Effect of Cold Shock on Sperm Storage in Female *Drosophila melanogaster*

Thesis submitted in partial fulfilment of the requirements of

five year BS-MS Dual Degree Programme

By

Ruchika Choudhary

Indian Institute of Science Education and Research, Mohali

Under the guidance of

Dr. N.G. Prasad,

Department of Biological Sciences,

Indian Institute of Science Education and Research, Mohali

Certificate of Examination

This is to certify that the dissertation titled "Effect of Cold Shock on Sperm Storage in Female *Drosophila melanogaster*" submitted by Ms. Ruchika Choudhary 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. T. R. Rao | Dr. R. R. Choudhury | Dr. N. G. Prasad (Supervisor)

Dated: April 22, 2016

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.

Ruchika choudhary
(Candidate)

Dated: April 22, 2016

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)

Acknowledgement

I would like to thank Dr. N. G. Prasad for the opportunity to work on this project of mine for MS thesis and his invaluable guidance. I am thankful to my lab members Zeeshan, Saudamini, Tj, Manas, Radhika, for their friendly guidance, Nitika, Komal and Aathira for helping me in stock maintenance. I thank Karan for teaching me basics of fly handling. I am happy that I worked among Martik, Rohit and Aprajita who made the experience more fun.

I am also grateful to all my friends who kept me sane all this while.

I am thankful to DRL IISER Mohali for letting me use Fluorescence microscope. Specially, I would like to thank Saikat.Gosh.

Finally, I would like to thank IISER Mohali and my family for their support.

Contents

Abstract

The ability to resist variation in temperature is an important component of fitness in *Drosophila melanogaster*. Given that it is a costly trait, it certainly has a cost effect on other life history traits. Cold stress can affect both male and female reproductive fitness leading to a decrease in progeny production, egg viability and adult mortality. Cold shock can affect female reproductive fitness by killing both eggs and stored sperm. In this study we have explored effect of cold shock on sperm storage in females and its evolution. We found that females selected for cold resistance do not store more sperms rather they remove the dead sperm faster than control females. Hence selected females can mate faster than control females after cold shock, increasing the number of progeny produced.

List of figures

List of tables

Introduction

Females of many reptiles, birds, insects and some mammals are known to store sperm received during mating. The site of storage and duration is variable among different animal taxa. There are many studies focused on the occurrence of prolonged sperm storage, but few are directed towards explaining the mechanism and adaptive benefits of it. One such study is published by Birkhead & Moller (1993) which extends Sandell's (1990) hypothesis that, "…In mammals, delayed implantation has evolved to allow females to time both their copulation and birth seasons optimally" As females seek opportunities to obtain the best male, Birkhead & Moller (1993) suggest that sperm storage might have evolved through sexual selection, enabling females to choose the best male to sire most of her progeny. Another study on crickets has shown that controlled sperm storage helps promiscuous species to decrease inbreeding and increase genetic diversity. (Bretman, et al., 2009). In one such promiscuous species, *Drosophila melanogaster*, sperm storage is advocated to be a great tool for increased fecundity, fertility and decreased cost associated with multiple matings (Qazia, *et al.*, 2003).

In female *Drosophila melanogaster* sperm can be kept alive for about two weeks by storing them in dedicated storage organs. The *D. melanogaster* possesses two types of storage organs, seminal receptacle (SR) and spermathecae (ST). As Miller described, the seminal receptacle is a long, close ended tube, located at the anterior of the uterus. On the other hand, spermathecae is a pair of mushroom like structures which is connected to the uterus by long slender ducts. (Lefevre & Jonsson, 1962). In *Drosophila melanogaster* 60-80% of the sperms transferred by a male are stored in the seminal receptacle and are used before those of the spermathecae (Lefevre & Jonsson, 1962). However, not all *Drosophila* species are able to use spermathecae as a storage organ.(Pitnick, et al., 1999).

During copulation, male releases sperm in the uterus. Displacement of sperm from uterus to storage organs begins 20-30 minutes after copulation (Qazia, *et al.*, 2003). Some of

these stored sperm are used for delayed fertilization and rest is dumped by the female over the time (Snook & Hosken, 2004). While dumping, sperm is moved back to uterus and ejected out of the female body.

