JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12364

Dose-dependent effects of an immune challenge at both ultimate and proximate levels in *Drosophila melanogaster*

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Keywords:

disease;
dose dependence;
eco-immunology;
immune elicitor;
life history;
lipopolysaccharide;
trade-off;
sperm competition.

Abstract

Immune responses are highly dynamic. The magnitude and efficiency of an immune response to a pathogen can change markedly across individuals, and such changes may be influenced by variance in a range of intrinsic (e.g. age, genotype, sex) and external (e.g. abiotic stress, pathogen identity, strain) factors. Life history theory predicts that up-regulation of the immune system will come at a physiological cost, and studies have confirmed that increased investment in immunity can reduce reproductive output and survival. Furthermore, males and females often have divergent reproductive strategies, and this might drive the evolution of sex-specific life history trade-offs involving immunity, and sexual dimorphism in immune responses *per se*. Here, we employ an experiment design to elucidate dose-dependent and sex-specific responses to exposure to a nonpathogenic immune elicitor at two scales – the 'ultimate' life history and the underlying 'proximate' immune level in *Drosophila melanogaster*. We found dose-dependent effects of immune challenges on both male and female components of reproductive success, but not on survival, as well as a response in antimicrobial activity. These results indicate that even in the absence of the direct pathogenic effects that are associated with actual disease, individual life histories respond to a perceived immune challenge – but with the magnitude of this response being contingent on the initial dose of exposure. Furthermore, the results indicate that immune responses at the ultimate life history level may indeed reflect underlying processes that occur at the proximate level.

Introduction

All animals possess immune capability, whether it be a simple system reliant on innate immunity, as typical of invertebrates, or the acquired (adaptive) systems of vertebrates (Little & Kraaijeveld, 2004; Kurtz, 2005; Sadd *et al.*, 2005; Male *et al.*, 2012). However, regardless of immune system type, the efficiency by which an individual can mount an immune response to pathogenic challenge will vary across a range of factors – some of which are intrinsic to the individual (such as age, sex or genotype of the individual) and some of which are associated with the external environment in which the individual finds itself in (Lazzaro & Little, 2009; Winterhalter & Fedorka, 2009). Hence, immune function is likely to be subject to considerable context dependence, and immune responses tightly entwined with individual life histories (Roff, 2002).

Life history theory is grounded in the idea that the increased expression of one life history trait will come at a cost to the expression of another trait (Roff, 2002). This idea has been corroborated in the context of immunity, by empirical studies in both vertebrate (Zuk & Stoehr, 2002; Hanssen *et al.*, 2004; Martin *et al.*, 2008; Downs *et al.*, 2013) and invertebrate systems (Fellowes & Godfray, 2000; Moret & Schmid-Hempel, 2000; Cotter *et al.*, 2004; Burger *et al.*, 2007; Stoehr, 2007; Ayres & Schneider, 2009; Boggs, 2009). Indeed, the idea of the immune defence invoking physiological costs is embedded in several key evolutionary theories, for example, the parasite-mediated hypotheses of sexual selection (Hamilton & Zuk, 1982; Folstad & Karter, 1992).

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In sexually reproducing species, males and females often vary in their expression of classic life history traits. Much of this variance is likely to be underpinned by the fact that there are typically stark differences in the strategies that each sex adopts to maximize its reproductive potential (Bateman, 1948), and these differences have driven the evolution of sexual dimorphism of many traits, to the extent that some traits are completely sex-limited in expression (Andersson, 1994). Accordingly, because survival, and hence life time reproductive success, is tightly entwined with the ability to resist or tolerate disease, life history trade-offs involving immunity might also be optimized differently across the sexes (Bateman, 1948; Rolff, 2002; Rolff *et al.* 2005; McKean & Nunney, 2005; Nunn *et al.*, 2009). For example, whereas males can rapidly increase their reproductive success as a function of the number of females that they mate with, female reproductive success is constrained by the number of ova a female can produce and thus should not increase greatly with mating rate (Bateman, 1948). These discrepancies in maximal reproductive output could therefore drive sex differences in immune investment, with males investing more heavily into immediate reproduction, at the expense of immune system maintenance, relative to females (Rolff, 2002). Several studies have supported such predictions, by showing female-biased expression of immune activity in response to immune challenges (Zuk, 1990; Zuk & McKean, 1996; Rolff, 2002; Nunn *et al.*, 2009).

It is currently unclear whether sex-specific life history responses to immune challenge are driven mainly by the pathological effects that are directly attributable to the pathogen, or whether the initial reaction of the host to the incoming pathogen can itself impact on the ensuing life history response (Jacot *et al.*, 2004; Robb & Forbes, 2006; Zanchi *et al.*, 2011; Grindstaff *et al.*, 2012; Serra *et al.*, 2012; McNamara *et al.*, 2013). For example, a pathogenic infection can invoke disease phenotypes via pathogen replication, manipulation of host resources and cellular environment and interference in host signalling pathways, as well as direct tissue damage – all of which will exert costs on host physiology (Bhavsar *et al.*, 2007; Fedorka & Mousseau, 2007; Agudelo-Romero *et al.*, 2008; Paschos & Allday, 2010; Haq *et al.*, 2012; Sadd & Siva-Jothy, 2007; Zhong *et al.*, 2012). But, when faced with an incoming pathogen, the host should also experience direct costs due to the need to redeploy limited cellular resources away from other physiological tasks to thwart the infection before it can become established, and possibly also through the side effects of auto-immunity (Roff, 2002; Lee, 2006). These are costs that are brought about directly through activation and deployment of the immune system, which are set in place at the initial time of exposure in an effort to immediately clear the infection. The magnitude of these initial host-induced costs of an immune challenge can be explored within an experimental context, by administering test subjects with a 'nonreplicating' immune challenge, for example pure peptidoglycans (PGN) and lipopolysaccharide (LPS), both of which are components of the bacteria cell wall (Kim *et al.*, 2005; Lemaitre & Hoffmann, 2007), or by using heat-killed bacteria (Adamo *et al.*, 2001; Robb & Forbes, 2006; Roth & Kurtz, 2009; Zanchi *et al.*, 2011), followed by the monitoring of life history and physiological responses.

