No apparent evidence of reproduction-immunity trade-off in *Drosophila melanogaster*

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A *dissertation submitted for the partial fulfilment of BS-MS dual degree in Science*

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Dedicated to…

My Parents, my brother, and my sister

For their advice, their patience, and their faith

Certificate of examination

This is to certify that the dissertation titled "No apparent evidence of reproduction-immunity trade-off in Drosophila melanogaster" submitted by Mr. Nitin Bansal (Reg. No. MS14045) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Manjari Jain Dr. Rhitoban Ray Choudhury Dr.N.G.Prasad

(Supervisior)

Dated: April 25, 2019

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. N. G. Prasad at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Nitin Bansal

(Candidate)

Dated: April 25, 2019

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. N. G. Prasad

(Supervisor)

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List of Figures and Tables

Contents

Abstract

A trade-off between organismal traits competing for shared resources is a fundamental assumption of the life-history theories. Investment into reproduction vs. immunity has been a topic of much discussion in the literature (Lawniczak et al., 2006). Reproduction-immunity trade-offs are considered to be condition dependent (McKean and Nunney, 2001) with an overarching hormonal control (Lazzaro et al., 2017). The ubiquity of such trade-off has been questioned too (Fedorka et al., 2007). In this study we describe the evolution of the correlation between reproduction and immunity, using replicate *Drosophila melanogaster* populations having a common ancestor. The study is unique because of its use of lab adapted, outbred populations harbouring substantial genetic variation. We measured immunocompetence of virgin and mated flies of both sexes; immunocompetence being defined as the survivorship post infection with a live pathogen. Two Gram-positive (*Enterococcus faecalis* and *Staphylococcus succinus*) and two Gram-negative (*Pseudomonas entomophila* and *Providencia rettgeri*) bacteria were used as pathogens. We did not find any evidence of trade-offs. The results indicate that, for either sex, mating improves post-infection survivorship of flies, irrespective of the pathogen used. This study adds to the work done previously on the ancestors of the present populations (Gupta et al., 2013)

Chapter 1

Introduction

A trade-off arises when two traits in an organism compete for resources from a common finite pool. If allocation in one is increased causing a change in fitness; it has to be decreased in the other (Stearns 1998, Prasad and Joshi 2003). There are several studies describing the phenomenon of trade-off in an organism. In some species, mating can lead to an increase in lifespan (Arnqvist & Nilsson 2000, Holmes et al. 2001) or a decrease in lifespan without any significant effect on egg production in the Mediterranean fruit fly, *Ceratitis capitata* (Chapman et al. 1998).

This study investigates the correlation between reproduction and immunity in *Drosophila melanogaster* males and females. In one set of studies, mated Drosophila females suffered higher mortality and lower immune gene expression than virgins after infection with a pathogenic bacterium (Fedorka *et al.* 2007) and in flour beetle *Tenebrio molitor*, mating suppresses an immune effector system (phenoloxidase) in both sexes (Rolff and Siva-Jothy, 2002), or immune activity can be condition dependent in *Drosophila melanogaster* males (McKean & Nunney 2001). Another set of studies shows an increase in an aspect of immunity after mating for eg. mated Drosophila females show an upregulation of immunity related genes shortly after mating without any infection (Lawniczak & Begun 2004) and in female crickets *Gryllus texensis*, where mated females have a higher post infection survivorship then virgin females on infection with a Gram-negative bacterium *Serratia marcescens* (Shoemaker et al. 2006). However, no difference was seen in the ability to clear non-pathogenic bacteria after mating in *Drosophila melanogaster* females but there was a significant difference in bacterial clearance abilities of *D. melanogaster* males (McKean & Nunney 2005).

The immune system of *Drosophila melanogaster* is well understood and genes regulating immune response (Lemaitre & Hoffman 2007) along with a hormonal role in mediating immunity has been studied in *D. melanogaster* (Schwenke and Lazzaro 2017, Flatt 2008) and in other insect species (Rolff & Siva-Jothy 2002). Juvenile hormone is an endocrine hormone, synthesized in the corpus allatum and has a role in the development of oocytes and yolk protein deposition (Soller M, Bownes M, Kubli E 1999). Sex peptide (Acp-70)

transferred through males after mating activates the synthesis of Juvenile hormone, causing immunosuppression in *D. melanogaster* females (Moshitzky et al. 1996, Short & Lazzaro 2012) through receptor Germ Cell Expressed (GCE).

