Effect on sperm storage after cold shock and rapid cold hardening in female *Drosophila melanogaster*

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Certificate of Examination

This is to certify that the dissertation titled "*Effect on sperm storage after cold shock and rapid cold hardening in female Drosophila melanogaster*" submitted by Mss. Ateesha Negi (Reg. No. MS12058) 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.

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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.

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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.

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List of Abbreviations

CS: Cold Shock

RCH: Rapid cold hardening

NS: No shock

SR: Seminal receptacle

ST: Spermathecae

UT: Uterus

BRB: Blue Ridge Baseline

RFP: Red Fluorescent Protein

T: Time

ANOVA: Analysis of Variation

DF: Degrees of freedom

CO_2: Carbon dioxide

PBS: phosphate buffered saline

ABSTRACT

For ectotherms like *Drosophila melanogaster* the ability to resist variation in temperature is an important component of fitness. Cold shock (CS) can affect both male and female reproductive fitness leading to a decrease in progeny production, egg viability, adult mortality and effects female reproductive fitness by killing both eggs and stored sperm; however females of *D. melanogaster* remove these dead sperm, so as to let in fertile sperm by re-mating. Rapid cold hardening (RCH) which is short exposure of cold stress prior to chilling injury or CS, is sufficient to increase cold tolerance in *Drosophila melanogaster*. In this study I have explored the effect of CS and RCH, for which two different laboratory populations of *Drosophila melanogaster* were taken. The base line population which were subjected to different treatments namely RCH, RCH followed by CS and CS. It was found the females of baseline population which were subjected to RCH followed by CS, remarkably remove dead sperm from storage organs than control females as well as females of other treatment. This clearly shows that RCH indeed has an effect on sperm storage when given prior to chilling injury.

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CHAPTER 1

Introduction

1.1 Sperm storage in Female *D. melanogaster*.

Emergence many internally fertilizing organisms, including reptiles, fish, birds, mammals have the ability to store sperm. As females in promiscuous species are potentially under selection to fertilize their eggs with the sperm of the 'best' male, sperm storage ability might have evolved through sexual selection, enabling females to exercise cryptic mate choice (Birkhead and Moller, 1993). However the benefits associated with such processes vary across species. For example, in Drosophila, female sperm storage enables sperm competition, temporal separation of insemination from fertilization, manipulation of ejaculates, controlled fertilization and cryptic choice are few to name (Qazia, et al., 2003). Thus sperm storage is advocated to be a great tool for investigating sexual conflict and evolution of reproductive traits. *D. melanogaster* possess two type of storage organs, (Fig: 1,2,3,4 and 5) seminal receptacle (SR) and spermathecae (ST). (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 structure which is connected to the uterus by long slender ducts. (Lefevre and Jonsson, 1962). 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 sperm are used for delayed fertilization and rest are dumped by female over time (Snook and Hosken, 2004). While dumping, sperm is moved back to uterus and ejected out of the female body.

Effects of male factors as well as female factors on sperm storage are well documented. Transfer of male derived proteins such as Acps is essential for sperm transfer and sperm survival. Along with that, female factors including reproductive tract environment, female derived proteins are crucial for sperm storage. However, very few studies have investigated the role of environmental variations on female sperm storage.

