Genetic basis of improvement in the immune response in populations of *Drosophila melanogaster* selected against a gram-negative bacterium *Pseudomonas entomophila*

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Dedicated to Aniket,

for asking me to do so

Certificate of Examination

This is to certify that the dissertation titled "**Genetic basis of improvement in the immune response in populations of *Drosophila melanogaster* selected against a gram-negative bacterium *Pseudomonas entomophila***" submitted by Manas Arun Samant (Reg. No. MS10064) 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. T. R. Rao Dr. Rhitoban Ray Choudhury Dr. N. G. Prasad
(Supervisor)

Dated: November 30, 2015

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.

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Dated: November 27, 2015

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)

Acknowledgements

I must thank Dr. N. G. Prasad, who, apart from being my supervisor for this thesis, has also been my mentor at IISER Mohali.

The reader will notice that this thesis has been written in first person plural. The reasons are simple. The experiments described in the following pages would not have been possible without the team work of several persons. Among all the members of the evolutionary biology lab (EBL), who helped this project in their own little ways, I must mention V. Saudamini, Syed Zeeshan Ali and Sharmi Sen, who actually carried out these experiments with me.

List of Figures

List of Tables

Contents

Abstract

We investigated the genetic basis of improvement in the immune response in populations of *Drosophila melanogaster* selected against systemic infection by a gram-negative bacterium, *Pseudomonas entomophila* using two separate experiments.

Firstly, we tested whether the improvement in immune response in the selected populations, which had happened in a sex-specific manner, had occurred as a result of selection acting on X-linked immunity related loci. To that end, we set up crosses (two parental crosses and two reciprocal hybrid crosses) involving selected populations and their respective controls and measured the immune response of the F1 offspring in terms of survivorship post infection. We did not detect any effect of X chromosome on the immune response, as males from the two reciprocal hybrid crosses had indistinguishable immune responses. The nature of genetic variation underlying the improvement in immune response in selected populations appeared to be largely additive in both sexes, but with a slight trend in the direction of being recessive in males.

Secondly, we performed an experiment to test whether adaptive evolution is repeatable over short time scales. We set up crosses between replicate selected populations and measured the immune response of the F1 offspring in terms of survivorship post infection. Our results hint that improvement in the immune response might have involved different genetic changes in replicate selected populations.

Chapter 1

Introduction

In sexually reproducing organisms, a divergence in the evolutionary interests between the two sexes leads to sexual conflicts [1, 2]. Qualitatively, such conflicts have been divided into two kinds. *Inter*-locus sexual conflict is characterized by a situation in which selection leads to antagonistic evolution at *different* loci in the two sexes. This results in, typically, males on the one hand, becoming more manipulative and coercive with respect to acquiring mates while females on the other hand, becoming more resistant to male coercion [1, 2]. *Intra*-locus sexual conflict, which we will focus on in this section, deals with situations where the fitness optima in the two sexes for traits with a common genetic architecture differ [1, 2]. In other words, selection acts in opposite directions in the two sexes for a given locus. The underlying genetic variation, with respect to which there is a negative correlation between fitness in the two sexes, is said to be sexually antagonistic. Sex-specific expression of antagonistic alleles, leading to sexual dimorphism, is one of the ways in which intra-locus sexual conflict can be resolved [2].

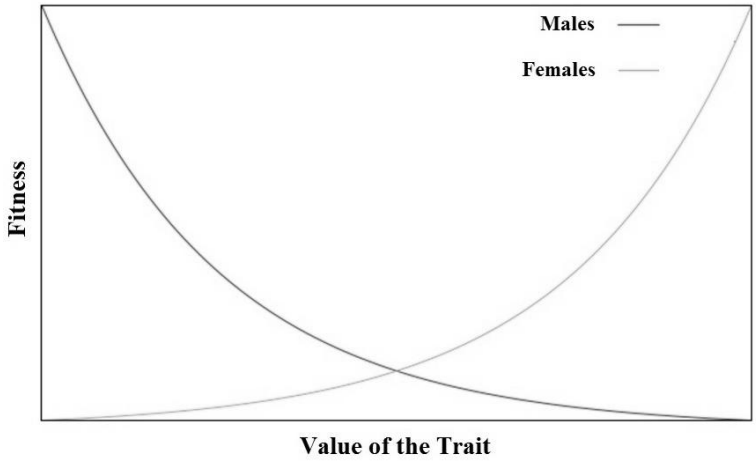

Figure 1.1 Intra-locus sexual conflict

Empirical evidence, both using laboratory systems as well as natural populations, for intra-locus sexual conflict is plentiful and from a diverse set of taxa ranging from fruit flies [3] to red deer [5]. Some studies have demonstrated negative correlations between the fitness of the two sexes. Some other studies have explored actual phenotypes which could lead to such correlations.

Chippindale et al (2001) cloned 40 haploid genomes from a laboratory population of *Drosophila melanogaster* and found a negative correlation between male fitness and female fitness [3]. Haploid genomes that gave rise to high fitness females on an average

gave rise to low fitness males and vice versa. Fedorka and Mousseau (2004) showed that in the cricket *Allonemobius socius* the fitness of a male is positively correlated with the fitness of his sons but negatively correlated with the fitness of his daughters [4]. Foerster et al (2007), in a long-term study on a wild population of red deer, *Cervus elaphus*, observed a negative correlation between the fitness of males and their daughters [5]. In an experimental evolution study Prasad et al (2006) expressed hemi-genomes sampled from a laboratory population of *Drosophila melanogaster*, only as males for several generations with the result that these hemigenomes gave rise to males with a significantly increased fitness, but females with a lower fitness relative to control populations [6].

Numerous traits have been shown to be under intra-locus sexual conflict. Long and Rice (2007) were able to identify locomotory activity as one such trait which could mediate intra-locus sexual conflict in laboratory populations of *Drosophila melanogaster* [7]. They showed that the correlation between fitness and locomotory activity was positive in males but negative in females. Price and Burley (1994), working on laboratory populations of Zebra Finches, *Taeniopygia guttata*, demonstrated that the correlation between the beak colour score and fitness was positive in males and negative in females [8]. In a field study on a population of collared flycatchers *Ficedula albicollis* Merila et al (1997) reported that selection (with respect to survival to adulthood) favoured large body sizes in females but the opposite in males [9].

Immunity, a trait which is the focus of this thesis, is one such trait which is reported to be involved in intra-locus sexual conflict. Svensson et al (2009) observed in a wild population of side-blotched lizards, *Uta stansburiana*, that a trait combination (orange throat colour and high antibody response) that increased survival in males decreased female fitness [10]. However, with respect to immune defence the genetic correlation between the two sexes was negative suggesting that antagonistic selection had led to the evolution of dimorphism of some sort. Similarly, in a laboratory study in *Drosophila melanogaster* Vincent and Sharp (2014) were able to show a negative genetic correlation between the two sexes for resistance (a measure of the host's ability to eliminate pathogens) as well as tolerance (a measure of the host's ability to maintain fitness in presence of pathogens) [11]. However, it must be noted that they did not explicitly demonstrate a negative correlation between the sexes for total fitness.

