Studying male mate choice and non-genetic inheritance in laboratory-adapted populations of *Drosophila melanogaster* evolved for higher immunity against a gram-negative bacterium *Pseudomonas entomophila*

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

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Certificate of Examination

This is to certify that the dissertation titled "Studying male mate choice and non-genetic inheritance in laboratory-adapted populations of *Drosophila melanogaster* evolved for higher immunity against a gram-negative bacterium *Pseudomonas entomophila*" submitted by Mr. Temura Chinmay Krishna Yadav (Reg. No.MS14159) 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.

Prof. Somdatta Sinha Dr. Manjari Jain Dr. N. G. Prasad

(Supervisor)

Dated: April 26, 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 acknowledgment 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.

Temura Chinmay Krishna Yadav
(Candidate)

Dated: April 26, 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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IISER Mohali had been an amazing place and a home away from home and I would like to put forward my sincere appreciation toward the administration of IISER Mohali for facilitating it to happen. Secondly, I would like to thank my masters' committee members, Professor Somdatta Sinha and Dr. Manjari Jain for providing with insightful directions in my research.

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Abstract

Females are ‘choosy’ while males are ‘flashy’ among most organisms. There are widespread pieces of evidence which suggest that males produce ornamental characters while females choose males with the best ornaments. Exhibiting choosiness often has adaptive value and in the past few decades, males exhibiting choice has been predicted and documented when some conditions are met. When males invest higher in reproduction or when they can perceive a variance in quality of the females, they do exhibit choice but there is no study on the impact on the outcome of choice brought by the ability of females to alter its quality. This study aims to address the question by using laboratory-adapted populations of *Drosophila melanogaster* evolved for higher immunity, whether the outcome of male’s choice depends on female’s infection status as reported in previous findings and whether the female’s ability to clear off pathogen can affect the outcome of the choice. Our study does not report any choosiness shown by males.

Inheritance of characteristics can happen in a non-genetic manner along with classic Mendelian genetic inheritance. A form of non-genetic inheritance of traits is the inheritance of characters by the stepchildren from the stepfathers. Body size of the offspring has been shown to be inherited from the stepfathers in Neriid flies. We made an attempt to examine if immunity related traits could inherit from the stepfathers to the stepchildren in laboratory-adapted populations of *Drosophila melanogaster*. We have not found any evidence of such an effect.

Chapter 1

Introduction

Study of evolutionary biology with a focus on the evolution of immunity had brought important insights in the fields of medicine by understanding how pathogens developed resistance against drugs. But with the empirical findings of Hamilton and Zuk (1982), the focus of evolutionary biologists working on the evolution of immunity expanded even to understand other life-history traits of an organism and their interactions with immunity. Hamilton and Zuk (1982) argued that it is through showy characteristics males could advertise their 'good genes' of immunity or resistance against pathogens and undergo inter-sexual selection while females gaining indirect benefits of producing fitter offspring. This hypothesis had a huge impact on the study of sexual selection and has been expanded to include the direct benefits (Folstad and Karter 1992; Kirkpatrick and Ryan 1991). The direct benefits of employing choosiness include a lower risk of pathogen transmission during reproduction.

Interactions between immunity and other life-history traits like traits related to reproduction were also widely studied. Stearns (1992) theorized a trade-off model in which he argued that life-history of an organism is shaped by the trade-offs made in the investment of resources that are limited. As per this hypothesis, reproduction and immunity which are costly traits must trade-off with each other. Empirical shreds of evidence across many invertebrate species were in line with this prediction (Mckean&Nunney 2008, Simmons et al. 2010, Hangartner 2015).

As time progressed Evolutionary biologists working on the evolution of immunity began to study immunity of insects. Mechanisms related to innate immunity are conserved across invertebrates and vertebrates and Vilmos and Kurucuz (1998) reviewed the conserved mechanisms of innate immunity among insects and mammals.

The first line of defense is the anatomical barriers like exoskeleton made up of chitin and acidic gut lining (Lemaitre and Hoffman 2007). The components of innate immunity have been studied extensively in *Drosophila melanogaster* and are divided into cellular (predominantly during larval stages) and humoral components (predominantly during adult stages) (Kounatidis&Ligoxygakis 2012). Cellular components include hemocytes which are sub-divided into plasmatocytes which help in phagocytosis of pathogens, crystal cells functioning in melanization and wound repairing and lamellocytes in engulfing foreign bodies (Lemaitre and Hoffman 2007). Humoral components include the production of anti-microbial peptides by the fat bodies. ‘Toll' pathway is activated to fight against gram-positive bacteria by producing ‘defensins' and ‘imd' pathway against gram-negative bacteria by producing ‘drosocins', ‘diptericins' and ‘attacins' (Lemaitre and Hoffman 2007).

Various researchers have used different means to quantify immune responses ranging from levels of phenoloxidase activity or lytic activity but these findings don’t generally correlate with survivorship post-infection (Adamo 2004).

For my Masters' dissertation have used laboratory-adapted populations of *Drosophila melanogaster* to address two broad questions revolving around immunity and their interactions with life-history traits. I have studied male mate choice in response to the infection status of females and the effect of female's pathogen clearing ability on the outcome of the choice, which I presented in Chapter 3 of my thesis.

I have examined survivorship post-infection of stepchildren of males of different populations which manifest different levels of immune response to validate if the

non-genetic inheritance of immunity-related traits could happen from stepfathers to stepchildren. I presented my findings in Chapter 4 of my thesis.

Chapter 2

Materials and methods

Experimental System: ***Drosophila melanogaster***

(Phylum: Arthropoda, Class: Insecta, Order: Diptera, Family: Drosophilidae)

Drosophila melanogaster, commonly known as the fruit fly is a holometabolous insect which undergoes complete metamorphosis and consist of 4 distinct life stages – Egg, Larva, Pupa and Adult. The larval stage is a very important stage as most of the nutrition is acquired. In the wild, these flies feed forage on rotten fruits and generally lay their eggs on them. The laboratory populations are grown on standardized food medium (the composition of the food medium provided at the end of this chapter). When grown at 25 ^{0}C, 60-90% relative humidity, *D. melanogaster* eggs take about 24 hrs to hatch, to form 1st instar stage of larvae. After a day they transform into 2nd instar stage. Another day after this, they transform into a 3rd instar stage and remain in this stage for the next 2-3 days. The larvae then, emerge from the food and search for a dry site to pupate. Formation of pupa involves immobilization of larvae by the formation of a brown sheath called puparium which helps in adhering to a surface. This stage lasts for the next 4-5 days, during which larvae undergo metamorphosis by getting rid of the old larval tissues and developing tissues anew from the imaginal disks. The pupal stage is characterized by the transition of pupal color from light yellow to dark brown. The flies then start to eclose from the pupae, approximately

9-10 days post egg laying. Post eclosion, it takes 6-8 hrs for the males and females to become sexually active.

