***Drosophila melanogaster* survival after infection with *Beauveria bassiana* depends on sex, mating status, and diet**

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What is already known: *Drosophila melanogaster* are sexually dimorphic in their response to several bacterial pathogens and to the fungal pathogen *Beauveria bassiana*, with females dying faster from infection compared to males. Mated females die faster from infection compared to virgin females when inoculated with several bacterial pathogens. This mating-immunity tradeoff is thought to be a female trait, resulting from female exposure to male ejaculate.

What this study adds: We challenge the previously commonly held notion that trade-offs between immune defense and mating are a female trait in *Drosophila melanogaster*. Instead, we show that a mating-immunity trade-off exists in both males and females. Mated males and females die faster after inoculation with *B. bassiana* than virgins, and continuous mating further reduces survival compared to one-time mating. Moreover, we challenge the previous observation that females are more susceptible to infection compared to males. Instead we show that whether females or males die faster from infection depends on their mating statuses, specific fungal strain, and fly genotype. Lastly, we show that survival after fungal infection is largely influenced by diet, and that post-infection dietary improvements can help enhance survival.

**Abstract**

Post-mating immunosuppression has been widely accepted as a female trait in *Drosophila melanogaster*. In experiments that use bacterial pathogens, it is shown that mating suppresses immune defense in females, but not in males. Our results challenge this notion by presenting a mating-immunity trade-off in males as well as in females. When inoculated with the fungal pathogen, *Beauveria bassiana*, both males and females die faster compared to inoculated virgins, and survival is lower when inoculated flies are continuously mated compared to a single day of mating. Past studies with *Beauveria bassiana* have shown females to be more susceptible to infection than males. Our results challenge this finding as well, showing that the direction of sexual dimorphism in immune defense depends on mating status, specific *Beauveria bassiana* strain, and fly genotype. Moreover, we show that survival after fungal infection is largely influenced by diet, and that post-infection dietary improvements can help enhance survival. Post mating suppression in *Drosophila* survival of *B. bassiana* infection presents study opportunities with potential applications for biological control of insect vectors of human disease and insect crop pests.

# Introduction

Mosquito disease vectors can be catalysts for the spread of malaria, dengue fever, and other devastating diseases within human populations (Heinig et al. 2015; Robert et al. 2016). The entomopathogen *Beauveria bassiana*, which grows naturally in soil and infects a broad range of insect hosts, has been demonstrated to have potential uses against mosquito disease vectors as well as insect crop pests that threaten food security (Feng and Pu 2005,Heinig et al. 2015; Id et al. 2019), and even bed bugs (Barbarin et al. 2012).

*Drosophila melanogaster*, the laboratory fruit fly, has an immune system that is similar to insect disease vectors and pests, contributing to its value as a model organism. Moreover, the *D. melanogaster* immune system has many similarities to the mammalian innate immune system (Hoffmann and Reichhart 2002). Similar to human skin, the fly cuticle provides a physical barrier of defense against pathogens. Upon pathogen entry into the hemocoel, a humoral response may be activated, largely through expression of genes in the Toll pathway, leading to the production of antimicrobial peptides (Buchon et al. 2014). Cellular responses, such as phagocytosis (Pham et al. n.d.) and melanization (Tang 2009), also help to ward off pathogens. The ease of maintenance and short life cycle of *D. melanogaster* make them an optimal candidate for experiments involving many organisms and multiple replicates.

*D. melanogaster* exposure to *B. bassiana* generally happens on the cuticle surface. Upon contact with the cuticle, spores germinate and penetrate the cuticle, growing as hyphae inside the host, and later sporulating on the cadaver (Ortiz-Urquiza and Keyhani 2013). Female *D. melanogaster* are more susceptible than males to five tested strains of *B. bassiana* (Shahrestani et al. 2018). This sexual dimorphism is ablated by mutations in the Toll pathway, and by mutation of the gene *Relish*, which is in the IMD pathway (Shahrestani et al. 2018). The IMD pathway has classically been thought to be involved in fighting Gram negative bacteria, but not fungi, whereas the Toll pathway is known to be involved in fighting Gram positive bacteria and fungi (Buchon et al. 2014). Therefore, this result suggests not only that the sexual dimorphism in defense is, at least in part, controlled by humoral defenses, but also that defense against fungal infection is more complex than previously thought, involving at least one gene in the IMD pathway. Better understanding sexual dimorphism in insect defense against *B. bassiana* can be important for developing effective biological control efforts that can target reproductive female insects.

