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Reciprocal costs of infection and reproduction in *Drosophila melanogaster*

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Trade-offs occur when an organism has to allocate limited resources to multiple biological processes. How organisms allocate their resources and whether one trait gets priority over another is poorly understood. Prior work has shown that reproductive investment reduces the capacity of *Drosophila melanogaster* to mount an effective immune response against subsequent bacterial infection. However, it has not been tested whether the observed trade-off was unidirectional with reproductive fitness given primacy over immunity, or whether it might also occur in the reciprocal direction with an active prior immune response reducing reproductive output. In this work, we delivered bacterial infection to female *D. melanogaster* prior to mating and tested whether reproductive capacity became reduced. We found that infected females produced the same number of eggs as uninfected females, but the eggs from infected females exhibited lower survivorship to adulthood. Additionally, we found that mating destabilizes chronic bacterial infections, stimulating additional host death and increasing variance in pathogen burden. Together, our results suggest the cost of reproduction and infection in *Drosophila* females is reciprocal, regardless of the order in which they occur.

1. Introduction

Biological processes are energetically demanding and the resources available to organisms are often limited. Unequal allocation of resources into different processes can lead to trade-offs where higher investment in one trait leads to a detrimental effect in another trait [1–4]. Trade-offs between reproduction and immunity have been observed in multiple systems [5–11]. In *Drosophila melanogaster*, reproductive investment limits the female ability to respond to pathogenic bacterial infections, resulting in a higher pathogen burden and reduced survival of infection in reproductively active females [7,12–16]. This reduced resistance to infection can be seen within 2.5 h of mating and persists for at least 10 days post-mating. Post-mating reduction in immune capacity is dependent on the presence of the germline [13,17], but it has remained unclear whether this effect is unidirectional with reproductive fitness given primacy or whether prior infection may also constrain reproductive output.

Drosophila produces egg yolk proteins in the fat body, which is the tissue also responsible for systemic immune response [18–20]. Mating and insemination stimulate female *D. melanogaster* to lay eggs, which in turn stimulates renewed investment in oocyte production [21]. A recent study by Gupta *et al*. suggested that post-mating susceptibility to infection arises due to a failure of the fat body to meet the demands of the immune system when it is already actively engaged in reproductive provisioning [22]. However, that study (and ones before it) did not test whether the demands of the immune response reciprocally limit reproductive fitness through decreased egg number or quality. A previous study by Howick & Lazzaro showed a transient reduction in egg production by *D. melanogaster* females immediately following experimental bacterial infection, with a return to baseline rates of

egg production within 72 h despite continued infection [23]. A second study by Kutzer & Armitage found a sustained decrease in egg production through 72 h after infection with different bacterial pathogens, but with no decrease in the quality of those eggs as a consequence of infection [24]. However, in those *Drosophila* experiments, infection was delivered subsequent to or simultaneous with mating, so there was no opportunity to test the effect of sustained immunological activity on reproductive fitness. An immune challenge prior to mating in dung beetles, *Euoniticellus intermedius*, resulted in smaller brood size compared to control females [25]. Similarly, sustained immunopathology against a cestode tapeworm is associated with reduced reproductive success in males and reduced ovary mass in female threespine stickleback fish [26].

In this study, we test whether reproductive fitness is impacted by a bacterial infection sustained prior to active reproduction in *D. melanogaster*. Specifically, we ask whether flies that are carrying an established infection prior to mating (i) exhibit an increase in the rate of infection-induced mortality subsequent to mating relative to infected flies that remain unmated, (ii) have reduced egg production, and/or (iii) produce lower quality eggs than flies that were not infected prior to mating. We found that mating destabilizes sustained bacterial load in flies that have survived infection, triggering additional mortality and leading to a reduction in the quality of eggs produced.

2. Methods

(a) Fly strain and husbandry

We used Canton S flies for all experiments unless stated otherwise. The flies were reared at room temperature (22–23°C) with a 12 : 12 h light : dark cycle. Groups of 10 male and 10 female flies were reared and allowed to lay eggs in plastic vials containing cornmeal–sucrose medium (weight by volume in 1 l of H_2O: 0.7% agar, 6% Brewer's yeast, 6% cornmeal and 4% sucrose; 100 g Tegosept in 26.5 ml of 95% ethanol and 12 ml of 0.04% phosphoric acid and 0.4% propionic acid mixture added to inhibit microbial growth) [17]. Larval density was maintained at approximately 150 larvae per food vial. Adult offspring were sorted by sex within 8 h of emergence from the pupal case and were maintained separately before mating.

