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

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Summary

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

Key-Words

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and Methods

FLY STOCKS AND REARING

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

yeast, in single-sex groups of 10 using light CO_2 anaesthesia the day before immune challenge. Each vial of flies was only used in one of the following experiments.

IMMUNE CHALLENGES

The impacts of microbial injection on fly fecundity, metabolic rate, food ingestion and body weight were studied. Microbes were prepared as both live and dead suspensions to permit separation of the physiological effects of immune activation from those of microbial replication. *B. bassiana* spores were grown on potato dextrose agar (PDA) from an existing strain using standard procedures (Tinsley, Blanford & Jiggins 2006); live and dead conidiospores were suspended in oil (87.5% Shellsol T, 12.5% Ondina EL). *E. coli* were cultured overnight in Luria Broth (LB) at 37 °C with continuous shaking, both live and dead *E. coli* were suspended in sterile LB. Heat-killed suspensions of *B. bassiana* and *E. coli* were prepared by boiling at 100 °C for 15 min. The absence of growth was confirmed for heat-killed suspensions by plating 100 µl on PDA and LB agar respectively; the viability of live suspensions was similarly verified. Live fungal spores (2.0×10^6 spores ml^{-1}), heat-killed fungal spores (2.0×10^7 spores ml^{-1}) and 2.0×10^6 cells ml^{-1} of live and heat-killed bacteria were used to trigger immune responses in flies by injection into the thorax using a fine tungsten wire needle. The terminal 0.3 mm of the needle was bent slightly to provide a marker ensuring consistent penetration. Flies received CO_2 anaesthesia for injections, but then not during any subsequent assays. Four day old flies received one of six treatments: gas control (GC, flies anaesthetised with CO_2 but not injected), injection control (IC, flies injected with a needle dipped in blank oil), dead fungal (DF, dead *B. bassiana* injection), live fungal (LF, live *B. bassiana* injection), dead bacterial (DB, dead *E.* coli injection) and live bacterial injection (LB, live *E. coli* injection). Microbial suspensions were vortexed frequently to prevent microbes settling and needles were sterilised with ethanol and flaming.

EFFECT OF IMMUNE CHALLANGE ON FECUNDITY

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

MEASURING METABOLIC RATE

This study was conducted using 410 independent 10-fly groups in seven blocks; each block contained multiple replicates of five or six of the different immune treatments. The effect of immune activation on fly metabolic rate was assessed by respirometry, measuring CO_2 production with an infrared gas analyser (IRGA: EGM-4, PP Systems). Day one measurements were made on 5 day old male and female flies 16-18 h after immune treatment; further measurements were made at 24 h intervals. Flies were housed in a plastic chamber connected in a circuit to an IRGA with tubing (total system volume 40.5 cm^3). Air circulated within this sealed system and CO_2 accumulation was measured. In each assay the metabolic rate of a group of 10 flies was measured at 25 °C over 5 min, recording CO_2 every 1.6 seconds; data from the first 2 min whilst flies settled were discarded. Measurements on each 10-fly group were repeated on three or four consecutive days. CO_2 efflux per minute was calculated by linear regression, then converted to nmole CO_2 min^{-1} fly^{-1} using knowledge of the apparatus volume.

EFFECT OF IMMUNE CHALLENGE ON FOOD INTAKE

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

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

EFFECT OF IMMUNE CHALLENGE ON FLY BODY MASS

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

STATISTICAL ANALYSIS

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

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

For analyses involving repeated measures on vials of flies over successive days the term 'vial' was included as a random effect, whilst temporal changes were assessed using the fixed effect of 'day' and its two-way interaction with treatment. With the exception of fecundity studies, models also included fly 'gender' and a 'gender by treatment' interaction. When analysing metabolic rate data, models contained an additional random effect of 'block', accounting for variation between the seven blocks over which the investigation was conducted. We also tested the impact of time of day and the air CO_2 concentration when each metabolic rate measurement was made. Finally, for investigations of variation in food ingestion after immune challenge, 'vial' was used as a random effect to associate the two five-fly batches from each vial. The number of flies in assay vials for fecundity, metabolic rate and body weight experiments varied slightly due to escapes; in each case we tested whether fly number influenced the trait measured.

