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A single mating is sufficient to induce persistent reduction of immune defense in mated female *Drosophila melanogaster*

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ABSTRACT

In many species, female reproductive investment comes at a cost to immunity and resistance to infection. Mated *Drosophila melanogaster* females are more susceptible to bacterial infection than unmated females. Transfer of the male seminal fluid protein Sex Peptide reduces female post-mating immune defense. Sex Peptide is known to cause both short- and long-term changes to female physiology and behavior. While previous studies showed that females were less resistant to bacterial infection as soon as 2.5 h and as long as 26.5 h after mating, it is unknown whether this is a binary switch from mated to unmated state or whether females can recover to unmated levels of immunity. It is additionally unknown whether repeated mating causes progressive reduction in defense capacity. We compared the immune defense of mated females when infected at 2, 4, 7, or 10 days after mating to that of unmated females and saw no recovery of immune capacity regardless of the length of time between mating and infection. Because *D. melanogaster* females can mate multiply, we additionally tested whether a second mating, and therefore a second transfer of seminal fluids, caused deeper reduction in immune performance. We found that females mated either once or twice before infection survived at equal proportions, both with significantly lower probability than unmated females. We conclude that a single mating event is sufficient to persistently suppress the female immune system. Interestingly, we observed that induced levels of expression of genes encoding antimicrobial peptides (AMPs) decreased with age in both experiments, partially obscuring the effects of mating. Collectively, the data indicate that being reproductively active versus reproductively inactive are alternative binary states with respect to female *D. melanogaster* immunity. The establishment of a suppressed immune status in reproductively active females can inform our understanding of the regulation of immune defense and the mechanisms of physiological trade-offs.

1. Introduction

Reproduction and immunity trade off evolutionary and physiologically in diverse organisms such as mammals, birds, and insects (Rose & Bradley, 1998; Schmid-Hempel, 2003, 2004; Schmid-Hempel & Ebert, 2003; Schwenke et al., 2016; Sheldon & Verhulst, 1996; Wigby et al., 2019; Zera & Harshman, 2001). A physiological trade-off may occur if the demands of mounting an immune response conflict with the demands of pre- and/or post-mating traits required for reproduction. Conflict could arise if a limited resource, such as nutrients, are required for both immune defense and reproduction. Consequently, organisms may develop mechanisms to direct the allocation of resources to enhance their fitness, resulting in reduced immune capacity in reproductively active individuals. These mechanisms may be dynamically regulated as an organism responds to the environment across their lifespan, resulting in plasticity for the physiological trade-off. An evolutionary trade-off can occur if genetic variation exists within a population for the mechanisms of resource allocation or the cost of either trait. Natural selection may then act on the underlying genetic variation, depending on relevant environmental conditions such as pathogen prevalence. In this study, we investigate the dynamics of the physiological trade-off between female reproduction and immunity in *Drosophila melanogaster*.

Studies in insects have detected both positive and negative effects of mating on immune defense (reviewed in Schwenke et al., 2016). In the mealworm beetle, *Tenebrio molitor,* mating enhanced resistance to

Abbreviations: AMP, antimicrobial peptide; SP, Sex Peptide; JH, juvenile hormone; CS, Canton S.

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infection with the fungus, *Beauveria bassiana* (Valtonen et al., 2010). Likewise, following mating, the cricket *Gryllus texensis* was more resistant to the bacterium *Serratia marcescens* (Shoemaker et al., 2006). Conversely, the aphid *Acyrthosiphon pisum* was more susceptible to parasitoid attack after mating (Gwynn et al., 2005). We and others have shown that when infected with pathogenic bacteria, mated female *D. melanogaster* are less likely to survive (Fedorka et al., 2007; Short & Lazzaro, 2010), have higher bacterial loads (Short et al., 2012; Short & Lazzaro, 2010), and express lower levels of antimicrobial peptide (AMP) genes than unmated females (Short et al., 2012). Short & Lazzaro 2010 showed that while females harbor significant genetic variation for immune capacity after mating, variation in male genotype did not affect female post-mating immune defense, indicating this trait is not driven by ongoing sexual conflict. Instead, these results are most consistent with a female-specific physiological trade-off between mating status and immune defense.

Along with sperm and other molecules, males transfer seminal fluid proteins like Sex Peptide (SP) to the female during mating, which rapidly and extensively changes female physiology, gene expression, and behavior (reviewed in Avila et al., 2011; Perry et al., 2013). Previous studies showed that females mated to either SP-null males or males lacking the N-terminus of SP do not suffer reduced immune defense (Schwenke & Lazzaro, 2017; Short et al., 2012). The N-terminus of SP is linked to increased production of juvenile hormone (JH) in the corpora allata, which in turn promotes egg production by stimulating the synthesis and incorporation of yolk proteins into stage 10 oocytes (Soller et al., 1997, 1999). JH is immune suppressive (Flatt et al., 2008; Schwenke & Lazzaro, 2017) and application of the JH analog methoprene to unmated females mimics the effects of mating on immune defense (Schwenke & Lazzaro, 2017).

Interestingly, many studies have also identified that mating and/or transfer of SP to females increases the expression of immune related genes such as AMPs in the whole body and the reproductive tissues (Delbare et al., 2017; Domanitskaya et al., 2007; Gioti et al., 2012; Innocenti & Morrow, 2009; Lawniczak & Begun, 2004; McGraw et al., 2004; Peng et al., 2005b). Short et al. 2012 measured the expression of several AMP genes in the whole body of females at 0, 4, 12, and 24 h after systemic infection. At both 4 h and 12 h after infection, mated females expressed lower levels of AMP transcripts than unmated females. Twenty-four hours after infection, however, mated females expressed higher levels of AMP transcripts than unmated females. These results indicate that given a systemic infection, mated, infected females induce expression of AMP genes at a lower initial level over a slower trajectory than unmated, infected females. The difference in rate of induction of AMP gene expression may contribute to the increased pathogen burden and decreased survivorship of mated females.

