimental groups) and the male then removed
222 prior to presenting a mouse carcass in order that results were not confounded by his
223 presence (Cotter & Kilner 2010a). On day 4 of the breeding bout (bout duration is
224 from carcass presentation to larval dispersal) haemolymph samples were collected
225 and processed to determine PO levels. Haemolymph samples were obtained from 51
226 individuals. Day 4 of the breeding bout is a time of intense larval care and lytic
227 activity peaks at this time (Cotter et al. 2013).

228 **b) Changes in personal immunity (AMP, defensin) across lifespan in breeding and non-**
229 **breeding beetles**

230 Potential changes in defensin expression across lifespan provide us with a
231 proxy for investment into the humoral arm of the personal immune system as the
232 organism ages. Due to the nature of humoral immunity and the fact that it is largely
233 induced upon challenge (defensin expression is low or absent in unchallenged
234 individuals), in this part of the study all individuals were challenged with an immune
235 elicitor in order to upregulate defensin expression. Female beetles were assigned to
236 three age classes: 2 weeks, 5 weeks and 8 weeks. Within each age class, beetles were
237 split into either breeding or non-breeding sub-groups. This resulted in 6 groups, with
238 9 individuals per group. Elicitor (1mg of lipopolysaccharide (LPS) (Sigma-Aldrich)
239 and 2.5mg of peptidoglycan (PEP) (Sigma-Aldrich) were suspended in 1ml of sterile
240 insect ringer's solution and 1ul of this solution injected into each beetle using a
241 Hamilton syringe) was injected into the cuticle behind the pronotum 24 hours prior to
242 sampling (to upregulate defensin to the greatest extent (Reavey et al. 2014)) and in the
243 case of the breeding beetles this occurred on day 3 of the breeding bout, with
244 sampling taking place on day 4 (males for all experimental groups were aged 2 weeks
245 and were removed after mating). RNA was extracted from the beetles and defensin
246 upregulation was measured in accordance with the protocol below. Total body tissue
247 from each beetle was pooled during extraction (to maximise samples with a given
248 extraction effort); 3 beetles were pooled resulting in 3 overall samples per group. Due

249 to death in the week 8 group, the sample size was diminished (6 beetles of the initial 9
250 survived in both the breeding and non-breeding group). 1 sample was omitted from
251 the two-week old breeding beetles experimental group due to potential error
252 introduced during the extraction process.

253 **c) Changes in social immunity across lifespan in breeding beetles**

254 Lytic activity was measured in breeding beetles across lifespan. Lytic activity
255 is only upregulated in the presence of a breeding resource (Cotter & Kilner 2010a).
256 Six age classes were used, beetles aged 2 weeks to 7 weeks at weekly intervals, with
257 each age class consisting of a discrete group of female beetles. 10 beetles were
258 allocated to each age class and paired at the appropriate time for breeding (males were
259 aged 2 weeks for all experimental groups). Beetles were mated and the male then
260 removed prior to presenting a mouse carcass in order that results were not confounded
261 by his presence (Cotter & Kilner 2010a). On day 4 of the breeding bout, exudate
262 samples were obtained from all beetles and processed to determine lytic activity
263 levels. Exudate samples were obtained from 51 individuals.

264 **Experiment 2: The effect of wounding on immunosenescence**

265 In conjunction with measuring PO and lytic activity in breeding beetles across
266 lifespan (Experiment 1), a manipulative experiment was also carried out to determine
267 whether wounding with a sterile 0.5mm needle at various stages of lifespan affected
268 the trade-off between personal and social immunity (Cotter et al. 2013). The
269 experimental set up was as described in Experiment 1c, except that a further group of
270 beetles for each age class was used to test the effects of wounding. On day 3 of the
271 breeding bout, the beetles in the wounded treatment group were wounded on the
272 cuticle behind the pronotum with a sterile 0.5mm needle, while those in the non-
273 wounded group were handled. On day 4 of the breeding bout, exudate samples and
274 haemolymph samples were obtained from all beetles and processed to determine lytic
275 activity and PO levels. Exudate samples and haemolymph samples were obtained
276 from 94 individuals, predominantly due to mortality in the later groups. This enabled
277 us to consider if immune insult through wounding at different age classes (shown
278 previously to upregulate PO (Reavey et al. 2014) while downregulating lytic activity
279 (Cotter et al. 2013)) results in a change in the balance of personal and social immunity
280 across lifespan.

281 **Haemolymph sampling**

282 Haemolymph was obtained from *N. vespilloides* by piercing the cuticle behind
283 the pronotum with a sterile 0.5mm needle and then collecting the haemolymph as it
284 was released with a pipette (approximately 5ul haemolymph is released). The
285 haemolymph was then diluted with an equal volume of anticoagulant buffer to
286 prevent it from forming a solid mass (EDTA anticoagulant in PBS - pH 7.4) and then
287 stored in a freezer (-20°C) prior to analysis.