Previous work on the ancestors of the present population (Vanika Gupta, Ph.D. thesis, 2015) shows no effect of sexual activity on immunity in *Drosophila melanogaster* males when the males are infected with two kinds of pathogen; i.e. *Pseudomonas entomophila* (Pe) and *Staphylococcus succinus* (Ss). This present study will add on to the work done previously and will help in getting a clear concise of the effect of sexual activity on immunity in my host population.

I used the Blue Ridge Baseline population of *D. melanogaster* as my model system. It is a large outbred population having five replicates, maintained in the lab for about 200 generations. Each replicate is genetically distinct and the total sample represents a picture of naturally occurring genetic variation in this population making these replicates a good fit for this study. I am specifically interested in (a) determining whether there is any effect of sexual activity on the post infection survivorship of *Drosophila melanogaster* males and females when host (flies) are infected with different kinds of pathogenic bacteria, and (b) determining whether there is any role of Juvenile hormone in governing this phenomenon in my host population.

Chapter 2:

Materials and Methods

2.1. Experimental population

BRB is a large outbred population which was established from the 19 isofemale lines. These isofemale lines were set up from flies caught at Blue Ridge, USA and hence the name. After 10 generations in the lab, BRB was divided into five replicates (BRB_{1-5}), which are independent replicate populations that do not interbreed and thus harboring substantial genetic variation among each replicate population. These independent replicates are maintained in a 14-day discrete generation cycle, 12 hours: 12 hours Light: Dark cycle at 25° C temperature and 60-70 % Humidity. A generation starts with transferring eggs into cylindrical glass vials (25mm diameter × 90mm height) containing standard banana-jaggery food (Prasad et al. 2003). These eggs are collected at a density of 70 eggs per vial with 40 vials per replicate. On 12th day post egg collection the eclosed flies are transferred into cuboidal Plexiglass fly cages (25cm length × 20cm width × 15 cm height) having a food plate. The number of individuals while transferring to cages is around 2800 per block. On the 14th day, after giving a prior oviposition window of 18 hours, eggs are collected for the next generation which represents a sample of the no. of eggs laid in the 18-hour window. This population is under no selection pressure.

2.2. Bacterial populations

Bacterial culture was setup in Luria Bertini Broth (LB) medium at a bacterial specific temperature (refer table 1), 150 rpm, overnight, a day before the infections. Secondary subculture was setup by diluting the primary culture to 50 fold. To obtain the desired OD at 600 nm (OD_{600}=1) culture was pelleted down and suspended in an equal volume of 10mM $MgS0_4$.

Four bacterial populations, i.e. two gram-positive bacteria (*Enterococcus faecal* (Ef) and *Staphylococcus succinus* (Ss)) and two gram-negative bacteria (*Pseudomonas entomophila* (Pe) and *Providencia rettgeri* (Pr)) were used in this study. The optimum temperature for

growth and time taken for primary culture and secondary subculture to grow is shown below (refer table 1).

Bacteria	Optimum growth temperature(°C)	Time for growing primary culture (hours)	Time for growing secondary subculture (hours)
Pe	**27**	**8-10**	**4-6**
Ss	**37**	**5-6**	**2-3**
Ef	**37**	**5-6**	**2-3**
Pr	**37**	**5-6**	**2-3**

Table 1: Bacterial growth parameters

2.3. Experimental setup

Eggs were collected at a density of 70eggs/ vial and were kept at 12:12 L: D cycle at 25°C temperature. Flies were isolated as virgins on 9^{th} and 10^{th} day post egg collection within 6 hours of eclosion and were maintained in single sex vials. Flies were kept in single sex vials at a density of 10 per vial. For treatment involving sexually active males and females, flies were kept in their rearing vials till the day of infection (i.e. 12^{th} day post egg collection). On the day of infection, virgin and sexually active males and females were grouped into four sets and each set was further divided according to the type of treatment i.e. the kind of pathogen the flies are infected with (refer table 2). For example, 100 males and 100 females of both virgin and mated sets were infected with Pe and a similar procedure was done for other bacteria. The flies were then housed in separate cages and observed for mortality. A total of 6 blocks (Four BRB replicates and two repeats) were used in this study. For each block, 100 flies/sex/mating status/bacteria were infected.