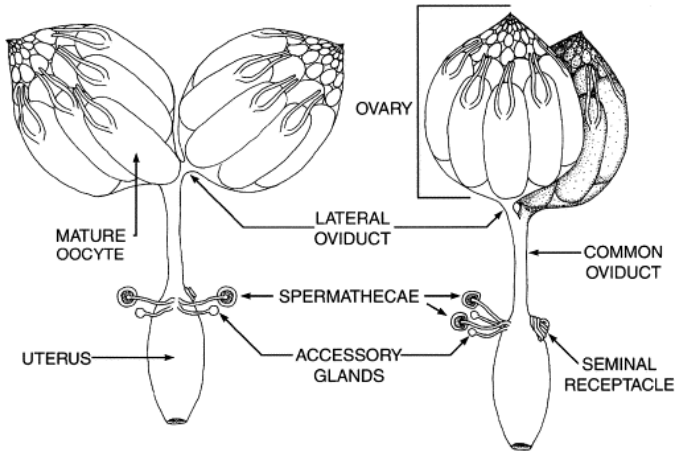


Figure 1: Female *Drosophila melanogaster* storage organs source: https://amasianv.wordpress.com/2013/03/13/battle-of-the-sperms/

There are many environmental as well as physiological factors which can have an effect on sperm storage. Female's own physiology can affect the storage as well. One such factor is temperature. High temperature (heat stress) is known to cause a decrease in sperm storage in males and a decrease in female fecundity (Jorgensena & Sorensen, 2006). Similarly, exposure to sub-zero temperatures has also been reported to result survival-reproduction trade-off in *Drosophila melanogaster* (Marshall & Sinclair, 2009). More specifically, low temperatures are reported to kill sperm present in males and females (Novitski & Rush, 1949). Upon dissecting out the reproductive tract of Cold-Shocked females, Novitski & Rush (1949) did not find any motile sperm present and a low storage of immotile sperm was found in cold subjected females. Results from such studies suggest that cold shock deseminates females and dead sperms are expelled out of the female body.

Karan Singh selected replicate populations of *Drosophila melanogaster* for resistance to cold stress. The selected populations evolved higher post cold-shock egg viability and higher post-cold shock mating frequencies relative to control populations. Post cold shock, selected females recovered faster in terms of reproductive behavior and produced more progeny. Males from selected population have higher fertility, higher sperm offence ability, greater progeny production and are able to transfer functional sperms within 4

hours. While it is clear that selection for improved resistance to cold stress has resulted in evolution of reproductive traits in both males and females of selected populations, the underlying mechanisms of higher fitness of selected populations post cold shock relative to control populations remain largely unexplored.

In the present thesis, I investigated whether greater post cold shock fitness of selected females can be (at least partly) explained by evolution of sperm storage.

Major questions addressed, in order to answer the question of evolution of sperm storage in females in response to cold shock were: -

- Are selected females better at protecting stored sperms from cold shock?
- Are selected females better at removing dead sperm from reproductive tract?

Methodology

For my study I have used *Drosophila melanogaster* as experimental model system due to short life cycle, ease of handling.

Ancestral Populations:

The experiments were carried out using Freeze Shock Resistant populations (FSB) and their Controls (FCB) which were originally derived from replicates of BRB populations adapted to standard laboratory conditions. BRB (Blue Ridge Baseline) is a large outbred population of *Drosophila melanogaster* that was established from 19 iso-female lines. The iso-female lines were founded by 19 females caught in the wild from Blue Ridge, USA. There were five replicates of BRB population (BRB_{1-5}) which were maintained on a 14 days discreet generation cycle at 25℃ on a standard banana--yeast-jaggery food. The flies were kept at 12 hours alternate light-dark cycle under 50-60 % humidity conditions. BRB populations were maintained under these conditions for 35 generations with N_e of 2800 individuals.

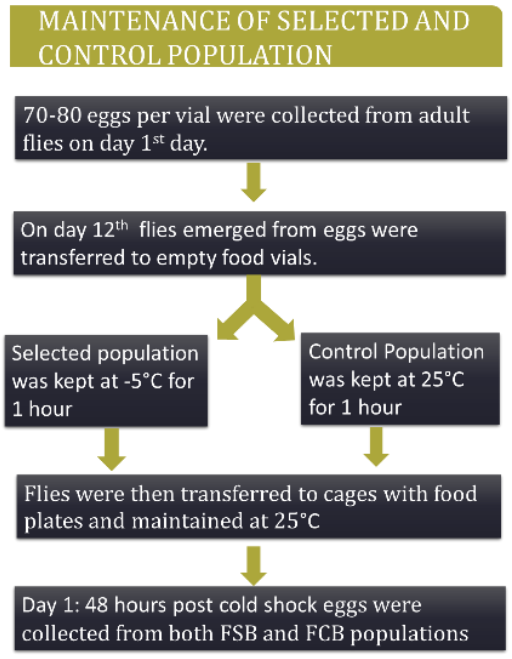
Experimental Populations:

After 35 generations, two populations from each BRB population were established, named FSB and FCB where FSB refers to Freeze Shock resistant, population selected for resistance to Cold stress while FCB refers to Controls. Populations derived from same BRB population were given the same subscript and handled together as they were more closely related to each other. For example, FSB_{-1} and FCB_{-1} were derived from BRB_{-1} and termed as Block-1. 5 populations of FSB (FSB_{1-5}) and 5 populations of FCB (FCB_{1-5}) were obtained. The derived populations of FSB and FCB were maintained on 13 days discrete selection regime under 25ºC, 50-60% RH, 12:12 light: dark cycle with N_e of around 1400 individuals.