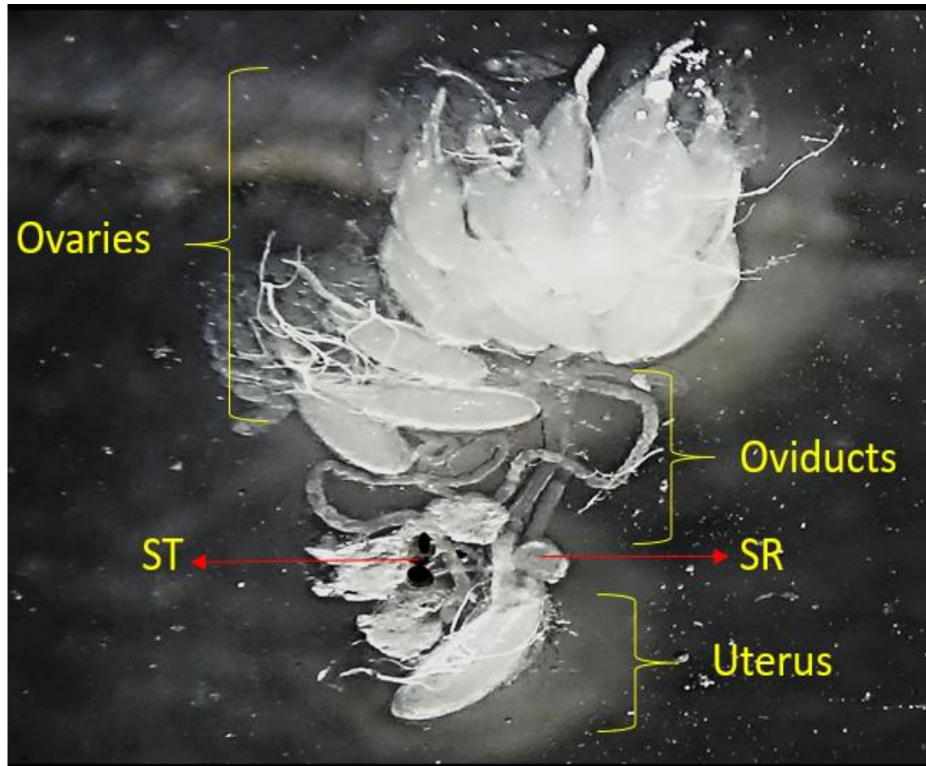

Fig.1: Female *Drosophila melanogaster* storage organs

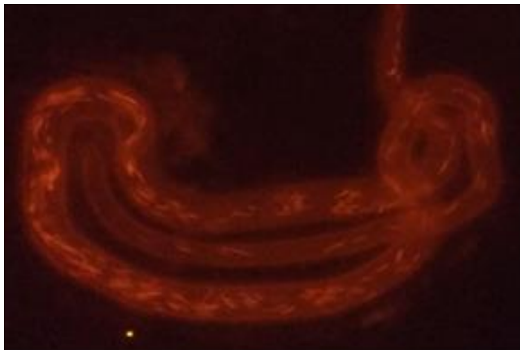

Fig.2: SR with RFP tagged sperm

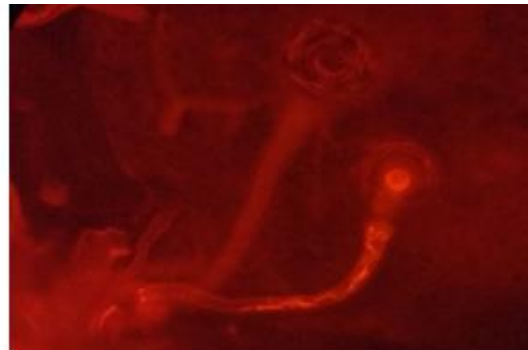

Fig.3: ST with RFP tagged sperm

Fig.4: SR of virgin female

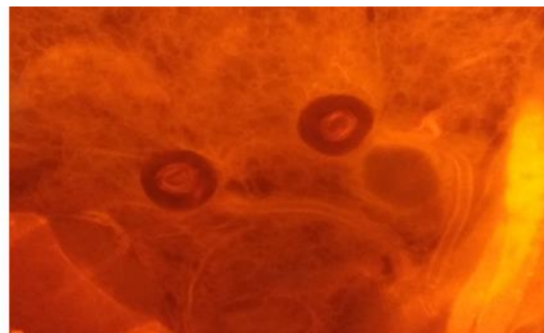

Fig.5: ST of virgin female

1.2 Cold shock:

Cold shock (CS) and Chilling injury are relative terms; the temperature range which CS represent, vary depending on the species in question. For a tropical species, 10 ◦C may be sufficiently low to cause chill coma (a loss of locomotory capacity due to impaired neuromuscular function), while a polar or alpine species may be able to walk normally at −5 ◦C or below (e.g. a Himalayan midge can walk at −16 ◦C (Kohshima, 1984)). Some of the studies also report that exposure to sub-zero temperature results in survival reproduction trade off in Drosophila melanogaster (Marshall and Sinclair, 2009) by deactivating sperm present in males and females (Novitski and Rush, 1949). Result from such studies suggested that cold shock disseminates females and dead sperm are expelled out of the female body. In *Drosophila melanogaster* exposure to sub-zero temperatures is eventually lethal. It has been found out that mortality caused by cold shock is not due to internal ice formation as this internal ice formation occurs above their supercooling point (temperature at which the body fluid changes into ice) (Lee and Denlinger, 1985; Knight et al., 1986). Exposure to -5 ◦C for two hours induces 50% mortality in Drosophila melanogaster, although the supercooling point known for this species is around -20 ◦C (Czajka and Lee, 1990).

1.3 Rapid cold hardening:

Drosophila melanogaster, can markedly increase their capacity towards chill (cold shock) tolerance through a quick cold hardening or acclimation process, termed as rapid cold hardening (RCH). RCH is a protective effect gained by prior exposure to sub-lethal low temperatures or by gradual cooling; it has been demonstrated that RCH improves the ability of several species of insect to withstand cold shock (Meats, 1973; Lee et al., 1987; Chen et al., 1987; Larsen and Lee, 1994; Burks and Hagstrum, 1999; Kelty and Lee, 1999, 2001; Powell and Bale, 2004; Overgaard et al., 2005, 2006;). RCH can be effective varying from minutes to hours, RCH is known to improves certain important fitness traits such as survival and reproduction during cold exposure (Chen et al., 1987; Czajka and Lee, 1990; Kelty and Lee, 1999; Shreve et al.,2004).

In D. melanogaster, RCH lowers the lowest temperature at which the flies can remain active (critical thermal minimum) (Kelty and Lee, 1999), There have been studies which demonstrate that the ability to survive RCH is likely to be of ecological relevance in *D.melanogester*.