2.4. Infection Protocol

Flies were infected on the lateral side of the thorax with the help of a fine needle (Minutein pin 0.1 mm, Fine Science Tools, CA) after lightly anesthetizing using CO_2. For Bacterial infections, flies were pricked with a fine needle dipped in bacterial suspension (bacteria suspended in 10Mm $MgSO_4$) on the thorax. For Sham infections, flies were pricked with a

fine needle dipped in 10Mm $MgSO_4$ on the thorax, which served as a control for the experiment. Flies were maintained at 25°C throughout the experiment.

2.5. Monitoring the flies for survivorship

After infections, flies were separated and housed in different cages according to the type of treatment (see Figure 1), and mortality was monitored every 4 hours for 96 hours post infection. There were 20 experimental cages for each block.

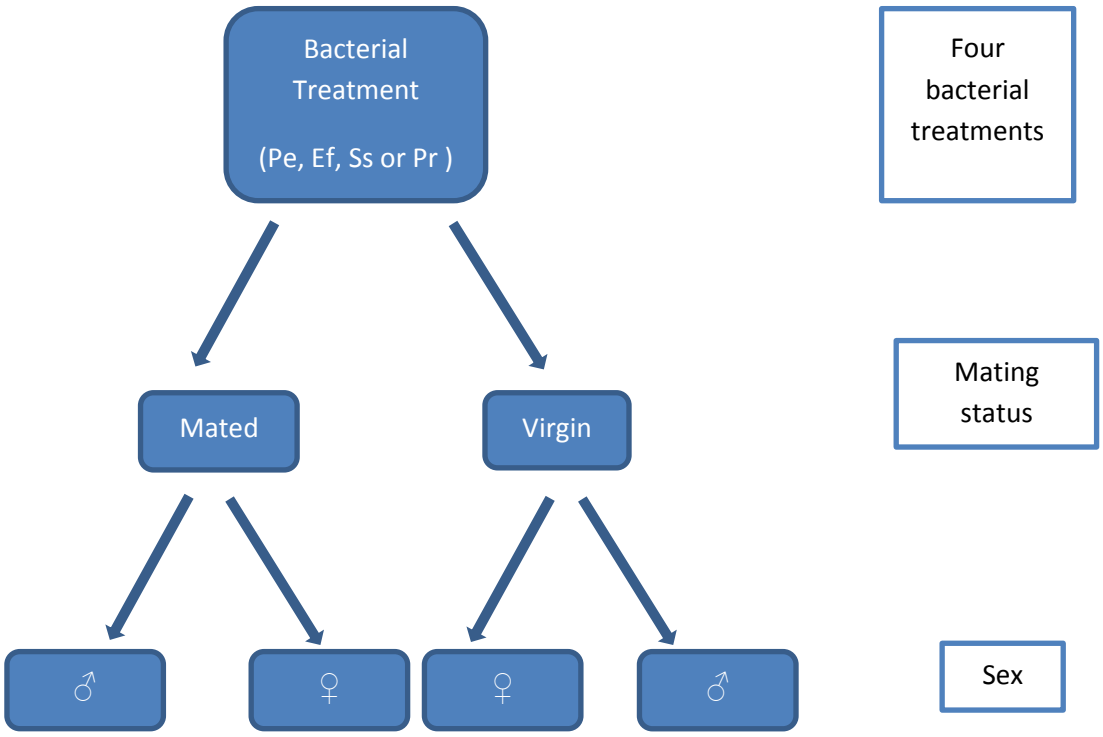

Figure 2.1: Experimental Setup

Next, I went to check if there is any role of Juvenile hormone in my host population.

2.6. Juvenile hormone extraction protocol:

I used the below-mentioned protocol (Protocol modified from Sugime *et al.* 2017 and Vanika *et. al.* unpublished data) to quantify the amount of Juvenile hormone in my host population. The protocol needs to be standardized.

Before starting the extraction process

1. After the experimental treatment, flies were flash freeze using liquid N_2 and were stored at -80°C until ready for extraction.
2. Methanol and hexane were kept at -20°C at least for 12 hours before the extraction process.

