

1 **Immune response costs are associated with changes in resource acquisition and not**
2 **resource reallocation**

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12 The causes of immune defence costs

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29 **Summary**

- 30 1. Evolutionary ecologists frequently argue that parasite defence is costly because
31 resources must be reallocated from other life-history traits to fuel the immune
32 response. However, this hypothesis is rarely explicitly tested. An alternative
33 possibility is that immune responses impair an organism's ability to acquire the
34 resources it needs to support metabolism. Here we disentangle these opposing
35 hypotheses for why the activation costs of parasite resistance arise.
- 36 2. We studied fecundity costs associated with immune stimulation in *Drosophila*
37 *melanogaster*. Then, by measuring correlated changes in metabolic rate, food
38 consumption and body weight, we assessed whether responses were consistent with
39 immunity costs originating from altered resource allocation or from impaired resource
40 acquisition.
- 41 3. Microbial injection resulted in a 45% fecundity decrease; it also triggered a mean
42 decline in metabolic rate of 6% and a mean reduction in food intake of 31%, body
43 weight was unaffected. Metabolic rate down-regulation was greater in males than in
44 females, whereas declines in food ingestion were of similar magnitude in both sexes.
45 These physiological shifts did not depend on whether microbial challenges were alive
46 or dead, thus they resulted from immune system activation not pathogenesis.
- 47 4. These costs of immune activation are significant for individuals that successfully
48 resist infection and might also occur in other situations when immune responses are
49 upregulated without infection.
- 50 5. Whilst we found significant activation costs of resistance, our data provide no
51 compelling evidence for the popularly argued hypothesis that immune deployment is
52 costly because of reallocation of energetic resources to the immune system. Instead,
53 reduction in resource acquisition due to 'infection-induced anorexia' may be the
54 principal driver of metabolic changes and fecundity costs resulting from immune
55 response activation.

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58 **Key-Words**

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60 Appetite, *Drosophila melanogaster*, energetic trade-off, fecundity, immunity, infection-
61 induced anorexia, life-history, metabolic rate, parasite resistance.

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64 **Introduction**

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66 Immune responses are generally considered to be costly: a fact that is central to many
67 fundamental concepts in evolutionary ecology, such as sexual selection (Hamilton & Zuk
68 1982), the maintenance of genetic variation for parasite resistance (Flor 1956) and host-
69 parasite coevolution (Haldane 1949). These costs of immunity can be divided into two broad
70 categories. First, the costs of forming and maintaining constitutive immune mechanisms,
71 such as barrier defences and immune cell populations (Fellowes, Kraaijeveld & Godfray
72 1998; Kraaijeveld, Limentani & Godfray 2001). Second, the costs of activating inducible
73 immune responses upon infection, such as immune molecule synthesis and fever
74 development (Schulenburg *et al.* 2009; Martin, Hawley & Ardia 2011). Here we focus on the
75 activation costs of immunity.

76 Life-history concepts suggest that immune activation costs are principally energetic
77 or nutritional, involving reallocation of resources to parasite defence at the expense of other
78 fitness-related traits (Moret & Schmid-Hempel 2000; Schulenburg *et al.* 2009). However,
79 evolutionary trade-offs may be governed both by variation in resource allocation between
80 different traits and also by variation in resource acquisition ability (van Noordwijk & de Jogn
81 1986). The relative magnitudes of variation in resource allocation and resource acquisition
82 can profoundly shape population responses to selection and the nature of associations
83 between life-history traits (Reznick, Nunney & Tessier 2000). Here we use this evolutionary
84 framework to investigate the causes of immune activation costs, testing the relative

85 importance of resource budget reallocation and alterations in resource acquisition ability in
86 driving the costs of immune system deployment. This distinction is important because
87 resource reallocation can adaptively withdraw resources from particular traits to minimise
88 overall fitness loss, whereas the consequences of impaired resource acquisition are
89 potentially more widespread.

90 Costs of immunity are primarily realised as a decline in the quality or quantity of an
91 individual's offspring. In *Drosophila melanogaster*, immune-challenged females suffer
92 reduced fecundity; study of flies with genetically manipulated immune responses
93 demonstrates that this cost arises specifically from immune system activation (Zerofsky *et al.*
94 2005). Similarly, in *Anopheles gambiae* immune stimulation with lipopolysaccharide (LPS)
95 significantly reduces fecundity (Ahmed *et al.* 2002). As well as fecundity effects, immune
96 challenge by LPS injection reduced survival of bumblebee workers under starvation
97 conditions (Moret & Schmid-Hempel 2000). However, it is not clear why activating the
98 immune system to attack parasites should invoke these fitness costs.

99 Total resource expenditure can be assessed by measuring metabolic rate. Some
100 studies suggest that energetic resources are indeed reallocated, perhaps from stored
101 reserves, to support immune system activity. Antibody production following immune
102 challenge in collared doves increased basal metabolic rate by 8.5% 7 days after injection
103 (Eraud *et al.* 2005). Similarly, in invertebrates, cabbage white butterfly pupae increased
104 metabolic thermogenesis by 8% in response to the immunogenic stimulus of a nylon filament
105 implant (Freitak *et al.* 2003). Nevertheless, in the collared dove study the authors concluded
106 this metabolic cost was small and of similar magnitude to other normal homeostatic
107 processes (Eraud *et al.* 2005). Furthermore, mice did not experience elevated metabolic rate
108 when injected with immune elicitors, either in standard conditions, or under hypoxia
109 designed to cause metabolic stress (Baze, Hunter & Hayes 2011). Thus, immunity-induced
110 increases in metabolism are not universal; whether increased resource expenditure in the
111 immune system is the major factor which causes declines in other fitness traits remains to be
112 determined.

113 Mounting an immune response may also alter resource acquisition, changing the
114 ability of organisms to support fecundity. Some studies have suggested that organisms
115 increase food consumption when infected by pathogens to fuel the immune response (Moret
116 & Schmid-Hempel 2000). However, the opposite, reduced food intake, is a common
117 behaviour in animals upon immune challenge; a phenomenon termed infection-induced
118 anorexia (Exton 1997). It is counterintuitive that animals as diverse as humans, mice and
119 flies should adaptively decrease their food intake when infected. Nevertheless, the
120 suggestion that this is a maladaptive symptom of illness has been challenged by work in
121 *Drosophila*, which indicated that survival of flies following infection by some (but not all)
122 pathogens is enhanced by this anorexic response (Ayres & Schneider 2009).

123 Although immune costs are frequently argued to be resource-mediated (DiAngelo *et*
124 *al.* 2009), non-energetic costs can have significant fitness effects. Immune defence
125 molecules produced to attack parasites can also cause collateral damage to host tissues,
126 including inflammatory responses. In invertebrates, the cellular encapsulation response can
127 attack host tissues causing pseudo-tumours (Govind 1996; Minakhina & Steward 2006), the
128 synthesis of melanin for immunity can cause dispersed tissue damage (Sadd & Siva-Jothy
129 2006) and immune responses against enteric microbes frequently cause extensive damage
130 to the gut lining (Buchon *et al.* 2009).

131 Here we investigate costs of immune upregulation in *D. melanogaster*. Studying this
132 model ectothermic invertebrate enabled us to investigate metabolic changes specifically
133 associated with immune system deployment whilst avoiding the potentially confounding
134 thermal impact of fever, which is commonly associated with pathogen infection in
135 endotherms. The *D. melanogaster* immune system mounts a complex attack on invading
136 microbes comprising coordinated cellular and humoral responses. Two key signalling
137 cascades principally drive this attack: the Toll and the immune deficiency (IMD) pathways.
138 The Toll pathway is activated preferentially by fungi and Gram positive bacteria, whilst the
139 IMD pathway is stimulated primarily by Gram negative bacteria (Lemaitre & Hoffmann 2007).
140 Each pathway triggers transcription of an appropriate subset of the fly's antimicrobial genes

141 to defend against the type of microbe encountered (Hoffmann 2003). The enzyme
142 phenoloxidase (PO) catalyses melanin production, which possesses cytotoxic properties as
143 well as assisting wound healing and clotting (Eleftherianos & Revenis 2011). In adult
144 *Drosophila*, cellular immune responses involve phagocytosis and parasite encapsulation by
145 plasmatocyte cells circulating within the haemolymph (Williams 2007).

