Evolution of female mating behavior in a population of Drosophila melanogaster selected for increased immunity

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

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Dedicated to my parents

Certificate of Examination

This is to certify that the dissertation titled "Evolution of female mating behavior in a population of *Drosophila melanogaster* selected for increased immunity" submitted by Ms. Biswajit Shit (Reg. No. MS14106) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The dissertation work represents original research carried out at IISER, Mohali under the supervision of Dr. N. G. Prasad, Associate Professor, Department of Biological Sciences during the academic year 2018-2019. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. N.G. Prasad Dr. Rhitoban Roychaudhury Dr. Manjari Jain

Dated: 26th April, 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.

Biswajit Shit
(Candidate)
Dated: 26th April, 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)

Acknowledgement

Firstly, I would like to express my sincere gratitude to my advisor Dr. N.G. Prasad for the continuous support of my master's thesis and related research, for his patience, motivation, and immense knowledge. His excellent way of teaching and friendly attitude towards student really inspired me. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study.

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Abstract

Females' gain in reproduction, with increase in number of mating, is minimal. More mating also make females prone to mate-harm by males. Thus females should evolve to minimize their mating rate. But evolution of elevated mating rate is possible if females can gain benefits from these extra mating. We tested this hypothesis using *Drosophila melanogaster* populations selected for better survivorship against bacterial infection. In these populations, it has already been reported that mated females gain in terms of increased survivorship when infected compared to virgin females. I thus predicted that in these selected populations, since the females benefit from mating, females should evolve higher mating rate compared to the control population. As reported, I found that female flies from I (selected) populations survived better when challenged with bacteria than flies from S (sham control) populations. Overall females from IRS (reverse selected) populations had similar survivorship compared to the unhandled controls across all the blocks after 120 generations of reverse selection. The results suggest that I females have higher mating rate than the S in three out of four blocks as was predicted. IRS females had no fixed patterns for mating rate. Interestingly in my experiments I discovered that the 'benefit due to mating' gained by females in I populations is not substantially different from that of the control and reverse selected populations, contrary to our hypothesis.

Chapter 1: Introduction

Reproductive behaviour and immune response are two very important life history related traits for an organism's life. The most interesting thing is they are functionally related and disease resistance is a critical component of life history (Zuk and Stoehr, 2002). Like other life history traits (such as fecundity, life-span, age, reproductive maturity, growth pattern), the ability to defend a pathogenic attack and mounting an immune response against it, is a very important component that shape an organism's fitness. The maintaining and deploying of life history traits needs resources because they are expensive. So, in resource limiting condition they can be very costly and can show trade off.

Previously, several studies have investigated about the trade off between reproductive behaviour and immune response with different model organism. In insects, increased sexual activity has been shown to be costly for several immunity related components like, phenoloxidase activity, haemolytic activity etc. (Fedorka et al., 2004; Mckean and Nunney, 2001; Rolff and Siva- Jothy, 2002). During mating, male ejaculates can be a reason behind reduced defence against infection (Short et al., 2012). However, the trade off between sexual activity and immunity can't be seen always. A study in male crickets has shown no difference in lytic activity even when sexual activity was increased (Dowling and Simmons, 2002).

Other studies have shown that mating has many beneficial effects on immunity. Mating enhances resistance against pathogen in field cricket (Shoemaker et al., 2005). Another study on mealworm beetle has shown that immunity can be increased because of copulation (Valtonen et al., 2009). In *Drosophila melanogaster*, male ejaculates (Sperm or seminal fluid) can upregulate immunity related genes and antibacterial proteins can also be transferred along with male accessory gland proteins (Peng et al., 2005; McGraw et al., 2004; Lung et al., 2000). Another study of *Drosophila melanogaster* reported increased resistance to bacteria, in males with increased sexual activity (Gupta et al., 2013). In a *Drosophila melanogaster* population selected for increased immunity, it was shown that mated females of selected population have higher immunity compared to their virgin counterpart and there was clear role of male identity

(Radhika, 2016, MS thesis). All these studies clearly indicate benefits of reproduction in mounting immune response.

As a promiscuous species, the most important part of *Drosophila melanogaster* reproductive behaviour is multiple mating. According to Bateman's principle, females don't get much benefit from multiple mating in terms of reproductive success compared to males (Bateman, 1948). This thing become a common observed example of sexual conflict, where males get benefit because of increased mating rate and different direct and indirect cost of mating favours a lower mating rate for females (Chapman et al., 2003, Rowe et al., 1994., Kazancioglu and Alonzo, 2012). High numbers of mating, leads females to be more prone to mate harm (Parker, 2006). Several other costs are also associated with this multiple mating and they affect female fitness directly and indirectly. The ecological cost of mating includes general time and energy costs, increased predation rate, risk of physical injury and parasite or pathogen infections (Reviewed by Arnqvist and Nilsson, 2000). All these costs have different indirect effect in terms of female egg production rate and life span. The accessory substances transferred to females with male ejaculates have several complex effects on female reproductive behaviour (Eberhard and Cordero 1995). Sometimes those accessory substances are toxic for females.

Along with all these costs, multiple mating can give several direct and indirect benefits to the females (Arnqvist and Nilsson, 2000). Such direct benefits include refilling of depleted sperm supplies (Arnqvist, 1989; Siva-Jothy, 2000), the transfer of nuptial gifts and nutrients (Wedell, 1997; Wiklund et al. 2001). Reproduction is beneficial for females in terms of stimulation in egg production rate, increased female fertility, increased female fitness by stimulating egg maturation and egg laying (Arnqvist and Nilsson, 2000). Females can get indirect genetic benefits from multiple mating in terms of increase in offspring fitness and egg viability (Yasui, 1998). In general multiple mating is of two types – Monoandrous and polyandrous. Both of them are beneficial for the females. But Polyandrous females show higher relative fitness compared to monoandrous females as polyandry provides additional opportunity to gain genetic fitness (Fedorka and Mousseau, 2001).

Multiple mating has several costs and benefits and most of the time the benefits outweigh the costs. Indirect genetic benefits are strong examples of such kind of benefits. Based on above mentioned positive (benefits) and negative effects (costs) and their simple assumptions, a net

optimum mating rate is predicted for females that reflects the trade-off between several costs and benefits of mating (Arnqvist and Nilsson, 2000). But these optimal mating rates tend to be higher if they get more benefits (Arnqvist and Nilsson, 2000). This evolution of mating rate is possible as female insects can modulate their mating rate in response to different environmental factors like, operational sex ratio, population density, presence of predators, food availability and phenotype of their previous mates (Arnqvist and Nilsson, 2000). The determining factors of mating rate evolution are mainly based on the male accessory substances and the corresponding female receptor (Eberhard and Cordero, 1995).

The benefit of post mating immunity is already discussed. As evolution of mating rate is possibly based on different direct and indirect benefits so, because of post mating improvement of immunity female mating rate can be increased. Therefore, in this study, I tested the evolution of female mating rate and benefit of post mating improvement of immunity using a population of Drosophila (I, U, S populations, discussed later) selected for increased immunity against *Pseudomonas entomophila* bacteria. The previous data on this population has shown that, mated females of this population has better immunity compared to their virgins and also selected population has better immunity compared to their controls (Radhika, 2016). So, for my study, I explored how female mating behaviour can be evolved because of benefits from post mating improvement of immunity.

Mainly, I have asked two major questions:

1. Does mated female get any extra benefit from post infection mating?
2. Is there any effect of post mating infection on reproductive behaviour (mating rate) in females of selected (I) and control (S, IRS) populations? (to see the evolution of mating rate)

Chapter 2: Materials and Methods

Model Organism:

I used the fruit fly, *Drosophila melanogaster*, as model system for my study. These are holometabolous insects (undergo complete metamorphosis during their life time) which belong to the order Diptera and used extensively as a model organism. Short life cycle, easy handling, experimentally manipulation of laboratory ecology and availability of genetic information make them very popular and powerful model organism for evolutionary studies (Prasad and Joshi, 2003).