Here, we expose *Drosophila melanogaster* to a nonreplicating immune elicitor, crude LPS (i.e. nonpurified LPS, also containing peptidoglycans, proteins and DNA), to explore the effects of initial immune activation at two scales – the ultimate life history scale (through components of reproductive success and survival), at which selection will act, and the proximate mechanistic scale (via measurement of a humoral immune response trait). Although there have been recent suggestions that the LPS molecule itself may participate in activating certain parts of the immune response in *Drosophila* (Shi *et al.*, 2012, Anand *et al.*, 2012), other evidence show that it is predominantly by-products present in crude LPS (specifically, peptidoglycans) that is responsible for LPS-induced immune reactions (Lemaitre & Hoffman, 2007; Kaneko *et al.*, 2004). Regardless of the underlying mechanism, crude LPS is sufficient for the purposes of triggering a general immune response in our study. Hence, using a nonreplicating immune elicitor rather than a live bacteria or virus, we sought to remove the effects on the host that would be brought about by sustained pathogenic infection, and home in on the question of whether the costs of the hosts' initial and direct response to challenge, in terms of the activation and deployment of the immune arsenal, are large enough in themselves to trigger sex-specific responses at the ultimate or proximate scale. We also investigate whether any such effects are contingent on the magnitude of the initial exposure experienced by the host, by administering the immune elicitor at several doses differing in their concentration. Despite a growing interest in eco-immunological questions, few studies to date have adopted a multidose design, and very few have done so in both males and females (McKean & Nunney, 2005; Lefevre *et al.*, 2010, Ramsden *et al.*, 2008, Altizer & Oberhauser, 1999, Gray, 1998). Yet, it is well known that disease dynamics frequently exhibit clear dose dependence (Ben-Ami *et al.*, 2010), and furthermore, that many pathogens require a minimum infection dose for the host to develop a tangible immune response, for example (Paulo *et al.*, 2010, Leggett *et al.*, 2012). Moreover, as outlined above, life history theory predicts that the expression of immunity will trade off with other fitness-related traits, in a sex-specific manner. Hence, some of the evident drawbacks associated with 'single-sex and single-dose' studies include the risk of inaccurately extrapolating results generated in one sex to both sexes, and the risk of

administering doses that are not ecologically relevant, as well as the innate constraints associated with generalizations of results beyond a particular dose and study (Ayres & Schneider, 2012).

Materials and methods

Fly stocks

The base stock population was founded by 60 wild-caught nonvirgin females, collected from three different localities within Coffs Harbour, NSW Australia, in February 2010. Ten sons and ten daughters of each of these 60 females were added to those of the other 59 females to create a single mass bred population. The stock population is maintained across twelve 10 dram vials, on a 12:12 hour light/dark cycle, at 25 °C. The flies are kept on a potato-dextrose-yeast-agar medium that also contains 20 mg live yeast. Each vial in the population is propagated by 20 four-day-old pairs over a 20-h egg-laying period, and all eclosing offspring are admixed with those of the other vials each generation prior to their sorting into new vials.

The base population has been propagated on a discrete generation life cycle since February 2010. At the time of this assay, the population had been reared for over 50 generations under the conditions described above, selected for an early life schedule. That is, all individuals contributing genes to the subsequent generation were young adults, of 4 or 5 days of age. Effectively, a female's entire lifetime reproductive success in this laboratory population is decided by her ability to produce numerous viable offspring during a 20-h period early in life.

From the laboratory population described above, we initiated two independent replicate populations in February 2012. The resulting replicate populations were each reared across two 250-mL bottles at 25 °C, with each population temporally separated, such that experimental focal flies were emerging on a weekly cycle. To propagate the replicate populations, 4- to 5-day-old adult flies emerging from the two bottles per population were admixed every generation, to avoid divergence within each population replicate.

Experimental design

Immune challenge

All experiments centred around a challenge with a nonreproducing immune elicitor, consisting of five increasing concentrations (0%, 0.05%, 0.1%, 0.5% and 1%). Hence, to mimic a relevant bacterial infection, we used crude LPS (phenol-extracted lipopolysaccharide; Sigma-Aldrich, L6136) from *Serratia marcescens*, which is a highly pathogenic gram-negative bacterium that naturally infects *Drosophila* (Lemaitre *et al.*, 1997). Crude LPS contains, in addition to the LPS macromolecule itself, other components of the cell wall such as fragments of peptidoglycan (PGN), proteins and DNA (Kaneko *et al.*, 2004; Sullivan & Belloir, 2014). Whereas the LPS molecule is clearly responsible for inducing innate immune response and antibody production in mammals and some insects (Meng *et al.*, 2010, Janeway *et al.*, 2001, Esko *et al.*, 2009), studies have shown that it is primarily peptidoglycans that activate the main antimicrobial response in *Drosophila*, through the activation of the Imd pathway (Kaneko *et al.*, 2004; Lemaitre *et al.*, 1995). However, the exact role of the LPS molecule in the *Drosophila* immune response remains unclear (Kounatidis & Ligoxygakis, 2012; Zhu & Zhang, 2013). For example, it has been shown that specific proteins (NPC2) can bind to both the outer cores of the LPS molecule and to the underlying endotoxin lipid A, suggesting a potential role in the fly immune response (Shi *et al.*, 2012). LPS has also been found to play a role in the release of antibacterial histone from its bounded site at cytosolic lipid droplets in *Drosophila*, again suggesting a role in the innate immune reaction (Anand *et al.*, 2012). The initiation of the latter process could potentially also interfere with other physiological processes associated with lipid droplet-bound histones, such as the 'buffering' of harmful free-floating histones (Cermelli *et al.*, 2006). Note that most available evidence suggests that any effect of LPS on the expression of physiological or life history traits in *Drosophila* will be indirectly or directly related to immune pathways. Yet, we acknowledge that it is possible that LPS administration might interfere with other (nonimmune) related pathways – through, for example, a general toxicity or stress effect (Sano *et al.*, 2005). Such a caveat is, however, relevant to any study that uses an ecological or medical treatment capable of inducing a condition-dependent response.