Extraction of Juvenile hormone

3. Flies (100) were homogenized in 400 µL of methanol containing 30ng Fenoxycarb in a microcentrifuge tube. Fenoxycarb serves as an internal standard and would tell the efficiency of extraction.
4. To this fly homogenate, 100 µL of 2% sodium chloride solution was added.
5. After addition of 300 µL of hexane to this fly homogenate, the sample was vortexed properly. A crucial step for hormone extraction process.
6. This mixture was centrifuged at 3000 rpm for 5'.
7. The hexane phase (top layer ~300 µL) was transferred to a clean glass tube.
8. Steps# 5-7 was repeated four more times (total five). Hexane phase was pooled from a given sample into the same tube.
9. Hexane mixture was dried in a centrifugal concentrator and the pellet was dissolved in 100 µL of methanol.
10. Each sample was filtered through a 0.22 µm spin filter separately.

LC-MS/HPLC was done.

2.7. Statistical analysis:

Survivorship data was analyzed using the Log-Rank model where death was recorded for each fly and flies not dead by the last time were treated as censored data. The analysis was implemented on SAS JMP v 7.0.1.

Chapter 3

Results

Sexual activity enhances survivorship when infected with either gram-positive (*Enterococcus faecalis* and *Staphylococcus succinus*) or gram-negative (*Pseudomonas entomophila* and *Providencia rettgeri*) bacteria: On day 12th, four sets i.e. sexually active males and females, and virgin males and females were infected with different kinds of bacteria by pricking with a fine needle dipped in bacterial culture for the experimental treatment and in 10 mM MgSO4 for both virgin and mated controls. The number of dead individuals was recorded at regular time intervals, i.e. every 4 hours for 96 hours post infection. I found that virgin females had lower survivorship and higher mortality rate compared to sexually active females when flies were challenged with Pe, Ef, Ss or Pr (Figure 3.1). The similar trend was obtained in case of males, as the mortality rate was high in virgin males as compared to sexually active ones when the flies were challenged with Pe, Ef, Ss or Pr (Figure 3.2). Both the sham treatments (mated and virgin control) survived in this experiment.