288 **Phenoloxidase (PO) assay**

289 Following defrosting of the haemolymph samples, 2µl of
290 haemolymph/anticoagulant buffer solution was added to 500µl of PBS (pH 7.4).
291 100µl of this solution was placed in a well of a 96-well microplate with 100µl of
292 10mM dopamine. While many researchers use L-dopa as a substrate for PO
293 reactions, for insect POs, dopamine is the preferred substrate over L-dopa. It is the
294 natural substrate for insects and is more soluble than L-dopa (Sugumaran 1998).
295 Readings were taken every 10 seconds for three minutes at 490nm and 25°C on a
296 Thermo Scientific Multiscan Spectrum spectrophotometer. The maximum rate of
297 reaction across 6 windows of change (absorbance readings) was then used as an
298 approximation of PO level.

299 **Exudate sampling**

300 When disturbed or handled, most of the beetles produce an exudate from their
301 abdomen. Tapping the abdomen gently often results in the production of exudate.
302 This can then be collected in a capillary tube, blown into an eppendorf and stored
303 until processing. Lytic activity of the samples was measured as described below.

304 **Lytic assay**

305 Bacterial agar plates were used and clear zones measured to determine lytic
306 activity. The agar plates consisted of 10ml of 1.5% agar:potassium phosphate buffer
307 (2:1) and 50mg of freeze-dried *Micrococcus luteus*. *M. luteus* was selected as it is a
308 soil bacterium, which is the breeding environment of the burying beetle. The exudate
309 samples were processed by punching 20, 2mm diameter holes in each agar plate and
310 filling each well with 1µl of exudate. Two technical replicates were processed per
311 sample. The plates were incubated at 33°C for 24 hours and the resulting clear zones

312 were measured using digital callipers to determine the magnitude of lytic activity.
313 Lytic activity (mg/ml) was then calculated from a standard serial dilution of hen egg
314 white lysozyme.

315 **Antimicrobial peptide (AMP) assay**

316 Due to its ubiquity, we chose the AMP *defensin* as our measure of humoral
317 immunity. RNA was extracted 24 hours after injection of the elicitor and qRT-PCR
318 used to determine any changes in *defensin* expression across the age classes and with
319 breeding status. RNA was isolated using Trizol® Reagent (Invitrogen, Life
320 Technologies) in accordance with the manufacturer's instructions. DNA was
321 removed by treatment with TURBO™ DNase (Invitrogen, Life Technologies) and
322 RNA converted to cDNA using a High Capacity RNA-to-cDNA kit (Applied
323 Biosystems, Life Technologies). Primers were designed for *defensin* and the
324 housekeeping gene *beta tubulin* from ESTs (Expressed Sequence Tags) known for *N.*
325 *vespilloides* (Vogel et al. 2011) (See Supplementary information). 10µl of SYBR,
326 0.4µl FWD primer, 0.4µl REV primer, 7.2µl of water and 2µl of 25ng/µl of cDNA
327 was used in each PCR reaction. Real time PCR was carried out using a Biorad
328 Thermo Cycler with the following conditions; 95°C for 3 mins, and 50x (95°C for 10
329 seconds, 52°C for 10 seconds and 72°C for 20 seconds) with a melt analysis from
330 65°C to 95°C ramping at 0.5°C. Primer efficiency (PCR efficiency as other
331 conditions were constant) was determined using a feature on the thermo cycler
332 machine, for use in the data analysis (*defensin*: 1.9, *tubulin*: 2.0). The Pfaffl equation
333 was used as the model for data analysis.

334 **Statistical analyses**

335 All statistical analyses were carried out in R 3.1.3 (Development Core Team,
336 2013). General linear mixed models were used in all analyses to control for the effect
337 of family, apart from Experiment 1b where a generalized least squares model was
338 carried out due to the unequal variance. In Experiment 1b, values from the Pfaffl
339 equation were normalised for use in the model. The assumptions of the models were
340 tested by visual inspection of the diagnostic plots. PO and lytic activity data were log
341 transformed to approximate normality. The statistics presented are estimations from
342 the minimum adequate model following stepwise deletion of non-significant
343 variables, i.e. the model only contains variables that are significant, unless statistics

344 for non-significant terms are quoted, in which case the non-significant term is
345 included last in the model.