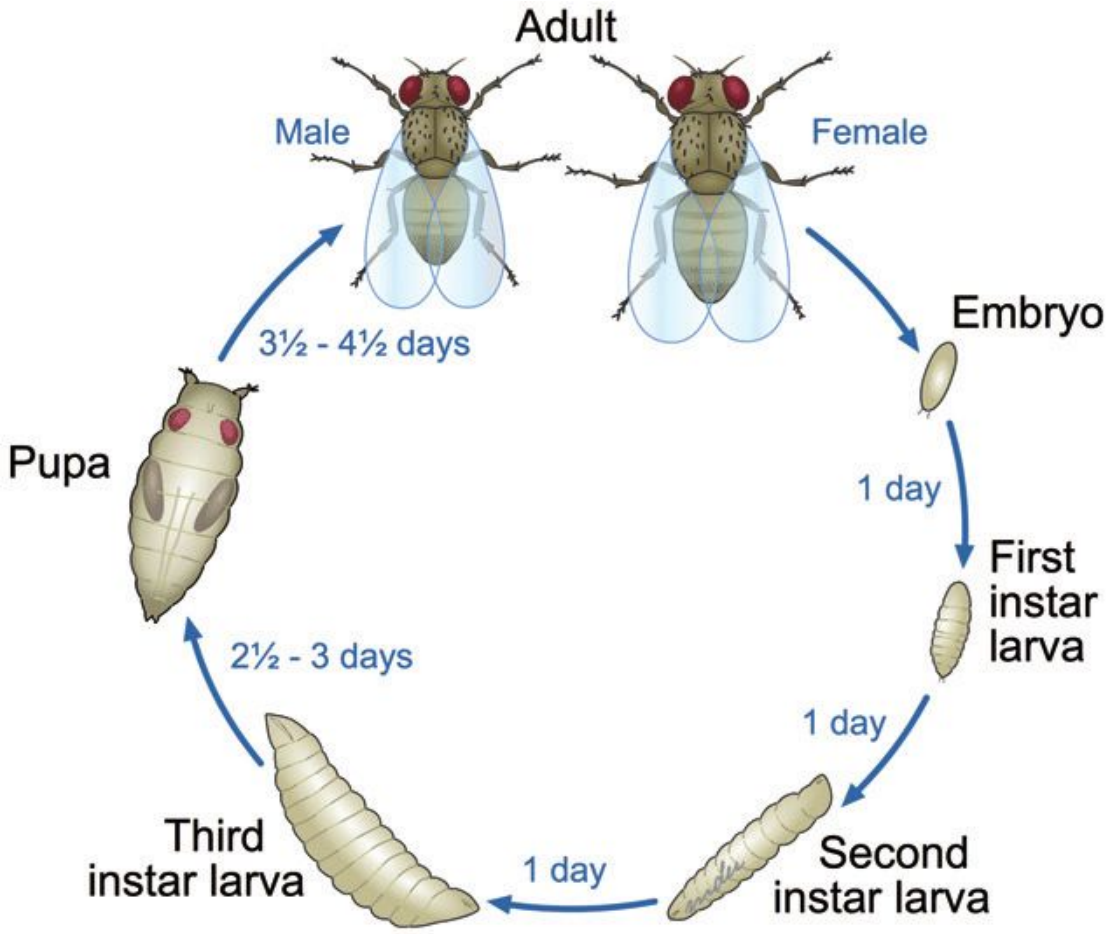


Figure 2.1: the Life cycle of *Drosophila melanogaster*

Source: https://www.researchgate.net/figure/The-whole-life-cycle-of-the-fruit-fly-Drosophila-is-relatively-rapid-and-takes-only_fig1_264127592

In my experiments, I used the following laboratory populations of *D. melanogaster*.

Blue Ridge Baseline (BRB) and its derivatives:

BRB population:

BRB_{1-5} are five individual, replicate, baseline populations of *D. melanogaster* that are large and outbred. Initially, a BRB population was established from 19 iso-female lines acquired from the Promislow lab, University of Washington. After 10 generations, the replicate populations were set up. These populations are the populations maintained on a 14-day discrete generation cycle under standardized laboratory conditions. The maintenance protocol is as follows:

On Day 1, 60-80 eggs are collected in 8-dram vials (25mm diameter x 90mm height) containing 8-10 ml of banana-jaggery food (Table 2.1). Eggs are collected into 40 such vials and incubated for the next 12 days at 25 ^{0}C, 60-90 % RH, 12/12 Light/Dark hrs. By Day 12 post egg collection, most of the flies eclose and these are transferred into a plexiglass fly cage (25cm length x 20cm width x 15cm height) provided with a Petri-plate consisting of the standard banana-jaggery food with an excess of yeast paste spread on the surface of the food. On Day 14 post egg collection, a fresh food plate is provided for the next 18 hrs, which is the window of oviposition. Eggs are collected from this plate and these form the next generation of the population.

IUS selection regime:

Three independent regimes each were derived from BRB_{1-4}, namely Infected or I regime, Unhandled or U regime and Sham infected or S regime. There are therefore four independent replicate blocks (IUS_{1-4}), maintained in a 16-day discreet generation cycle, under the same laboratory conditions as BRBs.

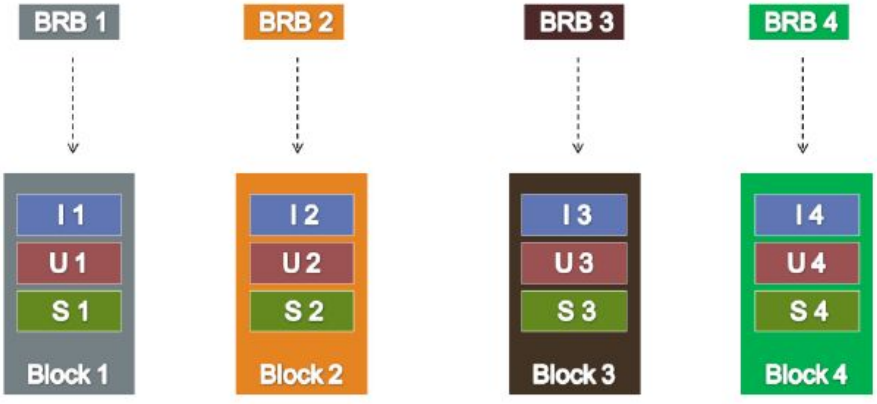


Figure 2.2: Schematic of the establishment of IUS populations. (ref: Radhika's thesis)

Infected or I regime:

The flies of this regime receive a systemic infection with the pathogen *Pseudomonas entomophila* on day 12 post egg collection every generation. 150 flies of each sex are mildly CO_2 anesthetized, pricked on the lateral side of thorax with a thin needle (*Minuetin pin* 0.1mm, Fine Science Tools, CA) dipped in a bacterial suspension (*Pseudomonas entomophila* of the desired OD suspended in 10mM $MgSO_4$ solution).

