Role of mitochondrial genome in sexual conflict in *Drosophila melanogaster*

Karan Bhatt

A dissertation submitted for the partial fulfillment of BS-MS Dual Degree in Science

Indian Institute Of Science Education and Research, Mohali

April, 2018

Certificate of Examination

This is to certify that the dissertation titled "Role of mitochondrial genome in sexual conflict in *Drosophila melanogaster*" submitted by Mr. Karan Bhatt (registration number MS13035) 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 20th, 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 acknowledgements of collaborative research and discussion. This thesis is a bona fide record of original work done by me and sources listed within have been detailed in the bibliography.

Karan Bhatt
Dated: April 20th, 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)

List of Figures

List of Tables

Contents

Acknowledgments

I would like to thank NGP for being an awesome guide and allowing me to join this wonderful lab/family called EBL.
I would also like to thank my committee members Dr. Manjari Jain, Dr.Rhitoban Ray Choudhury for their support. I Also thank the Evo-Gen lab, especially Manisha Gupta for helping me run the assays (or running them by herself) during this project.
I thank all the past lab members; Vanika di, Vinesh, Karan Singh, for teaching me fly handling and other lab work. & I am thankful to all the undergrads Tejju, Nitin, , Pareshnath, N.S., Meena, Vandana, Mayank, Poddar, who helped me during the experimental observations. I am grateful to have seniors/friends (Sharmi, Saudamini, Anubhuti, Radhike, Martik, Ekta, MTT, Punjani ji) who made my stay in the lab/campus memorable. I must thank Nagendra bhaiya for **a**. providing clean vials, cages, plates and **b.** the money I made in his absence. I would like to thank Aparajita, AKB, Reshma, Vaibhvi, Aatashi, Harsha, JJ, UDB, Komal, Neetus, Punjani ji , Ziggs for their support during this project.
Now I would like to thank some people because of whom I was able to complete not only my thesis project, but also the five year long journey at IISERM. Zeeshan (Badka ji)-from fly handling to life lessons, covering/correcting my mistakes, and as he says brother from another mother. Tj Chechi (bhai bhai bhai)-for all the life lessons, for south-indian movies, for always having my back. Manas bhai-for helping me even when he was high on his saste nashe. JJ-awesome wing-woman on whom I can count. Rohit Kapila-the funny guy in the lab. Prakhar-for disappointing me every single time. Agarwala-from writing mails to providing laundry coupons/ bandaid/ ya jo kuch bhi, drama. Broti – for all the panchayats, for helping me during data-entry (work I never wanted to do). Pratibha-for coffee, drama, data entry and telling me why one should write the acknowledgement.
I would like to thank Rohit and Idu (for telling me to join iiser), Kausal and Kalik ji-for all the guru gyan, Viru-the tubelight, Akhil-responsible guy in the group, Maansaab+Pala+Praveen-for COD, Sidharth-make me feel proud one day, Alkit- for saving the penalty.
And Yoda.

In the end, I would like to thank my family for their support and encouragement even though they have no idea what I was doing.

1. Abstract

Earlier Mitochondrial genome variations were considered to be neutral. Now many of the studies have shown the mitochondrial and cytoplasmic variation in life history and fitness traits.Mitochondrial genome interacts with the nuclear genome and form enzyme complexes(oxidative phosphorylation) which leads to ATP production.Genetic variation in the cytoplasmic gene can hence affect the metabolism and aging.From mitochondrial aging hypothesis, we know that reactive oxygen species plays a major role in senescence of the individuals.From these all studies we know that the Mt-DNA are not selectively neutral.Positive selection is there in the mitochondrial genome, and Fitness consequences arise from the mt-DNA variation.The production of high-quality sperm is itself energetically demanding. There are many other studies suggesting mitochondrial genome's role in sperm competition theory.

Mitochondrial variation can be maintained within/between the population as Mito-nuclear genotype where the nuclear genome is common in all population.Here using laboratory adopted populations of Drosophila melanogaster selected for the different level of sexual conflict were used to create these mitochondrial-nuclear lines where different mitochondrial genome was expressed in a common nuclear genome background.Now, these different families were used to check the role of the mitochondrial genome and see how the same has evolved under the sexual conflict. Different reproductive traits(sperm competitive ability and mating behavior) were measured between the two population.We do not find any difference in the sperm competitive ability between the families of two different population in all three blocks, the same pattern can be observed for the copulation duration. However, there is a difference in copulation duration in one block where F regime has higher copulation duration than M regime.

2. Introduction

Variation in mitochondrial DNA (mtDNA) has been traditionally assumed to be neutral(1). However, recent work has shown that mitochondria harbor significant fitness-related variation. These non-neutral mitochondrial variation can have many evolutionary implications(Dowling et al 2008). Furthermore, since oxidative phosphorylation, where mitochondria plays the primary role in Eukaryotes, requires coordination between mitochondrial and nuclear genes, there is scope for 'mito-nuclear fitness interactions, such that selection acts on composite mitochondrial-nuclear genotypes. Additionally, as a result of their asymmetric mode of inheritance, mitochondria are expected to accumulate alleles that benefit females
even if they harm males(8).

Many experiments have shown that these variations affect phenotype and life history traits(5,6). Mitochondrial DNA has a major role in energy metabolism, variation in the metabolic rate may be associated with variation in lifespan (Sacher 1977) and genetic variation in mtDNA could conceivably affect both metabolism and aging (Beckman and Ames 1998; Speakman 2005).

Interlocus Sexual conflict, a characteristic of promiscuous populations, stems from the differential investment made by the two sexes per bout of reproduction. Generally, females invest a lot more per bout of reproduction compared to males. Therefore, the optimum mating rate for females is much lower than that for males. This conflict over mating rates has been shown to lead to an antagonistic "arms race" between males and females. Both males and females evolve traits that increase their own fitness, even if its at the cost of the fitness of the other sex. Males evolve "persistence" traits that increase the average mating rate of the population, while females evolve "resistance" traits that bring down the average mating rate of the population.

Most of the theoretical work on interlocus sexual conflict has overlooked the possibility of the role played by the mitochondrial genome in male-female antagonistic coevolution, and there is a dearth of empirical studies investigating the same. In this study, we looked into the role of the mitochondrial genome in sexual conflict, using Drosophila melanogaster as model organism. Many studies have looked into the effects of these mitochondrial variations within a population,(3,4) Here we tried to investigate whether there is any difference when these variations are measured between two population.In the present study, we planned to experimentally investigate whether mtDNA has had a role to play in sexually antagonistic coevolution observed in laboratory populations of Drosophila melanogaster.