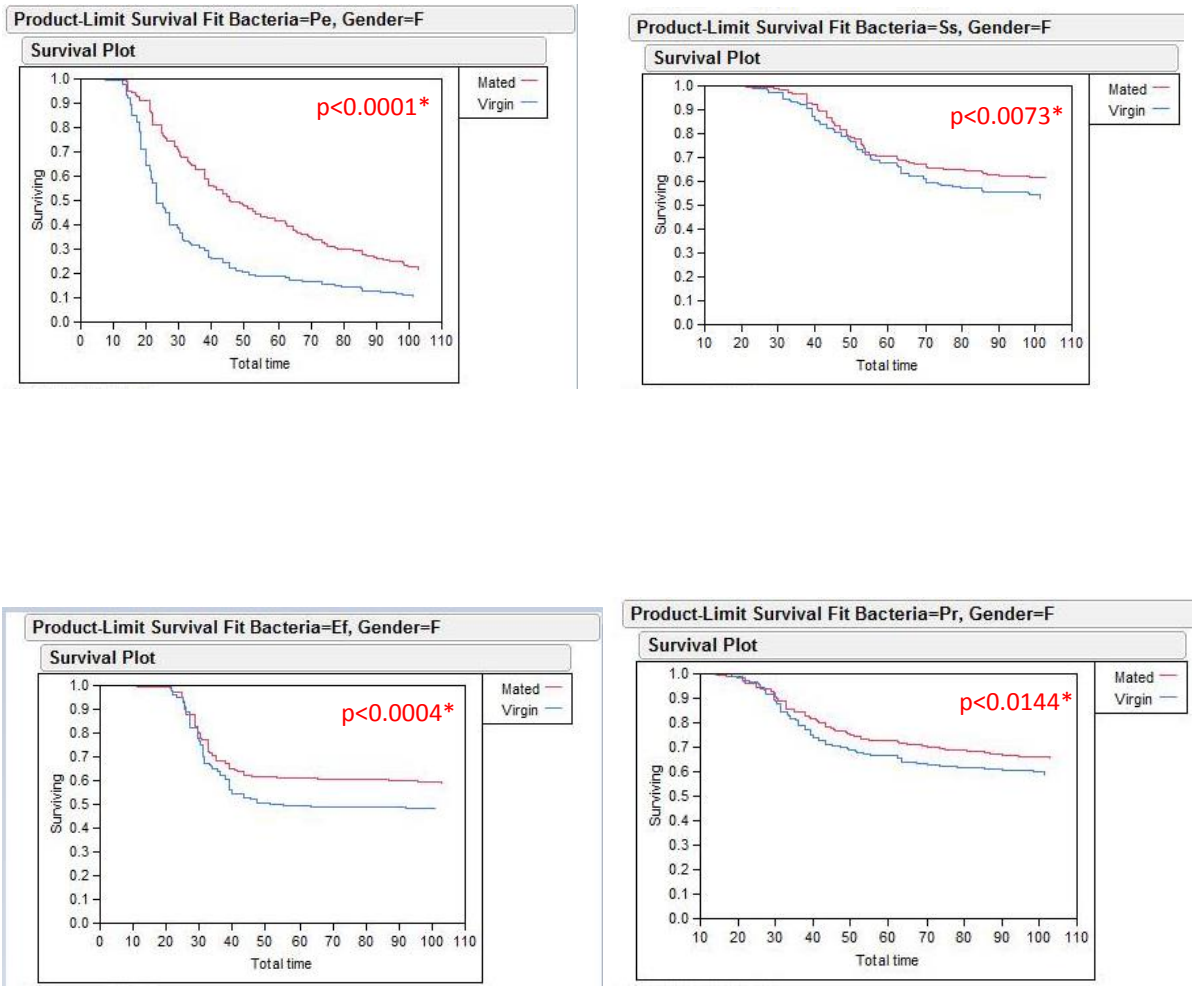

Figure 3.1: Effect of sexual activity on female survivorship after infection with *P. entomophila, S.succinus, E. faecalis, P. rettgeri.* Kaplan-Meier plots of survivorship of sexually active and virgin females. Sexually active females survived significantly better than virgins ($p<0.05$) for each of the four bacterial treatments. Log- Rank analysis was performed using SAS JMP v7.01. Separate sham infected controls for both virgins and mated flies were run. No mortality in any of the sham infected controls was observed during the experiment.