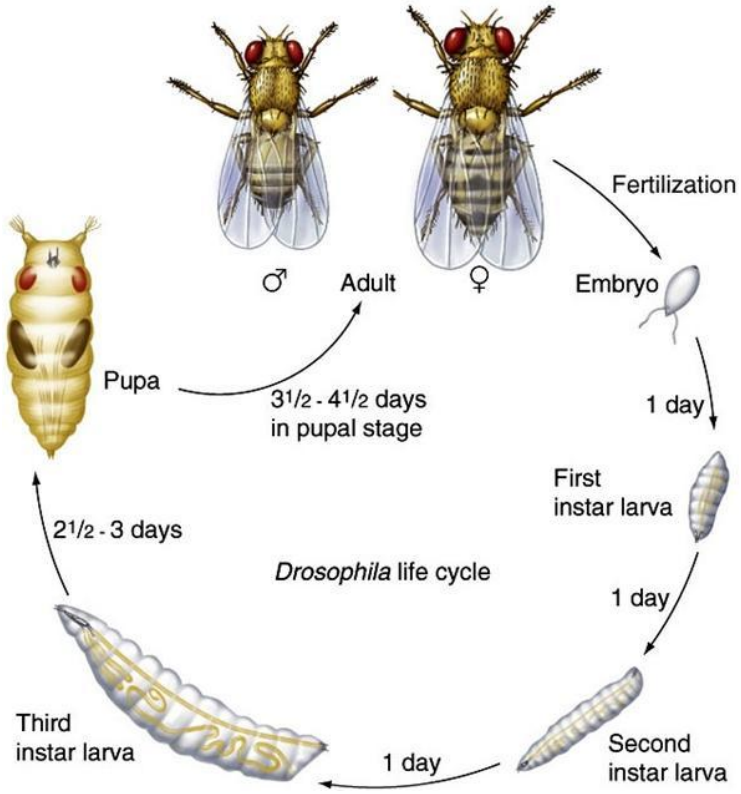

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Figure – 1: *Drosophila melanogaster* life cycle

D.melanogaster has an adult life-span of typically 35-40 days and can cycle from egg to egg in about 10 days on nutritious food medium at around 25° C (Prasad and Joshi, 2003). Drosophila life cycle consists of four stages: Egg, Larva, Pupa and Adult. The eggs are hatched into 1st instar

larvae around 18-24 hours after their eggs are laid, and then they molt into 2nd instar larvae after a day. They molt into 3rd instar larval stage after another day and after that they continue to feed and grow bigger until they start to pupate. They remain in pupal stage for around 4-5 days and after that they eclose as adults. The flies achieve their sexual maturity after about 6-8 hours of post eclosion (Prasad and Joshi, 2003).

Circadian clock has very important role in adult eclosion and attaining of sexual maturity of Drosophila (Prasad and Joshi, 2003). Adult females usually start laying eggs after 1-2 days of post eclosion (Prasad and Joshi, 2003).

Ancestral Population:

For this study, I have used populations of *D.melanogaster* flies derived from a large outbred population called Blue Ridge Baseline (BRB). This population was established from 19 isofemale lines which were generated from 19 females caught in the wild from Blue Ridge Mountain, USA.

The BRB population is maintained as 5 independent replicate populations on a 14- day discrete generation cycle, 12:12 light: Dark regime, 25℃ temperature and 60-70% relative humidity. Every generation eggs are collected from adult flies at a density of 70 eggs per glass vial (25mm diameter × 90mm height) containing 8-10 ml of standard Banana – Jaggery food. 40 such vials are set up for each replicate. On 12th day post egg collection, adult flies are transferred to plexiglass cages (25cm length × 20cm width × 15cm height) along with petriplate of standard Banana – Jaggery food supplement. On 14th day post egg collection, a fresh food plate is provided in the cage and eggs are collected18 hours later to start the next generation. The population size for each replicate is almost 2800 individuals per generation (Gupta et al., 2013).

Experimental Populations:

The populations used for this study are I_{1-4}, S_{1-4}, U_{1-4} and IRS_{1-4}, which are derived from the base line population BRB (BRB_{1-4}) (Figure- 2). From four replicates of BRB four replicates of those selection regimes are derived. These populations are derived to see the evolution of increased immune response. For maintenance of these selection regimes, egg collection is done at a density

of 70 eggs per vial (25 mm diameter × 90 mm height) in 8-10 ml of standard banana jaggery food. Ten such vials are set up for each selection regime per generations. These populations are maintained on a 16 day discrete generation cycle and under 12:12 light: dark cycle at 25°C temperature and 60-70% relative humidity. The details of these selection regimes (Figure -2) are as follows-

- I (Infected regime) - On 12^{th} day post egg collection, 150 males and 150 females flies are sorted under light CO_2 anesthesia and are infected by pricking on their thorax by fine needle (*Minutein pin* 0.1 mm, Fine Science Tools, CA) dipped in a bacterial solution of gram negative bacteria *Pseudomonas entomophila* (Bacteria suspended in 10mM of $MgSo_4$ solution). The bacterial concentration used for the infection is measured in terms of Optical Density (OD_{600}) and it is modified at regularly to maintain mortality rate under 30% every generation. After infection flies are transferred into plexiglass cage (14 cm length × 16 cm width × 13 cm height) provided with a petriplate of standard Banana-Jaggery food and mortality rate is monitored. On 16^{th} day of post egg collection, the cages are provided with a fresh food plate (cut plate) from the survivors to start the next generation.
- S (Sham infected) – Actually it is a pricking control. On 12^{th} day post egg collection randomly 100 males and 100 females from every generation are pricked with a needle dipped in a sterile 10mM $MgSo_4$ solution using light CO_2 anesthesia. There is no mortality in this regime. After pricking of flies, the rest of the steps is same as that of I flies.
- U (Unhandled) – This is a control population. In this case, on 12^{th} day post egg collection, randomly 100 males and 100 females are sorted under light CO_2 anesthesia and transferred to cages for every generation. The next steps are common as other two regimes.
- IRS (Infected Relax Selected) – After 40 generation of forward selection one set of I flies underwent no selection pressure. This was done to find out the cost of immunity by figuring out time taken by IRS flies to revert back to the ancestral condition. Stronger the selection pressure quicker is the reversion. As a maintenance regime of this population, on 12^{th} day post egg collection, 100 males and 100 females are sorted under light CO_2 anesthesia and transferred to cages for every generation. The next steps are common as

other selection regimes. Currently it is in 122 generations whereas I, S, U are in 162 generations.

