Change in relative size of accessory glands with days in *Drosophila melanogaster* populations adapted to larval crowding.

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Certificate of Examination

This is to certify that the dissertation titled " Change in the relative size of accessory glands with days in *Drosophila melanogaster* populations adapted to larval crowding" submitted by Ms. Adheena Reji (registration number MS14114) for the partial fulfillment 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 15th, 2019

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. N. G. Prasad at the Indian Institute of Science Education and Research, Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgements of collaborative research and discussion. This thesis is a bonafide record of original work done by me and sources listed within have been detailed in the bibliography.

Adheena Reji

Dated: April 15th 2019

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

Dr.N.G.Prasad

(Supervisor)

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Contents

Abstract

According to life history theory, natural selection and other evolutionary forces shapes organisms in a way that leads to optimization of their survival and reproduction when they face ecological challenges from the environment. It explains how natural selection work to shape the way in which organisms parcel their resources into making offspring (Daniel Fabian & Thomas Flatt. Life History Evolution, 2012. Nature Education). Fitness of an organism would be maximum when its survival and reproduction is maximum. But this is not the case in nature due to various constrains like limitation of resources and trade-offs. Trade-off exist when an increase in one trait which improves the fitness is coupled to a decrease in another trait which thereby leads to a decline in fitness so that the fitness benefit is balanced with a fitness cost .One of the most commonly seen trade-off is the trade-off between reproduction and survival. A previously conducted study *on Drosophila melanogaster* that has experimentally evolved adaptation to larval crowding suggests that these populations have evolved an increased life span. Using the same model population this study investigates the existence of any reproductive trade -off in them with respect to their investment in accessory glands (in terms of relative accessory gland size).

The study reports that there is a significant effect of adaptation to larval crowding on relative accessory gland size.

1. Introduction

The study is conducted on laboratory populations of *Drosophila melanogaster* which have experimentally evolved adaptation to larval crowding. The model organism used, *Drosophila melanogaster* belong to the family *Drosophilidae*. It is commonly known as common fruit fly and is widely used for biological research purposes especially in genetics, life history evolution and physiology. The life span of *Drosophila melanogaster* is about 50 days under optimal environmental conditions (25^{o} C) which starts from egg to death (Linford, Nancy J.; Bilgir, Ceyda; Ro, Jennifer 2013). Under normal conditions a female lays almost 400 eggs on a convenient substance which may be decaying food matter (like mushrooms or sap fluxes) that can act as a food source for the hatched larvae. The eggs that are 50 mm long get hatched after 12 -15 hours (at 25^{o} C) (Thompson JN (1978), Golic KG, Hawley RS (2005).).The hatched larvae grows for 4 days and undergoes two stages of molting to form second - instar larvae and third - instar larvae at about 24 hours and 48 hours after hatching. During these 4 days of growth they feed on the sugar available on the food matter and on the microorganisms that decomposes the food matter. Then the larvae undergo encapsulation in the puparium and undergo metamorphosis (at 25°C) which is a 4 day long process. The adults then eclose out of the puparium (Thompson JN (1978), Golic KG, Hawley RS,2005). .

Being a holometabolous insect the environment that it get exposed as larvae can have direct consequences on their fitness as adults. Previous studies shows that the resources acquired as larvae can determine the adult fitness components (Chippindale, Leroi, Kim and Rose 1993). Thus larval crowding which varies the quality and quantity of resources available for them as larvae can act as an important ecological stressor in the life history of *Drosophila melanogaster*. During larval crowding they also get exposed to toxic nitrogenous wastes like ammonia and urea. Studies show that tolerance to these toxic substances is an energy requiring process (Borash, Gibbs, Joshi and Mueller 1998). This usage of energy limits the usage of resources for other functions and development.

High larval density cultures produces adults which are smaller in body size than adult flies emerging from low larval density cultures (Vinesh Naresh Shenoi et. al 2016). Thus larval crowding have major adult fitness consequences through its direct effects on body size.

The laboratory experimental evolution studies on r and k populations of *Drosophila melanogaster* (Mueller & Ayala, 1981*)* and on CU and UU populations of *Drosophila melanogaster* (Mueller et al., 1993) have helped to study the outcomes of density dependent selection in *Drosophila melanogaster*. The results and observations from these studies (reviewed in Nagarajan et al., 2014) show that laboratory populations of D. melanogaster that are selected for adaptation to larval crowding evolve a set of larval traits. These include increased larval competitive ability (Mueller, 1988), larval feeding rates (Joshi & Mueller, 1988, 1996), locomotor activity during feeding (Sokolowski et al., 1997), urea tolerance (Borash & Ho, 2001), growth rate during post-critical size period (Santos et al., 1997) and minimum food requirement for pupation (Mueller, 1990; Joshi & Mueller, 1996).

A series of studies conducted also shows that larval crowding can affect the reproductive traits which include courtship behaviour and mating. Larval density is known to effect both the pre-copulatory and post-copulatory success in male and female Drosophila *melanogaster*. Males that emerge from low larval densities have large body size than males emerging from high larval densities. These larger males are able to mate at a faster rate and remate more frequently than the smaller males (Partridge, Ewing, and Chandler, 1987; Partridge and Farquhar 1981; Partridge , Green and Fowler, 1987; Turiegano, Monedero, Pita, Torroja and Canal 2013; Wigby, Perry, Kim, and Sirot, 2015) . Similarly the females emerging from low larval densities are larger in body size than the females emerging from high larval densities. These larger females have higher mating rate and are able to remate more frequently than smaller females (Amitin and Pitnick, 2007; Wigby et al., 2015)

In a study conducted by Bangham, Chapman and Partridge in 2002 it could be observed that larval density through its direct consequences on body size can affect the post-copulatory success in *Drosophila melanogaster*. The study found that larger males had higher post-copulatory success. Larger males transfer higher quantities of ejaculate into the female genital tract during copulation (Simmons, Parker and Stockey, 1999) and they have

higher sperm competitive ability (Amitin and Pitnick, 2007). In another study conducted it was seen that females emerging from low larval density cultures are better at avoiding males during courtship and thereby showing reluctance to mate (Turiegano et al., 2013). This observation is consistent with the fact that increased male fitness is achieved at a cost of female fitness (Females that mate with larger male have shorter life span and lay fewer eggs (Friberg and Arnqvist, 2003 ; Pitnick and Garcia-Gonazalez, 2002)) and these antagonistic interactions are expected to select females that evolve strategies that minimizes the harmful effects of males. The harmful effects of males are mediated through the transfer of seminal proteins during mating (Chapman, Liddle, Kalb, Wolfner and Partridge, sss1995) and persistent courtship (Long, Pischedda, Nichols and Rice, 2012; Turiegano et al., 2013) .

All the above mentioned studies were focused on the correlation between larval densities and adult pre/post- copulatory reproductive behavior by manipulating the larval densities for a single generation. Hence the observations and results from these studies shows the phenotypic correlation that need not point to the genetic correlations.

In a further study conducted by Vinesh Naresh Shenoi et al.,2016 the evolution of courtship and mating behavior in response to larval crowding was investigated. The *Drosophila melanogaster* populations involved in the study were the same populations used in this study (the population selected for larval crowding- CU (Melanogaster Crowded as larvae Uncrowded as adults) and the ancestral non selected population- MB (Melanogaster Baseline)). The study showed that males from populations adapted to larval crowding (CU) showed a significantly higher frequency in courtship than males from the ancestral population, the population that was not selected for adaptation to larval crowding (MB). When reared at high and low larval densities, the males from population adapted to larval crowding showed a higher frequency of courtship. It was also observed that soon after yeast supplementation CU male and females mated more often than MB males and females. Since CU populations are exposed to high larval densities and low nutrition for generations we would expect them to adapt these conditions. Thus they may behave better and emerge with better body conditions at low nutritional conditions. Hence these findings could be as a result of CU males being able to maintain better body conditions even at lower larval densities and thus courting better.

When grown at high larval densities CU male are smaller than MB males (Shenoi et al.) and smaller males are known to have deficient courtship when compared with lager males (Ewing, 1961). Therefore the increased frequency in courtship may also be a way of compensating for their smaller size.

Another study conducted by Vinesh. N. Shenoi, S. Z. Ali & N. G. Prasad, on MCU and MB populations of *Drosophila melanogaster* shows that the population adapted for larval crowding had evolved an increased longevity in both males and females.