346 **Results**

347 **Experiment 1: Changes in personal and social immunity across lifespan**

348 **a) Changes in personal immunity (PO) across lifespan in breeding and non-breeding** 349 **beetles**

350 *Non-breeders:* PO levels decreased across lifespan in non-breeding beetles in
351 a linear manner, dropping as the beetle aged (GLMM: estimate = -0.035 +/- 0.015, t_{119}
352 = -2.31, $P = 0.023$; Fig. 2a). There was no effect of sex on PO levels (GLMM:
353 estimate = 0.093 +/- 0.065, $t_{126} = 1.43$, $P = 0.155$) or the age*sex interaction (GLMM:
354 estimate = 0.025 +/- 0.031, $t_{125} = 0.80$, $P = 0.425$). Beetles were also analysed from 0-
355 4 weeks in order that selection for long-lived beetles was not occurring (age may
356 correlate with PO), and due to small sample sizes for the later groups. PO levels still
357 decreased across the lifespan of a beetle (GLMM: estimate = -0.081 +/- 0.027, $t_{82} = -$
358 3.06, $P = 0.003$). No effect of sex on PO was observed (GLMM: estimate = 0.048 +/-
359 0.075, $t_{86} = 0.64$, $P = 0.526$) or the age*sex interaction (GLMM: estimate = -0.057 +/-
360 0.054, $t_{85} = -1.06$, $P = 0.291$).

361 *Breeders:* In contrast, age did not affect PO levels in breeding beetles
362 (GLMM: estimate = 0.048 +/- 0.035, $t_{47.89} = 1.37$, $P = 0.176$; Fig. 2a).

363 **b) Changes in personal immunity (AMP, defensin) across lifespan in breeding and non-** 364 **breeding beetles**

365 Defensin levels increased with age for breeding beetles, but there was no
366 change in expression with age for non-breeding beetles, as observed in the
367 age*breeding status interaction ($F_{1,11} = 13.13$, $p = 0.004$, Fig. 2b).

368 **c) Changes in social immunity across lifespan in breeding beetles**

369 Lytic activity initially increased until female beetles were around 4 weeks of
370 age, before decreasing as the beetles aged further (GLMM: age = 0.864 +/- 0.243,
371 $t_{44.13} = 3.55$, $P < 0.001$, $\text{age}^2 = -0.106 +/- 0.028$, $t_{44.87} = -3.85$, $P < 0.001$; Fig. 2c).

372 **Experiment 2: The effect of wounding on immunosenescence**

373 PO was upregulated following wounding (GLMM: estimate = 0.189 +/- 0.082,
374 $t_{85.84} = 2.293$, $P = 0.024$; Fig. 3a) and this effect did not change significantly with age
375 (age*wounded interaction: GLMM: estimate = -0.029 +/- 0.052, $t_{84.26} = -0.570$, $P =$
376 0.570; Fig. 3a). There was no effect of wounding on lytic activity (GLMM: estimate
377 = -0.061 +/- 0.101, $t_{90} = -0.609$, $P = 0.544$; Fig. 3b) and no interaction between age
378 and wounding (GLMM: estimate = -0.053 +/- 0.370, $t_{88} = -0.144$, $P = 0.886$), or age²
379 and wounding (GLMM: estimate = 0.017 +/- 0.043, $t_{88} = 0.402$, $P = 0.688$). There
380 was no correlation between PO and lysozyme activity for either wounded ($F_{1,41} =$
381 2.55, $p = 0.118$) or non-wounded beetles ($F_{1,47} = 1.24$, $p = 0.272$).

382 **Discussion**

383 Here, to the best of our knowledge, for the first time in any taxa we assess
384 immunosenescence in both personal and social immunity. We show that while
385 personal immunity is maintained (defensin) or declines (PO) with age in non-
386 breeders, breeding beetles maintain (PO) or even increase (defensin) their investment
387 in personal immunity. Social immunity on the other hand, which is present only in
388 breeding beetles, peaks in middle aged beetles before starting to fall as beetles age.

389 As hypothesised, PO was found to decline with lifespan in non-breeding *N.*
390 *vespilloides*, the pattern occurring in both sexes. This species seems to follow a
391 'typical' pattern of immunosenescence; the decline of immune function as the
392 organism ages. Indeed, it supports other studies across taxa showing a decline in PO
393 across lifespan (Adamo et al. 2001; Moret & Schmid-Hempel 2009; Whitehorn et al.
394 2011; Roberts & Hughes 2014). However, in our experiment PO decreases even in
395 very young beetles, with the highest activity occurring just after emergence and
396 declining steadily throughout life. As these beetles have a pre-reproductive period of
397 approximately 2 weeks, the highest levels of investment correlate with the period
398 before they have the opportunity to reproduce. From an investment perspective a
399 younger organism is selected to invest in their immune system to aid chances of
400 survival to adulthood and future breeding opportunities. The initially high PO levels
401 could also be due to sclerotisation of the cuticle, which is relatively soft immediately
402 after eclosion. Also, due to the soft cuticle forming a less effective barrier to
403 microbes, younger beetles may be selected to invest more in immune function.
404 However, this would only be relevant for the first 2 days post emergence as the

405 cuticle hardens rapidly after eclosion. In older organisms, due to a limited duration of
406 lifespan ahead, the optimal strategy may be to conserve resources for reproduction.
407 Furthermore, older individuals may be constrained further by a decline in condition
408 and damage to tissue with age.