2.1 Study System:

Our study system consists of a total of six populations, all of which trace their ancestry to a large lab-adapted population "LHst", which has a benign and recessive "scarlet eye" marker (in the LH genetic background). Three populations, called M1-3, are maintained at adult sex ratios of 3 males: 1 female. The remaining three populations, called F1-3, are maintained at adult sex ratios of 1 male: 3 females. The M populations experience elevated levels of sexual selection and interlocus sexual conflict relative to the F populations. Males and females from the M populations have been shown to have undergone rapid sexually antagonistic coevolution: relative to F males, M males court more frequently, are more active and have higher sperm offence and defense abilities; they are also more harming to baseline females (the evolution of these costly reproductive traits appears to have come at the cost of reduced longevity); relative to F females, M females have higher resistance to male mate-harm, which has come at the cost of basal levels of fecundity and longevity(7).

In order to assess the evolution of the mitochondrial genome in this sexually antagonistic coevolution, we established 25 mitochondrial families from each of the six populations (M1-3 and F1-3). First, we collected 25 virgin females from each population and crossed them (a single male and a single female) to males from the LHst population. This resulted in a total of 150 families, each with a unique mitochondria. In subsequent generations, we collected 10 virgin females from each family and crossed them to 10 LHst males, to bring all the families inthe common nuclear background. Every generation, on an average, the proportion of the original nuclear genome decreased by a factor of two. We continued this back-crossing procedure for nine generations, by the end of which we had families with mitochondria from either M1-3 or F1-3 populations and nuclear genome from the LHst population.

2.2 Experiments performed:

As explained earlier, before starting with the experiments, we had to bring all the 150 families into the common LHst background, which required around 6 to 7 months of backcrossing the females generated from the mitochondrial lines, with the common LHst males. After managing to bring the common nuclear genome background, in all the mitochondrial families, we performed the following assays using males from these families:

1. Sperm competitive ability (sperm offense and sperm defense) -Sperm competition is considered as a major evolutionary force in male-male competition(Parker, 1970; Birkhead & Møller, 1998; Simmons, 2001) Production of high-quality sperm is energetically demanding and sensitive to ROS production. Sperm motility is also associated with mitochondria present in the sperm(midpiece and tail region) for energy production(8-9). There are many other studies suggesting mitochondrial genome's role in sperm competition theory. In this experiment, we measured the sperm competitive

ability(sperm offence & defence) in the mitochondrial lines generated from the sex-biased population, when expressed in the common nuclear genome.

2. Mating behavior (copulation duration)- Sperm competitive ability can be associated with the mating behavior of the individual. The increase in the sperm competitive ability can be explained because of the difference in the copulation duration in the two population.In order to check this behavior, the copulation duration for the mating pairs was calculated.

3. Materials and Methods

3.1 Making of mitochondrial(CYTO)-nuclear lines:

We used a laboratory adapted population of *Drosophila melanogaster* selected for different level of sexual-conflict,M(Male biased 3:1), C(Control 1:1) and F(Female biased 3:1).The first step was to create different mitochondrial lines representing different mitochondrial genome. As we know that the mitochondria are transferred from the mothers to their progeny along with the cytoplasm, so the females of the sex-biased population were used to make the different cytoplasmic lines. Since the mitochondria is transferred along with the cytoplasm each cytoplasmic line represent the single mitochondrial line . For the details of the MCF refer to the chapter 2 of the thesis of Dr.Bodhisatta Nandy(2013). To express the common nuclear background, the females of these population were backcrossed with baseline population (LHst) males for 10 generations assuming the whole nuclear genome is then replaced with the LHst genome and hence creating different mitochondrial lines containing same nuclear genome.

3.2 Initiation of Mito-nuclear(MN) Lines:

First, the eggs were collected(150/vial) 5 vials of each 3 blocks of M, F respectively on cornmeal molasses food. (for food composition refer to table 1). For the preparation of food media refer to the thesis of Dr.Bodhisatta Nandy(2013). We know that the mitochondrial genome is transferred from females only. After eclosion of flies on the 12th day, 25 females were sorted and single females were transferred to cornmeal molasses vials(containing small yeast granules (to increase the fecundity). After 18 hr incubation window, the flies were discarded, and eggs(Gen.0)were reared on the same vials. So in total 150 females were used to create 150 different mitochondrial lines(25 families 0f M, F each*3 blocks). The LHst baseline population is used for the collection of males. These males were used during the backcrossing of focal females.About 150 eggs/ vial, total 30 vials of LHst were collected on cornmeal molasses food.Now we have 150 different mitochondrial lines each line represent the mitochondrial genome of respective female from which the family is derived.

3.3 Stock maintenance.

On day 9-11, 16 virgin females were collected with the help of CO2 anesthesia and kept in the small pour food vials. On the 14th day, the LHst males were sorted 5 males per vial in large pour cornmeal food containing small yeast granules. From the 16 virgin females , 5 females per vial were combined with males.On day 16 the flies were transferred to fresh large pour food vials for egg collection (stock).After the incubation of 18hrs flies were flipped again in fresh vials for backup egg collection. The egg density in stock vials was trimmed to nearly 150 eggs. For the maintenance representation refer to figure 1,2. Now that we finally have 25 families/population and 2 vials of each family. Total 150*2 different families were then maintained with the ratio of 5m:5f for four generations. The virgin females were collected from the stock vials and on 14th day were backcrossed with LHst males. Again the same routine is followed for egg collection as mentioned above. For next five generation the flies were then reared at the ratio of 16:12(male, female).

Earlier studies have pointed out that the effects caused by mitochondrial genome may be the result of the bacteria present in the cytoplasm(Hoffmann et al., 1986). To eliminate this, the flies were treated with TCA solution(Hoffmann et al., 1986) in cornmeal food during the egg-laying window in 7th generation. Next generation, DNA was isolated from the flies from all the populations and was checked for the presence of cytoplasmic bacteria, Wolbachia. The results showed that no Wolbachia is present in TCA treated flies. After one generation the flies were again treated with the TCA.

From generation 10, the flies were then maintained in the ratio of 16:16 and instead of backcrossing the virgin females with the baseline population males, the males of the same line were used. Now the flies were cultured (i.e., the mated flies were sorted in the ratio of 16:16 with the help of CO2 anesthesia) on 14th day and for stocks the eggs were collected on the 16th day.

After six months of time, we were able to make 150 different cytoplasmic(mitochondrial) lines having same (LHst)nuclear genome, with half the lines (75 lines) having mitochondria from M regime (25 families per block of M regime) and half the lines (75 lines) having mitochondria from F regime (25 families per block of F regime), (Table 1). Now from these lines, experimental flies were generated(refer to experiment setup) on which different assays were performed to measure the different male reproductive traits.

MN M1 1-25	MN F1 1-25
MN M2 1-25	MN F2 1-25
MN M3 1-25	MN F3 1-25

Table 1. 25 families/ block/ regimes . A total of 150 families.

S.NO	**INGREDIENT**	**AMOUNT (PER LITRE OF FOOD)**
1.	Water	1100ml
2.	Agar	14.8gm
3.	Molasses	100ml
4.	Corn Meal	100gm
5.	Baker's yeast	41.2gm
6.	Propionic Acid	8ml
7.	p-Hydroxymethyl Benzoate	2.25gm
8.	Ethanol	22.5ml

Table 2. The composition of corn meal-molasses food.