146 In this study we use a fungus (*Beauveria bassiana*) and a bacterium (*Escherichia*
147 *coli*) to trigger either Toll-dependent or IMD-dependent immune responses. First we assess
148 the magnitude of fecundity costs associated with these immune defences. Then we study
149 how the resource budget of flies alters during immune system deployment by quantifying
150 correlated changes in metabolic rate, food intake and body mass. We use these measures
151 to dissect the importance of altered resource allocation and resource acquisition in mediating
152 fecundity declines. We predicted that if immune activation costs are principally due to
153 resource reallocation, then either there would be no change in overall metabolic rate (if
154 resources are withdrawn from non-essential traits and perfectly reallocated to immunity), or
155 alternatively metabolic rate might go up if resources are reallocated from stored reserves to
156 be spent on immune function. However, if compromised energy acquisition underpins
157 immune costs, immune activation should be accompanied by reduced feeding rate and
158 potentially a decline in other metabolic-related traits.

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161 **Materials and Methods**

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163 FLY STOCKS AND REARING

164 The wildtype genotype Samarkand (from Bloomington Stock Centre) was used throughout.
165 Flies were bred in bottles; all rearing and experimentation was on Lewis food medium (Lewis
166 1960) at 25 °C, 70% RH on a 12 h L/D cycle. For all the experiments flies were allowed to
167 mate following eclosion, then 3 day old flies were sorted into vials without additional live

168 yeast, in single-sex groups of 10 using light CO₂ anaesthesia the day before immune
169 challenge. Each vial of flies was only used in one of the following experiments.

170

171 IMMUNE CHALLENGES

172 The impacts of microbial injection on fly fecundity, metabolic rate, food ingestion and body
173 weight were studied. Microbes were prepared as both live and dead suspensions to permit
174 separation of the physiological effects of immune activation from those of microbial
175 replication. *B. bassiana* spores were grown on potato dextrose agar (PDA) from an existing
176 strain using standard procedures (Tinsley, Blanford & Jiggins 2006); live and dead
177 conidiospores were suspended in oil (87.5% Shellsol T, 12.5% Ondina EL). *E. coli* were
178 cultured overnight in Luria Broth (LB) at 37 °C with continuous shaking, both live and dead
179 *E. coli* were suspended in sterile LB. Heat-killed suspensions of *B. bassiana* and *E. coli* were
180 prepared by boiling at 100 °C for 15 min. The absence of growth was confirmed for heat-
181 killed suspensions by plating 100 µl on PDA and LB agar respectively; the viability of live
182 suspensions was similarly verified. Live fungal spores (2.0 x 10⁶ spores ml⁻¹), heat-killed
183 fungal spores (2.0 x 10⁷ spores ml⁻¹) and 2.0 x 10⁶ cells ml⁻¹ of live and heat-killed bacteria
184 were used to trigger immune responses in flies by injection into the thorax using a fine
185 tungsten wire needle. The terminal 0.3 mm of the needle was bent slightly to provide a
186 marker ensuring consistent penetration. Flies received CO₂ anaesthesia for injections, but
187 then not during any subsequent assays. Four day old flies received one of six treatments:
188 gas control (GC, flies anaesthetised with CO₂ but not injected), injection control (IC, flies
189 injected with a needle dipped in blank oil), dead fungal (DF, dead *B. bassiana* injection), live
190 fungal (LF, live *B. bassiana* injection), dead bacterial (DB, dead *E. coli* injection) and live
191 bacterial injection (LB, live *E. coli* injection). Microbial suspensions were vortexed frequently
192 to prevent microbes settling and needles were sterilised with ethanol and flaming.

193

194 EFFECT OF IMMUNE CHALLENGE ON FECUNDITY

195 Immediately following the four immune challenge and two control treatments, groups of 10
196 female flies were allowed to oviposit in vials containing standard fly food containing blue food
197 colouring (0.1% v/v) to aid egg counting. Flies were tipped into fresh vials after two 24 h
198 periods, providing fecundity estimates for three consecutive days after immune treatment.
199 Flies that escaped or died during vial transfers were recorded and fecundity measures
200 adjusted accordingly. After oviposition vials were frozen and eggs counted later under a
201 stereo microscope. In total 300 flies were studied: five independent groups of 10 flies for
202 each of treatment.

203

204 MEASURING METABOLIC RATE

205 This study was conducted using 410 independent 10-fly groups in seven blocks; each block
206 contained multiple replicates of five or six of the different immune treatments. The effect of
207 immune activation on fly metabolic rate was assessed by respirometry, measuring CO₂
208 production with an infrared gas analyser (IRGA: EGM-4, PP Systems). Day one
209 measurements were made on 5 day old male and female flies 16-18 h after immune
210 treatment; further measurements were made at 24 h intervals. Flies were housed in a plastic
211 chamber connected in a circuit to an IRGA with tubing (total system volume 40.5 cm³). Air
212 circulated within this sealed system and CO₂ accumulation was measured. In each assay the
213 metabolic rate of a group of 10 flies was measured at 25 °C over 5 min, recording CO₂ every
214 1.6 seconds; data from the first 2 min whilst flies settled were discarded. Measurements on
215 each 10-fly group were repeated on three or four consecutive days. CO₂ efflux per minute
216 was calculated by linear regression, then converted to nmole CO₂ min⁻¹ fly⁻¹ using knowledge
217 of the apparatus volume.

218

219 EFFECT OF IMMUNE CHALLENGE ON FOOD INTAKE

220 Food consumption assays followed protocols of previous authors by measuring pigment
221 intake from food (Libert *et al.* 2007; Ayres & Schneider 2009). Immediately after
222 administering one of the six treatments, 70 groups of 10 flies were transferred to food

223 medium comprising 0.5% v/v bromophenol blue (Sigma), 5% w/v sugar, 5% w/v yeast, 2%
224 w/v agar, and water. After 24 h the head of each fly was removed using a scalpel (to exclude
225 red eye pigments), then bodies were homogenised on ice in five-fly groups in 500 µl ice-cold
226 TE buffer. Homogenate samples were centrifuged at 13362 x g at 4 °C for 10 min; the
227 supernatant was then similarly re-centrifuged. The amount of blue pigment in 100 µl
228 supernatant was measured in 96-well plates using a Versa Max microplate reader
229 (Molecular Devices) to record absorbance at 520 nm. The flies in each original vial were split
230 between two five-fly replicates; these replicates were measured in different 96-well plates.
231 To convert absorbance values into food mass eaten per fly a calibration relationship was
232 determined by measuring the absorbance of serial dilutions of a known food mass ($n = 6$
233 samples). The linear regression equation for this mass-absorbance plot ($y=0.0008x-0.0048$)
234 had an R^2 value of 0.9998.

235

236 EFFECT OF IMMUNE CHALLENGE ON FLY BODY MASS

237 Flies were divided into 120 single-sex 10-fly groups when 3 days old and weighed whilst
238 anaesthetised on a PI 225D balance (Denver Instruments) reading to 0.01 mg. The next day
239 each group received one of three injection treatments: injection control, dead fungal spores
240 or dead bacteria. On the three subsequent days each group was reweighed; flies that died or
241 escaped were recorded and each weight was converted to a per-fly mass. Flies were
242 maintained on Lewis medium throughout.

243

244 STATISTICAL ANALYSIS

245 All analyses were conducted in R version 2.15.1 (R Development Core Team 2013); linear
246 mixed effects models were executed using lmer from the lme4 package (Bates, Meachler &
247 Bolker 2013). Our principal aim was to assess the impact of the six immune challenge
248 treatments on fly life-history traits. Data from the six treatments were progressively pooled by
249 a systematic process to produce minimally complex models that adequately explained trait
250 variation. We concluded that the treatment differences were important if the more complex

251 model had improved explanatory power (see below). The impact of breaching the cuticle
252 was tested by pooling data from the gas control and injection control treatments. We tested
253 whether trait variation was due to pathogenesis or immune activity by pooling data from live
254 and dead microbial treatments, and we tested if microbial identity influenced immune costs
255 by pooling bacterial and fungal treatments. Finally, tests for a general effect of immune
256 stimulation compared control groups to data pooled from across all microbial injected flies.