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

Results

FECUNDITY COSTS OF IMMUNE ACTIVATION IN *D. MELANOGASTER*

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

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

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

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

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

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

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

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

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

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

Discussion

In this study we investigated the validity of the hypothesis that the costs of defending against parasites arise because resources normally invested in other physiological processes must be diverted to fuel the demands of the immune response. We demonstrated clear immune 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

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

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

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

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

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

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

Data will be uploaded to Dryad prior to publication.

Legends

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

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

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

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

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

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

References

Adamo, S.A., Roberts, J.L., Easy, R.H. & Ross, N.W. (2008) Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *Journal of Experimental Biology*, **211**, 531-538.

Adamo, S.A., Bartlett, A., Le, J., Spencer, N. & Sullivan, K. (2010) Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Animal Behaviour*, **79**, 3-10.

Ahmed, A.M. & Hurd, H. (2006) Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. *Microbes and Infection*, **8**, 308-315.

Ahmed, A.M., Baggott, S.L., Maingon, R. & Hurd, H. (2002) The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. *Oikos*, **97**, 371-377.

Arnold, P.A., Johnson, K.N. & White, C.R. (2013) Physiological and metabolic consequences of viral infection in *Drosophila melanogaster*. *Journal of Experimental Biology*, **216**, 3350-3357.

Ayres, J.S. & Schneider, D.S. (2009) The role of anorexia in resistance and tolerance to infections in *Drosophila*. *PLoS Biology*, **7**, e1000150.

Bates, D., Meachler, M. & Bolker, B. (2013) Lme4: Linear mixed-effects models using S4 classes. R package version 0999999–2. http://cran.r-project.org/web/packages/lme4/index.html

Baze, M.M., Hunter, K. & Hayes, J.P. (2011) Chronic hypoxia stimulates an enhanced response to immune challenge without evidence of an energetic trade-off. *Journal of Experimental Biology*, **214**, 3255-3268.

Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S. & Lemaitre, B. (2009) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host & Microbe*, **5**, 200-211.

Burr, M.J., Hunter, A.S. (1969) Effects of temperature on Drosophila- V. Weight and water, protein and RNA 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 Freitak, 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.

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

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

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

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

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

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

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

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

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

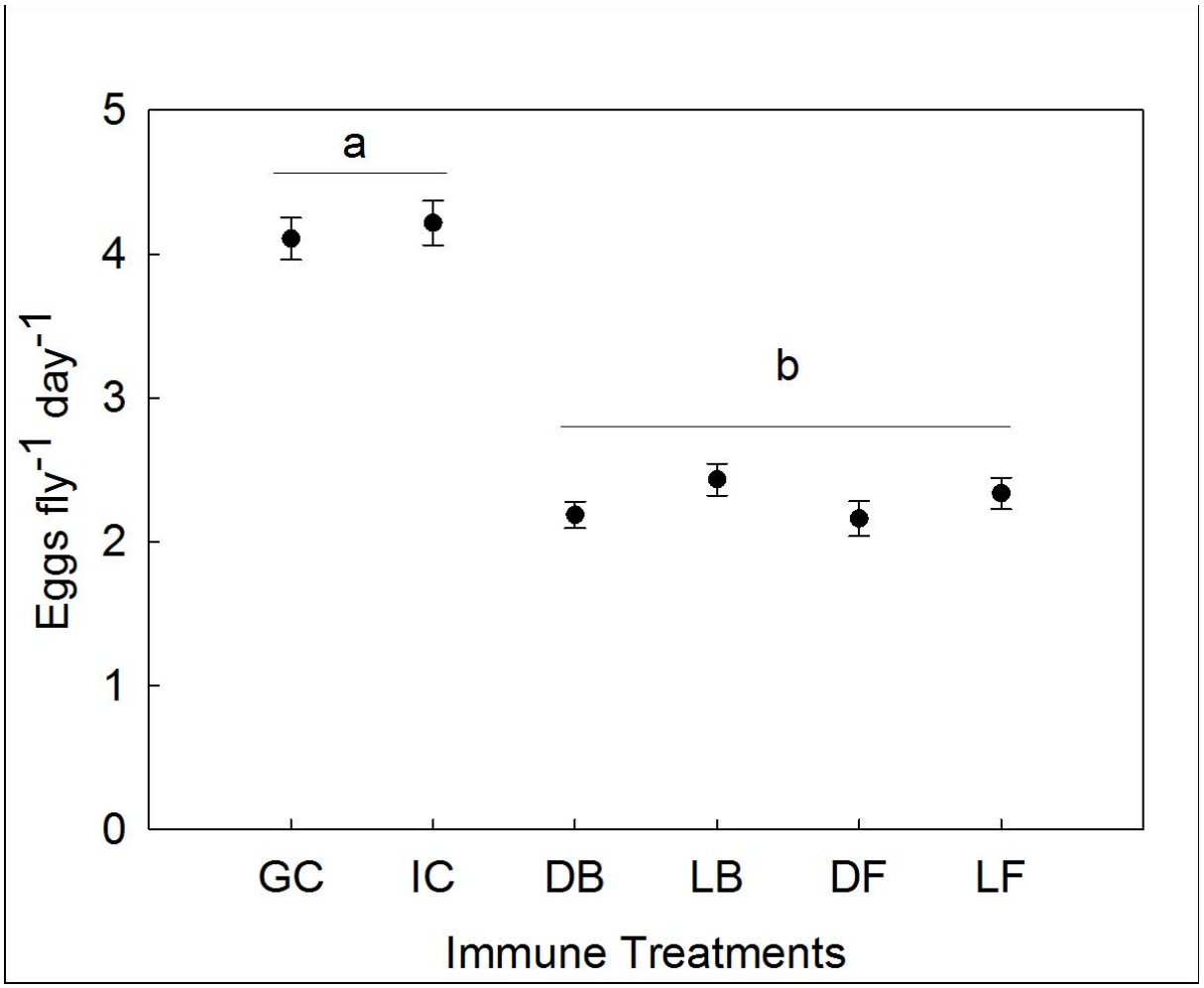
Tinsley, M.C., Blanford, S. & Jiggins, F.M. (2006) Genetic variation in *Drosophila melanogaster* pathogen susceptibility. *Parasitology*, **132**, 767-773.