The physiological adjustments associated with RCH are very important for insects' ability to adjust with the varying temperature and sustain their activities at low temperature, activity including courting/ mating therefore preserves reproductive behavior (Lee et al., 1987; Kelty and Lee, 1999, 2001; Koveos, 2001; Shreve et al., 2004).

1.4 Aim the Research:

As there have been no studies investigating the effect of RCH and Cold shock on sperm storage ability. In light of previous evidences. I, in the present study investigate the effect of RCH post cold shock on the sperm storage patterns in females and checked if RCH can provide a protective response against injuries caused by cold shock on sperm processing, sperm dumping and sperm storage.

Specifically the following questions asked:

1. Does RCH influence the manner in which *D. melanogaster* females from a baseline laboratory population process sperm post cold shock before eventually dumping them?
2. Does RCH influence motility of the sperm post cold shock?

CHAPTER 2

EXPERIMENTAL SECTION

2.1 Experimental fly population:

To investigated the effect of cold shock and rapid cold-hardening on sperm storage ability in female D. melanogaster; experiments were performed on adult flies from two populations (i) baseline population (BRB) free from any selection regime (reared at constant 25 ◦C) and (ii) RFP population.

2.1.1 Baseline population (BRB):

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 (BRB1-5) 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. This population is well adapted to the laboratory conductions.

2.1.2 RFP population:

For my experiment RFP males were chosen to mate with BRB females so that the sperms could be easily visible under fluorescent microscope. As sperm heads of RFP males are tagged with Red fluorescent protein. RFP males were chosen as baseline males to cross with females. 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)

2.2 Experimental Design:

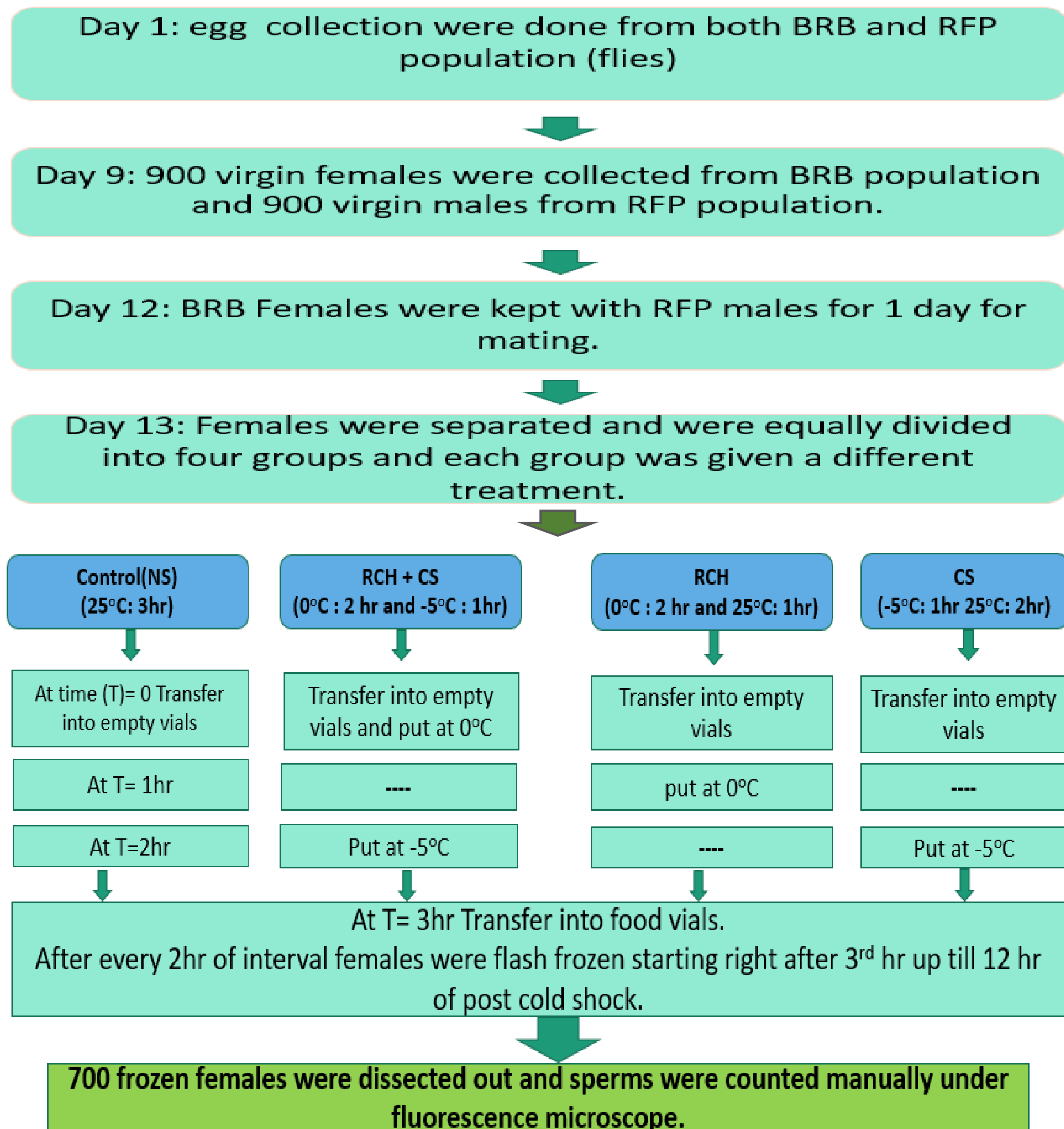

Fig.6: Flow diagram showing experimental design.