According to life history theory natural selection and other evolutionary forces shapes organisms in a way that leads to optimization of their survival and reproduction when they face ecological challenges from the environment. It explains how natural selection work to shape the way in which organisms parcel their resources into making offspring (David Reznick , 2010) (Daniel Fabian & Thomas Flatt. Life History Evolution, 2012. Nature Education). Fitness of an organism would be maximum when its survival and reproduction are maximum during all the stages of its life. Therefore it is expected that all life history traits should always get evolved in order to maximize both survival and reproduction (Houle 2001). But this situation would immediately lead to the evolution of "Darwinian demons" (Law 1979).Darwinian demons are organisms that start to reproduce immediately after they are born, produce an infinite number of offspring, and live forever(Daniel Fabian & Thomas Flatt. Life History Evolution, 2012. Nature Education). Such organisms, however, do not exist in the real world since resources are always finite and limited, and life history traits are subject to and are affected by many types of constraints. Therefore it is not possible that natural selection favours for maximizing all life history traits and achieving fitness beyond limits. We call such limits evolutionary constraints (Stearns 1992, Houle 2001).

Trade-offs are known to be one such major constrain (Stearns 1992, Roff 1992, Flatt and Heyland 2011). A trade-off exists when an increase in one life history trait which improves the fitness of the organism is coupled to a decrease in another life history trait which leads to a decline in the fitness of the organism, so that the fitness benefit is balanced against a fitness cost. A huge number of previously conducted studies show the evidence for genetically based life history trade-offs (Stearns and Partridge 2001, Flatt and Schmidt

2009, Flatt 2011). Many of these studies could find a negative correlation between early fecundity and adult lifespan. Trade- offs can be well explained by the Y – model proposed by van Noordwijk & de Jong (1986) (Fig 1.)

Fig 1 - Y – model of resource allocation depicting trade-off between two traits

The most important example of a trade-off is life-time fecundity vs. life-span. Many of the previous studies had shown the existence of a trade- off between life span and reproductive investment patterns (Fowler and Partridge 1989; Cordts and Partridge 1996; Kuijper et al., 2006). For example direct artificial selection in laboratory populations of *Drosophila melanogaster* causes the evolution of increased adult lifespan but this is coupled to decreased early reproduction (Zwaan et al. , 1995).

Hence a study was conducted by Vinesh Shenoi et al. which looked into the existence of a trade-off between life- time-fecundity and life span in the population adapted to larval crowding. Results showed that CU population had evolved an increased life span and CU males showed a significantly higher frequency in courtship than MB males.The results

could not find any evidence of a trade-off since the pre-copulatory reproductive behavior (courtship in males and mating in females) of CU's didn't compromise with their increased life span.

Taking these results into account, Vinesh Shenoi et al. conducted a further study based on the hypothesis that there might be intrinsic trade-off of lifespan with the investment in reproductive tissue. The study examined the relative sizes of adult male reproductive organs (relative testis size) and female reproductive organs (relative seminal receptacle length and ovariole number) in selected population (CUs) and control populations (MBs) by growing them at low and high larval densities. The results from this study showed that investment in reproductive tissue does not trade off with increased life span in the selected populations (CU's). Moreover it was seen that selection for adaptation to larval crowding has led to a correlated increase in relative testis size in males.

Thus, none of the previous studies conducted could find any trade-off between lifespan and reproduction in the CU population (population adapted to larval crowding). Expecting such a trade-off, this study investigates the effect of adaptation to larval density on investment in accessory glands, an important reproductive tissue in adult male *Drosophila melanogaster* (investment is studied in terms of relative size of accessory glands).

Accessory glands.

The accessory glands of *Drosophila melanogaster* which is a secretory tissue in the male reproductive system, produces and secretes a complex mixture of proteins that form components of the seminal fluid. Male accessory gland proteins (Acps) and are transferred to females during copulation along with the seminal fluid (Chen 1984; Monsma and Wolfner, 1988). Accessory gland proteins enhance the female's egg production, increase her rate of ovulation, reduce her sexual receptivity, assist in the female's storage of sperm, stimulate the rate of egg production , affects the receptivity as well as longevity of the mated female (reviewed in Wolfner, 2002; Gillott, 2003; Ravi Ram and Ramesh, 2003; Chapman and Davies, 2004). There are multiple targets for a single Acp and its pattern of localization is unique. The known targets of Acps include ovary, uterus, oviduct, oocytes,

sperm storage organs like spermatheca and seminal receptacle. Certain Acps may move beyond the reproductive tract and get released into the hemolymph. Clearly accessory gland proteins play a key role in reproductive success of *Drosophila melanogaster* by changing the sexual behavior of female, supporting sperm transfer and storage (P. S. Chen, 1996). Therefore changing the patterns of investment in accessory glands can be a form of lifespan - reproduction trade-off if such a trade-off exists in the population. In this study, the investment in accessory gland is investigated in terms of its relative size.

Changes in larval density for both males and females can have well pronounced consequences for adult male seminal protein production and transfer, and for female remating patterns (Stuart Wigby, Jennifer C. Perry, Yon-Hee Kim and Laura K. Sirot, 2016). Hence larval crowding can be considered as a factor affecting the investment in accessory glands in adult flies (i.e. affecting the relative accessory gland size as far as this study is concerned).

Effect of age on reproductive success in males.

Mating rate in male *Drosophila melanogaster* decreased significantly with increasing male age (Hanna Ruhmanna,b, Mareike Koppika, Mariana F. Wolfnerc, Claudia Frickea, 2018). A decrease in reproductive capacity in males with an increase in age is known to be due to senescence of the reproductive tissues in them and thereby leading to impaired fertility (reviewed in Johnson and Gemmell, 2012).The male's ability to mate and fertilise eggs decreases rapidly with increasing age. Male's mating probability decreases and latency-time to mating increases with reproductive aging.. With an increase in age males become less efficient in inducing female post-mating changes. Since a "well-composed" ejaculate (Perry et al., 2013) is necessary for male competitiveness and reproductive success, this reduction in male reproductive success can be attributed to the diminished capacity of males to produce enough high quality seminal fluid proteins

It is also known that seminal fluid proteins produced in the male accessory glands significantly increase the male reproductive success (Stuart Wigby et al., 2009). Since male reproductive success decreases with age (Hanna Ruhmann, Mareike Koppik, Mariana F.

Wolfner, Claudia Fricke, 2018) and selection on accessory gland size affects male competitive reproductive success through its effect on seminal fluid protein production and transfer (Stuart Wigby et al., 2009), it is reasonable to expect a correlation between age and accessory gland size in *Drosophila melanogaster*.

Hence this study also investigates the effect of age of the fly on investment in accessory glands in adult male *Drosophila melanogaster* (investment is studied in terms of relative size of accessory glands).

For the purpose of analysis, the null hypotheses for this experiment are

1) The absence of any difference in relative accessory gland size in the selected population (CU- selected for adaptation to larval crowding) and ancestral population (MB) of adult male *Drosophila melanogaster*.

2) The absence of any difference in the relative accessory gland size across different age groups of adult male *Drosophila melanogaster*.

2. Materials and Methods

2.1 Base line populations and stock maintenance (Vinesh Shenoi N, thesis, IISER Mohali (2016)).

This study is carried out on eight populations of *Drosophila melanogaster*, four selected for adaptation to larval crowding (CU 1-4 - crowded as larvae uncrowded as adult) and four control populations (MB 1-4 - melanogaster baseline). The MB and CU populations were originally derived in the laboratory of Prof. Amitabh Joshi at Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore. The fly populations MB 1-4 and CU 1-4 were derived from JB populations (laboratory populations of *Drosophila melanogaster*) (Sheeba et al., 1998). The JB populations (JB 1-4- four replicates) have been maintained under well defined laboratory conditions - 60-80 eggs/vial density, 25°C temperature, 90% Relative humidity (RH), constant light, standard banana-jaggery food, on a 21-day discrete generation cycle. These four replicates of JB populations were then mixed together to form a single population which was named as MB (Melanogaster Baseline). This MB population was separated into 4 replicates named as MB 1, MB 2, MB 3, MB 4 after 10 generations.

2.1.1 Maintenance of MB (Melanogaster baseline) Populations (MB 1-4)

The four replicates of MB populations (MB 1-4) were maintained on a 21-day discrete generation cycle. They were fed on standard corn meal-charcoal food. Each generation was initiated by collecting eggs from 12-day old females. These eggs were transferred into glass vials (25mm diameter × 90mm height) containing 6-8 ml of corn meal-charcoal food at a density of 60-80 eggs/vial (no larval crowding). These vials containing collected eggs were incubated at 25°C temperature, 90% RH and constant light. The flies start eclosing by day 9 post egg collection. Peak eclosion is on day 10. By day-12 post egg collection eclosion is complete in all the vials i.e almost all the adults come out in all the vials. These adult

flies were then transferred into a Plexiglas cage (24 x 19 x 14 cm) containing corn meal-charcoal food plate and wet cotton for maintaining high RH levels. The number of adults was approximately 2500 per population per generation. In each cage fresh food plate was provided on days 14 and 16 post egg collection. On day 18 post egg collection, the flies were provided with a fresh food plate with *ad libitum* live yeast paste. The flies were provided with a fresh food plate (which is a cut plate for offering the flies vertical surface to lay eggs) after 2 days and were allowed to oviposit for 18 hours. These eggs were then used to start the next generation. Before deriving the selected populations from MBs they were maintained under standard laboratory conditions for 15 generations. The selected populations (Selected for adaptation to larval crowding) were named as CU 1-4 (Crowded as larvae, Uncrowded as adults). There were 4 replicates of CU population (one selected population derived from each of the MB populations). Each replicate of CU was since then maintained as separate population (Shenoi VN, Ali SZ, Prasad NG 2016). Hence they were treated as blocks (statistical blocks) during analyses.