Sexual dimorphism in post-infection survival of D. melanogaster has also been seen with the bacterial pathogens *Providencia rettger*i and *Providencia alcalifaciens* (Short and Lazzaro)*.* With these pathogens, not only are females more susceptible than males, but mated females are more susceptible than virgin females (Short and Lazzaro)*,* with inoculated mated females surviving less than infected virgins and carrying higher bacterial loads (Short and Lazzaro n.d.). Given this mating-immunity trade-off, a potential explanation for the increased susceptibility to infection in females relative to males is that females allocate more resources to reproduction (Schwenke, Lazzaro, and Wolfner n.d.). Females are thought to enter a post-mating state, which is induced by receiving sex peptide from male seminal fluid (Wigby and Chapman 2005). These studies focused on the mating-immunity trade-off as being a female-specific trait. As such, virgin females are thought to have more male-like, or better, immune defense (Short and Lazzaro n.d.).

However, when comparing sexual dimorphism in defense against *B. bassiana* across 297 recombinant inbred fly lines (the DSPR lines of King et al. n.d.)), we found that the direction of the sexual dimorphism is genotype-dependent (unpublished). In some fly lines, females are more susceptible to infection than males, in some lines there is no sexual dimorphism, and in other lines males are more susceptible than females (unpublished). In these experiments, flies were mated with males and females cohabiting, suggesting that sexual dimorphism in immune defense is not caused by the suppression of immune defense in females due to receiving sex peptide during mating. It is possible that males also experience a mating-immunity trade-off. Indeed, McKean et al. (2001) showed evidence for a trade-off between male sexual activity and male immunity.

Here we show that both females and males exhibit a tradeoff between mating and post-infection survival. Flies that mated for 24 hours had worse post-infection survival than virgins, and flies that continuously mated throughout adulthood had even worse survival. Post-infection survival was also sexually dimorphic, but the direction of this dimorphism depended on mating status; among virgins and flies that mated continuously, females survived better than males, but among flies that mated for only 24 hours prior to infection, females were more susceptible to males. We also show that the direction of sexual dimorphism in post-infection survival depends not only on mating status, but also on fly genotype, fungal strain, and diet.

**Methods**

*Drosophila melanogaster Population*

Ninety-six *D. melanogaster* isofemale lines from five geographic areas (Greenberg et al. n.d.), Beijing (Begun, Aquadro, and Begun 1995), Netherlands (Bochdanovits and Jong 2003), Ithaca NY, Tasmania, and Zimbabwe (Begun and Aquadro 1993), were round-robin crossed, first per population, then across populations, making a genetically diverse population. We obtained the resulting megapopulation from the laboratory of Andy Clark at Cornell University. We then maintained a large population, named Population C3, with non-overlapping 14-day generations at population size of 2,000 flies at ~25° C, 12h light:12h dark cycles. For the past 72 generations, population C3 was maintained on a Cornmeal diet (12 g/L agar, 29 g/L yeast, 0.710 g/L cornmeal and 0.092 g/L molasses) and prior to that it was maintained on a Glucose diet (100g/L yeast, 100g/L glucose, 1% Drosophila agar). At the start of each experiment, flies were reared at densities of 60-80 eggs per vial on Cornmeal diet.

*Fungal Pathogen*

Two strains of the entomopathogenic fungus *Beauveria bassiana* were used: ARSEF 12460 (USDA Agricultural Research Service Collection of Entomopathogenic Fungi, Ithaca, NY), which was isolated from one *Drosophila melanogaster* that was infected with ARSEF 8246 (which itself was isolated from the shorefly, *Scatella tenuicosta*), and GHA, which was obtained from Mycotech, Inc. (now Bioworks, Inc., Victor NY, lot number TGA1-96-06B). Fungal spores were stored at -4° C. Prior to use, spores were allowed to warm to room temperature.