(b) Bacterial infection

Adult female *D. melanogaster* were infected with a Gram-negative bacterium *Providencia rettgeri* [12] at 5–7 days after eclosion from the pupal case. At this age, all tissues are developmentally adult, including the fat body [27]. To prepare the bacteria, a single colony of *P. rettgeri* was used to inoculate an overnight culture grown in Luria–Bertani broth at 37°C with shaking. The next morning, a subculture was inoculated with 250 µl of overnight culture in fresh LB and grown to log phase. The log culture was diluted to $A_{600} = 1.0$ and was then used to infect flies. The flies were infected under light CO_2 anaesthesia and infection was delivered by pricking the sternopleural region of the thorax with a 0.15 mm needle dipped in the bacterial culture, as explained in [28]. Infected females were allowed to recover in food vials. Injury control females were pricked with sterile needles and uninfected controls were simply subjected to CO_2 anaesthesia.

(c) Experimental design

Since we wanted to test the effect of the timing of mating relative to infection, we divided flies into four main treatment groups: (i) M24I, flies allowed to mate 24 h prior to infection; (ii) I0M, flies infected and immediately mated; (iii) I24M, flies were infected and mated 24 h later; (iv) I120M, flies infected then mated 5 days later. We used multiple control groups in our experimental design as well: (i) M24−, mated, held for 24 h, then given sterile wound to act as controls for the M24I files; (ii) −0M, given a sterile wound and immediately mated as a control for the effects of infection on the I0M and I24M flies; (iii) M0, females mated on the day of infection but not wounded as a control for the combined effects of injury plus infection in the I0M treatment (the M0 flies can also be considered as an injury control for the −0M treatment); (iv) −120M, given a sterile wound and then mated 5 days later as a control for the I120M flies; and (v) unmated virgin flies given a sterile wound. All flies used in the experiments were aged 5–7 days post-eclosion at the time of bacterial infection or sterile wounding. We confirmed successful infection by measuring pathogen burden (realized inoculation) in a subset of 5–10 flies from each treatment group immediately after bacterial injection ($t = 0$) using the method described below. Infection was successfully delivered to every pricked fly. Independent sets of flies were used for the survival assay, the egg and offspring count assay and the bacterial load assay.

(d) Survival assay

Post-infection survival was measured in three independent experimental blocks, each consisting of 5–7 vials per treatment, with five females housed within each vial. Survivorship was recorded for 10 days post-infection, with females transferred to new food vials every third day. If the flies were to be mated, the flies were transferred to new food vials on the day of the mating and the males were removed 24 h later.

(e) Egg and offspring count assay

Eggs and offspring were counted in three independent experimental blocks for all treatment and control conditions except for unmated virgin flies, which produce very few eggs, none of which hatch. In order to ensure successful mating, five female flies

were housed with eight males for 24 h and then the males were removed. Females were transferred to new vials every 24 h for 5 consecutive days and eggs were counted manually under a light microscope immediately following transfer. The vials were then left at room temperature until adult offspring emerged. Each replicate block included 3–5 vials of females per treatment. Adult offspring were transferred to empty vials 24 h after the emergence of the first adult offspring and frozen until they were counted. Our adult offspring counts could be underestimations if some viable pupae failed to emerge during the collection window.

(f) Bacterial load assay

We quantified bacterial load in chronically infected females to test whether mating has any impact on previously established pathogen burden (and also to quantify the initial inoculation, as mentioned above). Individual females were anaesthetized with CO_2 and homogenized in 500 µl sterile 1 × phosphate-buffered saline (PBS). Fifty microlitres of the homogenate were plated on an LB agar plate using a WASP2 spiral plater (Microbiology International). The plates were incubated overnight at 37°C and the bacterial colonies that developed were counted using the ProtoCOL plate counting system (Microbiology International). There were 13 plates that had colony densities too high to be resolved by the counting software. Those plates were assigned the highest measurable value (112 500 colony forming units (CFUs) per fly) observed across all three experimental blocks.