Many theoretical studies have predicted a non-random distribution of sexually antagonistic alleles with respect to whether they are on autosomes or sex-chromosomes. Using a single-locus population genetics model Rice (1984) showed that the conditions required for maintenance of polymorphisms involving sexually antagonistic alleles are much less restrictive when the locus is X-linked as opposed to when it is on an autosome [12]. Therefore sexually antagonistic variation is expected to be over-represented on X chromosomes when compared to autosomes. Although some workers have argued that under certain conditions autosomes are expected to accumulate sexually antagonistic variation (eg. Fry 2010 [13]), several empirical studies have documented X-linked sexually antagonistic variation. Gibson et al (2002) cloned 20 X chromosomes from a laboratory population of *Drosophila melanogaster* and estimated that X chromosomes harbour around 45% of total fitness related genetic variation and, more importantly, 97% of sexually antagonistic genetic variation [14]. In a study that is more relevant to this thesis, Hill-Burns and Clark (2009) documented considerable immunity-related variation on the X chromosome in *Drosophila melanogaster*. They sequenced several known immunity related genes on the X chromosome and were able to identify several SNPs, some of which they showed to be associated with immunity phenotypes in a sex-specific or even sexually antagonistic manner [15].

In a laboratory evolution study, Vanika Gupta [16] selected replicate populations of *Drosophila melanogaster* against systemic infection by *Pseudomonas entomophila*, a gram-negative bacterium, and found a quick response to selection. The improvement in the immune response in the selected populations was however sex-specific in nature. In selected populations, females had evolved (at least) increased *resistance*, while males had evolved increased *tolerance.* This sexual dimorphism prompted her to hypothesise that a large fraction of the loci involved in the improvement of immune response in these populations would be X-linked.

In the present thesis, we carried out experiments to test whether there is a significant effect of X chromosome in the immunity selections lines started by Gupta. We set up reciprocal crosses between selected and control populations and measured the immune response of the F1 offspring.

As an additional experiment we were also interested in understanding whether, in Gupta's populations, the improved immune response had involved similar genetic changes in

independent blocks of her selection regime. Kawecki and Mery (2006), working on populations of *Drosophila melanogaster* selected for improved learning response, set up crosses between replicate selected populations and measured the learning response of the F1 progeny. They reported that in some of the crosses the F1 offspring displayed learning responses significantly lower than those of the selected populations [17]. They showed that the increased learning response in the selected populations had involved allelic substitutions that were recessive in nature and had occurred at *different* loci in different replicate lines, thereby suggesting that adaptive evolution over short time scales need not be repeatable. We carried out an experiment similar to that of Kawecki and Mery on populations selected for improved immune response.

Chapter 2

Materials and methods

2.1. Experimental Populations: We carried out our experiments on laboratory populations of *Drosophila melanogaster*. All experimental flies trace their ancestry to four laboratory adapted, outbred populations labelled BRB_{1-4}, where BRB refers to Blue Ridge Baseline, as the flies from which these populations have been founded were collected in the Blue Ridge Mountains. The details of these populations are described by Gupta et al 2012 [18]. But briefly, each of the four BRB populations is maintained on a 14 day discrete generation cycle with N_e of around 2800 individuals. In the year 2012, Vanika Gupta established three selection regimes from each of the four BRB populations. These populations were labelled I_i, S_i and U_i where the subscript refers to the subscript of the ancestral BRB population. Thus there were four I populations (I_{1-4}), four S populations (S_{1-4}) and four U populations (U_{1-4}). I populations are selected for improved immune response against systemic infection by *Pseudomonas entomophila*, S populations are the injury controls, while U populations are unhandled controls. It must be noted that all populations that bear the same subscript are more closely related to each other in terms of their ancestry as opposed to populations with different subscripts. For example, I_3 is more closely related to S_3 and U_3 than it is to I_2 or U_4. Also, during regular population maintenance, we always handle populations with common subscripts on the same day. For these reasons, in all analyses, populations with a common subscript form a statistical block. Thus, there are four independent blocks for the laboratory selection experiment started by Gupta.

We maintain all three selection regimes on a 16 day discrete generation cycle on a standard banana-yeast-jaggery diet. The flies experience a temperature of 25°C, a relative humidity of 60-70% and alternate 12 hour long light and dark periods. For each population, we collect eggs from a food plate introduced into the population cage 18 hours before egg collection. We set up 10 glass vials (25 mm diameter × 90 mm height), each containing around 70 eggs in close to 6 mL of food for each population. Typically, the larvae which hatch out of these eggs pupate 6 to 7 days post egg collection. Adults emerge from these pupae between 9 and 11 days post egg collection. The three selection regimes (I, U and S) differ in their handling only on one day (the 12^{th} day post egg collection) in their life cycles (see Figure 2.1). For U populations, we collect 100 adult

males and 100 adult females and transfer them into a Plexiglas cage (14 cm length × 16 cm width × 13 cm height) provided with a food plate. We use light CO_2 anaesthesia for sorting flies. For I populations, we infect 150 adult males and 150 adult females with *Pseudomonas entomophila* and transfer them to a Plexiglas cage having a food plate. In case of S populations, we sham infect 100 adult males and 100 adult females before transferring them to a Plexiglas cage having a food plate. For details of the bacterial stocks and the protocol used for infection as well sham infections, see below. Note that there is a difference in the number of flies transferred to the cage between I populations and S (or U) populations. In I populations, the bacterial dosage is adjusted in such a manner that infection causes close to a third of the flies to die within a span of 96 hours post infection. In S and U populations there is no mortality in this 96-hour window. Therefore, taking 150 males and 150 females, to begin with, in I populations ensures that eventually adult densities in cages between I populations and S (or U) populations are comparable. In order to start the next generation, on the 16th day post egg collection, we introduce a fresh food plate in the cages. Eggs that give rise to the next generation are a sample of the eggs laid in 18 hours after the introduction of this fresh food plate.

	Day 1	Day 12	Day 16
I_{1-4}	Egg Collection: 10 Vials of 70 eggs each	Infect 150 males and 150 female with *Pseudomonas entomophila*	Fresh food plate as substrate for egg collection
S_{1-4}	Egg Collection: 10 Vials of 70 eggs each	Sham infect 100 males and 100 female with 10 mM Magnesium Sulphate	Fresh food plate as substrate for egg collection
U_{1-4}	Egg Collection: 10 Vials of 70 eggs each	Sort 100 males and 100 females with carbon dioxide anesthesia	Fresh food plate as substrate for egg collection

Figure 2.1 The I, U and S selection regimes

2.2. Bacterial stocks: During maintenance of selection regimes as well as for experiments described in this thesis, we used *Pseudomonas entomophila* strain L48,

which was provided to us by Dr. Pierre Cornelis at the Free University of Brussels, Belgium. We maintain the stocks in glycerol, frozen at -80°C. While using the bacteria for infections, we set up an overnight culture in Luria Bertani Broth (LB) (HiMedia Laboratories Pvt. Ltd.) at 27°C at 150 RPM. The next morning, we set up a subculture by diluting the overnight culture 100 fold. Around 4 hours later, we centrifuge the subculture and discard the supernatant. We then use sterile 10 mM $MgSO_4$ to suspend the pellet of bacterial cells. We adjust the OD at 600 nm of this suspension to the required value, by adding appropriate quantities of sterile 10 mM $MgSO_4$.

When Gupta started the selection lines the OD_{600} used for infecting I populations was 1.0. However, as I populations rapidly evolved higher survivorships post infection, we subsequently increased OD_{600} of the bacterial suspension used for infections in a stepwise manner so as to keep the total mortality close to a third.

2.3. Protocol for infections/sham infections: We use a fine tungsten needle (Minutein pin 0.1 mm, Fine Science Tools, CA) for infections. We dip the needle in the bacterial suspension and prick the fly to be infected in the dorso-lateral part of the thorax. In order to immobilize the flies we use light CO_2 anaesthesia. For sham infections, the pricking protocol is similar, except we dip the needle in a sterile 10 mM $MgSO_4$ solution.

