Secret of their immunity:

Role of hemocytes in evolved immunity in *Drosophila melanogaster*

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MS13043

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

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Dedicated to my parents and brother

Certificate of examination

This is to certify that the dissertation titled "***Secret of their immunity*: Role of hemocytes in evolved immunity in *Drosophila melanogaster***" submitted by **Ms. Vaibhvi** (Reg. No. MS13043) 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 | Dr. Rhitoban Ray Choudhury | Dr.N.G.Prasad (Supervisor)

Dated: April 20, 2018

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.

Vaibhvi

(Candidate)

Dated: April 20, 2018

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. N. G. Prasad

(Supervisor)

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Contents

Abstract

Immune responses can be majorly classified as innate and adaptive. Invertebrates lack a proper adaptive immune response but have an elaborate innate immune system. Innate immunity can be further of two types - cellular and humoral. The humoral innate immune response is mediated through antimicrobial peptides specific for a class of pathogens, reactive oxygen species etc. while the cellular innate immune system comprises of the three types of hemocytes: plasmatocytes, crystal cells and lamellocytes.

I aim to inspect the differences in the hemocyte density in *Drosophila melanogaster* laboratory populations selected for a better immune response against systemic infection by gram negative bacteria *Pseudomonas entomophila*. In *Drosophila melanogaster*, hematopoiesis occurs in four stages: embryonic, larval, lymph gland and adult stage. The adult hemocytes are a mixture of all these four origin. I compared the hemocyte density in the wandering stage of the third instar larvae of selected and control populations in four separately maintained replicates. Results obtained indicate no differences in the number of hemocytes between the selected and control populations in three replicates while in one, the hemocyte density is higher in the control population. This could point towards the possibility that either the selected populations employ some pathway other than cellular immunity to increase their immune response or the hemocyte density increases in the adult stage when the selection pressure is applied. A protocol for adult hemocytes extraction was also standardized.

Chapter 1: Introduction

Immune system is responsible for protecting a multicellular organism from pathogenic attacks and is broadly classified as adaptive and innate immunity. The innate immune system works by the germline-encoded cascades to identify and get rid of foreign substances while the adaptive or acquired immune system is characterised by the B and T cells with antigen specific receptors generated by somatic gene rearrangement and the ability to have an immunological memory (Fearon,1997).

Most of the pioneer studies on immunology were focused on the adaptive immune system. The realisation that innate immune system serves as more than an evolutionary relic and interacts with adaptive immune system for immune response shifted the focus towards innate immunity in the 1990s. (Vilmoz, Kurucz,1998). A proper adaptive immune response is limited to Vertebrates, while other animals and plants rely solely on the innate immune system. However, recent studies indicate the presence of adaptive-like immune response (priming) in lower organisms like *Drosophila melanogaster* (Pham et al, 2007).

Drosophila serves as a great model for studying immune system because of the conservation of signaling molecules and pathways between insects and mammals (Kornberg and Krasnow, 2000). The well established techniques for genetic manipulations in *Drosophila*, which can be further employed in other higher organisms, make it a more compatible system (Rubin and Lewis, 2000).

The innate immune system of *Drosophila* is further classified as humoral and cellular. The epithelia serves as the first line of defence, which when ruptured, exposes the pathogens to hemocoel and internal organs. The humoral response is mediated through antimicrobial peptides (AMPs), reactive oxygen or nitrogen species, enzymatic cascades etc. and shows specificity to a class of pathogens. These AMPs are released in the fat bodies or the epithelia of systems like respiratory tract, reproductive tract, etc.. Some specific AMPs released in response to a class of target pathogens include Defensin and Metchnikowin (gram-positive bacteria), Diptericin, Attacin and Drosocin(gram-negative bacteria) and Drosomycin and Metchnikowin (fungi). There are specific immune pathways which are involved in the recognition of particular pathogen classes and release of these AMPs, with the two main pathways being: *Imd* for gram negative bacteria and *Toll* pathway for gram positive bacteria, fungi and viruses (Lemaitre and Hoffman 2007, Schneider and Chambers 2008).

The cellular immune response on the other hand lacks specificity and is mediated through following three types of blood cells or hemocytes (Parsons and Foley, 2016; Banerjee et al 2003):

1. **Plasmatocytes**: These make up to 90-95% of the total hemocyte populations and range from 8-10µm in diameter. They are circular in shape and extend pseudopodia. These are analogous to vertebrate macrophages in function and phagocytose the foreign particles, pathogens or apoptotic debris. Plasmatocytes are long lived and survive through the entire lifespan of *Drosophila*.
2. **Crystal cells**: These are the second most abundant blood cells in *Drosophila*. They are bigger than plasmatocytes, ranging from 10-12µm in diameter. Their name comes from the presence of crystalline structure inside the cells which are responsible for releasing prophenoloxidase enzyme. This enzyme catalyses the process of melanin deposit on the injury site.
3. **Lamellocytes**: These are the largest blood cells with size in the range 15-40 µm and flat shape. Their function is to encapsulate the foreign particles that are too big to be phagocytosed. Lamellocytes are only found during the larval stages and in very small numbers. Their count increases in case of parasitic challenges or an unhealthy host.

Hemocytes function separately, but are also involved with the humoral immune system and mediate the process of AMP release.