(g) Statistical analysis

All statistical analyses were performed using R (v. 4.1.1) [29]. Survival analysis was carried out using the 'survival' package (v. 3.2-11) [30], for which we used a mixed effect Cox proportional hazards model with treatment as a fixed effect and experimental block as a random effect. In order to compare survival between treatment groups, we used the same Cox proportional hazard model within the emmeans() function from the package emmeans (v1.8.3), which uses Tukey's HSD for *p*-value corrections.

$$\text{Surv(Hours, Censor)} \sim \text{Treatment} + \text{Block}$$

For bacterial load, we first calculated CFU per fly, which was then natural log transformed for subsequent analysis. Since some of our CFU per fly values were zero, we added one to all the values prior to the log transformation. We first compared two mixed effects models, one with block as a random effect and another without block to test the effect of the experimental block using the lme4 package in R. We treated mating status as fixed effects in both models.

$$\text{ln\_count} \sim \text{Block} + \text{Mating status}$$

$$\text{ln\_count} \sim \text{Mating status}$$

Since we did not see a significant effect of Block (electronic supplementary material, table S1), we pooled the data from all three blocks together and since the transformed data were not normally distributed, we used Levene's test to compare the variance between treatments. Because treatments did not have equal variances, we performed Welch's *t*-test to compare between means of mated and unmated treatments. Similarly, Fisher's exact test was used to compare between the number of dead individuals between two treatment groups.

To estimate per-fly egg and offspring count, the total number of eggs and offspring was divided by the total number of females in each treatment group within each experimental block. Egg-to-offspring ratio was calculated by dividing the total number of offspring by the total number of eggs for each vial in each experimental block. We then compared two mixed effect models (i) with block as a random effect and (ii) without block to test the effect of block on our data using the lme4 package in R. In both models, treatment groups were used as fixed effects.

$$\text{Egg to offspring ratio} \sim \text{Treatment} + \text{Block}$$

$$\text{Egg to offspring ratio} \sim \text{Treatment}$$

Since we did not observe an effect of block (electronic supplementary material, table S2), we carried out pairwise differences between treatments using the pairwise *t*-test() function in R with Bonferroni correction.

3. Results

(a) Mating triggers mortality in infected females

In order to test whether mating would affect the probability of surviving a previously established bacterial infection, we infected *D. melanogaster* females with *P. rettgeri* and allowed them to mate at either 0, 24 or 120 h after infection (I0M, I24M and I120M). We also included a set of females that were mated 24 h prior to infection (M24I) as was done in previous studies (e.g. [7,12–15]). We tracked survival for 10 days after infection. As expected, infection significantly reduced the probability of survival in all mating treatments (figure 1A). Interestingly, we found that females who were already carrying a previously established infection exhibited increased death within 24–48 h after mating (figure 1A, electronic supplementary material, table S3), even in the I120M group where the infection was delivered 5 days before mating and was expected to be chronic and stable [23,31].