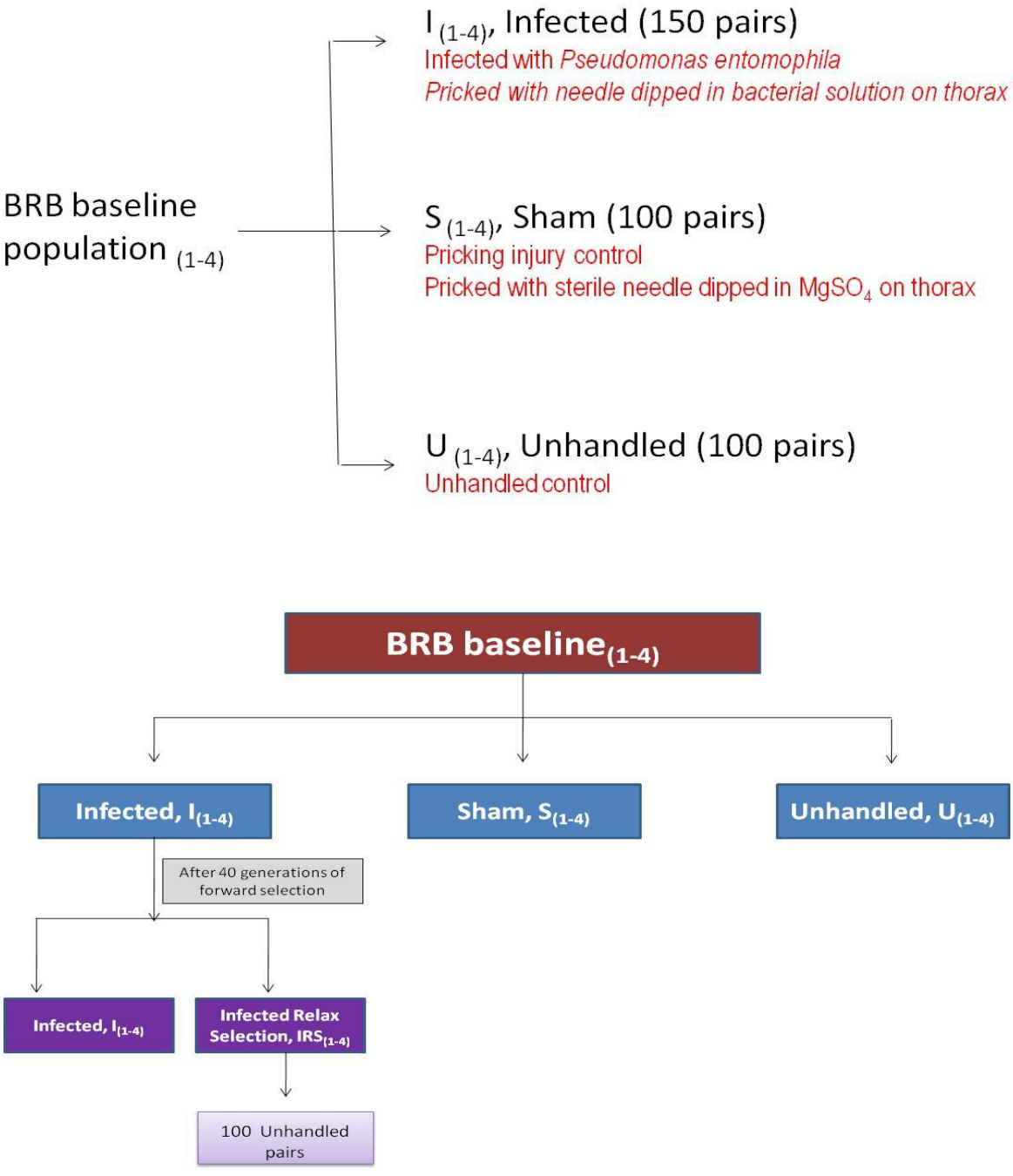


Figure – 2: Selection protocol

Bacterial stocks:

The bacterial stock used to infect the flies for stock maintenance as well as for this study, is *Pseudomonas entomophila* strain L48. It is a gram negative bacterium and is considered as a natural pathogen for *Drosophila melanogaster* flies (Vodovar et al., 2005). For infections of selection regimes and experiments, bacteria are cultured in Luria Bertani Broth medium overnight (~10 hours) at 27˚C, 150 rpm. The next morning secondary culture (~4 hours) is put from this primary culture by diluting it 1000 fold. The sub culture is then centrifuged to be pelleted and re suspended in 10 mM MgSo4 solution to obtain required OD (presently 2.9).

Standardization:

Non-genetic parental effects can affect fitness related traits. So, this kind of effect should be avoided to conduct experiments. These effects are avoided by doing standardization , which is carried out by rearing I, U, S, and IRS flies under similar condition (with no infection or injury) for one generation before conducting experiments. For this, eggs are collected from stock populations at a density of 70 eggs per vial. On 12^{th} day post egg collection, around 250 males and 250 females are transferred to cages. To generate experimental flies, egg collection is done from these cages.

Experimental design:

In order to compare the mating rates of three selection regimes (I, S, IRS) in presence and absence of *Pseudomonas entomophila* bacteria and also to understand benefit of immune response in these three selection regimes, mating observation followed by infection and remating observation were done.

The eggs were collected (70 eggs/vial) from BRB baseline (BRB_5) and standardized I, S and IRS populations. On 9^{th} - 10^{th} day post egg collection virgin males were collected from BRB and virgin females were collected from I, S, and IRS populations. On 12^{th} day post egg collection, in the morning females of I, S, and IRS are mated in group of 8, means there was 8 females and 10 males in one vial. Males were common (BRB_5) for all selection regimes. 40 such vials were set

up for mating of each selection regimes. 4 hours post first mating females were infected (20 vials for each selection regime) with *P. entomophila*. Females were also sham-infected (10 vials for each selection regimes) with sterile $MgSo_4$ as control. Then 2 hours of recovery period was given. Thereafter males were reintroduced into some vials and remating observation was done in following treatments –

1. Mated Infected ♀ x uninfected ♂

2. Mated Sham ♀ x uninfected ♂

3. Mated infected ♀ without ♂

4. Virgin infected ♀ without ♂

Vials were monitored for five days. Observation was done every hour for mating pairs and dead flies.

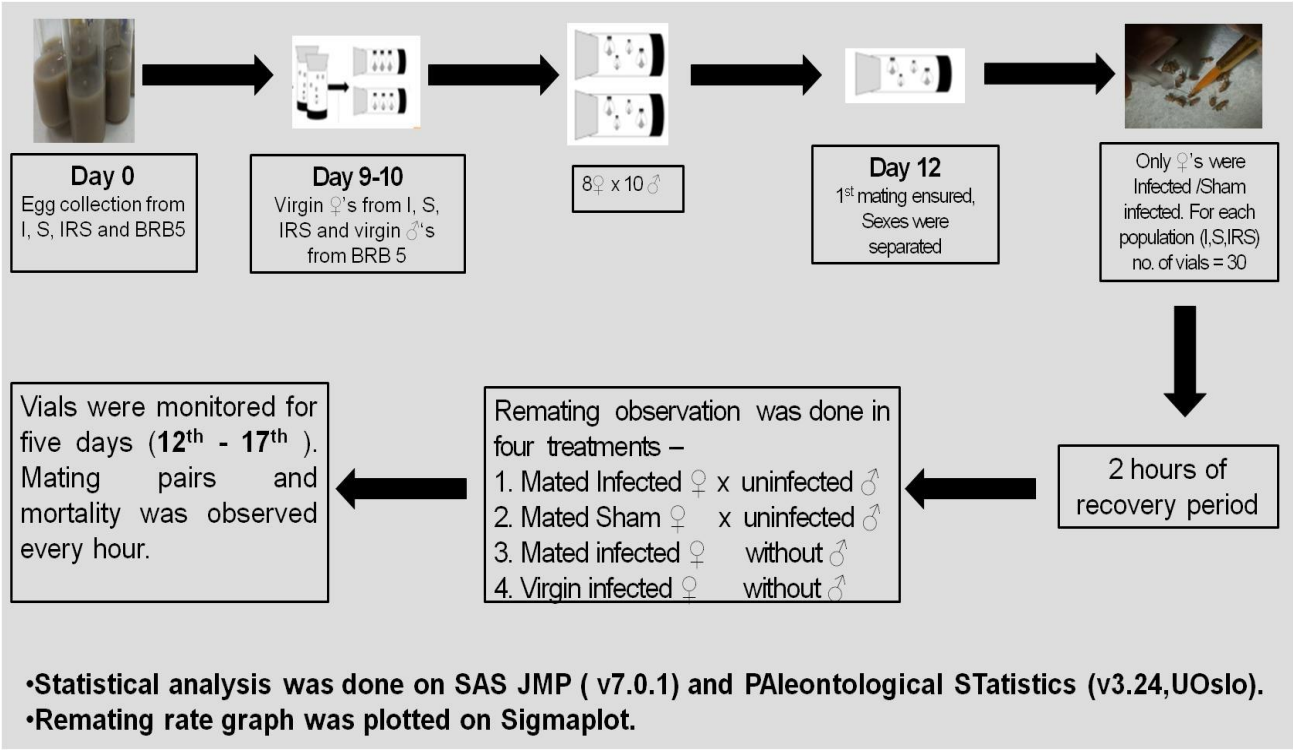


Figure – 3: Experimental design

Statistical analysis:

All the statistical analysis was carried out using SAS JMP (v 7.0.1). For survivorship analysis, Cox-propotional hazards analysis was run in JMP using selection, treatment and selection*treatment as fixed factors. Survivorship curves were generated using Kaplan – Meier estimator. For the comparison of mating rate between selected and control populations, repeated measures ANOVA was done in JMP using selection, treatment as fixed factors and block as a random factor.