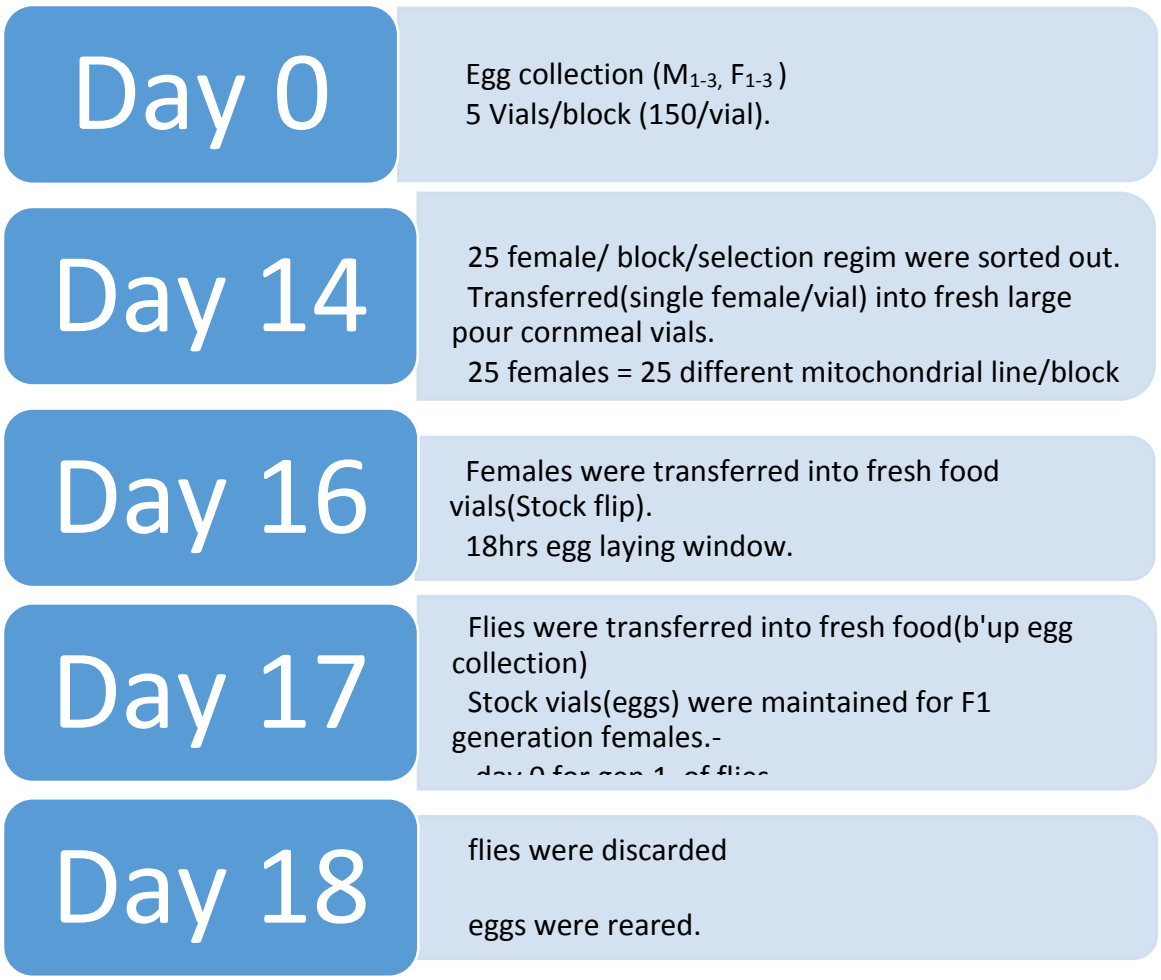

Figure 1. Representation of **A**.Making 25 different mitochondrial lines from each block (M_{1-3}, F_{1-3}). **B.** Egg collection for generation one of these flies.

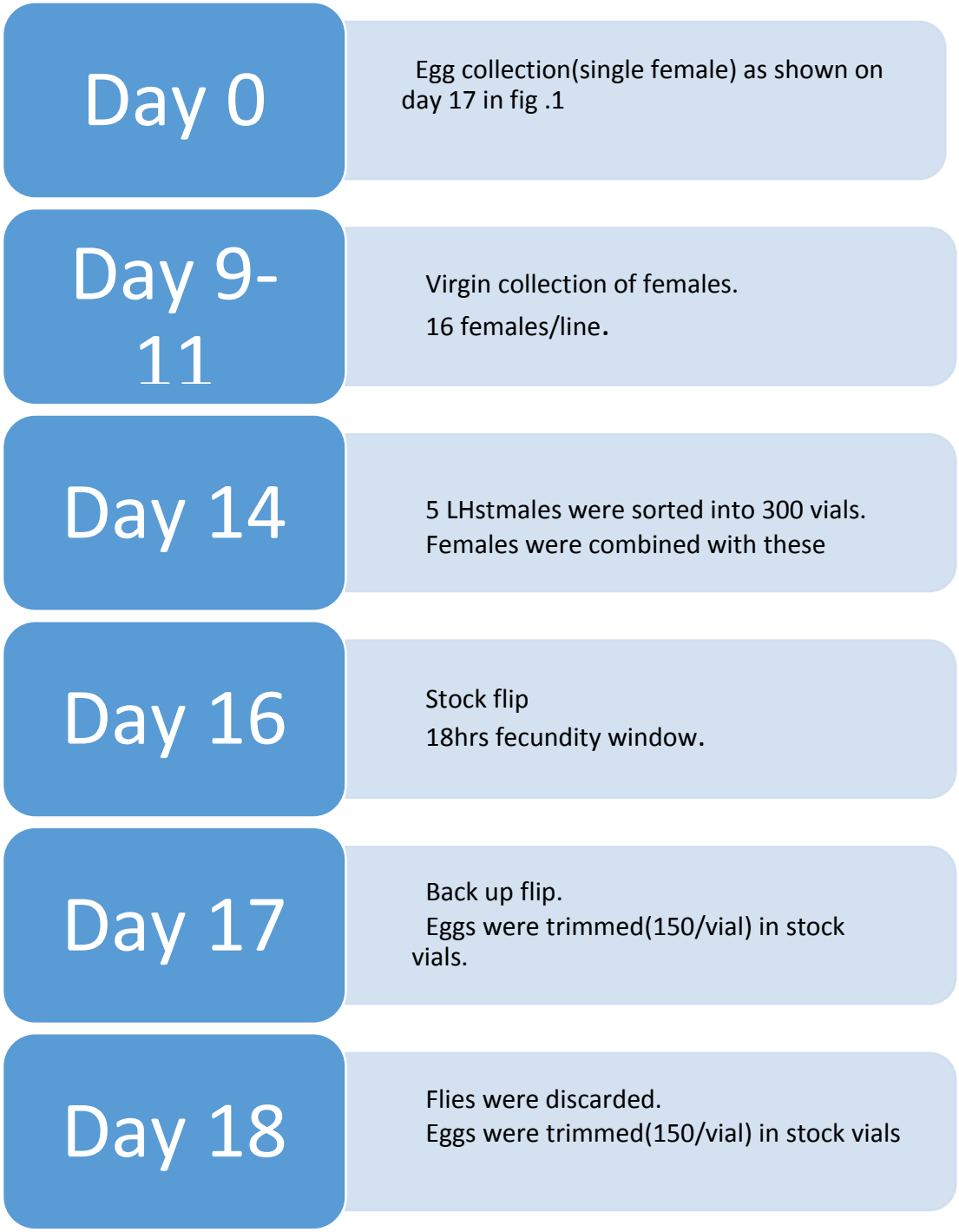

Figure 2. Representation of different mitochondrial lines(M,F) into a common nuclear(LHst)background.