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

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

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

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



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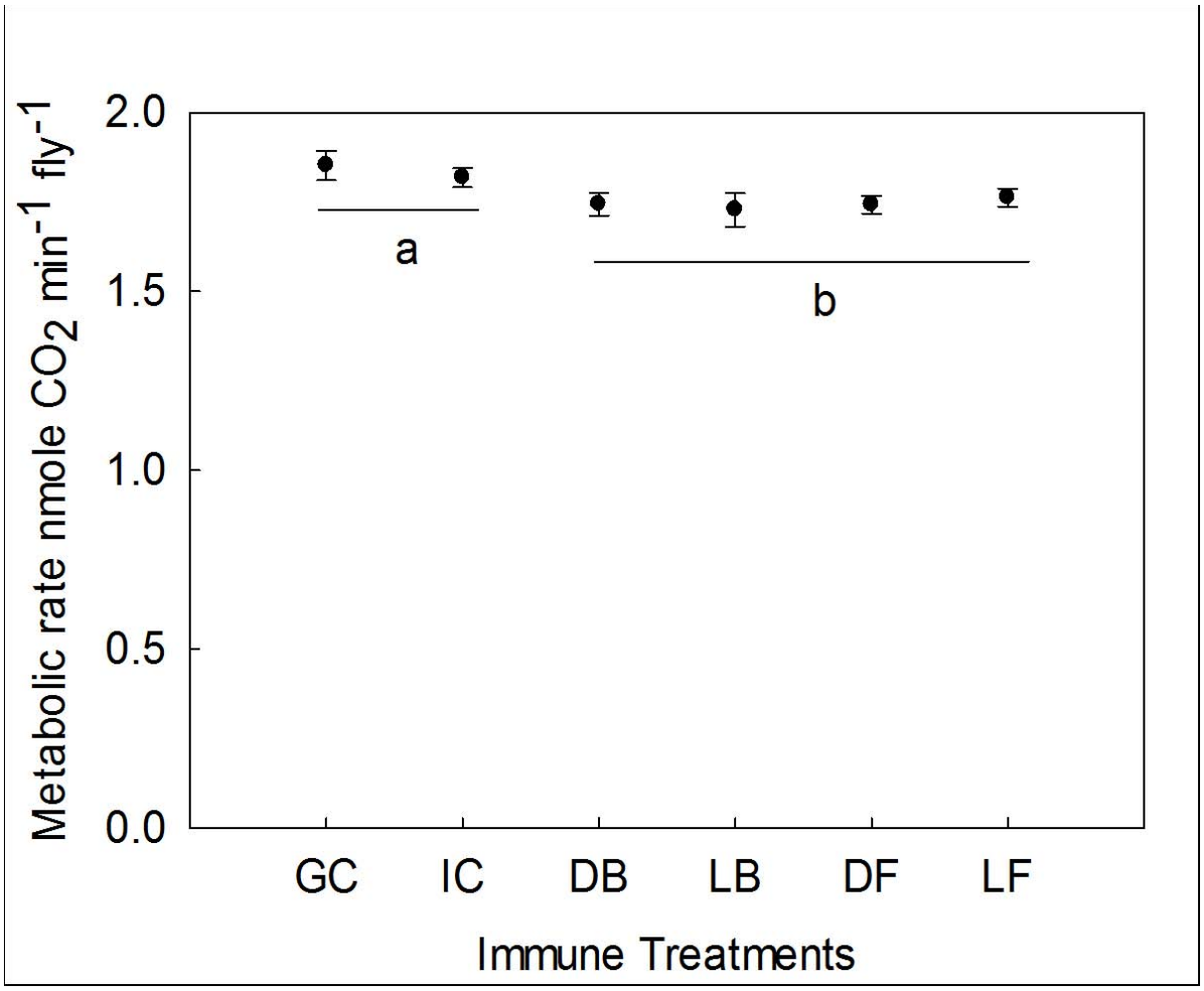
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1.0
0.5
0.0
a
b
GC
IC
DB
LB
DF
LF
Immune Treatments

