Study of interplay between immune function and reproductive behaviour in populations of *Drosophila melanogaster* selected for increased pathogen resistance.

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DECLARATION

I hereby declare that the matter embodied in the report entitled "*Study of interplay between immune function and reproductive behaviour in populations of Drosophila melanogaster selected for increased pathogen resistance*" are the results of the investigations carried out by me at the Department of Biological Sciences, IISER, Mohali, under the supervision of Dr. N. G. Prasad and the same has not been submitted elsewhere for any other degree.

Signature of Student

Date: 28.3.2016

CERTIFICATE

This is to certify that this dissertation entitled "*Study of interplay between immune function and reproductive behaviour in populations of Drosophila melanogaster selected for increased pathogen resistance*" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by Radhika R. at IISER Mohali under the supervision of Dr. N. G. Prasad, Associate Professor, Department of Biological Sciences during the academic year 2015-2016.

N. G. Prasad

Signature of Supervisor

Date: 28/3/2016

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INTRODUCTION

The fitness of an organism is determined by its ability to survive and reproduce. Several life-history traits such as longevity, fecundity, resource acquisition capabilities, *etc.* majorly impact the survival and reproduction of organisms. However, the benefits of favourable reproductive traits may be reaped only in the presence of traits that enable an organism to survive at least upto an age of reproductive maturity. Immune function is one such major contributor that helps an organism ward off pathogens and parasites, thus enhancing the prospect of its survival and thereby its reproductive success.

Trade-offs, a scenario where beneficial changes in one trait is negatively correlated with another, are commonplace in life-history studies (Stearns 1989). Van Noordwijk and de Jong (1992), in their revolutionary paper, put forth the 'Y model' to explain this phenomenon. Using variation in the acquisition and allocation of resource as the basis for the existence of trade-offs (de Jong and Van Noordwijk 1989), this model posits that trade-offs are often masked when the resources available to organisms are plentiful and may be unmasked under resources limited conditions. The predictions of this model, considered to be a cornerstone of life-history evolution, have been tested and found to be sound (King et al, 2011; Brown 2003; Christians 2000).

Immune function, being a resource intensive trait, has been shown to trade-off with various other traits in a variety of taxa including birds (Hanssen *et al.* 2005), rodents (Demas *et al.* 2003), fishes (Ohlberger *et al*. 2011) and even plants (Lozano-Durán *et al.* 2013). Particularly in insects, immunity is known to trade-off with longevity (Ye *et al.* 2009), offspring viability (Ye *et al.* 2009), developmental time (Rantala and Roff, 2005) and reproductive behaviour (McNamara, Wedell and Simmons 2013).

One of the most intriguing aspects of immunity related trade-offs happens to be with reproduction related traits (reviewed in Schwenke *et al.*2016; Rose and Bradley 1998).

The cost of reproduction on immunity has been extensively documented. Reproduction has been shown to negatively impact longevity (Nordling et al 1998; Fedorka *et* a. 2004) and bacterial clearing abilities (McKean and Nunney 2001). Also, mating seems to negatively impact various components of cell-mediated and humoral immunity such as phenoloxidase activity (Rolff and Siva-Jothy 2002), hemocyte load (Fedorka *et al*. 2004), lytic activity (Fedorka *et al.* 2004), and encapsulation ability (Siva-Jothy *et al* 1998). Also, it has been shown that the detrimental effect of mating on immunity is temporary and wares off with time (Fedorka *et al*. 2007).

However, there are some studies, albeit fewer in number, that show report a positive effect (Johansson *et al.* 2004; Shoemaker *et al.* 2006; Gupta *et al.* 2013) or no effect (Short and Lazzaro 2010; McKean *et al.* 2008) of reproduction on immune function. Additionally, expression of immunity related genes are known to be upregulated in response to mating (McGraw et al. 2004; Peng et al. 2005b).

The main objective of my thesis was to look for the possible effects of immunity and reproduction on each other. These studies were carried out using laboratory populations of *Drosophila melanogaster* selected for higher survivorship against a gram-negative bacterium, *Pseudomonas entemophila.* I attempted to address the following questions:

- What kind of reproductive strategies have evolved in the selected populations?
- Does reproduction have different effects on the immunity of selected populations in comparison with that of the controls?

MATERIALS AND METHODS

Model system

For my thesis, I worked with populations of *Drosophila melanogaster* adapted to laboratory conditions. These flies have a holometabolous life cycle; *i.e*. they undergo complete metamorphosis during their lifespan and possess four distinct life stages: egg, larva, pupa and adult. At 25^0C, *Drosophila melanogaster* eggs hatch about 20-24 hours post oviposition to give rise to 1^{st} instar larvae. These molt into 2^{nd} instar larvae which in turn molt into 3^{rd} instar larvae. While the first two larval stages last about 24 hours each, the 3^{rd} instar larvae last for 2-3 days. Through these three stages the larvae continue to feed and grow bigger until the 3^{rd} instar larvae move away from the food and enter the pupal stage, which lasts for about 3-4 days. During this time the larvae undergo metamorphosis by ridding themselves of most of the larval tissue through lysis and the adult body is generated anew via differentiation of imaginal disks. Once the metamorphosis is complete, the pupae darken in colour and on the $9\text{-}10^{th}$ day post egg laying fully developed adults eclose from the pupal casing.