Mating and receipt of SP shifts females behaviorally and physiologically from an unmated to mated state, resulting in increased egg production, increased food intake, successful release of sperm from storage organs, and decreased receptivity to remating, among other responses (Avila et al., 2010, 2015; Chapman et al., 2003; Gioti et al., 2012; Peng et al., 2005a). Some of these changes are short-term, lasting less than a day, (Kalb et al., 1993; Kubli & Bopp, 2012; Peng et al., 2005b; Ravi Ram & Wolfner, 2007), after which females return to unmated levels of egg production and receptivity to mating (Heifetz et al., 2001; Kalb et al., 1993; Liu & Kubli, 2003). Long-term responses are primarily caused by retention of SP for 10 to 14 days via binding of the SP N-terminus to stored sperm (Findlay et al., 2014; Peng et al., 2005a; Ravi Ram & Wolfner, 2009; Singh et al., 2018). The active region of SP is gradually released from sperm, maintaining high levels of egg production, decreasing rates of re-mating, increasing food intake, and slowing digestion (Apger-McGlaughon & Wolfner, 2013; Avila et al., 2011; Carvalho et al., 2006; Cognigni et al., 2011; Gioti et al., 2012; Peng et al., 2005a).

Previous studies demonstrating the suppressive effect of mating (Fedorka et al., 2007; Short & Lazzaro, 2010) and transfer of SP (Schwenke & Lazzaro, 2017; Short et al., 2012) on female immune defense did not test whether this was a short- or long-term response. Here, we tested whether female immune defense has two binary states (unmated and mated) or can be dynamically modified. First, by extending the interval between mating and infection, we asked whether female defense is persistently suppressed after mating or whether the suppressive effect can wane with time. Second, by performing more than one mating event, we asked whether the level of immune suppression remains constant after a shift from unmated to mated state or whether multiple matings (and therefore multiple doses of seminal fluid proteins like SP) compound the suppressive effect of mating on immune defense.

2. Material and methods

2.1. Fly stocks and husbandry

All flies were raised on Cornell cornmeal-sucrose medium (weight by volume in 1L 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 on a 12:12 h light:dark cycle at room temperature (22–25 °C). All female flies were of the wild-type strain Canton S (CS). Males used to assess the persistence of post-mating immune suppression were also CS. In experiments to test the effects of multiple matings, males were either *CS pBac{3xP3-EGFP, ProtB-EGFP}16B*) (Manier et al., 2010) or CS.

2.2. Infection Procedure:

All female flies were infected with the Gram-negative bacterium *Providencia rettgeri,* a natural bacterial pathogen of *Drosophila melanogaster* with moderate pathogenicity (Galac & Lazzaro, 2011; Juneja & Lazzaro, 2009). *D. melanogaster* resistance to *P. rettgeri* infection is reduced by mating (Short & Lazzaro, 2010). For all experiments described, bacterial cultures were started from a single colony of *P. rettgeri* picked from a Luria Bertani (LB) – agar plate to begin an overnight LB culture at 37 °C. The following morning, the saturated overnight culture was diluted 1:3 and grown for 3 h at 37 °C. This new growth culture was centrifuged for 5 min at 3000 rpm and resuspended in ~ 600uL of sterile phosphate-buffered saline (PBS). The bacterial resuspension was diluted in sterile PBS to a working solution of A_{600} = 1.0.

All females were anesthetized with CO_2 during infection. Sterilely wounded control flies were pierced in the thorax with a 0.15 mm anodized steel needle. Infected flies were pierced with the same needle dipped into the dilute bacterial culture. For a demonstration of this technique, see (Khalil et al., 2015). Following infection, all flies were placed on fresh food.

2.3. Experimental set ups

2.3.1. Persistent effects of mating on immune defense

For each block of the experiment, female CS flies were collected within 8 h of eclosion from the pupal case and separated from males before they were sufficiently mature to mate. Females were aged for 3 days in groups of ~ 10 per vial and then split equally into five treatment groups corresponding to the number of days after mating when infection would be delivered: unmated, 2 days, 4 days, 7 days, and 10 days. The experiment was designed such that all females were age-matched (14 days post-eclosion) when infected, and all females in each block were infected on the same day (Fig. 1A). On the fourth day of the experiment (10 days prior to infection), 15 CS males (3–5 days post-eclosion) were added to vials of females designated for the 10 days mating treatment. The males were left with the females for 8 h and then removed. On the eighth day of the experiment (7 days prior to infection), 15 CS males (3–5 days post-eclosion) were added to the vials of females designated