To generate different concentrations, LPS was diluted according to a serial dilution protocol with PBS (Sigma-Aldrich tablet P4417, pH 7.4) to each of the five different concentrations and administered in a 41.4 nL dose using a nano-injector (Nanoject; Drummond Scientific Company, Broomall, PA, USA) inserted into the abdomen of adult flies. All flies were injected 24 h after their eclosion, while anaesthetized under light CO_2 exposure.

Female reproductive fitness and longevity

We measured early life fecundity and clutch viability of females, to mimic the conditions under which female fitness had been evolving in this laboratory population over numerous (approximately 50) generations. Our measure of female fecundity was the number of eggs ovipositor by a female over an 18-h period of opportunity, when 4 days post-eclosion. Clutch viability was gauged as the proportion of these eggs that hatched and developed into reproductively mature adults.

To ensure temporal consistency of the effects, the assay was conducted over several ($n = 12$) sampling blocks, each separated temporally by 1 week. Virgin females were collected within 5 h of their eclosion into adulthood and stored in groups of 8 ± 0.2 (SE) individuals per vial. The females of each group were subjected to one of the five levels of the immune treatment, by microinjection 24 h after eclosion. Injected females were put back into their respective vials with *ad libitum* access to live yeast, where they entered a 72-h recovery. After these 72 h, the whole cohort of females per vial (all of whom had received the same level of the immune treatment) was exposed to an equal number of virgin males for 2 h, during which time they were freely able to mate. Each female was then placed in an individual 'oviposition' vial for 18 h (without live yeast) to enable ovipositioning. Immediately following the 18-h period, females were transferred to fresh vials, and the number of eggs laid per oviposition vial counted. The longevity of each female was subsequently monitored, with routine checks every 3–4 days, at which time all females were transferred to fresh vials of standard food medium, but without access to live yeast.

Oviposition vials were retained at 25 °C and 12 days later scored for the number of eclosing adult offspring and the sex of each eclosing offspring. This provided a measure of early life clutch viability (i.e. egg-to-adult viability per clutch, which is a proxy of fertility) and allowed us to screen for sex ratio biases amongst the resulting brood of adults.

The full data set contained 1761 females sampled across 12 blocks, where each block was represented by a mean of 147 ± 22 females, and an average of 30 ± 3 (SE) females were sampled per treatment level within each block. However, not all females produced eggs. Hence, in the analysis of clutch viability, we only included the data points where at least one egg had been laid (1048 females). In the analysis of fecundity however, we included the whole data set ($n = 1761$). To further disentangle any influence of immune treatment on female egg production, we conducted a simple binary analysis of egg laying, again using the full data set of 1761 individuals (i.e. response variable 'no eggs' vs. '> 0 eggs'). The associated longevity assay only contained 1734 females, because some female data points were lost over the course of the assay (i.e. flies escaped or accidently got stuck in the food medium after CO_2 exposure). Limiting the survival analyses to only those females that had successfully laid at least one egg (1033 females; a few more data points were lost because females either lacked survival data or viability data) did not qualitatively alter the results. Finally, we also looked at short-term survival (survival to 72 h) of females across the treatment levels, in a subsample of the data ($n = 604$, four blocks). The primary focus on this assay was to determine whether there were dose-related effects on mortality during the time between the immune treatment and the reproductive assays, which could have biased the subsequent sample of flies entering into the reproductive assays.

Male reproductive fitness and longevity

Focal males were generated according to a similar protocol to that described above for females, with the exception that each male was kept in its own individual vial throughout the immune challenge.

Male reproductive fitness was estimated via a sperm competitiveness assay. The species is polyandrous, with females typically mating with several males, successively, early in life. This behaviour invokes strong reproductive competition between males for copulations, as well as strong post-copulatory sexual selection on the male ejaculate (Simmons, 2001). Here, we tested male sperm competitiveness in the 'offensive' role, as defined by the ability of a focal male, who has successfully coerced a once-mated female to re-mate, to fertilize the ova of this female when in competition against the sperm of the rival competitor male (i.e. a 'tester' male). This assay involved exposing an individual standard-aged (4–5 days old) virgin 'tester' female with one standard-aged (4–5 days old) virgin 'tester' male for 150 min. During this time, virtually all females will mate once and only once (Friberg & Dowling, 2008), and this applies to this population of flies (D. K. Dowling, personal observations). The tester flies were created from two distinct homozygous brown-eyed lines (Yee *et al.*, 2013), each originally derived from an outbred copy of the LH_M population (Rice *et al.*, 2005), into which an autosomal recessive brown-eyed mutation had previously been backcrossed (Friberg & Dowling, 2008). Virgin females from one of these homozygous lines were crossed to virgin males of the other line, nonreciprocally, to derive the tester males and females in the F_1 generation, each of which possessed a standard heterozygous genotype. Thus, the tester flies were heterozygous genome-wide (genetic variation existed within the individual), but devoid of genetic variation across individuals (all flies of a given sex possessed the same genotype). Standard-aged and standard genotype tester flies were continually created via the nonreciprocal cross between the homozygous lines, for each block of the assay.