409 The finding that PO does not decline in female breeding beetles was initially
410 surprising; PO declines in non-breeders and is suppressed in young breeding beetles
411 (Reavey et al. 2014). As the experiments on non-breeding and breeding beetles were
412 carried out at different times, an element of caution must be used when comparing
413 results. However, it appears that PO is indeed downregulated in young breeding
414 beetles (2-3 weeks) relative to PO in virgins of the same age but then maintained or
415 upregulated in older breeding beetles. The fact that PO is held at the same level
416 during breeding across lifespan or indeed *upregulated* compared to the decline in non-
417 breeders indicates that personal immunity may be important when beetles gain their
418 first breeding opportunity in later life. Although PO is suppressed during breeding in
419 young beetles, perhaps a further decline in older beetles would fully compromise
420 standing immunity at a time when the individual is investing heavily in lifetime
421 reproductive success. However, it may be that a breeding attempt, which could be the
422 final opportunity to reproduce, calls for the organism to invest both in the brood, but
423 also in 'staying alive' for the duration of the parental care period.

424 Considering our measure of humoral immunity, defensin expression following
425 an immune challenge was found to remain at constant expression levels throughout
426 lifespan in female non-breeders. A limited sample size and only scope for three age
427 classes means that we must be cautious when interpreting the results. However,
428 although the variation is high we know that gene expression studies using a range of
429 methodologies across taxa show that some of the most dramatic transcriptional
430 changes that occur during ageing are associated with immunity, and so this variation
431 may be expected (DeVeale et al. 2004). It seems that age, encompassing a decline in
432 state, does not affect humoral investment as measured by defensin expression in non-
433 breeders. Knowledge on how PO investment and defensin investment compare with
434 regards to costs and benefits would be interesting, considering that PO declines with
435 age (albeit PO in unchallenged individuals). However, levels of defensin expression
436 increase in breeding beetles across lifespan. An increase in immune response genes
437 with age has also been observed in *Drosophila* (Pletcher et al. 2002; Zerofsky et al.
438 2005). Also, the process of mating has been shown to increase AMP expression

439 (Peng et al. 2005). If this is occurring in *N. vespilloides*, perhaps mating is
440 differentially affecting female immunity in different age classes. It is of note that the
441 levels of defensin expression for breeding beetles at younger age classes are lower
442 than that of non-breeders. PO is suppressed during breeding (Reavey et al. 2014), and
443 this may also be occurring for humoral immunity, with the suppression lifted at older
444 age classes when all resources may be invested in both reproduction and ‘staying
445 alive’ to complete the breeding bout.

446 Lytic activity, the social immune response (Cotter & Kilner 2010b), increased
447 in female breeding burying beetles up to middle age, before decreasing in old age.
448 Different patterns of reproductive investment with age exist across taxa, with a
449 common pattern being an initial increase in investment in early-middle aged class,
450 before a decline in old age. Hypotheses for these changes include: the selection
451 hypothesis (Curio 1983; Mauck et al. 2004), the constraint hypothesis (Curio 1983;
452 Korndeur 1996; Pärt 2001) and the restraint hypothesis (Williams 1966; McNamara et
453 al. 2009). As there was no significant mortality in our experimental beetles up to 5
454 weeks, which would allow less-fit individuals to be removed, the selection hypothesis
455 does not support the initial increase in lytic activity with age. It is more likely that the
456 constraint or restraint hypotheses support the changes we observe in lytic activity.
457 For example, there may be physiological constraints present with regard to lysozyme
458 production; this process may require maturation and indeed the age at which the
459 beetles normally produce their first brood in the field is unknown. The restraint
460 hypothesis provides another possible explanation for changes in reproductive
461 investment: young individuals provide less reproductive effort as the value of the
462 first/early brood is lower than that of expected future offspring. Life-history theory
463 predicts increased reproductive effort when residual reproductive value decreases
464 (Trivers 1972). There is evidence of reproductive restraint in burying beetles (Cotter
465 et al. 2010a), and elements of this theory may apply to the changes in lytic activity:
466 for young individuals (2 weeks), the value of the brood is low relative to future
467 broods and may not merit such a high investment in lysozyme production, which is a
468 costly resource (Cotter et al. 2010b). While the pattern of lytic activity differs slightly
469 to that of first time reproductive investment in this species (Cotter et al. 2010a), the
470 common pattern is for breeding performance to improve in the early years of life,
471 reaching a maximum at middle age (Reid et al. 2003), which is exactly the pattern
472 observed for lytic activity.

