Adult immunocompetence against infection by *Enterococcus faecalis* and larval phenol-oxidase activity in the population of *Drosophila melanogaster* adapted to larval crowding

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

Indian Institute of Science Education and Research Mohali

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Certificate of Examination

This is to certify that the dissertation titled **"Investigating adult immunocompetence against infection by *Enterococcus faecalis* and measuring phenol-oxidase activity in the population of *Drosophila melanogaster* adapted to larval crowding"** submitted by **Mr. Soumyadip Poddar** (Reg. No. MS16018) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Manjari Jain

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(Supervisor)

Dated: April 09, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Professor 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.

Soumyadip Poddar

Dated: April 09, 2021

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.

Professor N.G Prasad

(Supervisor)

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ABSTRACT

The environment experienced by an organism during their juvenile stages is known to impact their adult stage. In holometabolous insects like the model organism of this study, *Drosophila melanogaster*, most of the resource acquisition happens during the larval stages. Larval crowding is a condition in which larvae are exposed to an environment which has scarcity of food and high amount of accumulated toxic waste. As adult traits are affected by the developmental environment during larval stages, in larval crowding like conditions, adult traits are prone to get affected. Though, the effect of scarcity of nutrition and poor developmental environment on adult immune response has been studied extensively, the effect of adaption to larval crowding like conditions has not been studied, therefore this study investigates the evolution of ability to survive infection in adult stage as a correlated response to adaptation to larval crowding environments. Using four populations of *Drosophila melanogaster* adapted to larval crowding for more than 250 generations and their respective control populations, this study shows that there is no difference between populations adapted to larval crowding and their respective controls in post infection survivability against infection by a gram-positive bacteria *Enterococcus faecalis.* Therefore, the results suggest that adaptation to larval crowding has no effect on adult immunocompetence measured in terms of survivorship post infection against *Enterococcus faecalis.* In the latter part of the study Phenol-oxidase (PO) activity levels of the populations adapted to larval crowding and their respective controls were compared as various studies have used PO activity as an immune measure to determine the degree of immunocompetence of an organism. The data till now reports that a significant difference in PO activity is present between the selected and control population. This result in turn suggests that PO activity cannot be used as a general immune measure to determine the degree of immunocompetence of an organism.

1. INTRODUCTION

A sequence of events regarding survival and reproduction which occur throughout an organism's life cycle (birth to death) constitute life history of the organism. According to the life history theory evolutionary forces like natural selection shape organisms in a way which leads to the optimization of their survival and reproduction to increase the fitness when the face challenging environments. Life history theory also predicts how natural selection should shape the way in which organisms parcel their resources into making babies (Fabian D, 2012). Fitness of any organism would become maximum when its survival and reproduction are at the maxima and to achieve that all the life history traits should evolve continuously (Houle D, 2001). But this would result in the creation of "Darwinian demons" which are organisms that can reproduce as soon as they are born, are able to produce an infinite number of progenies and can live forever (Law, 1979). However, such organisms do not exist in reality because of two main reasons: i) finite resources; ii) "trade-offs" exist between different life history traits. As a result, every organism has to optimize different life history traits to achieve maximum fitness overcoming the challenges posed by nature. When resources are limited, increased investment in one trait which would result an increment in fitness can have reduced investment in another trait which would lead a decrement in fitness. So, the fitness advantage is balanced against a fitness cost. Various studies have shown the evidence of genetically linked life history trade-offs (Flatt T and Heylad A, 2011) (Stearns S. C. & Partridge L, 2001). Trade-off between life time fecundity and life-span is one of the most important examples of such life history trade-offs. Such trade-offs can be explained by the 'Y-model of resource allocation' proposed by van Noordwijk and de Jong (1986) (Fig 1). According to the theory of density-dependent natural selection at extreme population densities, due to trade-off evolution creates alternative life histories. The reason behind these trade-offs is that genotypes which have highest fitness at low population densities will not maintain high fitness at high population

densities and vice versa (Mueller LD, 1991). In the context of shaping the life-history of organisms, density is considered to be one of the important ecological stressors.

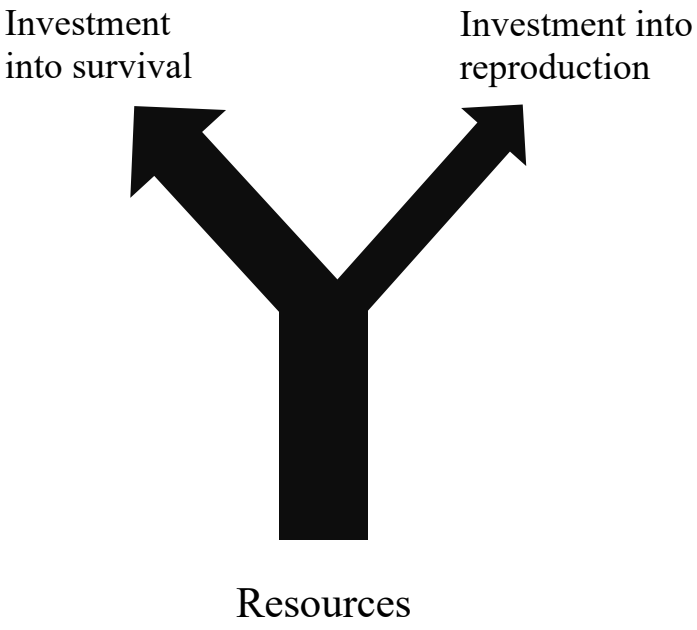


Fig 1: Y-model of resource allocation illustrating trade-off between two traits

The theory of "Developmental origin of health and diseases" states that nutrition and environment available to any organism during its critical developmental phases can have a potentially permanent effect on its resistance to various diseases. (Palmer, 2011). Also, early-life environmental conditions and nutrition available to any organism are essential factors in deciding its level of susceptibility to diseases (Moore SE, 2004). There are various studies on human immune traits which suggest that events in early life can have crucial role in permanently programming the components of immune system (Moore SE, 2004) (Moore SE C. T., 1999) (Moore SE F. A., 2012). Many studies also found that poor nutritional conditions during development result in poor adult immune response in both vertebrates (Hoi-Leitner M, 2001) (Brzęk P, 2007) (Chin EH, 2005) and invertebrates (Rolff J, 2004) (Okech BA, 2007) (Moret Y, 2009).