Maintenance of selected and control populations:

For each generation eggs from adult flies were collected on banana-yeast-jaggery food plates. The food plates were introduced into cages 18 hours before egg collection.20 glass vials with egg density of 70-80 eggs per vial (7-8ml of food) were set up for each population. Typically, adult flies start emerging on day 9 post egg collection and till day 11 all the adults are eclosed. On day 12, flies from selected populations were subjected to selection pressure (Cold Shock).

On day 12, adult flies were flipped from food vials to empty vials. The cotton plug was pushed down to confine the flies in a smaller area. Flies from FSB populations were subjected to cold shock by immersing the vials into salt-water-ice slurry maintained at -5^{o}C for 1 hour. Similarly, flies from control populations were kept at 25^{o}C for one hour. After one-hour flies are transferred to Plexiglas cage and provided with a banana-yeast-jaggery food plate.

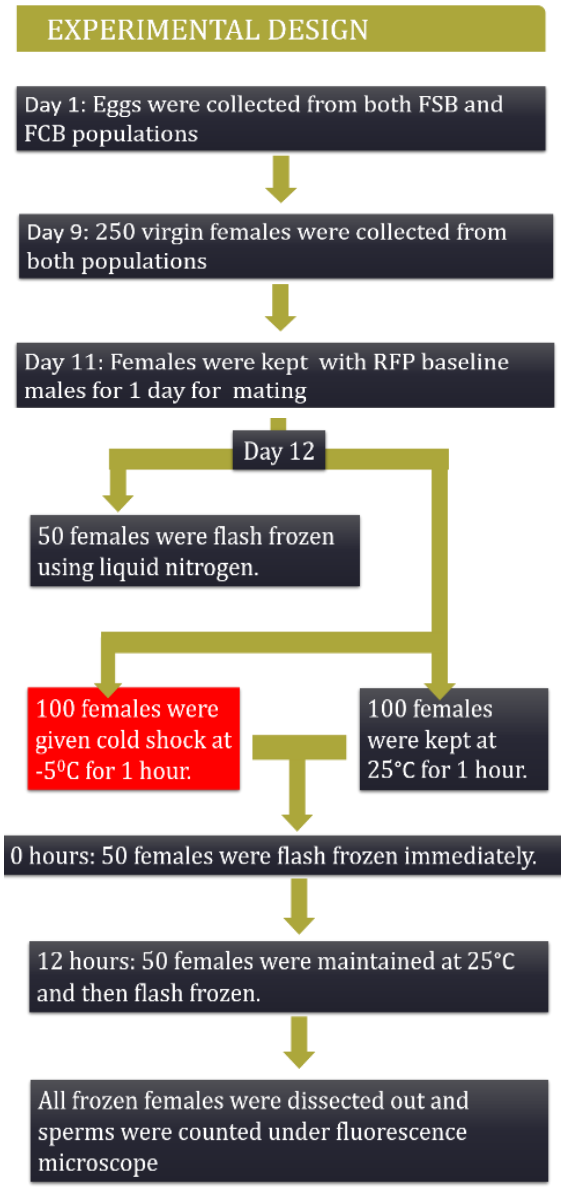


Further, these populations were maintained at 25^{o}C and eggs were collected 48 hours post cold shock to start another generation.

RFP Males:

RFP males were chosen as baseline males to cross with females. Sperm heads of RFP males are tagged with Red fluorescent protein. To make RPF males, Manier *et al.*(2010) fused RFP with Mst35Ba and Mst35Bb in LH Flies which encodes similar sperm-specific chromosomal protein. RFP population was kindly provided by Scott Pitnick and was reared on cornmeal food at standard laboratory conditions. A detailed description is presented in (Manier, *et al.*, 2010)

Experimental Design:



The aim of the study was to look at the effect of cold shock on sperm stored by mated females. For that, virgin females from FSB and FCB populations were crossed with baseline RFP males. Females were subjected to cold shock post -mating and were dissected out at different time points and sperm was counted. As the sperm heads of RFP males is tagged with Red fluorescent protein it made counting of sperm stored in female storage organs.

Before carrying out the experiment flies were standardized to account for differences due to non-genetic effects. For that selection pressure was relaxed for one generation and both FSB and FCB populations were maintained at baseline population conditions. Flies thus generated are called standardized flies.Eggs were collected from standardized flies to perform the experiment. 250 females were collected as virgins using CO_2 anesthesia and were housed in single sex groups of 10 for 2 days. On day 11, females were housed with same-age RFP males for mating (10 females: 12 males). On day 12, males were removed by aspiration.