Based on their mobility, hemocytes are further classsified as circulating or sessile populations. The process of hematopoiesis in *Drosophila* is reported to occur in three waves: embryonic, larval and lymph gland hematopoiesis (Bruckner and Gold 2014). Embryonic hematopoiesis occurs in the head mesoderm region of the early embryo stage and largely completes by 7 hours post-fertilisation. Here the progenitor cells differentiate into two main cell types i.e. plasmatocytes and crystal cells. The second wave starts in the larval development stage and results in the rapid expansion of cell number from 200 in first instar stage to nearly 5000 in the third instar stage (Lanot et al 2001). Lineage tracking shows that these new hemocytes are formed from the proliferation of already differentiated hemocytes. The third wave of hematopoiesis occurs during late larval stage in a dedicated hematopoietic organ the lymph gland. These cells proliferate and differentiate in the gland during the larval stages and only in case of immune challenge are released in the circulation in the larval stage itself. On the onset of metamorphosis these hemocytes are released into circulation. Until recently it was thought that there is no hematopoiesis in adult flies, but recent studies indicate the presence of hematopoietic hubs in the adult flies as well (Ghosh et al 2015). This concludes that the hemocytes in adult flies are a mixture of

those derived from embryonic, larval, lymph gland and some from adult hematopoietic origin (Parsons et al 2016, Bruckner and Gold 2014, Lanot et al 2001, Banerjee et al 2003).

In this project we aim to explore the cellular innate immunity of laboratory adapted phylogenies of *Drosophila melanogaster*. These populations have been selected for increased immunity (in terms of survivorship) in response to systemic infection by gram negative bacteria *Pseudomonas entomophila*. Earlier studies have looked at expression of genes involved in *toll* and *imd* pathways in these populations but there is no difference in the expression as compared to the control populations (Gupta 2015). Comparing the metabolome of the selected flies to the control indicate the presence of higher amount of metabolites including AMPs even in the absence of infection, indicating a basal level increase of the metabolites in these populations (Gogna et al 2017).

This study aims to look at the basal level differences in hemocyte count in selected vs control populations. The amount of hemolymph in adults drops drastically as compared to the larval stages and most of the hemocytes are sessile rather than circulating. The extraction of hemolymph from adults is very difficult. Most of the protocols reported can extract very minimal amount of hemolymph ranging from 10-25 nL (Piyankarage et al 2012, Broughton et al 2004, Folk et al 2001). The highest amount of hemolymph extracted from one fly has been 50nL using the protocol reported by Macmillan and Hughson 2014.

On the other hand, one third instar larva can be easily used to extract more than 300nL of hemolymph. As hematopoiesis in the adult stage and lymph gland is less as compared to the embryonic and larval stage and the adult hemolymph is a combination from all the four stages of hematopoiesis, we expect the differences in adult hemocyte number to show in the larval stage and hence I tried to look for basal level differences in the hemocyte count in third instar larvae of selected vs control population.

The extraction of adult hemolymph was tried using several protocols, out of which one protocol worked and was standardized, which is also mentioned in the methods section. Due to time constraint, experiments could not be conducted on adult *Drosophila*.

Chapter 2: Materials and Methods

2.1 Model organism

The following study was conducted on laboratory adapted populations of *Drosophila melanogaster*. It is a holometabolous insect belonging to the order diptera. Its life cycle consists of four discrete stages, namely: egg, larvae, pupae and adults (Fig 1). Diploid fertilized eggs, post-oviposition take 18-24 hours to hatch into first instar larvae. These molt into second and subsequently third instar larvae, with each stage taking around 24 hours. They remain in the third instar stage for 2-3 days, during which they feed for the first 48-60 hours and grow exponentially in size. Then they move out of the food and wander around looking for a dry clean space to pupate. This stage of third instar larvae is called the 'wandering stage'. In lab conditions, where these stages usually happen in glass vials containing food, on entering the wandering stage the larvae move upwards towards the cotton plug and pupate close to the plug. Initially the pupae are transparent or white in color, same as larvae. Gradually they turn darker in shade, from yellowish to the final color before eclosion being dark brown to black. They eclose 2-3 days post pupation and once eclosed they gain sexual maturity in 6-8 hours.

2.2 Ancestral populations

The experimental populations selected for a better immune response are derived from the laboratory adapted ancestral baseline populations BRB (Blue Ridge Baseline). These are large outbred populations established by putting together 19 isofemale lines with 100 males and 100 females from each line. These isofemale lines were set up from flies caught at Blue Ridge, USA and hence the name. After 10 generations in the lab, BRB was divided into five replicates (BRB 1-5). These independent replicates are maintained in a 14-day discrete generation cycle, 12 hours:12 hours Light:Dark cycle at 25° C temperature and 60-70 % Humidity. They sustain on standard banana-jaggery food (Prasad et al. 2003). A generation starts with transferring eggs into cylindrical glass vials (25mm diameter – 90mm height) containing food. These eggs are collected at a density of 70 eggs per vial with 40 vials per replicate. On 12th day post egg collection the eclosed flies are transferred into cuboidal Plexiglass fly cages (25cm length – 20cm width – 15cm height). The number of individuals while transferring to cages are around 2500 per block. On 14th day, after giving a prior oviposition window of 18 hours, eggs are collected for the next generation.