Invertebrate organisms are popular as a model to understand immune response of organisms because they are less complex than vertebrates and highly tractable. Among different invertebrates, *Drosophila* has been used frequently in numerous immunological studies. *Drosophila* is holometabolous insect, and like other holometabolous insects in case of *Drosophila* also resource acquisition for adult stage happens mainly during the larval stages. *Drosophila* females generally choose decaying food matters as the egg laying site and the environment during larval stages is confined largely to these egg laying sites (Atkinson, 1977). So this can lead to a resource limited and crowded environment which would result in lesser amount of nutritional uptake and exposure to an environment full of toxic compounds (Urea, Ammonia) during the larval stages (NAGARAJAN, 2016). Some studies also suggest that larval crowding conditions negatively affects adult traits like adult body size, fecundity, etc. (C Roper, 1996).

Survivorship post infection is very costly trait in terms of resource allocation or resource intensive trait (Ian P.F. Owens, 1999) (Schwenke RA, 2016) and it trade-off with other important life history traits (Kraaijeveld A. R., 2001) female fecundity, egg viability, reproductive output (Schwenke RA, 2016). Hence, it can be expected that in resource limited environment like larval crowding, organisms would have poor immunocompetence in their adult stage (Kraaijeveld A. R., 2001). Also, to mount an immune response organisms have to generate various non-specific toxins which poses a risk of damaging their (host's) own tissues (Lars Råberg, 1998) (Imroze Khan, 2017). This can have a very detrimental effect on organisms which have developed under larval crowding due to which have a poor body condition.

It is well accepted that single generation of larval crowding affects adult traits like adult body size, longevity, fecundity (C Roper, 1996). Some recent studies have also reported that adaptation to larval crowding can lead to the correlated evolution of a set of adult traits such as increased adult longevity, increased pre-copulatory reproductive behaviour (Shenoi V N, 2016) (Shenoi VN B. S., 2016). Although, no study has looked at the consequence of adaptation to resource limited developmental environment like larval crowding on the adult survivorship post infection.

This study is conducted on eight laboratory populations of *Drosophila melanogaster*, among them four of the populations are adapted to larval crowding and four of the

populations are control (discussed in detail in chapter 2). Using this model system I have tried to answer the following questions:

1) Do the resources available to an organism during developmental stages affect its ability to survive infection in the adult stage ?
2) Does adaptation to poor developmental environment (larval crowding) result in the evolution of immunocompetence in the adult stage ?

For answering these questions, I have used laboratory populations of *Drosophila melanogaster* that have evolved for more than 250 generations in resource limited developmental environment (larval crowding) (MCU populations) and their low density controls (MB populations). Larvae from both regimes were grown at low and high densities (discussed in detail in chapter 2) and then I have measured their survivorship post infection in adult stage against infection by a gram-positive bacterium *Enterococcus faecalis* which has been reported to be extracted from *Drosophila melanogaster* and considered as natural pathogen for fruit flies (Chapman JR, 2020). This bacteria is known to cause systemic pathogenic infection along with causing severe damage to the gut (Chapman JR, 2020).

I have also performed another experiment to measure the Phenol-oxidase activity (PO activity) levels in third instar larvae of MCU and MB populations (discussed in detail in chapter 2). Insects and other invertebrates do not have certain characteristic of the adaptive immune system like the vertebrates have which provide them pathogen specific immunity and immune memory, so they are dependent on innate immune responses to defend themselves against various pathogens (Ferrandon D, 2007). In invertebrates, melanization is one of the important processes of the innate immune system which plays a crucial role to defend them from microbial infections (Hillyer J.F., 2003) (Mavrouli M. D., 2005) (Johansson M. W., 1996). This melanization involves rapidly synthesizing and depositing a blackish brown coloured pigment called melanin at the site of infection and injury. Melanin and other intermediates of this cascade of melanization prevent the invasion of the microbial pathogens into the body of the host and help in wound healing (Jiravanichpaisal P, 2006) (Nappi AJ, 2005). Phenol-oxidase is a key enzyme in this cascade of melanization (Söderhäll K, 1998) (Cerenius L, 2004). Also, various studies have used PO activity as an immune measure to determine the degree of immunocompetence of an organism (Leicht K, 2013) (Boisseaux P., 2018) (Sauer, 2001) (Schmid-Hempel, 2001) (Shelley A. Adamo, 2001). However, many recent studies have also claimed that there is no correlation between

PO activity and the immunocompetence of an organism (Kasianov NS, 2017) (Adamo, 2004). Hence, I have decided to perform a PO activity assay on the third instar larvae of MCU and MB populations to find out if there is any effect on the PO activity level due to adaptation to larval crowding and if there exists any kind of correlation between the PO activity and adult immunocompetence against the gram-positive bacterium *Enterococcus faecalis*