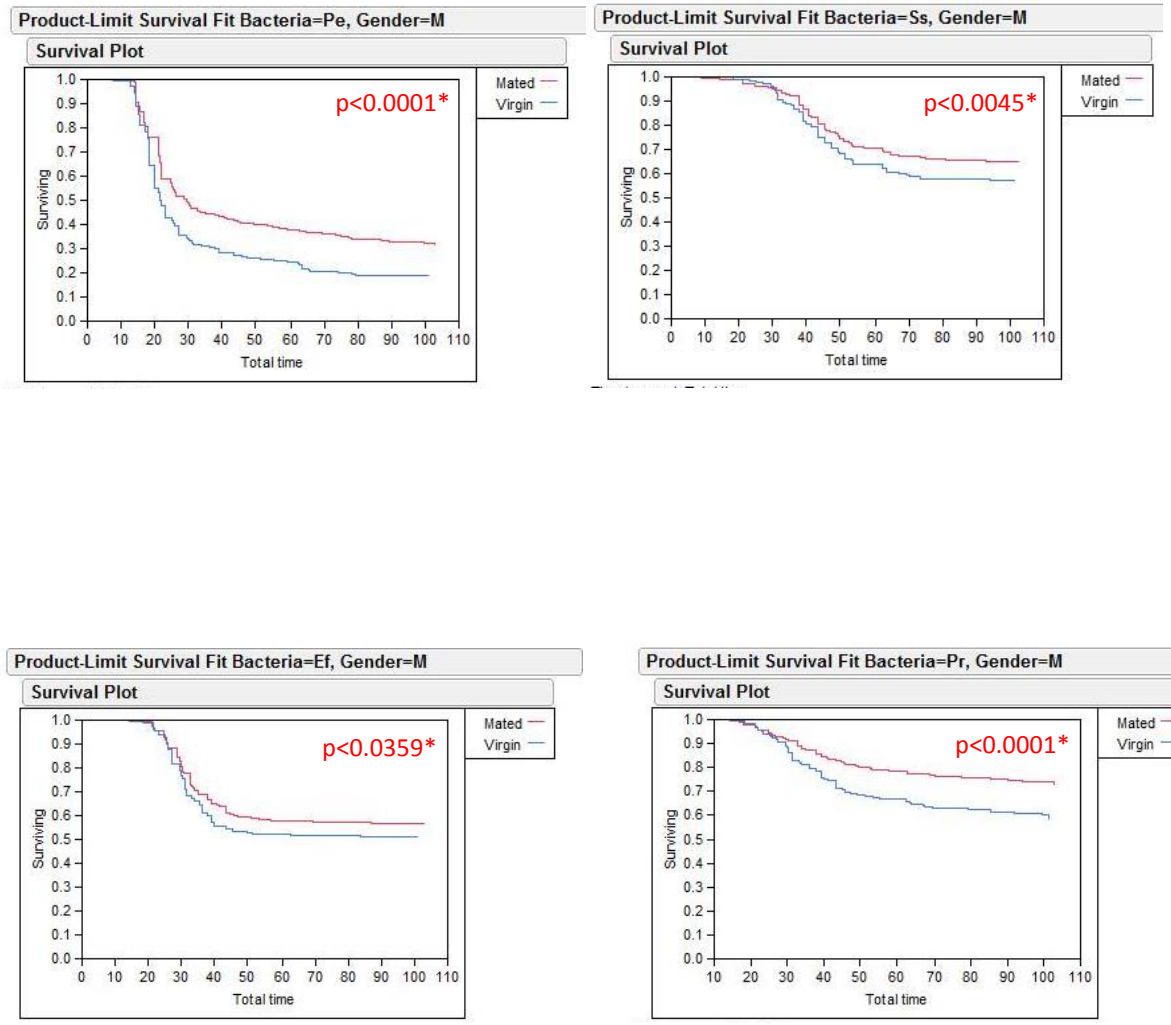

Figure 3.2: Effect of sexual activity on female survivorship after infection with *P. entomophila, S.succinus, E. faecalis, P. rettgeri.* Kaplan-Meier plots of survivorship of sexually active and virgin males. Sexually active males survived significantly better than virgins ($p<0.05$) for each of the four bacterial treatments. Log- Rank analysis was performed using SAS JMP v7.01. Separate sham infected controls for both virgins and mated flies were run. No mortality in any of the sham infected controls was observed during the experiment.

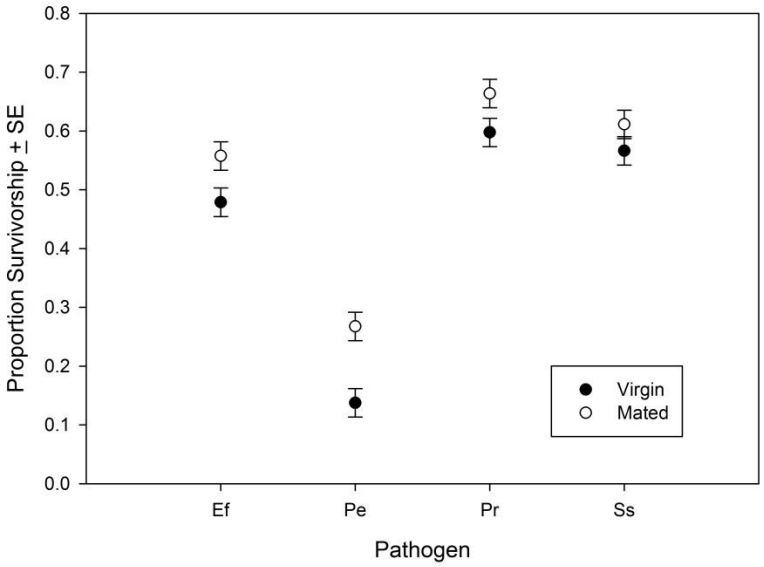

Figure 3.3: Proportion survivorship of sexually active (○) and virgin (●) flies infected with different kinds of pathogen: This data was pooled across all blocks keeping block as the random factor. Proportion survivorship of flies was lowest in the case of *P. entomophila* followed by *E. faecalis*. However, the proportion survivorship of virgin and mated flies was not significantly different after infection with *P. rettgeri* and *S.succinu*s. Statistical analysis was performed using SAS JMP v7.01 and plotted using Gnuplot.