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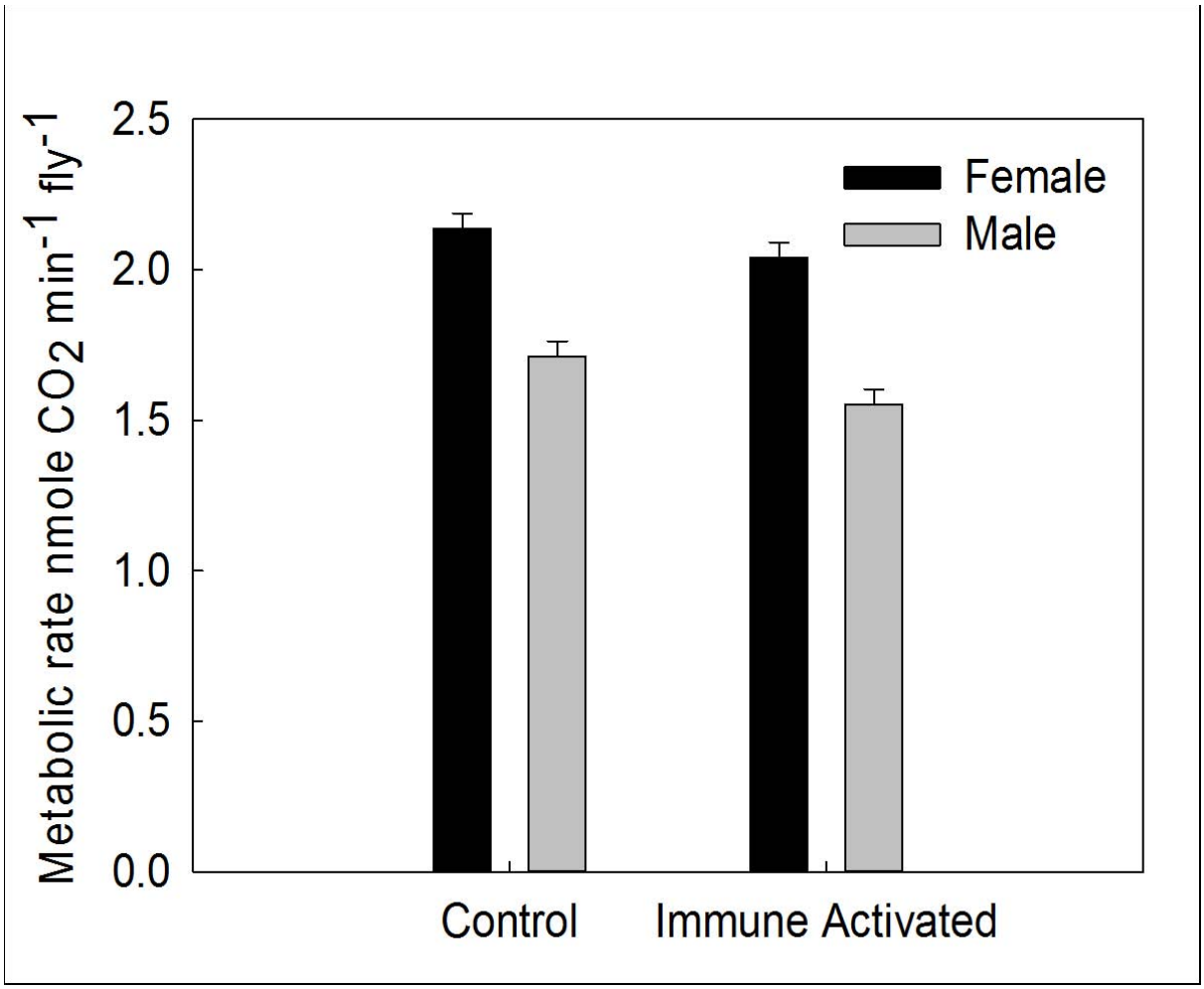
Metabolic rate nmole CO_2 min^{-1} fly^{-1}
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1.0
0.5
0.0
Female
Male
Control
Immune Activated