Following the matings between tester flies, the tester males were discarded, and the tester females were immediately transferred to a vial with a 4-day-old focal male for an ensuing 45-h period. During this time, the focal males could attempt to coerce each once-mated female to re-mate, in which case the focal male's sperm would be placed in competition with those of the tester males, for fertilizations of the tester female's ova. Immediately after this 45-h period, the focal males were transferred to individual vials where they entered a longevity assay, as described above for females.

Simultaneously, tester females were transferred to fresh vials, containing no live yeast (similar to focal female protocol), in which to oviposit for 24 h. Tester females were then discarded, but the ovipositioning vials retained at 25 °C for 12 days, at which point the paternity of the focal males was assigned, by scoring the eye colour phenotype in each of the offspring. Offspring sired by the focal males were wild type (red-eyed), whereas those sired by the tester males possessed brown eyes.

The male assay was run over 12 blocks, with a mean of 84 ± 16 (SE) males per block and 17 ± 0.3 (SE) individuals per treatment level and block. Cases where tester females failed to lay any eggs were discarded. Our main point of interest lay in specifically measuring male sperm competitiveness of the focal males. Hence, we omitted cases in which no red-eyed offspring were produced. Although it is technically possible that focal males had mated in these instances, but failed to achieve any paternity success, this is unlikely given the high second male sperm precedence in the species (80%) (Friberg & Dowling, 2008). When focal males achieved 100% paternity success, we were able to verify whether or not the tester male had achieved an initial mating by examining whether any brown-eyed offspring eclosed from the vial that had been used to house the tester female during her 45-h exposure to the focal male. Cases where no brown-eyed offspring had been produced were omitted from the analysis ($n = 66$, which represents six per cent of the total sperm competitiveness sample size). Finally, we also conducted a binary analysis of male reproductive success [success (i.e. at least one offspring produced) vs. (no success)].

The number of focal males assayed for sperm competitiveness was 1014. The binary analysis of reproductive success contained 1656 data points. The longevity assay based on all males contained 1670 data points. Limiting these analyses to only those males that successfully produced at least one offspring under sperm competition did not qualitatively alter the results. Finally, similarly to female short-term survival, we also assessed short-term survival (to 72 h post-injection) in males, across the treatment levels. This analysis was conducted for a subsample of the data ($n = 763$, six blocks).

Immune trait

The *Drosophila* antimicrobial immune response consists of two main components; the humoral and the cellular response (Hedengren *et al.*, 1999; Hoffmann, 2003). The humoral response involves the production of antimicrobial peptides (AMPs) and other peptides within the fat body which are secreted into the hemolymph, where they fight invading microorganisms (Hoffmann & Reichhart, 2002; Hoffmann, 2003; Meister & Lagueux, 2003; Brennan & Anderson, 2004). In contrast, the cellular immune response involves the *Drosophila* blood cells, the haemocytes, which are of three types: plasmatocytes (90–95% of adult haemocytes), lamellocytes and crystal cells (only found in larvae). Plasmatocytes and lamellocytes are largely responsible for phagocytosis and encapsulation of invading microorganisms, whereas crystal cells are involved in the melanization process (Meister & Lagueux, 2003; Lemaitre & Hoffmann, 2007). In *Drosophila*, only embryos and larvae have haematopoietic organs (i.e. blood forming organs), although it has been suggested that adults may, despite lacking haematopoietic organs, still possess circulating prohaemocytes (blood progenitors) (Crozatier & Meister, 2007; Grigorian *et al.*, 2011). Because we assayed adult flies, we choose to focus on the antimicrobial activity of the hemolymph, as an indicator of the proximate immune responses induced by the immune treatment. This was conducted using a lysozyme-like assay, which provides a measure of how much bacteria an individual's hemolymph can clear in a standard amount of time, hence providing a gauge of the strength of the humoral immune response (Kurtz *et al.*, 2000).

Antimicrobial assay

This assay was used to estimate the general antibacterial activity, by measuring the zone of inhibition around a 3-mm filter paper containing sample fly hemolymph. The protocol was modified from Mohrig and Messner (Mohrig & Messner, 1968) and Wiesner and colleagues (Wiesner *et al.*, 1998). 1.5% Agar was diluted with distilled water to a concentration of 1.5%, autoclaved and incubated in a water bath at 48 °C. Meanwhile, a bacterial solution consisting of *Micrococcus luteus* cells was prepared by mixing freeze-dried bacteria with a 0.2 M potassium phosphate buffer (pH 6.4). One fifty microlitres of streptomycin was added to the bacterial solution, and the resulting solution was mixed with the prepared Agar solution. Six millilitres of this solution was added to 90-mm sterilized petri dishes, which were left to set at 25 °C for 30 min. Plates were then placed in an incubator (33 °C) for 17 h to allow the bacteria to form an even lawn, after which they were placed in the fridge at 4 °C. At the time of assay, 12 sterilized filter papers (3 mm) were evenly distributed across each plate.