2.4. Experimental Design: We carried out our experiments using flies from I and S regimes.

2.4.1. Experiment 1: We carried out this experiment between generation 65 and 75 after the selection regimes were begun. In order to start the experiment, we provided I and S populations with a fresh food plate. (It must be noted that prior to this, we had provided these flies with *ad libitum* yeast for two days in order to boost their fecundity.) 18 hours later, we collected eggs from these plates at a density of around 70 eggs per vial containing close to 6 mL of food. We set up 20 vials each for I and S populations, which were then maintained in an incubator at standard conditions mentioned above. We collected virgin males and females from these vials and housed them in separate food vials at a density of 10 individuals per vial. On the 12^{th} day post egg collection, we set up the following four crosses in Plexiglas cages by transferring, for each cross, 100 virgin females and 100 virgin males of the appropriate population:

1. I ♀ × I ♂ (henceforth called I*I)
2. S ♀ × S ♂ (henceforth called S*S)
3. I ♀ × S ♂ (henceforth called I*S)
4. S ♀ × I ♂ (henceforth called S*I)

After providing these cages with *ad libitum* yeast for two days, we introduced a fresh food plate in each of these cages and 18 hours later collected eggs at a density of around 70 eggs per vial, with each vial containing around 6 mL of food, after which we incubated the vials under standard conditions. On the 12^{th} day post egg collection, by which time all eggs had developed into adults, we carried out infections with *Pseudomonas entomophila*. For infections the OD_{600} of the bacterial suspension was adjusted to 1.5. We divided all infections between three persons. Each person infected 50 males and 50 females from each of the four crosses and transferred them to a Plexiglas cage containing a food plate. Thus, for each cross there were three independent cages, each containing 50 males and 50 females. In addition to the cages containing infected flies, we also sham infected 50 males and 50 females per cross and transferred them to a similar cage. Thus, the experiment consisted of a total of 16 cages, 12 'infected' and 12 'sham infected'. We observed all 16 cages for the next 96 hours and recorded mortality. We removed dead flies while taking observations. We introduced fresh food plates in these cages, two days after carrying out infections. Note that this experiment was repeated for each of the four blocks of I and S populations.

Since males from the I*S cross inherit their X chromosome from I females and males from the S*I cross inherit their X chromosomes from S females, an effect of X chromosome in the immune response would be indicated if males from the I*S cross had a better immune response than males from the S*I cross.

2.4.2 Experiment 2: We carried out this experiment between generations 70 and 80 after the selection regimes were begun. Experiment 2 was similar to Experiment 1, except that instead of setting up crosses between I and S populations, we set up crosses between I populations belonging to different blocks. This experiment was divided into four smaller subexperiments, each of which consisted of setting up crosses between Ii and Ij (where j= i+1, except when i=4; in that case j=1) and measuring the survivorship post infection of the F1 offspring obtained from two parental (Ii&Ii and Ij*Ij) and two hybrid (Ii*Ij and Ij*Ii) crosses. Thus we carried out four sub-experiments, one for I1 and I2 (referred to as

subexperiment 1), one for I2 and I3 (referred to as subexperiment 2), one for I3 and I4 (referred to as subexperiment 3) and one for I4 and I1 (referred to as subexperiment 4). For each experiment, we collected eggs at a density of around 70 per vial (with each vial containing around 6 mL of food) from flies belonging to Ii and Ij populations provided with a fresh food plate for 18 hours. We set up 20 vials for each of the two populations. We collected virgin males and virgin females from these vials and on the 12^{th} day post egg collected combined them to set up four crosses. Each cross consisted of 100 virgin males and 100 virgin females in a Plexiglas cage. The four crosses were as follows:

1. Ii ♀ × Ii ♂ (henceforth called Ii*Ii)
2. Ij ♀ × Ij ♂ (henceforth called Ij*Ij)
3. Ii ♀ × Ij ♂ (henceforth called Ii*Ij)
4. Ij ♀ × Ii ♂ (henceforth called Ij*Ii)

We provided these flies *ad libitum* yeast for two days and then collected eggs from food plates introduced in the cages for 18 hours. We transferred around 70 eggs each into vials having 6 mL of food. For each cross we collected 10 vials worth of eggs. On the 12^{th} day after collecting eggs from the crosses, we carried out infections using *Pseudomonas entomophila*. We adjusted the OD_{600} of the bacterial suspension to 2.0. For each cross, we set up four cages, three containing 50 infected males and 50 infected females and one containing 50 sham infected males and 50 sham infected females. Thus there were a total of 16 cages. We provided these cages with food plates, which we replaced with fresh ones two days later. We recorded mortality in all cages for 96 hours post infection.

2.6. Statistical Analyses: For Experiment 1, we performed separate analyses for the two sexes. We analysed the data in two different ways. Firstly, we calculated the proportion of flies alive at the end of the 96 hour observation window for each cage. Therefore we had three survivorship values per cross per block. We used a mixed model ANOVA with blocks as a random factor on these survivorship values and a post-hoc Tukey's HSD for multiple comparisons.

Secondly, we compared actual survivorship curves between different crosses by fitting a Cox's proportional hazards model. For this purpose, we pooled the data across all the three different cages for each cross and analysed each block separately. In order to test whether cross had a significant effect, we used a log likelihood ratio test. For multiple comparisons, we used a Chi-squared test on risk ratios to test whether they were

significantly different from 1. While making multiple comparisons we did a Bon-Feroni correction.

Experiment 2 did not have blocks, but four different subexperiments. We analysed the data independently for each subexperiment with the two sexes being examined separately. Like Experiment 1, we performed two analyses: a one-way ANOVA on proportion of flies alive at the end of the 96 hour observation window in each cage and a Cox's proportional hazards model to analyse the survivorship curves. We used a log likelihood ratio test to examine the effect of cross on survivorships and a Chi squared test on risk ratios for multiple comparisons. For multiple comparisons we used Bon-Feroni correction. All analyses were performed on Statistica and JMP 12.

We plotted all graphs using Gnuplot Version 4.6 (Copyright (C) 1986 - 1993, 1998, 2004, 2007 Thomas Williams, Colin Kelley.)

Chapter 3

Results

3.1. Experiment 1: While analysing survivorship values using a mixed model ANOVA, there was no effect of block for either sex. Therefore, we pooled the data across all blocks and repeated the analyses. There was a significant effect of "Cross" in both males ($p<0.001$) and females ($p<0.001$). We found that both in males as well as in females, individuals belonging to the I*I cross had significantly higher survivorships as compared to individuals from S*S cross (see Figure 3.1). In neither sex were the two hybrid crosses (I*S and S*I) different from each other. However, there was a difference between the two sexes with respect to the survivorship of individuals from the hybrid crosses relative to individuals from the two parental crosses (I*I and S*S). In females (see Table 1), individuals from the two hybrid crosses had survivorship that were significantly lower than those of individuals from the I*I cross but higher than those of individuals from the S*S cross. In males (see Table 2), on the other hand, although individuals from the two hybrid crosses had significantly lower survivorships than the I*I cross, there was no significant difference in the survivorships of individuals from the hybrid crosses and those of individuals from the S*S cross.