4. Experimental Assays and Set Up

Once the mitochondrial lines were 10 generation old, we measured the different male reproductive traits in these lines. The comparison was made between the lines of the two populations which were selected for the different level of sexual conflict.

We performed different assays to check:

1. Mating behavior (copulation duration).

2. Sperm competitive ability.

    a. Sperm defense

    b. Sperm offense.

Generation of Experimental flies:

The eggs were collected from the stock vials by taking an extra flip (transferring the adults into the fresh vial(8-10ml food)) on the 18th day and giving them egg laying window for 18 hrs. After that flies were discarded and eggs (for focal male) were trimmed to the estimated density of 150eggs/vial. On the same day (18th) 3.6 bunches(1bunch = 10vials) of eggs were collected of LHst(for common female) & LH(for competitor male) from their cages by giving them fresh food plate for 8hrs(fecundity window), with exact 150 eggs per vial. The eggs were first counted and collected on agar and then transferred to the vials. From 8-10th day post egg collection, virgins were collected with the time gap of 5-7 hrs. All the virgin focal males(MN line male) were collected(4 vials, 8males/vial, per family) in vials containing 1-3ml corn-meal molasses food.Similarly, 20 bunches of LHst virgin females(8/vial) and 4.5 bunches of LH virgin males (8/vial) were collected.All the experiments were performed on 11th day.

1. Mating behavior (copulation duration)

On day 11 the experiment was performed to check the copulation duration of the focal males. Both male(focal) and female vials(8 individual/vial) were flipped into a fresh food vial. For each line, two vials were used during this combination flip.The vials were then observed for 70 min. and the total number of mating pairs(maximum 8 pairs) were counted every 1 min. Observations were taken until all the mating pairs value come to 0(from 8 to 0 because 8 pairs were used).The experiment was performed at standard room temperature 25c.

2a. Sperm defense (P1)

Virgin common females(LHst) were combined with the focal males(8males:8females),once all the females have mated the males were then discarded using CO2 anesthesia. The females were then given a 2hr recovery time from CO2. After 2hrs the females were then combined with competitor male(LH) and vials were reared in standard lab conditions for 48 hrs.After this, the males were discarded again, and the females were transferred to test tubes containing the fresh food.After 18 hrs egg laying window, the females were discarded, and test tubes were maintained under lab conditions for 11-12 days.On 11-12th day the test tubes were checked for making sure that all the flies have eclosed and then the test tubes were frozen in -20 for progeny count.

Since our focal male have scarlet eye color and the competitor male(LH) is red-eyed, we used the eye color as a marker to identify the progeny that was sired by the focal male(scarlet eye) and competitor male(red eye).The proportion of scarlet eyed individuals in total progeny can be observed as P1. Experiment setup's detail is well described in the thesis of Dr.Bodhisatta Nandy(2013).

2b. Sperm offense (P2)

The setup for this assay was similar to the earlier one (P1), but here the females were first combined with the competitor male and then the focal male. But when the females were combined with the focal male a single pair of flies was aspirated into the fresh vial(total 400vials, 8pair/line). These vials were observed for 40-48hrs for remating. Once the mating is complete, the female was immediately transferred to the test tube for laying eggs, and after 18 hrs females were discarded and test tubes were maintained for the progeny count.