473 With regard to the effect of immune challenge on personal and social
474 immunity we see that wounding upregulates PO in female breeding beetles across
475 lifespan. This supports data, showing beetles can still upregulate PO while breeding
476 (Reavey et al. 2014). The fact that the response is of a similar magnitude as the
477 organism ages may indicate the importance of responding to a challenge at any age
478 while breeding. In contrast, when considering changes in lytic activity in response to
479 wounding across lifespan in female beetles, no effect of wounding was observed. We
480 initially thought this was odd as the experiment was based on the results of Cotter *et*
481 *al.* (2013) with the expectation that personal and social immunity would trade-off.
482 However, on closer inspection (Fig. 3b), it can be observed in our study that this
483 trade-off exists only in week 3 beetles, which is the age class used in the Cotter *et al.*
484 (2013) experiment. Why this trade-off exists at this age and none of the other age
485 classes is unclear; it may be that at other ages a trade-off occurs with different traits or
486 that as lytic activity is lower at other age classes, it is not as costly and does not
487 require a decrease in response to wounding.

488 Future experiments should consider if the response of PO to wounding in non-
489 breeders changes with age. This was not considered as the focus of this study was
490 initially on whether the trade-off between personal and social immunity (only present
491 in breeding beetles) changed with age. Furthermore, measurements of both proPO as
492 well as PO could be of interest as they might show different patterns with age (e.g.
493 Armitage & Boomsma 2010). Changes in lytic activity in male burying beetles with
494 age would also be interesting. Males have lower lytic activity levels than females
495 (Cotter & Kilner 2010a); we might expect a similar pattern, but lower absolute levels.
496 The responses to different immune challenges would be interesting to observe. It
497 would also be useful to measure a greater number of AMPs.

498 In summary, both personal and social immunity change across lifespan but
499 how they change depends upon the immune traits measured and the breeding status of
500 the individual. These changes are likely a result of the decline of the organism
501 alongside strategic changes in immune investment with age. While senescence is not
502 an adaptive process, and indeed in the wild animals generally do not live long enough
503 for senescence to be the cause of mortality, some patterns of decline may be adaptive
504 responses due to ‘time left to further fitness’, resulting in changes in resource
505 allocation and immune trait expression.

506 Our results regarding PO in non-breeders generally support other findings in
507 the literature, suggesting that the decline with age may be a conserved strategy across
508 species. Changes in PO with age in taxa while breeding has not been researched in
509 detail; the long breeding bout in burying beetles lends itself to its examination. The
510 maintenance/upregulation of defensin is also similar to immune response gene studies
511 in the literature, where it seems the transcripts often are at high levels in older age
512 classes. To the best of our knowledge, our study is the first to consider changes in
513 social immunity with age in a reproductive insect. As study on the area of social
514 immunity is fairly recent, as research in this field grows, further studies across taxa
515 will yield interesting findings with regards to how much variability in the pattern
516 exists and what drives the trends. Age related investment in immune function
517 contributes to how well an organism can resist or moderate infection at various stages
518 of their lifespan, which has consequences for host parasite dynamics. Recognising
519 changes in immune function, both personal and social, with age is important both for
520 understanding evolutionary theory as well as providing clues regarding factors
521 affecting animal health.

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528

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687 **Figure legends**

688 **Figure 1.** A *Nicrophorus vespilloides* female, courtesy of Steve Collett.