2.1.2 Maintenance of CU (melanogaster crowded as larvae uncrowded as adult) Populations (CU 1-4)

Similar to that of MBs, the CU populations were also maintained on a 21-day discrete generation cycle at 25°C temperature, 90% RH and constant light. In CUs also, eggs laid by 12 day old females were collected and transferred into glass vials (25mm diameter × 90mm height) containing 1.5ml of corn meal-charcoal food at a density of 800 eggs/vial (larval crowding). These vials containing collected eggs (twenty four vials per population) were then incubated at standard laboratory conditions. Out of a total of 800 eggs, only 70 - 80 adults eclose in each vial and thus the pre-adult mortality is high in this population compared to MB population. The eclosion pattern in CUs is much spreaded than MBs. This is due to the increase in developmental time with the increase in larval densities in which they are cultured (Santos et al., 1997). In CU population eclosion of adult flies start from day 8 post egg collection and continues till day 18 post egg collection. To prevent the crowding of adult flies in the vials, young adults were transferred into Plexiglas cages (24 x 19 x 14 cm) as soon as they eclose. This was done daily once (almost on the same hour of the day) starting from day 8 post egg collection until day 18 post egg collection. On

every alternate day fresh food plate of cornmeal charcoal food was provided starting from day 8.Wet absorbent cotton was also provided in each cage for maintaining high RH levels. On day 18 post egg collection, the flies were provided with a fresh food plate with *ad libitum* live yeast paste. After 2 days the flies were provided with a fresh food plate (cut plate was given in-order to offer the flies vertical surfaces to lay eggs). The flies were then allowed to oviposit on it for 18 hours. These eggs were collected for starting the next generation (Shenoi VN, Ali SZ, Prasad NG 2016).

The following schematics clearly shows the lineage of MB and CU Populations.

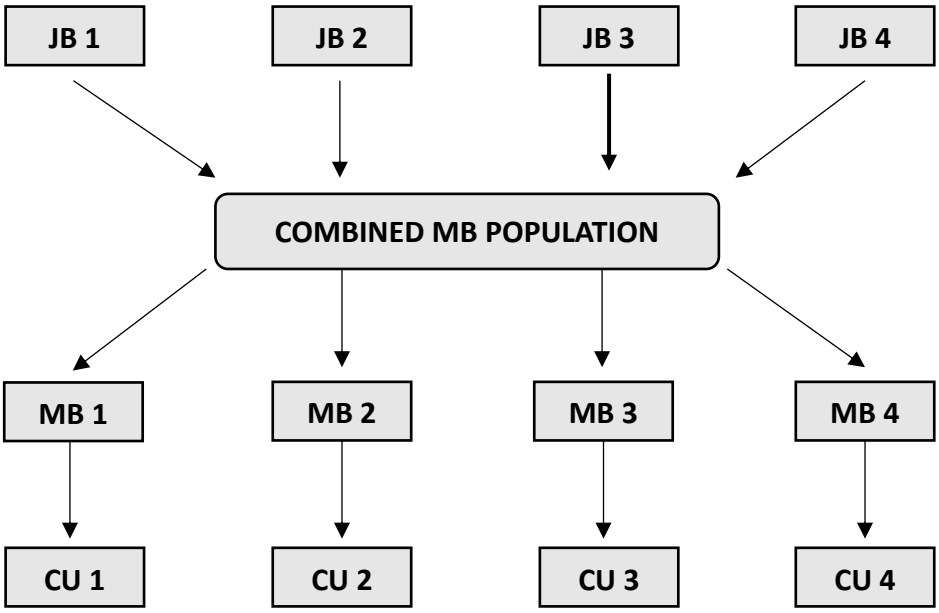


Fig 2. – Lineage of MB and CU population

2.2 Experimental populations

All the experiments were conducted on the backup populations of the MBs and MCUs (MB-1 backup, MB-2 backup, MB-3 backup, MB-4 backup, CU-1 backup, CU-2 backup, CU-3 backup, MCU-4 backup) which are the standardized flies. Flies for experiments are always cultured after standardization. Standardization is done by making CUs and MBs to pass through a generation of common rearing regime. This is done to remove any non-genetic parental effect if present. The backup population of CU and MB is generated by collecting eggs from CU and MB populations respectively and culturing them at a density of 300 eggs in approximately 30 ml of charcoal-cornmeal food. Thus the backup populations from which we are culturing experiment flies are already standardized. The experiments were conducted between 251 – 257 generations of MB population and between 236- 242 generations of MCU population. All the flies used for experiment are maintained on a 21 day life cycle (i.e. egg collection from each population occurs on the 21^{st} day and the next generation is started) and are fed on charcoal – cornmeal food.

For all the experiments carried out in this study four blocks of CU and MB population were considered. Two different levels of density treatments were created: 600 eggs in 2 ml of charcoal – cornmeal food (which is called as high density treatment or hd treatment) and 60 eggs in 6 ml of charcoal –cornmeal food (which is called as the low density treatment or ld treatment). All these treatments are done for 3 age groups of the flies where the age of the fly is given in days. For block 1 and 2 the days considered were day 19, day 22, day 25. For blocks 3 and 4 the days considered were day 18, day 21, day 24 Thus there were 4 blocks each having 12 treatments i.e. 2 density treatments X 2 selection regimes X 3 age groups (3 days).

2.3 Experimental Procedure

The experimental flies are fed with charcoal- cornmeal food supplemented with *ad libitum* live yeast for 48 hours which is followed by an egg laying window of 6 hours. The egg

laying window is made by providing them with a cut plate with charcoal – cornmeal food. A cut plate is provided rather than a normal flat large plate with charcoal – cornmeal food because the cut plate offers vertical surfaces for egg laying which is preferred by the flies than horizontal surfaces. The egg laying window is followed by egg collection. After egg collection the vials containing eggs are stored in incubator that provides 25^{o}C temperature for the collected eggs. The rates of eclosion are different for different density treatments. For high larval densities the eclosion peak is delayed than the normal since development time increases in higher larval densities (Santos et al., 1997). For high density treatments, eclosion starts on the day 8 post egg collection and continues till day 18 post egg collection. In order to avoid crowding of adult flies in food vials containing 2 ml of charcoal - cornmeal food i.e. high density treatment, the flies were dumped into Plexiglas cages (24 × 19 × 14 cm) each day starting from day 8 post egg collection till day 18 post egg collection. This dumping is done daily once almost during the same hour of the day (from day 8 till day 18). For low density treatments, eclosion starts on day 9 post egg collection with a peak eclosion on day 10 post egg collection. By day 12 post egg collection, almost all the adult flies are eclosed in all the low density experimental vials. The eclosed adults from low density treatments are then dumped into Plexiglas cages (24 × 19 × 14 cm). After dumping the flies into cages they are maintained under standard conditions and provided with fresh charcoal – cornmeal food plate on every alternate days. For blocks 1 and 2, male flies were randomly aspirated (collected) from the cages into vials containing 2 ml of banana jaggery food on days 19, 22 and 25 post egg collection. For blocks 3 and 4, male flies were randomly aspirated (collected) from the cages into vials containing 2 ml of banana jaggery food on days 18, 21 and 24 post egg collection. The male flies that were collected in banana- jaggery food vials were froze in -20^{o}C each day. These flies were stored in – 20^{o}C until used for dissection. When male flies were collected an equal number of female flies were also collected and removed from each cage on each day so that the normal male to female sex ratio of the cage of the cage is not disturbed. The male flies that are stored in – 20^{o}C are later used for dissection. During this study a total of 960 male flies were assayed i.e a sample size of 20 (n=20) was assayed for each selection regime X block X density treatment X day.