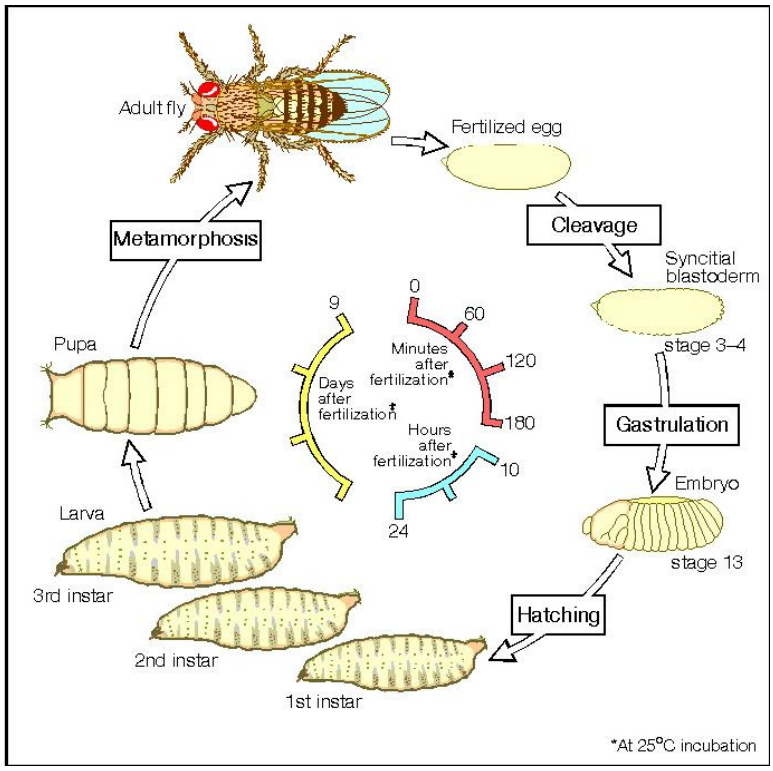

Fig 1: Life cycle of *Drosophila melanogaster*. *Source: http://myfruitfly.weebly.com/about.html*

Both males and females remain sexually inactive upto 6-8 hours post eclosion at 25^0C, thus making the collection of unmated flies fairly simple.

Fly stocks and their maintenance protocol

Blue Ridge Baseline (BRB): These are five large, outbred replicate populations of *Drosophila melanogaster* established from 19 iso-female lines that were acquired from the Promislow lab at the University of Washington. BRBs are maintained under a 14 day discreet generation life cycle where eggs are collected from adult flies and dispensed into vials containing about 8 ml of standard banana–jaggery food (Table 1) at a density of approximately 70 eggs per vial. On the 12^{th} day post egg collection adult flies from each replicate population, about 2800 individuals, are transferred into plexiglass cages. The cages are provided with plates containing fresh banana–jaggery food every alternate day and maintained under 12:12 light-dark cycle at 25°C and 50-60% RH.

I,U,S selection regime: This selection line was undertaken to investigate and better understand the evolution of immunity in *Drosophila melanogaster* in response to systemic infection. To establish the selection regime, flies were derived independently from four blocks of BRBs (BRB 1-4) to establish four blocks of the selection line (I,U,S 1-4). Within each block of the selection line, three separate regimes were established. This procedure ensured that flies from the three different regimes within each block shared common ancestry (Fig 2).

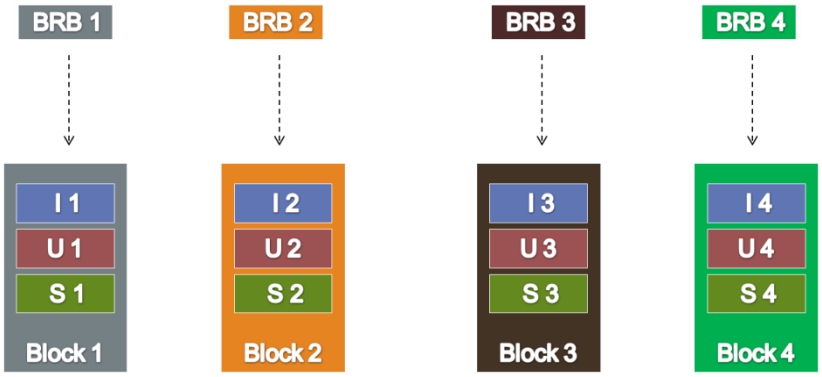

Fig 2: Schematic representation of the common ancestry of the different I,U,S regimes.

The three separate regimes are handled in the following manner (Fig 3):

- **Infected regime (I)**: Flies in this regime experience a systemic infection by a bacterium, *Pseudomonas entemophila* (PE) every generation. Infections are carried out by exposing the flies to mild CO_2 anaesthesia and pricking on the lateral side of their thorax with a thin needle (*Minutein pin* 0.1 mm, Fine Science Tools, CA) dipped in a bacterial suspension of the desired Optical density (OD). Here, 150 pairs of flies are infected on the 12^{th} day post egg collection (2-3 days old as adults) and the OD for infection is chosen so as to maintain a mortality rate of about 33%, thus ensuring the survival of 100 pairs of flies. On the 16^{th} day post egg collection, eggs are collected from the survivors to start the next generation.

- **Sham Infected regime (S)**: In this regime, 100 pairs of flies are pricked in the thorax with a needle dipped in sterile $MgSO_4$ (10mM) solution. This regime serves as a control for injury as well as the effects of $MgSO_4$, which is used to prepare the bacterial suspension.