689

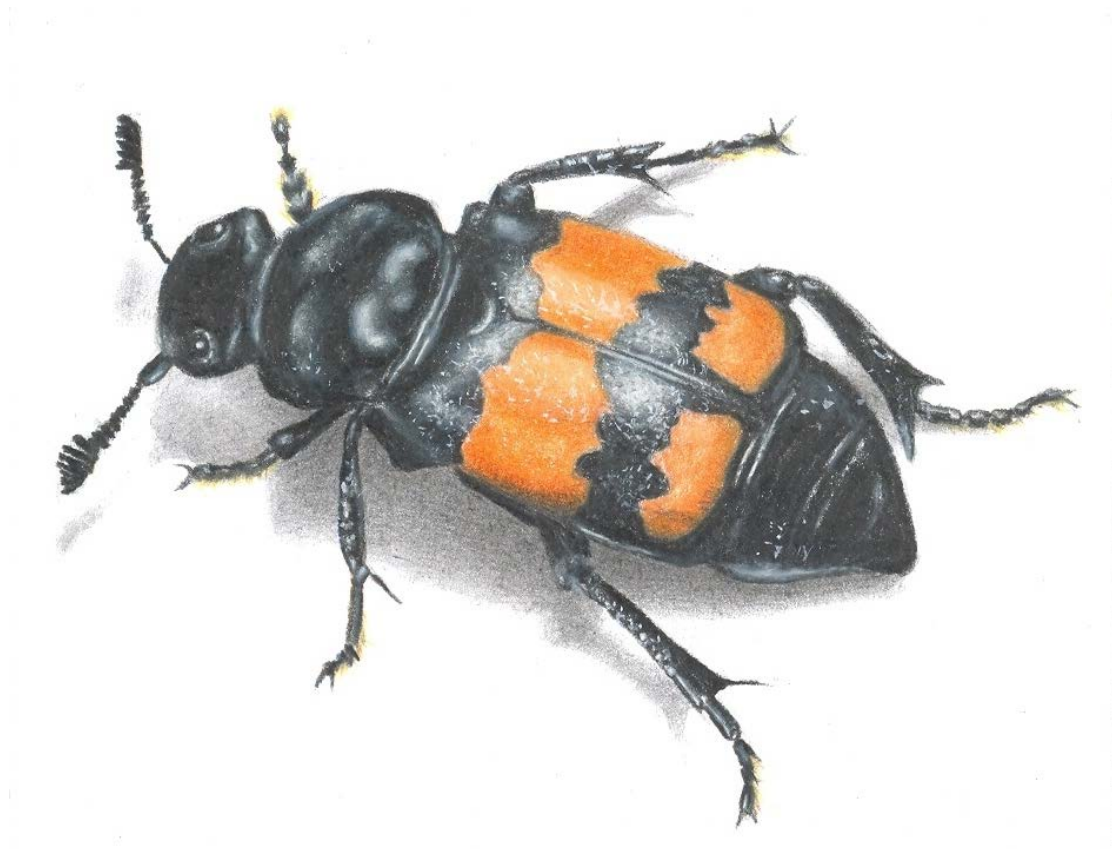
690 **Figure 2.** Changes in personal and social immunity across lifespan, **a)** The
691 relationship between PO activity and age in non-breeding and breeding beetles
692 (Experiment 1a). The raw data for PO are in open grey circles for the non-breeding
693 beetles and open black circles for breeding beetles against the age in weeks of the
694 beetle. Means and SE are shown for the raw data, alongside a fitted line of the model
695 in grey for the relationship between age and PO activity in non-breeders. **b)** The
696 relative level of defensin expression against the age in weeks of the beetles
697 (Experiment 1b). Raw data for breeding female beetles are shown in black circles and
698 for non-breeding female beetles in grey circles. The fitted line of the model for
699 defensin expression in breeders with age is included in black. **c)** Lytic activity against
700 beetle age (Experiment 1c). Raw data are presented in open black circles. The data
701 are produced from female beetles. The line shows the fitted values of the model
702 across lifespan.

703

704 **Figure 3.** The effect of wounding on immunosenescence, **a)** PO activity against
705 beetle age (weeks) for breeding beetles is shown with both control (grey circles) and
706 wounded (black circles) groups (means and SE of the raw data) (Experiment 2). The
707 data is produced from female beetles. The raw data are also presented in the
708 respective colours with open circles. **b)** Lytic activity against beetle age (weeks) is
709 shown with both control (grey circles) and wounded (black circles) groups (means
710 and SE of the raw data) (Experiment 2). Raw data are also presented in the respective
711 colours in open circles. The data are produced from female beetles. The line shows
712 the fitted values of the model across lifespan.