Composition of 1 litre standard Banana-Jaggery food:

Ingredient	Amount
Banana(g)	205
Barley flour(g)	25
Jaggery(g)	35
Yeast(g)	36
Agar(g)	12.5
Ethanol(ml) (to mixed with yeast)	45
Water(ml)	180
p-Hydroxymethyl benzoate(g)	2.4
Ethanol(ml)	36

Chapter 3: Results

In order to measure and compare the benefit of mating between mated and virgins of I, S, IRS and also between the singly mated selection regimes and multiply mated selection regime, in the presence (infected with bacterial concentration of OD 1.5) and absence of pathogen, survivorship analysis was done. Remating rates of all three populations were compared to see the evolution of mating rate.

Remating rate analysis:

When repeated measures ANOVA was done in all three populations, selection had significant effect (P = 8.58E-06) across all the four blocks. When data was analysed block wise, then a significant effect of selection was found on remating rate in three blocks (Block 1 – p = 0.000533, Block 2 – p = 0.010704, Block 4 – p = 0.035424).). In Block-1 remating rate of I was significantly higher than S and IRS but S and IRS was not significantly different from each other. Block 2 and 3 were showing same trend. In these two blocks I has significantly higher remating rate than S but IRS had similar remating rate with I and S (Figure -6).

Treatment (Infected and Sham) had significant effect (p = 0.002195) across all the four blocks. But when the analysis was done by blocks then, only one block was showing significant effect (Block 2 – p = 0.044813). So, overall we can say that remating rate has no difference in presence or absence of bacteria (i.e., infected and sham infected conditions) (Table – 1, p-19)

Selection*Treatment had no significant effect for all the four blocks (Table -1).

Day had significant effect on remating rate across all the blocks (p = 0.001499). During block wise analysis Block 2 (p = 0.025693) and Block 3 (p = 0.002603) were showing significant results (Table – 1). But there were no fixed patterns for remating rates across all the blocks (Figure – 6).

The interaction between selection and day had no effect on remating rate (Table -1). The trend for the mating rate was same for all the populations for different days (Figure – 5).

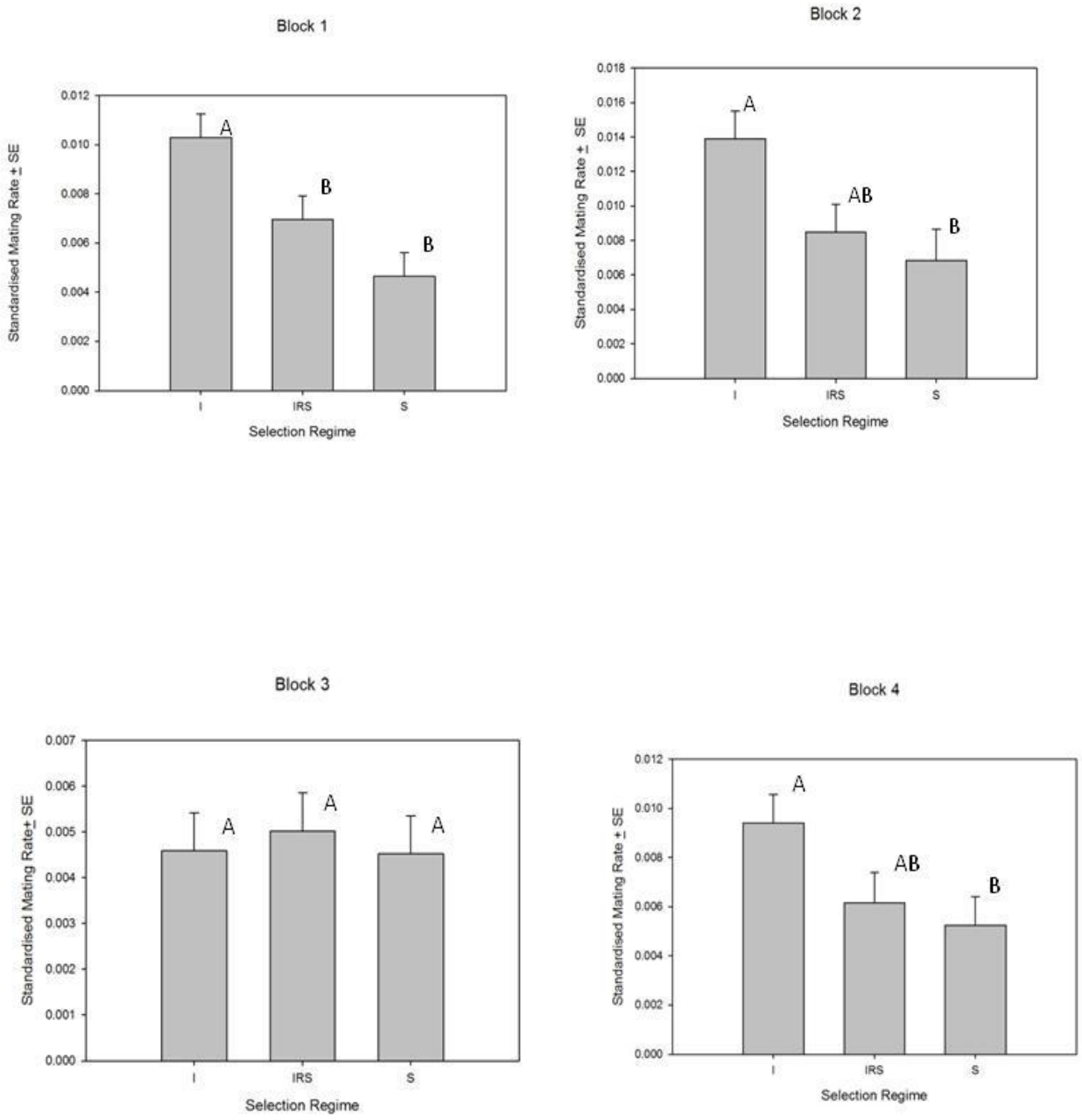
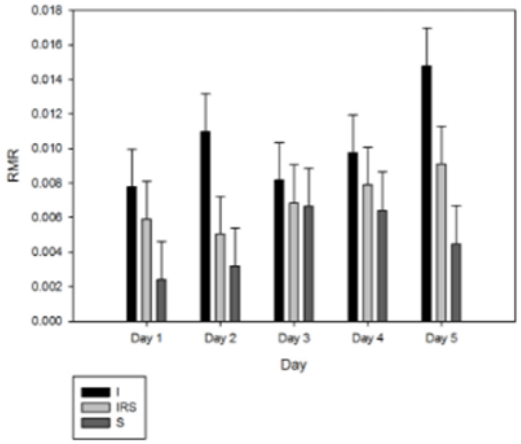
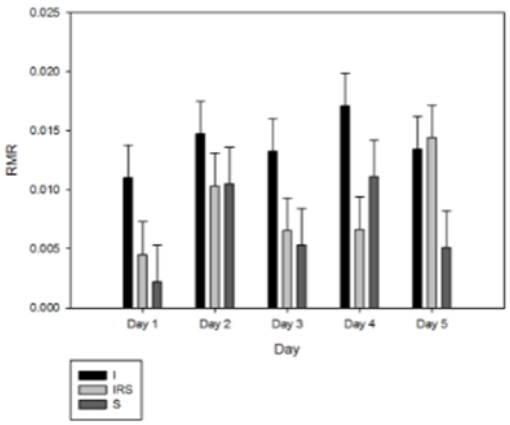