2.3 Experimental populations

The experimental lines are derived from BRB populations and are selected for increased survivorship in response to systemic infection by *Pseudomonas entomophila*. After twenty two generations of maintaining the BRB (1-4) populations in laboratory conditions, the experimental lines were established. Each of the four replicates of the ancestral BRB (1-4) population was used to derive their respective experimental population block (1-4), with each block having three regimes: I, U and S. This implies that the populations in one block share common ancestry and are more closely related to each other than to populations of different blocks.

The three regimes are I (Infected by bacteria), U (Unhandled Control) and S (Sham infected). These populations are maintained in a 16 day discrete generation cycle on banana-jaggery food. They are kept at 12:12 L:D cycle at 25°C temperature and 60-70% humidity (Fig 2).

A generation starts with transferring eggs into glass vials at a density of 70 eggs per vial with 10 such vials per regime. On 12^{th} day post egg collection the selection pressure, as per the population, is applied. 'I' flies are anesthetized using CO_2 and then pricked on their thorax with a tungsten needle dipped in a bacterial suspension prepared in 10mM $MgSO_4$. 15 mating pairs are infected from each of the 10 vials and these 300 flies are then transferred into plexiglass cages. 'S' flies are similarly anesthetized and pricked on their thorax with a needle dipped in 10mM $MgSO_4$. 10 mating pairs are poked from each vial and these 200 individuals are transferred to cage. 'U' flies are anesthetized using CO_2, 10 mating pairs per vial are sorted and these are transferred into a cage. Every alternate day the food in the cages is changed. On 16^{th} day post egg collection, eggs are collected for the next generation from these cages by giving a prior oviposition window of 18 hours. A four day gap between infection and egg collection makes sure that only the flies that survive the bacterial infection can contribute their progenies to the next generation.

2.4 Bacterial populations

Pseduomonas entomophila is a gram negative bacteria, which is a natural pathogen of *Drosophila melanogaster*. Strain used for infections in the selection regime is *Pseudomonas entomophila* L48 (Pe). The night before infections, a primary culture is setup by suspending the bacteria in Luria Bertani Broth and incubating it overnight at 27° C temperature and 150 rpm. In the morning, this culture is diluted 50 fold to setup a secondary subculture which is incubated until it reaches the required OD and is then pelleted down and suspended in 10mM MgSO4 maintaining the same OD. This bacterial slurry is further used for infections.

The OD value is such that the post-infection mortality is 30%. During first few generations the OD was 1, which was then incremented after every few generations as the mortality decreased, with the current value being 2.3.

2.5 Standardization of experimental populations

Non genetic effects owing to the variation in environmental conditions can affect the traits being observed. To account for such effects, identical conditions are provided to all the experimental populations for one generation so that the observed results are solely due to genetic effects. Eggs are collected from the stock cages at a density of 70 eggs per vial and on 12^{th} day post egg collection around 500 individuals are transferred into cages. Experiment is conducted on progenies of these flies.

2.6 Larval hemocyte count

The experiment aims to compare the basal hemocyte count in third instar larval stage of I and S populations. As the immune system can be triggered by small variations in the environment, which could further change the hemocyte count, conditions were kept same as the usual stock maintenance regime.

2.6.1 Experimental conditions

Eggs were collected from the standardized flies of I and S selection regime at a density of 70 eggs per vial (after giving a prior oviposition window of 18 hours). These were kept at 12:12 Light:Dark cycle at 25°C temperature and 60-70% humidity. Hemolymph was collected from third instar larvae in wandering stage. The third instar stage is reached 3 days post oviposition and it takes further 2-3 days to attain wandering stage, after which they pupate within 6-8 hours. The experiment was conducted when approximately 50% of the larvae in a vial had exited the food, which roughly represents the peak development time.

For each block, 16 slides per selection regime were prepared, which were divided into two days. This was achieved by collecting eggs for one block on two consecutive days so that the approximate difference between the development times for the two sets was one day. In order to reduce any variations due to handling, same two handlers prepared slides for all the four blocks and half slides per treatment were made by each. For all blocks, ‘S’ population has a smaller development time than ‘I’ population, hence for each set the slides for ‘S’ population were prepared first.

2.6.2 Hemolymph extraction and measurement

Larvae were taken out of the vial using a brush and were put into 1xPBS in a petriplate. These were washed again in clean PBS to remove any food stuck to their surface. The larvae were then put on a clean slide under a Dissection microscope and using two fine dissection needles the cuticle was carefully pierced such that the hemolymph oozes out without damaging any organs. This hemolymph was collected using a 50nL Drummond microcapillary tube (Sigma-Aldrich) and the amount taken from each larva was measured. Usually this amount was around 25nL-50nL, but if it was less than 5nL, then that sample was discarded and another larva was taken. This hemolymph was released into a 10 µL PBS drop on a one-cavity slide . For each slide hemolymph was extracted from three larvae.