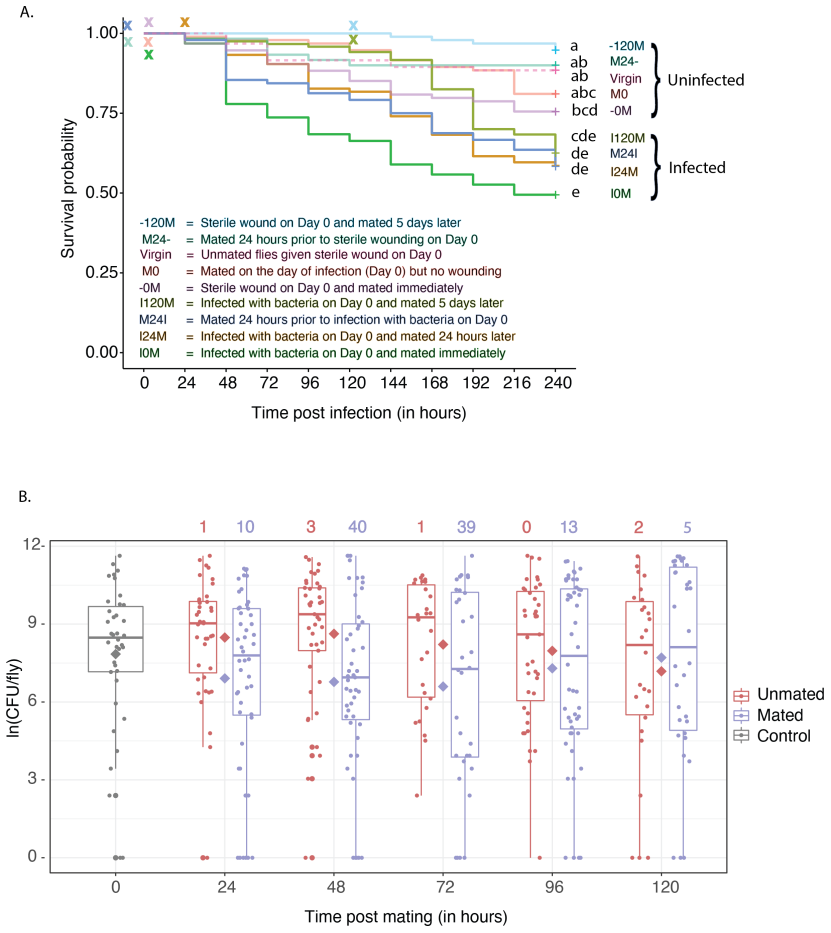

Figure 1. Effects of mating on survival and chronic bacterial load. (A) Mating affects survival in infected females. Flies were infected with *P. rettgeri* (A_{600} = 1.0) by septic pinprick [28]. Results from three experimental blocks were combined for graphical representation. Treatments with different letter signs are significantly different from each other (Tukey's test). 'X' indicates the timing of mating for each treatment group, which is relative to the timing of infection as indicated on the *x*-axis. (B) Mating destabilizes chronic bacterial load, resulting both in the clearance of infection and loss of infection control. Bacterial load was measured from individual flies subsampled at 5 days after mating, where mating happened 5 days after infection. The numbers at the top of the plots indicate the number of dead individuals observed on each day. Diamond shapes represent average bacterial load per treatment group for each time point. Control refers to chronically infected flies prior to being divided into mated/unmated groups.

(b) Mating destabilizes chronic bacterial load

Providencia rettgeri infections have previously been suggested to stabilize after 3 days post-infection with a constant chronic bacterial burden and no additional mortality [23,31], so it was surprising to us that mating at 5 days post-infection stimulated additional death. We therefore performed an independent experiment where we gave flies a single infection and allowed it to establish for 5 days [23,31], then mated the surviving flies on the fifth day after infection (I120M). We measured bacterial load daily in subsamples of the females for an additional 5 days post-mating to test whether the chronic bacterial burden remained stable as previously reported [23,31,32]. We found a significant effect of mating on bacterial load (β = 1.1096, s.e. = 0.3271, d.f. = 377.1892, t = 3.392, $p < 0.001$). In addition, we found a significantly higher variance in bacterial load of mated individuals compared to unmated individuals (Levene's Test, d.f. = 1, F = 16.907, $p < 0.001$; figure 1B), indicating that mating destabilizes the chronic bacterial load. We also found that mating stimulated significant additional death in chronically infected females (Fisher's exact test; $p < 0.001$; electronic supplementary material, table S4). Interestingly, we also observed that some mated individuals were able to clear the pathogen completely following mating (electronic supplementary material, table S5). Previous work has shown that the individuals with the highest bacterial loads are the ones that die from their infections [32]. It therefore seems likely that the variance in bacterial load of mated-post-infection females may be larger than we observe, since individuals that died were removed from the experiment and not measured for bacterial load. Across the entire data set, mean chronic bacterial load became lower in mated females compared to unmated females (Welch's t-test, $p < 0.001$), presumably due to the combined effects of death (and hence removal from the assay) of high-load individuals and appearance of individuals who cleared their chronic infections, both of which occurred disproportionately in the mated condition.

(c) No correlation between reproductive output and bacterial load

To test whether there was a trade-off between severity of infection and reproductive output, we counted the number of eggs laid for 24 h after mating by individual 120 M females and then measured the pathogen burden of these females at the end of the 24