2.2.1 Methodology:

To study the effect of RCH on sperm stored by mated females. For which experimental flies were generated from BRD populations. 900 virgin females were collected and on day 12 these virgin females were housed with baseline RFP males of same age for mating (10 females: 12 males). On day 13, males were removed by aspiration and females were divided into four groups having 210 females in each group. Each group was transferred into empty vails (30 females per vail) for the following treatment:

1. **Control or no socked (NS) females:** these females were not subjected to RCH or CS, rather were kept in empty vails for 3hr at 25°C.
2. **RCH + CS females**: these females were subjected to RCH for 2hr at 0°C followed by CS at -5°C for 1hr.
3. **CS females:** these females were first kept in empty vails for 2hr at 25°C and then subjected to CS at -5°C for 1hr.
4. **RCH females:** these females were first kept in empty vails for 1hr at 25°C and then subjected to RCH at 0°C for 2hr.

All these above treatments were performed simultaneously on the same day.

After completion of treatments (end of 3rd hr) these females were transferred into food vails. 30 females from each of these group were flash frozen after every interval of two hours starting right from the end of the treatment, extending up to 12th hour therefore there were seven time points when 30 females from each group was flash frozen.

All the flies of both the populations were flash frozen using liquid nitrogen to preserve the tissue at highest possible condition without further degradation. These flies were stored at -20°C till taken out for dissections.

2.2.2 Sperm Counting Method:

1. Take the stored females (flash frozen) and place the female in 1X PBS on the slide.
2. Pull out the ovaries using forceps, seminal receptacle, spermathecae and uterus were will eventually come out along with it.
3. Tease apart seminal receptacle with a tungsten needle. Put on the cover slip for well separated seminal receptacle, spermathecae and uterus.
4. Then image these tissues under fluorescence microscope with attached camera under 10X. The sperm was counted manually at 40X magnification.

2.2.3 Sperm recording Method:

1. Take the females from the treatments (live) and anesthetize them with CO_2.
2. Place the female in *Drosophila* Ringer's (50% Drosophila Ringer's Solution at final concentrations of 91 mM KCl, 23 mM NaCl, 1.5 mM CaCl2, and 5 mM Tris- HCl (pH 7.2)).
3. Tease apart seminal receptacle with a tungsten needle. Put on the cover slip for well separated seminal receptacle, spermathecae and uterus (don't press the coverslip too hard).
4. Then record immediately after the tissues are dissected, under fluorescence microscope with attached camera under 10X. The sperm was counted manually at 40X magnification.

2.2.4 Statistical analysis:

Data was analyzed using one-way analysis of variance. All the statistical analysis were carried out using Statistica. All analyses were done at $\alpha = 0.05$ level of significance.

CHAPTER 3

OBSERVATIONS & RESULTS

3.1 Observation of BRB females subjected to RCH, RCH+CS and CS:

Sperm present in seminal receptacle and uterus was counted at seven different time points: starting from 0 hours post cold shock up till 12 hours post cold shock, after every 2hr of interval.