2.6.3 Slide preparation

The hemolymph collected on a slide, diluted with 1xPBS, was kept undisturbed for 30 minutes in a humified chamber to let the cells settle down and adhere to the bottom. After 30 minutes the area in which PBS-hemolymph mixture was spread was marked on the back side of the slide using a permanent marker. PBS was removed by tilting the slide and 4% formaldehyde prepared in 1xPBS was added for fixation. After 10 minutes fixative was removed and 0.3% PBT (tween 20 in 1xPBS) was used to wash the slide twice, 5 minutes each. All the subsequent steps were done in dark. Phalloidin stain (Alexa Flour 488 - Thermo Fisher Scientific) of 150 nM (in 1xPBS) concentration was added to the slide and was kept for an hour long incubation. This was followed by two 0.3%PBT washes, five minutes each. DAPI (Thermo Fisher) of 300 nM concentration (in 1xPBS) was added for counterstaining with a 2-3 minute incubation period. Slide was finally washed with 1xPBS for 2 minutes. PBS was used for mounting and transparent nail paint was used to seal the slide after putting coverslip.

2.6.4 Imaging

Once prepared, the slide was kept at 4°C in dark and was imaged within 3 days. Fluorescence microscope (Zeiss Axio Scope.A1) was used to image the slides. Phalloidin and DAPI stains were visualized in Rhodopsin and DAPI channel respectively. Releasing hemolymph using microcapillary into PBS on the slide, mixes and homogenises the suspension. Hence, each slide was imaged at five fixed coordinates in the marked region of the cavity. Taking (0,0) as the coordinates of the center of the marked area, images were taken at (0,0), (0,2), (0,-2), (2,0), (-2,0) (unit millimeters) at 100x magnification. At the same magnification, micrometer was imaged to measure area covered in one frame of view.

The whole region in which cells dispersed was marked using a permanent marker and was imaged using a ZEISS steni microscope fitted with a AxioCam ICc1 camera. This gave the total area for calculating

the total number of hemocytes in the given volume. Micrometer was imaged here as well to get the area in meters.

2.6.5 Image analysis

New identities were given to the pool of images from all the blocks to perform a blind analysis. All images were analysed separately by two handlers to account for handler effect.

The number of cells in each image were counted manually using ImageJ (National Institutes of Health, http://imagej.nih.gov/ij/). The two images of the same site captured under DAPI and Rhodopsin channel were merged using ImageJ (Fig 3). Cells were identified as almost circular in shape with most of them having visible pseudopodia. Only the ones with both DAPI and Phalloidin were counted. The images taken in light microscope were used to measure the area in which cells were dispersed. Using area measurement function in ImageJ, the area marked by permanent marker was manually marked and measured. This measurement was converted into meters using a micrometer image. The total area covered by fluorescence microscope in one frame was also calculated using micrometer image.

In order to compare the basal number of hemocytes in selected vs control populations, the quantity 'hemocytes per nL' was calculated. The average number of cells in one image taken for one slide was obtained was taking the average of the values obtained for the five images taken for each slide. This number was divided by the area of one frame covered in fluorescence microscope and then multiplied by the area of the whole marked region. This value was then divided by the total amount of hemolymph added on that slide. So, the number of hemocytes per nL of hemolymph, for each slide, was calculated using the following formula:

$$\textit{Number of hemocytes per nL of hemolymph} = \frac{\textit{Average number of cells in one frame}}{\textit{Area of one frame}} \times \frac{\textit{Total area in which cells spread}}{\textit{Volume of hemolymph taken}}$$

2.7 Adult hemocytes extraction

2.7.1 Unsuccessful protocols

Due to very less amount of hemolymph in the adult flies the extraction becomes very difficult. Following is an account of the protocols which were unsuccessful in extracting hemocytes from adult flies:

1. 80-100 flies were pricked on their thorax using a tungsten needle and were kept in the silica bed spin columns usually used for DNA purification. The tubes were centrifuged at 9000g for 5 minutes at 4°C (Park et al 2014). Hemolymph was successfully obtained in the collection tube but it was cell-free.
2. A small 0.5mL MCT was taken and a very small opening was made at its bottom. This tube was further placed in a 1.5 mL MCT. 80-100 flies were pricked on their thorax, transferred into the upper tube and the tubes were centrifuged. This method gives hemolymph with hemocytes, but there is a lot of debris (Youtube video: Drosophila hemolymph collection procedure).
3. A one-cavity slide was taken and a bubble of 1xPBS and anticoagulant mixture was taken in the cavity. One fly was taken with its wing removed and was transferred into the bubble. It was pricked on the thorax using a needle and left in the solution for some time. Same was repeated for 9 more flies (Modified from Evans et al 2014). Very minimal amount of hemolymph was collected which can't be successfully used for further cell count.
4. A few flies were frozen at -20℃ and then were pricked in the thorax region. These flies were kept in 1xPBS plus anticoagulant solution and thawed by keeping at room temperature. This freeze thaw method was expected to give hemolymph mixed in the buffer solution (Miyazawa and Arakawa, 1997). No cells were observed using this method.

2.7.2 Standardized protocol

Mentioned here is the protocol that successfully gives hemocytes from adult flies. This is adopted from Haselton and Fridell 2011.