*Fungal Inoculation by Spray*

Flies were briefly anesthetized with CO2 and then moved onto Petri dishes placed on ice to maintain anesthesia while they were sprayed either with 5mL of a control fungal-free suspension of 0.03% Silwet in autoclaved DI water, or with 5mL of a fungal suspension of 0.3g of *Beauveria bassiana* spores (either ARSEF 12460 or GHA) suspended in 25 mL of 0.03% Silwet (Plant media, a division of bio world). Sprays were done using a custom-built spray tower (Vandenberg 1996). The spray dose was double checked by placing a microscope slide cover next to the flies during inoculation, then resuspending the slide and counting the number of spores in a 10uL suspension. Each spray introduced ~103 spores/mm2 onto the surface of anesthetized flies. After the spray, flies from each treatment were moved to separate acrylic cages (Volume: 450 cm³), fed with a Petri dish of fly medium, and kept at ~100% humidity for 24 hours. In high humidity conditions, fungal conidia germinate, and the hyphae penetrate the insect cuticle and grow in the hemocoel (Clarkson and Charnley 1996). After 24 hours, humidity was reduced to 60% for the duration of each assay.

*Mortality*

Dead flies were removed daily from all cages to avoid secondary inoculation of live flies by spores on the cuticles of the deceased flies and to record the numbers of dead females and males. Food plates were replaced with fresh ones daily.

*Experiment 1: Effects of mating and sex on post-infection survival of D. melanogaster when inoculated with B. bassiana strain ARESEF 12460*

To test the effects of mating on post-infection survival, three mating conditions (virgin, mated, and cohabit) were established as follows. To ensure virginity of flies, on day five from egg, third instar larvae were transferred from rearing vials into individual straws using a paint brush. The straws had Cornmeal food on one end and were sealed with pipette tips on both ends. On day 12 from egg (2-3 days post eclosion), emerged flies were sexed while still in their straws, and the food in the straw was replaced. On day 16 from egg, flies were moved from straws to vials at densities of 30 flies/vial, using brief CO2 anesthesia. At this stage, the 30 flies in the vial were either all male or all female for the virgin groups, or half male and half female for the mated and cohabiting groups. After 24 hours, on day 17 from egg, the flies were sprayed with fungus or control suspension. For each spray, 60 flies were anesthetized using carbon dioxide for 5 minutes on a CO2 pad to allow sorting by sex, then placed on ice for the ~2 minutes of the spray time. For the virgin groups, males and females were sprayed separately and kept in separate cages after the spray. For the mated groups, after 24 hours of mating in vials, males and females were sprayed separately and maintained in separate cages. Lastly, for the cohabiting groups, after 24 hours of mating in vials, males and females were sprayed together and cohabited in the same cages after the spray. Flies were in cages at densities of ~60 same sex flies/cage, or 30 males and 30 females per cage. At least 257 flies per sex per treatment were tested. Food plates in cages were replaced daily, and eggs laid on the plates by cohabiting and 24-hour mated females were kept and incubated for 7 days until pupation, at which point the number of pupae were counting as a proxy of offspring count. Mortality and fecundity were followed for 21 days post spray.

*Statistical Analysis of Experiment 1*

All analyses were performed in the statistical software R (<https://www>.r‑project.org/). To determine the effects of mating on the survival of female and male *D. melanogaster* when inoculated with the entomopathogenic fungus, *B. bassiana*, we used the Cox Proportional Hazard model (Cox n.d.) with a novel approach via Bootstrap for creating confidence intervals for survival probabilities of the model. Before building the model, we tested the difference among all the replicates by Kaplan-Meier survival function (Kaplan and Meier 1958) as well as log-rank test (Mantel 1966) and found no differences among the four replicates. To check the proportional hazards assumption, a scaled Schoenfeld residual was plotted and a test using the Schoenfeld residuals against the transformed time was conducted for each covariate. If any covariate broke the assumption, a time-dependent Cox PH model was proposed, in which the time splitting point was selected by a stratified model. With additional interaction terms, a best time-dependent Cox PH model was chosen by both likelihood test score and Akaike information criterion (Akaike 1998). To detect any influential outliers, every observation was assessed by its delta-beta value for each predictor of the best model. Influential outliers were kept in the model as we had no reason to think they resulted from error. The Cox PH model not only allows for hazard ratio estimates but also for predicted survival probabilities. Rather than constructing confidence intervals in a traditional method using standard errors, we resampled observations of each fly group 1,000 times with replacement to construct 95% confidence intervals for a linear combination of the covariates in the model.