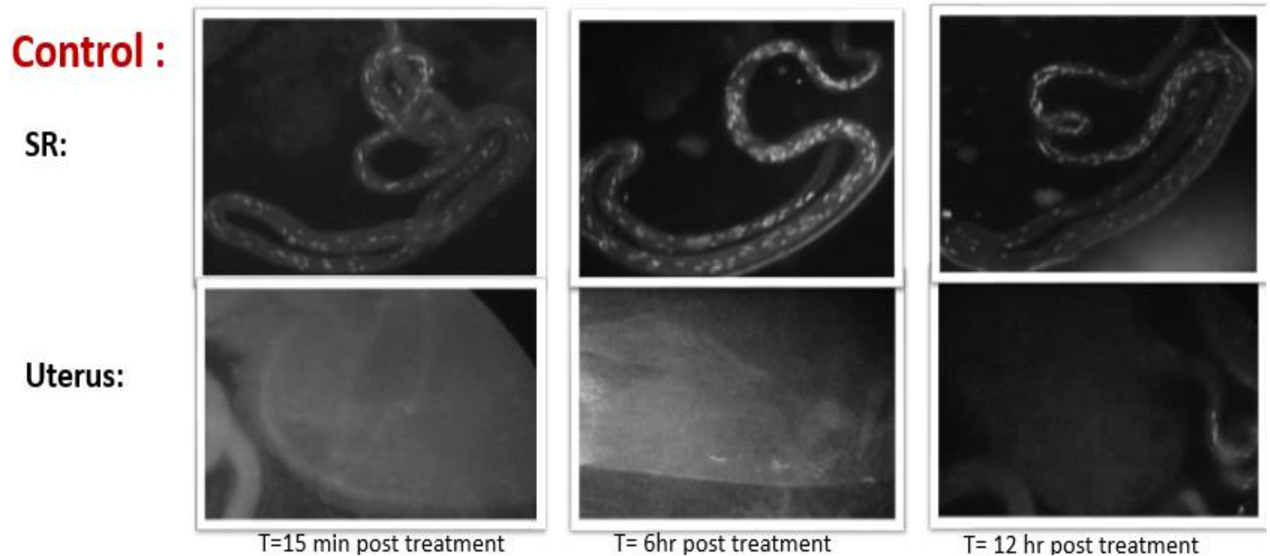

Fig.7: SR and UT of control females over time. Here Majority of the sperms were located in SR whereas uterus was found to be completely empty; indicating that none of the sperms were pushed out to the uterus for ejecting them (no dumping of sperm).

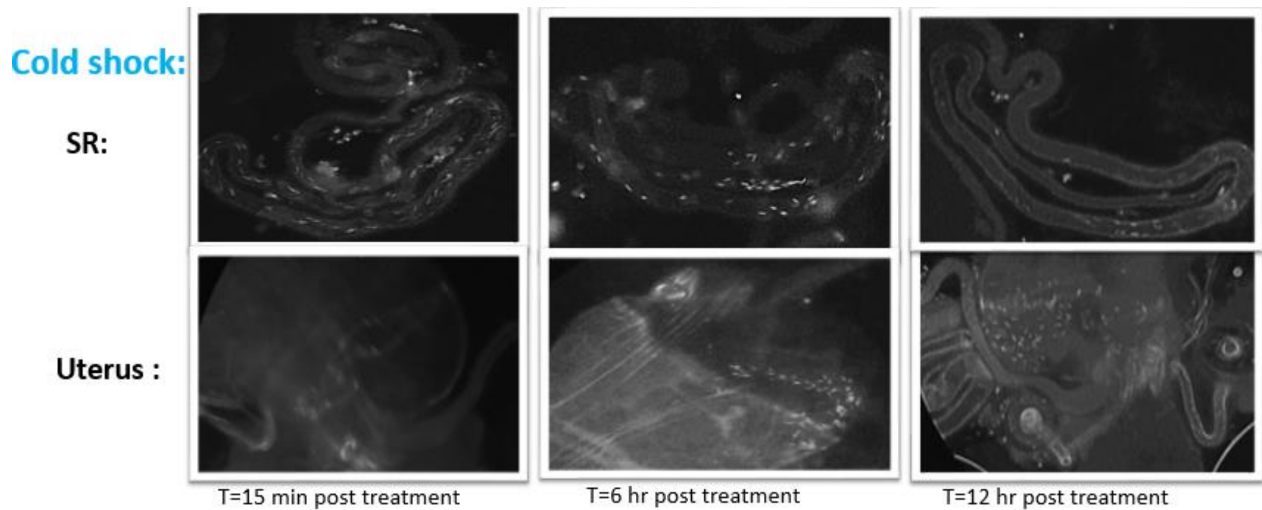

Fig. 8: SR and UT of cold shock females over time. Here half of the sperms were transferred into the uterus from SR up till six hour post CS treatment and at 12 hr post CS treatment very few sperms were found in SR whereas uterus was filled with sperms; indicating that sperms were pushed into the uterus in order to eject them out of the body (dumping of sperm).