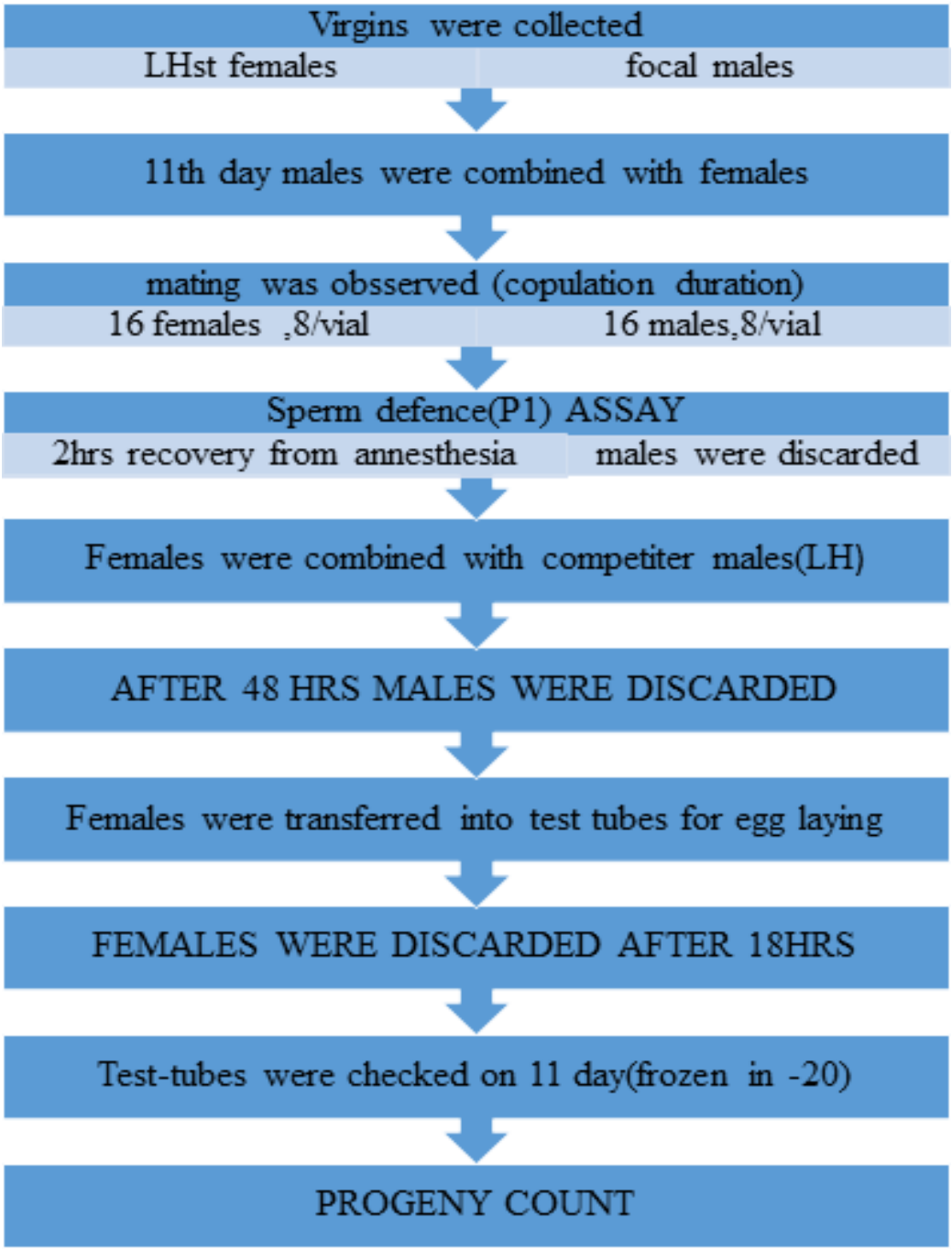

Figure 3. Experiment setup - Sperm defence (P1) & Copulation duration.

5. Data Analysis and Results

All data was analysed using JMP 7. Graphs were plotted with the help of SIGMA plot.

1. Sperm defence(P1): P1 values were arcsin square root transformed. Blocks were analysed separately. Selection was modeled as a fixed effect. Line/family was modeled as a random effect nested within Selection.
2. Sperm ofence (P2): Analysis for P2 was similar to P1.
3. Copulation Duration: Blocks were analysed separately. Selection was fixed and Line was a random factor nested within Selection.

A. P1 (fig.4.1,4.2,4.3)

In alL three blocks there is no differecnce in the sperm defence ability between the two population.

Factor		Nparm	Df	DfDen	F Ratio	p	
SELECTION		1	1	48.2264	0.000137	0.990719	**Block 1**
SELECTION		1	1	47.61439	0.951903	0.334167	**Block 2**
SELECTION		1	1	47.80538	2.941095	0.092829	**Block 3**

	Mean	SE	Mean	SE	Mean	SE
	Block 1		Block 2		Block 3	
F	0.562844	0.023897	0.604128	0.021399	0.491585	0.021296
M	0.562445	0.024439	0.574432	0.021645	0.440569	0.020771

B. P2(FIG. 5.1,5.2,5.3)

In all three blocks there is no difference in the sperm defence ability between the two population**.**

Factor	Nparm	Df	DfDen	F Ratio	p	
Selection	1	1	46	1.670714	0.202618	Block 1
Selection	1	1	48	0.487098	0.488592	Block 2
Selection	1	1	46	0.013535	0.907891	Block 3

	Mean	SE	Mean	SE	Mean	SE
	Block 1		Block 2		Block 3	
F	0.802887	0.029876	0.846352	0.034309	0.881821	0.021164
M	0.857499	0.029876	0.812488	0.034309	0.878339	0.021164

C. Copulation Duration(fig.6.1,6.2,6.3)

There is no difference in block 1,2 but in block 3 F regime have more copulation duration than M regime.

	Nparm	**Df**	**DfDen**	**F Ratio**	**p**	
Selection	1	1	48.15504	0.053614	0.817871	Block 1
Selection	1	1	48	2.976586	0.090911	Block 2
Selection	1	1	45.93323	8.542228	0.005369	Block 3

	Mean	SE	Mean	SE	Mean	SE
	Block 1		Block 2		Block 3	
F	15.43836	0.363429	16.92302	0.328644	16.56436	0.344112
M	15.31884	0.366522	17.72488	0.328644	15.13983	0.345177

Figures:

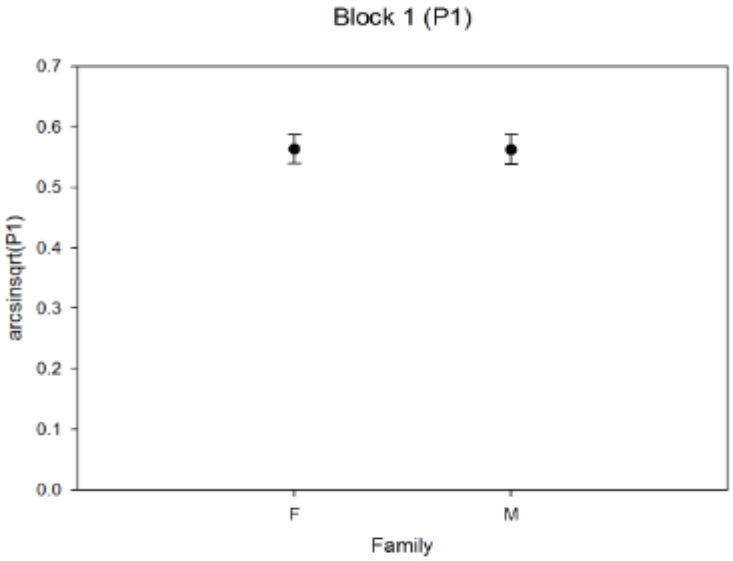

Fig.4.1. Sperm defence(p1) ability of block one(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=0.990719.

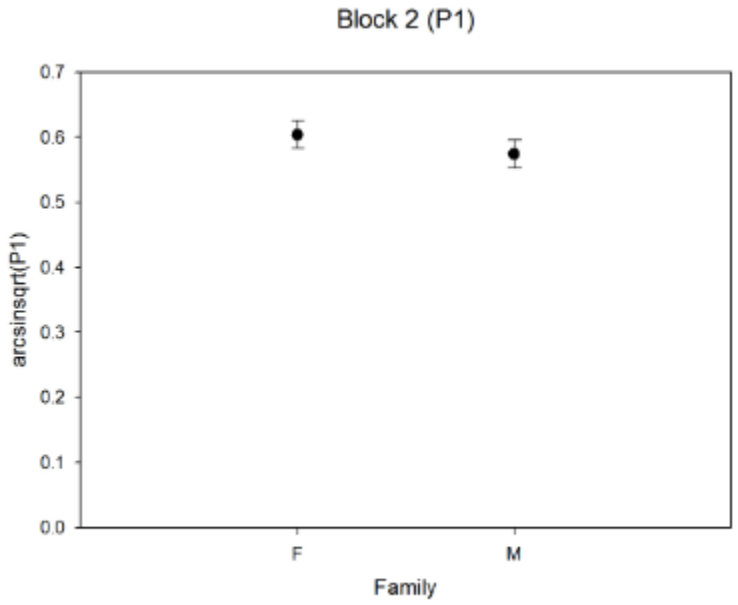

Fig.4.2. Sperm defence(p1) ability of block two(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=0.334167.