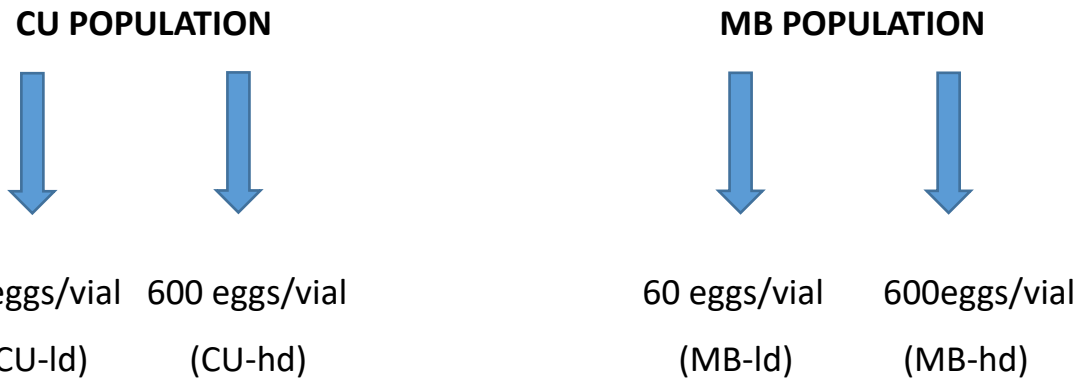
Dissections were performed using 1X PBS (phosphate buffered saline). Each male fly was transferred using a brush to a glass slide containing one or two drops of 1X PBS. The glass

slide was then kept under a microscope and the accessory glands and wing were dissected out. The two glands that were initially attached to each other are then separated carefully using a fine dissection needle. This glass slide containing accessory glands and wing was transferred to Leica microscope for imaging. Before imaging the wing and the glands, a standard scale was set in the microscope using a micrometer. The image of wing and accessory glands was then taken using the camera attached to the Leica microscope.

The images captured are analyzed using the software imagej

The experimental procedure is summarized below.

DAY 0 – Egg collection from CU and MB Population - egg collection done in both high density (hd-600 eggs in 2 ml food) and low density (ld- 60 eggs in 6 ml food) treatments.



DAY 8 - Start dumping the high density flies.

DAY 12 - Dumping the low density flies.

DAY 18 - Continue dumping of high density flies till the eighteenth day

DAY 19 -Aspirate (collect) random male flies from the cages and freeze them for dissection

Repeat the collection of random male flies for 3 more days

Freezing of flies is followed by the dissection of male flies to measure the accessory gland area and wing length. The relative size is calculated by dividing the two measurements. In this experiment, flies of 3 age groups are dissected namely early, mid and late age which are denoted as Day 1, Day 2 and Day 3 respectively

Fig 3. (a) – Accessory glands

Fig 3. (b) - Wing

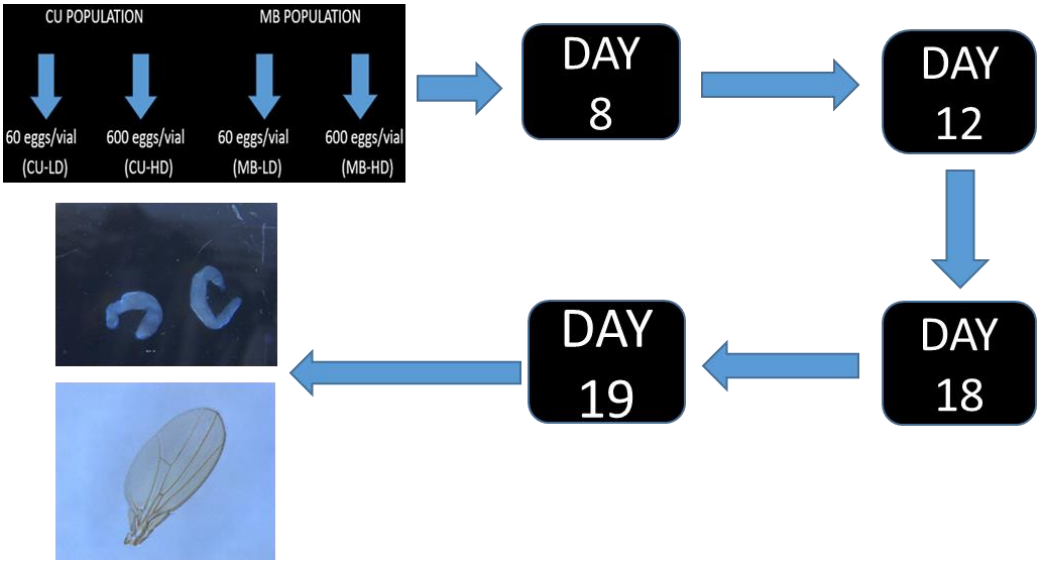


Fig. 4 - Experimental procedure

For blocks 1 and 2 flies were collected on days19, 22, 25 post egg collection. For blocks 3 and 4 flies were collected on days 18, 21, 24 post egg collection.

For the purpose of analysis the age of the flies dissected are named as Day 1- Early age, Day 2-Mid age, Day 3 - Late age. For block 1 and 2, day 19, day 21 and day 25 are early, mid and late ages respectively. For block 3 and 4 day 18, day 21 and day 24 are early, mid and late ages respectively.

For every block egg collection, eggs were collected into 10 vials containing charcoal – cornmeal food (6 ml food for ld treatment and 2 ml food for hd treatment) for each density treatment from each section regime. Thus there were 10 vials containing collected eggs each of CU-hd, CU-ld, MB-hd and MB-ld. These 10 vials is further divided into two groups A and B each containing five vials and are dumped separately into cages when flies start to eclose (i.e 2 cage replicates for each selection X treatment) . This was done to avoid any cage effect if present. Thus for each block there were 8 cages to maintain. For each block the 8 cages were labelled as CU-hd A, CU-hd B, CU-ld A, CU-ld B, MB-hd A, MB-hd B, MB-ld A and MB-ld B (CU- hd A is the cage replicate of CU- hd B and so on).

3. Results

3.1 Analysis

A total of 960 flies were sampled in the whole experiment i.e. a sample size of 20 (n=20) was assayed for each selection regime X block X density treatment X day. Area of accessory glands and wing length was analyzed using the software imagej. The relative size of accessory glands in mm is calculated by dividing these two measurements.

Relative size of accessory gland = accessory_gland area in mm^2 / wing length in mm

The relative size of accessory gland was subjected to an ANOVA test using the following characters as factors in a full factorial model:

- Selection regime (CU or MB)
- Treatment (ld or hd)
- Day (Day 1 or Day 2 or Day 3)

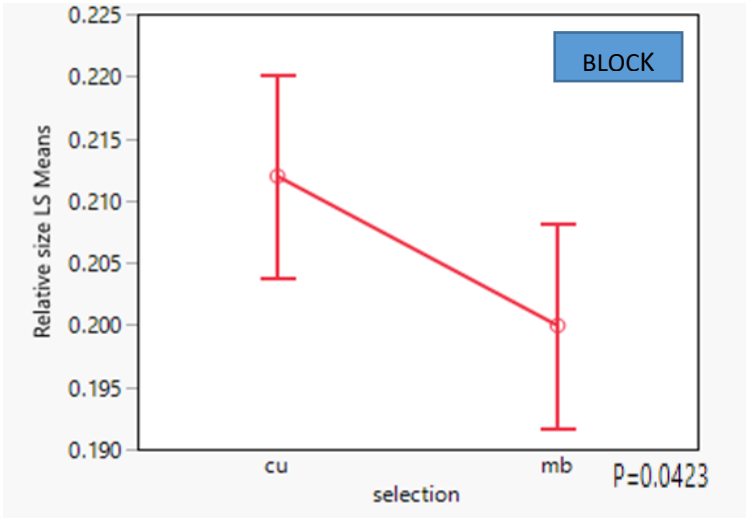
The ANOVA test was done for each block separately and also for all the four blocks combined. When test was performed by combining all the 4 blocks, block was considered as arandom factor.

The α value for all statistical tests in this study is 0.05.

3.2 Observations

3.2.1 Effect of selection – Block wise.

In all the four blocks, adaptation to larval crowding had a significant effect on the relative accessory gland size (with **P=0.0423, P< 0.0001**, **P< 0.0001**, **P< 0.0001** for blocks 1,2,3,4 respectively) (Fig 5.). The CU population is seen to have a significant increase in the relative size of accessory glands than the MB population. Hence CUs do not trade off the accessory gland investment with their increased life span



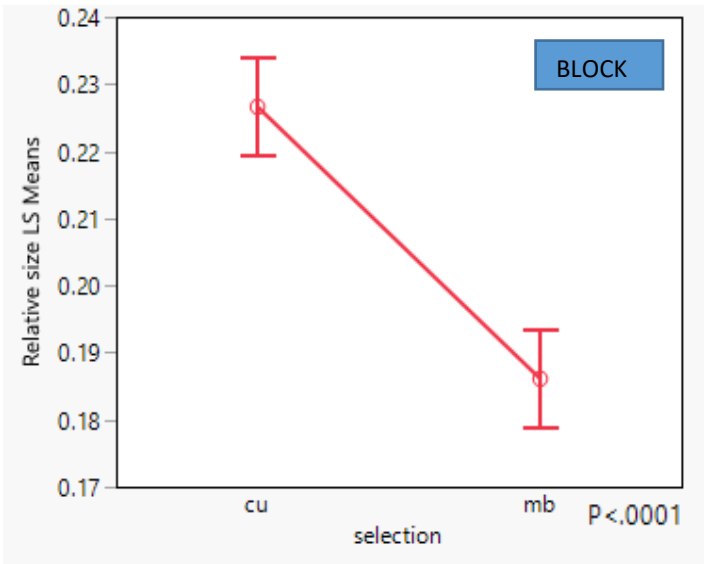
LSMeans Differences Student's t

α= 0.050 t= 1.97042

LSMean[j]