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Mating	0.07875	0.07875	1	24.1476	**0.0161**
Treatment	2.22359	0.5559	4	21.9840	**<.0001**
Mating*Treatment	0.03947	0.00987	4	2.3717	0.1107
Block&Random	0.36339	0.12113	3	5.3016	**0.0293**
Mating*Block&Random	0.00978	0.00326	3	0.5446	0.6693
Treatment*Block&Random	0.30344	0.02529	12	3.9327	**0.0090**
Mating*Treatment*Block&Random	0.04992	0.00416	12	2.4237	0.0696
Gender	0.01711	0.01711	1	4.0030	0.1392
Mating*Gender	0.0021	0.0021	1	0.5928	0.4974
Treatment*Gender	0.02376	0.00594	4	1.4900	0.2661
Mating*Treatment*Gender	0.01679	0.0042	4	2.4458	0.1033
Block*Gender&Random	0.01282	0.00427	3	0.7352	0.5688
Mating*Block*Gender&Random	0.01063	0.00354	3	2.0651	0.1585
Treatment*Block*Gender&Random	0.04783	0.00399	12	2.3222	0.0794
Mating*Treatment*Block*Gender&Random	0.0206	0.00172	12	.	.

Table 2: Mixed model ANOVA for proportion survivorship: with fixed factors (Treatment, Mating status, Gender) and Block as the random factor.

Chapter 4
Discussion

In this study, we measured the immunocompetence (defined as the survivorship post infection with a live pathogen) of sexually active and virgin flies when challenged with different kinds of bacteria. Previous studies on *Drosophila melanogaster* show that sexual activity and immunity (measured as bacterial load) are negatively correlated when the flies are challenged with pathogenic bacteria (McKean and Nunney 2001, Fedorka *et al.* 2007).

Male transfers sex peptide during mating which results in immunosuppression in mated females (Fedorka *et al.* 2007, Short & Lazzaro 2012) through activation of Juvenile hormone (Schwenke and Lazzaro 2017). We expected the immune response (measured in terms of mortality rate over 96 hours post- infection) of sexually active females to be lower than virgin females if there is any cost associated with mating in our host population. We found that sexually active females have higher post infection survival rates than virgins when the flies are infected with either *Pseudomonas entomophila* (Pe), *Staphylococcus succinus* (Ss), *Enterococcus faecalis* (Ef) or *Providencia rettgeri* (Pr). This trend was statistically significant for each treatment.

A study done on *D. melanogaster* males using BRB as the host population (Vanika Gupta, Phd Thesis, 2015), where the sexually active and virgin males were infected with either *Pseudomonas entomophila* (Pe) or *Staphylococcus succinus* (Ss) shows better survivorship of sexually active males when infected with Pe, having no significant difference in case of Ss. Adding to this previous study, we found that survivorship of virgin males was lower than sexually active ones when the flies are challenged with different kinds of pathogen treatment, i.e. *Pseudomonas entomophila* (Pe), *Staphylococcus succinus* (Ss), *Enterococcus faecalis* (Ef) or with *Providencia rettgeri* (Pr).This trend was statistically significant for each treatment.

In this study, we did not find any evidence of reproduction- immunity trade-off in our host population. There can be various possible reasons for the improved bacterial resistance observed in mated individuals relative to virgins. For instance, the trade-off between immunity and sexual activity might be host and pathogen specific. Given the virulence level

of each pathogen is different; the significance level between virgin and mated flies with each bacterium is also different. Secondly, immune assays of single immune components do not always reliably reflect overall immune function (Adamo 2004a), and their meaning can be ambiguous. In this study, survivorship post infection as an overall measure of the health of the organism is measured and its Darwinian fitness. Other studies have measured phenoloxidase activity, encapsulation ability, etc.

The juvenile hormone causes post mating immunosuppression in *D. melanogaster* females through a receptor, Germ Cell Expressed (Schwenke and Lazzaro 2017) or by inhibiting phenoloxidase activity, a component of immune system (Rolff & Siva-Jothy 2002) in both the sexes. In our host population, where immunocompetence of sexually active flies is better than virgins, the role of Juvenile hormone needs to be assayed by measuring the JH titer values in adult flies.

Overall, my results indicate that immunity trade-offs might be mediated through complex interactions between host, pathogen and the environment and whether the correlation between immunity and reproduction is typically positive, negative or neutral across species will need to be explored further.

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