713 **Figures**

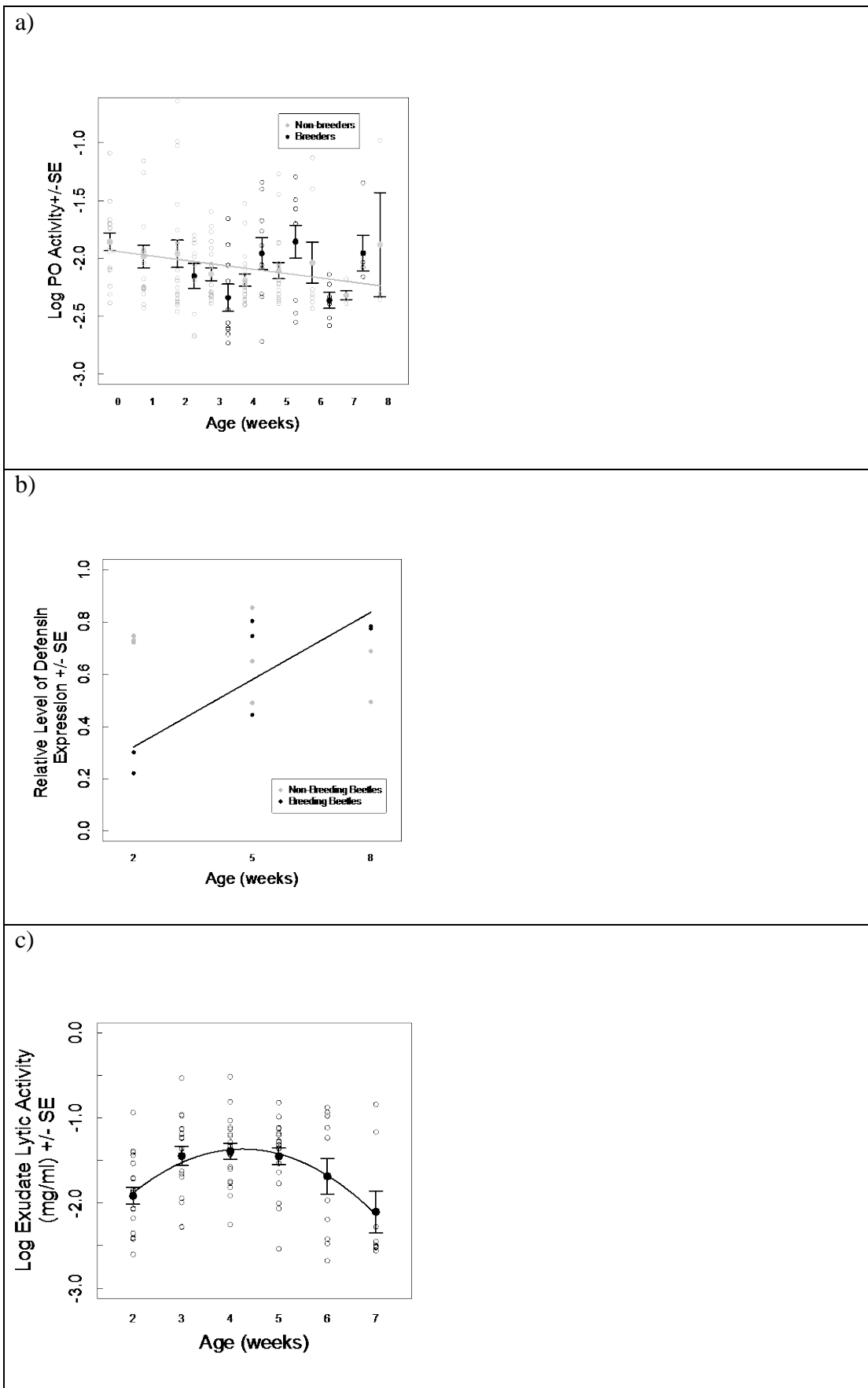
714 **Figure 1.**



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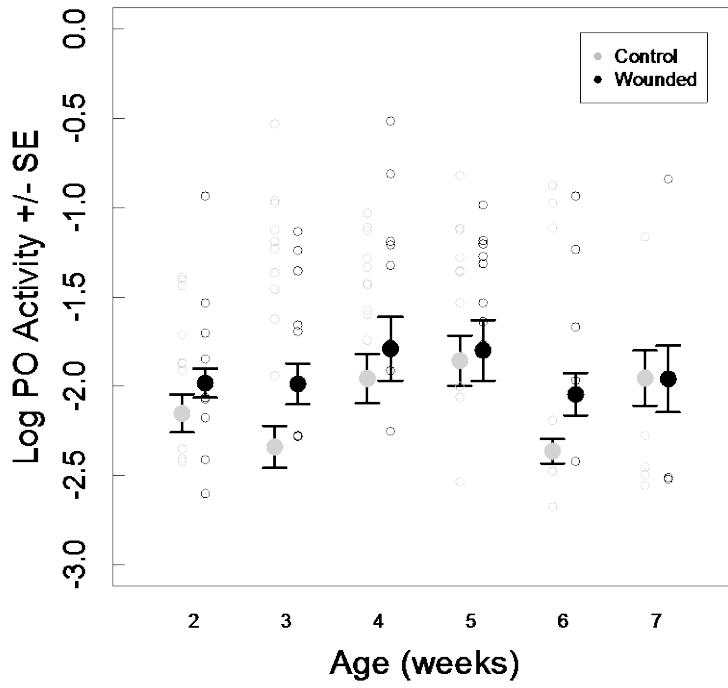
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719 **Figure 3.**