257 For analyses involving repeated measures on vials of flies over successive days the
258 term 'vial' was included as a random effect, whilst temporal changes were assessed using
259 the fixed effect of 'day' and its two-way interaction with treatment. With the exception of
260 fecundity studies, models also included fly 'gender' and a 'gender by treatment' interaction.
261 When analysing metabolic rate data, models contained an additional random effect of
262 'block', accounting for variation between the seven blocks over which the investigation was
263 conducted. We also tested the impact of time of day and the air CO₂ concentration when
264 each metabolic rate measurement was made. Finally, for investigations of variation in food
265 ingestion after immune challenge, 'vial' was used as a random effect to associate the two
266 five-fly batches from each vial. The number of flies in assay vials for fecundity, metabolic
267 rate and body weight experiments varied slightly due to escapes; in each case we tested
268 whether fly number influenced the trait measured.

269 All models employed Gaussian errors. Models were serially simplified by eliminating
270 terms for which inclusion did not enhance model explanatory power by 2 AIC units.
271 Likelihood-ratio tests comparing models with and without the term of interest were used to
272 calculate *P*-values. Results are presented as means ± standard errors.

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279 **Results**

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281 **FECUNDITY COSTS OF IMMUNE ACTIVATION IN *D. MELANOGASTER***

282 Fecundity was recorded from 30 groups of 10 flies, observed daily for three days after
283 receiving immune treatments. Immune stimulation by microbes was associated with a major
284 reduction in fecundity (Fig. 1; control vs immune challenged flies, $\chi^2_1 = 72.42$, $P = 2.2 \times 10^{-16}$).
285 Mean fecundity of flies receiving a microbial injection of any type was 2.16 eggs fly⁻¹ day⁻¹
286 (± 0.109), approximately half that of flies receiving control treatments, which laid 4.16 eggs
287 fly⁻¹ day⁻¹ (± 0.150). The egg output of control injected IC flies (4.22 eggs fly⁻¹ day⁻¹ ± 0.153)
288 was not different from the control anaesthetised GC flies (4.12 eggs fly⁻¹ day⁻¹ ± 0.146),
289 demonstrating the injection process itself had no significant effect on fecundity ($\chi^2_1 = 0.41$, P
290 $= 0.521$). The flies receiving microbial immune challenges all responded similarly, with no
291 individually significant differences between treatments ($\chi^2_3 = 3.44$, $P = 0.329$). Indeed there
292 was no significant fecundity difference between flies injected with live and dead microbes (χ^2_1
293 $= 3.08$, $P = 0.079$), nor between flies injected with bacteria and fungi ($\chi^2_1 = 0.26$, $P =$
294 0.613). Fecundity did not change notably across the days of the experiment (day, $\chi^2_1 = 1.71$,
295 $P = 0.190$) and the fecundity reduction associated with immune stimulation remained
296 significant three days after microbial injection ($\chi^2_1 = 17.71$, $P = 2.2 \times 10^{-5}$). The exact number
297 of flies in each vial varied slightly (mean = 9.56, SE = 0.133), however this variation did not
298 influence the per-fly fecundity ($\chi^2_1 = 0.27$, $P = 0.60$).

299

300 **IMMUNE ACTIVATION DECREASED THE METABOLIC RATE OF *D. MELANOGASTER***

301 To investigate the effects of immune upregulation on metabolic rate 4100 flies in single-sex
302 groups of 10 were subjected to metabolic rate measurements after immune challenge or
303 control treatment. The metabolic rate of immune activated flies was 6% lower than control
304 flies (Fig. 2): a highly significant decline ($\chi^2_1 = 25.42$, $P < 4.0 \times 10^{-7}$). The four microbial
305 treatments reduced metabolic rate by similar amounts ($\chi^2_3 = 1.16$, $P = 0.763$). There was no
306 difference either between live and dead microbial injections ($\chi^2_2 = 0.67$, $P = 0.717$), or

307 between fungal and bacterial injections ($\chi^2_2 = 0.80$, $P = 0.671$). As with fecundity
308 experiments, metabolic rate declines were associated with microbe exposure, not the
309 injection process: metabolic rate of control injected IC flies ($1.83 \text{ nmol min}^{-1} \text{ fly}^{-1} \pm 0.045$) was
310 almost the same as anaesthetised GC flies ($1.85 \text{ nmol min}^{-1} \text{ fly}^{-1} \pm 0.041$) and the difference
311 was not significant ($\chi^2_1 = 1.60$, $P = 0.206$). The metabolic rate reduction associated with
312 immune activation persisted during our experiment: following initial reduction there was no
313 consistent metabolic rate change across the three days post-treatment ($\chi^2_1 = 0.63$, $P =$
314 0.427).

315 The CO_2 levels in the laboratory fluctuated naturally during the study; higher CO_2
316 concentrations at the start of an assay were associated with slightly lower metabolic rates
317 ($\chi^2_1 = 8.40$, $P = 0.003$): an increase of 1 ppm CO_2 was associated with a metabolic rate
318 decrease of $0.004 \text{ nmol min}^{-1} \text{ fly}^{-1}$ (95% CI 0.003 - 0.007). Time of day at which measurements
319 were taken did not affect fly metabolic rate ($\chi^2_1 = 0.01$, $P = 0.918$). A very small number of
320 flies escaped from vials during transfers, therefore the mean flies per vial was 9.99; (SE =
321 0.002), this variation had no effect on the per-fly metabolic rate ($\chi^2_1 = 0.51$, $P = 0.477$). The
322 metabolic rate of male flies was significantly less than females (Fig. 3; $\chi^2_1 = 25.42$, $P = 2.2 \times$
323 10^{-16}). Furthermore, a significant gender by immune activation interaction demonstrated that
324 immunity-induced metabolic declines were 50% greater in males than females (Fig. 3; $\chi^2_1 =$
325 8.55 , $P = 0.003$). For females, control metabolic rate was $2.14 \text{ nmol min}^{-1} \text{ fly}^{-1}$ (± 0.050),
326 which declined by an average of $0.10 \text{ nmol min}^{-1} \text{ fly}^{-1}$ following immune activation; whereas in
327 males control metabolic rate was $1.71 \text{ nmol min}^{-1} \text{ fly}^{-1}$ (± 0.050) and immune treatments
328 caused a $0.16 \text{ nmol min}^{-1} \text{ fly}^{-1}$ decline.

329 IMMUNE ACTIVATION REDUCED THE FOOD INTAKE OF *D. MELANOGASTER*

330 We measured food intake by assessing pigment uptake into the gut from coloured food.
331 There were 70 independent feeding assays, each on a single-sex group of 10 flies; each
332 group was then split in half for 140 pigment assays on five-fly samples. Microbe injected
333 flies ate $72.4 \mu\text{g fly}^{-1} \text{ day}^{-1}$ (± 2.65), 30.9% less than control flies, which ate $104.7 \mu\text{g fly}^{-1} \text{ day}^{-1}$

334 ¹ (± 3.75) (Fig. 4; $\chi^2_1 = 60.89$, $P = 6.0 \times 10^{-15}$). There were no significant differences in
335 feeding rate between bacterial and fungal treatments ($\chi^2_1 = 1.41$, $P = 0.235$), live and dead
336 microbial injections ($\chi^2_1 = 0.58$, $P = 0.445$), nor between the IC and GC control groups ($\chi^2_1 =$
337 0.11 , $P = 0.74$). Whilst male flies ate significantly less than females ($\chi^2_1 = 34.98$, $P = 3.3 \times$
338 10^{-9}), the extent of the feeding decline was of similar magnitude in both sexes (Fig. 5; $\chi^2_1 =$
339 0.02 , $P = 0.888$): males and females suffered 32.9 and $31.5 \mu\text{g fly}^{-1} \text{ day}^{-1}$ reductions
340 respectively.

341

342 IMMUNE ACTIVATION DID NOT AFFECT BODY MASS IN *D. MELANOGASTER*

343 Experiments testing the impact of immune activation on fly body weight assessed mass for
344 10-fly groups of males ($n = 64$) and females ($n = 62$). In this case we only compared injection
345 control, dead bacteria and dead fungal spore treatments. Flies were weighed the day before
346 immune challenge and for three days afterwards. Female flies gained 7.6% weight during
347 the experiment, whereas male flies lost 3.9% weight (Fig. 6; sex by day interaction ($\chi^2_1 =$
348 327.97 , $P < 2.2 \times 10^{-16}$). However, considering just the post-injection data, the immune
349 treatments had no effect on absolute weight, nor on the temporal pattern of weight change
350 for either sex (treatment effect, males $\chi^2_2 = 1.57$, $P = 0.456$, females: $\chi^2_2 = 0.50$, $P = 0.778$;
351 day by treatment interaction, males: $\chi^2_2 = 0.74$, $P = 0.691$, females: $\chi^2_2 = 0.32$, $P = 0.854$).
352 There was slight variation in the exact number of flies in each vial (mean = 9.95, SE = 0.014)
353 but this did not affect the per-fly body weight ($\chi^2_1 = 0.29$, $P = 0.589$).