A few flies were mildly anesthetized using CO_2 and affixed in a row on a double sided tape on CO_2 station with their dorsal side down. Applying a little pressure using the back of a brush, the wings were stuck to the double sided tape so that the flies don't mobilize. Under a Dissection microscope, the proboscis was gently held using forceps and pulled forward to make the top of the head region visible. Using a tungsten needle in the other hand, the head was punctured just above the ptinial suture. Care was taken not to insert the needle too deep into the head, as it will result in damage to other organs and possible contamination of hemolymph with other fluids. All the flies in a row were first punctured . Then one wing of the flies was removed from the double sided tape and it was positioned laterally. Again, pressure was applied on the thorax region using the back side of a brush, which results in a

bubble of hemolymph oozing out of the punctured portion in head. The drop was collected using a 50nL Drummond microcapillary tube (Thermo Fisher) and was released into 10 µL 1xPBS drop taken on a one-cavity slide. Same was repeated for 10 flies per slide. For the time of collecting hemolymph for one slide, the slide was kept on an ice pack. Once hemolymph was collected, same steps for fixation and staining were repeated as mentioned before.

Chapter 3: Results

In order to compare the difference in the density of hemocytes between the selected and control populations, ANOVA was done using JMP 7.0.1. The data of hemocyte density was first normalized by logarithmically transforming. The data obtained for the four independent replicate populations was analyzed separately in order to not lose the inter-block variations. For each block the experiment was conducted separately on two days. This 'day' component was kept as a 'random factor' for analysis. Figure 4 shows the difference in hemocyte density between the two populations for the four blocks. The graph shows the actual values of hemocyte density. Table 1 shows the p value of differences between I and S population.

Block 1, 2 and 4 show no significant difference in the hemocyte density between I and S populations ($p > 0.05$). While Block 3 shows a significant difference in the density ($p < 0.05$) with S having almost double the density as I.

Chapter 4: Figures and Tables

Figure 1: Life cycle of *Drosophila melanogaster*

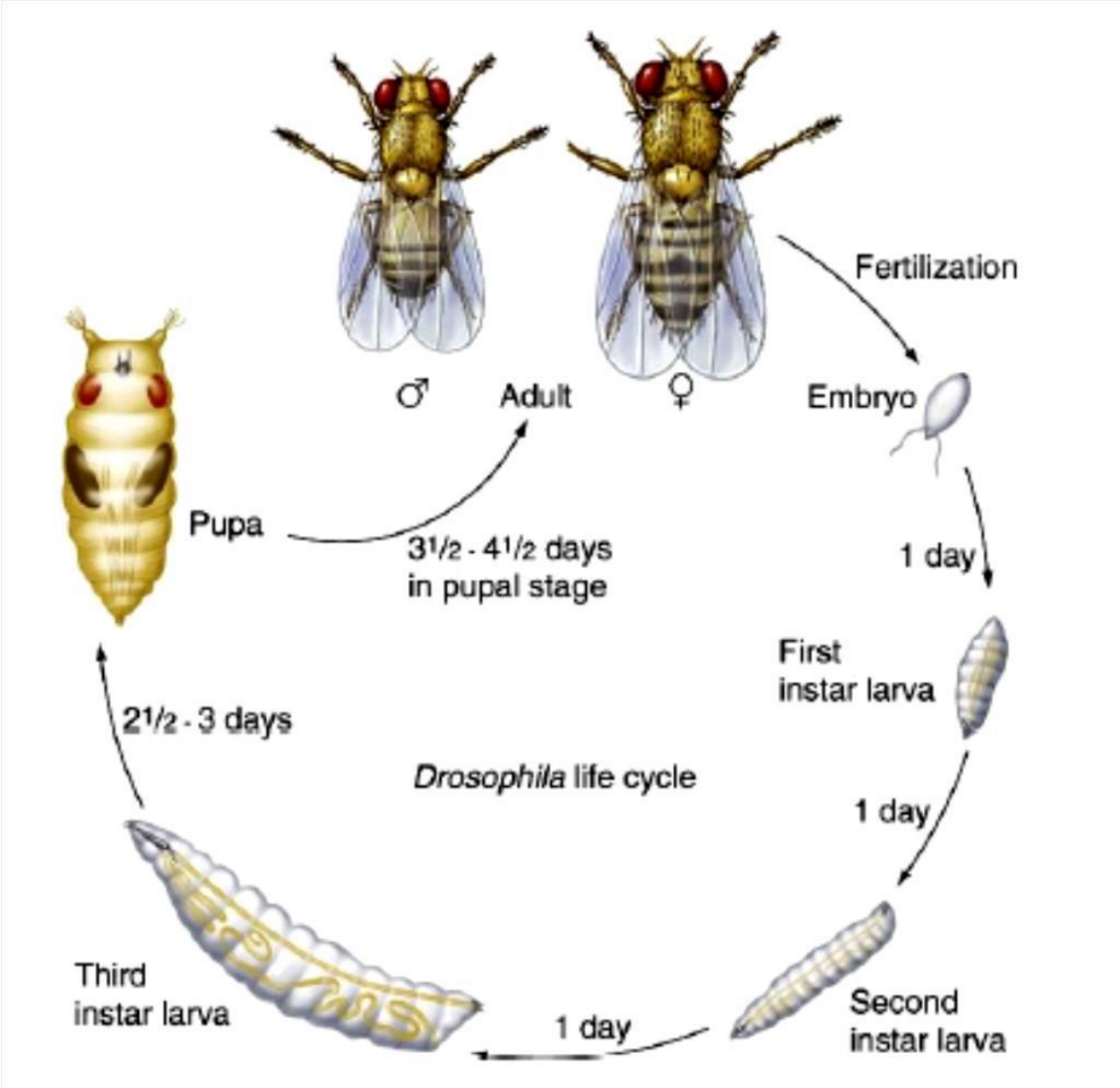

Figure 2: Flowchart showing maintenance regime of Experimental populations (Taken from Gupta, 2015)