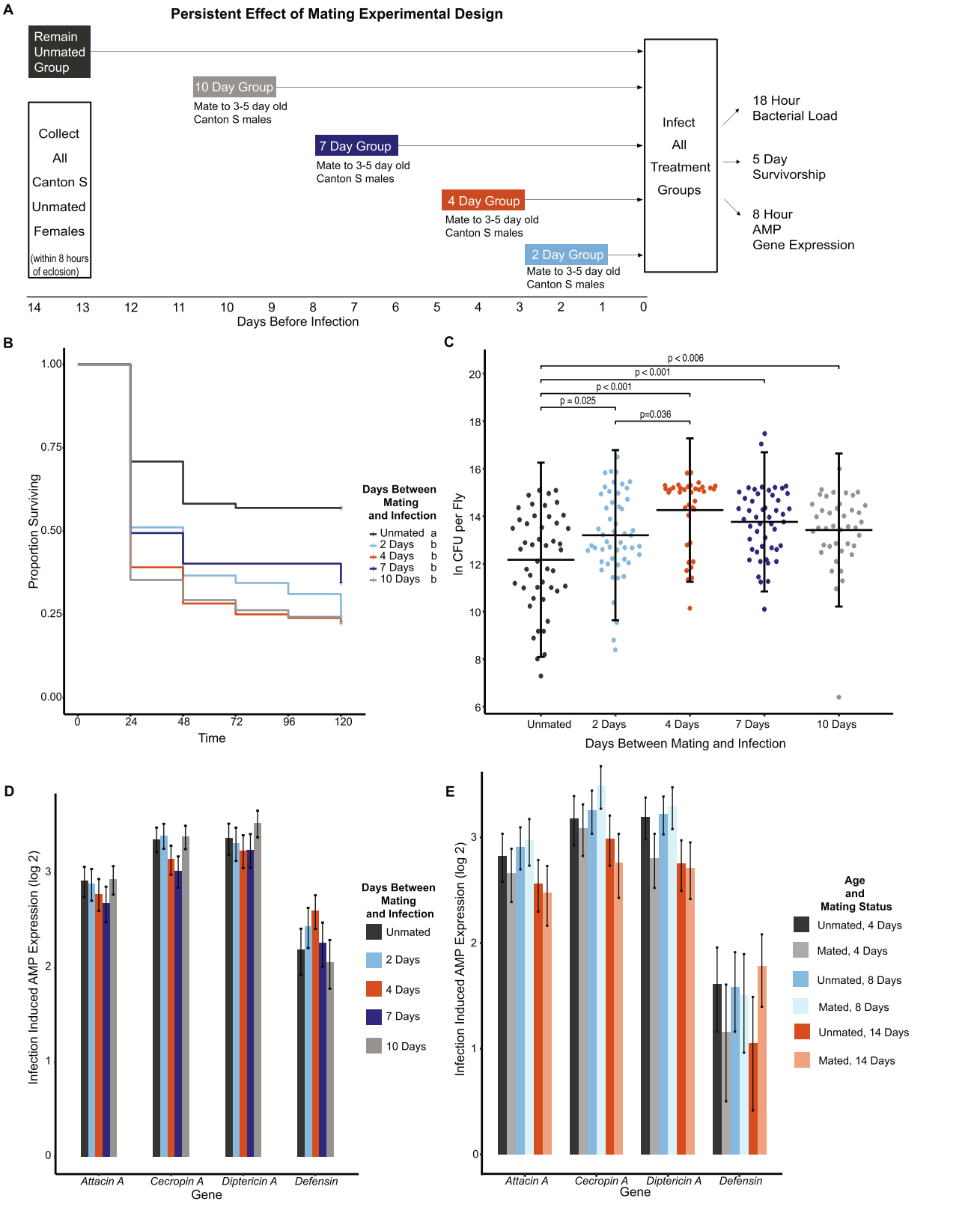

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Fig. 1. Mating suppresses immune defense for up to 10 days after mating. (A) Design for experiments testing the persistence of immune suppression after mating. All females were age-matched and infected on the same day. The four intervals between mating and infection were 2, 4, 7, and 10 days. An additional group remained unmated throughout the experiment. (B) All mated females had significantly lower survivorship than unmated females (n = 79) regardless of the interval between mating and infection (2 day interval, $p = 0.001$, n = 90; 4 day interval, $p < 0.001$, n = 92; 7 day interval, $p = 0.026$, n = 87; 10 day interval, $p < 0.01$, n = 99). There was no statistical difference in survivorship between the four mated groups. Statistical significance of all pairwise comparisons are represented by letter. The graph illustrates combined data from two biological-replicate blocks, which did not significantly differ. (C) All mated females had significantly higher bacterial loads than unmated females (n = 47) regardless of interval between mating and infection (2 day interval, $p = 0.025$, n = 51; 4 day interval, $p < 0.001$, n = 37; 7 day interval, $p < 0.001$, n = 49; 10 day interval, $p = 0.006$, n = 41). Mated females had similar bacterial loads, regardless of the interval between mating and infection, except 2 day and 4 day intervals ($p = 0.036$). Only significant differences are represented with brackets. Each data point represents a single female. (D) Infection-induced expression of AMP genes in unmated, 2 day, 4 day, 7 day, and 10 day interval mated females. Infection of mated and unmated females induces AMP gene expression statistically equivalently ($p > 0.05$), regardless of the interval between mating and infection. (E) No statistically significant ($p > 0.05$) differences in AMP gene expression we observed between 4 day, 8 day, and 14 day post-eclosion females, regardless of mating status.

for the 7 days mating treatment, left for 8 h, and then removed. This process was repeated on the eleventh and thirteenth days of the experiment for the 4 days and 2 days mating treatment groups, respectively. All treatment groups were infected on the same day (the 15th day of the experiment) and placed on fresh food. Every vial of every treatment group (including unmated) was transferred to fresh food every 3 days.

2.3.2. Effects of multiple matings on immune defense

On the first day of the experiment, female CS flies were collected within 8 h of eclosion. Flies were allowed to mature in single sex groups of 15–20 for three days. On the fourth day of the experiment, all females were briefly anesthetized with CO_2, placed in individual vials, and separated into unmated, one-mating, or two-mating treatment groups (Fig. 2A). On the fifth day, a single three-day-old *CS pBac{3xP3-EGFP, ProtB-EGFP}16B* male was aspirated into each of the two-mating treatment vials. Vials were observed and any females who failed to mate within 1 h were discarded. After mating ceased, males were removed by either aspirating them out of the vial or crushing them with a flat metal stick. On the sixth day of the experiment, two three-day-old CS males were aspirated into the two-mating treatment vials and left overnight to provide the female with an opportunity for a second mating. Simultaneously, one three-day-old CS male was aspirated into each of the one-mating treatment vials. On the seventh day of the experiment, all males were aspirated out or crushed within the vial. Thus, the timing of mating in the one-mating treatment matches the timing of the second mating in the two-mating treatment. The following day, females from all treatment groups were infected in random order with *P. rettgeri* and placed into fresh food vials. Male flies were discarded.