The analysis using Cox's proportional hazards model, in each block for each sex, we observed a significant effect of the cross in the log likelihood ratio test (see Table 2 and Table 3). We obtained consistent trends after making multiple comparisons in block 2, block 3 and block 4, with block 1 being markedly different from the rest of the three blocks (see Table 5, Figure 3.2, Figure 3.3). In both males and females, across all blocks, individuals from the I*I cross had higher survivorships compared to individuals from the S*S cross and the survivorships of individuals from the two hybrid crosses were not significantly different from each other. In males, for block 2, block 3 and block 4 the survivorships for the hybrid crosses was significantly lower than that of the I*I cross but significantly higher than the S*S cross. In females as well an identical pattern was observed for block 3 and block 4 and although the trend was similar in block 2, the difference between the I*I cross and the I*S cross was not significant. In block 1, for males the two hybrid crosses had survivorships which were significantly lower than the I*I cross but not different from the S*S cross. In block 1 females, the two hybrid crosses were neither different from each other nor from the S*S cross. One of the hybrid crosses,

the I*S cross was not significantly different from the I*I cross, while the other hybrid cross S*I cross was different from the I*I cross in terms of survivorship.

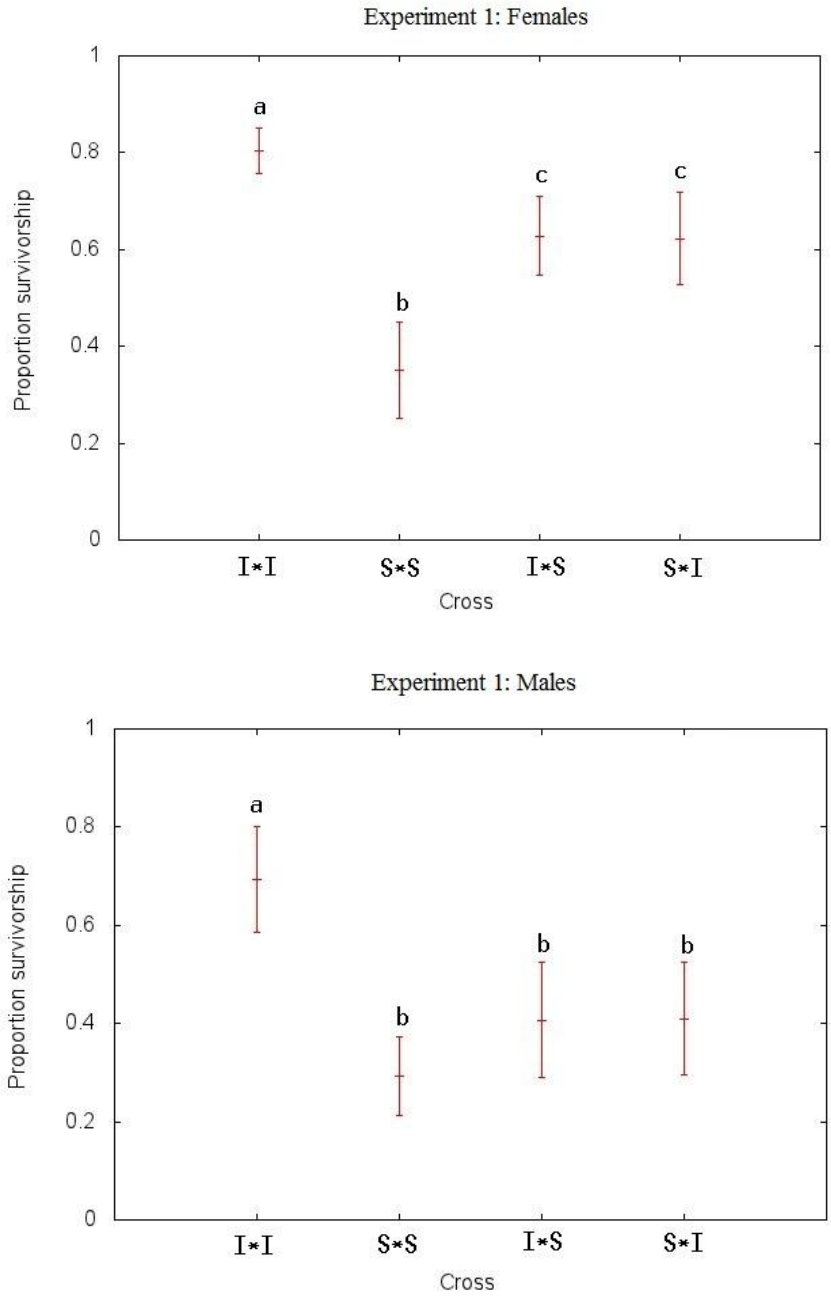

Figure 3.1. Experiment 1: Effect of Cross on proportion survivorship (crosses without common letters are significantly different). Error bars indicate 2*SE

	I*I	I*S	S*I	S*S
I*I		**0.024405**	**0.018262**	**0.000169**
I*S	**0.024405**		0.999538	**0.000309**
S*I	**0.018262**	0.999538		**0.000373**
S*S	**0.000169**	**0.000309**	**0.000373**	

Table 1 Experiment 1**:** Multiple comparisons for proportional survivorship in females. (The numbers indicate p-values for Tukey's HSD)

	I*I	I*S	S*I	S*S
I*I		**0.002270**	**0.002572**	**0.000181**
I*S	**0.002270**		0.999971	0.436230
S*I	**0.002572**	0.999971		0.410627
S*S	**0.000181**	0.436230	0.410627	

Table 2. Experiment 1: Multiple comparisons for proportional survivorship in males. (The numbers indicate p-values for Tukey's HSD)

	Number of parameter	df	LR ChiSq	p
Block 1	3	3	25.49822	**<.0001**
Block 2	3	3	80.21543	**<.0001**
Block 3	3	3	78.83939	**<.0001**
Block 4	3	3	153.821	**<.0001**

Table 3. Experiment 1**:** Log likelihood ratio test for effect of Cross on female survivorship using Cox's proportional hazards model

	Number of parameters	df	LR ChiSq	P
Block 1	3	3	16.38132	**0.0009**
Block 2	3	3	59.91014	**<.0001**
Block 3	3	3	79.80066	**<.0001**
Block 4	3	3	128.4987	**<.0001**

Table 4. Experiment 1: Log likelihood ratio test for effect of Cross on male survivorship using Cox's proportional hazards model

Level 1	Level 2	Risk Ratio	p
I*S	I*I	1.6192454	0.0305
S*I	I*I	2.2558863	**0.0001**
S*I	I*S	1.3931713	0.0828
S*S	I*I	2.5818037	**<.0001**
S*S	I*S	1.5944487	0.0115
S*S	S*I	1.1444742	0.4406

Table 5a. Block 1 Females

Level 1	Level 2	Risk Ratio	p
I*S	I*I	1.794661	0.0114
S*I	I*I	2.204	**0.0004**
S*I	I*S	1.228087	0.2954
S*S	I*I	5.144865	**<.0001**
S*S	I*S	2.866762	**<.0001**
S*S	S*I	2.33433	**<.0001**

Table 5b. Block 2 Females

Level 1	Level 2	Risk Ratio	p
I*S	I*I	3.16217	**<.0001**
S*I	I*I	2.630326	**<.0001**
S*I	I*S	0.83181	0.2616
S*S	I*I	5.016332	**<.0001**
S*S	I*S	1.586357	**0.0021**
S*S	S*I	1.907114	**<.0001**