2. MATERIALS AND METHOD

2.1 Fly population and stock maintenance (Vinesh Shenoi N, thesis, IISER Mohali 2016)

I have carried out this study on eight laboratory populations of *Drosophila melanogaster*, among them four are selected for adaptation to larval crowding (MCU 1-4: **M**elanogaster **C**rowded as larvae, **U**ncrowded as adults) and four are control populations (MB 1-4: **M**elanogaster **B**aseline). These MCU and MB populations were first derived in the laboratory of Prof. Amitabh Joshi at Evolutionary and Organismal Biology Unit at Jawaharlal Nehru Centre for Advanced Scientific Research in Bangalore. The MB 1-4 populations were derived from JB populations which are laboratory populations of *Drosophila melanogaster* (Sheeba, 1998). The four replicates of the JB population (JB 1-4) have been maintained under well-defined laboratory conditions which are 60-80 eggs/vial density, 25^{o} C temperature, 90% relative humidity (RH), constant light, standard banana-jaggery food (6-8 ml food/vial). All the replicates of JB population have been maintained on a 21 day discrete generation cycle. These four replicates JB(1-4) were then mixed together to form a single population and named as **M**elanogaster **B**aseline (MB) population. After maintaining this population for 10 generations it was spilt into four populations to form four replicate of MB population (MB 1, MB 2, MB 3, MB 4).

2.1.1 Maintenance regime of control populations (MB 1-4 populations)

The four replicates of MB population (MB 1-4) are maintained on standard cornmeal-charcoal food on a 21 day discrete cycle. In each generation, egg collection is done from 12-day old females. These eggs are then transferred to into glass vials (25 mm diameter x 90 mm height) containing 6-8 ml of cornmeal-charcoal food at a density of 60-80 eggs/vial. For each of the replicate 40 such vials are collected. Then all the vials are incubated at 25^{o}

C temperature, 90% RH and constant light. The flies start eclosing on 9th day post-egg collection and till 12th day post-egg collection almost all the flies are eclosed with peak eclosion on 10th day post-egg collection. On 12th day post-egg collection the eclosed adult flies are transferred into a Plexiglas cage (24 cm × 19 cm × 14 cm) containing a petri plate of cornmeal-charcoal food and a piece of wet absorbent cotton for maintaining high RH levels. The adult population size in each cage is approximately 2500 for each replicate. On every alternate day, fresh food plates are provided in the cages. On 18th day post-egg collection, in each cage a fresh food plate supplemented with *ad libitum* live yeast paste is provided. After 48h (on 20th day post-egg collection) the flies in each cage is provided with a fresh food plate and allowed to lay eggs for 18h. Finally, these eggs are collected to start the next generation.

2.1.2 Maintenance regime of selected populations (MCU 1-4 populations)

The MCU populations were derived from the MB populations, after maintaining them for 15 generations under standard laboratory conditions (mentioned above). From each of the replicate of MB population a MCU population has been derived (MCU 1 is derived from MB 1 and so on). Since then each replicate of MCU population is maintained as separate population (Shenoi V N, 2016). Each MCU replicate connected to the MB replicate by the same replicate number are the direct descendants and that is why more closely related to each other. Hence they are treated as statistical blocks in analyses.

Similar to the MB populations, MCU populations are also maintained on a 21 day discrete generation cycle, 25^{o} C temperature, 90% RH and constant light. In MCU populations eggs are kept in glass vials (25 mm diameter x 90 mm height) containing 1.5 ml of cornmeal-charcoal food at a density of 800 eggs/vial. For each of the replicate 24 such vials are collected and then incubated in standard laboratory conditions as mentioned above. Adult flies start eclosing from 8th day post-egg collection and they keep eclosing till 18th day post-egg collection. Compared to the MB populations the eclosion pattern of the MCU populations is spread out. Due to the low amount of food and very high density pre-adult mortality is very high in this regime, only 100-120 adults eclose in each vial (out of 800 eggs). Hence the population size of each replicate of MCU population is approximately 2500. To avoid the condition of adult crowding in the vials, on a daily basis the eclosed

adults in the vials are transferred into a Plexiglas cage (24 cm × 19 cm × 14 cm) containing a petri plate of cornmeal-charcoal food and a piece of wet absorbent cotton for maintaining high RH levels starting from 8th day post-egg collection till 18th day post-egg collection. On every alternate day, fresh food plates are provided in the cages. On 18th day post-egg collection, in each cage a fresh food plate supplemented with *ad libitum* live yeast paste is provided. After 48h (on 20th day post-egg collection) the flies in each cage is provided with a fresh food plate and allowed to lay eggs for 18h. Finally, these eggs are collected to start the next generation.

This following schematic will show the lineage of MB and MCU populations clearly.