In the two-mating treatment group, the first male was homozygous for a transgene that expressed the dominant marker EGFP in his eyes and the heads of his sperm. Therefore, all of his progeny also expressed EGFP in their eyes. The second male was of the strain CS, so all of his progeny had wild type eyes. Females in this experiment were confirmed to have mated twice by the presence of progeny from both males. Over all blocks of the experiment, 85% of the two-matings and 94% of the single-matings were successful.

2.4. Phenotypes measured and analysis

2.4.1. Survivorship assay

The number of living flies was recorded every 24 h for 5 days after bacterial infection. Control flies that were sterilely wounded but not infected rarely died (~10% across all survivorship experiments).

For the experiments measuring the persistence of the mating effect, survivorship was performed in two experimental blocks. Block one sample sizes: $n_{unmated} = 27$; $n_{10\ days} = 41$; $n_{7\ days} = 31$; $n_{4\ days} = 33$; $n_{2\ days} = 33$. Block two sample sizes: $n_{unmated} = 52$; $n_{10\ days} = 58$; $n_{7\ days} = 56$; $n_{4\ days} = 59$; $n_{2\ days} = 57$. To assess the effect of our different mating treatments, we used a mixed effect Cox proportional hazards model, where mating treatment is a fixed effect and experimental block was considered to be a random effect. Experimental blocks did not vary significantly in the model. The model was used to perform all pairwise comparisons with a Tukey p-value correction. All statistical analyses were performed in R Studio (R version 3.4.2).

The experiment that tested the effects of multiple matings was performed in two replicate blocks. Block one sample sizes: $n_{unmated} = 42$; $n_{one\text{-}mating} = 40$; $n_{two\text{-}matings} = 26$. Block two sample sizes: $n_{unmated} = 37$; $n_{one\text{-}mating} = 36$; $n_{two\text{-}matings} = 28$. To test for differences in post-infection survival among the mating treatments, we used a mixed effect Cox proportional hazards model, with mating treatment as a fixed effect and experimental block as a random effect. When blocks were analyzed separately, the pattern of response for each mating treatment was the same, so the two blocks were combined. The model was used to perform all pairwise comparisons with a Tukey p-value correction.

2.4.2. Bacterial load

For all experiments, bacterial load was measured 18 h after infection. Single flies were briefly anesthetized and homogenized in 500uL of sterile PBS. Homogenates were stored at 4 °C for up to 48 h before plating. Homogenates were diluted 1:100 with sterile PBS. Fifty microliters of diluted homogenate was spiral-plated onto LB-agar plates using a WASP2 spiral plater (Microbiology International) and incubated overnight at 37 °C. 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 colony forming units (CFU) in the plated sample. Homogenates were retained at 4 °C after plating, and samples that grew fewer than 20 colonies were re-plated without dilution in PBS and re-counted. Samples where colonies grew too densely to be resolved in the initial plating were re-plated with a 1:1000 dilution in PBS, then recounted. Control flies that were sterilely wounded but not infected never yielded any colonies. For experiments to test the persistent effects of mating, bacterial load was measured in one experimental block. Block one sample sizes: $n_{unmated} = 47$; $n_{10\text{-}day} = 41$; $n_{7\text{-}day} = 49$; $n_{4\text{-}day} = 37$; $n_{2\text{-}day} = 51$. The plate count data were natural log transformed and an ANOVA was applied to determine the effect of mating treatment:

$$\log_e(\text{count/mL}) = \text{mating treatment}$$

We used Tukey's test to make pairwise comparisons between treatment groups and correct for multiple comparisons.

For experiments to test the effects of multiple matings, bacterial load was measured in two experimental blocks. Block one sample sizes: $n_{unmated} = 46$; $n_{one\text{-}mating} = 43$; $n_{two\text{-}matings} = 45$. Block two sample sizes: $n_{unmated} = 37$; $n_{one\text{-}mating} = 32$; $n_{two\text{-}matings} = 28$. The plate count data were natural log transformed and an ANOVA was applied to determine the effect of mating treatment and experimental block:

$$\log_e(\text{count/mL}) = \text{mating treatment} + \text{block}.$$

We used Tukey's test to make pairwise comparisons between treatment groups and correct for multiple comparisons.

2.4.3. Immune system activity

Immune system activity was assessed by measuring the expression of four genes encoding AMPs (*Attacin A, Cecropin A, Defensin, and Diptericin A*) normalized to the expression of the housekeeping gene *Actin 5C* in

