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Investigation

Trade-off between antibacterial immune defense and oogenesis progression in female *Drosophila melanogaster*

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Trade-offs between reproduction and immunity are common in animals, potentially due to preferential allocation of limiting resources. In *Drosophila melanogaster*, mating stimulates egg production but also triggers a rapid and persistent decrease in female immune defense. Proteins essential for both processes are produced in fat body tissue, which may result in competition for cellular resources that could drive a functional trade-off between reproduction and immune defense. We predicted that arrest of oogenesis prior to egg provisioning would alleviate postmating immune suppression because cellular stress would be relieved, but that postmating immune suppression would be observed in genotypes that fully provision eggs even if fertility is compromised. In the present study, we test these predictions by evaluating postmating immune competence in mated *D. melanogaster* mutants that arrest oogenesis either prior to, or subsequent to, vitellogenesis. Consistent with our prediction, we find that mated female immune defense is maintained when egg development is arrested prior to vitellogenesis. We find that progression through the vitellogenic stages of oogenesis results in postmating immune suppression, except in the case of a mutant with an egg-retention phenotype, where we infer that the failure to lay eggs results in feedback that inhibits subsequent egg development. We additionally show that elimination of yolk protein synthesis in the fat body and follicle cells of the ovary partially restores female immune capacity. Nevertheless, females that lack *yolk protein* genes still experience partially reduced immune capacity after mating, suggesting that other reproductive demands also suppress immune defense.

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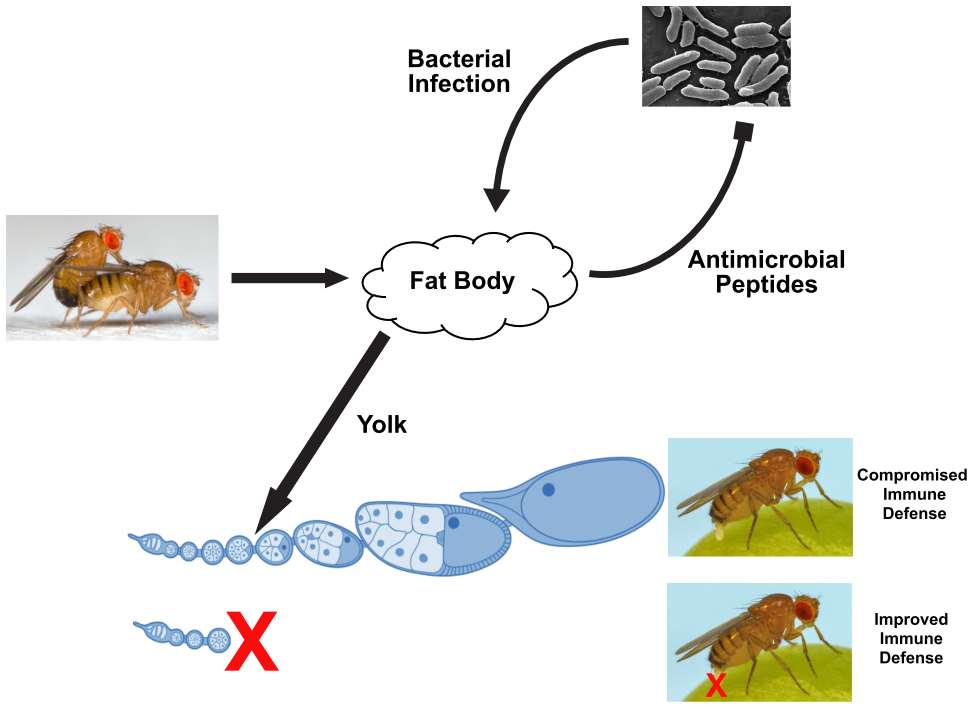
Graphical Abstract



Keywords: mating; reproduction; immune defense; life history; trade-off; yolk protein; antimicrobial peptides; *Drosophila*

Introduction

In many animal species, investment in reproduction can come at the expense of immune defense (Norris and Evans 2000; Martin et al. 2008; Abrams and Miller 2011; Schwenke et al. 2016). As predicted by life history theory, a physiological trade-off can occur if two energetically demanding traits depend on a limited resource or shared physiological constraint and mechanisms of preferential resource allocation direct limited resources toward one trait at the expense of another (Sheldon and Verhulst 1996; Stearns 2000; Schmid-Hempel 2003; Schwenke et al. 2016). However, identifying the limiting resource(s) and understanding the mechanisms controlling preferential resource allocation remains challenging.

In *Drosophila melanogaster*, mating and receipt of seminal fluid proteins increases egg production, but also triggers a rapid and persistent decrease in female defense against systemic bacterial infection. When systemically infected with pathogenic bacteria, mated females survive at lower proportions, have higher pathogen loads, and express fewer antimicrobial peptides (AMPs) than unmated females (Liu and Kubli 2003; Fedorka et al. 2007; Short and Lazzaro 2010; Short et al. 2012; Schwenke and Lazzaro 2017; Gordon et al. 2022; Shianiou et al. 2023). Postmating immune suppression in wild-type *D. melanogaster* persists for at least 2 wk after mating (Gordon et al. 2022).

Two previous studies investigated how investment in egg production after mating affects immune defense in *D. melanogaster*. Fedorka et al. (2007) showed that mated ovo^{D1} females that arrest early in egg development (stage 4) experienced a transient susceptibility to infection by *Pseudomonas aeruginosa* at 3 h after mating but that immune defense was normal by 27 h after mating. Short et al., (2012) showed that daughters of *tudor* females, which have no germline stem cells, maintain immune defense after mating. These studies suggested a physiological trade-off between egg production and successful immune defense.

D. melanogaster oogenesis is defined by 14 sequential, yet distinct stages (reviewed in King 1970; de Cuevas 2015; McLaughlin and Bratu 2015; Hinnant et al. 2020) (Fig. 1). Each of the two ovaries contains 15 to 20 ovarioles held together by a common ovarian sheath. Each ovariole forms an assembly line of oogenesis: germline stem cells divide within the germarium, giving rise to 15 nurse cells and one oocyte, which then grows within the egg chamber before ovulation through the oviduct. Although many of the contents of the oocyte are deposited by the nurse cells during growth, oocyte development is supported by somatic follicle cells, through which molecules made in maternal tissues beyond the ovary are transferred to the oocyte. For example, vitellogenesis occurs at stages 8 to 10 of oogenesis, when yolk proteins are incorporated into the developing oocyte. Yolk proteins are lipoproteins that are abundantly produced in the female fat body and follicle cells (Gavin and Williamson 1976; Brennan et al. 1982; Isaac and Bownes 1982; Minoo and Postlethwait 1985; Bownes and Blair 1986). After production by the fat body, the yolk proteins are secreted via the hemolymph to the follicle cells where they are internalized through receptor-mediated endocytosis (vitellogenesis) via the receptor Yolkless (Giorgi 1979; Sappington et al. 1996). Germline stem cell divisions continue through adulthood, allowing for continuous, abundant egg production. As such, making eggs requires extensive production of proteins including yolk proteins and chorion membrane proteins, as well as lipids, RNAs, and ribosomes, all of which must be deposited into the oocyte (reviewed in King 1970; Kühnlein 2012; Breznak et al. 2023).