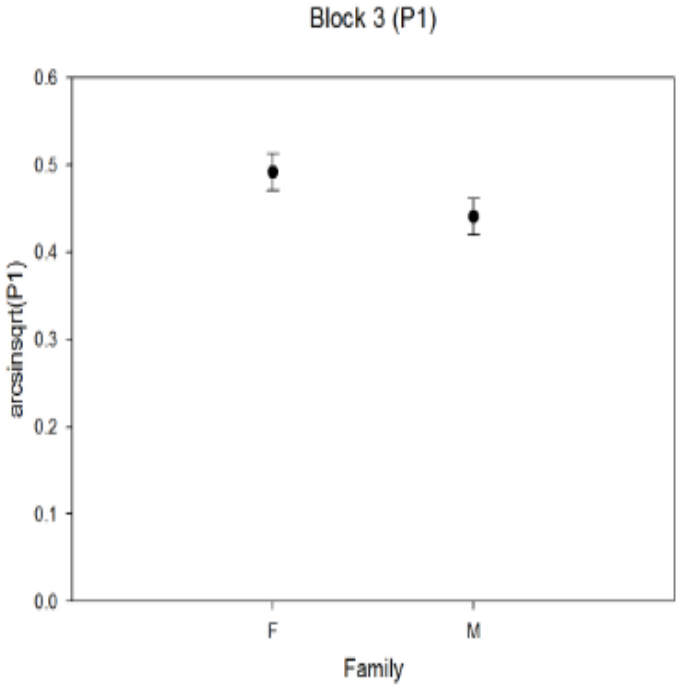

Fig.4.3. Sperm defence(p1) ability of block three(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=0.0928

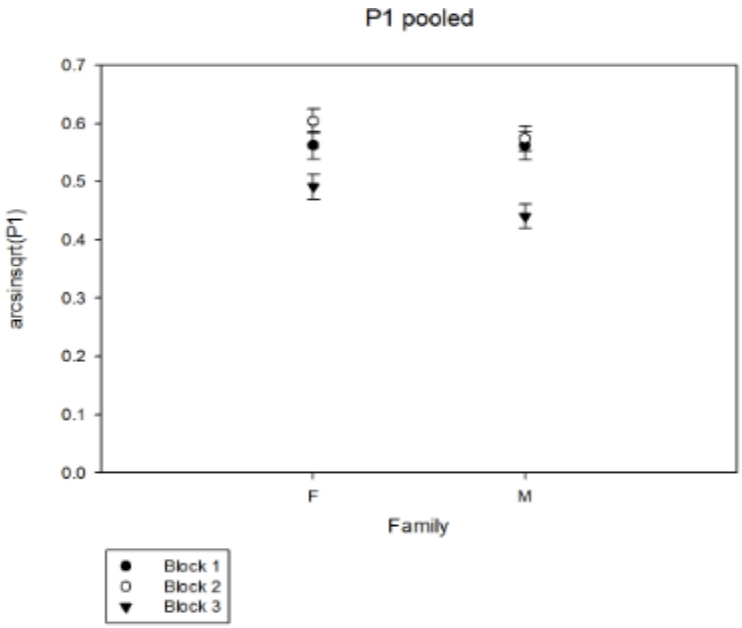

Fig.4.4. Sperm defence (P1) in b1,b2,b

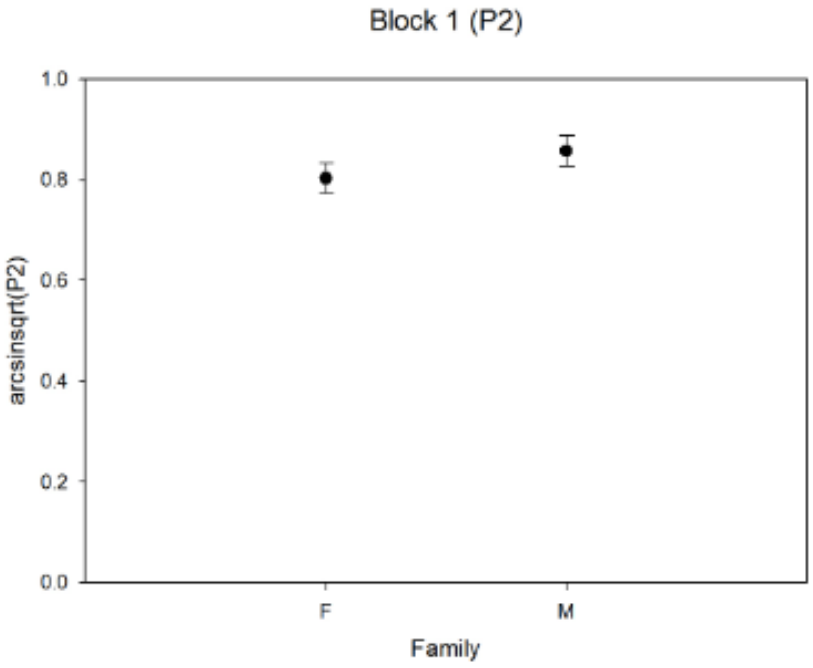

Fig.5.1. Sperm offence(P2) ability of block one(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=.0.202618.

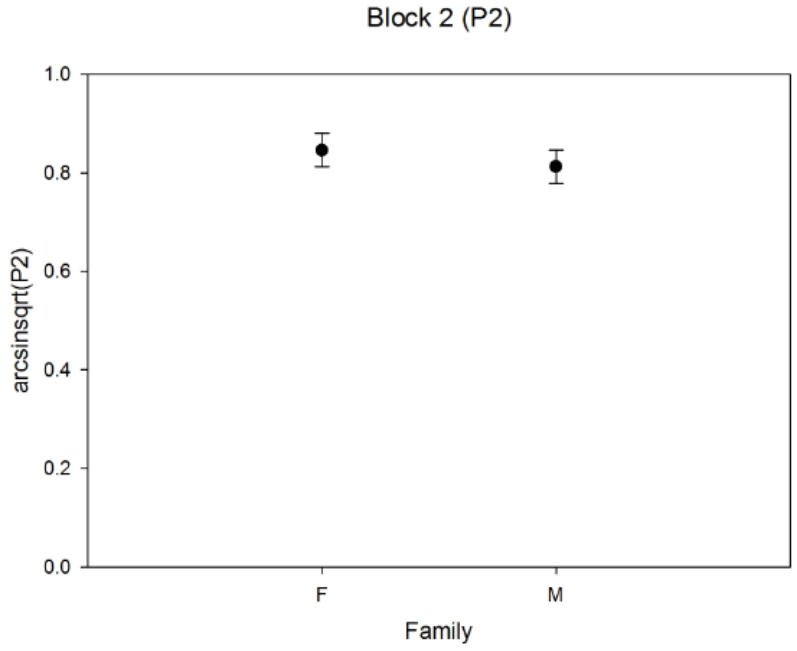

Fig.5.2. Sperm offence(P2) ability of block two. (F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=.0.202618.