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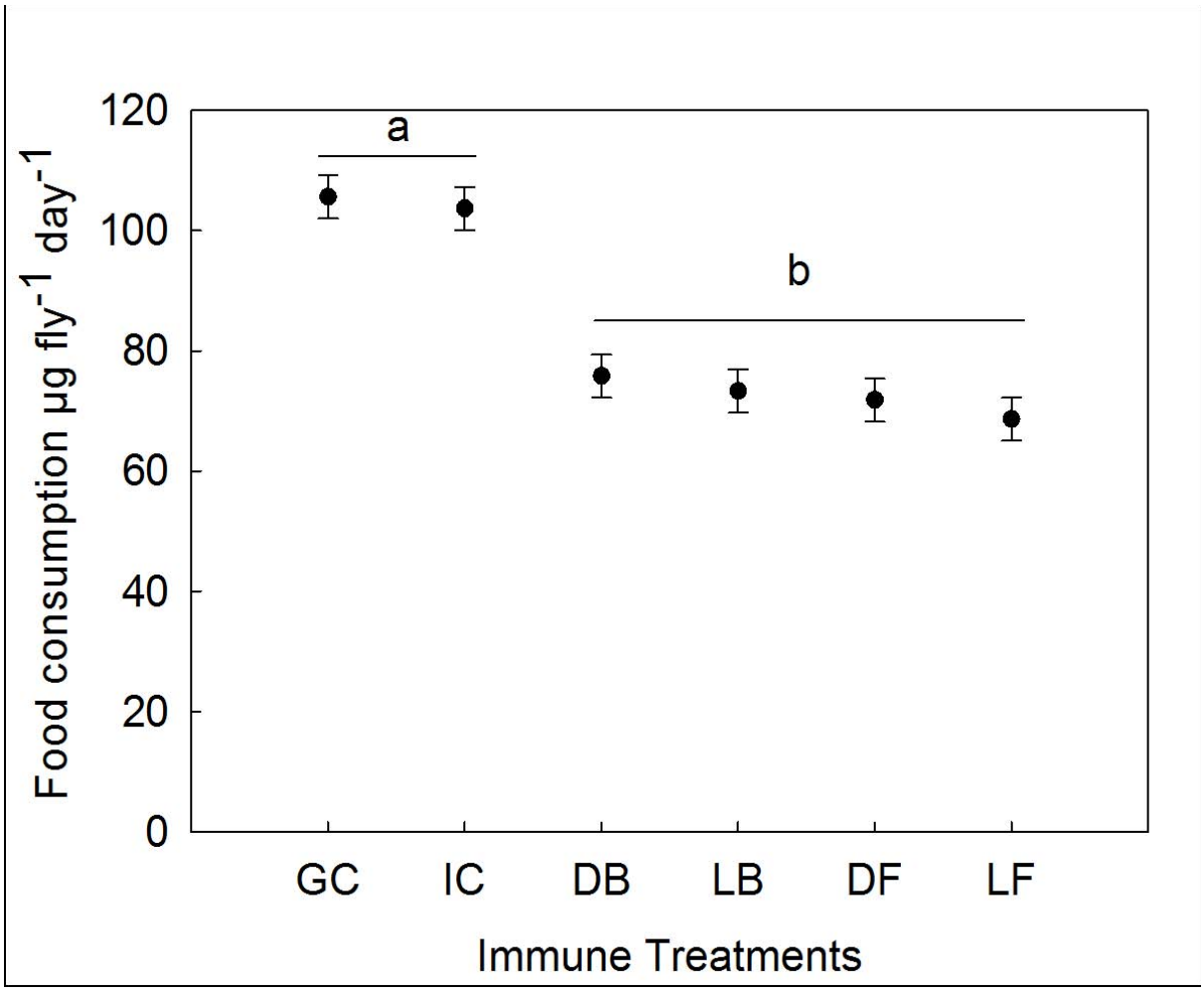
120
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Food consumption µg fly-1 day-1
a
b
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IC
DB
LB
DF
LF
Immune Treatments