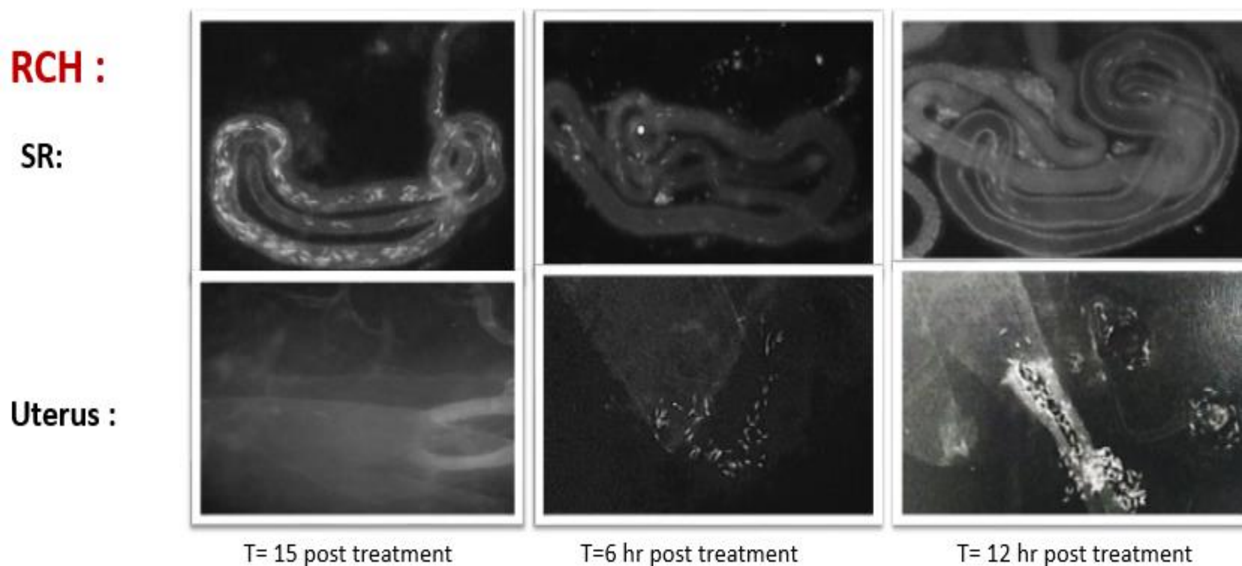

Fig. 9: SR and UT of RCH females over time. Here half of the sperms were transferred into the uterus from SR up till six hour post CS treatment and at 12 hr post CS treatment very few sperms were found in SR whereas uterus was filled with sperms; indicating that sperms were pushed into the uterus in order to eject them out of the body (dumping of sperm).

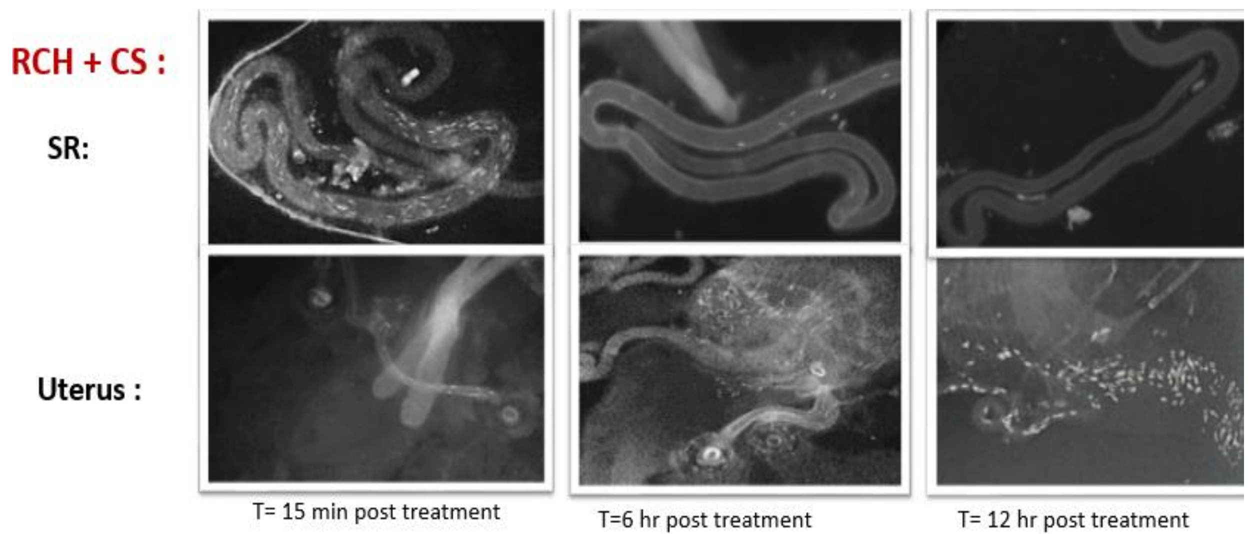

Fig. 10: SR and UT of RCH + CS females over time. Nearly all the sperms were transferred into the uterus from SR up till six hour post RCH +CS treatment and at 12 hr post CS treatment no sperms were found in SR also in the uterus was filled with sperms; indicating that sperms were pushed into the uterus in order to eject them out of the body (fast dumping of sperm).