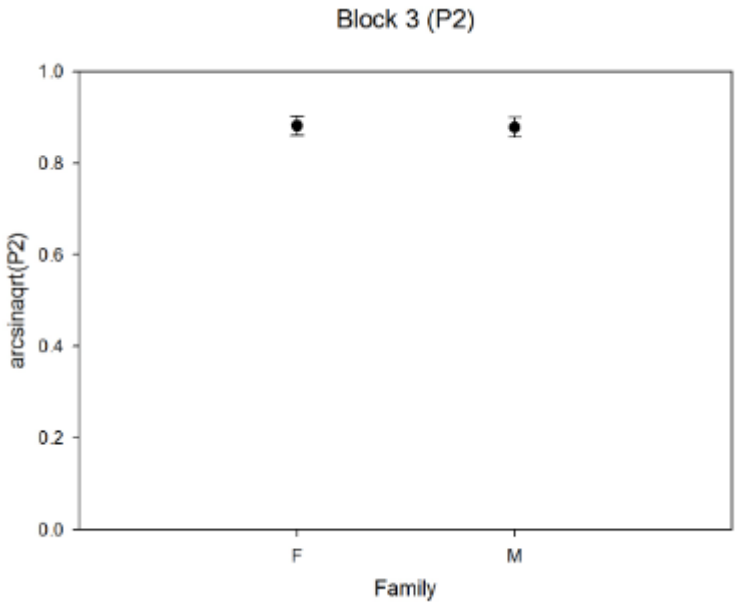

Fig.5.3. Sperm ofence(P2) ability of block three(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=.0.202618.

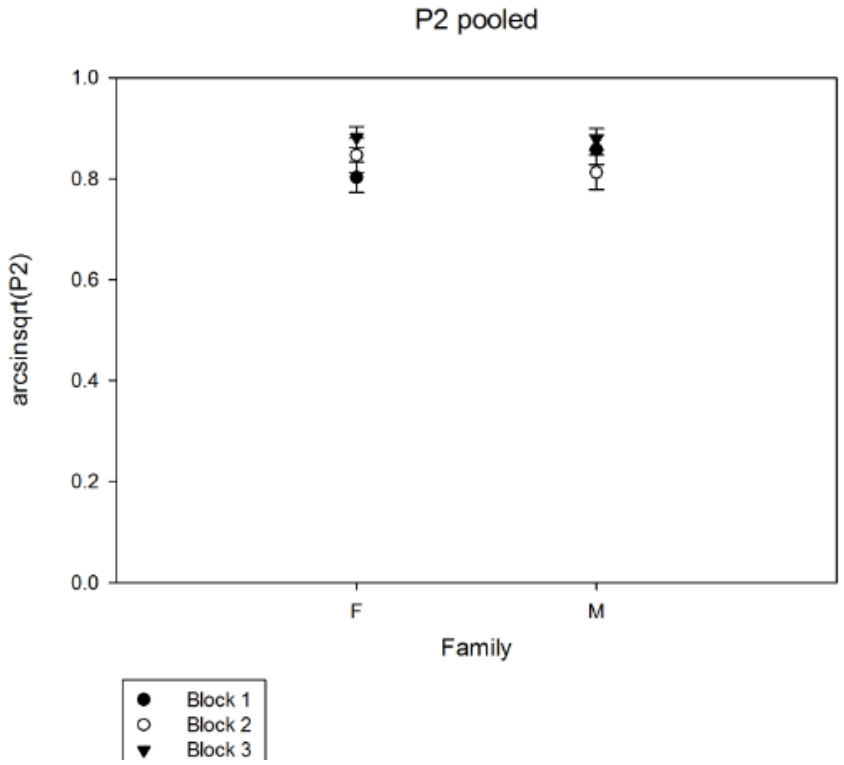

Fig.5.4. Sperm offence(P2) ability of block1-block3.

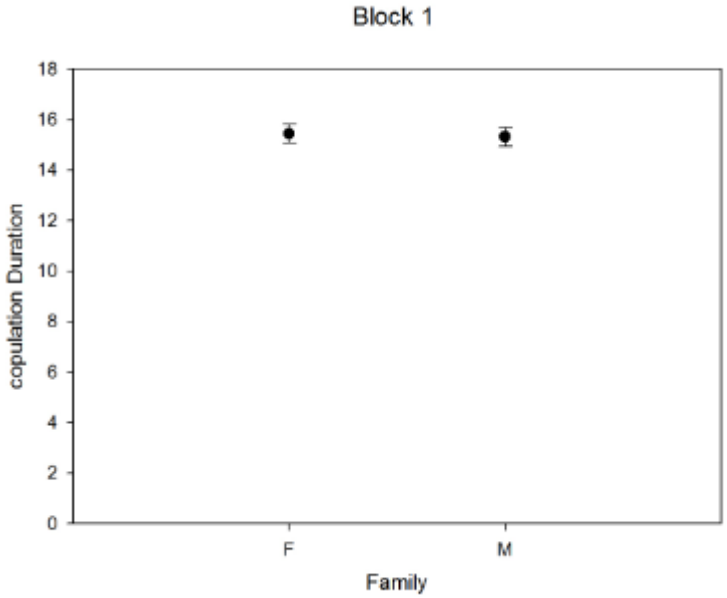

Fig.6.1. copulation duration of block one(F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=0.817871.

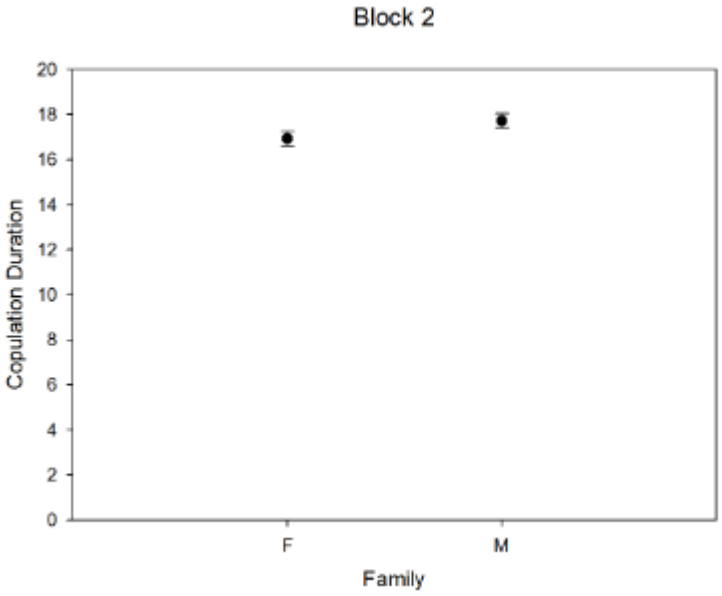

Fig.6.2. copulation duration of block two (F=mitochondrial genome of female boased regime,M=Mitochondrial genome of male biased regime).P=0.090911.