LSMean[i] Mean[i]-Mean[j] / Std Err Dif / Lower CL Dif / Upper CL Dif	cu	mb
cu	0	0.01201
	0	0.00588
	0	0.00042
	0	0.02359
mb	-0.012	0
	0.00588	0
	-0.0236	0
	-0.0004	0

Level		Least Sq Mean
cu	A	0.21196378
mb	B	0.19995700



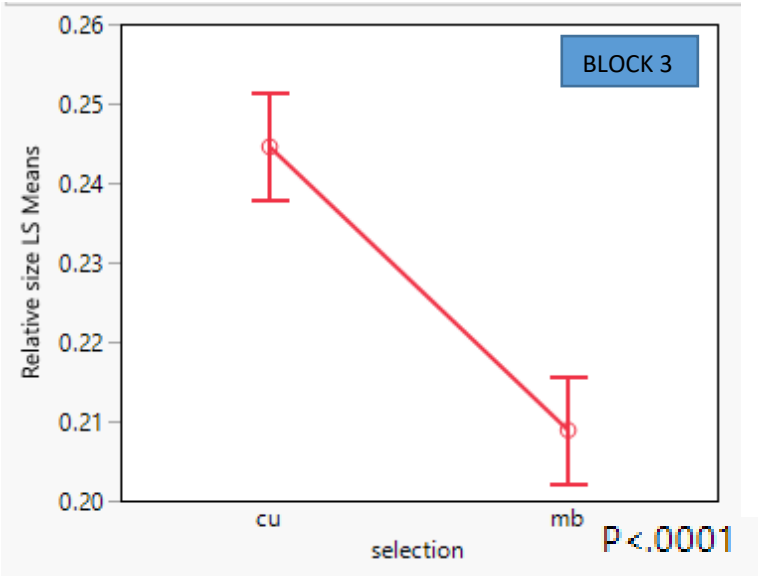
LSMeans Differences Student's t

α= 0.050 t= 1.97042

LSMean[j]

LSMean[i] Mean[i]-Mean[j] / Std Err Dif / Lower CL Dif / Upper CL Dif	cu	mb
cu	0	0.04051
	0	0.00526
	0	0.03014
	0	0.05088
mb	-0.0405	0
	0.00526	0
	-0.0509	0
	-0.0301	0

Level		Least Sq Mean
cu	A	0.22662542
mb	B	0.18611120



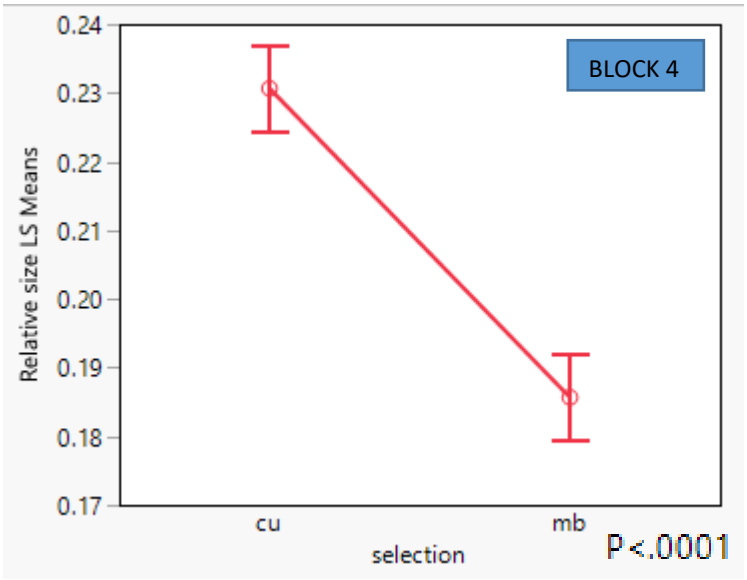
LSMeans Differences Student's t

α= 0.050 t= 1.97042

LSMean[j]

Mean[i]-Mean[j]	cu	mb
Std Err Dif		
Lower CL Dif		
Upper CL Dif		
LSMean[i] cu	0	0.03567
	0	0.00482
	0	0.02617
	0	0.04518
mb	-0.0357	0
	0.00482	0
	-0.0452	0
	-0.0262	0

Level		Least Sq Mean
cu	A	0.24456676
mb	B	0.20889261



LSMeans Differences Student's t

α= 0.050 t= 1.97042

LSMean[j]

Mean[i]-Mean[j]	cu	mb
Std Err Dif		
Lower CL Dif		
Upper CL Dif		
LSMean[i] cu	0	0.04498
	0	0.00455
	0	0.03601
	0	0.05394
mb	-0.045	0
	0.00455	0
	-0.0539	0
	-0.036	0

Level		Least Sq Mean
cu	A	0.23066499
mb	B	0.18568965

Fig 5. - Effect of selection on relative accessory gland size - Block wise result

3.2.2 Effect of treatment – Block wise.

The treatment (hd and ld) has a significant effect on the relative accessory gland size in each block (with **P< 0.0001** in each block). Both CU and MB population invest more in accessory glands when cultured at low larval densities than at high larval densities (Fig 6.).

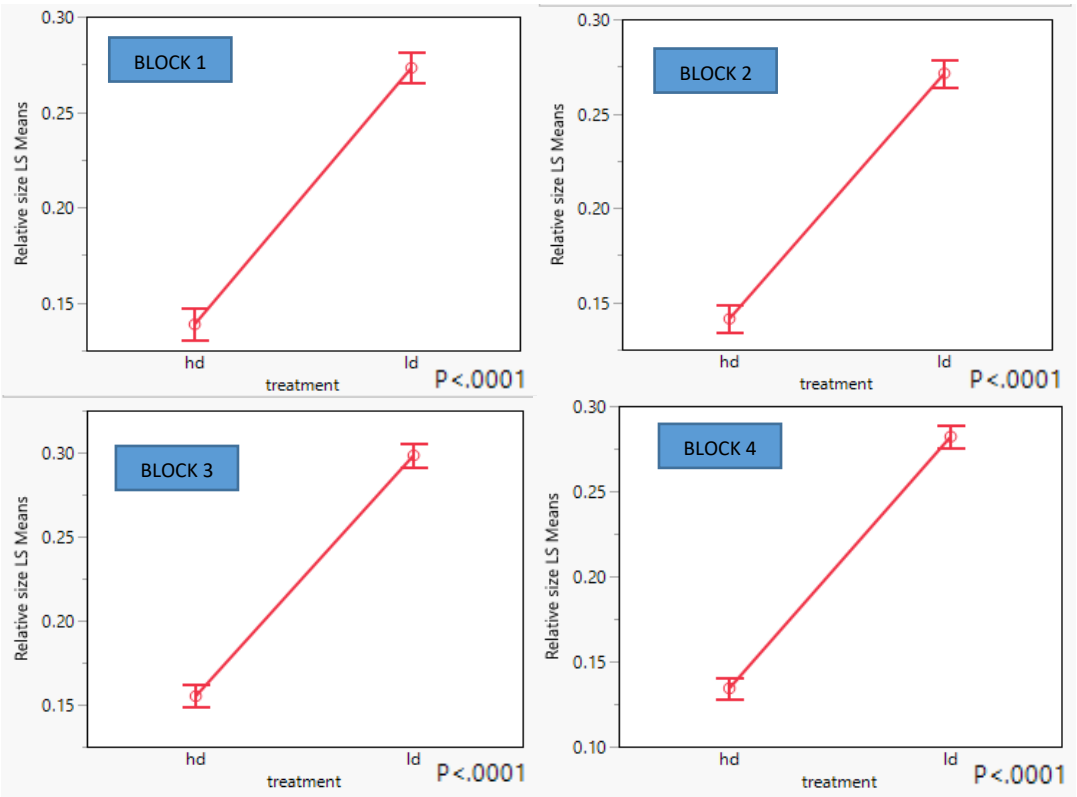
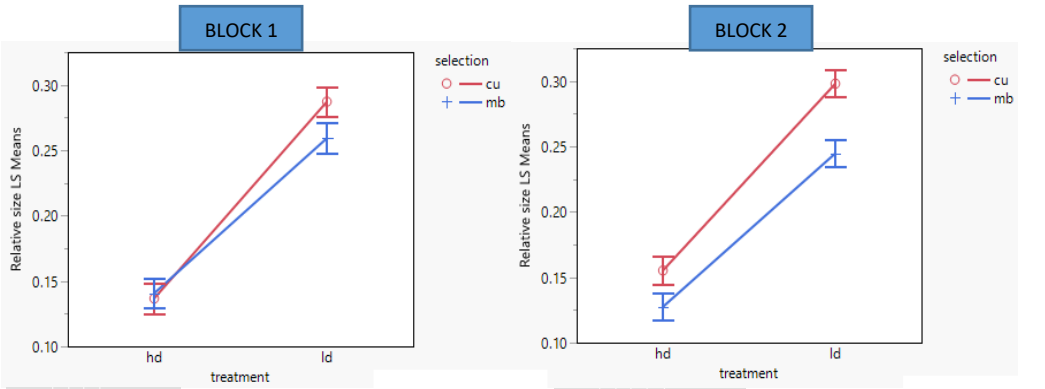


Fig 6. - Effect of treatment on relative accessory gland size – Block wise results.