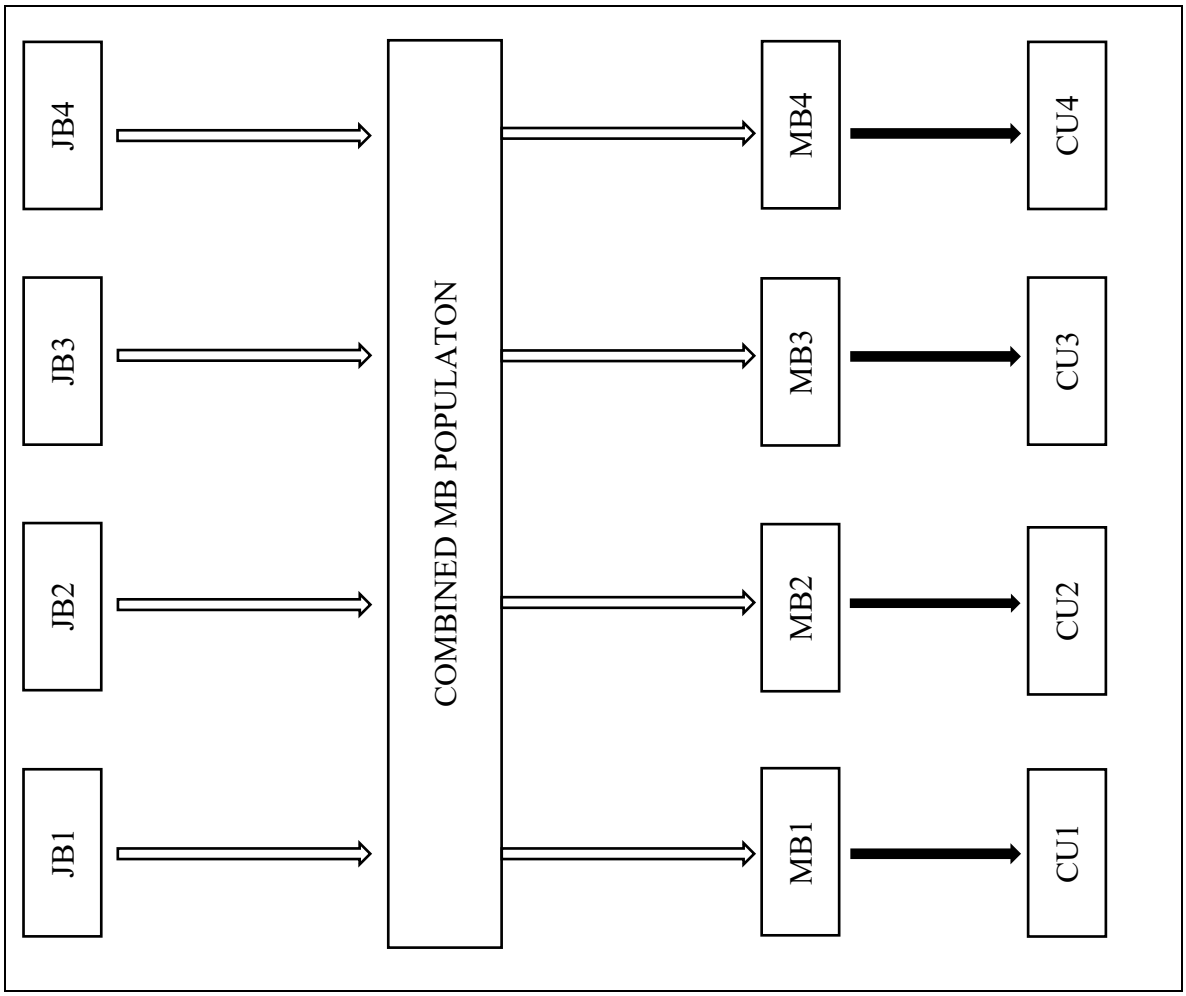


Fig 2: Lineage of MB and MCU population

2.2 Experimental populations

Both the MCU and MB populations are passed through one generation of common rearing regime. This process is called *'standardization'* and the flies generated by this process is called *'standardized flies'*. Standardization is done to account for potential non-genetic parental effects (Rose, 1984). For the process of standardization eggs are collected from stock population in fly culture bottles containing 50-60 ml cornmeal-charcoal food at a density of 300 eggs/bottle and incubated under standard laboratory conditions as mentioned above. For each replicate of MCU and MB populations 4 bottles are collected, hence each replicate of each population has a population size of 1200 flies. Like the MB populations, on 12th day post-egg collection these standardized flies are also transferred into a into a Plexiglas cage (24 cm × 19 cm × 14 cm) containing a petri plate of cornmeal-charcoal food and a piece of wet absorbent cotton for maintaining high RH levels. On every alternate day, fresh food plates are provided in the cages. For experiments eggs are collected from this standardized flies on or before 21st day post-egg collection (meaning egg collection should not be done from the standardized flies aged more than 12 days as adults). At the time of doing this study the MCU populations have been subjected to more than 250 generations of selection.

Before egg collection for the experiments the standard MCU and MB flies kept in the cages are provided with a food plate supplemented with *ad libitum* live yeast for 48 hours. Then a fresh plate is introduced into the cages and the standardized flies are allowed to lay eggs for a duration of 6h. Eggs laid during this time period is collected for experiments. Both larval crowding and adaptation to larval crowding affect the developmental time of *Drosophila melanogaster* populations (by increasing the variance and decreasing the mean) (Botella LM, 1985). Hence, egg collection for diffrernent populations and different treatments is done on different days to ensure that the age of the flies as adults is comparable on the doy of the experiment. For the experiments, each replicate of the MCU and MB populations has two treatments:

1. The high density (HD) treatment which has a density of 600 eggs per vial containing 2 ml of cornmeal-charcoal food
2. The low density treatment (LD) which has a density of 70 eggs per vial containing 6 ml of cornmeal-charcoal food

Maintenance of these experimental flies is same as stock flies i.e. high density (HD) flies are transferred into cages daily with ample amount of food after their eclosion within the vials and the low density (LD) flies are transferred into the cages on 12th day post-egg collection. Experiments for each of the blocks (replicates) were performed on separate days.