354

355

356 Discussion

357

358 In this study we investigated the validity of the hypothesis that the costs of defending against
359 parasites arise because resources normally invested in other physiological processes must
360 be diverted to fuel the demands of the immune response. We demonstrated clear immune
361 system costs in female flies, which suffered a sustained 45% reduction in fecundity across

362 the three days following immune challenge. However, our findings challenge the common
363 notion that this fecundity decline results from reallocation of resources to immunity.

364 We studied the three corners of the energy budget triangle: the rate at which energy
365 is used (metabolic rate), the rate at which energy is acquired (feeding) and the dynamics of
366 resource accumulation (body weight). We predicted that if mounting an immune response
367 requires mobilisation of additional stored resources then fly metabolic rate would increase
368 during immune system activity. Instead, metabolic rate fell by an average of 6% and
369 remained low up to four days after immune challenge. If resource expenditure were perfectly
370 reallocated from fecundity to immunity then this need not require an overall increase in
371 metabolic rate. However, at the same time, resource acquisition fell dramatically: flies
372 entered an anorexic state after immune challenge, with feeding rate falling by an average of
373 31%. Against this backdrop of depressed physiological activity we detected no effect of
374 immune stimulation on body weight, providing no evidence that metabolism during immune
375 activation depletes stored reserves. Nevertheless, flies are 70% water (Burr & Hunter 1969)
376 and may gain water and loose fat during lethal pathogenic infections (Arnold, Johnson &
377 White 2013). We cannot rule out that similar alterations could have occurred due to immune
378 activation by dead microbes in our experiments, potentially confusing detailed interpretation
379 of total body weight trends.

380 The most parsimonious explanation of our findings is that reduced food ingestion in
381 response to immune challenge restricts resource availability, resulting in depressed
382 metabolic rate and limited fecundity. Therefore, fecundity costs associated with immune
383 stimulation are probably not because the immune response requires increased energy
384 expenditure, but because anorexia induced by the immune system reduces acquisition of
385 resources that are normally required for egg production. This interpretation is supported by
386 comparison of physiological changes in males and females. The reduction in metabolic rate
387 was significantly greater in males than females, whereas feeding reductions were similar in
388 both sexes. We hypothesise that females mobilised energetic resources by resorbing eggs
389 from the ovarioles, as has been shown in both *Drosophila* and mosquitoes suffering

390 infections (Ahmed & Hurd 2006; Thomson, Schneemann & Johnson 2012). Egg resorption
391 may provide females with additional energetic reserves, not available to males, which
392 support metabolism when food acquisition is restricted during immune responses. We note
393 that this is a form of resource reallocation, but emphasise our conclusion that immune
394 activation costs originate from reduced food intake; if egg resorption occurs in this manner, it
395 only partially ameliorates some of these costs.

396 Our experiments only measured food intake for 1 day post-immune challenge,
397 whereas other traits were measured for three days. This was because the assay involved
398 sacrificing flies to measure food ingestion. This limits our understanding of how feeding
399 behaviour is affected by immune challenge beyond 24 hours. However, immune response-
400 dependent trends in fecundity, metabolic rate, and food intake established rapidly during the
401 first day post-challenge, and at least for fecundity and metabolic rate did not reverse by day
402 three.

403 If infection-induced anorexia is a key driver of the fecundity costs associated with
404 immune upregulation, this questions why the anorexic response exists. This phenomenon is
405 phylogenetically conserved, which perhaps points to a fundamental function and a variety of
406 adaptive benefits has been proposed (Exton 1997). Experiments in insects suggest anorexia
407 can enhance survival during pathogen attack (Ayres & Schneider 2009) and may function to
408 mediate conflicts between processing food and immune activity (Adamo *et al.* 2010).

409 One mechanistic factor shaping these immune-induced metabolic shifts is that some
410 immune system molecular pathways have pleiotropic roles in other physiological processes.
411 For example, in crickets, the lipid transport molecule apolipoprotein III is involved in immune
412 function, as well as in fuelling energetic demands of locomotion. This generates a trade-off
413 between lipid transport and immune defence causing immunosuppression following exercise
414 (Adamo *et al.* 2008). Also, the Toll pathway's immune activation role may conflict with
415 nutrient storage and growth as Toll activity can depress insulin signalling (DiAngelo *et al.*
416 2009). Therefore, a variety of proximate mechanisms may be responsible for metabolic rate
417 suppression in *D. melanogaster* following immune activation.

418 There were no differences in the responses of flies to live or dead microbes.
419 Therefore, surprisingly, the fecundity, feeding and metabolic rate reductions apparently all
420 resulted solely from activity of the immune response (or other downstream systems) and not
421 from infection pathology. Whilst *B. bassiana* is highly pathogenic to flies (Tinsley *et al* 2006),
422 *E. coli* inoculation does not normally cause mortality (Lemaitre & Hoffmann 2007); immune
423 responses caused by other pathogens or increased infection doses may cause different
424 effects. Our studies revealed no impact of immune stimulation on body weight; however,
425 here we only tested the effect of dead microbes, it remains possible that responses to live
426 microbial infection might be different. Recent studies have used *D. melanogaster* as a model
427 to understand the physiological changes which take place in the lead up to death by lethal
428 bacterial and viral infections (Chambers, Song & Schneider 2012; Arnold *et al.* 2013).
429 Chambers *et al.* (2012) reported that flies dying of *Listeria monocytogenes* suffered depleted
430 energy stores and underwent major changes in the transcription and activity of key metabolic
431 pathways. Arnold *et al.* (2013) concluded that pathology caused by *Drosophila C* virus
432 resulted in metabolic rate reduction. However, both these studies compared flies infected
433 with live microbes to unmanipulated flies. Our data challenge these conclusions, as we have
434 found that immune system activity alone can drive similar metabolic shifts of considerable
435 magnitude. Furthermore, our data show very similar costs and metabolic responses to fungal
436 and bacterial inoculation. Thus, it seems likely that these major physiological changes are
437 not specifically triggered by either the Toll or IMD immune signalling pathways, but represent a
438 generic response to immune activation.

439 The fitness reduction associated with immune system activation is potentially
440 substantial. Our data show that, not only does fecundity fall by 45% following immune
441 challenge, but also this fecundity depression persists for three days. Indeed Zerofsky *et al.*
442 (2005) showed fecundity was reduced for up to six days after immune activation. Thus,
443 depressed fecundity persists for a substantial fraction of a fly's life after acute immune
444 upregulation. Whilst some aspects of the fly immune response can be long-lived, IMD
445 pathway transcriptional upregulation following Gram negative bacterial challenge generally

446 only persists for ~24 hours (Lemaitre & Hoffmann 2007). Thus, the persistent nature of these
447 fecundity costs might possibly provide additional evidence against the hypothesis that
448 resource reallocation to immune molecule synthesis drives fecundity reduction. We note our
449 measures of fecundity are low for *D. melanogaster*, probably because our food vials were
450 not supplemented with live yeast. Fitness is determined by the quality as well as the quantity
451 of offspring; further studies might address the trans-generational impacts of immune
452 activation on general fitness traits.

453 For an organism that is infected by a potentially lethal microbe, these immune
454 activation costs may be worth paying; the inducible nature of these defences protects the
455 organism from these fitness consequences except when they are necessary. When
456 epidemics sweep through a population resistant individuals may survive, whilst susceptible
457 individuals die. Our data suggest that the survivors may still suffer considerable fitness
458 reduction as a consequence of resisting infection by deploying immune responses. Selection
459 should shape the magnitude of immune defence costs; high costs of resisting pathogen
460 infection may select for the alternative strategy of tolerance to the presence of microbes
461 (Little *et al.* 2010). Sizeable immunity costs may have profound consequences when the
462 immune system is activated in anticipation of infection. Some organisms adaptively
463 upregulate immune defence when environmental cues enable prediction of elevated
464 pathogen risk: for example density dependent prophylaxis in desert locusts (Wilson *et al.*
465 2002). Immune responses are also activated in the absence of pathogen infection during
466 courtship and in response to mating (McGraw *et al.* 2004; Immonen & Ritchie 2012). Thus,
467 immune system upregulation may be a major cost of copulation that could generate selective
468 forces governing the evolution of polyandry and female willingness to mate.