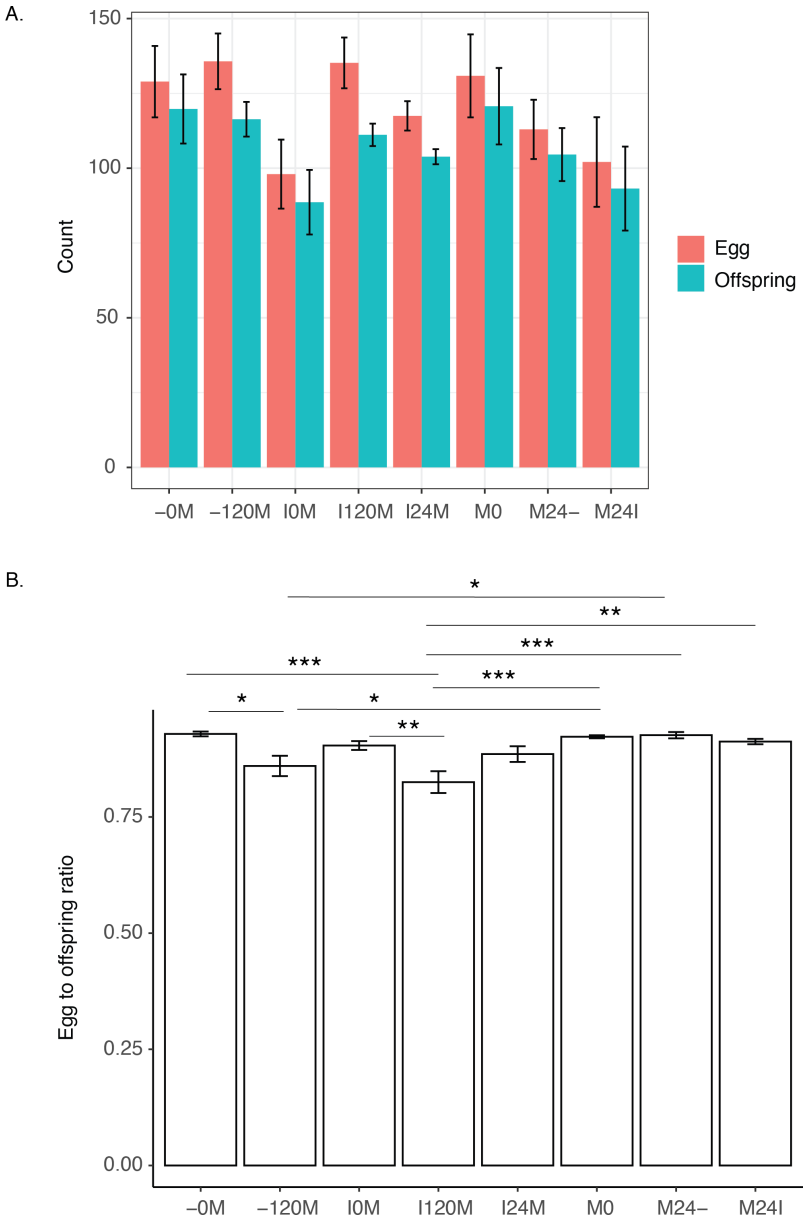

Figure 2. Egg-to-adult survival is reduced in chronically infected females. Error bars represent standard error across three experimental blocks. (A) No significant difference was observed in either egg (electronic supplementary material, table S7) or offspring count across conditions (electronic supplementary material, table S8). (B) Egg-to-offspring ratio was significantly lower in I120M females than in all other treatments except −120 M females. Asterisks indicate *p*-values obtained from pairwise *t*-test that remain significant after Bonferroni correction (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). −0M: sterile wound on day 0 and mated immediately, −120M: sterile wound on day 0 and mated 5 days later, I0M: infected with bacteria on day 0 and mated immediately, I120M: infected with bacteria on day 0 and mated 5 days later, I24M: infected with bacteria on day 0 and mated 24 h later, M0: mated on day of infection (day 0) but no wounding, M24−: mated 24 h prior to sterile wounding on day 0, M24I: mated 24 h prior to infection with bacteria on day 0.

h period. We found no correlation between pathogen burden and egg production ($R^2 = -0.02$, $p = 0.84$; electronic supplementary material, figure S1). We also tested whether there was a correlation between the number of eggs laid and time to death in chronically infected (I120M) females. We found that the daily number of eggs laid declined over the 5 days of observation and that females lay fewer eggs immediately before they die, but that egg production is not otherwise correlated with ultimate duration of survival (electronic supplementary material, figure S2).

(d) Reduced egg-to-adult survival in progeny of chronic-infected females

Finally, we tested whether the severity of chronic bacterial burden is correlated with the quality of the eggs that are produced by looking at the rate of adult emergence from eggs produced by uninfected versus infected females. We observed no correlation between the severity of pathogen burden and egg-to-offspring ratio ($R^2 = 0.036$, $p = 0.72$; electronic supplementary material, figure S3). Interestingly, we found that the females that were chronically infected (I120M) had a significantly lower egg-to-adult survival measured in terms of egg-to-offspring ratio than M24I (pairwise *t*-test with Bonferroni correction, $p = 0.0041$) and I0M (pairwise *t*-test with Bonferroni correction, $p = 0.0015$) females. Egg-to-offspring ratio was not significantly different between I120M and I24M (electronic supplementary material, table S6), suggesting prior infection reduced egg-to-adult survival in