These flies are then transferred into a plexiglass fly cage (14cm length x 16 cm width x 13 cm height) provided with a Petri-plate consisting of standardized banana-jaggery food. This plate is replaced by a fresh food plate every 48 hrs. Over the period of 96 hrs, a subset of the population dies and only the survivors get to lay eggs during the 18 hrs oviposition window that follows. Eggs are collected at a density 60-80 eggs per vial for a total of 10 vials. These form the individuals of the next generation.

Unhandled or U regime:

The flies of the U regime are maintained similar to the flies of the I regime except for the fact that only 100 males and 100 females are sorted under mild CO_2 and transferred to the plexiglass fly cage to follow maintenance protocol similar to I regime. These flies serve as the 'Unhandled' controls.

Sham-infected or S regime:

The flies of this regime serve the purpose as the pricking control to differentiate traits that have evolved in response to the bacterial infection from those that are caused by injury when pricked or by $MgSO_4$. 100 males and 100 females are anesthetized under mild CO_2, pricked on the lateral side of thorax with a thin needle that is dipped in 10mM $MgSO_4$ solution followed by maintenance protocol similar to U and I regimes.

LHst population:

It is derived from the LH population by introducing an autosomal recessive allele for scarlet eye color (st) by repeated backcrossing (Prasad et al. 2007).

The LH population was set up by Larry Harshman with 400 wild caught females from central California, the USA in 1991 (Chippindale and Rice 2001). This population is maintained on a 14-day discrete generation cycle under lab conditions of 25^0 C, RH 60-90%, 12h/12h Light/Dark cycle on standardized cornmeal-molasses food (Table 2.2). The LH population is maintained in 60 8-dram vials (25mm diameter x 90mm height), each with 6-8 ml of cornmeal-molasses food. Eggs are reared at a density of ~150 eggs/vial. On Day 12 post egg collection, under light CO_2 anesthesia males and females from different vials are mixed and 16 males and 16 females per vial are introduced into fresh vials provided with a limited amount of live yeast. Two days later the flies are transferred to oviposition vials and are housed for 18 hours after which, adults are discarded and the egg density is trimmed to 150/vial.

The LHst population is maintained similar to LH population but the population is made up of only 30 vials. LHst is backcrossed with LH periodically to maintain genetic uniformity across the two populations.

Bacterial stock:

To infect selection regime and experimental flies, a gram-negative bacteria called *Pseudomonas entomophila* strain L48 is used. This bacteria has been isolated from *D. melanogaster* and is considered a natural pathogen of *D. melanogaster* (Vodovar et al., 2005). The bacteria are grown in Luria Bertani Broth medium at 27 ºC, 150 rpm for a period of 8-10 hours. This culture is then sub-cultured by diluting it 1000 fold. It is monitored until for 3-4 hrs, after which it is pelleted and re-suspended in the required amount of 10mM $MgSO_4$ solution, according to the desired bacterial concentration.

Standardization:

It is essential to maintain all the populations on a similar maintenance protocol for one generation to distinguish genetic effects due to selection from non-genetic parental effects (Rose, 1984).

Statistical Analyses:

All statistical analyses were done using JMP7.0.1, STATISTICA, Excel 2016.

Table 2.1: Composition of banana-jaggery food

S.I no	**Ingredient**	**Amount (per liter of food)**
1	Water(ml)	1180
2	Agar powder(g)	12.4
3	Banana(g)	205
4	Jaggery(g)	35
5	Baker's yeast(g)	36
6	Barley flour(g)	25
7	p-Hydroxymethyl Benzoate(g)	2.4
8	Ethanol(ml)	45

Table 2.2: Composition of cornmeal-molasses food

S.I no	**Ingredient**	**Amount (per liter of food)**
1	Water(ml)	1100
2	Agar powder(gm)	14.8

3	Molasses(ml)	100
4	Cornmeal(gm)	100
5	Baker’s yeast(gm)	41.2
6	Propionic acid(ml)	8
7	p-Hydroxymethyl Benzoate(gm)	2.25
8	Ethanol(ml)	22.5

Chapter 3

Male mate choice and the impact of female's ability to clear off pathogen on the outcome of the choice

''We are, however, here concerned only with that kind of selection, which I have called sexual selection. This depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction." (Darwin 1871)

Introduction:

Darwin in his book, *The Descent of Man, and Selection in Relation to Sex* defined sexual selection as a mode of selection on the individuals of one sex which have an advantage over other individuals of the same sex, solely in terms of reproduction. He suggested that sexual selection can operate in two forms. 1. Intra-sexual selection in which members of the same sex compete among themselves to acquire mates. 2. Inter-sexual selection in which members of one sex display traits to be chosen by the other sex. Conventionally, males were considered as the 'flashy' and the females as the 'choosy' sexes respectively. Bateman (1948) argued that males possessing undiscriminating eagerness and females, discriminating passivity could be widespread in nature. This could be attributed to the fact that females invest relatively higher into

reproduction by producing costly eggs than males which invest very little by producing tiny sperm (Parker, Baker & Smith 1972). Recent studies in the past few decades have made an attempt to establish that 'Male mate choice', defined as a differential male sexual response to the different reproductively mature conspecific females could be very widespread across organisms (Bonduriansky 2001). The relative parental investment could be a key factor in determining the way sexual selection operates. Trivers (1972), predicted that the members of the sex that invest relatively lower in parental investment, compete among themselves to attain mates but if both the sexes invest equally, sexual selection would operate equally. Therefore, male mate choice could evolve in scenarios where males invest relatively high in parental care or courtship-feeding causing the fecundity (fitness component) of the females to be limited. This would result in 'complete sex-role reversal' (females undergo intra-sexual selection and males exhibit choice), as documented in the case of crickets and katydids, (Gwynne & Simmons, 1990; Gwynne, 1990, 1993; Simmons, 1993). The other forms of parental investment by males of an organism could be by incurring non-trivial costs while composing an ejaculate or performing courtship activity (Dewsbury, 1982; Pitnick and Markow, 1994; Cordts & Partridge, 1996).