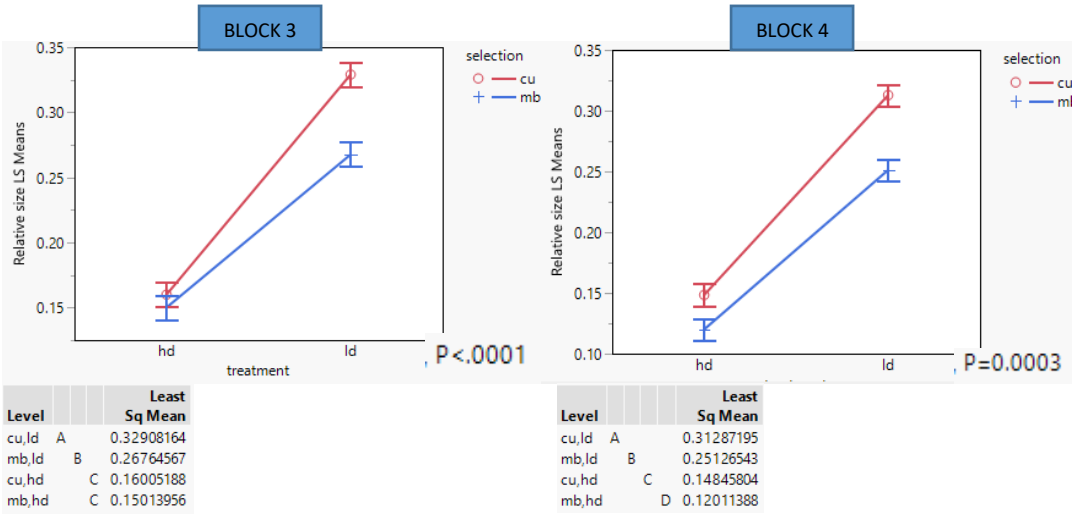


Level		Least Sq Mean
cu,ld	A	0.28717974
mb,ld	B	0.25922897
mb,hd	C	0.14068502
cu,hd	C	0.13674782

P= 0.0072

Level		Least Sq Mean
cu,ld	A	0.29799731
mb,ld	B	0.24474089
cu,hd	C	0.15525353
mb,hd	D	0.12748151

P= 0.0163



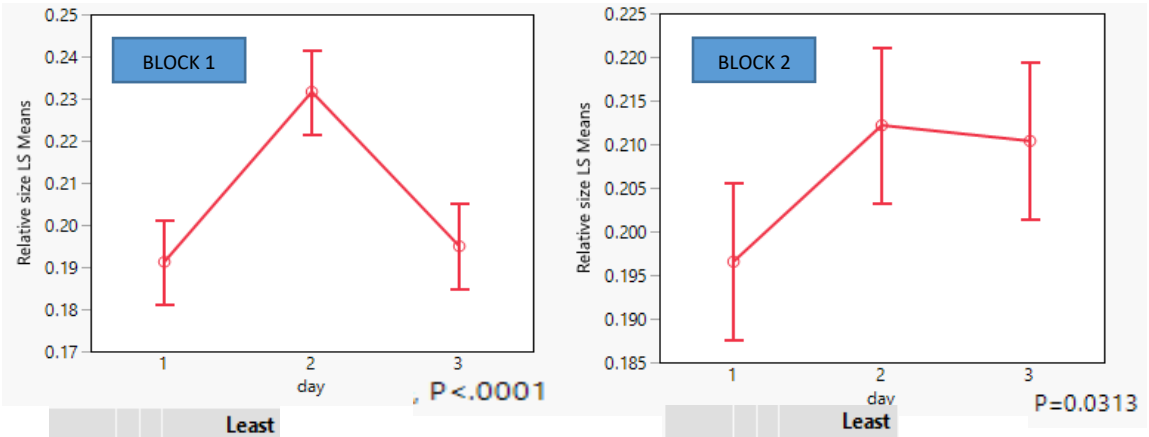
Level		Least Sq Mean
cu,ld	A	0.32908164
mb,ld	B	0.26764567
cu,hd	C	0.16005188
mb,hd	C	0.15013956

Level		Least Sq Mean
cu,ld	A	0.31287195
mb,ld	B	0.25126543
cu,hd	C	0.14845804
mb,hd	D	0.12011388

Fig 7. – Interaction effect of selection and treatment on relative accessory gland size. Tukey's HSD test results are shown below each graph

It is observed that there is significant selection regime x treatment interaction in each block (with **P=0.0072**, **P=0.0163**, **P< 0.0001**, **P=0.0003** in blocks 1,2,3,4 respectively) (Fig 7.). Low larval density treatment leads to greater investment on accessory glands in both CU and MB population.

3.2.3 Effect of age - Block wise.

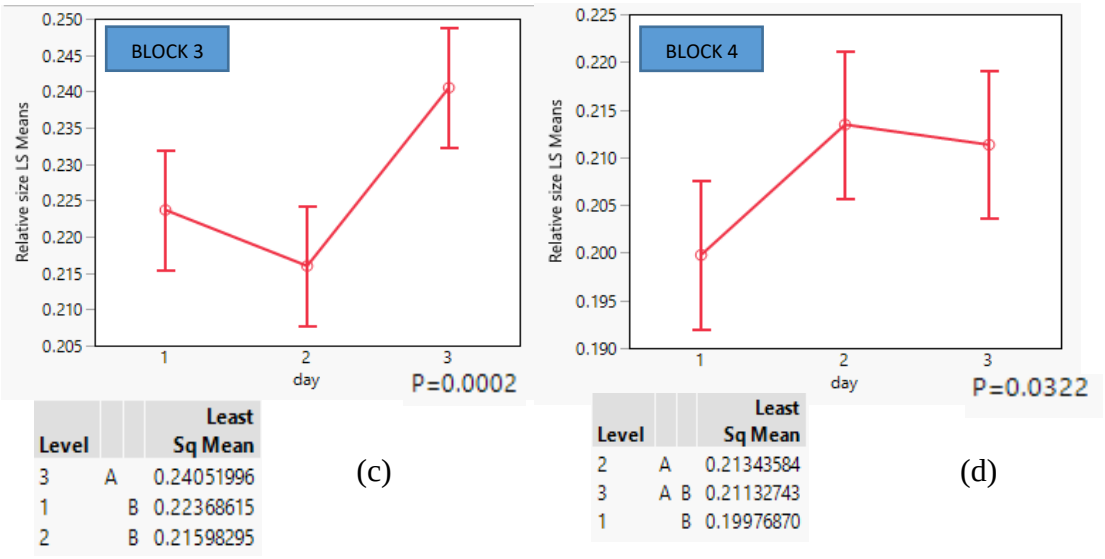


Level		Least Sq Mean
2	A	0.23163510
3	B	0.19498334
1	B	0.19126273

(a)

Level		Least Sq Mean
2	A	0.21217014
3	A B	0.21038143
1	B	0.19655336

(b)



Level		Least Sq Mean
3	A	0.24051996
1	B	0.22368615
2	B	0.21598295

(c)

Level		Least Sq Mean
2	A	0.21343584
3	A B	0.21132743
1	B	0.19976870

(d)

Fig 8. - Effect of day on relative size of accessory glands- Block-wise results. Tukey's HSD test results are shown below each plot.

Age of the fly (day) has a significant effect on the relative accessory gland size (with **P< 0.0001**, **P= 0.0313**, **P=0.0002**, **P=0.0322** in blocks 1,2,3 and 4 respectively) (Fig 8.). In blocks 1, 2 and 4 accessory gland attains a maximum size on day 2 i.e. during mid age of the fly (Fig 8 (a), (b), (d)). In block 3 accessory gland size attains a maximum value on day 3 i.e. during late age of the fly (Fig 8 (c).).