469 The sizeable nature of this immune response-induced fecundity cost has an
470 important applied dimension. Entomopathogenic fungi, such as *B. bassiana* which we used
471 here, are currently being trialled for control of the mosquito vectors of human pathogens,
472 such as the malaria parasite *Plasmodium*. Unlike the problems associated with the rapid
473 evolution of resistance to chemical insecticides in vector populations, these biopesticides

474 have been proposed to be ‘evolution proof’ (Read, Lynch & Thomas 2009). This is because
475 fungal biopesticides kill mosquitoes slowly. Thus although mosquitoes die before they can
476 transmit human infections, they still have substantial opportunities to lay eggs post-
477 exposure, reducing the fitness loss caused by pesticide control compared to conventional
478 chemical insecticides. However, our data demonstrate that immune system activation by
479 fungi results in a substantial fitness reduction. Similar findings have been reported for
480 *Anopheles* mosquitoes (Mouatcho, Koekemoer & Coetzee 2011). We therefore urge caution
481 that even if fungal biopesticides result in slow vector mortality, substantial fecundity loss
482 following exposure could still generate strong selection pressure for the evolution of novel
483 mechanisms to reduce mortality from biopesticides. Nevertheless, we acknowledge that our
484 experiments administered microbes by injection and that immune responses following
485 infection by natural routes could differ.

486 We hope that this study stimulates further critical evaluation of the role resource-
487 reallocation plays in generating the costs of life-history trait investment. It is appealing to
488 assume that fitness costs result from switches in resource allocation decisions. However, for
489 the activation costs of resisting parasite infection, immune system deployment causes major
490 impairment of resource acquisition, of sufficient magnitude to explain fecundity costs.

491

492

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494

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499

500

501 **Data Archiving**

502

503 Data will be uploaded to Dryad prior to publication.

504 **Legends**

505

506 **Fig. 1.** Microbial injections decreased the fecundity of *D. melanogaster*. Eggs were counted
507 from 30 groups of 10 flies for three consecutive days after immune treatments. Treatments
508 were gas control (GC), injection control (IC), dead bacteria (*E. coli*: DB), live bacteria (LB),
509 dead fungus (*B. bassiana*: DF) and live fungus (LF). Points represent daily means for each
510 treatment and error bars show mean standard errors. Different letters (a/b) denote
511 significantly different groups of treatments.

512

513 **Fig. 2.** Metabolic rate of *D. melanogaster* decreased after immune stimulation by microbial
514 injection. Letters (a/b) indicate that the control treatments (GC and IC) differed significantly
515 from the flies receiving bacterial (DB, LB) and fungal (DF, LF) immune challenges. Data
516 points show means \pm standard errors from 410 independent replicate groups of 10 flies
517 assayed daily for between two and four days after treatment.

518

519 **Fig. 3.** Immune stimulation caused a greater metabolic rate decline in male flies than in
520 females. Bars show mean metabolic rate of immune activated (DB, LB, DF, LF) and control
521 (GC, IC) flies with their standard errors.

522

523 **Fig. 4.** Microbial injection reduced food ingestion in *D. melanogaster*. Data points represent
524 means (\pm standard errors) from 140 measurements of food consumption on five-fly pools.
525 The letters (a/b) show that all immune challenged flies (DB, LB, DF, LF) responded similarly,
526 but were significantly different from control treatments (GC, IC).

527

528 **Fig. 5.** The extent of feeding reduction caused by immune challenge was the same for males
529 and females. Bars represent the mean food ingestion for immune activated (DB, LB, DF, LF)
530 and control treatments (GC, IC) with their standard errors.

531

532 **Fig. 6.** Immune activation had no detectable effect on the rate at which fly weight changed.
533 Flies were weighed the day before immune treatment (Day -1) and for three days afterwards
534 (Day 1, 2 & 3). Female flies gained weight, whilst male flies lost weight during this period.
535 However, immune challenge with dead bacteria (DB) or dead fungi (DF) did not influence
536 this temporal pattern compared to controls. 120 independent 10-fly groups were repeatedly
537 weighed; points show means $\pm 2 \times$ the mean standard error.

538

539 **References**

- 540 Adamo, S.A., Roberts, J.L., Easy, R.H. & Ross, N.W. (2008) Competition between immune function and lipid
541 transport for the protein apolipoprotein III leads to stress-induced immunosuppression in crickets. *Journal of*
542 *Experimental Biology*, **211**, 531-538.
- 543 Adamo, S.A., Bartlett, A., Le, J., Spencer, N. & Sullivan, K. (2010) Illness-induced anorexia may reduce trade-offs
544 between digestion and immune function. *Animal Behaviour*, **79**, 3-10.
- 545 Ahmed, A.M. & Hurd, H. (2006) Immune stimulation and malaria infection impose reproductive costs in
546 *Anopheles gambiae* via follicular apoptosis. *Microbes and Infection*, **8**, 308-315.
- 547 Ahmed, A.M., Baggott, S.L., Maingon, R. & Hurd, H. (2002) The costs of mounting an immune response are
548 reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. *Oikos*, **97**, 371-377.
- 549 Arnold, P.A., Johnson, K.N. & White, C.R. (2013) Physiological and metabolic consequences of viral infection
550 in *Drosophila melanogaster*. *Journal of Experimental Biology*, **216**, 3350-3357.
- 551 Ayres, J.S. & Schneider, D.S. (2009) The role of anorexia in resistance and tolerance to infections in *Drosophila*.
552 *PLoS Biology*, **7**, e1000150.
- 553 Bates, D., Meachler, M. & Bolker, B. (2013) Lme4: Linear mixed-effects models using Eigen and
554 version 0999999-2. <http://cran.r-project.org/web/packages/lme4/index.html>
- 555 Baze, M.M., Hunter, K. & Hayes, J.P. (2011) Chronic hypoxia stimulates an enhanced response to immune
556 challenge without evidence of an energetic trade-off. *Journal of Experimental Biology*, **214**, 3255-3268.
- 557 Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S. & Lemaitre, B. (2009) *Drosophila* intestinal response to
558 bacterial infection: activation of host defense and stem cell proliferation. *Cell Host & Microbe*, **5**, 200-211.
- 559 Burr, M.J., Hunter, A.S. (1969) Effects of temperature on *Drosophila*- V. Weight and water, protein and RNA
560 content. *Comparative Biochemistry and Physiology*, **29**, 647-652.