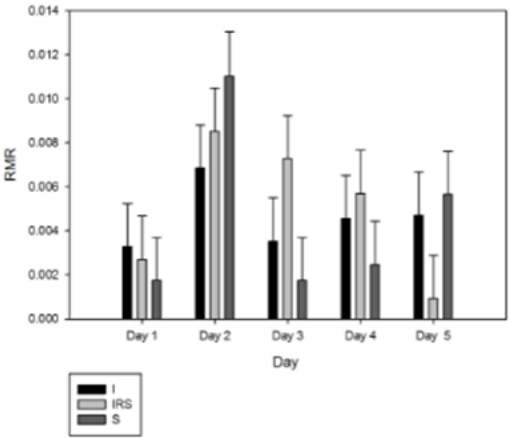
Figure -4: Remating rate graphs for selection



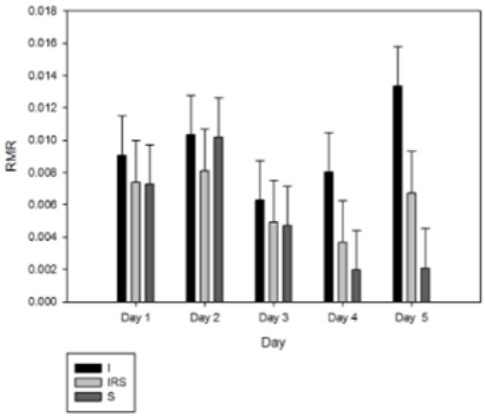
Block – 1



Block - 2

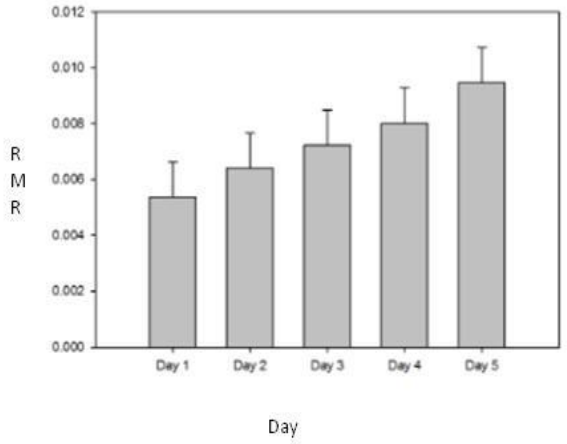


Block – 3

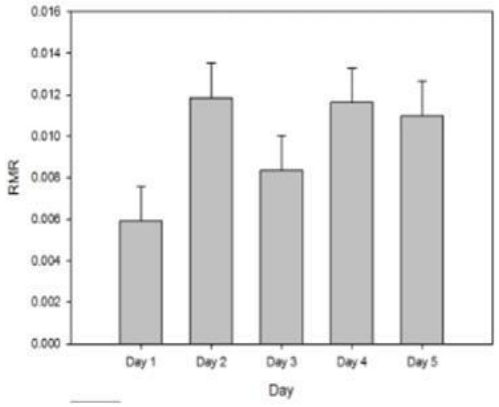


Block – 4

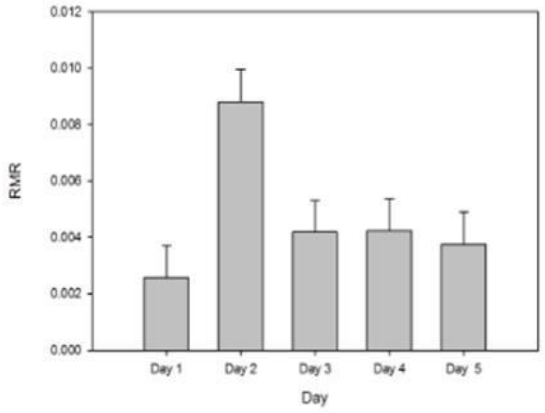
Figure – 5: Remating rate graphs for selection*days



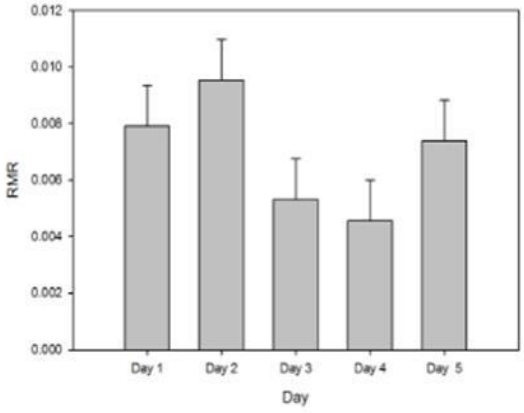
Block – 1



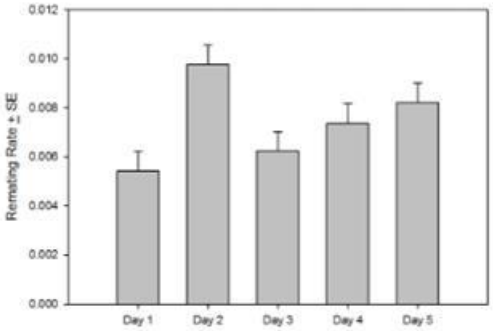
Block- 2



Block – 3



Block- 4



All blocks pooled together

Figure – 6: Remating rate graphs for days across all the populations.

Survivorship analysis:

When infected with bacterial concentration of OD 1.5, population had a significant effect on survivorship of females post infection with *Pseudomonas entomophila* bacteria, for all the infected treatments.i.e., Virgin infected, Single mated – infected and multiple mated - infected (Table 2). For all the treatments, I females were significantly better compared to S or IRS populations (Figure – 7, 8, 9). The survivorship of S and IRS was also significantly different (Figure – 7, 8, 9).

There was no effect of treatment (virgin and mated) and also of population*Treatment for all the blocks (Table – 2). It means virgins and single mated females were not different significantly for all the populations across all the blocks (Figure – 10.1, 10.2, 10.3).

When the Single mated – infected and multiple mated - infected (Treatments) were compared then also Treatment and Population*Treatment has no effect on survivorship for all the blocks (Table – 3). It means infected single mated and infected multiple mated females were not different significantly for all the populations across all the blocks (Figure – 11.1, 11.2, 11.3).