Virgin flies were assayed at 24 h post-injection, having been reared identically to those involved in the reproductive assays described above. All flies were surface-washed in 70% ethanol and rinsed with sterile water, prior to hemolymph extraction, to avoid introducing variation in the amount of bacteria an individual fly would have to clear due to surface contamination. After the wash, we decapitated the fly and 'bled' it directly onto a filter paper. The body was then further teased apart (using callipers) onto the filter paper to maximize the amount of hemolymph that was extruded. Pilot experiments had proven this method to

be the most efficient method of extracting workable hemolymph yields. Although we were unable to directly measure the amount of hemolymph extracted, we measured thorax size of each fly to use as a covariate in the subsequent statistical analyses, to account for variation in absolute hemolymph volume across flies.

Each plate contained a negative control consisting of PBS buffer, and a positive control of chicken lysozyme (0.1 mg mL^{-1} chicken lysozyme). Plates were incubated at 33 °C for 24 h. When the plates had completed the incubation, a photograph of each plate was taken under standardized conditions (equal light and distance), for subsequent analysis in ImageJ (http://rsweb.nih.gov/ij/index.html). Each inhibition zone diameter was encircled graphically using the outermost boundary at which the inhibition zone edge was still homogeneous in its appearance (clear and distinct edges), and the diameter was measured to the nearest 0.001 mm, at 33% enlargement. Only one person conducted these measurements, and technical accuracy was confirmed by testing for repeatability across three different measurements of the same zone across a subsample of the data. Repeatability was 99.2%.

Statistical analysis

All statistical analyses were conducted in SAS 9.3 or R 2.15.2 (R Development Core Team, 2012), and Akaike's information criteria (AIC) and log-likelihood tests have been used for model selection throughout the manuscript. Poisson-distributed female fecundity data (i.e. number of eggs on day 4) were overdispersed and zero-inflated. To confirm this, we formally tested whether the data set was zero-inflated by simulating (×1000) the 95% confidence intervals around the number of zeroes expected based on a fully fitted and reduced final Poisson model of the data set, which included an observation-level random factor to account for overdispersion. The results of this simulation confirmed that the data were zero-inflated (CI_{min} = 505, CI_{max} = 645, number of zeros = 713). Based on the above results, data were analysed in R, package glmmADMB (Fedorka & Mousseau, 2007; Skaug *et al.*, 2012), using a zero-inflated negative binomial model, NB1 fit [variance is calculated as ($\phi\mu$) that allowed the zero-count outcomes to be a mixture of structural and sampling zeros (Bolker, 2008; Fournier *et al.*, 2011)]. The model was fitted with immune treatment as a fixed factor; with block, and vial nested within block, as random factors. The best fit model was selected based on AIC fit statistics (zero-inflated models with the Poisson, binomial NB2 [variance = $\mu(1+\mu/k)$], and binomial NB1 families were compared). The original Poisson mixed model containing an observation-level random effect, in the *lme4* package (Bates & Maechler, 2009; Bates *et al.*, 2013), produced qualitatively identical results, but with an inferior model fit (based on residual patterns). Furthermore, we note that an analysis of female fecundity, which excluded 0 values, produced qualitatively similar results to the zero-inflated negative binomial model (immune treatment: $F_{4,167} = 2.71$, $P = 0.0317$). In addition, data on baseline fecundity (egg or no egg) and clutch viability were analysed with the same fixed and random factors as above, but using binary/binomial models in the Glimmix procedure (generalized linear mixed models, SAS 9.3).

Male offensive sperm competitiveness was analysed as a binomial model with a logit link (Proc Glimmix; SAS Institute, Cary, NC, USA), where the number of red-eyed offspring was set as the response variable (only including data points where males had been confirmed to have mated) with the total number of offspring as the binomial denominator. Immune treatment was fitted as a fixed factor, and block as a random effect (males did not share vials, so there was no nested level). Because the data showed significant signs of overdispersion that could not be accounted for by including additional explanatory parameters, we added a multiplicative over-dispersion variable to adjust standard errors and F statistics. Moreover, because variances differed substantially across blocks, we allowed the overdispersion variable to also vary at the level of block (group effect). The data also contained a number of residual outliers, some of which were extreme (three data points had studentized residuals > 3 IQR, representing 0.3% of the data). These outliers were excluded from the analysis to improve model fit (this did not alter the overall results qualitatively, however). Finally, as per female data, the analysis of binary male reproductive success (offspring vs. no offspring) was conducted using a binary model in SAS.

Longevity data for each sex were analysed using a Cox proportional hazard mixed effect model in the *coxme* package of R (Therneau, 2012), with immune treatment as a fixed factor, and block (and for females, vial nested in block) as random factors. Short-term survival (to 72 h) was analysed using a generalized mixed model with a binomial distribution in SAS 9.3.

Antimicrobial activity was analysed in mixed models using a Gaussian distribution. The size of the filter paper (3 mm) was subtracted from the values to facilitate a normal distribution of the data. Moreover, the response variable (inhibition zone, mm) was transformed to the log scale in the statistical analysis to account for skewedness and non-normality. 'Sex' and 'immune treatment' were entered as fixed factors, and 'block' and 'plate identity' nested in 'block' were entered as random factors. Thorax size was centred around the population mean to enable an appropriate estimation of the regression slope and fitted as a covariate in the model. All possible interactions were investigated, and best model fit was selected based on information criteria values (AIC) and residual model fit. We also tested for potential nonlinear polynomial effects. These effects

were never significant and were excluded from the final models.

Results

Short-term survival

The results showed an average post-injection survival of 0.74 ± 0.20 SE in females, with no treatment-mediated differences in survival over the recovery period (GLMM distribution = binary, $F_{4,586} = 0.18$, $P = 0.9487$, $n = 604$). Short-term survival in males was higher than that of females, 0.97 ± 0.006 SE, but, similarly to females, did not change across immune treatments ($F_{4,753} = 1.84$, $P = 0.1186$, $n = 763$).