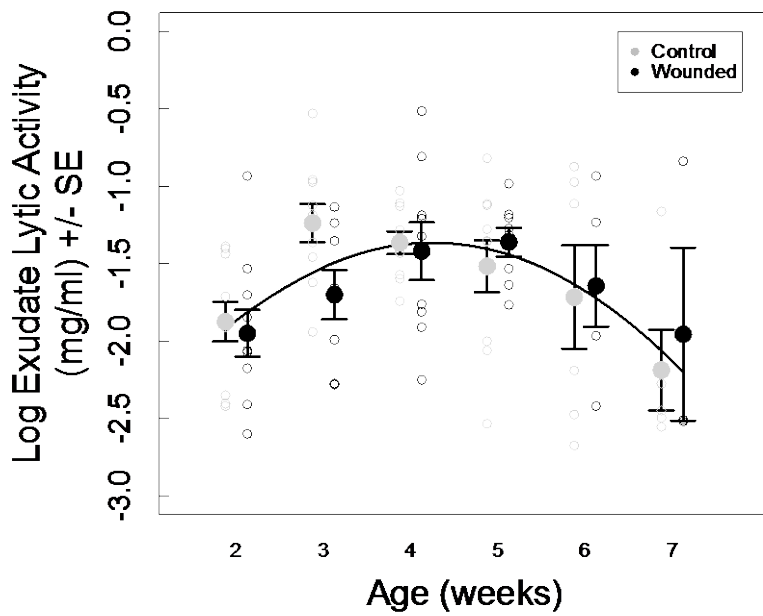
720

721 a)



722

723 b)



724

725 **Supplementary information**

726 **Primer design**

727

728 **Nicrophorus vespilloides Tubulin** **Accession Number (HO113191.1)**

729

730 ATCCAGAGCAGTTGATAACCGGAAAGGAAGATGCAGCCAACAATTACGC
731 TCGTGGTCATTACACCATCGGGAAAGAGCTCATCGATCAGGTATCTGATA
732 GGATTCGCAAAGTGC**GCGGATCAATGTCAAGGACT**TCAGGGTTTCCTGATT
733 TTCCACTCTTTTGGAGGAGGGACCGGTTCCGGTTTTACTTCCCTTTTGATG
734 GAAAGGTTGCCAGTGGATTACGGAAAGAAGAGCAAAGTGGAAATTTGCCG
735 TTTACCCGGCGCCTCAGGTCTCAACTG**CTGTGGTGGAGCCCTACAAT**TCGA
736 TCCTTACCACGCACACCACCCTTGAACACTCCGATTGCGCCTTTATGGTAG
737 ACAATGAAGCAATCTACGATATCTGCTTGAAGAATTTGGATATCCCTAGA
738 CCAGGATACTTGAATCTCAACAGACTCATCAGTCAGATCGTTTCATCTACG
739 ACCGCATCTTTGAGATTCGATGGAGCCATGAACGTCCATCTTACGGAATTC
740 CAAACGAATTTAGTTCCTTACCCACGTATACATTTTCCATTAATGACTTAT
741 GCACCAATCATTTTCAGCAGCGAAAGCCTACCACGAACAAATCTCAGTAGC
742 CGAAATCACAAACGCGTGCTTCGAACCCAACAACCAGATGGTGAAATGTG
743 ATCCTCGTCGAGGAAAG

744

745 Order

746 >Tub183Fwd: GCGGATCAATGTCAAGGACT (Tm = 57)

747 >Tub183Rev: ATTGTAGGGCTCCACCACAG (Tm = 57)

748

749 **Defensin (NIC-SSH ContigX1)** **Accession Number ()**

750

751 GATGGTTGCCAGCTTCGTGAGCGCTGGACCGGTTGAGCAAGATGCCGAAG
752 GACATGTTGTGGAAAGGGCCAACAGGCAACG**CAGGGTGACCTGCGATTTA**
753 **T**TGAGCGTATCGACGCCCTACGGTTCCGTCAACCATTCGGTCTGCGCCGCC
754 CACTGCCTCGCCATGCTGAAGGGTTTCAGAGGTGGAAGATGCATCGACGG
755 AGTCTGCAATTGCAGGAAGTAAAGGTGTTGTCGATTAATTGACTTCCACC
756 GATTGGACA**ATTGCCTCGATTGGAAGAGA**CCCCCTAACAGCTTTAATCC
757 CACAAGTTAATTAATTAGGTAACGAAAAAAAAAAGAAGTTTGCAATAAATA
758 AAACGTAGTTGTTACAAAAAAAAAAAAAAAAA

759

760 Order

761 >Def199Fwd: CAGGGTGACCTGCGATTTAT (Tm = 57)

762 >Def199Rev: TCTCTTCCAATCGAGGCAAT (Tm = 57)

763