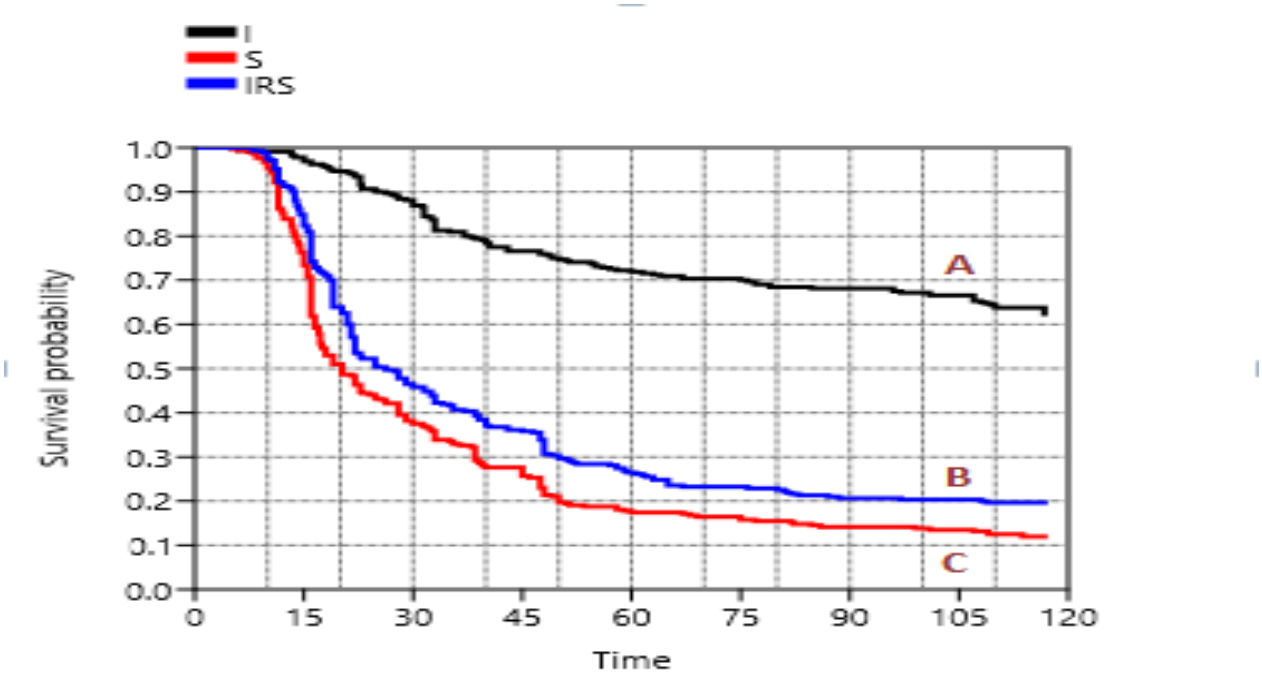


Figure –7: Survivorship curve across all blocks for infected-multiple mated treatment.

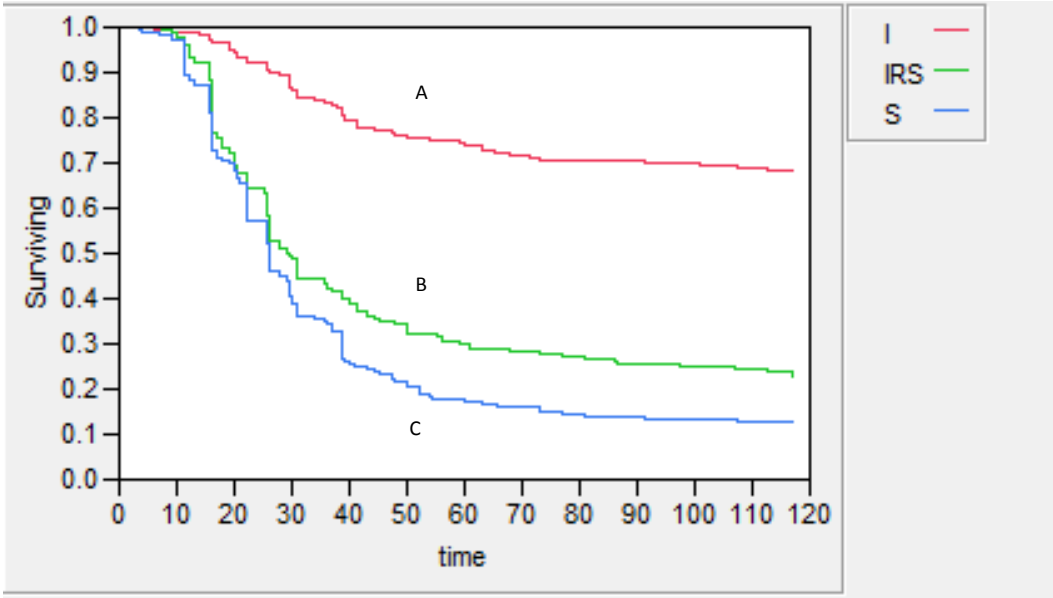


Figure – 8: Survivorship curve across all blocks for Virgin – infected treatment.

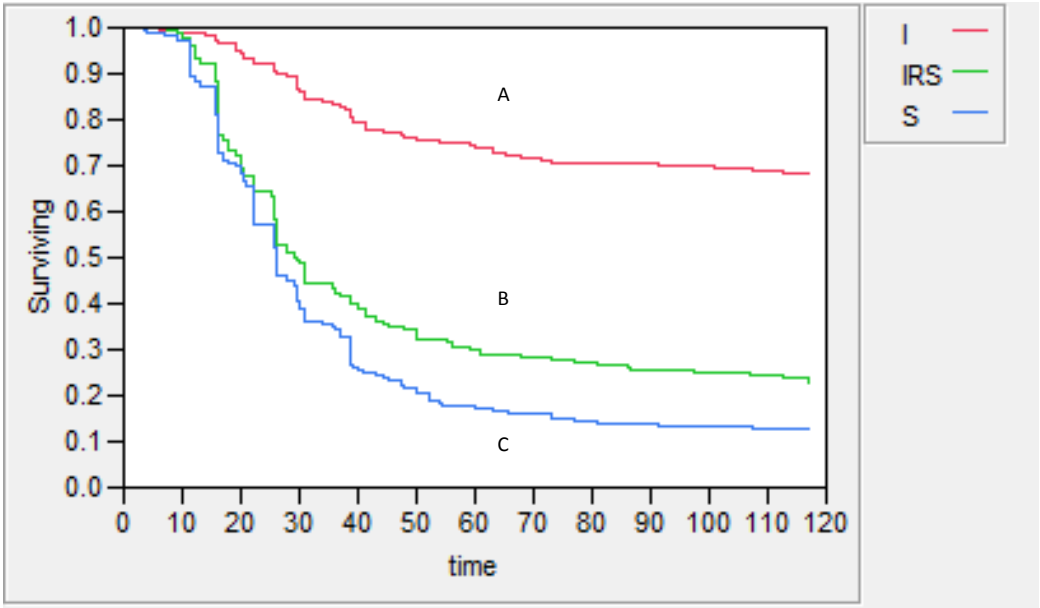


Figure – 9: Survivorship curve across all blocks for single mated – infected treatment.