Reproductive fitness and longevity

Female fecundity was affected by the immune treatment ($\chi^2 = 11.54$, d.f. = 4, $P < 0.0211$; random effects variance components: block = 0.092, vial identity = 0.094), decreasing with increasing immune treatment dose, but plateauing at mid- to higher doses (Fig. 1). There was no effect of immune treatment on whether females laid eggs or not ($F_{4,1527} = 1.78$, $P = 0.1297$), nor was there an effect of treatment on clutch viability (Fig. S1, $F_{4,817} = 1.76$, $P = 0.1342$).

Male sperm competitiveness was affected by the immune treatment ($F_{4,993} = 2.47$, $P = 0.0432$), exhibiting a reduction at the lowest immune treatment dose (0.05%) relative to the control (0%) and relative to the higher doses (Fig. 2). There was no effect of immune treatment on binary male reproductive success ($F_{4,1639} = 1.21$, $P = 0.3053$).

Neither female ($\chi^2 = 4.6984$, d.f. = 4, $P = 0.3197$) nor male ($\chi^2 = 6.2048$, d.f. = 4, $P = 0.1844$) survival was affected by the immune treatment.

Correlations between fitness traits and between fitness and the immune trait were examined across treatment levels ($n = 5$) using simple Pearson's correlation models. None of these correlations were statistically significant, but we note that they were low in power because they are based on only five data points (Fig S2, Table S1a, b).

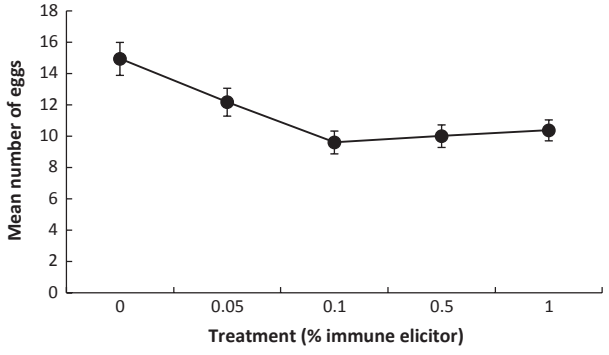

Fig. 1 Female fecundity (mean ± SE) at different concentrations of the immune treatments.

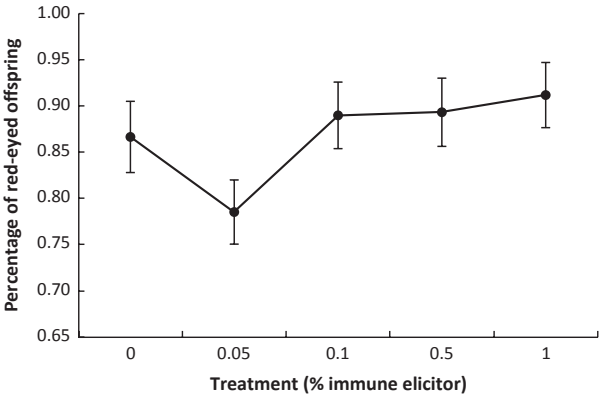

Fig. 2 Male sperm competitiveness (means ± SE) across the immune treatments.

Immune trait

There was no effect of immune treatment ($F_{4,446} = 1.35$, $P = 0.2492$) or sex ($F_{4,366} = 0.01$, $P = 0.9419$) on antimicrobial activity. However, the interaction between sex and treatment suggested a near-significant pattern of stronger dose dependence in male than in female antimicrobial activity ($F_{4,467} = 2.32$, $P = 0.0563$, Fig. 3). There was also a complex relationship between thorax size and treatment, where the magnitude of the slope between thorax size and antimicrobial activity changed across treatments ($F_{4,472} = 2.50$, $P = 0.0420$). However, the interaction between thorax size and sex was not significant ($F_{4,469} = 1.96$, $P = 0.1623$). Due to the relatively low sample size of the sex-specific

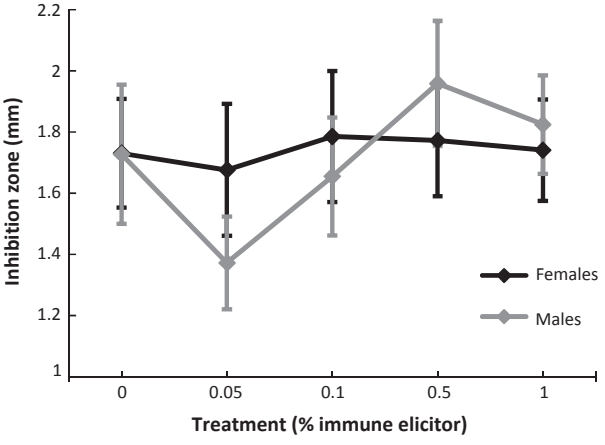

Fig. 3 Antimicrobial activity across treatments and sex (means ± SE).

immune data, we decided to explore the nature of these interactions further, by conducting separate analyses for each sex which enabled the analysis of the treatment-effect as a simple main effect. These analyses confirmed the trend that the immune treatment affected male, but not female, antimicrobial activity (Females: $F_{4,223} = 0.55$, $P = 0.6976$; Males: $F_{4,232} = 3.05$, $P = 0.0179$; however, Tukey's adjusted post hoc testing of all differences ($n = 10$) resulted in no significant differences between injection doses (Table S2). Moreover, the interaction between thorax size and treatment on antimicrobial activity was only significant in males (Females: $F_{4,233} = 0.69$, $P = 0.5988$; Males: $F_{4,234} = 2.62$, $P = 0.0355$).