Table 5c. Block 3 Females

Level 1	Level 2	Risk Ratio	p
I*S	I*I	3.552935	**<.0001**
S*I	I*I	2.682316	**<.0001**
S*I	I*S	0.754958	0.1166
S*S	I*I	9.502138	**<.0001**
S*S	I*S	2.674447	**<.0001**
S*S	S*I	3.542513	**<.0001**

Table 5d. Block 4 Females

Level 1	Level 2	Risk Ratio	p
I*S	I*I	1.702526	**0.0007**
S*I	I*I	1.755997	**0.0003**
S*I	I*S	1.031407	0.8295
S*S	I*I	1.495504	0.0116
S*S	I*S	0.878403	0.3742
S*S	S*I	0.851655	0.2712

Table 5e. Block 1 Males

Level 1	Level 2	Risk Ratio	p
I*S	I*I	2.163753	**<.0001**
S*I	I*I	2.134302	**<.0001**
S*I	I*S	0.986389	0.926
S*S	I*I	3.30392	**<.0001**
S*S	I*S	1.52694	**0.0023**
S*S	S*I	1.54801	**0.0016**

Table 5f. Block 2 Males

Level 1	Level 2	Risk Ratio	p
I*S	I*I	3.24267	**<.0001**
S*I	I*I	2.684102	**<.0001**
S*I	I*S	0.827744	0.2474
S*S	I*I	5.042372	**<.0001**
S*S	I*S	1.555006	**0.0031**
S*S	S*I	1.878607	**<.0001**

Table 5g. Block 3 Males

Level 1	Level 2	Risk Ratio	p
I*S	I*I	4.263954	**<.0001**
S*I	I*I	4.878496	**<.0001**
S*I	I*S	1.144125	0.3652
S*S	I*I	7.534569	**<.0001**
S*S	I*S	1.767038	**<.0001**
S*S	S*I	1.544445	**0.0019**

Table 5h. Block 4 Males

Table 5. Experiment 1: Multiple comparisons between crosses using Chi Squared test (at α=0.083) on risk ratios after fitting a Cox's proportional hazards model for females (**5a-5d**) and males (**5e-5h**)

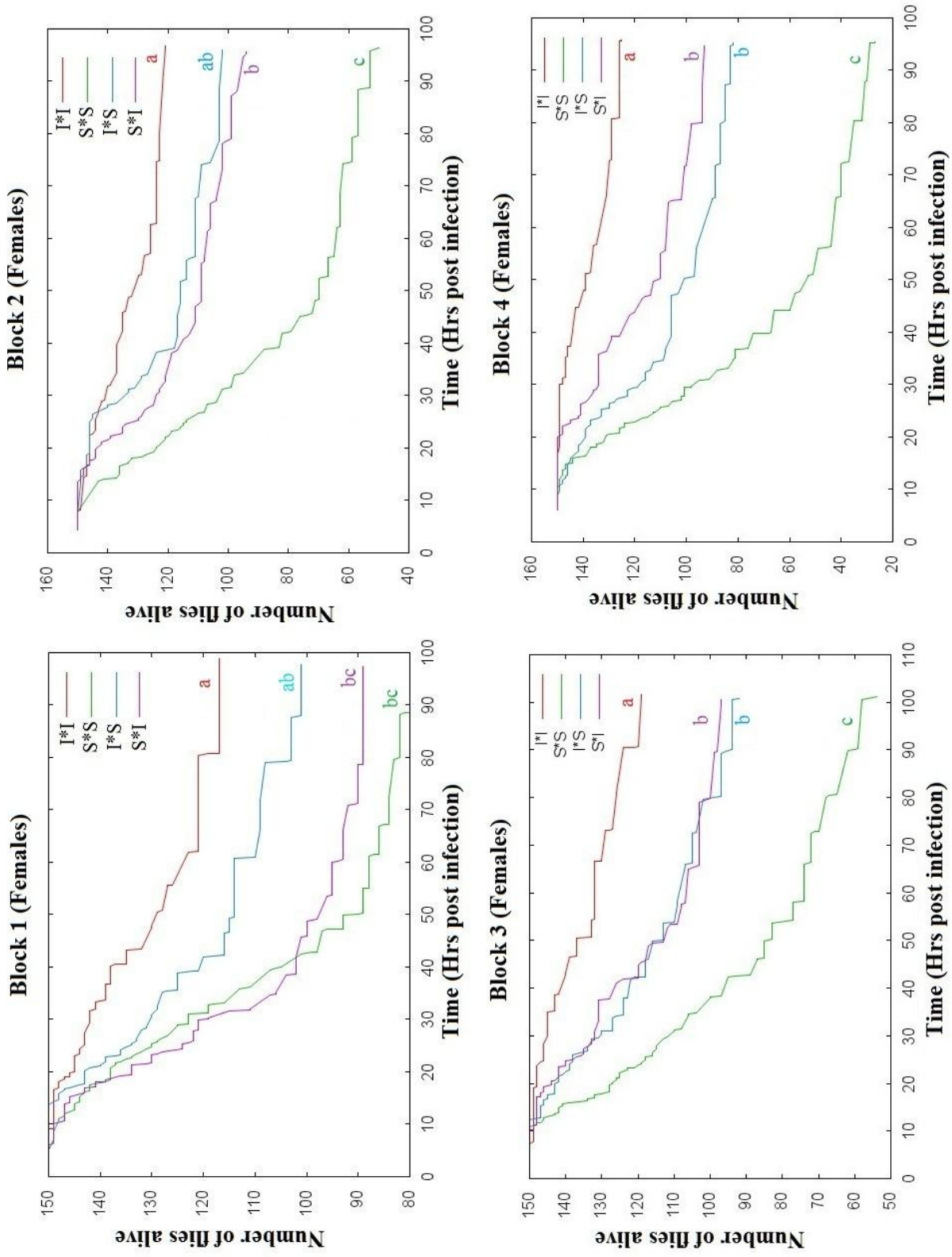

Figure 3.2. Experiment 1: Survivorship curves for females (curves without any common letters are significantly different)