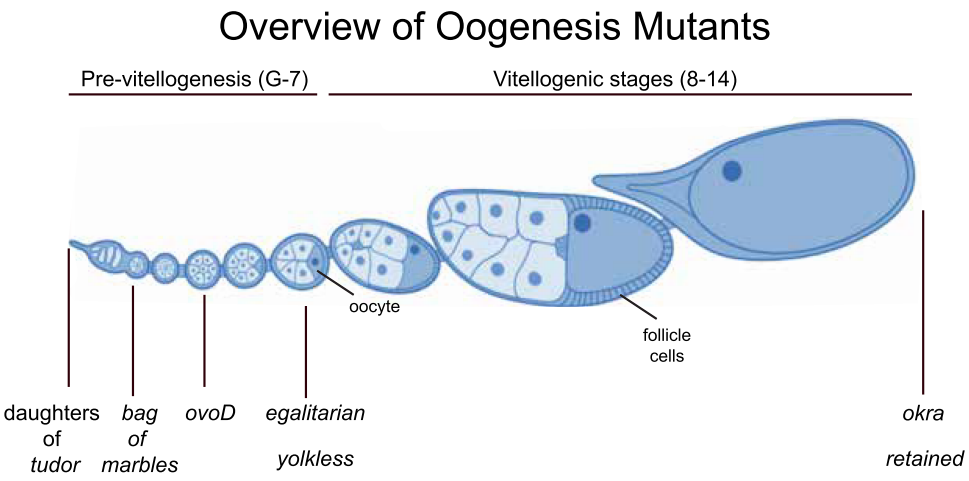


Fig. 1. Illustration of the arrest points of all mutants, both in the previtellogenic and vitellogenic stages of oogenesis.

Mounting an immune defense is also energetically demanding and requires rapid and intense production of immune effector molecules, including AMPs (Lemaitre and Hoffmann 2007; Buchon et al. 2014; Duneau et al. 2017). During a systemic infection, AMPs are primarily produced and secreted from the fat body (reviewed in Lemaitre and Hoffmann 2007; Arrese and Soulages 2010; Buchon et al. 2014). The demand of producing AMPs places more stress on the fat bodies of mated female *D. melanogaster* than of unmated females, potentially because it is layered on top of the investment in yolk production in mated females (Gupta et al 2022). Furthermore, both infection and mating cause shifts in lipid and carbohydrate metabolism in the fat body and other tissues (reviewed in Bland 2023; Darby and Lazzaro 2023). The centrality of the fat body for oogenesis, systemic immune responses, and metabolic control positions this tissue to mediate trade-offs among these processes.

We hypothesized that direct resource allocation to oogenesis, triggered by mating, limits female immune defense. We identified vitellogenesis (stages 8 to 10 of oogenesis) as a likely critical threshold for the trade-off between reproduction and immunity for two reasons. First, previous study showed that oogenesis will arrest prior to vitellogenesis under stressful environmental or nutritional conditions, suggesting that this process requires significant energy stores (Drummond-Barbosa and Spradling 2001; Terashima and Bownes 2004). Second, vitellogenesis and expression of the three *yolk protein* genes in the fat body and follicle cells is stimulated by an increase in juvenile hormone (JH) signaling after mating (Bownes 1989; Soller et al. 1999), and JH is also known to suppress immune defense (Flatt et al. 2008; Schwenke and Lazzaro 2017).

Under our hypothesis, we predicted that mutants which arrest before vitellogenesis would maintain immune defense after mating. We first confirmed previous observations that immune defense is maintained after mating in ovo^{D1} mutants (Fedorka et al. 2007) and germlineless daughters of *tudor* females (Short et al. 2012), both of which arrest oocyte development prior to vitellogenesis (Boswell and Mahowald 1985; Oliver et al. 1987). We then selected two additional mutants that arrest oogenesis previtellogenesis: *bag of marbles* and *egalitarian* (Fig. 1; Supplementary Table 1). *Bag of marbles* (*bam*) females overproliferate their germline stem cells and never differentiate an oocyte, resulting in sterility and a lack of vitellogenesis (McKearin and Spradling 1990; Bubnell et al. 2022). Females with mutations in *egalitarian* (*egal*) do not differentiate an oocyte or accumulate yolk proteins (Carpenter 1994).

Under our hypothesis of resource allocation as a direct cost, we also predicted that loss of yolk proteins would alleviate the translational demands on the fat bodies of mated females, allowing for increased immune defense. To test this, we assessed defense against bacterial infections by *yolk protein* mutants, which have reduced fertility (Tanaka et al. 2021) (Fig. 1; Supplementary Table 1). We additionally tested defense in *yolkless* mutants, which produce yolk proteins but cannot import them into developing oocytes, resulting in sterility (DiMario and Mahowald 1987).

Finally, we predicted that mutants whose egg development progresses through vitellogenesis would experience reduced immune defense. We tested the defense against bacterial infection by two mutants with egg-laying defects that arise postvitellogenesis: *okra* and *retained*. *Okra* females complete oogenesis and vitellogenesis but produce ventralized embryos that cannot hatch (Ghabrial et al. 1998) (Fig. 1; Supplementary Table 1). Females with mutations in *retained* complete oogenesis but do not oviposit (Schüpbach and Wieschaus 1991).

In line with our predictions, we found that all mutants that arrest oogenesis prior to vitellogenesis maintained their immune defense after mating. We additionally found that *yolk protein* mutants exhibited improved immune defense relative to controls. However, mated *yolk protein* mutants still experienced some reduction in immune defense relative to unmated *yolk protein* mutants, suggesting that other postmating changes in female physiology can also limit defense. As we predicted for mutants that complete vitellogenesis, we observed a postmating reduction in immune defense in *okra* mutant females. However, *retained* mutant females maintained their immune defense after mating, potentially suggesting a feedback dynamic that influences the reproduction-immunity trade-off. Taken together, our results broadly support the hypothesis that vitellogenesis and sustained egg production pose a direct cost that limits immune defense in *D. melanogaster*.

Methods

Fly husbandry

All flies were raised on cornmeal-sucrose medium (weight by volume in 1 L of H_2O: 0.7% agar, 6% Brewer's yeast, 6% cornmeal, and 4% sucrose with 26.5 mL of 100 g Tegosept in 95% ethanol and 12 mL mixture of 0.04% phosphoric acid and 0.4% propionic acid to inhibit microbial growth). All flies were kept in a 25 °C incubator on a 12:12 h light:dark cycle.

Genotypes

Full genotypes and stock numbers for all lines used are given in Supplementary Table 1. In brief, we assayed the immune defense of four previtellogenesis mutants (daughters of *tudor*[1], *ovo*^{D1}, *bag of marbles* [*bam*$^{null\text{-}3xP3}$], and *egalitarian* [*egal*[1]]) and two postvitellogenesis mutants (*okra*$^{17\text{-}11}$ and *retained*^{RO44}) obtained from the Bloomington Stock Center. We additionally measured the immune defense in females triple mutant for the three *yolk protein* genes (*yp1-3*$^{-/-}$), as well as females triple mutant for the *yolk proteins* and heterozygous for *apolipophorin B* (*apolpp*$^{-/+}$). The *egal*[1], *okra*$^{17\text{-}11}$, *retained*^{RO44}, and *yolkless*^{17} mutations result in sterility when homozygous, so these stocks are maintained as heterozygotes over balancer chromosomes. The presence of the balancers could conceivably allow recessive deleterious mutations to accumulate on the chromosome bearing the mutation. For our experiment, we therefore crossed each mutant to an appropriate molecularly defined deficiency line available through the Bloomington Stock Center. These progeny from these crosses are hemizygous for the mutant allele over the deletion on the homologous deficiency chromosome and are outbred for the remainder of the genome. Heterozygous controls were produced by crossing either the mutant or deficiency line to wild-type Canton S. Daughters of *tudor* mutants are *tud*[1] *bw sp*/CS, created by crossing *tud*[1] *bw sp*/*tud*[1] *bw sp* mothers to Canton S males, but they lack germ lines due to failure of the homozygous mutant mother to deposit *tudor* mRNAs into the daughter oocyte. Genetic controls have the same *tud*[1] *bw sp*/CS genotype but are made by crossing *tud*[1] *bw sp*/*CyO* mothers to Canton S males and have intact germlines due to maternal mRNA deposition into the egg. The *bag of marbles* mutant line was a gift from Dr. Jackie Bubnell and Dr. Charles Aquadro (Bubnell et al. 2022). *Yolk protein* and *apolippB* mutants were a gift from Dr. Tsubasa Tanaka and Dr. Akira Nakamura (Tanaka et al. 2021).