On day 12 post egg collection: -

1. One set of 50 mated females from both treatments were flash frozen using liquid nitrogen.
2. 100 females from selected as well as control populations were exposed to cold shock for an hour.
    2.1. Out of 100 Cold shocked females, 50 were flash frozen immediately after cold shock.
    2.2. Rest were kept in food vials at 25^{o}C for 12 hours and then frozen.
3. Another set of 100 females was kept at 25^{o}C for one hour instead of Cold shock
    3.1. Similarly, 50 flies were frozen immediately after cold shock.
    3.2. Rest were frozen 12 hours later.

Flies were flash frozen using liquid nitrogen to preserve the tissue at highest possible condition without further degradation. These flies were stored at -20^{o}C till taken out for dissections.

Sperm Counting:

The reproductive tract of stored females was dissected in 1X PBS using forceps. Further, seminal receptacle, spermathecae and uterus were dissected out from reproductive tract. The tissues were imaged under fluorescence microscope with attached camera under 10X. The sperm was counted manually at 40X magnification.

Statistical analysis:

Data was analyzed using two-way analysis of variance using selection as a fixed factor and block as a random factor. All the statistical analysis were carried out using JMP version 7.

Images

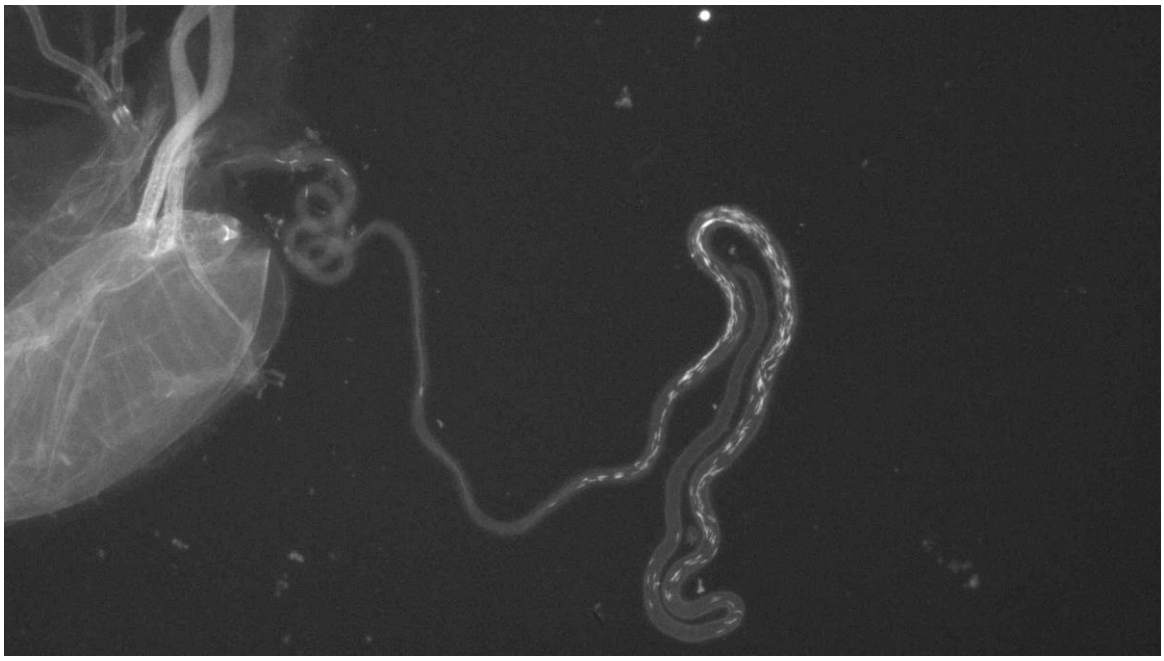


Figure 2: FSB-5, 1 hour before cold shock (non-shocked). Before the file is subjected to cold shock majority of the sperm is retained in SR.