Discussion

The immune treatment affected components of reproductive success in a dose-dependent, trait-specific and nonlinear manner. Whereas female fecundity decreased with increasing concentrations of the treatment, plateauing at higher doses, male fecundity only responded to the treatment when it was administered at the lowest concentration (0.05%). In males, the life history response was also underpinned by a weak but detectable response at the scale of the proximate immune trait (antimicrobial activity). In contrast, we did not detect a dose-dependent response in antimicrobial activity in females.

The costs to the host upon exposure to a pathogen can be partitioned into the cost of an immediate redeployment of resources to facilitate a rapid and effective immune response, as well as the longer-term costs of sustaining immune system activity against a replicating pathogen (McKean *et al.*, 2008). In our study, we used a design that enabled us to focus on the former effects – those of host redeployment of resources to an immune response. This design enabled us to assess the effects of the immune challenge on the host response, in the absence of any direct effects associated with infection by an actively replicating pathogen. We found negative effects of the immune treatment on certain components of reproductive success in each of the sexes, indicating that modifications to life history investment, caused by the host launching an immediate response to a perceived pathogenic threat, do exist.

The pattern of response of the reproductive traits to increasing doses of the immune treatment differed across the sexes. Generally, the effects were isolated to one component of reproductive success in each sex (fecundity in females, sperm competitiveness in males). Furthermore, the effects only consistently exhibited a downward negative trend in females, across the different doses of immune treatment. The effect of the treatment in activating the immune system was, however, not strong enough to drive any differential effects on survival, in either of the sexes. This held true both for survival in the short-term (i.e. the 72-h period immediately following the treatment and prior to the assay of reproductive success) and the long-term survival (the entire life span of the flies). Thus, while the immune challenge was strong enough to modify investment into reproductive investment in the immediate aftermath of the exposure to the treatment and, hence, represented a putative cost to early life reproductive success (at least in females), individuals were able to moderate their response to the challenge to ensure that it did not threaten their ongoing survival prospects.

Notably, there was a strong trend towards a significant interaction between treatment and sex on the expression of antimicrobial activity, whereby only males exhibited a detectable response at this mechanistic level. The pattern of this response was largely concordant with that observed in the focal male life history trait (sperm competitiveness). This was somewhat surprising because we had expected that decreases in investment at the life history level would be associated with increases in investment at the antimicrobial level if allocation trade-offs were at play, such that increasing doses of the immune challenge would results in increased antimicrobial activity and decreasing sperm competitiveness. Why we found a trend towards concomitant reductions in both antimicrobial and sperm competitiveness expression, only at a low dose of the immune treatment, is worthy of future attention. Note that the data on male life history and immune responses were generated from separate assays, utilizing different individuals, at different time points, hence reinforcing that these dose-specific patterns observed in males, albeit weak, are real and not simply statistical artefacts. Although speculative, this pattern, of positive response of male sperm competitiveness and underlying antimicrobial activity to the higher doses of the immune treatment, is consistent with the possibility of males following a strategy of terminal investment, in which they increase their investment into current reproduction (post-copulatory investment) under increased threat of imminent death (Bateman, 1948; Clutton-Brock, 1984; McKean & Nunney, 2005; Sadd *et al.*, 2006).

In contrast, the lack of a detectable immune response in females might be a consequence of a reallocation of resources away from current reproduction – as illustrated in the dose-dependent effect on fecundity – towards maintaining immunity at constant levels, regardless of immune stress. This result is arguably discordant with the idea of a general sex-specific trade-off hypothesis, in which investment in immunity should be female-biased given that an efficient immune defence is a key to increase life span (Zuk, 1990; Zuk & McKean, 1996; Rolff, 2002; Nunn *et al.*, 2009). However, we cannot rule out the possibility that the absence of a detectable immune response in females is associated with females possessing higher background

levels of immunity than males in general. Under such a scenario, females are likely to be less susceptible to a moderately sized immune challenge, such as the one administered in this study. Evidence for a general sex-difference in immune system levels was found in a recent meta-analysis by Nunn *et al.* (2009). The authors found that mammals displayed clear sex-specific differences in a key immune trait (white blood cell count), whereas the pattern for insects was slightly more inconsistent [sex differences in phenoloxidase (PO) levels, but not in haemocyte count (Nunn *et al.*, 2009)]. A potential caveat with inferring conclusions regarding background levels of immunity in insects from that study, however, is that the authors included studies that contained data based on 'natural' levels of immunity as well as data generated from immune challenged individuals.

It is worth pointing out that we used a commonly harnessed gram-positive bacterium in the media used for testing the antimicrobial activity of the hemolymph, see for example (Ahmed *et al.*, 2002; Jacot *et al.*, 2005; Freitak *et al.*, 2007, 2009; McNamara *et al.*, 2013). Given that flies were injected with gram-negative bacteria, the pathway primarily responding to gram-positive bacteria (Toll) is likely to have been subjected to a weaker up-regulation than the pathway primarily responding to gram-negative bacteria (Imd). Hence, it is possible that we would have observed a stronger response had we used gram-negative bacteria for this assay. We suspect it is unlikely, however, that this would have altered the actual pattern of the response, given that both pathways interact to increase the overall levels of immune-gene induction (in fact, septic injury alone is sufficient to trigger both pathways) (Lemaitre & Hoffmann, 2007). In addition, there are several other mechanisms, apart from the induction of antimicrobial peptides that are involved in the *Drosophila* immune response, hence influencing the overall antimicrobial response (Hoffmann & Reichhart, 2002).