3.2.4 Observations – 4 blocks combined

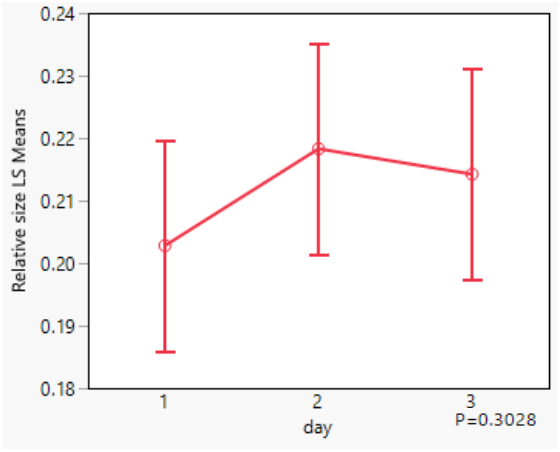
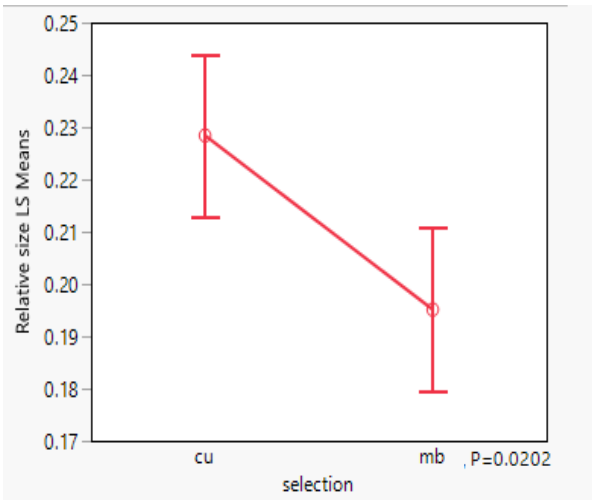
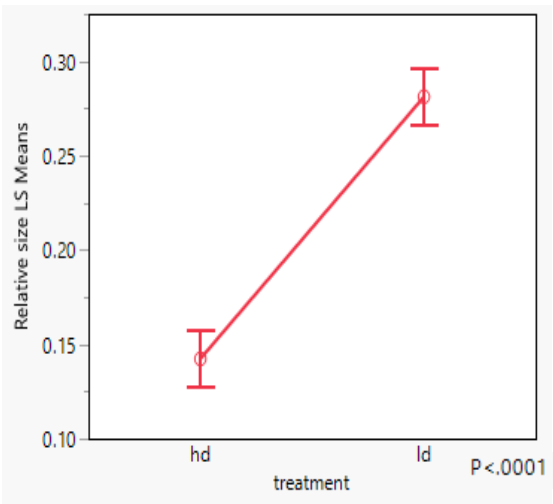


Fig 9. - Effect of day on relative accessory gland size



(a)



(b)

Fig 10 (a). – Effect of selection on relative accessory gland size

Fig 10 (b). – Effect of treatment on relative accessory gland size

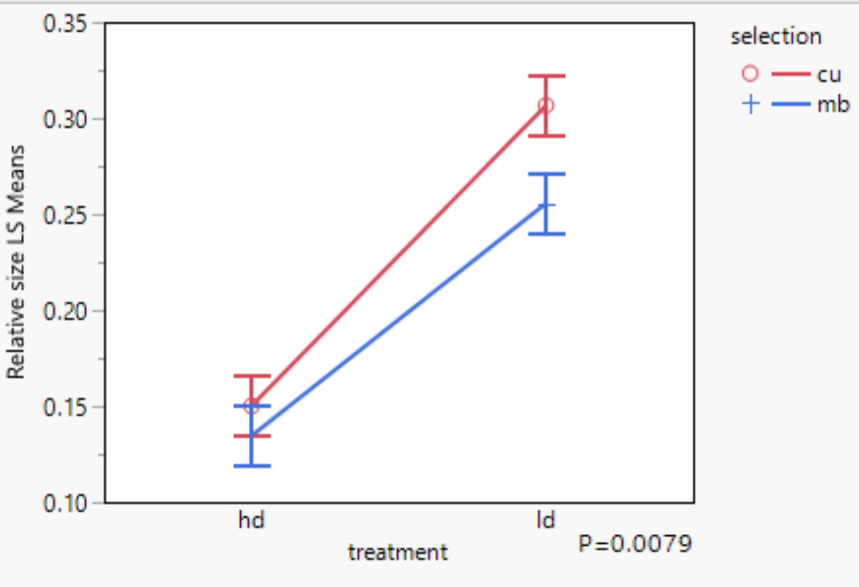


Fig 11. – Interaction effect of selection and treatment on relative accessory gland size

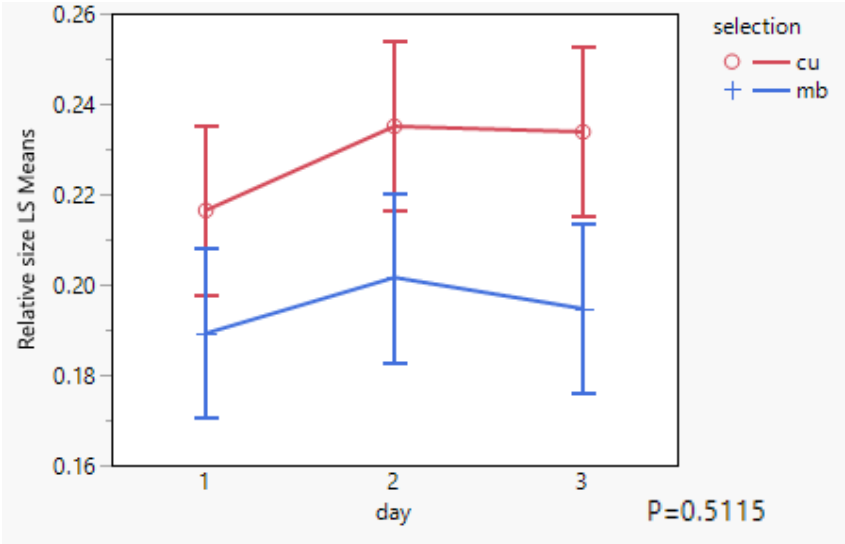


Fig 12. – Interaction effect of day and selection on relative accessory gland size. No Day x selection interaction is found

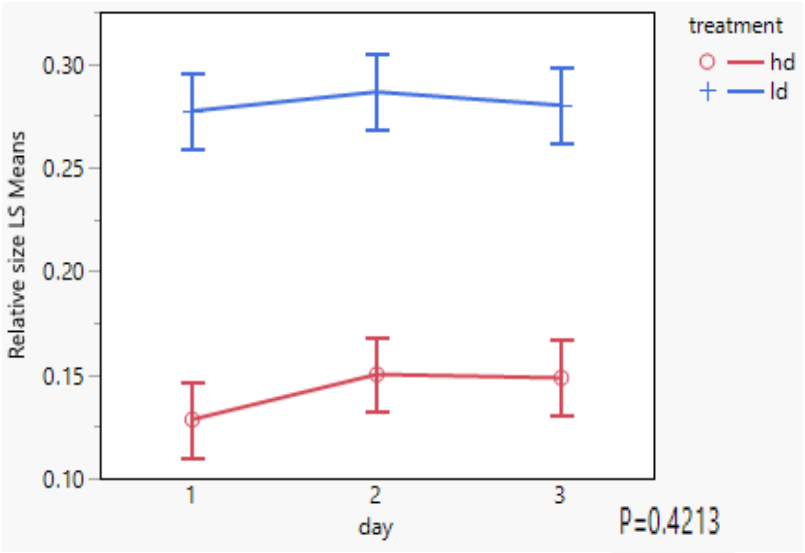


Fig 13. - Interaction effect of day and treatment on relative accessory gland size.

No Day x treatment interaction is observed.

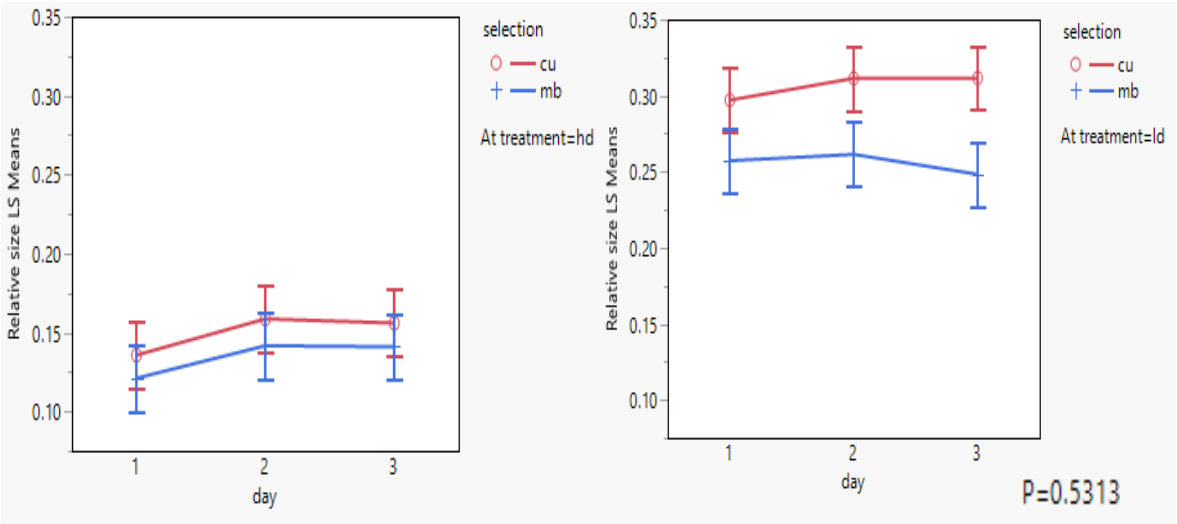


Fig 14. – Interaction effect of selection, day and treatment on relative accessory gland size.