- 561 Chambers, M.C., Song, K.H. & Schneider, D.S. (2012) *Listeria monocytogenes* infection causes metabolic shifts
562 in *Drosophila melanogaster*. *PLoS One*, **7**, e50679.
- 563 DiAngelo, J.R., Bland, M.L., Bambina, S., Cherry, S. & Birnbaum, M.J. (2009) The immune response attenuates
564 growth and nutrient storage in *Drosophila* by reducing insulin signalling. *Proceedings of the National Academy of
565 Sciences of the United States of America*, **106**, 20853-20858.
- 566 Eleftherianos, I. & Revenis, C. (2011) Role and importance of phenoloxidase in insect hemostasis. *Journal of
567 Innate Immunity*, **3**, 28-33.
- 568 Eraud, C., Duriez, O., Chastel, O. & Faivre, B. (2005) The energetic cost of humoral immunity in the collared
569 dove, *Streptopelia decaocto*: is the magnitude sufficient to force energy-based trade-offs? *Functional Ecology*,
570 **19**, 110-118.
- 571 Exton, M.S. (1997) Infection-induced anorexia: active host defence strategy. *Appetite*, **29**, 369-383.
- 572 Fellowes, M., Kraaijeveld, A. & Godfray, H. (1998) Trade-off associated with selection for increased ability to
573 resist parasitoid attack in *Drosophila melanogaster*. *Proceedings of the Royal Society of London. Series B:
574 Biological Sciences*, **265**, 1553-1558.
- 575 Flor, H.H. (1956) The complementary genetic systems in flax and flax rust. *Advances in Genetics*, **8**, 29-54.
- 576 Freitag, D., Ots, I., Vanatoa, A. & Horak, P. (2003) Immune response is energetically costly in white cabbage
577 butterfly pupae. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **270**, S220-S222.
- 578 Govind, S. (1996) Rel signalling pathway and the melanotic tumour phenotype of *Drosophila*. *Biochemical
579 Society Transactions*, **24**, 39-44.
- 580 Haldane, J.B.S. (1949) Disease and evolution. *La Ricerca Scientifica*, **19**, S68-S76.
- 581 Hamilton, W.D. & Zuk, M. (1982) Heritable true fitness and bright birds - a role for parasites. *Science*, **218**, 384-
582 387.
- 583 Hoffmann, J.A. (2003) The immune response of *Drosophila*. *Nature*, **426**, 33-38.
- 584 Immonen, E. & Ritchie, M.G. (2012) The genomic response to courtship song stimulation in female *Drosophila
585 melanogaster*. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 1359-1365.
- 586 Kraaijeveld, A.R., Limentani, E.C. & Godfray, H.C.J. (2001) Basis of the trade-off between parasitoid resistance
587 and larval competitive ability in *Drosophila melanogaster*. *Proceedings of the Royal Society of London. Series B:
588 Biological Sciences*, **268**, 259-261.
- 589 Lemaitre, B. & Hoffmann, J. (2007) The host defense of *Drosophila melanogaster*. *Annual Review of
590 Immunology*, **25**, 697-743.
- 591 Lewis, E.B. (1960) A new standard food medium. *Drosophila Information Service*, **34**, 117-118.
- 592 Libert, S., Zwiener, J., Chu, X., Vanvoorhies, W., Roman, G. & Pletcher, S.D. (2007) Regulation of *Drosophila* life
593 span by olfaction and food derived odors. *Science*, **315**, 1133-1137.
- 594 Little, T.J., Shuker, D.M., Colegrave, N., Day, T. & Graham, A.L. (2010) The coevolution of virulence: Tolerance
595 in perspective. *PLoS Pathogens*, **6**, e1001006.
- 596 Martin, L.B., Hawley, D.M. & Ardia, D.R. (2011) An introduction to ecological immunology. *Functional Ecology*,
597 **25**, 1-4.
- 598 McGraw, L.A., Gibson, G., Clark, A.G. & Wolfner, M.F. (2004) Genes regulated by mating, sperm, or seminal
599 proteins in mated female *Drosophila melanogaster*. *Current Biology*, **14**, 1509-1514.

600 Minakhina, S. & Steward, R. (2006) Melanotic mutants in *Drosophila*: pathways and phenotypes. *Genetics*, **174**,
601 253-263.

602 Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: the price of immune system activation for
603 bumblebee workers. *Science*, **290**, 1166-1168.

604 Mouatcho, J.C., Koekemoer, L.L., Coetzee, M. & Brooke, B.D. (2011) The effect of entomopathogenic fungus
605 infection on female fecundity of the major malaria vector, *Anopheles funestus*. *African Entomology*, **19**, 725-729.

606 R Development Core Team. (2013) R: A language and environment for statistical computing. R foundation for
607 statistical computing, Vienna, Austria. <http://www.R-project.org/>.

608 Read, A.F., Lynch, P.A. & Thomas, M.B. (2009) How to make evolution-proof insecticides for malaria control.
609 *American Journal of Tropical Medicine and Hygiene*, **81**, 165-165.

610 Reznick, D., Nunney, L. & Tessier, A. (2000) Big houses, big cars, superfleas and the costs of reproduction.
611 *Trends in Ecology & Evolution*, **15**, 421-425.

612 Sadd, B.M. & Siva-Jothy, M.T. (2006) Self-harm caused by an insect's innate immunity. *Proceedings of the Royal
613 Society of London. Series B: Biological Sciences*, **273**, 2571-2574.

614 Schulenburg, H., Kurtz, J., Moret, Y. & Siva-Jothy, M.T. (2009) Introduction. Ecological immunology.
615 *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **364**, 3-14.

616 Thomson, T.C., Schneemann, A. & Johnson, J. (2012) Oocyte destruction is activated during viral infection.
617 *Genesis*, **50**, 453-465.

618 Tinsley, M.C., Blanford, S. & Jiggins, F.M. (2006) Genetic variation in *Drosophila melanogaster* pathogen
619 susceptibility. *Parasitology*, **132**, 767-773.

620 van Noordwijk, A.J. & de Jong, G. (1986) Acquisition and allocation of resources - their influence on variation in
621 life-history tactics. *American Naturalist*, **128**, 137-142.

622 Williams, M.J. (2007) *Drosophila* hemopoiesis and cellular immunity. *Journal of Immunology*, **178**, 4711-4716.

623 Wilson, K., Thomas, M., Blanford, S., Doggett, M., Simpson, S. & Moore, S. (2002) Coping with crowds: density-
624 dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United
625 States of America*, **99**, 5471-5475.

626 Zerofsky, M., Harel, E., Silverman, N. & Tatar, M. (2005) Aging of the innate immune response in *Drosophila
627 melanogaster*. *Aging Cell*, **4**, 103-108.