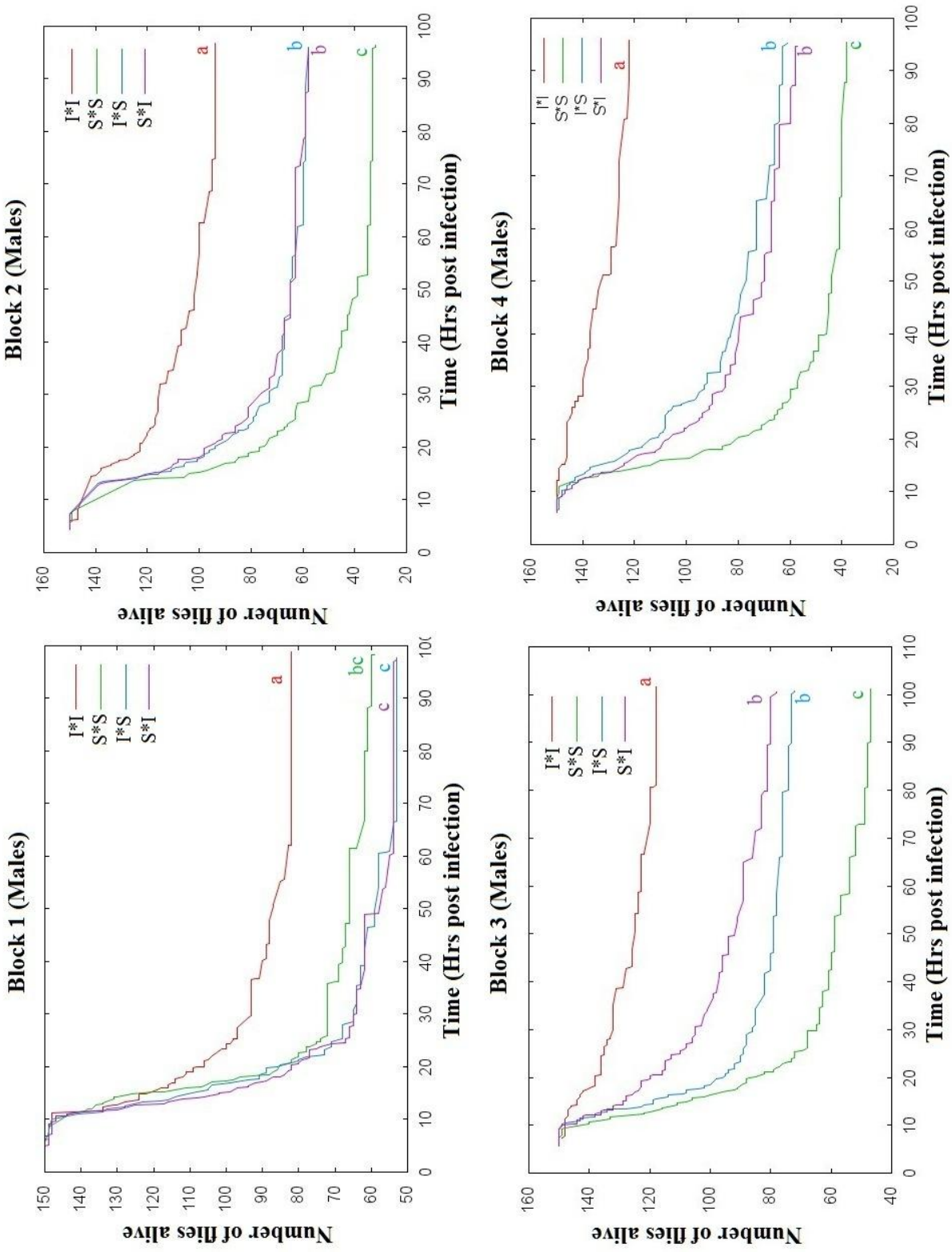

Figure 3.3. Experiment 1: Survivorship curves for males (curves without any common letters are significantly different)

3.2 Experiment 2: In neither sex was one way ANOVA able to detect any effect of Cross in terms of proportion survivorship at the end of the 96 hour observation window. This was true for all four sub-experiments. The patterns obtained from Cox's proportional hazards model, however, were considerably erratic across the four sub-experiments (see Table 8, Figure 3.4, Figure 3.5). In males we could not detect any difference between hybrid crosses and parental crosses in three out of four subexperiments. Only in subexperiment 1, the one involving I1 and I2 populations, did Cross have a significant effect. In subexperiment 1, males from I1*I1, I2*I2 and I1*I2 cross had survivorship curves that we could not differentiate between. But males from I2*I1 cross had significantly better survivorships. In case of females, in subexperiment 1 and subexperiment 2, there were no significant differences between in any of the crosses. In subexperiment 3, the two parental crosses were significantly different from each other with I3*I3 have superior survivorships when compared to I4*I4. We did not detect any difference in the survivorships between the two hybrid crosses (I3*I4 and I4*I3), nor was the difference between either of the two hybrid crosses and the two parental crosses significant. In subexperiment 4, I4*I1 had significantly lower survivorships than I1*I1 and I1*I4, which were not different from each other. We did not detect any difference between I4*I4 and any other cross.

	Number of parameters	**df**	**LR ChiSq**	**p**
Sub-experiment 1	3	3	5.446818	0.1419
Sub-experiment 2	3	3	6.028392	0.1102
Sub-experiment 3	3	3	12.65267	**0.0055**
Sub-experiment 4	3	3	10.14785	**0.0174**

Table 6 Experiment 2: Log likelihood ratio test for effect of Cross on female survivorship using Cox's proportional hazards model

	Number of parameters	**df**	**LR ChiSq**	**p**
Sub-experiment 1	3	3	35.45071	**<.0001**
Sub-experiment 2	3	3	3.376337	0.3372
Sub-experiment 3	3	3	2.902334	0.4069
Sub-experiment 4	3	3	6.59029	0.0862

Table 7 Experiment 2: Log likelihood ratio test for effect of Cross on male survivorship using Cox's proportional hazards model

Level 1	Level 2	Risk Ratio	p
I1*I2	I1*I1	0.550706	0.0201
I2*I1	I1*I1	0.793809	0.3287
I2*I1	I1*I2	1.44144	0.1779
I2*I2	I1*I1	0.755077	0.2424
I2*I2	I1*I2	1.371108	0.2525
I2*I2	I2*I1	0.951207	0.8439

Table 8a. Sub-experiment 1 Females

Level 1	Level 2	Risk Ratio	p
I2*I3	I2*I2	1.007019	0.9807
I3*I2	I2*I2	0.819814	0.5108
I3*I2	I2*I3	0.814101	0.496
I3*I3	I2*I2	0.478618	0.0316
I3*I3	I2*I3	0.475282	0.03
I3*I3	I3*I2	0.583812	0.134

Table 8b. Sub-experiment 2 Females

Level 1	Level 2	Risk Ratio	p
I3*I4	I3*I3	1.259295	0.5504
I4*I3	I3*I3	2.391695	0.0088
I4*I3	I3*I4	1.899233	0.0416
I4*I4	I3*I3	2.59009	**0.0036**
I4*I4	I3*I4	2.056777	0.0197
I4*I4	I4*I3	1.082951	0.7656

Table 8c. Sub-experiment 3 Females

Level 1	Level 2	Risk Ratio	p
I1*I4	I1*I1	0.99607	0.9889
I4*I1	I1*I1	1.938775	**0.0066**
I4*I1	I1*I4	1.946425	**0.0063**
I4*I4	I1*I1	1.264754	0.3846
I4*I4	I1*I4	1.269744	0.3767
I4*I4	I4*I1	0.652347	0.0671

Table 8d. Sub-experiment 4 Females

Level 1	Level 2	Risk Ratio	p
I1*I2	I1*I1	0.834688	0.3053
I2*I1	I1*I1	0.294434	**<.0001**
I2*I1	I1*I2	0.352747	**<.0001**
I2*I2	I1*I1	0.760287	0.1318
I2*I2	I1*I2	0.910864	0.6202
I2*I2	I2*I1	2.582202	**<.0001**

Table 8e. Sub-experiment 1 Males

Level 1	Level 2	Risk Ratio	p
I2*I3	I2*I2	1.617439	0.0831
I3*I2	I2*I2	1.351828	0.2981
I3*I2	I2*I3	0.835783	0.4916
I3*I3	I2*I2	1.150072	0.6395
I3*I3	I2*I3	0.711045	0.2041
I3*I3	I3*I2	0.850753	0.5641

Table 8f. Sub-experiment 2 Males

Level 1	Level 2	Risk Ratio	p
I3*I4	I3*I3	1.488508	0.1473
I4*I1	I3*I3	1.292525	0.366
I4*I1	I3*I4	0.868336	0.5849
I4*I4	I3*I3	1.51527	0.1299
I4*I4	I3*I4	1.017979	0.9432
I4*I4	I4*I1	1.172334	0.5384