No Selection x day x treatment interaction is observed.

When all the four blocks are combined, selection and treatment had significant effect on relative accessory gland size with **P = 0.0202** and **P < 0.001** respectively. Selection x treatment interaction is significant (**P=0.0079**) (Fig 11.). Effect of day on relative accessory gland size is not seen to be significant when all the 4 blocks are combined (Fig 9.). No Day x selection (Fig 12), Day x treatment (Fig 13.), Selection x day x treatment (Fig 14.) interactions are seen.

Table 1. Summary of results of a ANOVA on relative accessory gland size data using selection, treatment and day as fixed factors, crossed amongst themselves for block 1.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
selection	1	1	0.0086352	4.1718	0.0423*
day	2	2	0.0796081	19.2297	<.0001*
selection*day	2	2	0.0149899	3.6209	0.0283*
treatment	1	1	1.0833963	523.3997	<.0001*
selection*treatment	1	1	0.0152270	7.3563	0.0072*
day*treatment	2	2	0.0096440	2.3295	0.0997
selection*day*treatment	2	2	0.0184874	4.4657	0.0125*

Table 2. Summary of results of a ANOVA on relative accessory gland size data using selection, treatment and day as fixed factors, crossed amongst themselves for block 2 .

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
selection	1	1	0.0984841	59.2535	<.0001*
day	2	2	0.0116880	3.5161	0.0313*
selection*day	2	2	0.0040141	1.2075	0.3008
treatment	1	1	1.0140246	610.0934	<.0001*
selection*treatment	1	1	0.0097418	5.8612	0.0163*
day*treatment	2	2	0.0128758	3.8734	0.0222*
selection*day*treatment	2	2	0.0049406	1.4863	0.2284

Table 3. Summary of results of ANOVA on relative accessory gland size data using selection, treatment and day as fixed factors, crossed amongst themselves for block 3.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
selection	1	1	0.0763587	54.7068	<.0001*
day	2	2	0.0251942	9.0251	0.0002*
selection*day	2	2	0.0082280	2.9474	0.0545
treatment	1	1	1.2315420	882.3322	<.0001*
selection*treatment	1	1	0.0398203	28.5291	<.0001*
day*treatment	2	2	0.0209558	7.5068	0.0007*
selection*day*treatment	2	2	0.0035593	1.2750	0.2814

Table 4. Summary of results of ANOVA on relative accessory gland size data using selection, treatment and day as fixed factors, crossed amongst themselves for block 4.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
selection	1	1	0.1213669	97.7312	<.0001*
day	2	2	0.0086624	3.4877	0.0322*
selection*day	2	2	0.0010315	0.4153	0.6606
treatment	1	1	1.3103841	1055.192	<.0001*
selection*treatment	1	1	0.0165958	13.3638	0.0003*
day*treatment	2	2	0.0074668	3.0063	0.0514
selection*day*treatment	2	2	0.0020088	0.8088	0.4467

Table 5. Summary of results ANOVA on relative accessory gland size data (for all the blocks combined) using selection, treatment and day as fixed factors, crossed amongst themselves and blocks as a random factor.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
selection	1	1	0.2634179	142.5798	<.0001*
day	2	2	0.0410198	11.1014	<.0001*
selection*day	2	2	0.0052779	1.4284	0.2402
treatment	1	1	4.6189250	2500.079	<.0001*
selection*treatment	1	1	0.0744070	40.2742	<.0001*
day*treatment	2	2	0.0131641	3.5627	0.0287*
selection*day*treatment	2	2	0.0050689	1.3718	0.2541

4. Discussion

The study is designed to study the change in relative size of accessory glands with days in *Drosophila melanogaster* populations adapted to larval crowding. We report a significant effect of adaptation to larval crowding on the relative size of accessory glands. In fact the populations that are adapted to larval crowding (CU population) shows a significant increase in the relative accessory gland size. Hence we concluded that selection for adaptation to larval crowding has led to a correlated increase in relative accessory gland size in males. We examined the relative accessory gland size in selected (MCUs) and control populations (MBs) by growing them at low larval densities (60 eggs in 6 ml food) and high larval densities (600 eggs in 2 ml food).It was observed that density negatively affects body size of flies as well as accessory gland size of males in both selected and control populations.

In the studies that are previously done on the same population, it was seen that CUs have evolved an increase adult longevity in both males and females as a correlated response to adaptation to larval crowding (Shenoi et al., 2015; Chapter 3). It was also observed that the increased longevity of CU males had no trade off with their courtship activity (Shenoi et al., 2015; Chapter 4a) and the investment in testis (in terms of relative testis size) ((Shenoi et al., 2015; Chapter 6). Hence we hypothesized that there might be a trade-off between longevity and investment in accessory glands in CU population. But surprisingly, we found that CU males have evolved larger relative accessory gland size than MB males. Thus the smaller CU males had a significantly larger accessory glands than MB males (which are remarkably larger in body size than CUs). Thus no longevity- reproduction trade-off could be observed in the CU population till now.

Nutrition plays a major role in determining of body condition of many organisms. In a holometabolous insect like *Drosophila melanogaster* where adult body size ,adult fitness and energy acquired are largely dependent on pre-adult nutritional levels, larval density can act as a major ecological stressor that can modify the life-history of these organisms. The

CU populations on which the study is done are always faced with low nutritional levels and high density during the larval stage. Since they are been under this selection for more than 240 generations it is expected that the CU populations are already adapted to such low nutrition conditions. This would lead us to assume that CU population is able to deal better with low nutrition conditions that MB populations and thus emerge with a better body condition. Therefore the larger accessory glands of CUs may be a reflection of their better body conditions.

It is observed that during low density treatments also CUs have significantly larger accessory glands than MBs. This could be because adaptation to high larval densities might have shaped CUs in such a way that they are able to maintain better body condition even at lower larval densities.

Larval density also have significant effect on relative accessory gland size. In both CU and MB population, males that are cultured in low larval treatments are seen to have significantly larger accessory glands than in high larval density treatments. Thus both the selected and control population invests more into accessory gland tissue when grown at a relaxed larval condition.

Effect of age of the fly (age in days) on relative accessory gland size was also observed to be significant in each of the four blocks. In blocks 1, 2 and 4 the size is minimum on day 1, increases to a maximum on day 2 (significant) followed by a decrease on day 3(significant in block 1 but not in block 2 and 4) . On day 2 on which the accessory gland attains maximum size, the fly is 21 days old starting from egg collection (22 days old in block 4). It is to be noted that CU and MB population are maintained on a 21 day cycle i.e. on day 21, eggs are collected from the present generation to start the next generation. In conclusion, day 21 is the day on which future generation is initiated from the present generation. In effect, the males whose progeny are selected for the next generation are the ones who have successfully contributed to the eggs laid by females on day 21. Therefore, accessory glands acquiring maximum size on day 21 could be explained as a correlated response of the 21 day maintenance cycle. This could be due to the increased investment

on accessory gland tissue on day 21 which is a crucial day in the life cycle of CUs and MBs. It is known that size of the accessory glands and testes reduces significantly after successive matings (Jon R. Linklater, Bregje Wertheim, Stuart Wigby, and Tracey Chapman, 2007). Hence, decrease in mating frequency on day 21 could be a possible reason of accessory glands being the largest on the same day. Further experiments can be performed in-order to find the mating frequency on different days. These experiments might give a better idea of why accessory glands are the largest on day 21 in these 3 blocks.

Effect of day (age of the fly) on relative accessory gland size is showing a different trend in block 3 .In block 3 accessory glands attain a maximum value on day 24. However day (age of the fly) is seen to have a significant effect on relative accessory gland size in all the four blocks.

However when all the four blocks were combined we couldn't find a significant effect of age of the fly on relative accessory gland size. Also we couldn't see any interaction effect of day x selection, day x treatment, and selection x day x treatment.

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