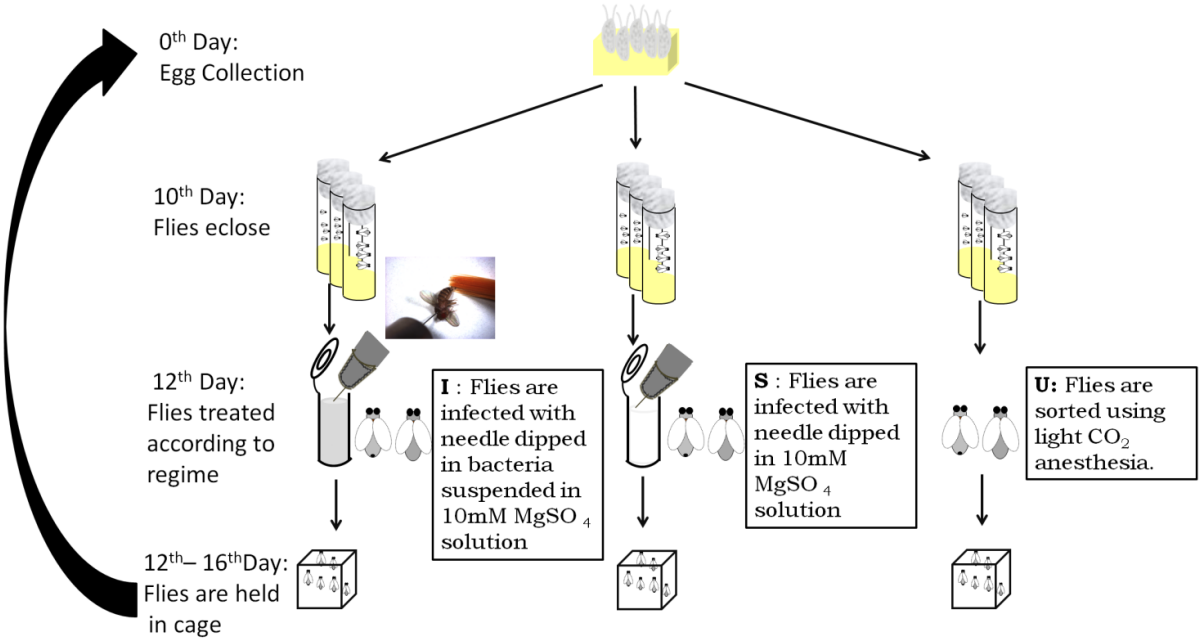

Figure 3: Examples of images taken for analysis: Image taken under fluorescence microscope at 100x magnification, showing hemocytes with their membranes stained by Phalloidin (yellow) and nuclei stained by DAPI (magenta).