To quantify the effect of mating on survival percent in the study, a time-dependent Cox Proportional Hazard model was proposed (Model 1).

. (Model 1)

In this Cox PH model, the 21-day experiment time was split into three time intervals: 0-5, 5-11, and 11-21. This transformed covariates Fungal, Mated, and Cohabit to time-dependent ones. Each time-dependent covariate has different coefficient estimates over the time intervals:

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The Cox PH model gave us a measure of the effect of mating on survival at any time point by looking at the hazard ratio estimates, and also compared survival probabilities over a span of 21 days visually.

We also investigated the effects of infection and mating status on offspring count via the analysis of variance (ANOVA)(Fisher 1932). We first calculated the number of offspring counts per surviving female fly and assessed the mean values of different groups of treatments and mating statuses. The analysis not only examined the main effects but also explored with the interaction effects between treatments and mating statuses. Based on the final model, the means with standard error and 95% confidence intervals were calculated. Also, multiple pairwise-comparison between means of groups were performed to indicate which groups were significantly different from others.

*Experiment 2: Sexual dimorphism in post-infection survival of D. melanogaster when inoculated with B. bassiana strain GHA*

On day 12 from egg, flies were transferred out of the rearing vials and into fresh Cornmeal vials in groups of 15 males and 15 females. To separate and count the flies by sex, we anesthetized them using a CO2 gun for 10 seconds and put them on the CO2 pad for ~3 minutes. Then on day 17 from egg, flies were inoculated with GHA or control suspensions. For each spray, 60 flies were anesthetized with CO2 for 15 seconds to immobilize them and placed on Petri dishes on ice for the ~2 minutes of the spray time. Males and females were sprayed together and cohabited together after the spray. Mortality was followed for 21 days post spray. At least 500 flies were tested per sex per treatment.

*Statistical analysis of Experiment 2:*

Sexual dimorphism in survival after GHA inoculation was analyzed similarly as in Experiment 1 (see Model 2).

(Model 2)

The time-dependent covariates have different coefficient estimates over the time intervals 0-8 days, 8-12 days and 12-21 days:

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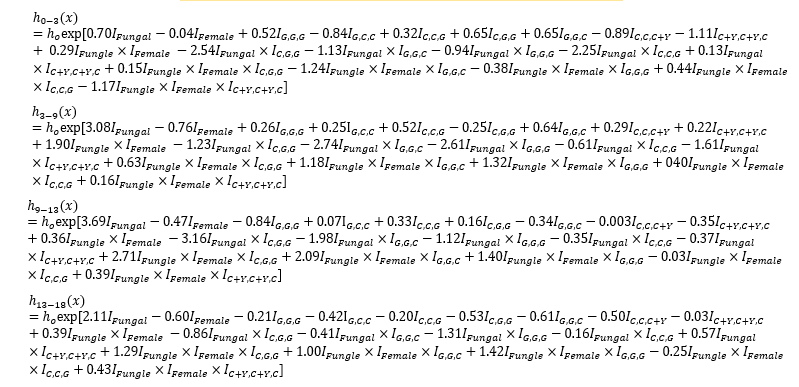
*Experiment 3: Effects of Cornmeal and Glucose diets on sexual dimorphism in post-infection survival of D. melanogaster inoculated with B. bassiana strain GHA*

The methods used were the same as in Experiment 2 with the following differences. On day 12, the flies were separated into vials that contained either Cornmeal diet or Glucose diet. Flies were sprayed with either GHA or control suspension on day 15 from egg, and then moved to cages and fed with either Cornmeal diet or Glucose

diet. The four dietary conditions tested were as follows:

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| --- | --- | --- |
| Treatment name on graphs | Day 12- 14 (before spray) | Day 15- 29 (after spray) |
| C/C | Cornmeal | Cornmeal |
| C/G | Cornmeal | Glucose |
| G/G | Glucose | Glucose |
| G/C | Glucose | Cornmeal |

*Statistical Analysis of Experiment 3*



*Experiment 4: Effects of yeast supplementation on sexual dimorphism in post-infection survival of D. melanogaster inoculated with B. bassiana strain GHA*

The methods used were the same as in Experiment 3, but the specific dietary conditions tested were as follows:

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| Treatment name on graphs | Day 12- 14 (before spray) | Day 15- 29 (after spray) |
| C/C | Cornmeal | Cornmeal |
| C/CY | Cornmeal | Cornmeal with yeast supplement |
| CY/C | Cornmeal with yeast supplement | Cornmeal |
| CY/CY | Cornmeal with yeast supplement | Cornmeal with yeast supplement |
| G/G | Glucose | Glucose |

To supplement the Cornmeal diet with yeast, a standard yeast paste was made using a ration of 1g of yeast per 5 mL of DI water. Then using a pipette, 5mg of yeast was added onto the surface of the Cornmeal food in each vial, and 80mg of yeast was added onto the Cornmeal food in each Petri dish (which gives the same amount of yeast per surface area for vials and plates).

*Statistical Analysis of Experiment 4:*

To investigate how the change of diets affects the survival of female and male *D. melanogaster* when inoculated with the entomopathogenic fungus, *B. bassiana* GHA we utilized the Cox Proportional Hazard model (Cox, 1972). Kaplan-Meier survival function (Kaplan et al., 1958) as well as log-rank test (Mantel, 1966)were applied to test the difference among four replicates. A scaled Schoenfeld residual plot and a formal test on Schoenfeld residuals against the transformed time of each covariate were used to detect any violation on the proportional hazards assumptions. If any covariate broke the assumption, the model was extended to a time-dependent Cox PH model, of which the time splitting points were chosen by a stratified model. With the consideration of potential interaction effects, the best time-dependent Cox PH model was selected by both likelihood test score and Akaike information criterion (Akaike, 1998). Then every observation was assessed by delta-beta score for each predictor of the best model to detect any influential outliers. Since there was no reasons to think such influential outliers resulted from data entry or other error, all observations were kept in the model.

A time-dependent Cox Proportional Hazard model was proposed to quantify the effect of diet change in terms of survival of female and male D. melanogaster (see Equation 2.1).

Equation 2.1

In this Cox PH model, a 14-day experiment time intervals was split into 3: 0-4, 4-9, and 9-14. This transformed covariates to time-dependent ones, including Fungal, Diet, interaction between Male and Diet C+Y, C+Y, and interaction between Fungal, Male and Diet C+Y, C+Y. Each time-dependent covariate has different coefficient estimates over time intervals (see Table 2.2).

Table 4. Coefficient Estimates for Cox Proportional Hazard Model for Males and Females of the Control and Fungal Infected Groups for Experiment 1.

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The proposed time-dependent Cox Proportional Hazard breaks the experiment time into 3 time intervals: 0-4, 4-9 and 9-14 days. The baseline is female without infection. The estimated hazard ratio () at a specific time point between any other group and the baseline can be obtained by the coefficient estimates

*Experiment 5: Effects of varying amounts of yeast supplementation on sexual dimorphism in post-infection survival of D. melanogaster inoculated with B. bassiana strain GHA*

The methods used were the same as in Experiment 4, except flies were maintained on Cornmeal diet for 14 days after the egg stage, were sprayed on day 15 from egg, and then were given cornmeal diets supplemented with varying amounts of yeast per 5mL of DI water: 0.5g, 1.0g, or 1.5g. Hence the dietary conditions were as follows:

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| --- | --- | --- |
| Treatment name on graphs | Day 12- 14 (before spray) | Day 15- 28 (after spray) |
| C/C | Cornmeal | Cornmeal |
| C/CY0.5 | Cornmeal | 0.5 g Yeast |
| C/CY1.0 | Cornmeal | 1.0 g Yeast |
| C/CY1.5 | Cornmeal | 1.5 g Yeast |