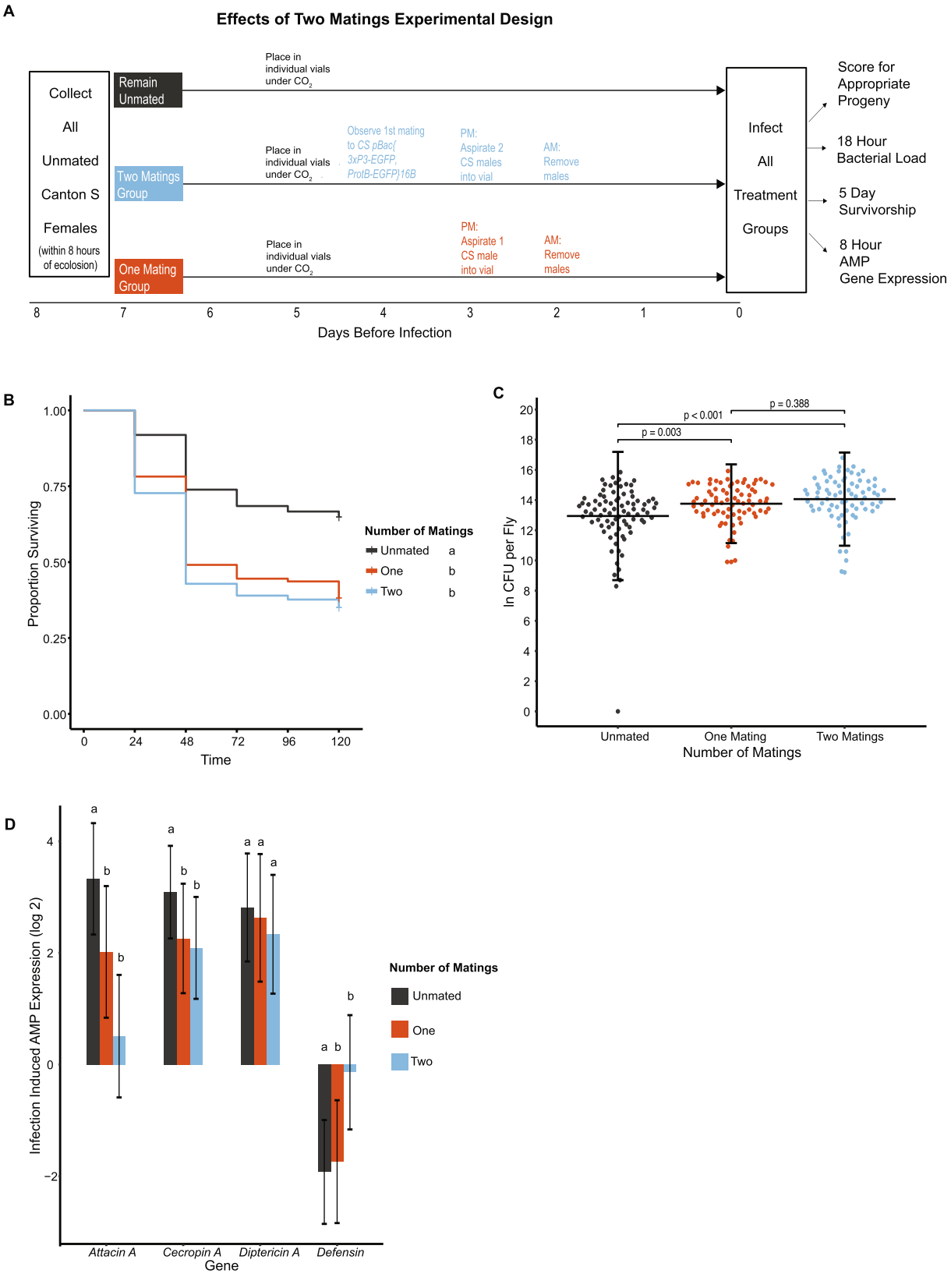

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Fig. 2. (A) Experimental design for experiments testing the effects of a second mating event on immune suppression. All females were age matched and infected on the same day. Females mated twice were first mated to *CS pBac{3xP3-EGFP, ProtB-EGFP}16B* (Manier et al., 2010), then CS. Females mated once were mated only to CS males. One group of females remained unmated throughout the experiment. (B) Mated females exhibited significantly lower survivorship than unmated (n = 79) females (two matings, $p = 1 \times 10^{-4}$, n = 55; one mating, $p = 0.005$, n = 76), with no statistically-significant difference in survivorship between the two matings and one mating groups ($p = 0.213$). Statistical significance of all pairwise comparisons are represented by letter. The graph illustrates combined data from two biological-replicate blocks. (C) Females mated once and twice both had significantly higher bacterial load than unmated (n = 83) females. (two matings, $p < 0.001$, n = 73; one mating, $p = 0.003$, n = 75). There was no difference in bacterial load between females mated once or twice ($p = 0.388$). Each data point represents a single female. Plots represent combined data from two independent blocks. (D) Infection increased the expression of AMPs relative to the control gene, *Actin5C*. Unmated females had higher levels of *Attacin A* and *Cecropin* transcripts (standardized by *Actin 5C* expression) than females mated either once or twice but had lower levels of *Defensin* transcripts than mated females. Unmated and mated females expressed similar levels of *Diptericin A* transcripts, regardless of whether they were mated once or twice.

both infected and uninfected females (see Supplementary Table A.1). Uninfected control females were anesthetized for the same length of time as the infected females but were not pricked with the needle. This allows us to measure expression of these AMP genes induced by both wounding and infection. In all gene expression experiments, groups of 7 females were pooled 8 h after infection and/or anesthetization and frozen at −80 °C. RNA was extracted using a TRIZOL/chloroform extraction method according to the manufacturer's instructions (Invitrogen) and purified by ethanol precipitation. RNA amounts and purity were assessed using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. Approximately 2ug of RNA was treated with DNase then reverse-transcribed to cDNA using M−MLV reverse transcriptase (Promega). Quantitative reverse transcription-polymerase chain reactions (qRT-PCR) were performed on a CFX Connect Real-Time Detection System (Bio-Rad) using the SsoAdvanced SYBR Green Supermix (Bio-Rad).

For the experiment testing the persistence of immune suppression, biological replicates (pools of 7 flies each) of females were collected in one block. Block 1 sample sizes: $n_{infected,\ unmated} = 6$; $n_{infected,\ 10\text{-}day} = 6$; $n_{infected,\ 7\text{-}day} = 6$; $n_{infected,\ 4\text{-}day} = 6$; $n_{infected,\ 2\text{-}day} = 6$; $n_{uninfected,\ unmated} = 5$; $n_{uninfected,\ 10\text{-}day} = 6$; $n_{uninfected,\ 7\text{-}day} = 6$; $n_{uninfected,\ 4\text{-}day} = 6$; $n_{uninfected,\ 2\text{-}day} = 6$.