A much recent prediction made by Parker (1983) and Gwynne (1991) suggests that individuals of one sex could exhibit choice even when there is no significant parental investment. Variance in quality among the members of one sex alone could be enough to drive choice to be exhibited by members of the other sex. This could result in systems where 'partial sex-role reversal' occurs where both males and females exhibit choice (Parker, 1983; Gwynne, 1991).

Numerous studies reported males of a population exhibiting choice when a variance of quality among the females existed, possibly by assessing the fecundity of a females (key component of fitness) as a component of quality through indicators like body size, fatness, gravid or non-gravid conditions (Pitafi, Simpson & Day, 1995; Gage & Barnard, 1996; Bonduriansky & Brooks, 1998; Lefranc & Bundgaard, 2000; Katvala & Kaitala, 2001; Byrne & Rice, 2006; Nandy et al. 2012). A component of quality could also be the pathogen load in a female and the variance in pathogen load could drive males to exhibit choice (Imroze & Prasad, 2013; Witmman & Fedorka, 2014).

Though above mentioned studies have documented male mate choice and weakened the notion of male being the 'flashy' sex while the female being the 'choosy' sex, none of the studies to my knowledge have made an attempt to study the impact caused by the ability of the female to improvise its quality on the outcome of choice exhibited by the males. I have tried to examine if the ability of a female to clear off pathogen can impact the outcome of the choice exhibited by males as this ability of females could possibly create an impact on the variance of quality and therefore on the choice exhibited by males.

To address this question, I considered laboratory-adapted populations of Drosophila melanogaster evolved for higher immunity against *Pseudomonas entomophila* (population I), and its baseline control (population U). Individuals of I population have been shown to have higher survivorship and females have been shown to have a greater pathogen clearing ability as compared to their baseline controls (Vanika Gupta, PhD thesis, IISER Mohali, 2015).

I hypothesized that males would exercise a strong choice when presented a choice between an infected U female and a sham-infected female (two choice setup) whereas a weak choice when presented with I infected and I sham-infected females in a similar setup.

<u>Experimental design:</u>

Male mate choice experiment with decapitated females:

Day 1: Eggs are collected from $I_{(1-4)}$, $U_{(1-4)}$, BRB4 populations at a density of 60-80 eggs/vial.

Day 9-11: 6 hours post egg collection, adult flies from I and U populations are sorted under light CO_2 anesthesia and collected into individual sex group vials at 10 individuals/vial. The same is done for BRB4 population but 30 females/vial are collected.

Day 12: Males of I and U population are transferred along with sperm-limiter BRB4 females to fresh vials for allowing the sperm-depletion of I and U males because research suggests that males exercise a stronger choice under resource depleted condition (Byrne and Rice, 2006). 12 hours later, males and females are separated under light CO_2 anesthesia, females are discarded and males are transferred to a vial.

Females of I and U population are randomly assigned to two treatments – infections or sham-infections. For infections- females are mildly CO_2 anesthetized, pricked on the lateral side of thorax with a thin needle (*Minuetin pin* 0.1mm, Fine Science Tools, CA) that is dipped in a bacterial suspension (*Pseudomonas entomophila* of 1.5 $OD_{600,}$ suspended in 10mM $MgSO_4$ solution). For sham-infections- females are anesthetized under mild C02, pricked on the lateral side of thorax with a thin needle that is dipped in10mM MgSO4 solution.

Females are dusted for 6-8 hrs with micronized dust of two different colors.

Day 13: 12 to14 hours post-infection, females are decapitated under light CO_2 anesthesia. Once the females recovered, observations are set up in the following manner in vials with fresh food. Females are decapitated to eliminate the potential confound on male's choice due to differential receptivity of females (Spieth 1966).

Table 3.1: Experimental design

Male	Female 1	Female 2
U	U infected	U sham
I	I infected	I sham
I	U infected	U sham
U	I infected	I sham

Observation of each vial was done for a maximum of 30 minutes and the following traits were recorded by an observer. The sample size for each treatment: 50 vials with reciprocal coloration (25 each) design.

The following traits were assayed:

1. Courts first (CF): The female the male chose to court with for the first time
2. Courtship latency (CL): The Time taken for the male to court a female of a particular kind.
3. Courtship frequency or Courts most (CM): Proportion of time a male courts female of a particular kind out of the total time the male spends on courtship.

Results:

Courts First or CF:

The x-axis indicates the treatment. For example, A x B implies B male given a choice between an infected and sham-infected A females.

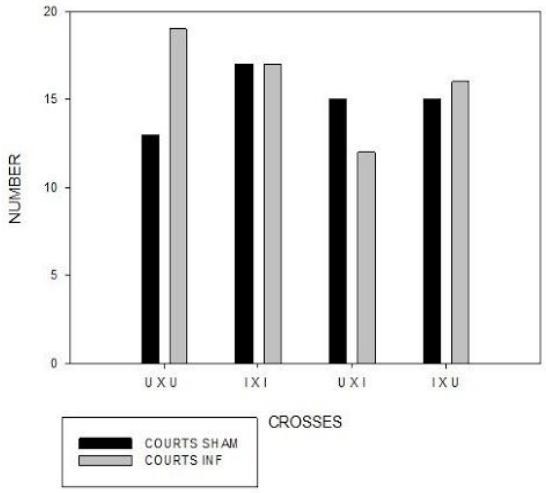
Y-axis indicates the number of vials that had males courting either sham-infected or infected females courting for the first time.

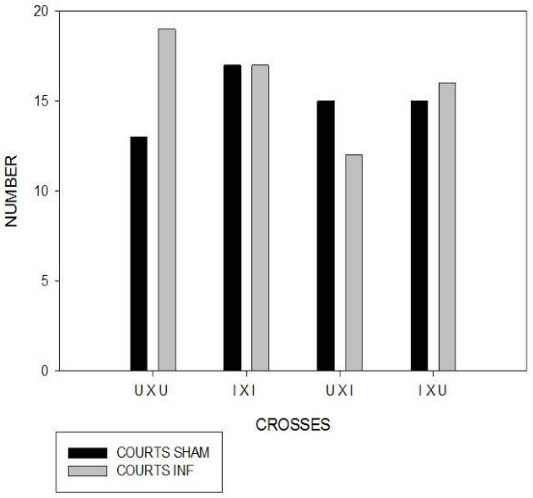
I used the binomial test in Excel 2016 to test if males 'courted first' females of a kind more. There was no significant preference shown by the males in the form of 'CF' for sham-infected or infected females of both U and I. This was consistent across all the blocks.