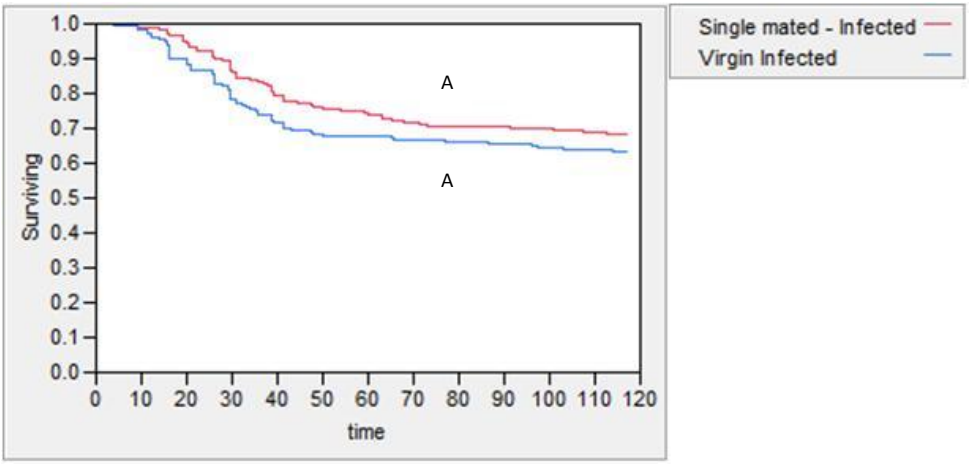
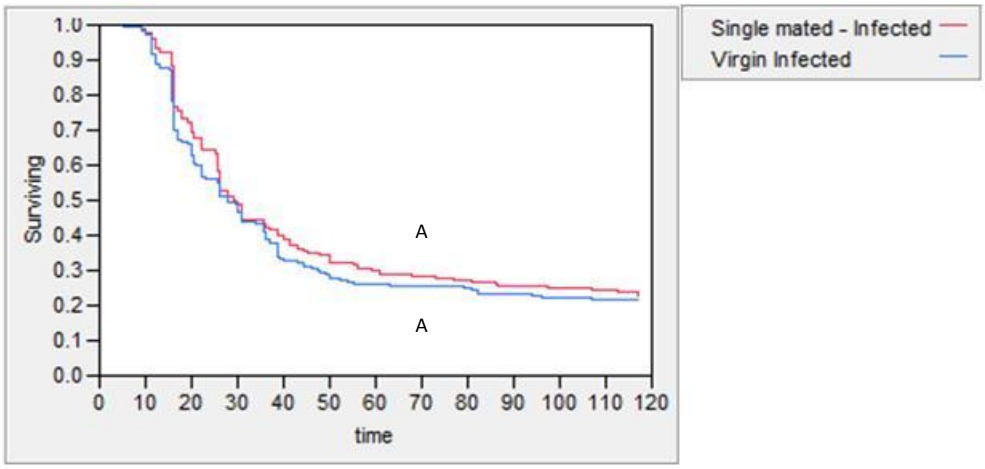
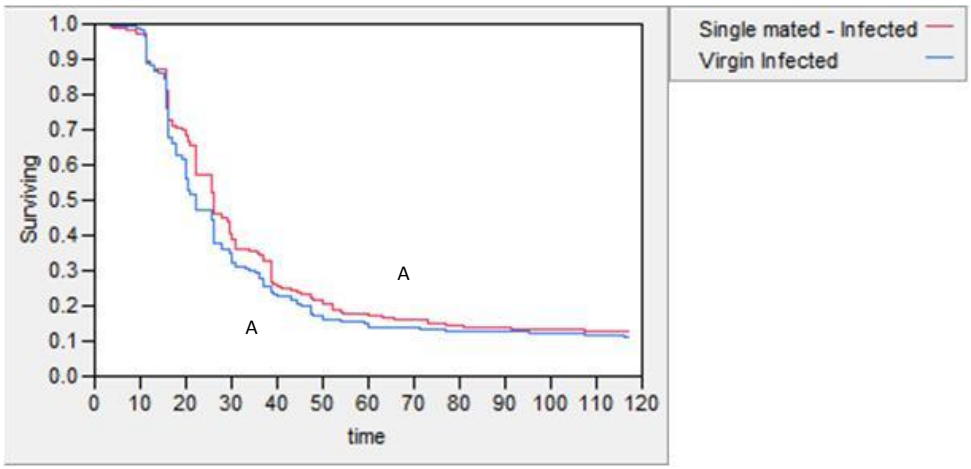


Figure –10.1: Survivorship curve for I population across all blocks for mated vs. virgin treatment



Figure–10.2: Survivorship curve for IRS population across all blocks for mated - virgin treatment



Figure–10.3: Survivorship curve for S population across all blocks for mated vs. virgin treatment

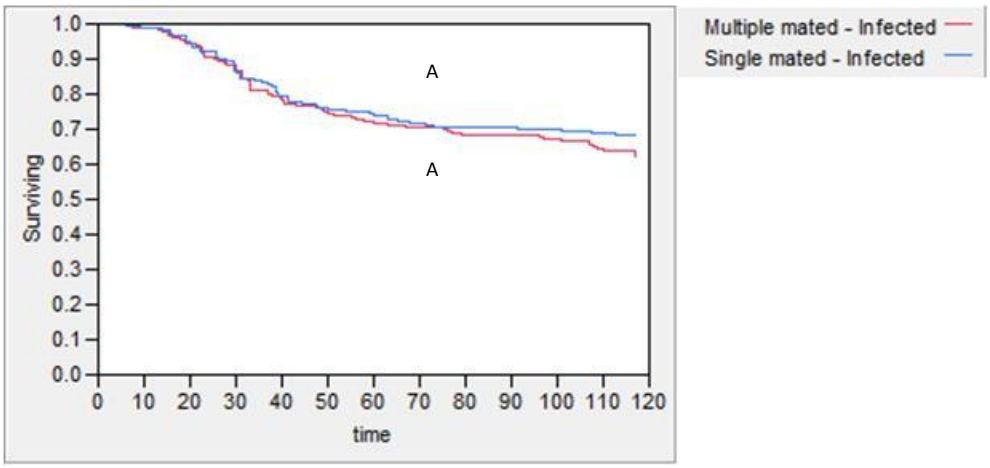


Figure – 11.1: Survivorship graph for I across all blocks for single – multiple mated treatments

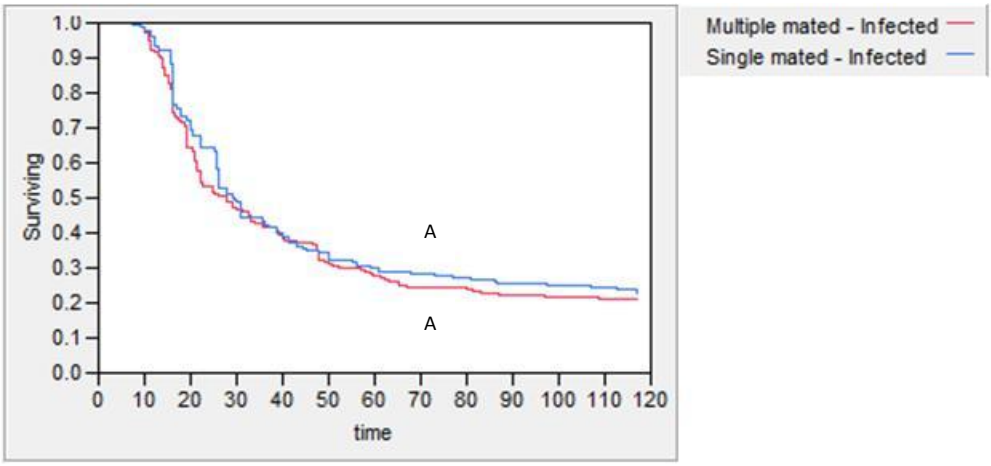


Figure – 11.2: Survivorship graph for IRS across all blocks for single– multiple mated treatments

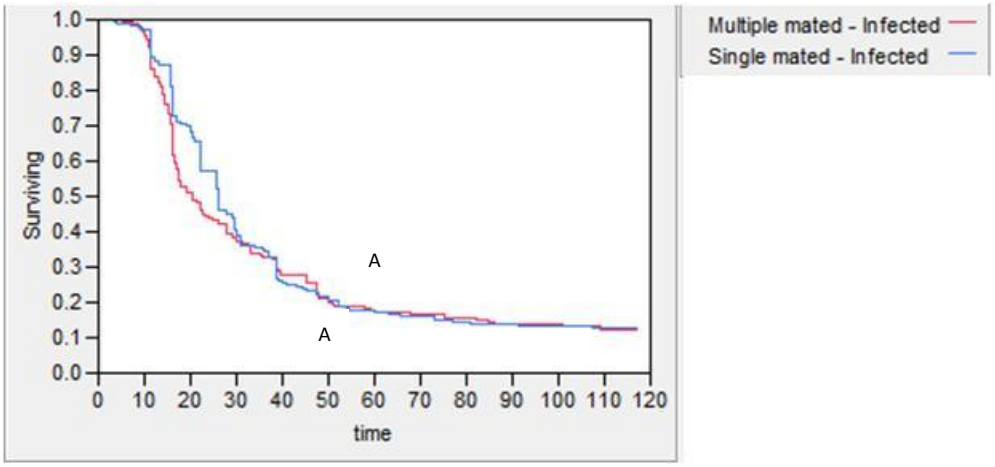


Figure – 11.3: Survivorship graph for S across all blocks for single – multiple mated treatments