To test for differences in infection induced gene expression between each of the five mating treatment groups, the following model was applied to the average of all biological replicates to achieve a corrected Ct value for each AMP gene expression measured:

$$Y_{ij} = \mu + Actin5C + \text{Mating Treatment}_i + \text{Infection Status}_j + \text{Mating Treatment}_i\text{*Infection}_j$$

Mating Treatment (i = 1,5) and Infection Status (j = 1,2) were treated as fixed effects. From these models, the least squares means for the Mating Treatment × Infection interaction terms were extracted. We then subtracted the least-squares mean value for uninfected flies from the least-squares mean estimates for the infected flies in each class. This difference indicates the $\log_2$ change in AMP gene expression level after infection, hereafter referred to as infection-induced expression. We performed marginal means comparisons of these infection-induced expression values between each pair of the five mating treatment groups with Tukey's corrections for p-values. These statistics were shown in a compact letter display where non-statistically significantly different ($p > 0.05$) infection-induced expression levels are assigned the same letter.

For the experiment testing the effect of multiple matings, because success of the mating had to be determined several days after infection, all flies were first frozen individually in a 96 well plate then subsequently pooled into groups of 7 flies. Block 1 sample sizes: $n_{infected,\ unmated} = 8$; $n_{infected,\ one\text{-}mating} = 8$; $n_{infected,\ two\text{-}matings} = 7$; $n_{uninfected,\ unmated} = 3$; $n_{uninfected,\ one\text{-}mating} = 3$; $n_{uninfected,\ two\text{-}matings} = 3$.

To test for differences in infection induced gene expression between each of the three mating treatment groups, the following model was applied to the average of all biological replicates to achieve a corrected Ct value for each AMP gene expression measured:

$$Y_{ij} = \mu + Actin5C + \text{Mating Treatment}_i + \text{Infection Status}_j + \text{Mating Treatment}_i\text{*Infection}_j$$

Mating Treatment (i = 1,3) and Infection Status (j = 1,2) were treated as fixed effects. From these models, the least squares means for the Mating Treatment × Infection interaction terms were extracted. We then subtracted the least-squares mean value of uninfected flies from the least-squares mean estimates for the infected flies in each class to calculate infection-induced differential expression. We performed marginal means comparisons of these infection-induced expression values between each pair from the three mating treatment groups with Tukey's corrections for p-values. These statistical groupings are shown in a compact letter display where non-statistically significantly different ($p > 0.05$) infection-induced expression levels are assigned the same letter.

An additional experiment was performed to determine the effect that a female's age has on the expression of these AMP genes. Unmated CS females were collected 14, 8, and 4 days prior to infection. Half of each age group was mated to CS males while the other half remained unmated. Pools of 7 uninfected or infected females were collected as described above. Block 1 sample sizes: $n_{infected,\ mated\ 14\text{-}day} = 5$; $n_{infected,\ mated\ 8\text{-}day} = 5$; $n_{infected,\ mated\ 4\text{-}day} = 5$; $n_{uninfected,\ mated\ 14\text{-}day} = 5$; $n_{uninfected,\ mated\ 8\text{-}day} = 5$; $n_{uninfected,\ mated\ 4\text{-}day} = 4$; $n_{infected,\ unmated\ 14\text{-}day} = 5$; $n_{infected,\ unmated\ 8\text{-}day} = 5$; $n_{infected,\ unmated\ 4\text{-}day} = 4$; $n_{uninfected,\ unmated\ 14\text{-}day} = 5$; $n_{uninfected,\ unmated\ 8\text{-}day} = 5$; $n_{uninfected,\ unmated\ 4\text{-}day} = 5$.

To test for differences in infection-induced gene expression between each of the six mating treatment groups (age and mating status), the following model was applied to the average of all biological replicates to achieve a corrected Ct value for each AMP gene expression measured:

$$Y_{ij} = \mu + Actin5C + \text{Mating Treatment}_i + \text{Infection Status}_j + \text{Mating Treatment}_i\text{*Infection}_j$$

Mating Treatment (i = 1,6) and Infection Status (j = 1,2) were treated as fixed effects. From these models, the least squares means for the Mating Treatment × Infection interaction terms were extracted. We then subtracted the least-squares mean value for uninfected flies from the least-squares mean estimates for the infected flies in each class to estimate infection-induced differential expression. We performed marginal means comparisons of these infection-induced expression values between every pair of the six mating treatment groups with Tukey's corrections for p-values. These statistics were shown in a compact letter display where non-statistically significantly different ($p > 0.05$) infection-induced expression levels are assigned the same letter.

2.4.4. Data availability

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

3. Results

3.1. Immune suppression persists for at least 10 days after mating

Previous studies showed that females were less resistant to bacterial infection as soon as 2.5 h and for as long as 26.5 h after mating (Fedorka et al., 2007; Short et al., 2012; Short & Lazzaro, 2010). To test whether mating causes a longer-term, more persistent decrease in female immune defense or whether females can eventually recover to unmated levels of immunity, we extended the interval between a single mating and infection to 2, 4, 7, or 10 days (Fig. 1A). We assessed immune

defense in mated and unmated females in three ways: 5-day survivorship post-infection, bacterial load measured at 18 h post-infection, and AMP mRNA levels measured at 8 h post-infection. For each of the four intervals between mating and infection, we found that all mated females had significantly lower survivorship than unmated females (Fig. 1B; Cox proportional hazards model, pairwise comparisons between unmated and mated treatment groups, Tukey's p-value correction: 2 day interval, $p = 0.001$; 4 day interval, $p < 0.001$; 7 day interval, $p = 0.026$; 10 day interval, $p < 0.01$). There were no statistically significant differences in survivorship among each of the four intervals between mating and infection (2 and 4 day intervals: $p = 0.889$, 2 and 7 day intervals: $p = 0.903$, 2 and 10 day intervals: $p = 0.771$, 4 and 7 day intervals: $p = 0.392$, 4 and 10 day intervals: $p = 0.999$, 7 and 10 day intervals: $p = 0.259$). Additionally, mated females at all four intervals had higher bacterial loads than unmated females at 18 h post-infection (Fig. 1C; Tukey's pairwise comparison on ANOVA; 2 day interval, $p = 0.025$; 4 day interval, $p < 0.001$; 7 day interval, $p < 0.001$; 10 day interval, $p = 0.006$). Bacterial loads among each of the four intervals between mating and infection were not statistically different from each other (2 and 7 day intervals: $p = 0.468$, 2 and 10 day intervals: $p = 0.973$, 4 and 7 day intervals: $p = 0.670$, 4 and 10 day intervals: $p = 0.195$, 7 and 10 day intervals: $p = 0.876$) except for between the 2 day and 4 day intervals ($p = 0.036$).