- **Unhandled regime (U):** 100 pairs of flies are exposed to mild CO_2 anaesthesia. These serve as the unhandled controls.

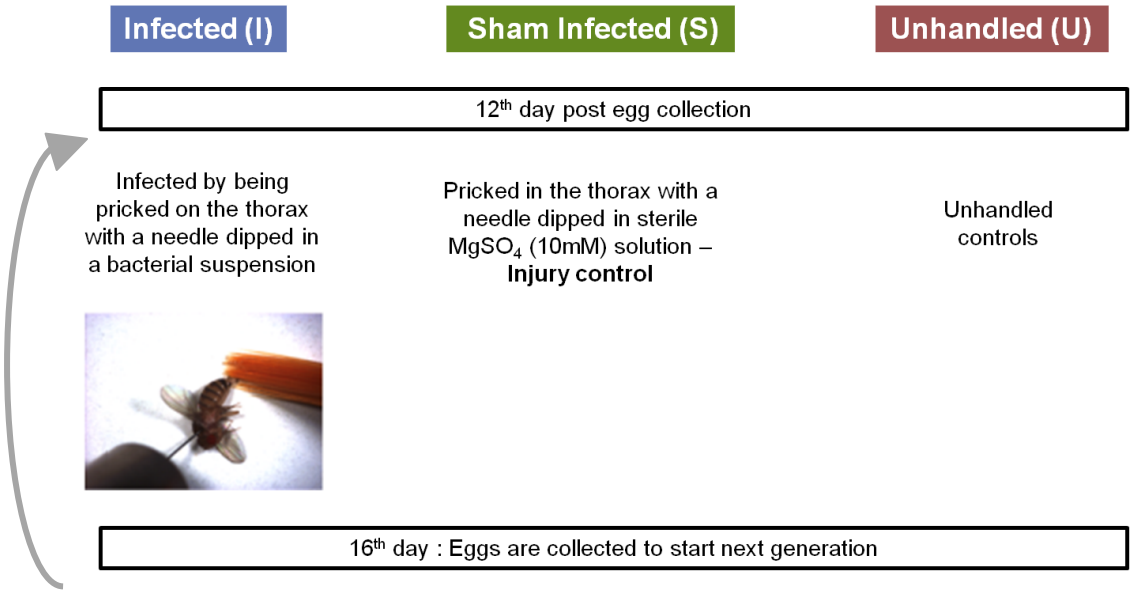

Fig 3: Schematic representation of the maintenance of the I,U,S selection line.

These populations are maintained on a 16-day discrete generation cycle. On the 12th day post egg collection, flies from each regime are subjected to the corresponding treatments, maintained in cages and provided with fresh food plates every alternate day. To start the next generation, eggs are collected from an 18 hour cut-plate given to flies on the 16th day post egg collection. Eggs are dispensed into vials containing about 8 ml of standard banana–jaggery food at a density of approximately 70 eggs per vial and incubated under a 12:12 light-dark cycle at 25°C and 50-60% RH (Gupta *et al.* 2015).

LH population: The LH are a long-term, laboratory adapted, outbred population established by Larry Harshman from 400 females collected from the wild in California, USA (Chippindale and Rice, 2001). This population is maintained under a 14 day discreet generation cycle in vials. To start the next generation, 16 pairs of adult flies are sorted under mild $C0_2$ anaesthesia and transferred into a vial containing molasses-cornmeal food (Table 2) and 60 such vials are set up. The flies are allowed to oviposit for about 18 hours and then dicarded. The density of the eggs laid by these flies is regulated roughly to 150 eggs per vial by trimming. Vials are incubated under a 12:12 light-dark cycle at 25°C and 50-60% RH.

LH_{st} population: This population has been derived from the above mentioned LH population by introducing a recessive scarlet eye colour marker via backcrossing. These flies are reared under conditions similar to that of the LH population.

Ingredients	Quantity
Banana	205 g
Barley flour	25 g
Jaggery	35 g
Yeast	36 g
Agar	12.4 g
Ethanol	45 ml
Water	1180 ml
p-Hydroxymethyl	2.4 g

Table 1: Ingredients and their quantities required for 1L of banana-jaggery food.

Ingredients	Quantity
Agar (g)	14.8 g
Cornmeal (g)	100 g
Molasses (ml)	100 g
Yeast (g)	41.2 g
p-Hydroxymethyl benzoate (g)	2.25 g
Ethanol (ml)	22.5 ml
Propionic acid (ml)	8 ml
Water (ml)	1100 ml

Table 2: Ingredients and their quantities required for 1L of cornmeal-molasses food.

Standardisation procedure:

Standardisation is a process where flies are passed through a generation of rearing under identical conditions. Standardised flies, about 500 in number, are maintained in plexiglass cages and provided with live yeast paste to increase oviposition. Two days later, eggs are collected from these standardised flies to give rise to experimental flies. One generation of common rearing is done in order to equalize the non genetic parental effects on the traits of experimental flies (Rose 1984).

Bacterial stocks

Pseudomonas entemophila (PE) strain L48 is a gram-negative bacteria and is a natural pathogen of *Drosophila melanogaster* (Vodovar et al., 2005). In order to prepare a bacterial slurry for experiments, bacteria is cultured in Luria Broth medium overnight at 27 ºC and 150 rpm. This primary culture is then used to seed a secondary culture which is incubated until its OD reaches ~1. The medium is then centrifuged for 10 min at 7200 rpm and 25^0C. The pellet obtained is re-suspended in $MgSO_4$ and diluted to obtain a slurry of the desired OD. Optical density measurements are carried out at 600nm.