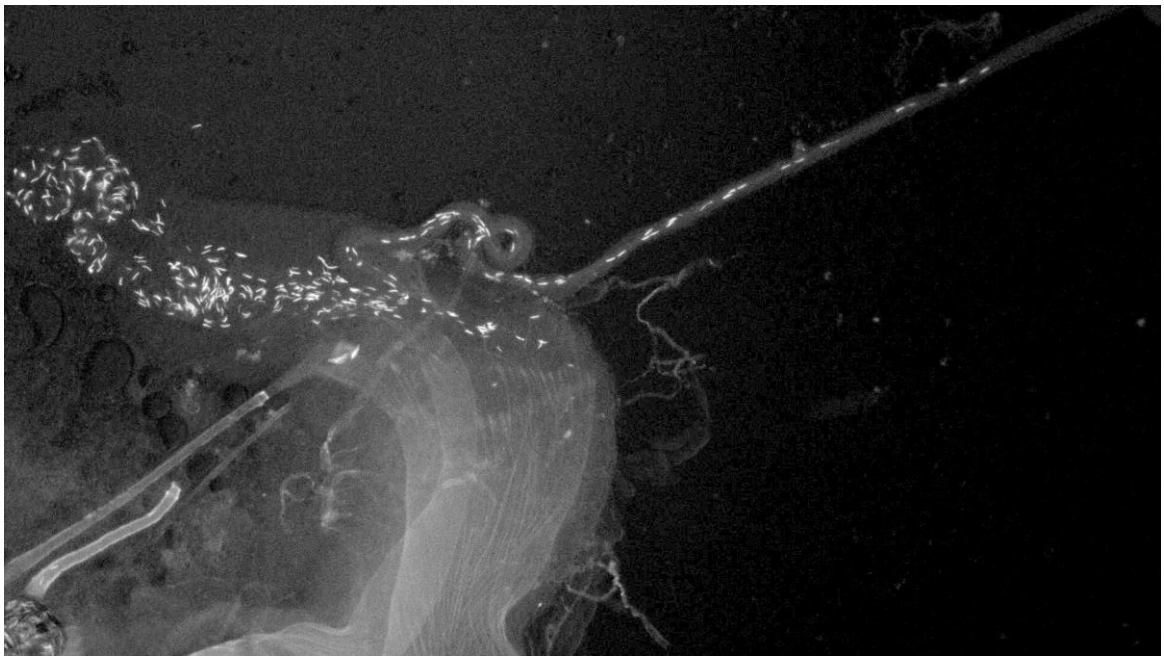


Figure 3: FSB-5, 0 hours post cold shock. FSB females are able to transfer 60% of the sperm to uterus immediately after cold shock.

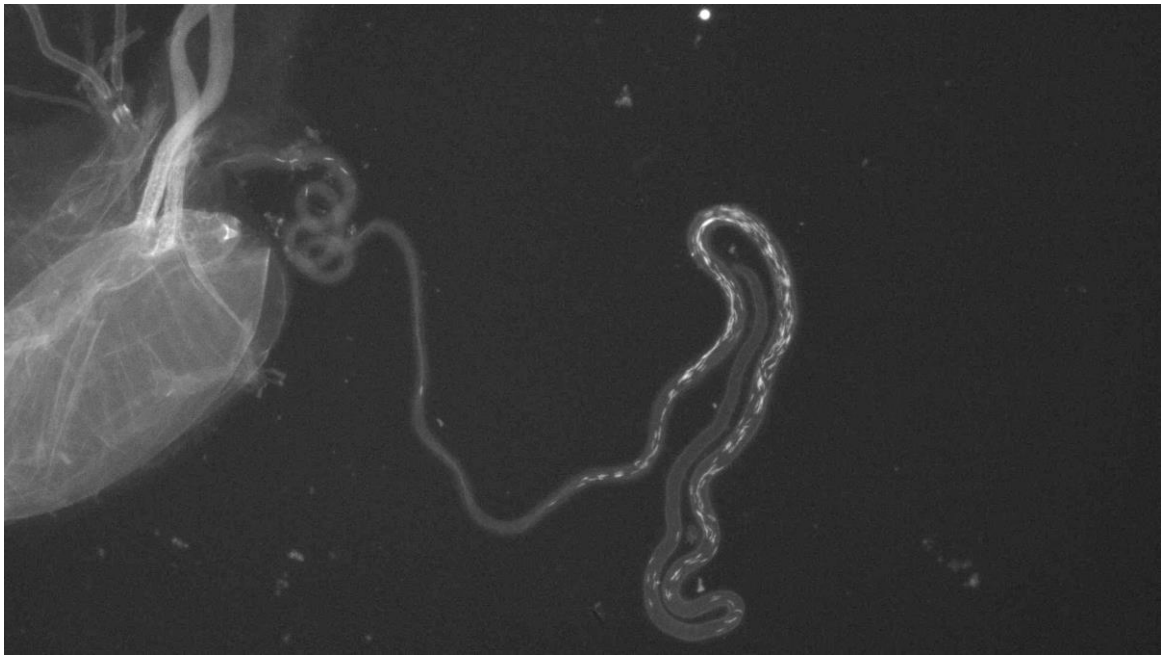


Figure 4: FCB-5, 0 hours post cold shock. Majority of the sperm is located in SR at 0 hours post cold shock.