Short et al. (2012) reported that females mated 2.5 h prior to infection express lower levels of AMP mRNA at 4 h and 12 h post-infection than unmated females do. By 24 h post-infection, bacterial loads of mated females exceeded those of unmated females and AMP mRNA levels became higher in mated females than in unmated females (Short et al., 2012). After establishing that mating causes a persistent, long-term effect on female survivorship and 18 h post-infection bacterial load, we asked in our current study whether the interval between mating and infection impacted the inducibility of the immune system. We used qRT-PCR to measure the infection-induced mRNA levels of four AMP genes (*Attacin A*, *Cecropin A*, *Defensin*, and *Diptericin A*) at 8 h post-infection in females representing each of the mating-to-infection intervals and in unmated females. Because our Gram-negative pathogen, *P. rettgeri,* activates both the Imd and Toll pathways (Troha et al., 2018), we selected these genes to capture immune signaling from both pathways. We observed significantly induced expression of all AMP genes in response to infection in every mating treatment. However, the magnitude of this induction was statistically equivalent between mated and unmated females in all cases (Fig. 1D, Supplementary Table A.5). This result was initially surprising given the clear reduction in survivorship and increase in pathogen burden in mated female flies. Prior experiments that detected a reduction in mated female AMP mRNA levels post-infection used female flies that were 3–5 days post-eclosion (Short et al., 2012). However, because we needed to test an extended interval between mating and infection in our current study, all female flies in the current experiment are 14 days post-eclosion. Genes encoding AMPs and other immune and stress response proteins are often upregulated as flies age (reviewed in Garschall & Flatt, 2018). We thus hypothesized that the older flies in the present study might already be expressing AMP genes at a higher baseline level prior to infection than the 3–5 days post-eclosion flies in Short et al. (2012). A higher baseline expression might then limit infection-induced expression and could mask differences between mated and unmated AMP gene expression. To test this hypothesis, we measured infection-induced mRNA levels of the same four AMP genes relative to *Actin 5C* in unmated and mated female flies at 4, 8, or 14 days post-eclosion. While we observed no difference in infection-induced mRNA level for any AMP gene between unmated and mated females at each of the three age groups (Fig. 1E), we noticed that the level of induction of AMP mRNA was lower at 14 days post-eclosion than at 4 or 8 days post-eclosion in both unmated and mated females. Consistent with this observation, uninfected females at 14 days post-eclosion had higher normalized baseline expression of AMP mRNA than uninfected females at 4 or 8 days post-eclosion (Supplementary Table A.10), so were starting from an initially higher level before reaching an apparent maximum.

3.2. Testing the effects of a second mating on immune suppression

Because immune suppression after mating is due to the transfer of seminal fluid proteins (Schwenke & Lazzaro, 2017; Short et al., 2012), we asked whether immune suppression would be greater in females who mated more than once and thus received multiple doses of seminal fluid and associated proteins. To test this, we contrasted 5-day survivorship post-infection, bacterial load measured at 18 h post-infection, and AMP mRNA levels measured at 8 h post-infection between unmated females, females mated once, and females mated twice (Fig. 2A).

We found that mated females were significantly less likely to survive infection than unmated females regardless of whether they were mated once or twice (Fig. 2B, Cox proportional hazards model, pairwise comparisons, Tukey's p-value corrections; two matings, $p = 1 \times 10^{-4}$; one mating, $p = 0.005$). There was no difference in survivorship between females mated once and females mated twice ($p = 0.213$). Similarly, mated females had higher bacterial loads than unmated females (Fig. 2C, two matings, $p < 0.001$; one mating, $p = 0.003$). Again, there was no difference in bacterial load in females mated once versus twice ($p = 0.388$).

Many studies have reported that mating and the receipt of seminal fluid proteins increases the expression of genes encoding antimicrobial peptides (Delbare et al., 2017; Fedorka et al., 2007; Innocenti & Morrow, 2009; Lawniczak & Begun, 2004; Mack et al., 2006; Peng et al., 2005b; Wigby et al., 2008). However, mated females show slower induction of AMP expression than unmated females in the first 12 h after infection (Short et al., 2012). Here, we tested whether expression levels of our four selected AMP genes measured at 8 h after infection differed between unmated females, females mated once, and females mated twice. Unmated females showed higher infection-induced expression of *Attacin A* and *Cecropin* than females mated either once or twice, but showed lower levels of *Defensin* expression. Unmated and mated females exhibited similar infection-induced expression of *Diptericin A*, regardless of whether they were mated once or twice (Fig. 2D).