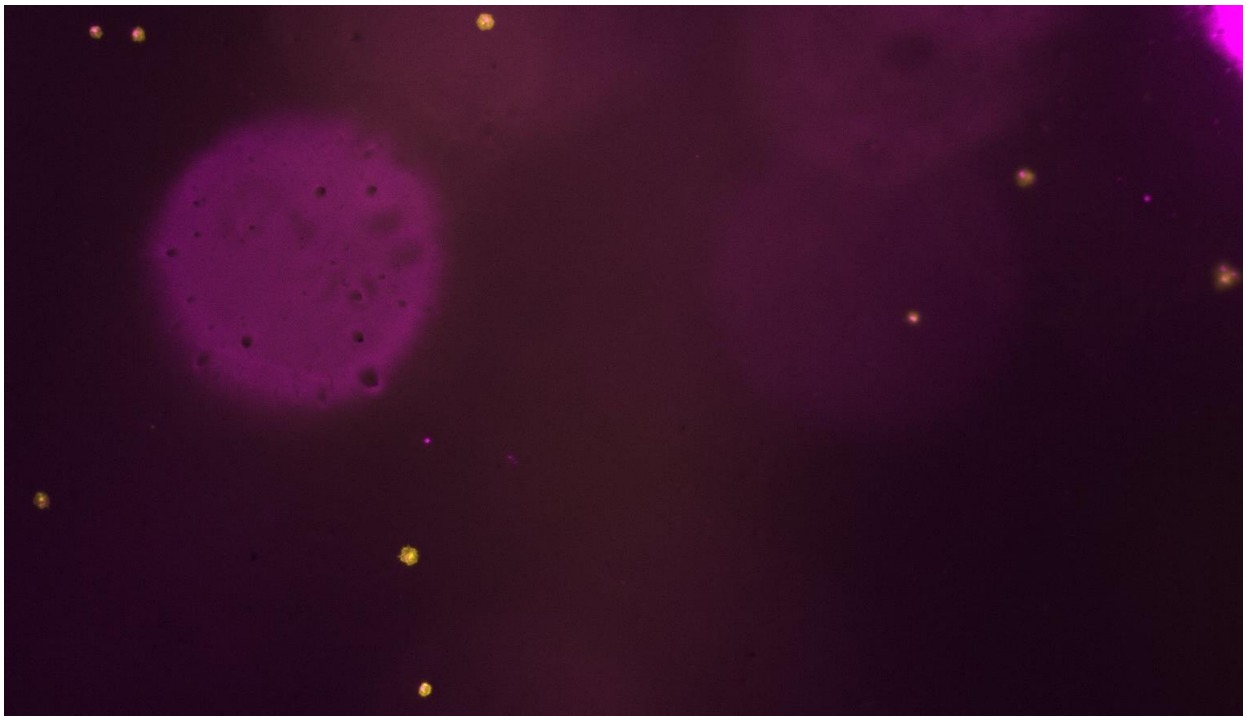

Figure 4: Graphs showing the differences in hemocyte density between selected (I) and control (S) populations for four replicates, analyzed separately: (a): Block 1, (b): Block 2, (c): Block 3 and (d): Block 4