Table 8g. Sub-experiment 3 Males

Level 1	Level 2	Risk Ratio	p
I1*I4	I1*I1	0.90591	0.6973
I4*I1	I1*I1	1.017377	0.9446
I4*I1	I1*I4	1.123044	0.6453
I4*I4	I1*I1	1.541189	0.0555
I4*I4	I1*I4	1.701259	0.0205
I4*I4	I4*I1	1.514864	0.0638

Table 8h. Sub-experiment 4 Males

Table 8. Experiment 2: Multiple comparisons between crosses using Chi Squared test (at α=0.083) on risk ratios after fitting a Cox's proportional hazards model for females (**8a-8d**) and males (**8e-8h**)

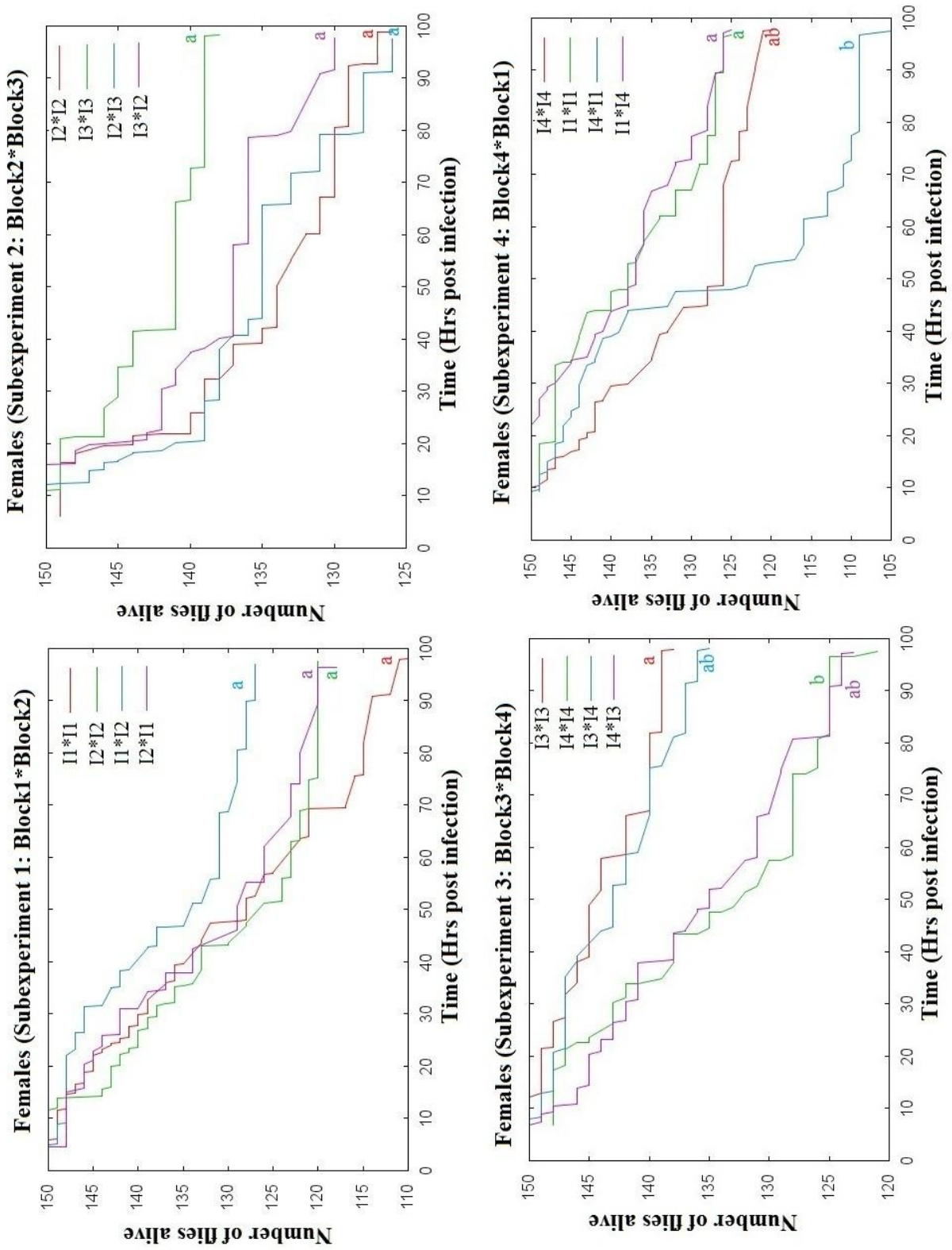

Figure 3.4. Experiment 2: Survivorship curves for females (curves without any common letters are significantly different)

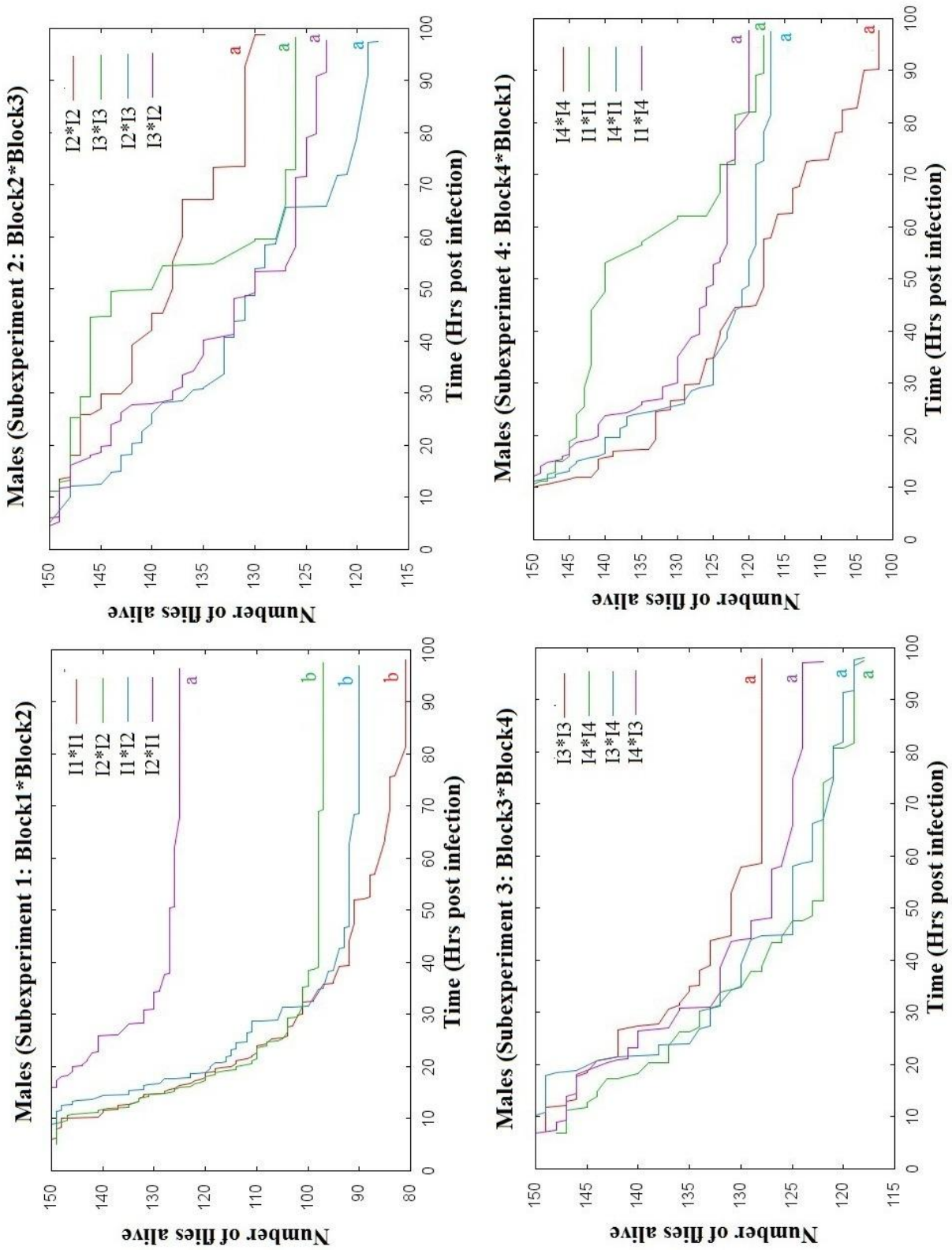

Figure 3.5. Experiment 2: Survivorship curves for males (curves without any common letters are significantly different)