Previous experimental studies that deployed nonreplicating immune challenges to estimate effects at the life history and the immune scale have generated mixed results, finding effects in some, but not all assayed traits. For example, Moret and Schmid-Hempel (2001) found that the immune challenge had no effect on haemocyte number, but did decrease antimicrobial activity and reproductive output, in bumblebees (*Bombus terrestris*). Jacot *et al.* (2005) found a raised antimicrobial activity and proPO activity (proPhenolOxidase activity), but no change in the PO activity, nor any effects on development, after an LPS challenge in crickets (*Gryllus campestris*). McNamara *et al.* (2013) found an increase in antimicrobial activity in larvae of the cotton bollworm moth (*Helicoverpa armigera*) after a challenge with LPS, but not in PO activity, in addition to effects on sperm transfer, development and pupal duration stage, depending on development stage (3rd or 6th instar). Similarly, Robb and Forbes (2006) identified sex-specific differences in immune response in damselflies (*Enallagma boreale* S.), where mature females demonstrated increases in both haemocyte load and antimicrobial activity upon immune challenge, whereas mature males only displayed a heightened haemocyte load. However, both males and females showed a decrease in survival after immune treatment (Robb & Forbes, 2006).

Our results were generated from assays that were conducted under benign and standardized conditions, in which the flies were well nourished and unaffected by density-dependent factors. A number of earlier studies have shown that the effects of an immune challenge are typically more pronounced when the test subjects are under dietary or environmental stress, and in fact, many studies fail to find a response under benign conditions (Rolff *et al.*, 2004; Jacot *et al.*, 2005; McKean & Nunney, 2008; Vale *et al.*, 2011; Simmons, 2012). The fact that we registered a response at both the ultimate and the proximate levels, despite keeping the flies in constant and benign conditions, clearly shows that generating an immune response exerts effects on the expression of other core traits, even in the absence of that added cost of disease, and suggests that these responses are thus costly.

The dose dependence in the expression of the reproductive traits to the immune challenge, extended to both sexes. This result is notable because it highlights the potential discrepancy in conclusions of studies that administer a gradient (i.e. more than two) of doses of an immune challenge, compared with those that only administer a single semi-arbitrarily determined dose that they then compare to a control (Ben-Ami *et al.*, 2010). The significance of this point is clear when considering the male sperm competitiveness responses to immune treatment in our data set, in which the males only demonstrated a negative response at the lowest dose relative to the control. If our treatment had consisted of a dose of 0.05% immune challenge vs. a control dose, our conclusion would be that sperm competitiveness is depressed upon exposure to an immune challenge. However, males subjected to all of the higher concentrations of the immune treatment (> 0.05%) enjoyed sperm competitiveness typical to that of the control males, complicating a simple interpretation. Similarly, this response was also discernable at the antimicrobial level in males. In light of this point, few previous studies that have used nonreplicating immune challenges such as LPS, PGN or heat-killed pathogens, to address an eco-immunological question, administered more than one dose (on occasion two) and a control, hence administering a dose gradient (Imler *et al.*, 2000; Ahmed *et al.*, 2002; Sadd & Schmid-Hempel, 2007). It is worth noting that the results of single-dose studies are mixed and inconsistent, and we suggest that

the nonlinear nature of the responses, as those shown here along the dose gradient, might help to account for some of the incongruence between studies that have previously tested the effects of an immune challenge on immune trait expression and other life history fitness traits (Lazzaro & Little, 2009).

Conclusion

We detected dose-dependent effects of an immune treatment on reproductive trait expression in both males and females. Because we used a nonreplicating immune elicitor rather than a replicating pathogen as the immune challenge, we can directly surmise that these effects are attributable to effects of up-regulating the immune system *per se* rather than any indirect costs associated with ongoing pathogenic infection (e.g. disease symptoms brought about by competition with a metabolically active and replicating pathogen). Furthermore, we found a sex-dependent signature of immune treatment at the proximate level, as indicated by the dose-dependent response trend in antimicrobial activity. However, we could not detect an effect of immune treatment on survival. In sum, our results suggest that the immediate investment made by individuals to deploying their immune arsenal, at the time of detecting a perceived immune challenge, can affect life history investment, but these effects are likely to abate in the days following the challenge, and with individuals fully recovering from the challenge. Furthermore, the study highlights that in accordance with findings using live pathogens to challenge individuals, the links between proximate and ultimate responses to immune challenge are far from simple.

Acknowledgments

We thank Katherine Sutton for laboratory work. We also thank Florencia Camus and Travis Johnson for advice and assistance during development of immunological assays. The study was funded by the Australian Research Council (Australian Postdoctoral Fellowship and Discovery Project, DP110104965) and Monash University Margaret Clayton – Women in Research Postdoctoral Fellowship, both to MN.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Clutch viability, across immune treatments, as gauged by the proportion eggs producing offspring (± SE).

Figure S2 Means (± SE) per immune treatment level for female fecundity (mean) and antimicrobial activity (mean ± SE).

Table S1 (a) Correlations across doses (group averages across immune treatments, $n = 5$), for female traits. Pearson correlation coefficient in upper part of the cell, and *P*-value in lower part of the cell. (b) Correlation across treatments (group averages across immune treatments, $n = 5$), for male traits. Pearson correlation coefficient in upper part of the cell, and *P*-value in lower part of the cell.

Table S2 Tukey–Kramer adjusted pairwise differences between treatment (% immune elicitor) doses in males. Estimates are based on Least square means.

Data deposited at Dryad: doi:10.5061/dryad.63qp0

Received 28 November 2013; revised 28 January 2014; accepted 25 February 2014