*Statistical analysis of Experiment 5:*

**Results**

*Mating Status Affects Survival After Inoculation with B. Bassiana ARSEF  
12460 in Both Males and Females*

Infected flies died significantly faster than control flies, suggesting the effectiveness of *B. bassiana* infection (see Figure 1, Table 3). Among infected flies, virgin females survived better than mated and cohabiting females in all time intervals (see Figure 1A, Table 3), and mated females survived better than cohabiting females (see Figure 1A, Table 3). For males, the same pattern was true in the 5-11 and 11-21 day intervals (see Figure 1B, Table 3). Even among control (uninfected) females, virgins showed a significantly higher survival than cohabits from day 5-21 and the same was seen with control males (see Figure 1B, Table 3).

*Survival After Inoculation with B. Bassiana ARSEF 12460 Is Sexually Dimorphic, but the Direction of the Dimorphism Depends on Mating Status*

There was a sexual dimorphism observed in all three mating statuses. Among infected cohabiting flies, females showed a better survival than males (*p*-value = 0.013, see Figure 3A, Table 4). The same trend was observed in virgins where males were more susceptible to fungal inoculation than females (*p*-value = 0.0016, see Figure 3B, Table 4). The trend was reversed in mated flies, where the males survived better than females (*p*-value = 0.0084; see Figure 3C, Table 4).

*Reproductive Output was Affected by Infection and Mating*

Mated and cohabiting females had offspring counts that declined with age over the span of 21 days post inoculation (see Figure 4A). The *p*-value of <0.0001 in the interaction between days and treatment (see Table 5) indicates that, as the flies age, the fungal groups showed a lower offspring counts than the controls (see Figure 4A). Females that cohabited with males laid more eggs than females that mated only for one day in control groups but not in the fungal inoculated groups (see Figure 4A).

*Survival After Inoculation with B. Bassiana GHA is Sexually Dimorphic, but only for the First Few Days After Inoculation*

Shahrestani et al. (2018) reported that when *D. melanogaster* Population C3 (same outbred population used here) was inoculated with GHA under cohabiting conditions, males survived better than females in the ten days post-inoculation observed in their study. Replicating their study, we found that after inoculation with GHA, males survived better than females for 12 days, after which male and female survival converged for the remainder of our 21-day study (see Figure 5, Table 6).

Key summary point of the diet change experiment

Control males and females showed a higher survival than the fungal inoculated males and females in all dietary conditions (see Figure 2.1). The flies that were fed with cornmeal diet before and after the spray showed a lower survival in fungal infected males (see Figure 2.1A, Table 2.3) and females (see Figure 2.1B, Table 2.3) compared to the fungal infected flies on other diets. Flies that were fed with cornmeal before spray and cornmeal + yeast after spray showed a rescue in survival in both males and females (see Figure 2.2, Table 2.4A). However, flies fed with cornmeal+ yeast before spray and cornmeal after spray showed the opposite results where the mortality rate increased (see Figure 2.2, Table 2.4B). Glucose and cornmeal + yeast diets given before and after the spray showed the highest survival rates (see Figure 2.2, Table 2.4C). There was a sexual dimorphism seen in all the dietary conditions where males survived better than females in fungal treatment (see Figure 2.2, Table 2.4).

Key summary point of the diet switch experiment

The results from experiment 1 show that flies on a glucose diet, performed better than those on cornmeal. For the second experiment, we chose to compares the effects of a diet switch with respect to the survival of both noninfected and infected flies. The results show that when diets switched from cornmeal to glucose, the survival presented a less negative outcome.

Key summary points of the yeast experiment

The results from experiment 2 show that flies on a cornmeal and yeast diet, demonstrated a less negative survival to those solely bred on cornmeal. The results were similar to that of glucose. Therefore, experiment 3 tested the variation of yeast supplementation for both infected and uninfected flies. The results show that diets with a greater yeast supplementation made from either 1.0 g or 1.5 g presented a less negative outcome.