Infection procedures and experimental set up

All flies were aged 3 to 5 d posteclosion and held in single-sex groups of ~10 to 15 per vial. Females of each genotype were randomly assigned to mated or unmated treatment groups. For mated treatment groups, 15 males were added to the vial 24 h prior to infection without any additional CO_2 anesthetization. During CO_2 anesthetization for infection, males were removed from the vials.

All female flies were infected with the Gram-negative bacterium *Providencia rettgeri*, a natural bacterial pathogen of *D. melanogaster* with moderate pathogenicity (Juneja and Lazzaro 2009; Galac and Lazzaro 2011) that has been previously used to investigate the mechanisms of postmating immune suppression (Short and Lazzaro 2010; Short et al. 2012; Schwenke and Lazzaro 2017; Gordon et al. 2022). *P. rettgeri* infection activates both of the major pathways controlling production of AMPs (Troha et al. 2018) and AMPs provide primary control of *P. rettgeri* infection (Unckless et al. 2016).

For all experiments, bacterial cultures were started from a single colony of *P. rettgeri* picked from a Luria Bertani (LB) agar plate, which was used to inoculate a culture grown in liquid LB overnight at 37 °C with shaking. The following morning, the saturated overnight culture was diluted 1:3 and grown for 3 h at 37 °C. This new subculture was diluted to a working solution of $A_{600} = 1$, centrifuged for 5 min at 3,000 rpm, and resuspended in sterile phosphate-buffered saline (PBS) to $A_{600} = 0.1$ unless otherwise noted. Females were anesthetized with CO_2 and a Nanoject II (Drummond) was used to inject 23 nL of bacterial solution into each fly as described in Khalil et al. (2015). Wounding control flies were injected with sterile PBS in parallel during each infection session. Females were placed on fresh food following infection treatment.

Survivorship

The number of living flies was recorded every 24 h for 5 d after infection. To assess the effect of mating on each genotype, we first modeled the survivorship data with a mixed effect Cox proportional hazards model (Breslow 1975) where genotype, mating treatment, and their interaction were fixed effects and experimental block was a random effect. There were two replicate experimental blocks for each genotype except *bam* and *retained*, which were measured in three replicate blocks.

$$Y_{ijk} = u + \text{Mating Status}_i + \text{Genotype}_j + \text{Mating Status}_i \times \text{Genotype}_j + (1|\text{Block}_k).$$

Next, we used marginal means comparisons to test for differences between mated and unmated females within a genotype. All statistical analyses were performed in R Version 2023.03.1+446.

Bacterial load

Bacterial load was measured 18 h after infection. Single flies were anesthetized and homogenized in 500 μL of sterile PBS, which was then diluted 1:100 with sterile PBS. For each individual fly, 50 μL of diluted homogenate was spiral-plated onto LB-agar plates using a WASP2 spiral plater (Microbiology International) and the plates were incubated overnight at 37 °C for colony growth. This instrument plates the sample with decreasing volume over a concentric spiral and the associated ProtoCOL plate counting system (Microbiology International) uses the number of colonies and their position on the spiral to estimate the number of viable bacteria (colony forming units; CFU) in the plated sample. Colonies were morphologically confirmed to be *P. rettgeri*. Control flies that were injected with PBS never yielded any colonies. The plate count data were natural-log-transformed and an ANOVA was performed to determine the effects of mating status, genotype, the interaction of mating status and genotype, and experimental block:

$$\log_e(\text{count/mL}) = \text{Mating Status} + \text{Genotype} + \text{Mating Status} \times \text{Genotype} + \text{Block}.$$

Two replicate experimental blocks were executed for each genotype. We used marginal means comparisons with Tukey's corrections to test for differences in mean CFU between mated and unmated females within a genotype. Analyses were performed in R Version 2023.03.1+446.

Egg counts with QuantiFly and progeny counts

QuantiFly, an automated tool to quantify *Drosophila* egg laying (Waithe et al. 2015), was tested and optimized for the egg-laying assays. QuantiFly uses advanced pattern recognition and machine-learning to count the number of *Drosophila* eggs on a surface. For the purposes of our study, we optimized image acquisition, algorithm training, and image analysis and implemented a custom QuantiFly protocol described in detail in Ray et al. (2023).

Cornmeal-sucrose medium was dyed green with 10% v/v of green food coloring from DyeCraft (UPC 860000500937). The day before matings, single 3-d posteclosion unmated females were placed into vials. Vials were randomly assigned to mated and

unmated treatment groups. Single 3 to 5 d posteclosion Canton S males were aspirated into the mated treatment group vials. Pairs were observed and the male was aspirated out after mating had finished. Females who failed to mate were discarded. Females were transferred to new vials every day for 3 d. Images were taken of the eggs on the food using an iPad (Apple) in square mode after fly transfer, then processed and counted in QuantiFly.

Egg laying for each genotype was completed in two blocks. Each dataset was filtered to contain only females that completed 4 d of egg laying. The total number of eggs laid per female was calculated as the sum of the 4 d of egg laying. To test for differences in the total number of eggs laid (Y_{ij}) between mated and unmated females of all genotypes, we applied a linear mixed model with mating status, genotype, and their interaction as fixed effects and block as a random effect:

$$Y_{ijk} = u + \text{Mating Status}_i + \text{Genotype}_j + \text{Mating Status}_i \times \text{Genotype}_j + (1|\text{Block}_k).$$

We used marginal means comparisons with Tukey's corrections to test for differences between mating status and genotype treatment groups.

For the *yolk protein* mutants, unmated females never produced any progeny. For the mated *yolk protein* mutant females, the total number of progeny per mated female was calculated as the sum of progeny in each of the four vials. The ratio of progeny to eggs was calculated by dividing the total number of progeny by the total number of eggs to estimate egg viability. We used a *t*-test to test for differences in the mean ratio of progeny to eggs between mutant and control females.

Results

Mutants that do not complete vitellogenesis maintain robust immune defense after mating

Daughters of $tudor^{1}$ mutant females have no germline and therefore produce no eggs (Boswell and Mahowald 1985). Consistent with a previous report (Short et al. 2012), we found that while mating reduced the immune defense of genetically matched controls (Fig. 2a, $P < 0.0001$; Supplementary Tables 2 and 3), mated daughters of $tudor^{1}$ females survived infection in similar proportions to unmated daughters of $tudor^{1}$ females (Fig. 2a, $P = 0.531$). A previous study found that mated ovo^{D1} females, which arrest oogenesis at stage 4 (Oliver et al. 1987), displayed no reduction in immune defense when infected with *P. aeruginosa* 27 h after mating (Fedorka et al. 2007). In our systemic infection with *P. rettgeri* administered ~24 h after mating, we also found that mated and unmated ovo^{D1} females survived in similar proportions (Fig. 2b, $P = 0.88$; Supplementary Tables 4 and 5) with similar bacterial loads (Fig. 2c, $P = 0.704$; Supplementary Tables 6 and 7).