The sample size for each treatment was around 30 replicated over four blocks.

Figure 3.1: A representation of how many times a female of a particular kind is courted first (CF).

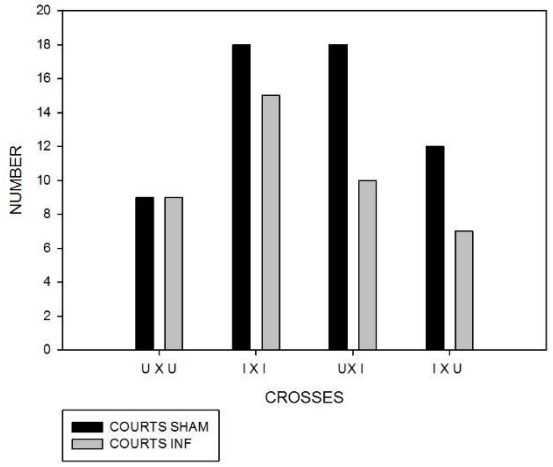
Block 1 Block 2





Block 3

Block 4



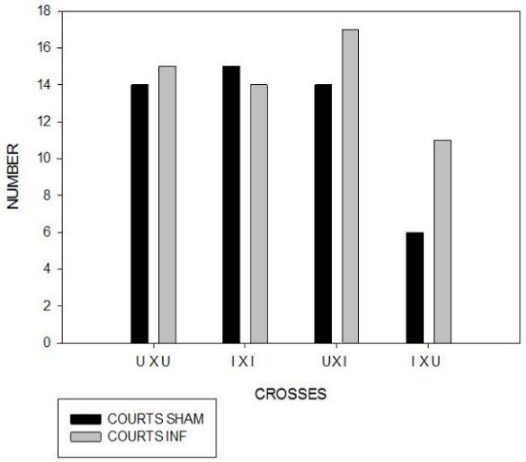


Table 3.2: Binomial test where k = number of successes (number of vials where a sham-infected female is courted); N= number of trials.

BLOCK	Treatment	K(sham-infected)	N	p-value
1	U X U	18	39	0.374629
1	I X I	21	32	0.055092
1	I X U	23	38	0.127938
1	U X I	12	26	0.422509
2	U X U	13	32	0.188543
2	I X I	17	34	0.567917
2	U X I	15	27	0.350554
2	I X U	15	31	0.5
3	U X U	14	29	0.5
3	I X I	15	29	0.5
3	UX I	14	31	0.36005
3	I X U	6	17	0.166153
4	U X U	9	18	0.592735
4	I X I	18	33	0.364166
4	UX I	18	28	0.092467
4	I X U	12	19	0.179642

Courtship latency or CL:

I used ANOVA from JMP 7.0.1 to test if the time taken by males to court female of a particular kind is significantly different from the time taken by males to court female of the second kind. I used male genotype, female genotype, infection status as fixed factors and block as a random factor.

There was no significant preference by the males toward sham-infected or infected females of both U and I population in terms of CL.

Significant effect of male genotype and female genotype but no significant interaction between these two factors.

Figure 3.2: Courtship latency vs infection status of female

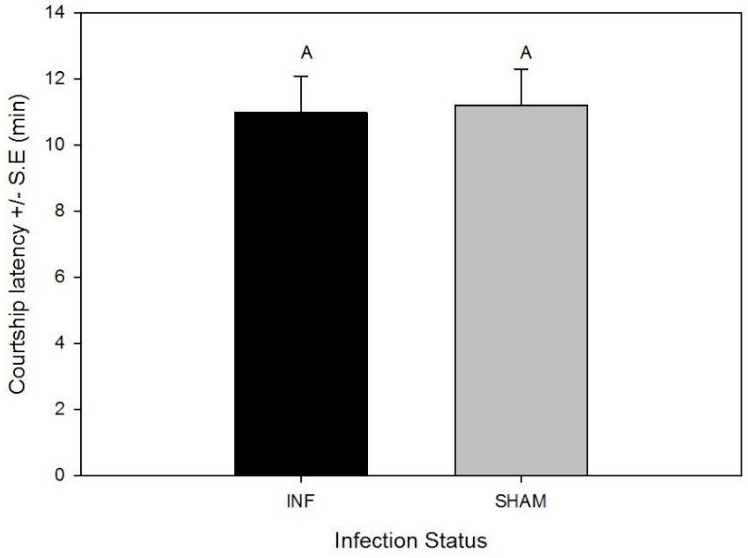


Figure 3.3: Courtship latency vs genotype of the male

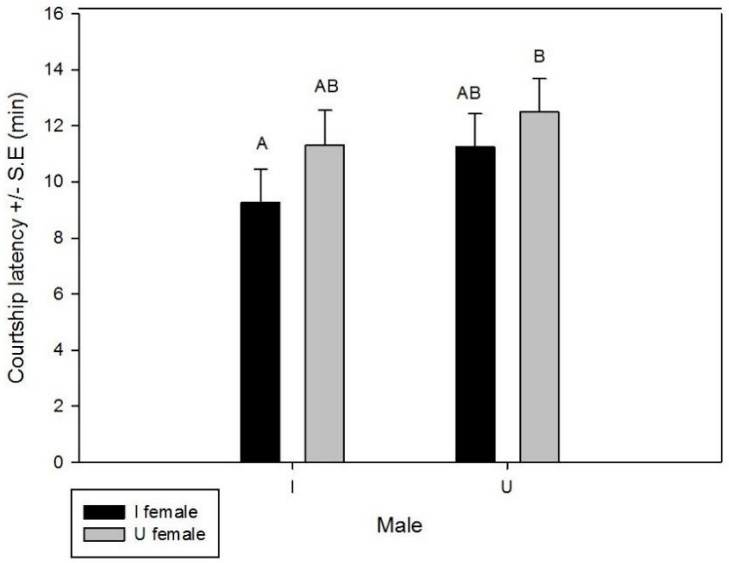


Table 3.3: ANOVA with male genotype, female genotype, infection status as fixed factors and their interactions; Block as the random factor

Source	**Nparm**	**Df**	**F-ratio**	**Prob> F**
INF STATUS	1	1	0.12546	0.723301
FEMALES	1	1	6.932389	**0.008666***
INF STATUS*FEMALES	1	1	0.614137	0.43352
MALE	1	1	6.494133	**0.011052***
INF STATUS*MALE	1	1	0.006074	0.937906
FEMALES*MALE	1	1	0.419571	0.51738
INF STATUS*FEMALES*MALE	1	1	0.133915	0.714526

Courtship frequency or Courts most (CM):

I used Student's single sample T-test to test if a female of a particular kind is courted more than the other by the male. There was no significant higher courtship frequency by males toward sham-infected or infected females of both U and I populations.