2.3 Experimental procedure

2.3.1 Infection experiment

2.3.1.1 Bacteria preparation

I have used a gram positive bacteria *Enterococcus faecalis* for the infection. This bacteria has been reported to be extracted from *Drosophila melanogaster* and this bacteria is considered as natural pathogen for fruit flies (Chapman JR, 2020). A day before infections, the primary culture was prepared by taking a small amount of bacterial inoculum from a cryovial into 10 mL of LB medium. This primary culture was incubated at 27^{o} C temperature at 150 rpm overnight. Then, a secondary culture was prepared by inoculating 10 mL of LB medium with 100 µl of primary culture. After that the secondary culture was also incubated under similar conditions as the primary culture. Then approximately 4h later the secondary culture was pelleted down and resuspended in 10 mM MgSO4 to get a bacterial suspension of OD_{600} =0.8 ± 0.1. This bacterial suspension was then used to infect the experimental flies.

2.3.1.2 Infection protocol

On the day of experiment, four days old (as adults) experimental flies were aspirated (collected) out of their cages from each combination of selection regime and treatment (MCU-HD, MCU-LD, MB-HD, MB-LD) and kept into sex separated vials. From each combination of selection regime and treatment 50 males and 50 females were collected for infection and 50 males and 50 females were collected for doing sham (injury control).

The experimental flies were infected in the lateral side of the thorax with the help of a fine needle (minutein pins, 0.1mm, *Fine Science Tools)* dipped into the prepared bacterial suspension (OD_{600} = 0.8 ± 0.1) (Vodovar N, 2005). The needle was dipped into the

bacterial suspension before infecting each fly. Sham infections were done as injury control by dipping the needle into 10 mM MgSO4 solution instead of the bacterial suspension before pricking each fly.

2.3.1.3 Observation

50 experimental flies per sex per selection regime per treatment were infected by *Enterococcus faecalis* and the same number of experimental flies were pricked by a needle dipped into 10mM MgSO4 solution (sham infection). Both the infected and sham flies were transferred into Plexiglas cages (16 cm x 13.5 cm x 13 cm) containing a cornmeal-charcoal food plate and a piece of wet absorbent cotton for maintaining high RH levels according to sexes (male or female), selection regime (MCU or MB), treatment (HD or LD) and type of infection (infected or sham). On every alternate day, a fresh cornmeal-charcoal plate was provided in each cage. For the next 96 h post infection mortality readings were taken in every one hour interval.

2.3.2 Phenol-oxidase activity experiment

I have collected hemolymph from wandering stage larvae (late 3rd instar). As it has been reported that maximum phenol-oxidase activity is achieved in late 3rd instar larval stage among the different developmental stages of *Drosophila melanogaster* (Mitchell, 1966). For collecting hemolymph I took out wandering stage larvae and cleaned them properly then put them into 1x PBS (Phosphate buffer saline) placed on a glass slide and kept the slide under a stereo microscope. A hemolymph extraction set up was made by taking a 2 ml/1.5 ml microcentrifuge tube and a 0.5 ml microcentrifuge tube and making a slit using a sharp blade under the 0.5 ml microcentrifuge tube then placing it into the 2 ml/1.5 ml microcentrifuge tube. With the help of a dissection needle I pricked the larvae near their mouth hook and ruptured the cuticle of the larvae in a subtle way so that no fat body came out of the larvae. Then pricked larvae were placed (7-8 larvae for LD treatment and 15-17 larvae for HD treatment) into the 0.5 ml microcentrifuge tube and the 2 ml/1.5 ml microcentrifuge tube along with the 0.5 ml microcentrifuge tube (kept inside the 2 ml/1.5 ml microcentrifuge tube) was centrifuged for 10-15 sec using a table top centrifuging machine. After centrifuging I collected the hemolymph (2 µl for each sample) accumulated in the 2 ml/1.5 ml microcentrifuge tube by using a micropipette and kept the collected hemolymph into ice to prevent it from coagulating.

PO activity was measured using Spectramax M2 plate reader. For recording the PO enzymatic activity, L-DOPA (Cat No. 333786, Sigma Aldrich) was used as a substrate and absorbance data was recorded. In each well of a 96 well flat bottom plate, 50 µl of hemolymph solution (25 times diluted in 1x PBS), 50 µl 1x PBS and 50 µl L-DOPA solution (3mg/ml of 1x PBS) were added and a blank (L-DOPA control) reaction was also set in one of the wells by putting 100 µl 1x PBS and 50 µl L-DOPA solution. For each treatment 5 hemolymph samples were loaded. Phenol-oxidase enzyme converts colorless L-DOPA into orange dopachrome and absorbance of this dopachrome produced in each sample was measured at 492 nm wavelength against the L-DOPA control. The absorbance readings were taken at 2 min interval for 2h at 25^0 C temperature (Kenneth Wilson, 2001)

3. RESULTS

3.1 Statistical analysis

For the survivorship analysis I have used Cox's Proportion hazard in R (Version 3.6.2). The flies that were alive by the end of the observation period were censored. A Cox model using the "Coxme" (Therneau, 2020) package of R was constructed. In this model, i) I have used survivorship post infection as a dependent factor; ii) selection regime and density treatment as independent factors and iii) block as a random factor. In the above model, I have analysed the sexes separately. On the result of the model, analysis of the deviance was done to determine to determine the factors that are affecting the survivorship post infection significantly. I have plotted the survivorship curves using the Kaplan-Meier method and using the 'Survival' and "ggusurvplot" (Terry M Therneau, 2020) packages in R. In the $MgSO_4$ pricked control treatments were not included in the analysis as there were negligible mortality.