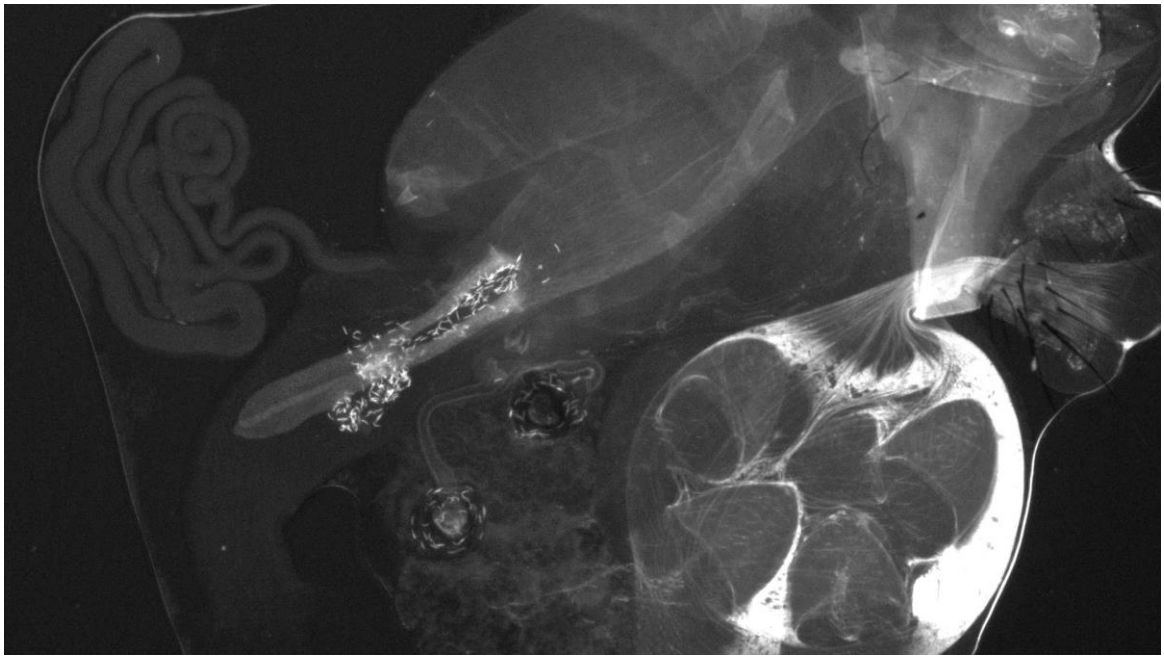


Figure 5: FCB-3, 12 hours post cold shock.SR is completely empty even in FCB. All of the sperm is pushed to uterus and is being ejected out of the body.

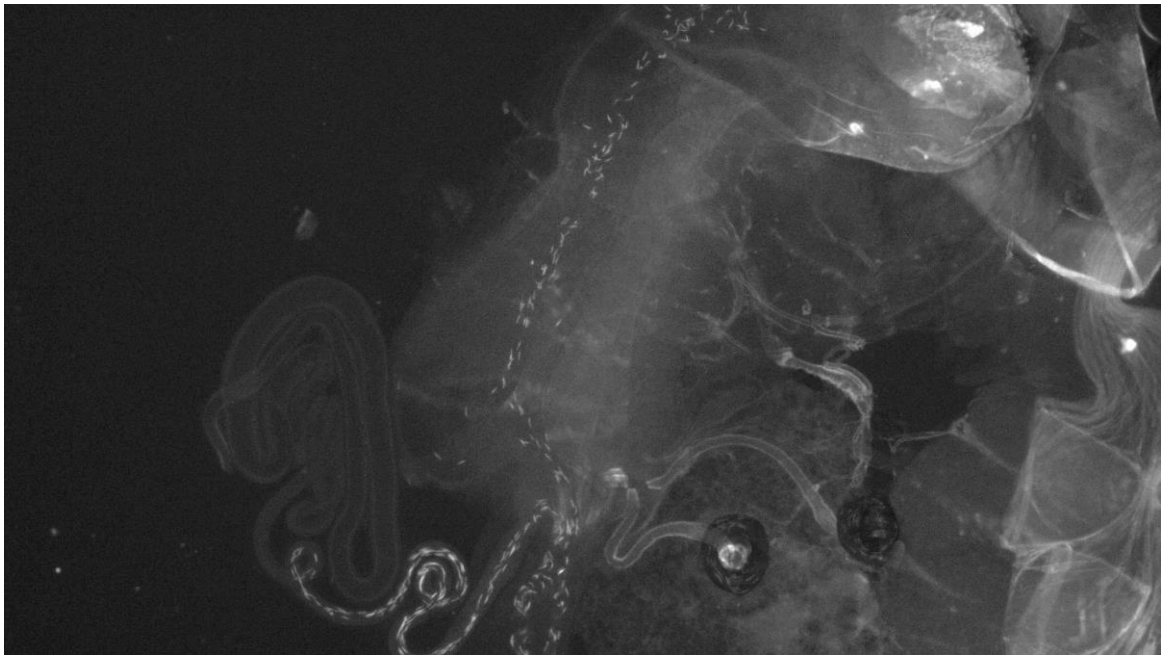


Figure 5: FSB-5, 12 hours post cold shock. Sperm is being removed from SR for temporary storage in uterus to be expelled out.