EXPERIMENTAL DESIGN

Drosophila melanogaster is a species that displays promiscuity. Additionally, females have the ability to store the ejaculate from multiple males they mate with (L.W. Simmons 2001). This attribute of the species leads to interesting possibilities when looked at in conjunction with the I selection regime. As described earlier, flies in the I regime are exposed to infections on the 12^{th} day post egg collection and eggs are collected from the survivors to start the next generation 5 days later, on the 16^{th} day. Thus, only those females that survive for a period of at least 5 days post infection successfully contribute to the gene pool of the next generation. However, a male could succumb to the infection much before the 16^{th} day and still manage to pass his genes to the next generation as long as the females he mated with live upto the 16^{th} day.

Since flies eclose on the 9-10^{th} day post egg collection and infections occur on the 12^{th} day, they have ample amount of time to mate prior to infections. Thus, one of the strategies males from the I regime could have evolved is to invest in reproduction prior to infection (9^{th} – 11^{th} day) and die without much investment post infection. Such a strategy would still ensure that their genes are passed on to the next generation via the sperm stored by females they have mated with prior to infection. If this were true, one would expect reduced sexual activity among the I flies post infection in comparison to that of S flies and/or a decline in competitive fitness of I males post infection. The two predictions mentioned above have been tested to decipher whether such a strategy has evolved in I males through experiments 1 and 2 respectively.

EXPERIMENT 1: Effect of immunity on reproductive behaviour

For this experiment, eggs were collected from standardized flies in vials containing 8 ml of banana–jaggery food at a density of 70 eggs per vial and incubated under a 12:12 light-dark cycle at 25°C and 50-60% RH. On 12^{th} day post egg collection, 150

pairs of flies from I regime were subjected to infection with PE (OD 2 ± 0.1) while 100 pairs of flies from S regime were subjected to sham infections with 10mM $MgSO_4$. Flies were maintained in plexiglass cages and the rate of mortality for each sex, number of courtships and number of mating pairs were monitored at hourly intervals between the 12^{th} and 16^{th} day post egg collection. This experiment was performed in triplicate for each of the four blocks.

EXPERIMENT 2: Effect of immunity on competitive reproductive success

To assay the competitive fitness of individual I and S males (focal male), they were pitted against a common competitor male (LH_{st}). To generate I and S males, eggs were collected from standardized flies in vials containing 8 ml of banana–jaggery food at a density of 70 eggs per vial and incubated under a 12:12 light-dark cycle at 25°C and 50-60% RH. Likewise, to generate LH_{st} flies eggs were collected from standardized flies in vials containing 8 ml of molasses-cornmeal food at a density of 150 eggs per vial and incubated under similar conditions.

On the 12^{th} day post egg collection, individual focal males (I or S) were combined with a common competitor LH_{st} male and given the same immunological treatment, *i.e.*, they were both either infected with PE (OD = 1.5 ± 0.1) or sham infected. Additionally, a single LH_{st} female was introduced into vials containing both the males. While the focal males had a dominant red eye colour marker, both the common competitor males and the females had a recessive scarlet eye colour marker. Thus, the reproductive success of I and S males could be tracked by the proportion of red eyed progeny. For reasons similar to that of experiment 1, the reproductive fitness of focal males were tracked between the 12^{th} and 16^{th} day post egg collection. This was done by transferring all three flies from each vial into fresh vials every day to yield daily a measure of reproductive fitness for 5 days. Once the red and scarlet eyed progeny of the experimental flies eclosed, the vials were frozen at -20^0C and the proportion of red eyed progeny were counted at a later time.

EXPERIMENT 3: Effect of male mate identity on the immunity of females

In the I,U,S regime, as mentioned earlier, the survival of a male upto the 16th day post egg collection is not imperative to ensure the passage of his genes to the next generation. Due to female *Drosophila melanogaster*'s ability to store sperms, males could sire progeny long after they are dead. A number of experiments have demonstrated that not only does mating impact the immune system of females but also that both the phenotype (Imroze and Prasad 2011) and genotype (Rice 1996) of a male could have a bearing on the immunity of a female. Thus, it is possible that males from the I regime have evolved a mechanism to enhance the immunity of the females they mate with. In order to test this hypothesis, the following experiment was performed.

Eggs were collected from standardized I and S flies in vials containing 8 ml of banana–jaggery food at a density of 70 eggs per vial and incubated under a 12:12 light-dark cycle at 25°C and 50-60% RH. On the 9th-10th post egg collection males and females from both the regimes were collected within 6 hours of eclosion to ensure virgin status. A factorial mating treatment was set up between I and S flies. Since it is known that effects of mating on females are mediated both by the Accessory Gland Proteins (ACPs) that are transferred by the males during mating (Chapman *et al.* 1995) as well as extended physical interaction with males (Lew *et al.* 2006), we chose to expose females to males for two different time periods.

Single mating: Here, vials containing virgin males and females were combined on the 12th day post egg collection to set up four crosses as depicted in Fig 4. Flies were allowed to mate exactly once before being segregated under mild CO_2 anesthesia. While the males were discarded, females were maintained in vials. Approximately 5-6 hours post mating, singly mated females from each cross were randomly allotted to one of the following two treatments: infection with PE (OD = 1.5 ± 0.1) or sham infection. While 70 females were subjected to infections with PE, 50 were sham infected per cross. Additionally, virgin I and S females were also subjected to

infection with PE and sham infection. These females were maintained in plexiglass cages for 96 hours post infection and their mortality was documented every 2-3 hours.