Next, we asked whether two additional mutants that arrest egg development prior to vitellogenesis could maintain immune defense after mating. We found that when challenged with a systemic bacterial infection, mated $bam^{null\text{-}3xP3}$ females not only suffered no decrease in immune defense, they survived at significantly greater proportions than unmated $bam^{null\text{-}3xP3}$ females (Fig. 3a, $P < 0.001$; Supplementary Tables 8 and 9). Bacterial load was similar between mated and unmated $bam^{null\text{-}3xP3}$ females (Fig. 3b, $P = 0.789$; Supplementary Tables 10 and 11). There was no difference in survivorship (Fig. 3a, $P = 0.741$; Supplementary Tables 8 and 9) or bacterial loads (Fig. 3b, $P = 0.399$; Supplementary Tables 10 and 11) of mated and unmated w^{1118} genetic background controls, with both showing clearly reduced defense relative to the mated $bam^{null\text{-}3xP3}$ females (Fig. 3a and 3b).

Similarly, we found that mating increased the survivorship of hemizygous $egal^{1}/Df(2R)$ females relative to unmated females (Fig. 3c, $P = 0.045$; Supplementary Tables 12 and 13) but had no effect on bacterial load (Fig. 3d, $P = 0.445$; Supplementary Tables 14 and 14). Heterozygous control $egal^{1/+}$ females suffered reduced survivorship (Fig. 3c, $P < 0.0001$; Supplementary Tables 12 and 13) and higher bacterial loads (Fig. 3d, $P = 0.003$; Supplementary Tables 14 and 15) after mating, although mating had no effect on the survivorship (Fig. 3c, $P = 0.838$; Supplementary Tables 12 and 13) or bacterial load of control $Df(2R)^{/+}$ females, which were more resistant to infection than the other genotypes (Fig. 3d, $P = 0.187$; Supplementary Tables 14 and 15). Considering all of the data in combination, we conclude that previtellogenic prevention of egg production alleviates the mating-induced reduction in immune defense.

Mating reduces immune defense of late-arresting oogenesis mutant *okra*, but not *retained*

We predicted that progression through vitellogenesis would reduce postmating immune capacity regardless of endpoint fertility or sterility. We therefore tested postmating defense against infection in two *D. melanogaster* mutant genotypes that progress egg development through vitellogenesis but have later-acting defects that eliminate fertility: *okra* and *retained*. We found that mating reduced the survivorship of hemizygous $okra^{17\text{-}11}/Df(2L)$ females (Fig. 4a, $P = 0.005$; Supplementary Tables 16 and 17) and increased their bacterial load, although nonsignificantly and to a lesser degree than in the heterozygous controls (Fig. 4b, $P = 0.17$; Supplementary Tables 18 and 19). Heterozygous control $okra^{1711/+}$ females also suffered reduced survivorship of infection after mating (Fig. 4a, $P = 0.006$; Supplementary Tables 16 and 17) and experienced higher bacterial loads (Fig. 4b, $P = 0.001$; Supplementary Tables 18 and 19). Mating also significantly increased the bacterial load of $Df(2L)^{/+}$ females (Fig. 4b, $P = 0.031$; Supplementary Tables 18 and 19) although the decrease in postinfection survivorship not significant (Fig. 4a, $P = 0.098$; Supplementary Tables 16 and 17).

We found that mating had no effect on the survivorship (Fig. 4c, $P = 0.332$; Supplementary Tables 20 and 21) or bacterial load (Fig. 4d, $P = 0.383$; Supplementary Tables 22 and 23) of hemizygous $retained^{RO44}/Df(2R)$ females, which complete oogenesis but do not oviposit (Schüpbach and Wieschaus 1991). The control $retained^{RO44/+}$ females suffered the expected reduction in survivorship (Fig. 4c, $P < 0.001$; Supplementary Tables 19 and 20) and higher bacterial loads (Fig. 4d, $P = 0.036$; Supplementary Tables 22 and 23) after mating, although mating unexpectedly had only a weak, nonsignificant effect on survivorship (Fig. 4c, $P = 0.083$; Supplementary Tables 20 and 21) and no effect on bacterial load (Fig. 4d, $P = 0.565$; Supplementary Tables 22 and 23) of $Df(2R)^{/+}$ control females.

Loss of yolk proteins and apolppB increases overall immune defense, but does not rescue decrease in immune defense after mating

Tanaka et al. (2021) identified an apolipophorin protein, apolppB, that is incorporated into the oocyte alongside yolk proteins via the *yolkless* receptor. Homozygous mutants of apolppB are non-viable, so we tested both $yp1^{41}$, $yp2^{7}$, $yp3^{111}$;;;$apolpp^{-/+}$ and $yp1^{41}$, $yp2^{7}$, $yp3^{111}$ mutants (Tanaka et al. 2021) for effects on postmating immunity. Consistent with the hatching assay reported in Tanaka et al. (2021), we found that $yp1\text{-}3^{-/-}$;;;$apolpp^{-/+}$ and $yp1\text{-}3^{-/-}$