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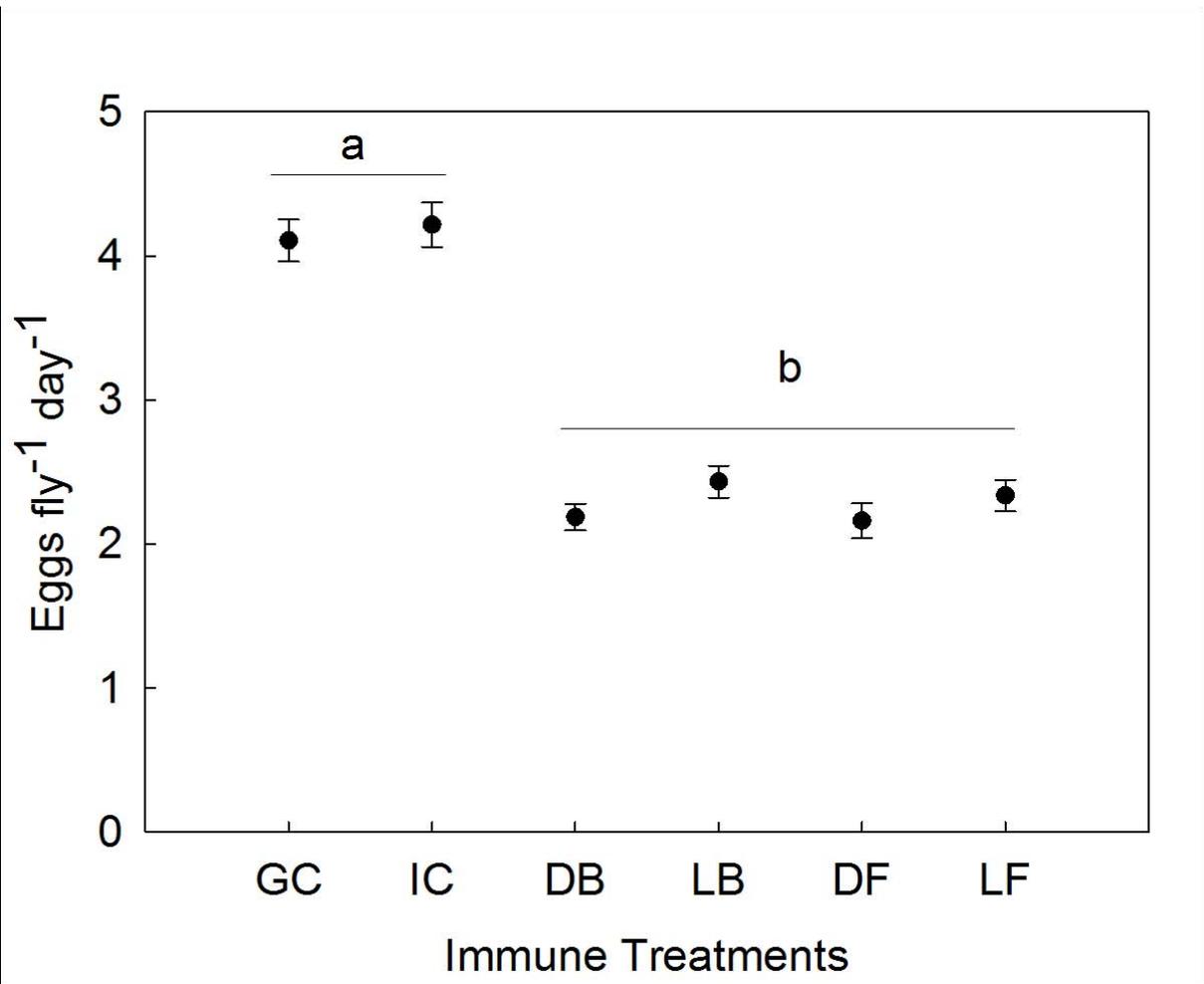
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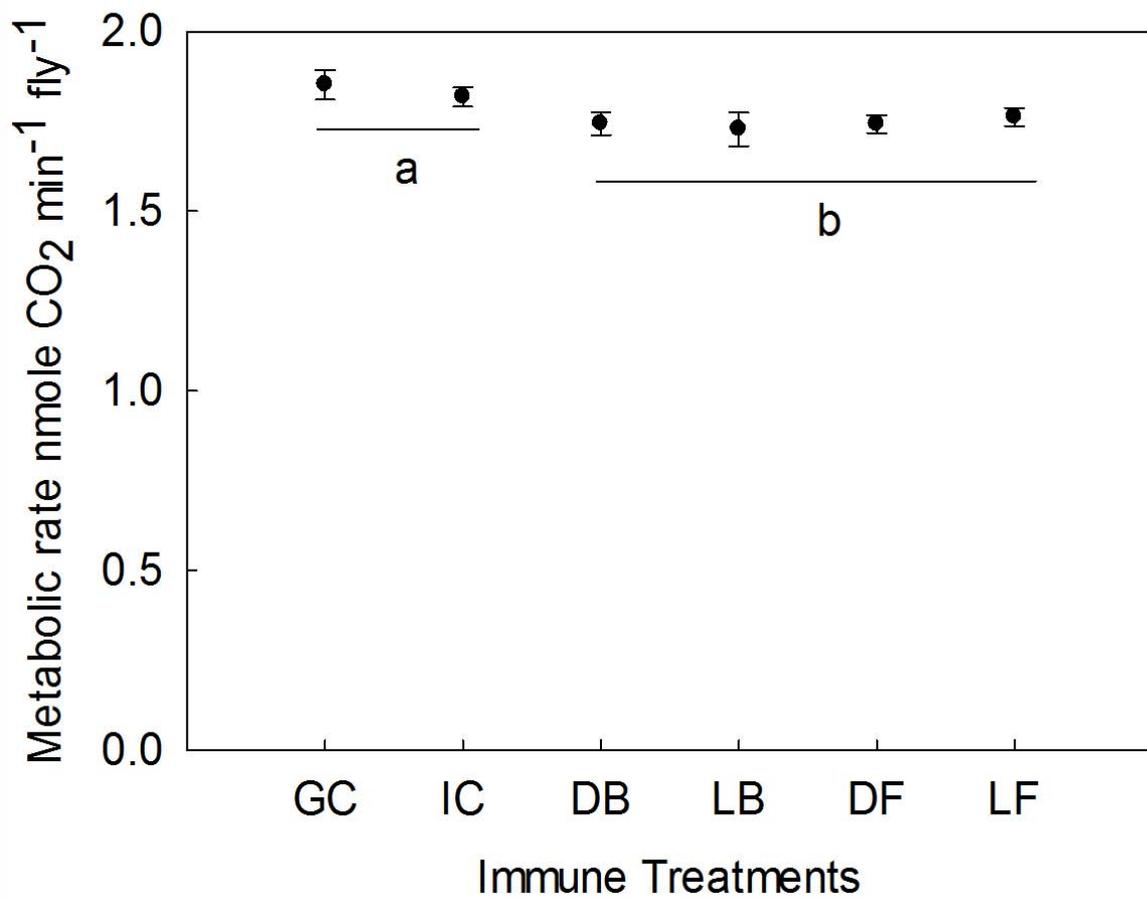
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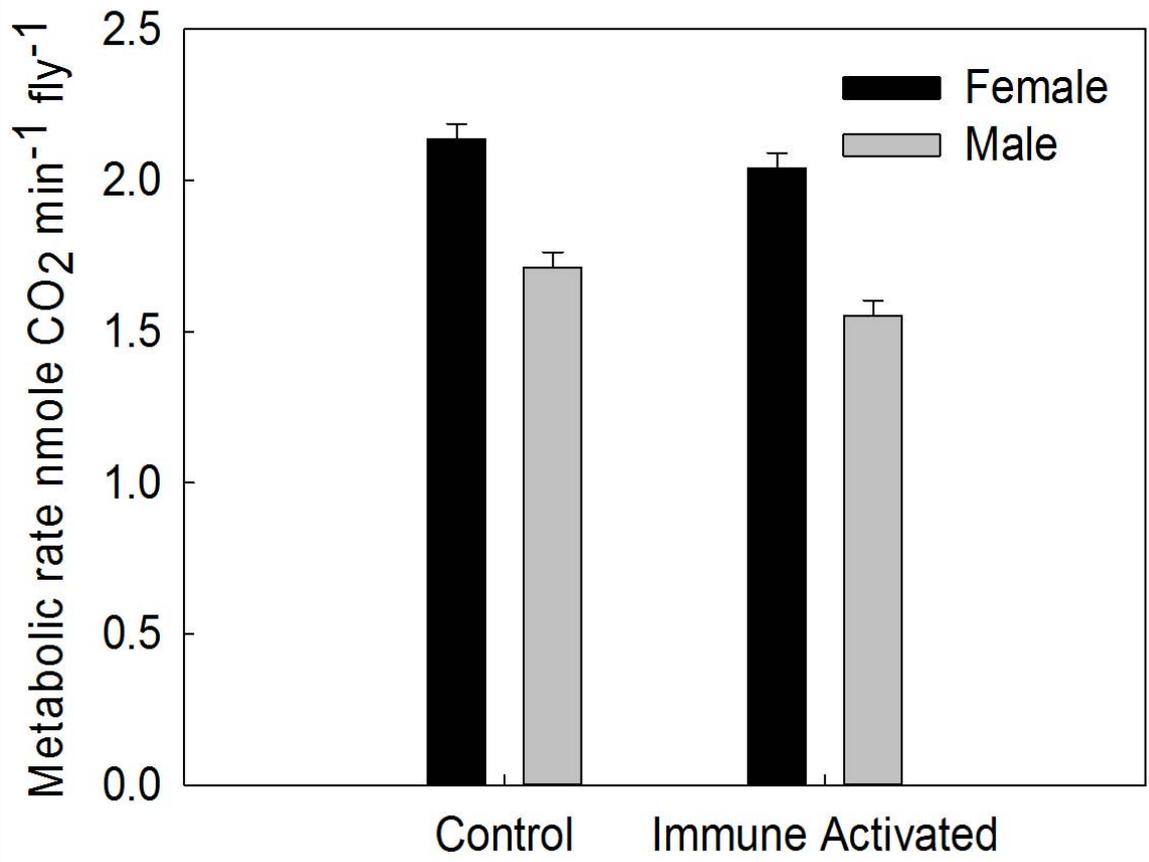