Continuous exposure or multiple matings: Here, vials containing virgin males and females were combined on the 10^{th} day post egg collection to form 4 crosses and allowed to interact for the next 48 hours. On the 12^{th} day, flies were segregated over mild CO_2 anesthesia and females from each cross were randomly subjected to either infection with PE (OD = 1.5 ± 0.1) or sham infection. While 70 females were subjected to infections with PE, 50 were sham infected per cross. Additionally, virgin I and S females were also subjected to infection with PE and sham infection. These females were maintained in plexiglass cages for 96 hours post infection and the mortality of flies was documented every 2-3 hours.

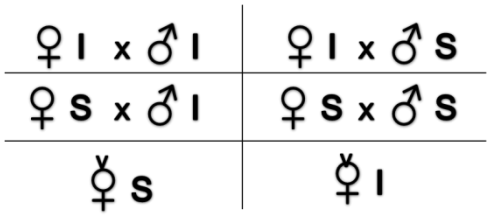

Fig 4: Schematic representation of the factoral mating treatment between male and female I and S flies.

RESULTS

EXPERIMENT 1: Effect of immunity on reproductive behaviour

Since the experiment was carried out over 5 days, data from each 24 hour window was binned together and assigned the labels Day 1-5. Analysis for this experiment was done using a three way mixed-model ANOVA with Block as a random factor and Selection and Day as a fixed factors. Over the course of the 5 days, Selection had significant effect on the courtship activity of flies (p=0.0095) but not on mating activity (p=0.0877). Courtship activity was seen to be greater in I than S but no such differences were found in number of mating pairs (Fig 5). No effect of Day was found on either courtship activity (p=0.1178) or number of mating pairs (p=0.7193) of flies. Also, a Block*Day (Random) interaction was observed for courtship activity

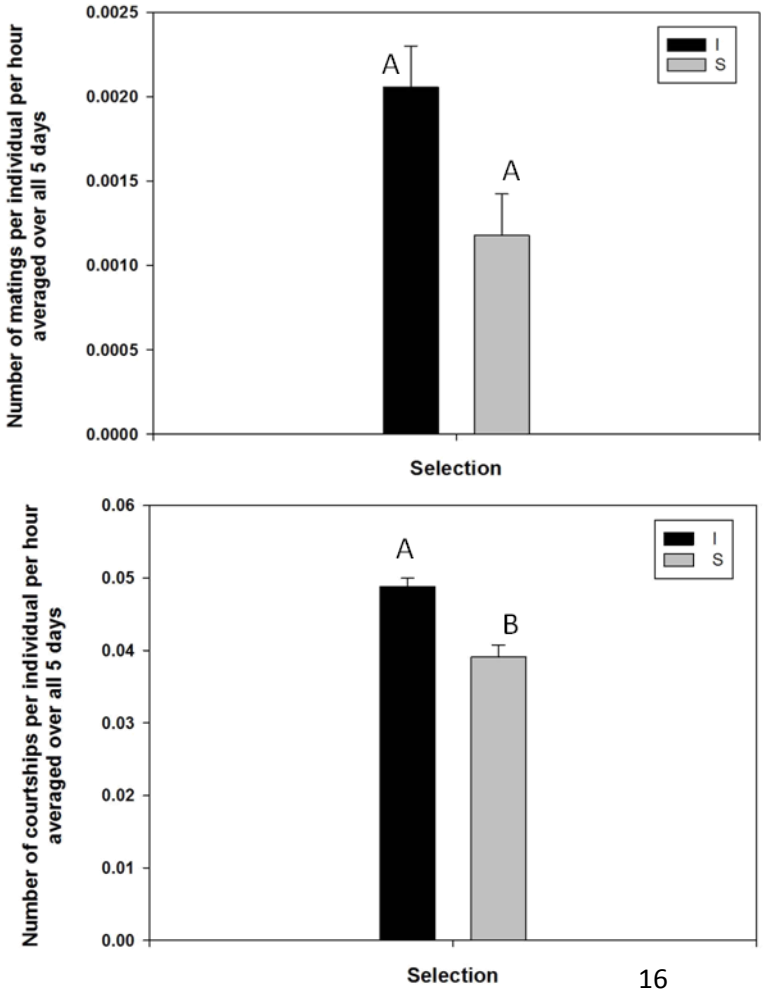

Fig 5: Average sexual activity displayed by per individual per hour avereged over five days. Fig 5a (above) depicts average number of matings per indiviual per hour while Fig 5b (below) depicts average courtship activity per indiviual per hour. Black bars represent I flies and grey bars represent S flies. Error bars denote standard error.

Source	MS Num	DF Num	DF Den	F ratio	Prob > F
Block & Random	0.005401	3	5.093576	1.740906	0.272334
Day	0.011479	2	6.004907	3.118278	0.117831
Selection	0.016923	1	3.023208	34.78056	**0.009524 ** **
Block*Day & Random	0.003687	6	6	5.214769	**0.032253 * **
Block*Selection & Random	0.000486	3	7.87503	0.683145	0.587223
Day*Selection	0.001349	2	6.025611	1.908409	0.228017
Block*Day* Selection & Random	0.000707	6	857	0.953127	0.45609

**p<0.01, *p<0.05

Table 3a: Effect of immune challenge on courtship behaviour in I and S flies. Summary of results from three way mixed model ANOVA with Block as a random factor and Selection and Day as a fixed factors.