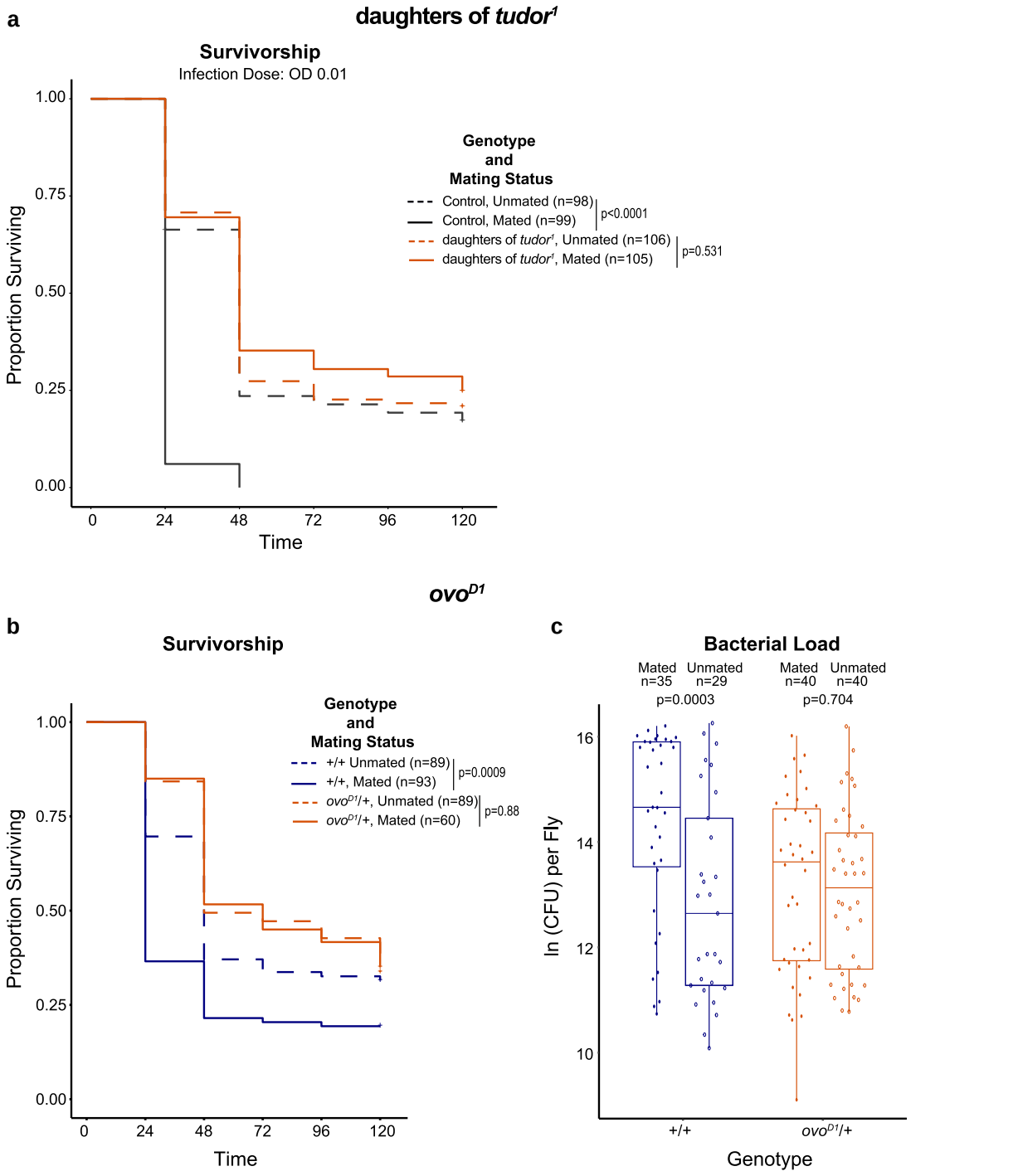


Fig. 2. Flies with genotypes that arrest oogenesis at early stages (daughters of *tudor*^{1} and *ovo*^{D1}) maintain robust immune defense after mating. a) Survivorship after infection for mated and unmated daughters of *tudor*^{1} females and controls. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. b) Survivorship after infection for mated and unmated *ovo*^{D1} and Canton S females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. c) Bacterial load 18 h after infection for mated and unmated *ovo*^{D1} and Canton S females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$.

females produced significantly fewer viable progeny than controls (Fig. 5a, $P = 2.2 \times 10^{-10}$, Supplementary Fig. 1, $P = 2.2 \times 10^{-16}$) but were not sterile. We additionally found no difference in the total number of eggs laid by either *yp1-3*$^{-/-}$;;;*apolpp*$^{-/+}$ or *yp1-3*$^{-/-}$ mutant females relative to controls (Fig. 5b, Supplementary Fig. 1). Thus, while these mutants do produce eggs, the eggs produced have reduced viability.

We tested the postmating immune defense of *yp1-3*$^{-/-}$ triple mutants and *yp1-3*$^{-/-}$;;;*apolpp*$^{-/+}$ mutants and respective *yw* and *yw;;;CiD*$^{/+}$ controls (Tanaka et al. 2021). We were concerned that mutation of the *yellow* gene, whose product is required for injury-related melanization (Biessmann 1985), might affect our infection results so we additionally created heterozygous controls by crossing *yp1-3*$^{-/-}$ and *yp1-3*$^{-/-}$;;;*apolpp*$^{-/+}$ and controls to

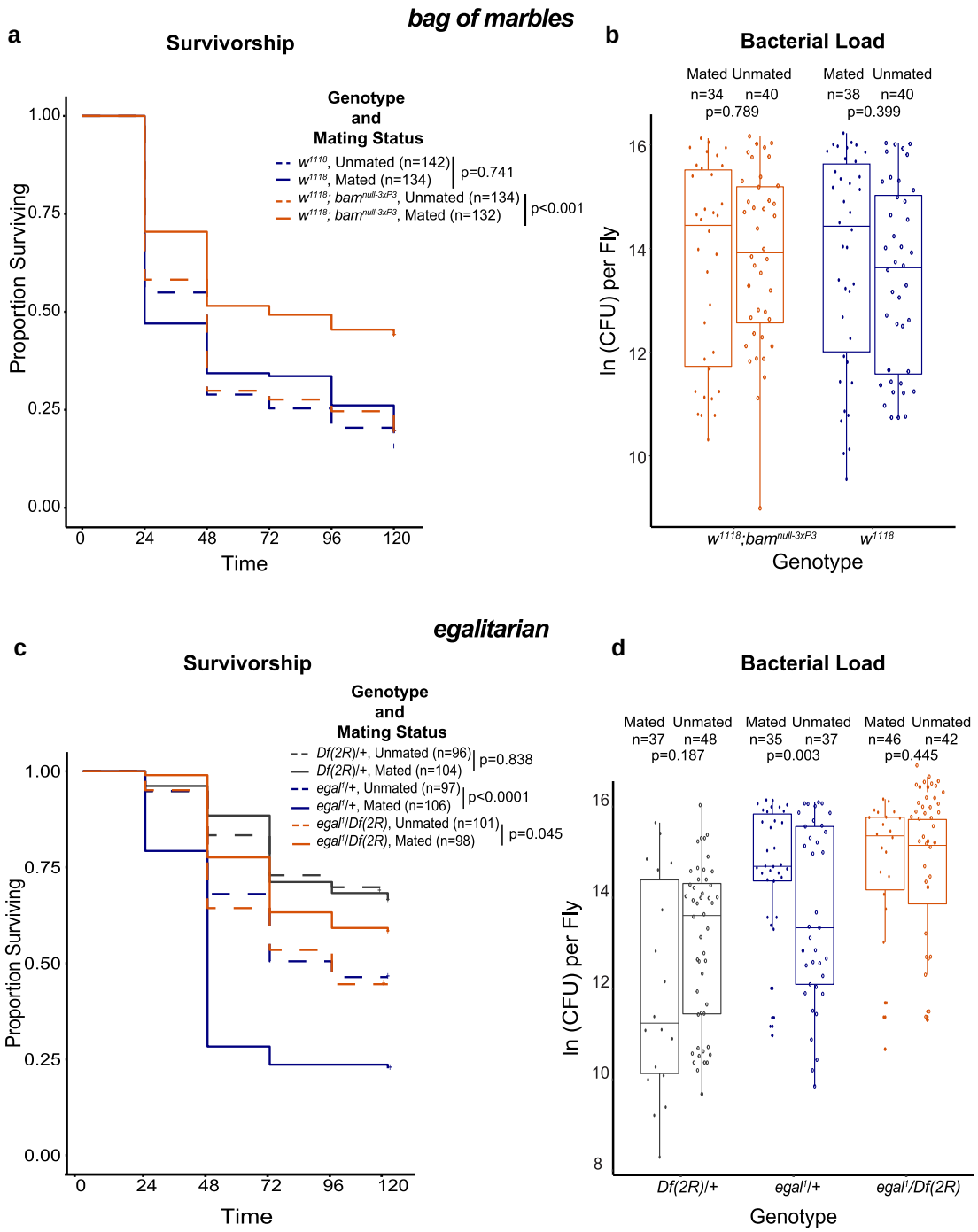


Fig. 3. Early arrest mutants of *bam* and mid-arrest mutants of *egalitarian* maintain robust immune defense after mating. a) Survivorship after infection for mated and unmated w^{1118}; $bam^{null\text{-}3xP3}$ and w^{1118}; +/+ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. b) Bacterial load 18 h after infection for mated and unmated w^{1118}; $bam^{null\text{-}3xP3}$ and w^{1118}; +/+ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. c) Survivorship after infection for mated and unmated $egal^{1}/Df(2R)$, $egal^{1/+}$, and $Df(2R)^{/+}$ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. d) Bacterial load 18 h after infection for mated and unmated $egal^{1}/Df(2R)$, $egal^{1/+}$, and $Df(2R)^{/+}$ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$.

wild-type Canton S flies. Mating reduced the survivorship of $yp1\text{-}3^{-/-}$;;;$apolpp^{-/+}$ females ($P = 0.006$; Fig. 5c; Supplementary Tables 24 and 25) but did not affect bacterial load relative to unmated $yp1\text{-}3^{-/-}$;;;$apolpp^{-/+}$ females ($P = 0.05$; Fig. 5d; Supplementary Tables 26 and 27). A larger proportion of $yp1\text{-}3^{-/-}$;;;$apolpp^{-/+}$ females survived infection than yw;;;$CiD^{/+}$ control females (Fig. 5c; Supplementary Tables 24 and 25). However, the proportion of survivorship for yw;;;$CiD^{/+}$ females was very low, with no difference observed in between mated and unmated females. Likewise, $yp1\text{-}3^{-/-}$ females survived in greater proportions than yw controls, but again the survivorship of yw controls was very poor, with all flies dead before 24 h (Supplementary Fig. 1c). Measurements of the bacterial load of yw controls at 18 h were not recorded as an insufficient number of flies survived to that timepoint. However, there was no