4. Discussion

Mating and receipt of seminal fluid proteins induce both short- and long-term changes in female physiology and behavior (reviewed in Avila et al., 2011; Perry et al., 2013). When systemically infected with bacteria, mated females exhibit reduced immune defense relative to unmated females (Fedorka et al., 2007; Short et al., 2012; Short & Lazzaro, 2010). Short et al. 2012 showed the onset of this immune suppression is rapid, with mated female immune defense declining as quickly as 2.5 h after mating. Mated females remained less likely to survive infection with greater pathogen burdens for 26.5 h after mating, but there was no test of whether mating causes longer-term suppression of female immune defense. Here, we showed that female immune defense has two binary states (unmated and mated), as females remain less likely to survive infection and carry higher pathogen loads than unmated females when infected up to 10 days after mating. Additionally, previous studies investigated the effects of a single mating on female immune defense. However, female *Drosophila* can mate with multiple males and therefore receive multiple doses of seminal fluid proteins. Additionally, previous studies have shown that multiple mating events (Fowler & Partridge, 1989) and even specifically repeated exposures to SP (Wigby & Chapman, 2005) can decrease female fitness and lifespan. Again, we showed that female immune defense has two binary states (unmated and mated), as mated females survived at lower levels with higher pathogen burdens than unmated females, regardless of whether they were mated once or twice. Twice-mated females were not more susceptible to infection than once-mated females, suggesting that an additional mating event or dose of seminal fluids does not further

suppress female immune defense. Together, the results of our experiments suggest that mating and receipt of seminal fluids cause a binary shift in female immune defense to a suppressed state. The mechanisms controlling and maintaining the shift from unmated to mated immune state remain to be elucidated.

Short et al. (2012) found that along with decreases in survivorship and increases in pathogen burden, mated females were slower than unmated females to induce the expression of AMP genes after infection. In the present study, we also observed a mating-driven decrease in survivorship and increase in bacterial load at every mating-to-infection interval. However, in contrast to Short et al. (2012), we saw little or no difference in the induction of AMP gene expression between mated and unmated females. Previously reported effects of mating on infection-induced immune gene transcription in *Drosophila* have been small and variable (Gupta et al., 2022; Rodrigues et al., 2021; Short et al., 2012; Short & Lazzaro, 2013), and any transcriptional effects may be amplified and compounded by a reduction in post-infection protein translation and AMP production in mated females (Gupta et al., 2022). Two recent papers modeling pathogen population growth and host immune induction found that small variations in the timing of immune activation at early stages of infection could result in large differences in probability of host survival (Duneau et al., 2017; Ellner et al., 2021). Delay or weakening in activation of AMP production due to transcriptional or translational impairment could therefore explain the dramatic decrease in survivorship and increase in pathogen loads in mated females relative to those of unmated females.

In our analysis of 4, 8, and 14-day old unmated and mated females, we found that the magnitude of infection-induced AMP gene expression assayed at 8 h after infection decreases in the oldest female flies because the baseline AMP expression level was higher in older females prior to infection. Studies on immune senescence in *Drosophila* have shown that AMP gene expression generally increases with age, possibly due to age-induced chronic inflammation (reviewed in Garschall & Flatt, 2018). Yet despite an overall increase in AMP gene expression, several studies have shown that older flies suffer worse infection outcomes than younger flies (Ramsden et al., 2008; Zerofsky et al., 2005). For example, Zerofsky et al (2005) found that while older flies induce higher levels of *Diptericin* mRNA than younger flies, older flies are delayed in their response, putting them at a disadvantage in combating infection. While the oldest flies we examined (14 days post-eclosion) were not senescent, the higher baseline levels (in uninfected flies) explain why females at 14 days post-eclosion have lower levels of induction of AMP mRNA in response to infection than females at 4 or 8 days post-eclosion. However, the small differences in level of induction of AMP mRNA between mated and unmated female flies of all ages again seems insufficient to explain the dramatic differences we observe in post-infection survivorship and bacterial load, and we suggest these small effects on mRNA expression level are probably amplified by differences in capacity for translation (Gupta et al., 2022).

The complete set of interactions between mating-triggered changes in female physiology and immune system function is not well understood. Our data is consistent with a hypothesis that long-term suppression of female immune defense is maintained by retention of sperm and associated SP. Consistent with this hypothesis, at our longest interval between mating and infection (10 days) females contain stored sperm (Kaufman & Demerec, 1942) and SP (S. Misra, personal communication). An alternative, but not mutually exclusive, explanation for the long-term immune effects that we report here is that SP could trigger secondary mechanisms that sustain suppression of female immune defense. Previous literature provides some support for this possibility. First, SP stimulates synthesis of JH, which is suppressive to the immune response (Flatt et al., 2008; Schwenke & Lazzaro, 2017). Schwenke and Lazzaro (2017) showed that applying the JH analog methroprene to unmated females mimics the effects of mating on immune defense. Second, sterile females are not immune suppressed. Short et al. (2012) showed that females without a germline, who produce no eggs, do not suffer a decrease in immune defense after mating. Another study, Fedorka et al. (2007) showed that equal proportions of mated and unmated sterile ovo^{D1} females survive an infection given 3 h or 9 h after mating. Together, these studies suggest changes in female hormone levels and elevated egg production triggered by mating limits post-mating immune defense. Still, specific interactions between JH and/or egg production related pathways and the immune system have yet to be identified.

In the present work, we establish that a single mating is sufficient and necessary to shift *Drosophila* female immune-system physiology from an unmated to a mated state, resulting in suppression of the immune defense for at least 10 days after mating. These findings reveal more about the dynamics of the physiological trade-off between mating and immunity in female *D. melanogaster* and inform future studies of the mechanisms controlling and conditions under which the physiological trade-off occurs. Identifying the mechanisms driving physiological trade-offs may allow us to understand how *D. melanogaster* and other insects evolve to maximize fitness while balancing costs to immune defense and reproductive capacity.

CRediT authorship contribution statement

Kathleen E. Gordon: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Mariana F. Wolfner:** Conceptualization, Methodology, Writing – review & editing. **Brian P. Lazzaro:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

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Data statement

All data files and code are available at https://github.com/WolfnerLab/Persistent_Mating_GordonK.git.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2022.104414.

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