Results

Sperm present in seminal receptacle and uterus was counted at three time points, 1 hour before cold shock, 0 hours post cold shock and 12 hours post cold shock.

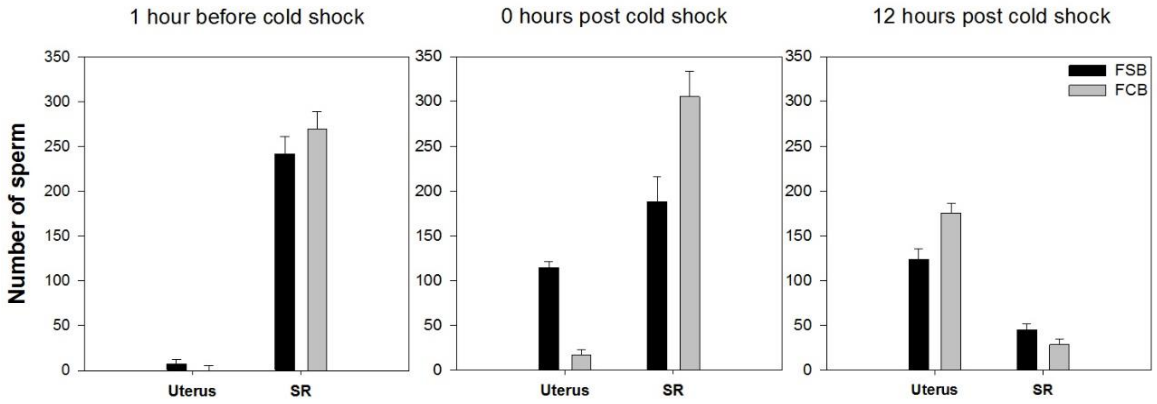


Figure 2: Effect of cold shock on number of sperm stored in seminal receptacle and uterus.

Effect of cold shock on sperm stored in seminal receptacle:

Number of sperm present in FSB and FCB female seminal receptacle, treated at different time points was analyzed. We did not find any significant effect of selection on sperm density in SR at 1 hour before cold shock and 12 hours post cold shock. However, there is a significant difference in SR sperm count at 0 hours post cold shock. (Table 1, Table 2, Table 3, Figure 2)

Table 1. Effect of cold shock on sperm count in SR at 1 hour before cold shock. There was no significant difference in FSB and FCB female sperm count in SR at 1 hour before cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF: degrees of freedom.

Source of variation	SS	DF Num	DF Den	F ratio	P
Selection	7812.025	1	1	0.9929	0.5011
Block	931.225	1	1	0.1184	0.7891
Selection*Block	7868.025	1	36	0.8182	0.3717
Total SS	362796		.	.	
Total DF	39				

Table 2. Effect of cold shock on sperm numbers in SR at 0 hours after cold shock. We found a significant difference in FSB and FCB female sperm count in SR at 0 hours post cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF: degrees of freedom.

Source of variation	SS	DF Num	DF Den	F ratio	P
Selection	137475.6	1	1	8.969	0.205
Block	22705.23	1	1	1.481	0.438
Selection*Block	15327.23	1	36	1.636	0.209
Total SS	512774.8		.	.	
Total DF	39				

Table 3. Effect of cold shock on sperm numbers in SR at 12 hours after cold shock. There was no significant difference in FSB and FCB female sperm count in SR at 12 hours after cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF: degrees of freedom.

Source of variation	SS	DF Num	DF Den	F ratio	*P*
Selection	1452.025	1	1	3.006	0.333
Block	5688.225	1	1	11.776	0.181
Selection*Block	483.025	1	36	0.104	0.749
Total SS	174671.8		.	.	
Total DF	39				

Effect of cold shock on sperm stored in uterus:

Data collected for sperm count in uterus in FSB and FCB females was analyzed using two-way ANOVA. There was no effect of cold shock on sperm numbers in uterus at 1 hour before cold shock, 0 hours post cold shock and 12 hours post cold shock. (Table 4, Table 5, Table 6, Figure 2).

Table 4. Effect of cold shock on sperm numbers in uterus at 1 hour before cold shock. There was no significant difference in FSB and FCB female sperm count in uterus at 1 hour before cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF: degrees of freedom.

Source of variation	SS	DF Num	DF Den	F ratio	P
Selection	497.025	1	1	1.000	0.500
Block	570.025	1	1	1.147	0.478
Selection*Block	497.025	1	36	1.189	0.283
Total SS	16610.98		.	.	
Total DF	39				

Table 5. Effect of cold shock on sperm count in uterus at 0 hours after cold shock. There was no significant difference in FSB and FCB female sperm count in uterus at 0 hours after cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF: degrees of freedom.