Source	MS Num	DF Num	DF Den	F ratio	Prob > F
Block & Random	4.66E-06	3	2.20114648	0.243105	0.861857
Day	1.59E-06	2	6.04090804	0.348107	0.719307
Selection	0.000135	1	3.0053369	6.242863	0.087667
Block*Day & Random	4.56E-06	6	6	0.611509	0.717402
Block*Selection & Random	2.17E-05	3	7.82735515	2.904417	0.102776
Day*Selection	4.34E-07	2	6.02499922	0.058279	0.943912
Block*Day* Selection & Random	7.45E-06	6	857	0.976426	0.439946

**p<0.001, *p<0.05

Table 3b: Effect of immune challenge on number of mating pairs in I and S flies. Summary of results from three way mixed model ANOVA with Block as a random factor and Selection and Day as a fixed factors.

EXPERIMENT 2: Effect of immunity on competitive reproductive success

The number of red and scarlet eyed progeny sired by the focal and common competitor male respectively was counted for each day and the percentage of progeny sired by the focal male on each day was calculated using the formula:

$$\text{percentage of progeny sired by thefocal male} = \frac{\text{no. of red eyed progeny}}{\text{no. of red eyed progeny + no. of scarlet eyed progeny}}$$

Analysis using a four way mixed model ANOVA with Selection, Treatment and Day as fixed factors and Block as a random factor shows a significant effect of Treatment ($p = 0.0112$) on the proportion of progeny sired by the focal male. Males from the infected treatment sired a greater proportion of progeny than sham infected flies. There is also a significant effect of Day ($p = 0.0135$) where proportion of red eyed progeny increased almost linearly from Day 1 to Day 5. A highly significant Treatment*Day effect was also observed ($p = 0.0006$). Tukey's HSD suggests that while the proportion of progeny sired by sham infectioned focal males did not change over time that of infected ones increased progressively from Day1 to Day5.

Lastly, a significant Selection*Treatment* Day ($p=0.0053$) interaction was also seen. Tukey's HSD revealed that the proportion of progeny sired by sham infected I and S males remains constant over 5 days and is not significantly different from each other (Fig 6). Also, the proportion of progeny sired by infected S males remains constant over 5 days and is not significantly different from that of either sham infected male on any day. Most importantly, infected I males significantly increase the proportion of progeny they sire over the five days. In fact the ratio doubled from 0.315 on Day 1 to 0.639 on Day 5. Performing a day-wise comparison between the proportions of progeny sired by infected I and S males showed no significant difference on Days 1-4. However, on Day 5, infected I males (0.639) sired a much higher (~1.5 times) proportion of progeny than infected S males (0.449).

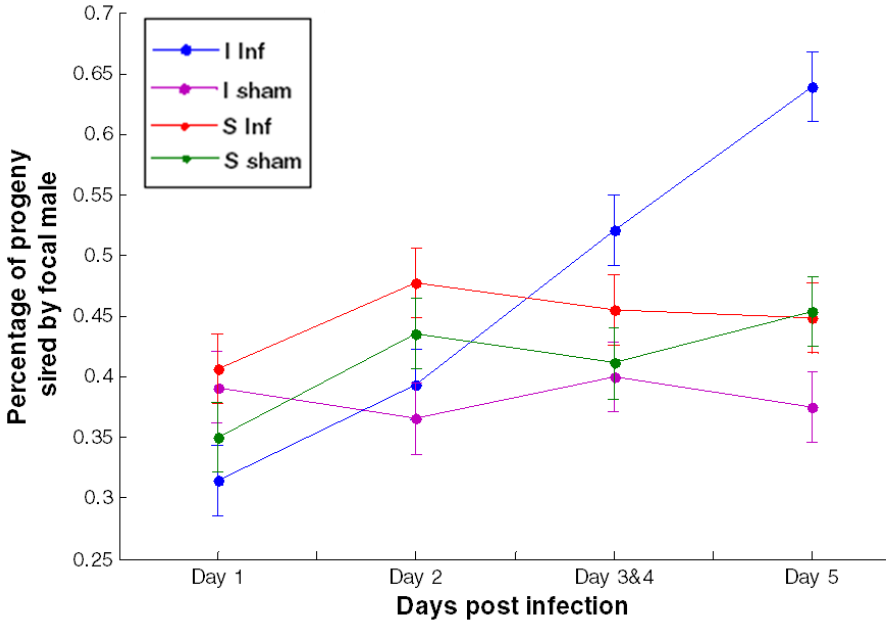

Fig 6: Proportion of progeny sired by I and S males (both infected and uninfected) over 5 days post infection when housed with a common competitor LHst male and a LHst female. Error bars denote standard error.

Source	MS Num	DF Num	DF Den	F ratio	Prob > F
Block & Random	0.006026	3	1.7692	0.318873	0.816426
Selection	0.000339	1	2	0.015246	0.909538
Block*Selection & Random	0.022212	3	1.409931	2.204895	0.200087
Treatment	0.056115	1	2	31.29044	**0.011285 ***
Block*Treatment & Random	0.001793	3	0.962459	0.510843	0.746755
Selection*Treatment	0.009916	1	2	1.672655	0.286487
Block*Selection* Treatment & Random	0.005928	3	6	1.774451	0.221827
Day	0.037165	3	6	6.308549	**0.013588 ***
Block*Day & Random	0.005891	9	0.100152	1.16229	0.492169
Selection*Day	0.013652	3	6	1.823591	.213014
Block*Selection*Day & Random	0.007486	9	6	2.240796	0.122577
Treatment*Day	0.014525	3	6	15.73311	**0.000635 *****
Block*Treatment* Day & Random	0.000923	9	6	0.276325	0.965541
Selection*Treatment* Day	0.028568	3	6	8.550677	**0.005322 ****

***p<0.001,**p<0.01, *p<0.05

Table 4: Proportion progeny sired over five days post infection. Summary of results from four way mixed model ANOVA with selection, treatment and day as fixed factors and block as a random factor.