both of these treatments. The egg-to-offspring ratio was lowest in I120M females (figure 2; electronic supplementary material, figure S4), which could suggest that chronic infection may have a cumulative cost, and we observed a consistent reduction in the number of adult offspring emerging from eggs produced by chronically infected females relative to females of the other infected classes regardless of the total number of eggs produced (electronic supplementary material, figure S5). However, the egg-to-adult ratio for chronically infected females is not significantly different than that of age-matched uninfected females (figure 2), suggesting that female age could also be an important driver of reduced egg-to-adult survival.

4. Discussion

Trade-offs occur when an organism has to allocate limited resources to energetically demanding processes such as reproduction and immunity. These trade-offs can be more obvious in organisms where a single tissue acts as an energy reserve [22]. Reproduction and immunity trade-offs have been observed in multiple taxa with mated individuals typically demonstrating poorer immune response compared to that of unmated individuals [5,6,8]. These studies have primarily focused on the effect of infection on individuals that have already started investing in reproduction, and there is less understanding of whether control of a pre-reproductive infection would be disrupted or if immunity would maintain priority over reproduction if infection was established first. Here, we allowed *D. melanogaster* females to mate at different time intervals following infection and tested whether infection prior to mating reduced reproductive fitness.

A study by Short *et al*. [13] showed no difference in survival of infection or pathogen burden between unmated and mated *D. melanogaster* in the absence of a germline, whereas egg-producing females had a higher bacterial load and lower survival, and a study by Howick *et al.* [23] showed a transient reduction in egg production following infection. These results suggest egg production is a costly process in relation to immunity. Here, we did not observe any difference in egg output of infected females compared with uninfected females, regardless of the time between infection and mating. We also found no correlation between the severity of infection and reproductive output measured either as egg count or as egg-to-adult survival. However, mating increased the probability of death from infection, regardless of whether mating or infection occurred first, suggesting joint cost of reproductive investment and infection. Furthermore, when mating and infection occur nearly synchronously (our I0M treatment), the female suffers most severely in both reproductive output and immune defence (figures 1 and 2), suggesting that the combination can be overwhelming when there is no opportunity for the fly to make physiological or behavioural adjustments (e.g. altered feeding). A recent study showed that pre-existing infection does not affect pre-copulatory behaviour of *Drosophila* and infected females remain sexually receptive [33]. Our findings suggest that the post-copulatory responses in *Drosophila* females could also be preserved, and that reproduction may be prioritized over immunity.

Unexpectedly, we found that mating destabilizes chronic bacterial infection, and that some females clear the infection completely while others succumb to the infection. The difference was profound with more than 40% of the chronically infected flies dying after mating compared with less than 4% death in chronically infected flies that remained unmated. Chronic infections are energetically costly and have been shown to deplete energy stores [31,34–36]. It is possible that our chronically infected flies were already limited in their energy reserves by the time they were allowed to mate. Activation of egg production following mating may have compromised the sustained immune response required to maintain control of infection, leading to increased bacterial load and eventual death in some individuals.

The observation of females that completely clear their infections after mating is even more surprising. Chronically infected *D. melanogaster* maintains upregulated expression of genes encoding antimicrobial peptides [31]. Mating has also been shown to upregulate the female immune system [37–41]. This mating-induced upregulation is small relative to infection-induced upregulation [42] and is thought to occur primarily in the reproductive tissue [6,41], so it has been unclear whether it makes a substantive contribution to defence against infection [13,42]. However, it is possible that mating-induced activation of the immune response combines with the maintained response to the chronic infection to enable clearance. Non-exclusively, physiological changes in the female associated with active reproduction might make the fly less hospitable to the infecting bacterium or augment the host immune response. Determining the exact mechanisms for bacterial escape to host lethality or host eradication of a chronic infection will require future study.

Overall, our data reaffirm the relationship between reproductive capacity and immune defence. Where previous studies in *D. melanogaster* have shown that prior mating compromises defence against subsequent infection, here we show that prior establishment of infection also compromises egg quality and realized fecundity. Thus, reproduction and immunity appear to be mutually limiting as a function of physiological constraints.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The data and code associated with this manuscript is available from the Dryad Digital Repository [43].

Supplementary material is available online [44].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. K.A.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; B.P.L.: conceptualization, funding acquisition, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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