For analysing the PO activity, I have compared the mean absorbance values of the treatments. I have plotted the data on excel and done an analysis of variance (ANOVA). I did not have sufficient data because I have only run one block out of four and in that block also I was unable to get data for one of the treatments due to some error. So I did the analysis for this partial data only.

3.2 Survivorship results

A sex specific effect of larval density was observed, males from the HD treatment survived longer and suffered less mortality than males from LD treatment (Table 1). However, no effect of selection history was observed in both males and females on survivorship against infection by *Enterococcus faecalis* (Table 2). These results were consistent across all the

four blocks having no effect of selection or larval density treatment in survivorship against infection by *Enterococcus faecalis.*

Treatment	Sample size	Number of deaths	Median death time	0.95 LCL	0.95 UCL
High density (HD)	400	202	54.5	35	NA
Low density (LD)	400	212	41	33	NA

Table 1: Showing total events (death), median death time for both selected and control populations in males against *Enterococcus faecalis*.

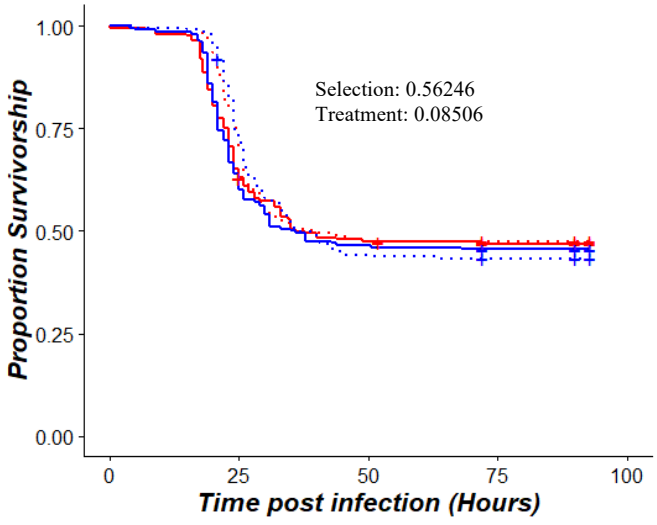
Selection	Treatment	Sample size	Number of deaths	Median death time	0.95 LCL	0.95 UCL
MB Males	HD	200	104	46.5	35	NA
	LD	200	102	50.5	29	NA
MCU Males	HD	200	98	NA	30	NA
	LD	200	110	35	30	NA
MB Females	HD	200	106	36	32	NA
	LD	200	105	39.5	30	NA
MCU Females	HD	200	109	35.5	29	NA
	LD	200	113	37	33	NA

Table 2: Showing total events (death), median death time for both selected and control populations in males and females against *Enterococcus faecalis*. HD is high density and LD is low density

Male	**loglik**	**Chisq**	**df**	**Pr(>|Chi|)**
NULL	-2635.1			
Treatment	-2628.9	12.4704	1	**0.000413**
Selection	-2628.9	0.0012	1	0.972566
Treatment x Selection	-2628.7	0.3182	1	0.572664
Female				
NULL	-2746.8			
Treatment	-2745.4	2.9655	1	0.08506
Selection	-2745.2	0.3355	1	0.56246
Treatment x Selection	-2745.2	0.0006	1	0.98024

Table 3: Summary table of Cox proportional hazards analysis showing effect of Selection, Treatment, and their interaction against *Enterococcus faecalis.* Significant terms are marked in bold.

a.



b.

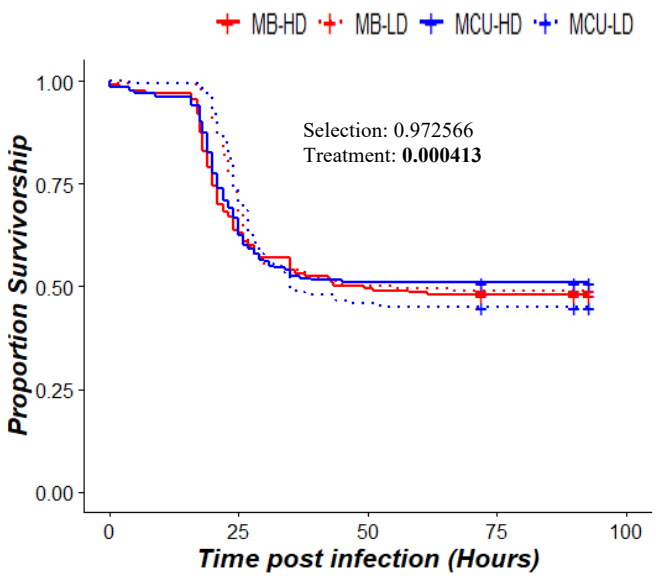


Figure 3 : Effect of selection and treatment on post infection survivorship of adults against *Enterococcus faecalis* (a) Females (b) Males. Dotted lines represent LD treatments and solid lines represents HD treatments. Red lines are survivorship curves for MBs whereas Blue lines are survivorship curves for MCUs

3.3 **PO activity results**

I have managed to run one out of the four blocks and I did not get data for MBLD treatment due to some error but I have compared the mean absorbance values of the other three treatments. MCU HD and MB HD were compared to determine selection effect (Figure 4) and MCU HD and MCU LD were compared to determine density treatment effect (Figure 5). A effect of selection history was observed, however there was no treatment density effect on PO activity.