EXPERIMENT 3: Effect of male mate identity on the immunity of females

The survivorship curves obtained from both the single mating and multiple mating (continuous exposure) show remarkably similar trends. Survivorship analysis was carried out using the Kaplan-Meier survival estimator followed by a Log-rank test to compare the survivorship curves (Fig 7a and 7b).

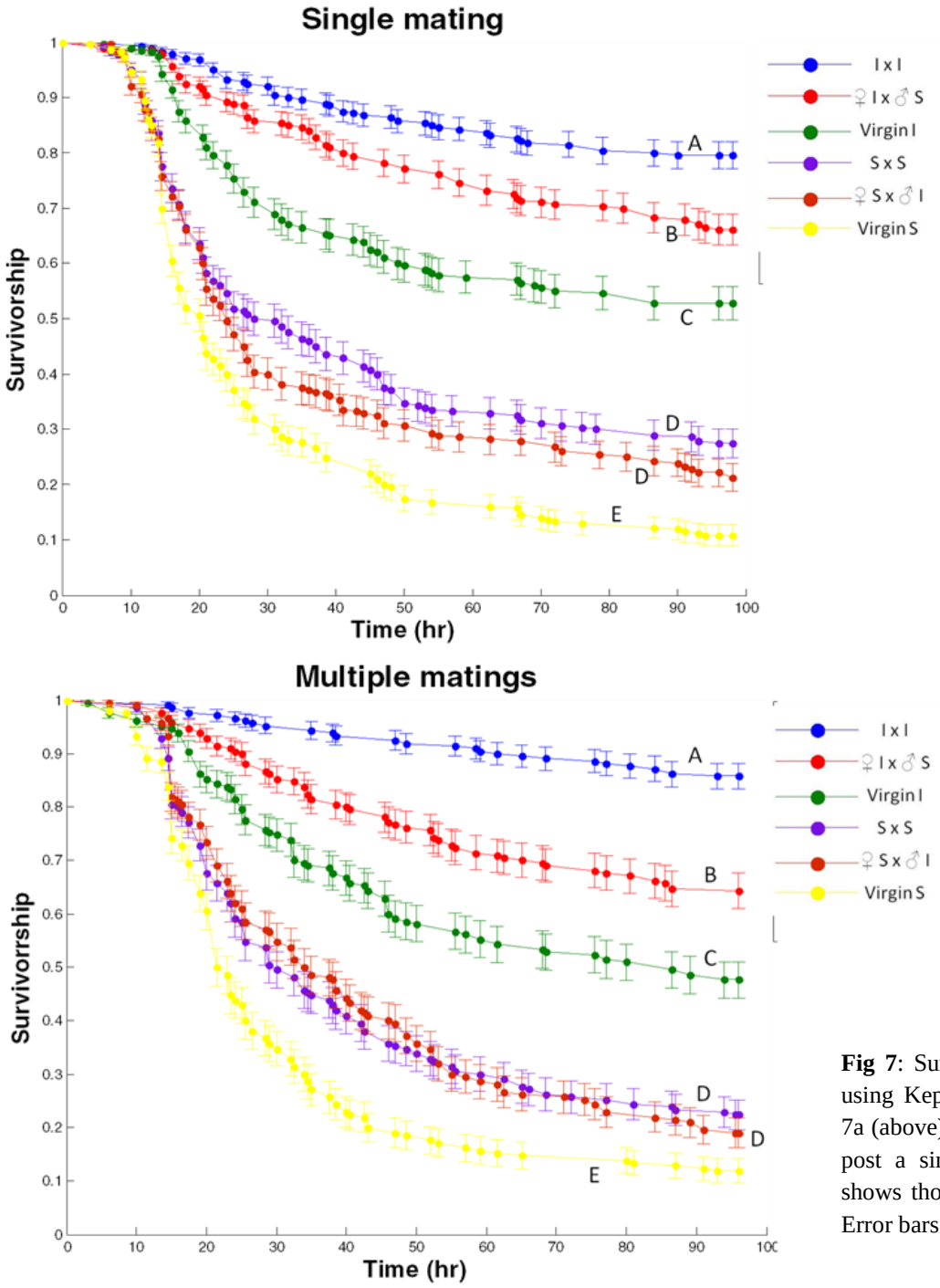

Fig 7: Survivorship curves plotted using Keplan-Meier estimator. Fig 7a (above) shows the survival plots post a single mating and Fig 7b shows those after multiple mating. Error bars denote standard error.

The results show that infected I and S mated females (both single and multiple) showed higher survivorship than the corresponding infected virgin females. Also, within the mated treatments, I females crossed with I males survived more than those crossed with S males. However, among the S females, though survivorship did improve on mating no male specific effects were observed. In all cases the sham infected controls showed near zero mortality. Table 5a and 5b shows the p values for pairwise comparisons of survivorship curves from each curve using the Log-Rank test. The Bonferroni correction for multiple comparisons sets the α for statistical significance at 0.0033.