Also, there was no significant difference in the amount of choice scores across different treatments. (ANOVA, JMP 7.0.1)

Table 3.4: Student's single sample T-test

Block	Treatment	Mean	Std.Dv.	N	Std.Err.	Reference	t-value	df	p-value
1	U X U	0.453756	0.358593	39	0.057421	0.500000	-0.805356	38	0.425625
1	I X I	0.626823	0.405765	32	0.071730	0.500000	1.768067	31	0.086892
1	I X U	0.524235	0.400693	38	0.065001	0.500000	0.372833	37	0.711400
1	U X I	0.504487	0.436490	26	0.085603	0.500000	0.052419	25	0.958612
2	U X U	0.517231	0.417000	32	0.073716	0.500000	0.233748	31	0.816718
2	I X I	0.476364	0.402046	34	0.068950	0.500000	-0.342790	33	0.733931
2	U X I	0.521629	0.450881	27	0.086772	0.500000	0.249262	26	0.805117
2	I X U	0.547363	0.385126	31	0.069171	0.500000	0.684726	30	0.498773
3	U X U	0.526507	0.434170	29	0.080623	0.500000	0.328770	28	0.744776
3	I X I	0.636923	0.394471	30	0.072020	0.500000	1.901170	29	0.067259
3	UX I	0.463822	0.428308	31	0.076926	0.500000	-0.470290	30	0.641549
3	I X U	0.431904	0.458916	19	0.105283	0.500000	-0.646791	18	0.525930
4	U X U	0.563251	0.407859	18	0.096133	0.500000	0.657950	17	0.519381
4	I X I	0.507709	0.457247	33	0.079597	0.500000	0.096856	32	0.923445
4	UX I	0.584606	0.375970	28	0.071052	0.500000	1.190762	27	0.244111
4	I X U	0.517671	0.424645	34	0.072826	0.500000	0.242651	33	0.809778

Figure 3.4: Courtship frequency of males of I and U with sham-infected females of I and U

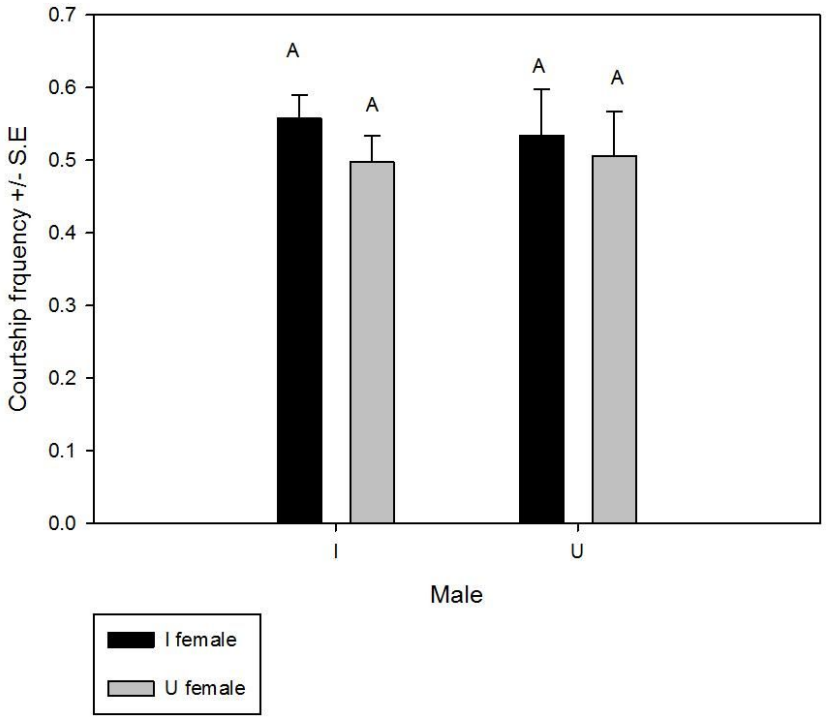


Table 3.5: ANOVA with male genotype and female genotype as fixed factors, their interaction and block as the random factor.

Source	Nparm	df	F-ratio	Prob> F
FEMALES	1	1	1.335139	0.248554
MALES	1	1	0.011693	0.916056
FEMALES*MALES	1	1	0.173234	0.677448

Discussion:

In this study, I used experimental evolution to show that male mate choice did not depend on the female's ability to clear off the pathogen.

There was no significant difference in courtship latency (CL) or courtship frequency (CM) or courtship first (CF) with infected or sham-infected females of both populations. It was hypothesized that a significant difference would be seen when a choice between infected or sham-infected females of baseline controls of population U was presented to the males. This finding is not in corroboration with the previous studies on baseline populations of *Drosophila melanogaster*(Imroze& Prasad 2013; Witmman&Fedorka 2014). It is very interesting that males haven't evolved any choosiness for sham-infected females of U population because around 70-90 % of the individuals of this population undergoes mortality on receiving infection OD_{600} 1.5 compared to individuals which undergo almost no mortality at OD_{600} 1.5 on receiving sham-infection. Males of *Drosophila melanogaster* have a high investment in courtship activity (Cordts& Partridge 1996) and therefore not showing any preference for sham-infected females could be very costly. It's important that variance of quality should exist for males to adopt choosiness and females used in this case have a differential pathogen load (Gupta.V PhD thesis, IISER Mohali 2016). It could be that males haven't evolved a mechanism to perceive the variance in quality of the females which is again striking. One hypothesis to explain this finding is that males don't incur significantly in reproduction as opposed to (Cordts& Partridge 1996) and

therefore haven’t evolved a mechanism to perceive the variance in quality in this case. The second hypothesis is that the two-choice set up that I used, involved male with no competitor males and therefore there's a chance that male does not perceive a sperm-competition risk and therefore isn't picky. This is less likely to be the case as (Nandy et al. 2012; Imroze& Prasad 2013; Witmman&Fedorka 2014) have shown males exhibiting choice in a two-choice setup. The third hypothesis I am making is more profound than the first two. Males generally assess the quality of females possibly by assessing how fecund a female could be through indicators like body size or fatness of abdomen or gravid/non gravid condition(Pitafi, Simpson & Day 1995; Gage & Barnard, 1996; Bonduriansky& Brooks 1998; Lefranc&Bundgaard, 2000; Katvala&Kaitala 2001; Byrne & Rice 2006; Nandy et al. 2012).Imroze& Prasad (2013) showed that infected females had a significant reduction in fecundity and possibly this was perceived by the males which drove them to exhibit choosiness. But the individuals of populations I, U, S, which I have used in our study, don’t have a reduction in fecundity on receiving infection with *Pseudomonas entomophila*(Gupta.V, PhD thesis, 2016). Therefore males were able to assess the quality of females but a variance in quality (fecundity) was absent in the design which drove the males to not exhibit choice.