Groups	Count	Sum	Average	Variance
MCU HD	5	0.591	0.1182	0.0033727
MB HD	4	0.144	0.036	0.00100867

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0150152	1	0.015015	6.363605	0.039658	5.591447
Within Groups	0.0165168	7	0.002359			

Table 4: ANOVA table of comparison between MCU HD and MB HD

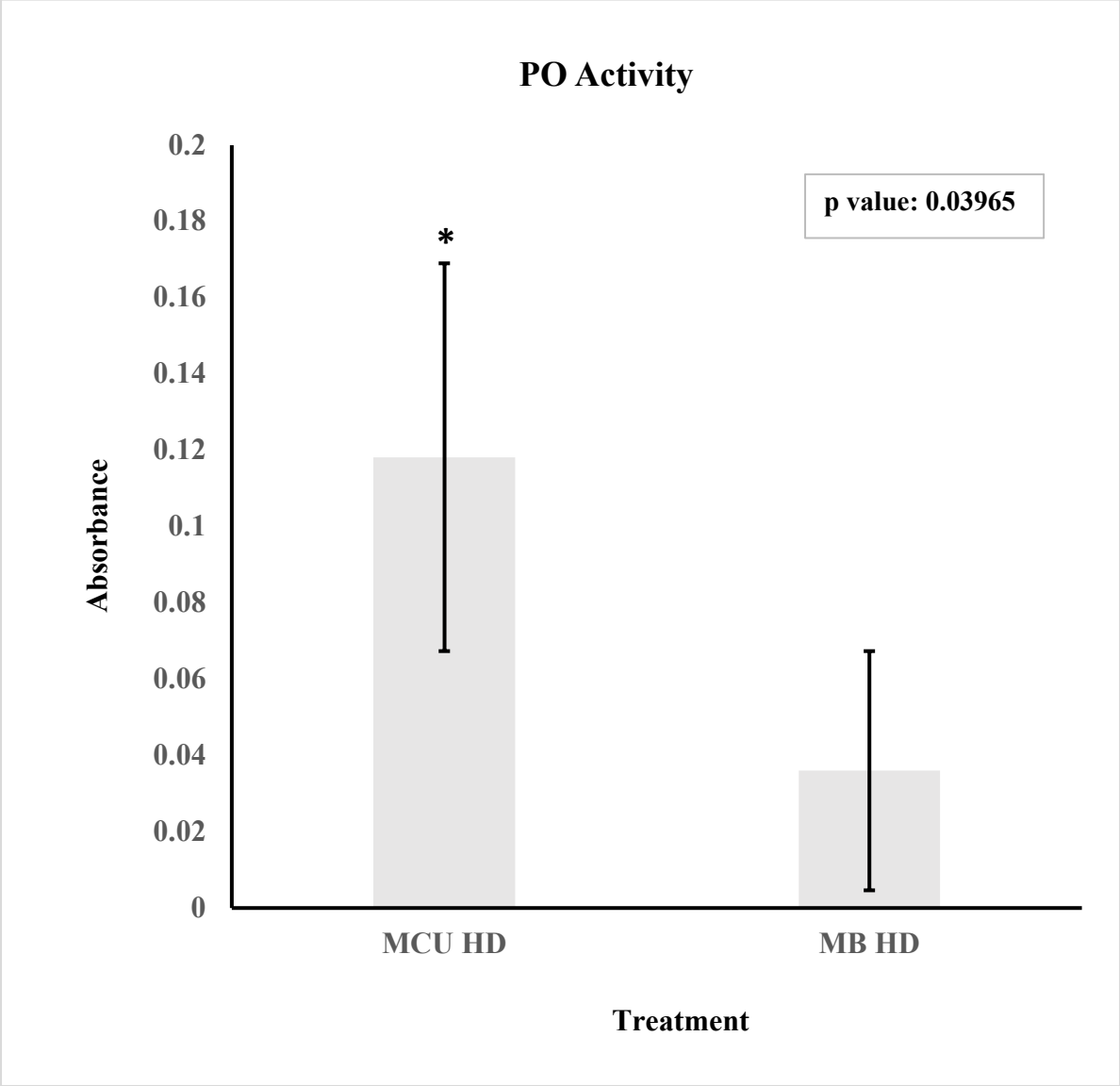


Figure 4: Comparison of mean absorbance values between MCU HD and MB HD

Groups	Count	Sum	Average	Variance
MCU LD	4	0.305	0.07625	0.00352292
MCU HD	5	0.591	0.1182	0.0033727

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.00391067	1	0.00391067	1.137789	0.321521	5.59144
Within Groups	0.02405955	7	0.00343708			