Groups being compared		**P value**
I x I	♀ I x ♂ S	**0.0003 *****
Virgin I	♀ I x ♂ S	**0.0003 *****
I x I	Virgin I	**< 0.0001 *****
S x S	♀ S x ♂ I	0.115
Virgin S	♀ S x ♂ I	**0.0003 *****
S x S	Virgin S	**0.0002 *****

***p<0.001,**p<0.01, *p<0.05

Table 5a: Pairwise comparisons of survivorship curves of singly mated females from each curve using the Log-Rank test.

Groups being compared		**P value**
I x I	♀ I x ♂ S	**< 0.0001 *****
Virgin I	♀ I x ♂ S	**0.0001 *****
I x I	Virgin I	**< 0.0001 *****
S x S	♀ S x ♂ I	0.951
Virgin S	♀ S x ♂ I	**0.0006 *****
S x S	Virgin S	**0.0006 *****

***p<0.001,**p<0.01, *p<0.05

Table 5b: Pairwise comparisons of survivorship curves of multiply mated females from each curve using the Log-Rank test.

DISCUSSION

Experiments 1 and 2 were performed to address the question whether flies in the I regime had evolved an alternate strategy of investment in reproduction wherein I males display reduced sexual activity and reproductive output post infection.

Data from experiment 1 show that not only do I males show mating activity comparable to that of S, but in fact show a higher courtship activity. Thus, I males show no reduction in sexual activity in comparison with those of controls.

Experiment 2 shows I and S males have comparable competitive fitness and reproductive outputs on the first 4 days post infection. However, on the 5^{th} day the reproductive fitness of I males is approximately 1.5 times that of S males. This result is significant for two reasons. Firstly, shows that I males do not have reduced reproductive output compared to the controls. This, coupled with the results of experiment 1, show that I males show similar investment in reproduction related activities compared to those from the control populations post infection with PE.

Secondly, and more importantly, a huge difference in reproductive success between I and S males is seen only on the 5^{th} day post infection *i.e.* the 16^{th} day post egg collection. This happens to be a very important day with regard to the I, U, S selection regime (see methods) since eggs are collected to start the next generation on this day. It is indeed very remarkable that we see such a finely tuned temporal increase only on the 5^{th} day post infection.

The phenomenon of last male precedence, which is observed in many species including *Drosophila,* can be used to explain this observation. If most males, but not all, in the population were to invest in reproduction pre infection but reduce reproductive effort or die post infection it is very likely that most of the progeny will be sired by those who survive and continued to invest in reproductive activity even after being exposed to infections. This could potentially serve as the reason behind why we see that males in the I regime have evolved to survive and reproduce post infection. Alternatively, it is also possible that males' ability to survive infections have

evolved as a by product of the females' ability to do the same. Please note that the female survivorship post infection is not expendable unlike that of males (see materials and methods).

While experiments 1 and 2 looked at the impact of immunity on reproduction in the I,U,S regime, experiment 3 looks at the opposite. We saw that mated females generally show greater survivorship than virgins. However, an overwhelming amount of extant literature suggests the opposite where reproduction seemingly has a cost on immunity (Nordling et al 1998; Fedorka *et* a. 2004; McKean and Nunney 2001; reviewed in Schwenke *et al.*2016; Rose and Bradley 1998). There are very few studies that report the same trend as we do (Johansson *et al.* 2004; Shoemaker *et al.* 2006; Gupta *et al.* 2013).

This disparity could be attributed to differences in the experimental design of these studies. Firstly, the method used to quantify immune activity is vastly different in each of these studies and range from survivorship analysis to measurement of proxies such as phenoloxidase activity or encapsulation ability. It is quite possible that the readout we get out of proxy measures of certain components of immunity may not always be representative of the state of the immune system of an organism as a whole. We believe that looking at survivorship is a better readout and have thus used it in our study. Secondly, it has been shown that differences in immunity of virgin versus mated flies seem to be pathogen specific (Gupta *et al.* 2013; Short and Lazzaro 2010). The same population of flies shows different survivorship patterns depending on the pathogen they have been exposed to. However, in both the studies mentioned above, a given pathogen elicited the same survivorship response across multiple populations. This leads one to believe that the effect of sexual activity on immunity cannot be generalised across or even within populations and results due to a complex interplay between the host immune system and the pathogen.

It has been demonstrated that both the phenotype (Imroze and Prasad 2011) and genotype (Rice 1996) of a male could have a bearing on the immunity of a female. In our experiment we observed that I females mated to I males survived more than those

mated to S males, but identity of male seems to have no effect on the survival of S females. This is indicative of a co-evolutionary mechanism within the I regime.The nature of such a fascinating mechanism is yet unreported and needs further study. However, one way in which such a mechanism could have evolved is via the activation of female immunity hormones in response to male ejaculate McGraw et al. 2004; Peng et al. 2005b).

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CONCLUSION

In this study we show that males belonging to populations selected for increased survivorship against pathogenic attack display increased sexual activity post infection. They also show a temporal increase in reproductive investment post infection. We also show that mating increases survivorship post infection in females in both I and S populations. Additionally, mating with I males confers a greater advantage on the survivorship of I females than with S males. However, identity of the mate did not significantly change survivorship in S females. This is indicative of a possible co-evolutionary machinery at play between I males and females.

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