3.2 Result of BRB females subjected to RCH, RCH+CS and CS:

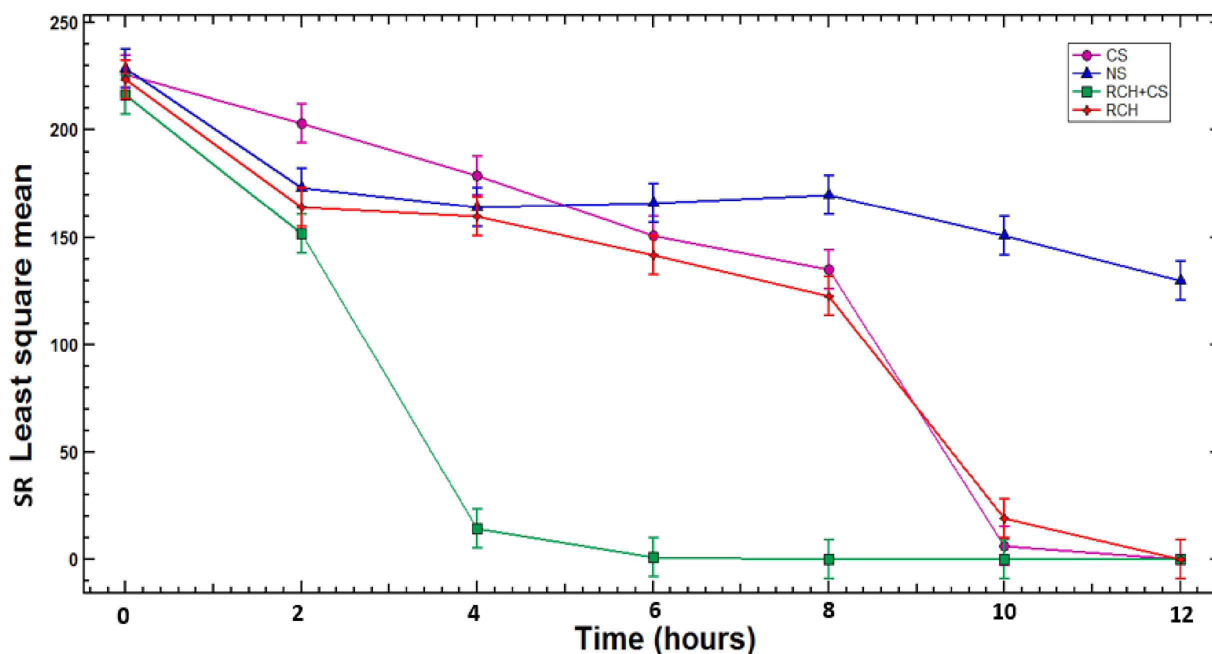

Fig.11: Effect of CS, RCH, RCH + CS on number of sperm stored in seminal receptacle in comparison with Control or no socked (NS) females.

Table 1: Effect of CS, RCH, RCH + CS and NS (treatment) on number of sperm stored in seminal receptacle over time was significant different. Summary of results from one-way ANOVA. DF: degrees of freedom. With confidence interval 95 percentage.

Source of variation	DF	Sum of squares	F-ratio	Prob.>F
treatment	3	944822.7	185.0798	<.0001*
time	6	2183793.5	213.8899	<.0001*
Treatment*time	18	689725.3	22.5182	<.0001*

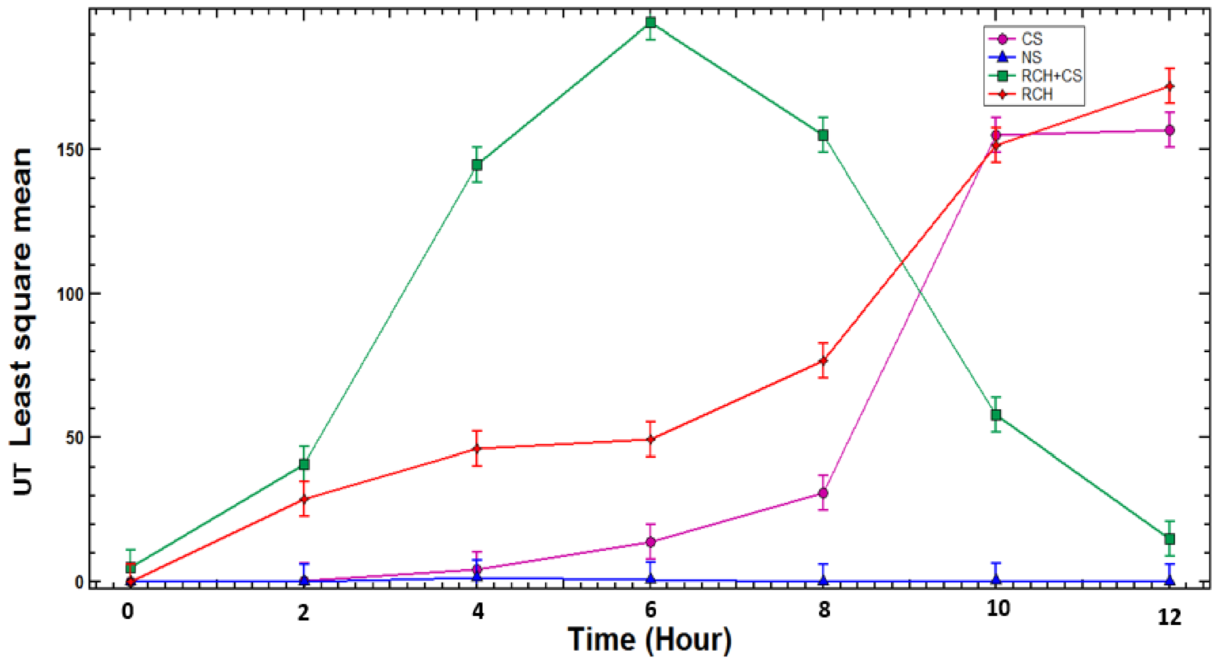

Fig.12: Effect of different treatments on number of sperm moved into uterus over time.

Table 2. Effect of CS, RCH, RCH + CS and NS (treatment) on number of sperm moved into uterus over time was significant different. Summary of results from one-way ANOVA. DF: degrees of freedom. With confidence interval 95 percentage.