Table 5: ANOVA table of comparison between MCU HD and MCU LD

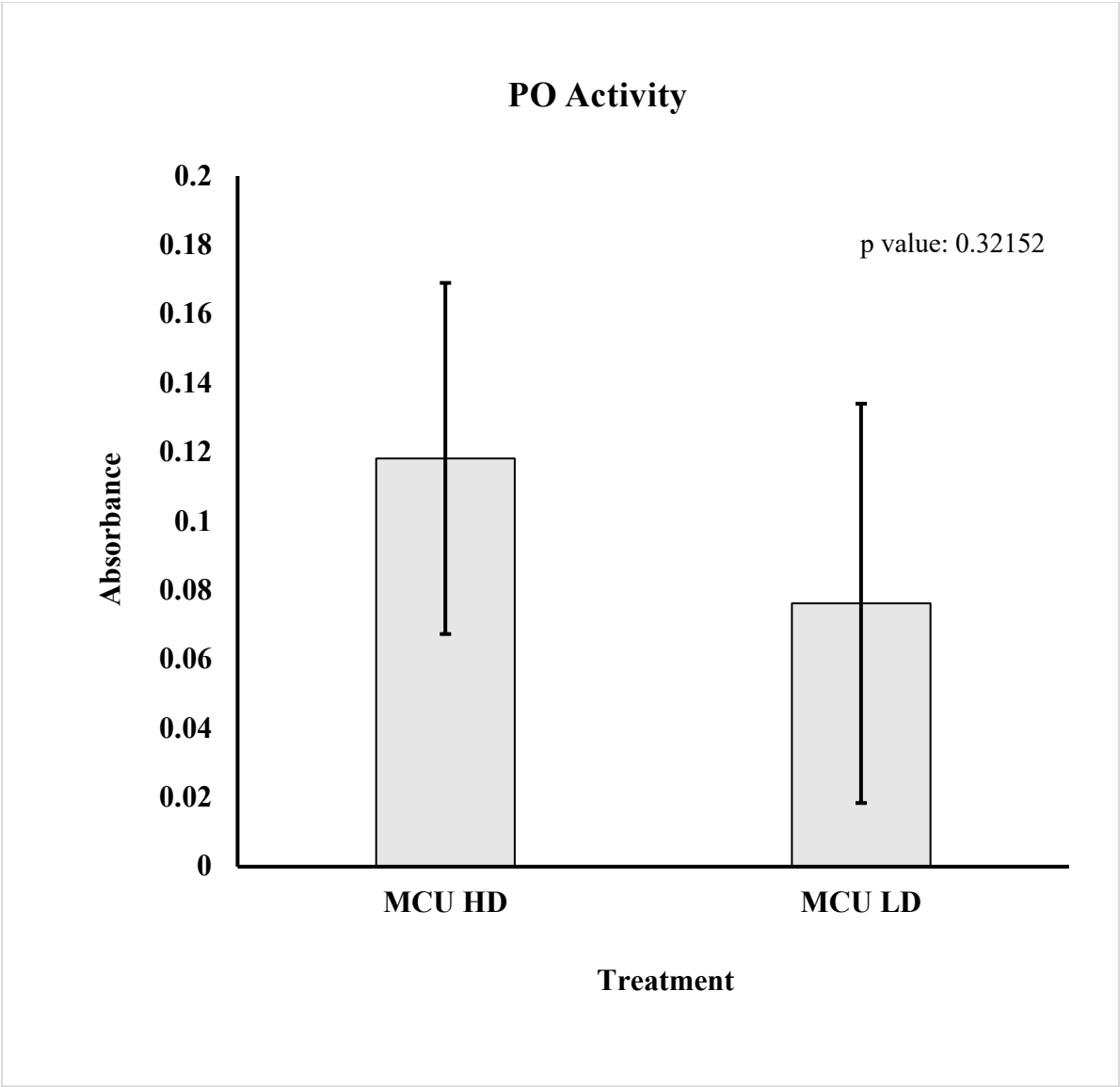


Figure 5: Comparison of mean absorbance values between MCU HD and MCU LD

4. DISCUSSION

4.1 Infection experiment

In this study I tried to answer the following questions:

1) Do the resources available to an organism during developmental stages affect its ability to survive infection in the adult stage ?
2) Does adaptation to poor developmental environment (larval crowding) result in the evolution of immunocompetence in the adult stage ?

Various studies have suggested that poor nutritional conditions during larval stages result in poor adult immune response due to a lack of resources and poor body conditions (Campero M, 2008) (Lee K.P, 2006) (Feder D, 1997) (Muturi EJ, 2011) (De Block M, 2008). In case of holometabolous insects, poor nutritional conditions during larval stages leads to reduced adult body size, which remains the same throughout their life once the adult stage is achieved (Miller RS, 1958) (Santos M, 1994) (Ireland S, 2006). The high larval density (HD) treatments used in this experiment leads to significantly smaller adult body size than low larval density (LD) treatments in these populations (Shenoi VN B. S., 2016). Although, same concentration of bacteria was used for infecting flies from both the treatments high density (HD) and low density (LD) but due to the smaller body size of the flies from high density (HD) treatments than the body size of the flies from low density (LD) treatments, the flies from high density (HD) treatments received a higher dose of bacteria per unit body mass than flies from low density (LD) treatments. Results from this study shows that in spite of receiving a higher dose of bacteria per unit body mass, the male flies from high density (HD) treatments survived longer and suffered less mortality than male flies from low density (LD) treatments and the female flies from high density (HD) treatments survived at par with the female flies. Hence, it can be concluded that the

developmental environment in the larval stages did not affect the post infection survivorship in the adult stage when infected by *Enterococcus faecalis*.

To find out the answer of the second question regarding the evolutionary effect of adaptation to the poor nutritional environment during larval stages on adult survivorship post infection, I looked at the survivorship of flies that has been adapted to larval crowding for more than 250 generations and their control populations. The results from this study suggest that no effect of selection history was present in both males and females on survivorship against infection by *Enterococcus faecalis.*

4.2 Phenol-oxidase activity

As I managed to do this experiment for only one of the blocks and also in that block due to some error I was not able to get the data for one of the treatments which is MB LD, it is not possible to arrive at a firm conclusion without the complete data. However, from the available data a significant difference in PO activity between the treatments MCU HD and MB HD was observed which suggests the presence of an effect of selection history on the PO activity. But there was no significant difference in PO activity between the treatments MCU HD and MCU LD which suggests that the developmental environment in the larval stages did not affect the PO activity in case of the population adapted to larval crowding. Also, if this trend would remain consistent across all the four blocks then it can be concluded that PO activity and adult immunocompetence have no correlation when immunocompetence is measured in terms of survivorship against infection by *Enterococcus faecalis.*

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