**Discussion**

Shahrestani et.al. (2018) found that *D. melanogaster* females from the outbred population C3 were more susceptible than males in the ten days following inoculation with *B. bassiana* strain GHA, under cohabiting conditions. To expand further on this result, we repeated this work but followed survival for 21 days post inoculation. While we replicated the results of Shahrestani et al. (2018) we additionally found that with this fungal pathogen, sexual dimorphism in defense began to disappear around 12 days post inoculation, such that cohabiting females and males had the same probability of survival from days 12-21 post inoculation. We next tested an additional *B. bassiana* strain, ARSEF 12460, that was shown to also result in sexual dimorphism in defense in Shahrestani et al. (2018). In our study with ARSEF 12460 the sexual dimorphism was reversed, such that among cohabiting flies, males were more susceptible to fungal infection than females, and this dimorphism persisted through the 21 days of the assay. Together these results suggest that the direction of sexual dimorphism is affected by the fungal strain used. Yet, in Shahrestani et al. (2018), females were reported to be more susceptible than males to infection with ARSEF 12460, which is opposite to our findings. One key difference in the studies is that they used the fly line Canton S in their study of susceptibility to ARSEF 12460 whereas we used the outbred population C3. This suggests, that the host genotype also influences the direction of sexual dimorphism in immune defense. Further evidence for this claim comes from our own unpublished results, in which we found that 297 recombinant inbred lines from the *Drosophila* Synthetic Resource Population have variation in both magnitude and direction of sexual dimorphism in defense against *B. bassiana*. Hence, there is no weaker sex when it comes to immune defense, and many factors, including pathogen and host genotypes affect sexual dimorphism in defense.

Another variable that has been previously shown to affect sexual dimorphism in immune defense against bacterial pathogens is mating (Short and Lazzaro n.d.). Mating was shown to suppress defense in female *D. melanogaster*, such that mated females survived infection with *Providencia rettgeri* and *Providencia alcalifaciens* less successfully than virgin females (Short and Lazzaro n.d.). This mating-immunity trade-off has been thought of as a female trait (Schwenke, Lazzaro, and Wolfner n.d.) and it is generally assumed that males do not experience the same mating-immunity trade-off, due to differences in resource allocation to reproduction and defense in females and males. It is traditionally expected that females will exert more energy during reproduction, thus producing an energy trade-off between reproduction and immune defense (Short and Lazzaro n.d.; Rolff and Siva-Jothy 2002). One hypothesis for mating-immunity trade-offs in females is that female defense is suppressed when exposed to the male ejaculate accessory gland proteins (Acp) or sex peptide (SP) (Wigby and Chapman 2005), due to the effects of juvenile hormone (Schwenke and Lazzaro 2017).

However, reproduction in male *D. melanogaster* also accrues costs (Harvanek et al. 2017), and it has been suggested that these costs trade-off with immune defense (McKean and Nunney 2001). We therefore expected that a mating-immunity trade-off would be present for both males and females, and that these trade-offs could influence the direction of sexual dimorphism in defense. For both males and females, we tested survival after inoculation with *B. bassiana* ARSEF 12460 for virgins, flies that mated for 24 hours before inoculation, and flies that mated both before and after inoculation (males and females that cohabited together).

We found that both females and males experience mating-immunity trade-offs. In both sexes, mating for 24-hours prior to inoculation made flies more susceptible to inoculation with ARSEF 12460 compared to virgins, and flies that cohabited with the other sex were more susceptible to inoculation than flies that only mated for one day. It is possible that the cause of the mating-immunity trade-off may be different for females and males. For females, we tested for differences in resource allocation by counting the number of offspring produced by females in different mating and infection conditions. Infected females produced fewer offspring than control females, suggesting that there is a reproductive cost to infection. Under uninfected conditions, females that cohabited with males produced more offspring than females that mated only once. However, under infected conditions, cohabiting and one-day mated females laid indistinguishable numbers of eggs, which may suggest that the increased susceptibility of cohabiting females was not due to increased egg laying. But it is also possible that, due to infection, the numbers of eggs laid were too low for differences to be picked up with our statistical analyses.

We did not measure male reproductive output. However, other factors, outside of resource allocation to reproduction could also potentially affect a trade-off with immune defense. In particular, mating itself is energetically costly, as is intrasex competition for mates. In our study, cohabiting flies were maintained in population cages with equal sex ratios. The stress and investment for successful mating could have affected male susceptibility to infection. It is worth noting that the fungus ARSEF 12460 is not itself transmitted by mating.