With the findings in our study, I hypothesize that males mainly assess the quality of females through indicators of fecundity and the status of infection is only relevant to the males if females incur a reduction in fecundity. It's worthwhile for one to validate this hypothesis where males are provided with a choice between females differing in fecundity/infection status.

Chapter 4

Telegony

Introduction:

Offspring inherit characteristics genetically from their biological parents. But there is a growing body of empirical evidence that inheritance of traits can also happen in a non-genetic fashion (Danchin et al. 2011; Bonduriansky 2012). Non-genetic inheritance is defined as any effect brought on the phenotype of offspring brought about by the transmission of factors other than DNA sequences from parents or other remote ancestors (Bonduriansky 2009). Among the many studies on non-genetic inheritance, a study on inheritance of traits from the stepfathers to the stepchildren forced researchers to revisit a phenomenon called as 'telegony' (Crean, Kopps&Bonduriansky 2014). 'Telegony' coined by Weismann, is a phenomenon in which the mother's previous mate can leave an influence on the mother which could translate to the offspring sired by the subsequent mates. This phenomenon had strong support in the nineteenth century but later lost support in light of the knowledge on Mendelian inheritance (Burkhardt 1979). Liu (2013) predicted mechanistic speculations for telegony to occur. Following this, shreds of evidence of stepchildren resembling stepfathers or stepfathers having an influence on the phenotype of the

stepchildren have been documented (Garcia-Gonzalez F & Dowling 2015; Crean, Kopps&Bonduriansky 2014; Eggert, Kurtz &Diddensde 2014) but only a few such studies exist till now.

Crean, Kopps&Bonduriansky(2014) have shown that the majority of the offspring were sired by the mother's second mate but they resembled the mother's previous mate in terms of their body size. They hypothesized that body size, a trait which could be inherited transgenerationally(Bonduriansky& Head 2007; Adler &Bonduriansky 2013), could be mediated by condition-dependent accessory gland products in the seminal fluid. The first male's seminal fluid could influence the immature ovules while the second male's semen couldn't influence the mature ovules (as once the ovules are mature, semen cannot influence ovules) but their sperm could fertilize the ovules resulting in majority of the children be sired by the mother's subsequent mate but resemble in terms of body size with the mother's previous mate.

I have made an attempt to understand how widespread this phenomenon could exist and tried to identify the existence of telegony in laboratory-adapted populations of *Drosophila melanogaster.* The survival ability of individuals of these populations post infection could be inherited in a transgenerational fashion (Manas, MS thesis, IISER Mohali, 2016). The survival ability of females post infection has been shown to depend also on the male genotype (Radhika MS thesis, IISER Pune, 2016). I hypothesized that the survival ability of the stepchildren post infection could possibly depend on the genotype of the stepfather.

To validate this hypothesis, I considered a female of a baseline population, LHst and allowed it to mate with males of populations of I or U or S. The mated females in each case were allowed to remate with a baseline male of LHst population. If the survival ability of the stepchildren(scarlet eyed) of I males is higher compared to stepchildren of U or S, it could be attributed to the difference in genotypes of the first mates (stepfathers) as population I had been shown to have higher survival ability against *Pseudomonas entomophila* than their unhandled controls (population U) or sham-infected controls (population S) (Gupta.V PhD thesis, IISER Mohali, 2016).

Experimental design:

Day 1: Egg collection from I, U, S populations at a density of 60-80 eggs/vial and from LHst at ~150 eggs/vial.

Day 9-11: Males collected as virgins from I, U, S and LHst populations every 6 hours post-eclosion (10 individuals/vial). Females collected as virgins from LHst population (8 females/vial).

Day 12: Infecting males of I, U, S populations with heat-killed bacteria (72^0 C for 30 min) of *Pseudomonas entomophila*to activate immune response but avoid mortality.

Randomly assigning these males to two treatments – A) First mating 6 hours post infection B) First mating 10 hours post infection

Males of I, U, S are housed with females of LHst in a group mating design in vials with cornmeal-molasses food. Only the vials which record the maximum mating pairs possible are taken for the next round of mating. Males are discarded and the mated females of LHst are housed with males of LHst in a single mating design in vials with cornmeal-molasses food. The remating observations are done for 12 hours. As soon as remating is complete in a vial, the male is aspirated out. After the remating observations are done, 6 females of a particular treatment are transferred to one oviposition vials containing 8-10ml of cornmeal-molasses food (oviposition window: 18 hours).

Day 1 of F1: Post oviposition window, eggs are trimmed to a density of ~150 eggs/vial.

Day 12 of F1: Infecting/sham-infecting the step-children of I, U, S males (scarlet eyed progeny) with OD_{600} 1.5 of *Pseudomonas entomophila* and 10mM $MgSO_4$ solution respectively.

Sample size for infections = 50 individuals/sex/genotype of step-father/treatment

Sample size for sham-infections = 30 individuals/sex/genotype of step-father/treatment

Day 12 – Day 16 of F1: Observations of mortality periodically for 96 hours in cages (14cm length x 16 cm width x 13 cm height).

Results:

Significant higher survivorship of stepdaughters of I and U populations compared to those of S population in 6-hour treatment.