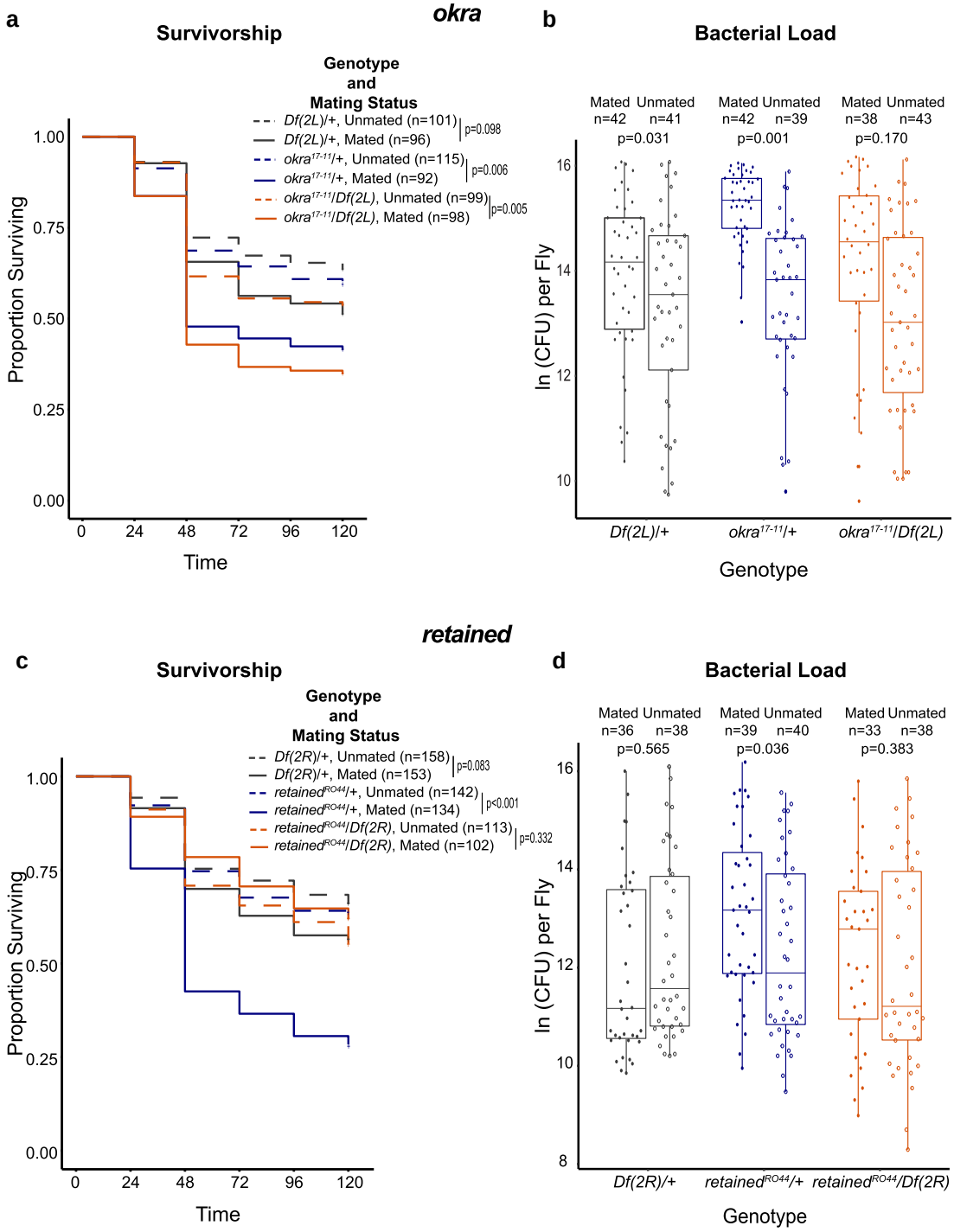


Fig. 4. Mated reduces immune defense of late-arresting oogenesis mutant *okra*, but not *retained*. a) Survivorship after infection for mated and unmated $okra^{17\text{-}11}/Df(2L)$, $okra^{17\text{-}11/+}$, and $Df(2L)^{/+}$ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. b) Bacterial load 18 h after infection for mated and unmated $okra^{17\text{-}11}/Df(2L)$, $okra^{17\text{-}11/+}$, and $Df(2L)^{/+}$ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. c) Survivorship after infection for mated and unmated $retained^{RO44}/Df(2R)$, $retained^{RO44/+}$, and $Df(2R)^{/+}$ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. d) Bacterial load 18 h after infection for mated and unmated $retained^{RO44}/Df(2R)$, $retained^{RO44/+}$, and $Df(2R)^{/+}$ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$.

apparent decrease in immune defense after mating for $yp1\text{-}3^{-/-}$ mutants, with no difference in survivorship or bacterial loads between mated and unmated $yp1\text{-}3^{-/-}$ females (Supplementary Figs. 1c and 1d; Supplementary Tables 28 to 31). For both $yp1\text{-}3^{-/-}$ triple mutants and $yp1\text{-}3^{-/-};;;apolpp^{-/+}$ mutants, the heterozygous control genotypes behaved as expected, with large decreases in survivorship and increases in bacterial load in mated females relative to unmated females (Supplementary Fig. 1, Figs. 5c and 5d; Supplementary Tables 28 and 29 and 30 and 31). Overall, we found that loss of yolk proteins in conjunction with heterozygosity