Source of variation	**SS**	**DF Num**	**DF Den**	**F ratio**	**P**
Selection	95648.4	1	1	108.249	0.061
Block	504.1	1	1	0.571	0.588
Selection*Block	883.6	1	36	0.053	0.819
Total SS	693097.9		.	.	
Total DF	39				

Table 6. Effect of cold shock on sperm numbers in uterus at 12 hours after cold shock. There was no significant difference in FSB and FCB female sperm count in uterus at 12 hours after cold shock. Summary of results from two-way ANOVA using selection as a fixed factor and block as a random factor. SS: Sum of squares, DF:degrees of freedom.

Source of variation	SS	DF Num	DF Den	F ratio	P
Selection	40832.1	1	1	21.441	0.135
Block	10368.4	1	1	5.444	0.258
Selection*Block	1904.4	1	36	0.162	0.690
Total SS	477119.9	.	.		
Total DF	39				

Discussion

Many studies have confirmed that cold shock/stress indeed does kill sperm stored in female storage organs (Novitski & Rush, 1949). It is also known that dead sperm need to be removed from the female seminal receptacle in order to make room for fresh fertile sperm by re-mating. Hence females need to re-mate to produce progeny post cold shock (Singh, *et al*., 2015). However, re-mated FSB females are found to produce more number of viable eggs in comparison to re-mated FCB population post cold shock. We hypothesize that such a difference in egg viability could be attributed to faster removal of damaged sperm by FSB females or better sperm protection by FSB females (Singh & Prasad, unpublished data). In this study we address the former hypothesis.

In our study, we first tested if the total number of sperm stored was different between the selected and control populations. We did not observe any significant difference in the total number of sperm stored prior to cold shock by FSB and FCB females. This indicates that there is no difference in the total number of sperm stored by FCB and FSB females prior to cold shock.

Our study shows that at 0 hours post cold shock, number of sperm in seminal receptacle (SR) is lower in cold shocked FSB females compared to FCB females. It could be because FSB females have adapted to remove dead sperm from seminal receptacle immediately after cold shock to store sperms from fresh matings and in turn used to fertilize eggs to produce more progeny. This result is supported by the observation that the number of sperm in the uterus 0 hours post cold shock is greater in FSB females. This indicates that FSB females have acquired the ability to quickly transfer stored sperm from their SR to the uterus for ejection post cold shock.

12 hours post cold shock, there is no significant difference in FCB and FSB females in number of sperm in the SR or uterus suggesting that by 12 hours both FSB and FCB females are able to expel most of sperm out of SR.

However, the question of sperm protection from cold shock is still unanswered which would be next logical experiment for the study. To address this question we will assay motility of sperm retained in SR by dissecting out live females post cold shock.

Bibliography

Birkhead, T. R. & Moller, A. P., 1993. Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society,* p. 295–311.

Bretman, A., Newcombe, D. & Tregenza, T., 2009. Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular ecology,* pp. 3340-3345.

Jorgensena, K. T. & Sorensen, G. J., 2006. Heat tolerance and the effect of mild heat stress on reproductive characters in Drosophila buzzatii males. *Journal of Thermal Biology,* pp. 280-286.

Lefevre, G. J. & Jonsson, U., 1962. Sperm Transfer, Storage, Displacement, and Utilization in Drosophila Melanogaster. *Genetics,* pp. 1719-1736.

Marshall, E. K. & Sinclair, B. J., 2009. Repeated stress exposure results in a survival–reproduction trade-off in Drosophila melanogaster. *Proceedings of Royal society B.*

Novitski, E. & Rush, G., 1949. Viability and Fertility of Drosophila Exposed to Sub-Zero temperatures. *Biological Bulletin,* pp. 150-157.

Pitnick, S., Markow, T. & Spicer, G. S., 1999. Evolution of Multiple Kinds of Female Sperm-storage Organs in Drosophila. *Evolution,* pp. 1804-1822.

Qazia, M. C. B., Heifetza, Y. & Wolfner, M. F., 2003. The developments between gametogenesis and fertilization: ovulation and female sperm storage in drosophila melanogaster. *Developmental Biology,* p. 195–211.

Singh, K., Samant, M. A., Tom, M. T. & Prasad, N. G., 2016. Evolution of Pre- and Post-Copulatory Traits in Male Drosophila melanogaster as a Correlated Response to Selection for Resistance to Cold Stress. *PLos One.*

Snook, R. R. & Hosken, J. D., 2004. Sperm death and dumping in Drosophila. *Nature,* pp. 939-941.

Singh & Prasad, unpublished data.