6-hour treatment:

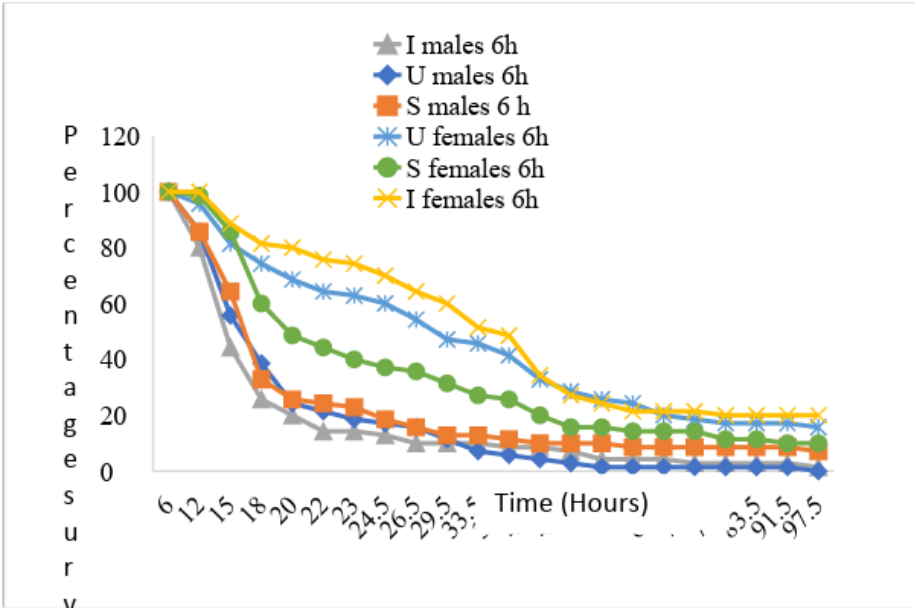


Figure 4.1: Cox proportional hazards survivorship curves of stepchildren of I, U, S males in the 6-hour treatment

10 Hour treatment:

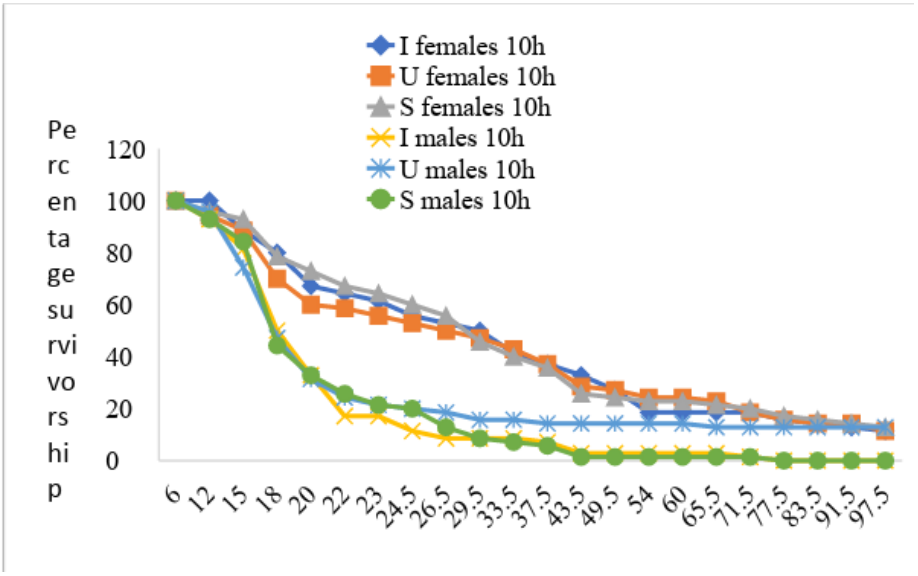


Figure 4.2: Cox proportional hazards survivorship curves of stepchildren of I, U, S males in the 10-hour treatment

Table 4.1: Log-rank and Wilcoxon test

Source	Treatment	Test	Chi-square	df	P > chi-square
Female	10 hour	Log-Rank	0.09584686	2	0.95320677
Female	10 hour	Wilcoxon	0.44794369	2	0.79933764
Male	10 hour	Log-Rank	2.46080881	2	0.2921744
Male	10 hour	Wilcoxon	0.06299175	2	0.96899495
Female	6 hour	Log-Rank	6.1005586	2	**0.0473457***
Female	6 hour	Wilcoxon	7.8218061	2	**0.02002241***
Male	6 hour	Log-Rank	3.72475768	2	0.15530275
Male	6 hour	Wilcoxon	3.92878881	2	0.14024079

Table 4.2: Wald Chi-square test with population, time point, and gender as fixed factors and their interactions

Source	Nparm	DF	Wald Chi-Square	Prob>ChiSq
Population	2	2	1.02465034	0.5991
Time Point	1	1	2.74508744	0.0976
Population*Time Point	2	2	0.595522	0.7425
Gender	1	1	108.163827	**<.0001***

Population*Gender	2	2	3.88284749	0.1435
Time Point*Gender	1	1	1.94209367	0.1634
Population*Time Point*Gender	2	2	7.92504192	**0.019***

Discussion:

In this study, we report no evidence of inheritance of traits related to immunity from stepfathers to stepchildren.

There was significant higher survivorship of the female progeny of all populations compared to males ($p < 0.0001$). This could be attributed to a difference in immune mechanisms of males and females as reported in previous studies.

There was no significant higher survivorship of the stepchildren of I males compared to those of U or S males except in one case where stepdaughters of I and U had higher survivorship than those of S males in the 6-hour treatment ($p = 0.019$). It must be noted that this experiment was run only in one block and must be replicated over in the remaining 3 blocks. The observation of higher survivorship of stepdaughters of I and U compared to those of S could be due to a random effect.

From the observations from only 1 block, it seems like telegony does not exist in *Drosophila melanogaster* with respect to immunity-related traits. There is a high chance that males of *Drosophila melanogaster* have components in the seminal fluid which can influence the female's response to infection, which increases the possibility of telegony to occur (Crean, Kopps&Bonduriansky 2013). In their study, condition-dependent accessory gland components of the first male could possibly have influenced the immature ovules of the females which were later fertilized by the sperm of the subsequent male. In our study, seminal fluid possibly cannot influence the immature ovules as there is structural compartmentalization in the female reproductive organs of *Drosophila melanogaster* due to which sperms/seminal fluid can only interact with the ovules once they are mature and released into the uterus.

Conclusion

In my masters' dissertation, I have addressed two questions which revolve around laboratory-adapted populations of *Drosophila melanogaster* evolved for higher immunity.

1. Understanding how male mate choice operates in these populations and whether the outcome of the choice is influenced by the infection status of the females and their ability to clear off pathogens.
2. Identifying the existence of telegony to check whether immunity related traits can inherit from stepfathers to stepchildren.

Infection status of females had no role to play in driving the males to show choosiness for sham-infected females possibly because males perceive fecundity as the quality of females. Populations of I, U and S do not incur a reduction in fecundity on receiving infection and therefore variance in quality was absent for the males to be forced to show choosiness.

Our study does not report any evidence for telegony found as the survivorship abilities of stepchildren of I, U and S males did not have any significant difference, possibly because of the compartmentalization in the female reproductive organs.

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