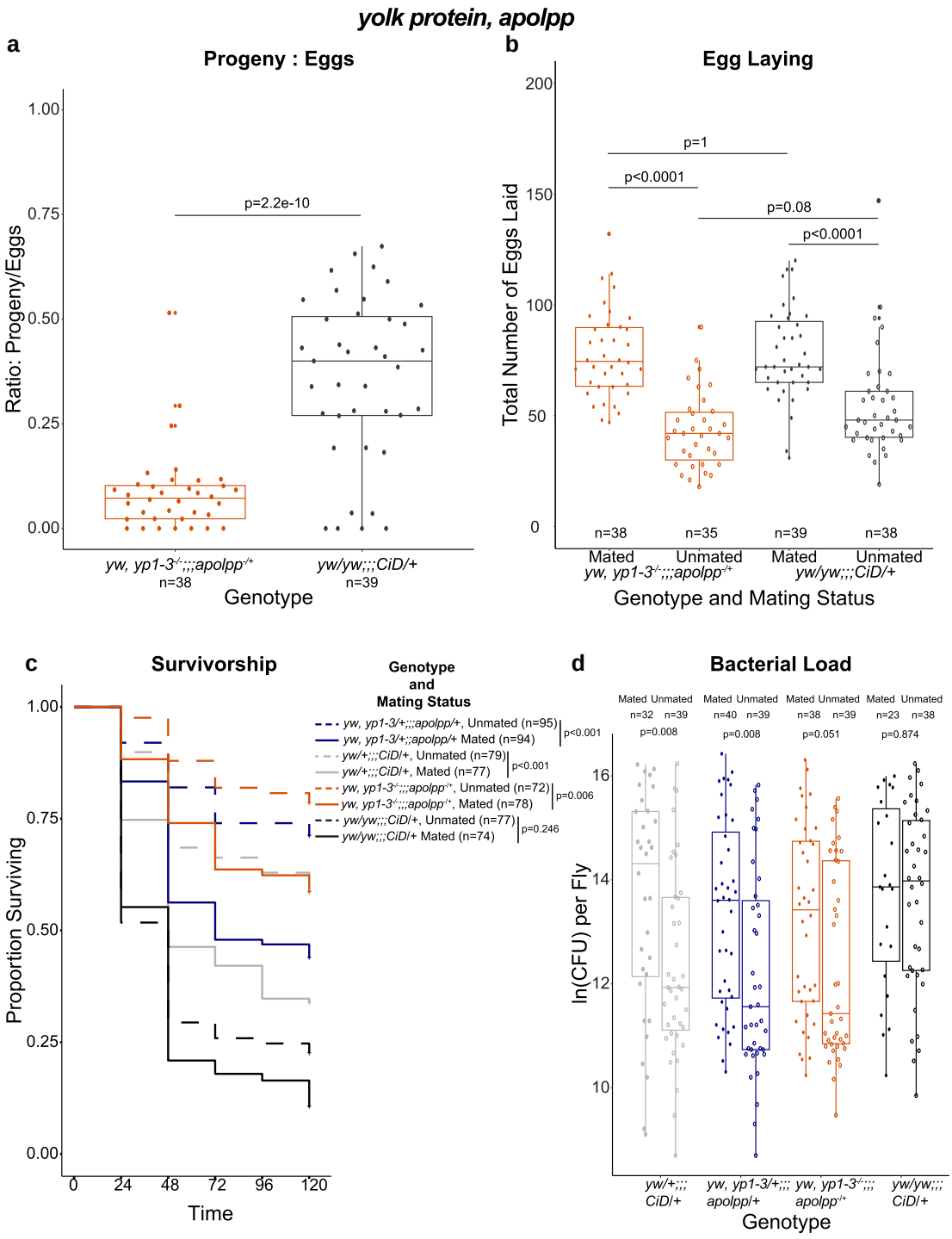


Fig. 5. Loss of yolk proteins and apolppB increases overall immune defense. a) Ratio of progeny to eggs laid by mated *yw*, *yp1-3*$^{-/-}$*;;;apolpp/CiD* and *yw/yw;;; CiD*$^{/+}$ females over 4 d. Student's *t*-test. b) Total number of eggs laid by mated and unmated *yw*, *yp1-3*$^{-/-}$*;;;apolpp/CiD* and *yw/yw;;;CiD*$^{/+}$ females over 4 d. Linear model, marginal means pairwise comparisons between genotype/mating status treatment groups, significance cut-off $P < 0.05$. c) Survivorship after infection for mated and unmated *yw*, *yp1-3*$^{-/-}$*;;;apolpp/CiD* and *yw/yw;;;CiD*$^{/+}$ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. d) Bacterial load 18 h after infection for mated and unmated *yw*, *yp1-3*$^{-/-}$*;;;apolpp/CiD* and *yw/yw;;;CiD*$^{/+}$ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$.

for *apolpp* mutations resulted in increased postinfection survival relative to *yw* and controls (Fig. 5c, Supplementary Fig. 1).

Mutants in the yolk protein receptor, *yolkless*, cannot complete vitellogenesis and are sterile (DiMario and Mahowald 1987). They nevertheless produce yolk in the fat body and, due to the block in uptake into follicle cells and developing oocytes, they accumulate yolk proteins in the hemolymph (DiMario and Mahowald 1987). We found that mating decreased the survivorship of hemizygous *yolkless*^{17}*/Df(1)* females (Fig. 6a, $P = 0.027$; Supplementary Tables 32 and 33) but had no effect on bacterial load (Fig. 6b, $P = 0.832$; Supplementary Tables 34 and 35). As expected, mating also reduced the survivorship of control heterozygous *yolkless*$^{17/+}$

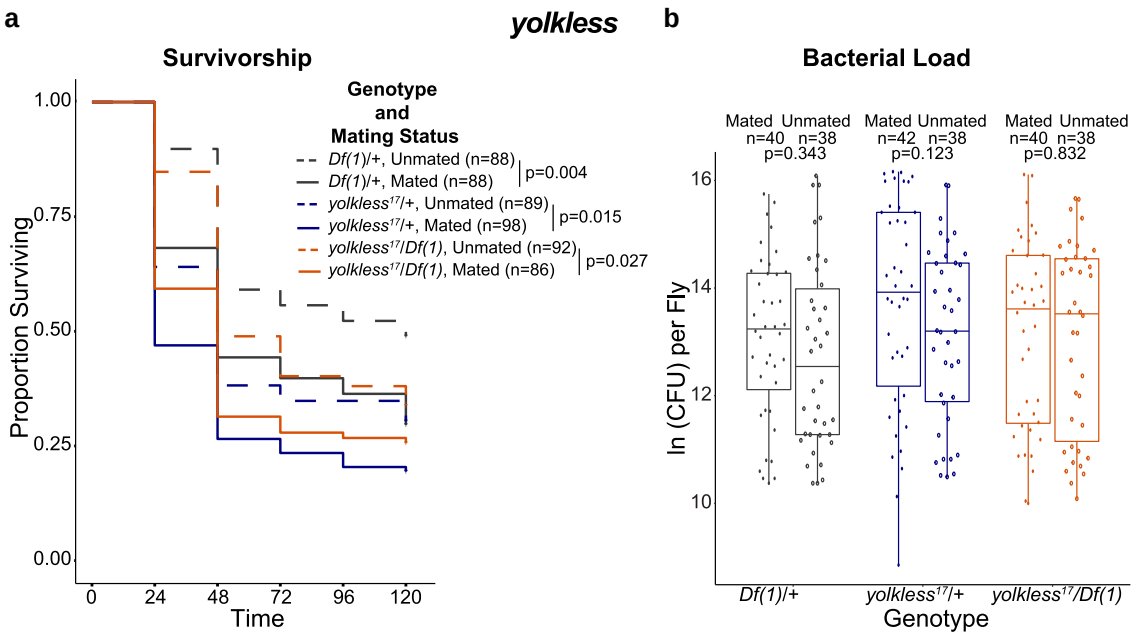


Fig. 6. Mating decreases immune capacity of *yolkless* mutants, the yolk protein receptor. a) Survivorship after infection for mated and unmated *yolkless*^{17}/*Df(1)*, *yolkless*$^{17/+}$, and *Df(1)*$^{/+}$ females. Mixed effect Cox proportional hazard model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$. b) Bacterial load 18 h after infection for mated and unmated *yolkless*^{17}/*Df(1)*, *yolkless*$^{17/+}$, and *Df(1)*$^{/+}$ females. Linear model, marginal means comparisons of mating status within a genotype, Tukey's corrections, significance cut-off $P < 0.05$.

females (Fig. 6a, $P = 0.015$; Supplementary Tables 32 and 33), although it had no effect on bacterial load ($P = 0.123$; Fig. 6b; Supplementary Tables 34 and 35). Similarly, mating reduced the survivorship of heterozygous control *Df*$^{(1)/+}$ females (Fig. 6a, $P = 0.004$; Supplementary Tables 31 and 32) but had no effect on bacterial load (Fig. 6b, $P = 0.343$; Supplementary Tables 34 and 35). Together, these results show that continuous production of yolk proteins, even without successful uptake into the oocyte, limits postmating immune defense.

Discussion

Physiological trade-offs between the energetically demanding traits of reproduction and immune defense are observed in many taxa (reviewed in Sheldon and Verhulst 1996; Schmid-Hempel 2003). In female *D. melanogaster*, mating causes a reduction in defense against pathogenic bacterial infections (Fedorka et al. 2007; Short and Lazzaro 2010; Short et al. 2012; Schwenke and Lazzaro 2017; Gordon et al. 2022; Shianiou et al. 2023). Two independent analyses of the transcriptomic response of females to mating and infection showed that the modest immune activation triggered by mating in the absence of infection is dependent on an intact germline (Short and Lazzaro 2013; Rodrigues et al. 2021). As previously reported (Fedorka et al. 2007; Short et al. 2012) and extended here, mated females with no germline or that arrest oogenesis in very early stages maintain robust immune defense after mating. A recent study found that the fat bodies of mated, infected females exhibit signatures of ER stress and impaired protein translation, potentially due to the concurrent demands of producing yolk proteins and AMPs (Gupta et al. 2022). Together, these results point toward a model in which mating triggers investment in egg production, which then manifests as a direct cost that inhibits immune defense.