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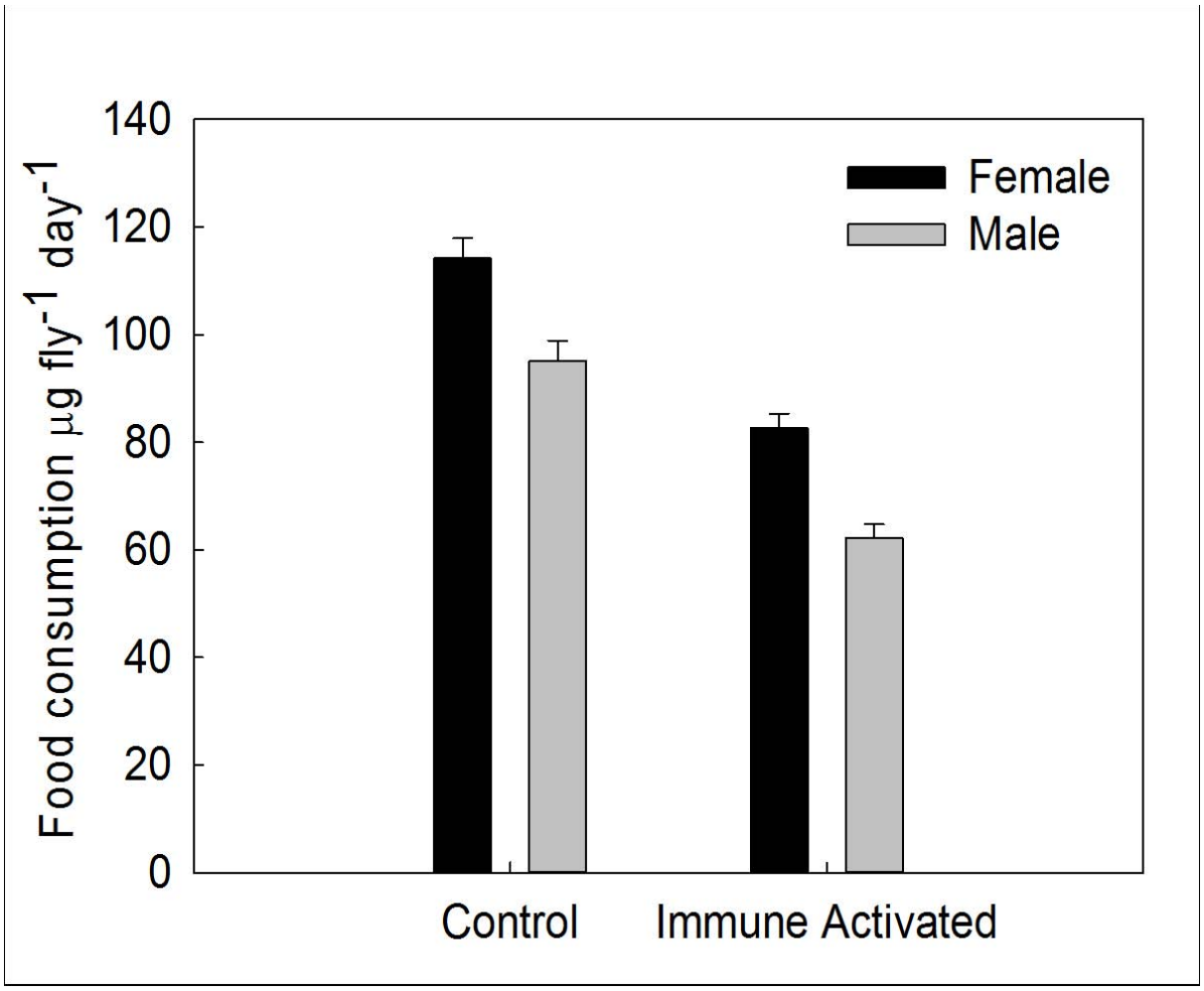
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Food consumption μg fly-1 day-1
Female
Male
Control
Immune Activated