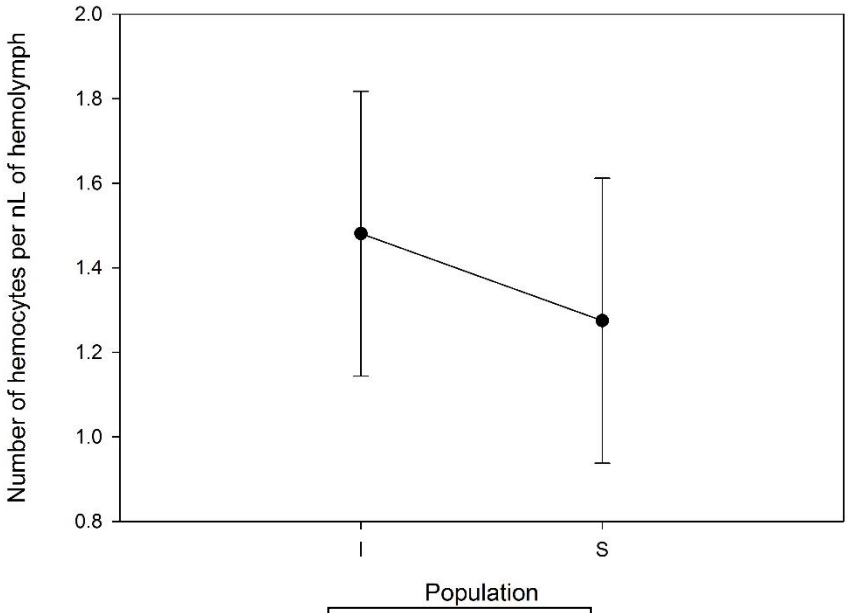

Figure 1a: Block 1

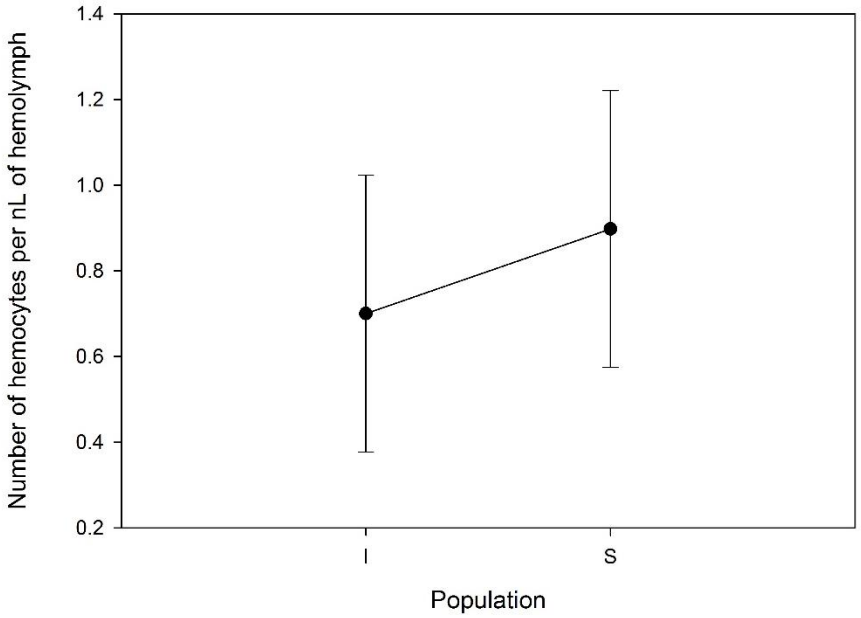

Figure 1b: Block 2

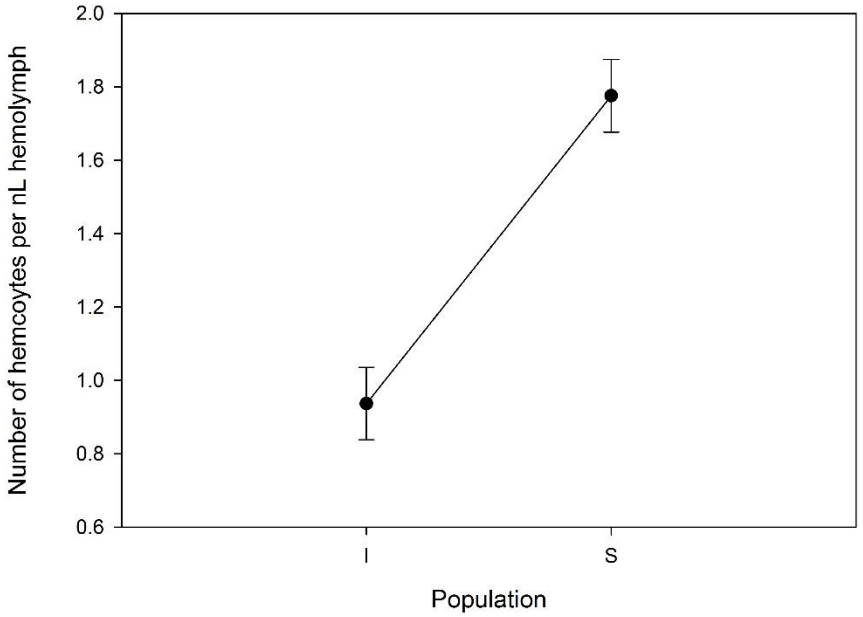

Figure 1c: Block 3

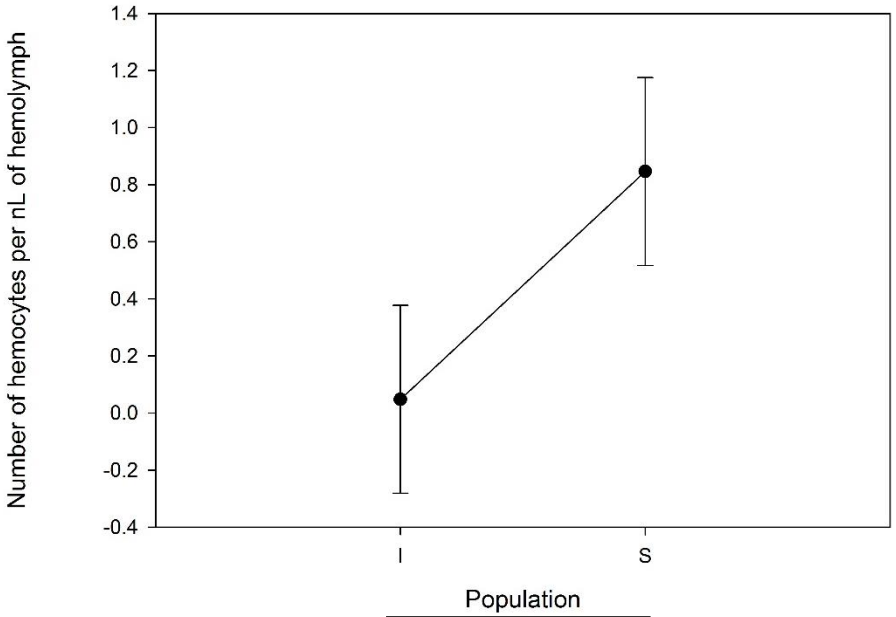

Figure 1d: Block 4

Table 1: ANOVA table for the analysis of hemocyte density differences between I and S populations showing the p-values for each block

Block	Source	Nparm	DF	DFDen	F ratio	Prob > F
Block 1	Population	1	1	1	0.13299816	0.77737374
Block 2	Population	1	1	1	0.80772364	0.53392082
Block 3	Population	1	1	1	259.479069	0.03947044*
Block 4	Population	1	1	1	11.8227745	0.18017838

Chapter 4: Discussion

In this study, I aimed to look for the differences in the basal hemocyte density in the populations selected for a better immune response (I) and control populations (S). A protocol for extracting and counting hemocytes from adult *Drosophila melanogaster* was successfully standardized in lab. Hemocyte density was compared for the late third instar larval stage (wandering stage). Out of the four replicate populations, three show no significant difference in I and S population which could be because the experiment was conducted on the larval stage while selection pressure is applied on the adult stage. Even though a major portion of hemocytes in the adult stage are embryonic and larval stages derived, immunity being a costly trait, it is possible that the hemocyte density increases only during the adult stage during the time period of application of selection pressure. The other explanation could be that increase in survivorship in response to pathogenic infection is not due to increased hemocyte concentration, but rather due to some other pathways like Antimicrobial Peptides.

For one block, the hemocyte density is higher in the control S population than the selected I population. This could be because of the possibility that the increased immunity is due to some other pathway which is being traded off with the hemocyte density, resulting in a lower hemocyte count for an increased immune response.

Future experiments which would provide a better picture about the cellular immune system include:

1. Basal hemocyte density at the adult stage: Hemocyte density should be measured for the adult stage, specifically at the 12th day post egg collection when the selection pressure is applied.
2. Hematopoiesis rate in the adult stage: Hemocyte density should be compare between the selected and control populations both pre and post infection to account for the factor that 'I' may be better at increasing their hemocyte density at a faster rate post bacterial infection.
3. Phagocytic activity of hemocytes: It is possible that the 'I' show a better immune response not because of an increased number of hemocytes but because of better phagocytic ability of the hemocytes. This can be tested with the help of a phagocytosis assay.

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