	Source	Nparm	DF	DFDen	F ratio	Prob >F
Block 1						
	Selection	2	2	51.0931	8.767475	0.000533
	Treatment	1	1	51.0977	2.468788	0.1223
	Selection*Treatment	2	2	51.0931	1.428236	0.24914
	Day	4	4	211.2554	1.502742	0.202537
	Selection*Day	8	8	211.239	0.706312	0.685819
	Treatment*Day	4	4	211.2554	2.438482	0.048123
	Selection*Treatment*Day	8	8	211.239	1.018884	0.422749
Block 2						
	Selection	2	2	50.01236	4.974855	0.010704
	Treatment	1	1	50.00533	4.235696	0.044813
	Selection*Treatment	2	2	50.01236	1.383899	0.260029
	Day	4	4	199.2229	2.833627	0.025693
	Selection*Day	8	8	199.2274	1.140987	0.337402
	Treatment*Day	4	4	199.2229	1.510201	0.20059
	Selection*Treatment*Day	8	8	199.2274	0.976753	0.455325
Block 3						
	Selection	2	2	54.2779	0.108904	0.897012
	Treatment	1	1	54.27972	2.705509	0.105782
	Selection*Treatment	2	2	54.2779	1.781521	0.178086
	Day	4	4	215.723	4.221758	0.002603
	Selection*Day	8	8	215.7178	1.345916	0.222195
	Treatment*Day	4	4	215.723	0.657773	0.622002
	Selection*Treatment*Day	8	8	215.7178	0.978106	0.454004
Block 4						
	Selection	2	2	52	3.56445	0.035424
	Treatment	1	1	52	1.699099	0.198148
	Selection*Treatment	2	2	52	0.06402	0.93806
	Day	4	4	208	2.031193	0.091278
	Selection*Day	8	8	208	0.91817	0.502319
	Treatment*Day	4	4	208	1.405939	0.233161
	Selection*Treatment*Day	8	8	208	1.945755	0.054907

Table -1: Summary statistics of remating rate analysis.

Block	Source	Nparm	DF	L-R chi square	Prob > chi square
1	Population	2	2	61.0563406	5.52E-14
	Treatment	1	1	0.44017492	0.50703804
	Population*Treatment	2	2	5.01822447	0.08134042
2	Population	2	2	69.4850274	8.16E-16
	Treatment	1	1	1.22457933	0.26846382
	Population*Treatment	2	2	2.05824233	0.35732085
3	Population	2	2	215.79221	1.38E-47
	Treatment	1	1	2.36172631	0.12434452
	Population*Treatment	2	2	0.99619458	0.60768581
4	Population	2	2	179.339427	1.14E-39
	Treatment	1	1	2.04918407	0.15228779
	Population*Treatment	2	2	0.88580883	0.64216859

Table -2: Cox Proportional Hazards analysis of Survivorship for Virgin vs. mated treatments.

Block	Source	Nparm	DF	L-R chi square	Prob > chi square
1	Population	2	2	91.6929066	1.23E-20
	Treatment	1	1	0.67420568	0.41158915
	Population*Treatment	2	2	8.54237508	0.01396519
2	Population	2	2	79.3417136	5.90E-18
	Treatment	1	1	0.00029514	0.98629341
	Population*Treatment	2	2	1.63782564	0.44091074
3	Population	2	2	202.669869	9.79E-45
	Treatment	1	1	4.85822219	0.02751467
	Population*Treatment	2	2	5.28333746	0.07124229
4	Population	2	2	176.151578	5.61E-39
	Treatment	1	1	0.77903057	0.37743779
	Population*Treatment	2	2	0.0442799	0.97810334

Table -3: Cox Proportional Hazards analysis of Survivorship for Single vs. multiple mated treatments.

Chapter 4: Discussion

This study was designed to see the evolution of mating rate in a population selected for increased immune response and to study the role of benefit of mating in terms of immunity on the evolution of mating rate. As hypothesized, mating rate has evolved in selected population but the survivorship data does not support the previously reported data (Radhika, 2016, MS thesis) of this population, which says mated flies are better in terms of immunity compared to virgins and Selected (I) population has better immunity compared to controls (S, IRS).

My study indicates that mating rate has evolved in this population i.e., I (Selected) population has higher mating rate compared to the S or IRS populations (Controls). Whereas, the survivorship data says that virgin and mated flies are not different in terms of immunity and also there is no extra benefit of multiple mating compared to single mating. It means, mating has no beneficial role in increasing immunity of selected populations which can't explain evolution of mating rate.

Female mating rate can be evolved if there are any kinds of benefit that reduces the different costs of mating (Arnqvist and Nilsson, 2000). Mating has several costs; females can counter-evolve to overcome such costs and because of which mating rate can be increased. Study on Bedbugs suggested that the protection against the physical injury and traumatic insemination caused by mating can be done by localizing damage to one area or by restricting the diffusion of the ejaculate inside the female or by reducing leakage of blood through the wound site or by restricting entry of pathogens into the bloodstream (Morrow and Arnqvist, 2003). Similarly, it might be possible that females in *Drosophila melanogaster* increase their mating rate by counter-evolving different costs of mating.

Another, possible explanation behind the increasing mating rate can be mate harm and mate harm resistance caused by mating. In promiscuous species like Drosophila, males try to increase their fitness by mating successfully with as many females available which usually leads to male-male competition. As a byproduct of this competition, males become more harming to the females by either physically or chemically, thus reducing female's ability to lay eggs or mate

with other male (Chapman et al., 1995). Female fitness depends upon no. of eggs they lays, so they develop strategies to overcome this harm, which is mate harm resistance. I (Selected) females might have evolved to nullify mate harm resistance strategy so, they are not avoiding mating. Multiple mating always cause mate harm. The control populations might have inherent mate harm resistant strategy so, they are avoiding more mating but I populations are evolved against mate harm and so, mate multiple times with increasing rate.

Day has significant effect in two blocks but there are no fixed patterns. So, we can't conclude anything with this day wise data of remating rate. The interaction between selection and day has no effect on remating rate. All the populations has same trend of remating for different days. This might be because of using common males for all the populations. All the populations were same facing same receptive background during the remating because of common males.

In this study, I have used common males (BRB5) to mate with I, S and IRS females. The reason behind using those was to reduce the mate choice effect and also to give a common background to the females, where the receptivity will be same for the females of all selection regimes. In earlier study on these populations mating was done in a full factorial way i.e., the treatments were I male*I female, I male*S female, S male*I female, S male*S female and in this case mated females were better than virgins in terms of immunity (Radhika, 2016, MS thesis). But in my study, this benefit of mating is vanished. Common males might have effect in this case. The females of selected population (I) might be more receptive towards common males (BRB), compared to their control females (S and IRS). So, they remate more even when the benefit of mating is not there.

Since, mating rate has evolved in selected populations but there is no role of immunity benefit, so, further study has to be done to investigate the other possible factors for mating rate evolution. Role of mate harm or the female adaptations against costs of mating or the role of common males and female response towards receptivity can be studied in this case.

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