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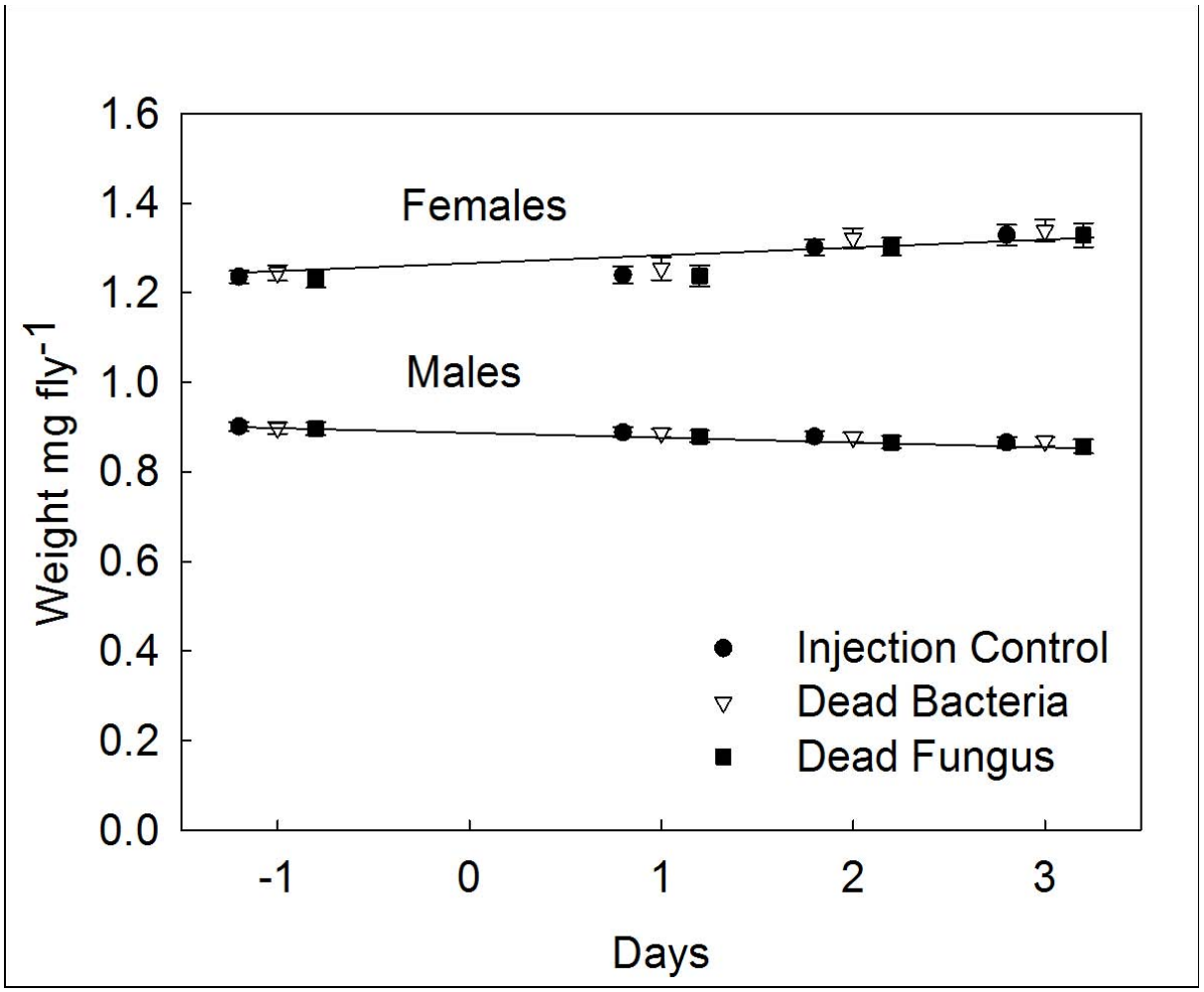
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Weight mg fly-1
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Males
Injection Control
Dead Bacteria
Dead Fungus
-1
0
1
2
3
Days

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