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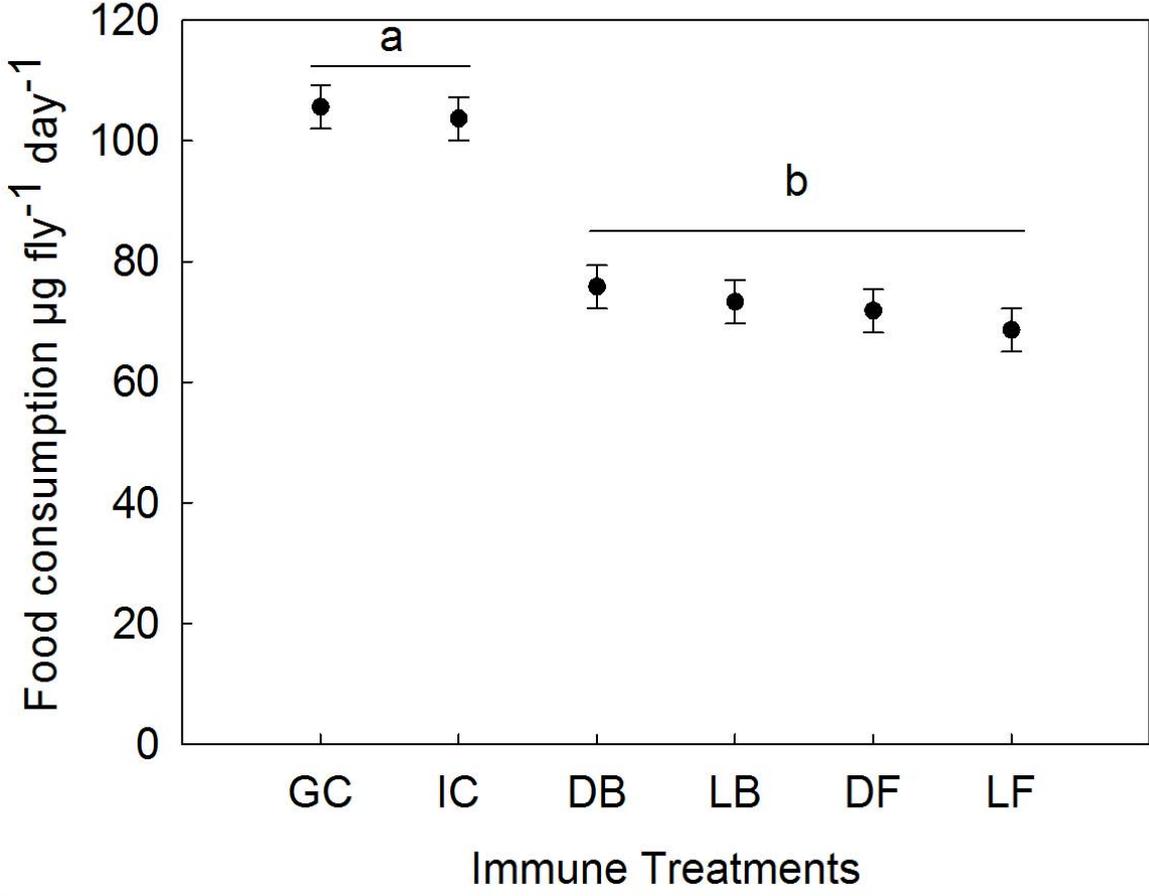
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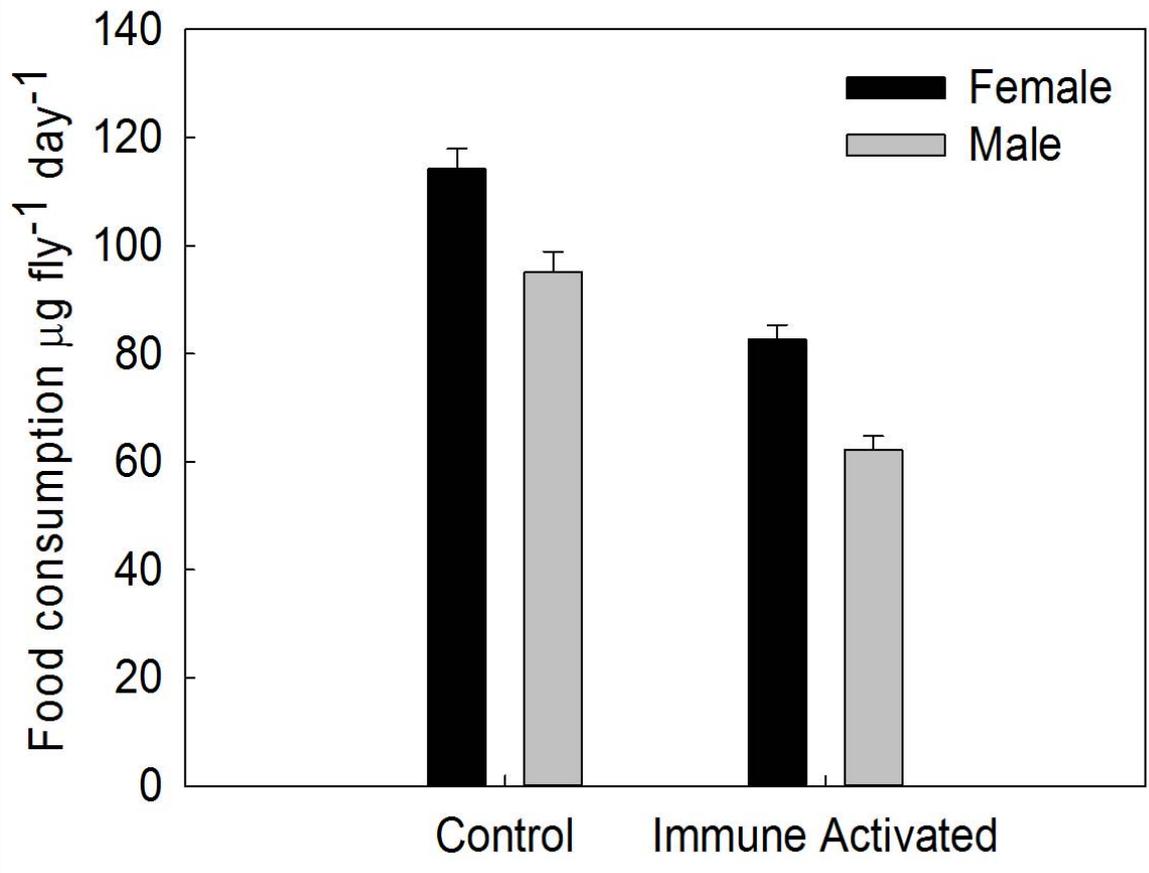
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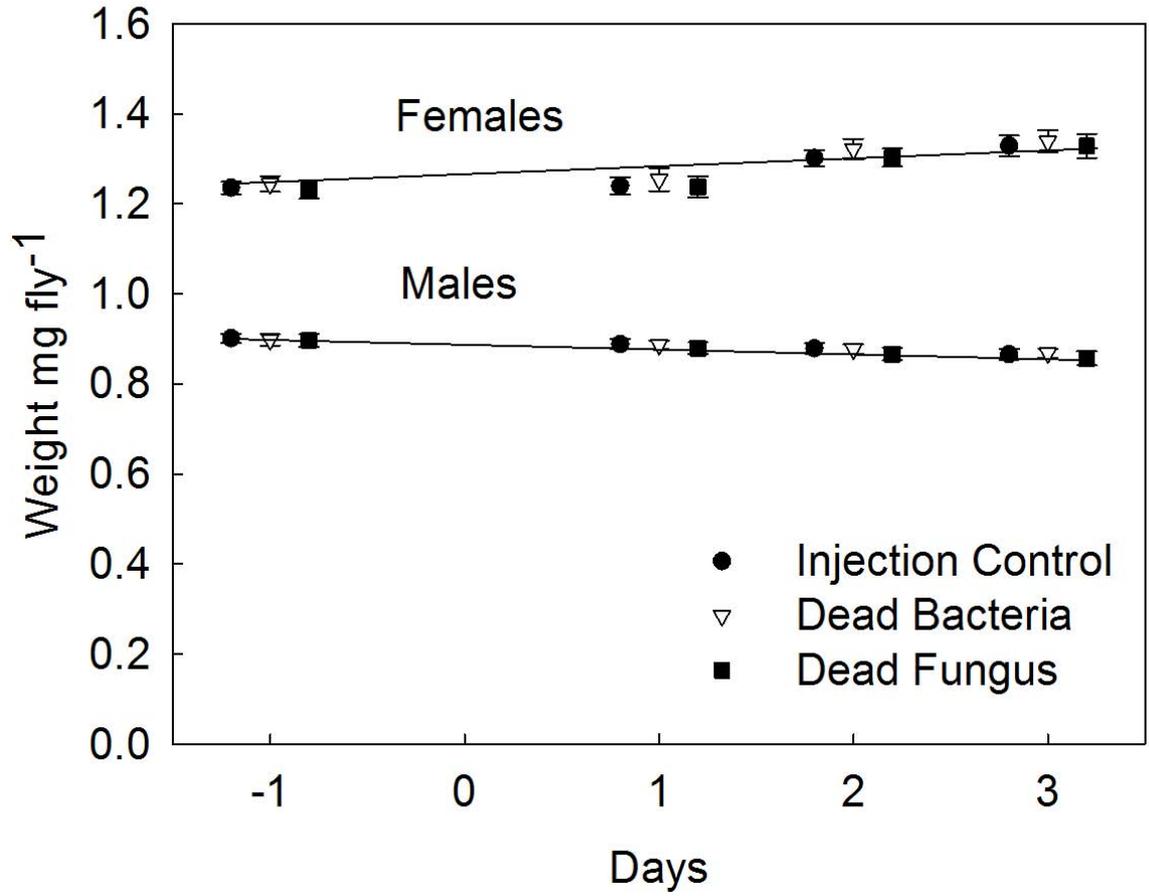
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