Prior to mating, both the mated and cohabiting flies were kept individually in straws to preserve virginity in early adulthood. The isolation and tight space within the straw may also cause stress, but this would be controlled among all three mating conditions. Flies were sexed in the straws before being divided among the treatments of this study, and this sexing was done while briefly anesthetizing the flies by placing the straws on ice. Each fly was anesthetized on ice for approximately two minutes. It is possible that this brief exposure to low temperature places high demands on the flies (Luckinbill 1998). As mentioned in the Luckinbill experiment (1998), rapid changes in temperature make it difficult for the organism to adjust to low temperatures. Their results show a vast reduction in populations of larva, pupa and adult when changes in temperature were drastic. In addition to early environmental stress, the limited food and space may contribute an exertion of energy (Guo, Mueller, and Ayalat 1991).

Interestingly, the direction of sexual dimorphism in defense against ARSEF 12460 varied by mating status. Among cohabiting flies, males were more susceptible to infection than females. However, when flies were mated for only 24-hours prior to inoculation, females were more susceptible to infection than males. Yet when virgin flies were compared, males were again more susceptible than females. This result supports the idea that the mating-immunity trade-off is at least in part affected by the frequency of mating, instead of being an inherent component of male and female biology.

Given the potential impacts of reproduction-related resource allocation on the mating-immunity trade-off and on sexual dimorphism in defense, our next avenue of research it to investigate the effects of dietary manipulations on these relationships. An experiment conducted by Lee, Kim, and Min (2013) observed the dietary preference of mated flies and its effect on survival. Varying ratios between proteins and carbohydrates were tested. Their results showed that mated females preferred a balanced diet compared to virgin females who preferred a more carbohydrate heavy diet. The lifespan of their flies varied on different diets, demonstrating a greater survival of mated females on a balanced diet compared to other diets. The three-way relationship between diet, mating, and immune defense remains an open area of study.

*Drosophila melanogaster* are natural decomposers whose fecundity increases with the increased ingestion of proteins (Lee et al., 2013). A high energy and high carbohydrate diet causes reduced eclosion in fruit flies and reduced survival in adults (Lindsey, 2017). Egg to adult time is seen to be fastest at Protein: Carbohydrate 8:1 and lowest at Protein: Carbohydrate 1:16 (Lihoreau et al., 2016). In males, a high carbohydrate and low protein diet increases the lifespan and reproductive performance. Moreover, in females, a high fat diet decreases their lifespan and makes them less receptive to mating, which can be rescued by changing the diet in females. When equal concentrations of sucrose and yeast are added in the standard food medium of fruit flies, the females tend to survive longer than males (Magwere, 2004). However, these studies did not look at the effects on susceptibility to infection under these diets.

An experiment by Lee et al. (2013) observed the sex-specific effects on diet preferences and life span for the wild-type population, Canton-S. While both experiments observed the effects of different mating statuses on sexual dimorphism, Lee et al. did not infect flies with a pathogen. Their results, however, presented that a balanced diet was preferred by mated female flies, ultimately demonstrating a greater median life span for them compared to virgin females and males altogether.

Most experiments observe the effects of either protein, carbohydrates and sugar; rarely looking at the factors together. For instance, the Howick and Lazzaro (2014) experiment observed the effects of low-sugar diets versus high-sugar diets on the survival of flies. A high sugar diet is seen to decrease immune defense against infection, resulting in a decrease in survival overall. In this experiment, our results are indicative that these ratios are imperative to the overall performance of flies. Our standard cornmeal diet contains approximately 77 g of molasses per 1 L of food, whereas our glucose diet contain 21 g of dextrose for every 1 L of food. The results in Figure 1 shows a highest survival for male flies in glucose diet and the highest survival for female flies in cornmeal and yeast diet in comparison with the regular cornmeal diet.

Upon analyzing all three experiments, the result show two narratives:

1. Ancestral diet demonstrates an improvement in immune defense and survival.
2. A more balance protein to carbohydrate diets improves….

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