Source of variation	DF	Sum of squares	F-ratio	Prob.>F
treatment	3	623647.3	278.371	<.0001*
time	6	542974.7	121.1811	<.0001*
Treatment*time	18	1266753.2	94.2380	<.0001*

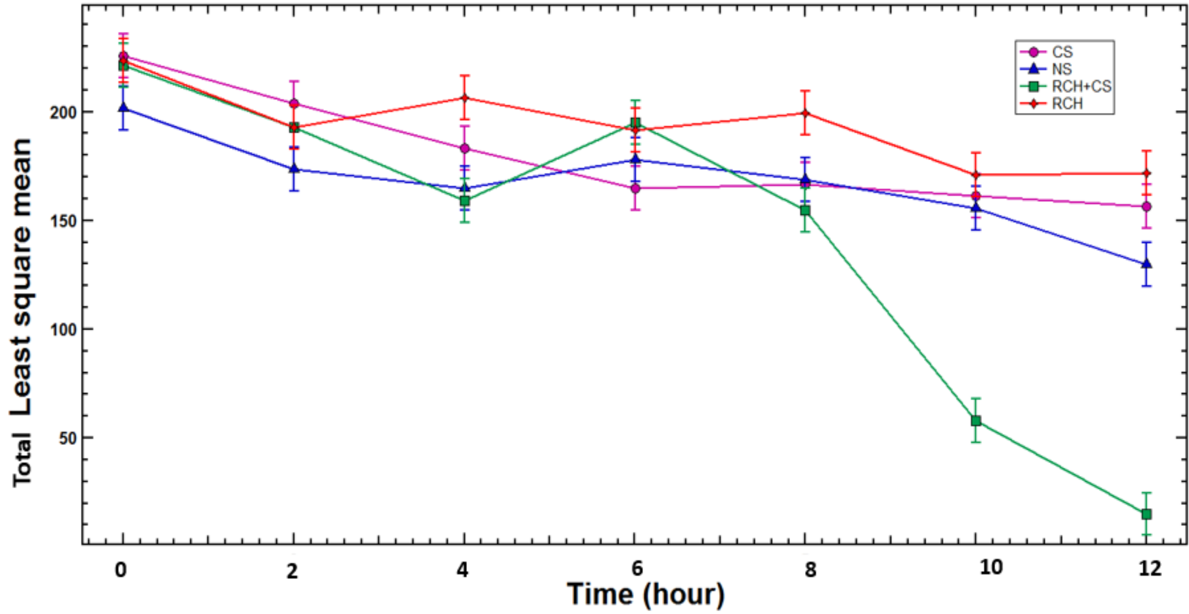

Fig.13: Total number of sperms in seminal receptacle and uterus over in different treatments.

Table 3. Total number of sperms in seminal receptacle and uterus in different treatment over time was significant different. Summary of results from one-way ANOVA. DF: degrees of freedom. With confidence interval 95 percentage.

Source of variation	DF	Sum of squares	F-ratio	Prob. > F
treatment	3	203031.92	33. 6929	<.0001*
time	6	542639.92	45.0252	<.0001*
Treatment*time	18	351327.04	9.7170	<.0001*

CHAPTER 4

DISSCUSSION

Low temperature affects insects differently based on the severity of the cold and the duration of exposure. There have been studies that have confirmed that cold shock/stress kill sperm stored in female storage organs (Novitski & Rush, 1949). Whereas there have been no study directed towards RCH killing or protecting the stored sperm in female. It is also known that dead sperm need to be removed from the female seminal receptacle in order to get fresh fertile sperm by re-mating. Hence females need to re-mate to produce progeny post cold shock (Singh, *et al*., 2015).

Current study shows that at 0 hours post cold shock, proportion of sperm in seminal receptacle is similar in all the treatments. Over time (up till 12th hr) all the treatments were significantly different, that is the number of sperm moved from SR to uterus over time significantly differs in all the treatments (table 1). In control females we found that majority of the sperms were located in SR whereas uterus was found to be completely empty (no dumping of sperm as the sperms were not damaged at all), clearly indicating that in normal conduction females rarely dump sperms. In CS treated females, half of the sperms were transferred into the uterus from SR up till 6th hour post CS treatment and at 12th hr post CS treatment very few sperms were found in SR whereas uterus was filled with sperms; Similar was the case with RCH treated females, both in RCH and CS treated females maximum sperm movement was found to be between 8th and 10th hour post treatment (fig.11); indicating that sperms were pushed into the uterus in order to eject them out of the body. In RCH followed by CS treated females we found that, nearly all the sperms were transferred into the uterus from SR up till 6th hour rather the sperm movement starts from 2nd hour and by 4th hour nearly all the sperms are found to be in the uterus (fig. 11), whereas at 12th hour there were no sperm were found in SR, indicating that sperms were pushed into the uterus in order to eject them out of the body and interestingly only few females had sperms in there uterus rest of the females have already ejected the sperm from out of their body (dumping of sperm was accomplished by these females). Therefore RCH indeed influence the manner in which *D. melanogaster* female handle the sperm before eventually dumping.

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