In testing that model, we first confirmed the previously reported results (Fedorka et al. 2007; Short et al. 2012) that two mutants that arrest oogenesis prior to vitellogenesis maintain immune capacity after mating, and we tested two additional mutants with previtellogenic oogenesis arrest. We found that both of these mutant females maintain full immune defense after mating. Thus, all four tested previtellogenic mutants (*ovo*^{D1}, daughters of *tudor*^{1}, *bam*$^{null\text{-}3xP3}$, *egal*^{1}; Supplementary Table 1) retain postmating immune performance, consistent with the model. Our analysis of *bam* null mutants is consistent with results reported by Rodrigues et al. (2021), in which ablation of the germline via overexpression of *bam* with a germline-specific driver increased the survival of mated females relative to controls. In contrast, in our tests of mutants whose defects act after vitellogenesis, we found that mated *okra*$^{17\text{-}11}$ females suffered reductions in immune defense after mating similar to wild-type, again consistent with the model predictions. However, we observed that *retained*^{RO44} females maintained robust immune defense after mating, which was not predicted. We chose *retained* as a late-arresting mutant because females make late stage oocytes but are unable to oviposit them (Schüpbach and Wieschaus 1991), so we presumed the flies would suffer the costs of oogenesis. However, the block in oviposition in this mutant causes the ovaries to become swollen with unlaid eggs (Schüpbach and Wieschaus 1991); we propose that this could result in feedback inhibition to curtail further oogenesis. Thus, *retained* mutant females may cease developing new oocytes and therefore not continue to invest resources in oogenesis. In that case, *retained* females would be more analogous to the previtellogenic mutants in our model, which did not experience postmating immune suppression.

Our results demonstrate that arresting investment in oogenesis prior to the vitellogenic stages in which yolk proteins are deposited into the oocyte prevents the postmating suppression of female immune defense. We additionally tested more specifically whether mating-induced production of yolk proteins is costly for immune defense. We found that disruption of yolk protein genes improved female immune defense relative to controls. However, mutants for the yolk protein receptor, *yolkless*, which produce

yolk proteins in the fat body but cannot import them into developing oocytes (DiMario and Mahowald 1987) exhibited reduced immune defense after mating. Thus, these results support our hypothesis that abundant synthesis of yolk proteins in the fat body, required by mated females for fertile egg production, limits defense against systemic infection. Although we have not directly tested it here, the AMP translation deficit shown to arise in the fat bodies of reproductively active females by Gupta et al. (2022) provides a plausible mechanism for the cost of yolk protein production. Also consistent with our results, a recent study found that reducing fat body gene expression of *yp2* and *yp3* individually increased survival after enteric infection with *P. aeruginosa* (Shianiou et al. 2023).

Interestingly, we found that *yp1-3*$^{-/-}$*;;;apolpp*$^{-/+}$ mutants still experience a reduction in immune defense after mating. This observation suggests two possibilities that are not mutually exclusive. First, it is possible that yolk protein production is not the only investment in oogenesis that limits immune defense. Tanaka et al. (2021) observed that *yolk protein* mutants can produce fertile stage 14 oocytes and hypothesized that the apolipophorin protein, apolppB could compensate for the loss of yolk and allow for preserved, though reduced, fertility. Like the three *yolk proteins* genes, *apolppB* is also expressed in the adult fat body (Leader et al. 2018) and may be costly to immunity. Because *apolppB* mutants are homozygous lethal, we were only able to test *apolpp*$^{-/+}$ for reductions in immune defense. It is possible that continued production of *apolppB* in the fat bodies of these mated *yp1-3*$^{-/-}$*;;;apolpp*$^{-/+}$ females continues to compete for cellular resources, constraining AMP production and decreasing the quality of mated female immune defense.

Second, given the difference in the response from *retained*^{RO44} and *yp1-3*$^{-/-}$*;;;apolpp*$^{-/+}$ mutants, immune defense in mated females could also be suppressed by mechanisms aside from competition between yolk protein production and AMP production within the fat body. The reduction in mated female immune defense is triggered by receipt of the seminal fluid protein, Sex Peptide (SP) (Short et al. 2012; Schwenke and Lazzaro 2017). Among other postmating changes, SP stimulates sustained egg production and the synthesis of JH in the female corpora allatum (Moshitzky et al. 1996; Fan et al. 2000; Liu and Kubli 2003; Peng et al. 2005). As previously noted, JH stimulates yolk protein production and progression through vitellogenesis (Soller et al. 1999). However, we and others have shown that JH can suppress female immune defense (Rolff and Siva-Jothy 2002; Flatt et al. 2008; Schwenke and Lazzaro 2017; Shianiou et al. 2023). Application of the JH analog, methoprene, mimics the suppressive effects of mating on unmated female immune defense (Schwenke and Lazzaro 2017) and ablation of the corpus allatum or knockdown of expression of the JH receptor, *germ cell expressed* (*gce*), restores immune capacity to unmated levels in mated females (Schwenke and Lazzaro 2017). Despite abundant evidence for JH suppression of immune defense, the mechanism behind this effect has yet to be identified. Given the robust immune responses of mated previtellogenesis mutants, additional mechanisms of immune suppression might be weak or be predicated on a modest investment into egg production, potentially in conjunction with the stimulation of JH production by SP.

For some of the mutants we tested, we observed differences in survivorship between mated and unmated females without significant difference in measured pathogen burden. Duneau et al. (2017) measured the trajectory of *P. rettgeri* infection in *D. melanogaster* in detail. In the first 6 to 8 h after infection, *P. rettegeri* grows at similar rates across individual hosts, but after ~8 h, stochastic and/or experimental variation in the host immune response and pathogen growth results in some hosts succumbing to infection with high pathogen burdens while others survive with chronic infections. The divergence in infection trajectories results in high among-individual variance in pathogen burden at 16 to 20 h postinfection but has smaller impact on the mean and median burdens among individuals. The single snapshot of bacterial load taken here, measured at 18 h postinfection, is methodologically consistent with our previous studies of postmating infections with *P. rettgeri* (e.g. Gordon et al. 2022), but may not have been sufficiently sensitive to capture differences in the dynamics of pathogen growth between oogenesis mutants and their respective genetic controls. The measurement of survivorship over 5 d, replicable over experimental blocks, offers a more robust contrast of overall defense against infection as a function of investment in reproduction.

We hypothesized that mating-induced suppression of immune defense in *D. melanogaster* arises as a direct cost of egg production, potentially mediated by competition within the fat body between the production of yolk proteins and AMPs. We found that production of yolk proteins and progression of oogenesis through the vitellogenic stages inhibits female immune defense, but that females who arrest oogenesis after vitellogenesis may still pay immunological costs. While our data are broadly consistent with our hypothesized model, other investments required for egg production and/or other postmating changes in female physiology may also contribute to postmating immune suppression. Our results support the interpretation that sustained egg production poses a direct immunological cost in *D. melanogaster* and provide insight into the mechanisms generating the physiological trade-offs between the complex, energetically demanding processes of reproduction and immune defense.

Data availability

All data and code are available at: https://github.com/WolfnerLab/Gordon_2025.

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Supplemental material

Supplemental material available at GENETICS online.

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Conflicts of interest

We report no conflicts of interest.

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