Chapter 4

Discussion

In Experiment 1 we measured the immune response of F1 offspring obtained by crossing flies from I and S populations of the same block in different combinations. We expected the immune response of males from the I*S cross to be stronger than that of males from the S*I cross, since the former inherited their X chromosome from I females while the latter inherited their X chromosome from S females. However, in neither of the two analyses (mixed model ANOVA on proportion survivorship and Cox's proportional hazards model), did we find a difference in the survivorships of males from the two reciprocal hybrid crosses, prompting us to reject our hypothesis. The results from Experiment 1 also provided some other insights. The mixed model ANOVA and post hoc multiple comparisons using Tukey's HSD test hinted that the nature of genetic variation responsible for improved immune response in I populations was different for the two sexes. In females, for instance, the two hybrid crosses, although not different from each other, were significantly different from both parental crosses (I*I and S*S). In males, on the other hand, the two hybrid crosses, which were not different from each other, were sufficiently close to the S*S cross to be statistically indistinguishable. They were of course significantly different from the I*I cross. This pattern of female hybrids being intermediate to the two parental crosses, but male hybrids being indistinguishable from the S*S cross indicated that the genetic variation responsible for improved immune response was, mostly, additive in females but recessive in males.

We fitted a Cox's proportional hazards model separately to each block. In both males and females, barring the exception of block 1, we obtained consistent results. In males, in blocks 2, 3 and 4, the two hybrid crosses, which themselves were indistinguishable, were significantly different from both the parental crosses. In females too, the hybrid crosses, which were indistinguishable from one another were intermediate to the two parental crosses. This trend was statistically significant in blocks 3 and 4. In block 2 the I*I cross was not significantly different from the I*S cross. The p value for Chi-squared test on risk ratios involving these two crosses was 0.0114. Note that as a result of Bon-Feroni correction we were using an α-value of 0.0083. Block 1, however was different from the rest. For example, in males the two hybrid curves were below the curve for S*S cross, although the difference was not significant. There are two possible reasons why block 1

might be behaving in an entirely different manner. It is, of course, possible that in the I1 population improvement in immune response has involved genetic changes which are different from those in the remaining three blocks, a concern we addressed in Experiment 2. Since we cannot rule out the possibility that the results obtained in block 1 are a consequence of a mistake in while carrying out the experiment, we have decided to repeat the experiment for block 1.

Overall the result of our analyses indicate that the variation responsible for the improvement in immune response in I populations is largely additive. In males, though, there is a definite trend that suggests that the variation might be recessive to an extent greater than in females. Qualitative comparisons between males and females show that the hybrids are closer to S*S in males than they are in females.

It can be argued that the inference from analysis on proportional survivorship values is influences by block 1, thereby biasing the two hybrid crosses closer to the S*S cross Because in block 1 the proportional survivorships of the two hybrid crosses (in males) are lower than the S*S cross. However, when we repeated the analysis excluding block 1, the results did not change. The hybrid males were still indistinguishable from the S*S cross and significantly different from the I*I cross. In females, however, this analysis could not distinguish between I*I and I*S ($p=0.0501$, Tukey's HSD).

The one conclusion that our results unequivocally support is that sex-specific evolution of immune response in our populations did not occur as a result of selection acting on X-linked immunity related loci. Below we have tried to address some possible reasons.

Our hypothesis was based on the following three points. Firstly, theoretical studies, such as Rice (1984)[12] have predicted that the X chromosome should be enriched in alleles that act in a sexually antagonistic manner. Secondly, empirical studies have documented intra-locus sexual conflict in *Drosophila melanogaster* (for example, [3]) and shown that X chromosome is a 'hot spot' for sexually antagonistic variation [14]. Lastly, there have been laboratory studies that have reported some degree of sexually antagonistic genetic variation with respect to immunity in *Drosophila melanogaster* [11],[15],[16]. If even one of these three lines of evidence was either incorrect or not relevant to our study, our hypothesis would be wrong.

Fry (2010) suggested that upon relaxing the assumption made by Rice (1984) of equal dominance in the two sexes, in certain cases, sexually antagonistic polymorphism can be maintained on autosomes for a much broader range of parameter values than on X chromosomes. Therefore, if there are immunity related alleles in our populations that act in a sexually antagonistic manner they may not necessarily be on the X chromosome.

Vincent and Sharp (2014) found a negative correlation between the two sexes in *Drosophila* for resistance and tolerance and Hill-burns and Clark (2009) found sexual antagonism for some X-linked immunity gene SNPs. Negative correlations between the two sexes for a certain trait need not necessarily lead to negative correlations in fitness, something that is essential for intra-locus sexual conflict. Therefore, assuming Rice's model to be correct, for the loci responsible for the sex-specific immune response observed in our population to be overrepresented on the X chromosome they must be associated with total fitness in a sexually antagonistic manner. If this is not true, then predictions made by models such as the one by Rice (1984) will not be applicable.

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In the second experiment, we tested if adaptive evolution is repeatable over short time scales using our populations. In both males and females, in most crosses involving replicate I populations, the hybrids were not significantly different from the parental crosses. If most of the loci involved in the improvement of immune response are overlapping across replicate I populations then one would expect the hybrid crosses to be indistinguishable from the parental crosses. We have already shown that the variation responsible for improved immune response in I populations is largely additive. Therefore, significant deviations either below or above (in terms of survivorships) the parental crosses would implicate the involvement of epistatic interactions between different loci from different lines. In males, only in the case of crosses involving I1 and I2 (i.e. subexperiment 1) were any differences observed, with one of the hybrid crosses- I2*I1- having significantly higher survivorships than all of the other three cross (I1*I1, I2*I2 and I1*I2). In females, in the case of crosses involving I1 and I4 (subexperiment 4) one of the hybrids- I4*I1- had significantly lower survivorships when compared to I1*I1, I1*I4. Although the survivorships of I4*I1 cross were lower than I4*I4 cross, the difference was not significant. These results suggest that improvement of immune response in I1 involved at least some loci that were not involved in I2 and I4.

One of the shortcomings of our experiment, however, was its inability to detect the involvement of different loci in different lines if the effects of alleles at these loci were purely additive.

Overall, we have conclusively shown that there is no effect of X chromosome in the improvement of immune in populations selected against *Pseudomonas entomophila*. We have also shown that the nature of the genetic variation involved in this improvement is largely additive, with a slight bias towards being recessive only in males. Our experiments also hint that in replicate selected populations improvement in immune response might have occurred as a result of different genetic changes.

Chapter 5

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