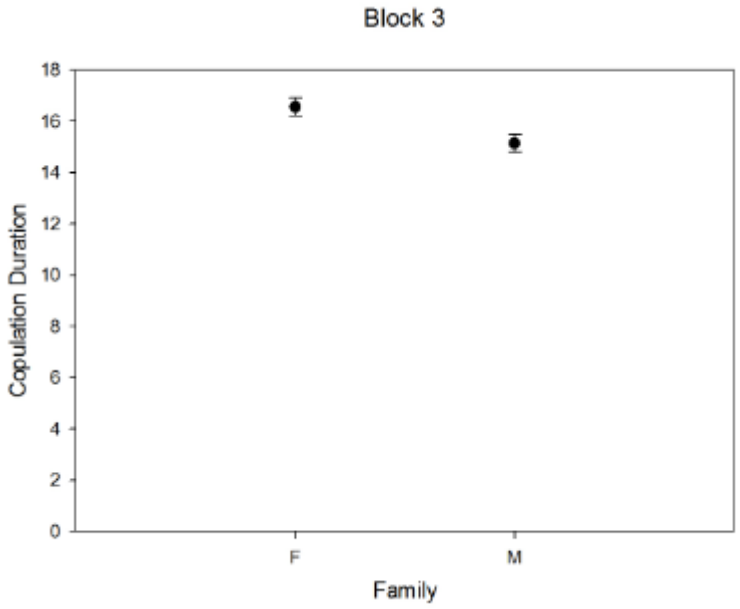

Fig.6.3. Copulation duration of block three (F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).P=0.005369.s

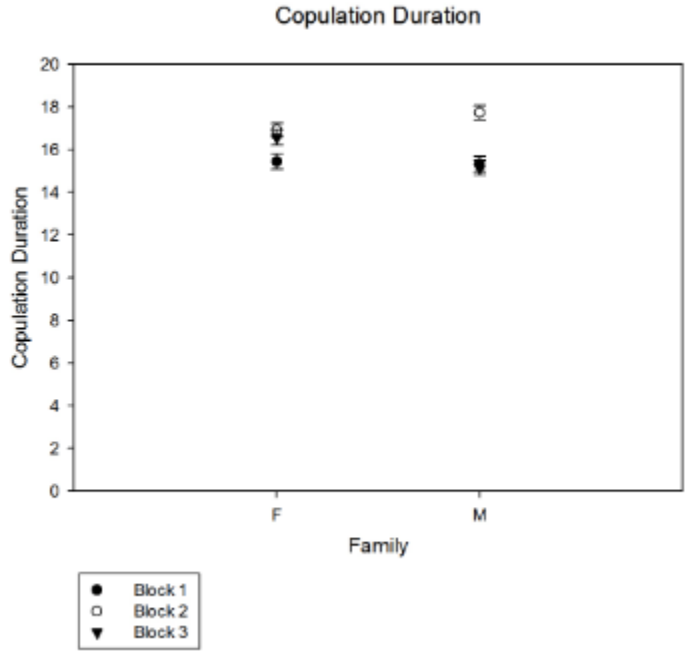

Fig.6.4. Copulation duration of block three (F=mitochondrial genome of female biased regime,M=Mitochondrial genome of male biased regime).

5. Discussion

To check the role of mitochondrial genome in the sexual conflict, we create different families having different mitochondrial genome of population of *drosophila melanogaster* selected for differential levels of sexual conflict. In these lines, we looked at the sperm competitive ability and copulation duration of the males. Many studies have used this system(cytoplasmic genomic variation).The variation in cytoplasmic genome affects female life span and aging within population of *Drosophila melanogaster*(Maklakov et al., 2006). No cytoplasmic variation was found when male offence or offensive sperm competitiveness was checked within the population (U. FRIBERG D. K. DOWLING(2008). Here in our study system we checked the cytoplasmic variation in reproductive traits between the populations.

In our experiment results we found that there is no difference in the sperm competitive ability between the 2 populations selected for different level of sexual conflict in all three replicates. Similarly there was no difference in copulation duration of these males in 2 blocks and in B3 the F regime have higher copulation duration.

We know that in these sex biased population the male mail traits have evolved in response to the sexual conflict. Males from F regime evolved reduced P1 and P2, Males in the male-biased regime evolved increased P1, but there was no significant change in P2. Increase in P1 was associated with an increase in copulation duration(Nandy et al 2013 Evolution (sperm competition)).From these results we can say that the nuclear genome have the role in the evolution of these traits in males but not the mitochondrial genome. Also since the mitochondria is transferred only from females and possibly the mitochondrial genome in these females have evolved because of the sexual-conflict.

Since sperm ‘behavior’ is likely to depend upon energy production, and mitochondria packed therein is the ‘powerhouse’ of sperm cells, we would also like to investigate energetic properties of the sperm, such as sperm velocity, amount of oxidative stress (reactive oxygen species), etc., from all the families.One can also measure the longevity of these population as we know from the mitochondrial aging hypothesis that ROS is responsible for the aging of the individual.

6. References

1. Ballard, J.W.O. and Kreitman, M. (1995) Is mitochondrial DNA a strictly neutral marker?

2. Dowling et all(2008) doi:10.1016/j.tree.2008.05.011

3. Rand, D.M. et al. (2001) Sexually antagonistic cytonuclear fitness interactions in *Drosophila melanogaster*. Genetics 159, 173–187

4. Dowling, D.K. et al. (2007) Intergenomic epistasis for fitness: within population interactions between cytoplasmic and nuclear genes in Drosophila melanogaster. Genetics 175, 235–244

5. Dowling et al., (2008,2014)

6. Ellison & Burton, 2008; Meiklejohn et al., 2013

7. Nandy, B., Chakraborty, P., Ali, Z.S. and Prasad, N.G. 2013. Sperm competitive ability evolves in response to altered operational sex ratio. Evolution. doi:10.1111/evo.12076.

8. Dowling, D.K. et al. (2007) Effects of cytoplasmic genes on sperm viability and sperm morphology in a seed beetle: implications for sperm competition theory? J. Evol. Biol. 20, 358–368

9. Blount,J.D.etal.(2001))Antioxidants, showy males and sperm quality. Ecol. Lett. 4, 393–396

10. Nandy B(2013)Of War and Love: A Study of Sexual Conflict and Sexual Selection Using. Drosophila melanogaster. Laboratory system. PhD Thesis,IISER

11. Maklakov, Alexei & Friberg, Urban & K Dowling, Damian & Arnqvist, Göran. (2006). Within-population variation in cytoplasmic genes affects female life span and aging in Drosophila melanogaster. Evolution; international journal of organic evolution. 60. 2081-6. 10.1111/j.0014-3820.2006.tb01845.x.

12. U. FRIBERG D. K. DOWLING(2008)No evidence of mitochondrial genetic variation for sperm competition within a population of Drosophila melanogaster https://doi.org/10.1111/j.1420-9101.2008.01581.