Biochemical mechanism for adaptation to cold stress in *Drosophila melanogaster*

Athira J Nair

MS11001

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

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

April 2016

Certificate of Examination

This is to certify that the dissertation titled "Biochemical mechanism for adaptation to cold stress in *Drosophila melanogaster*" submitted by Ms. Athira J Nair (Reg. No. MS11001) 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.

Professor 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.

Athira J Nair

(MS11001)

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)

Acknowledgements

First of all I owe my greatest gratitude to my supervisor Dr. N.G Prasad who helped me through all my difficult times and supporting me all through this for the successful completion of my thesis work. His patience, loving and caring behavior, understanding and encouraging words helped me to regain my confidence to a large extent. Without his help, I wouldn't have been able to finish this on time. Thank you so much Sir for all your help.

I am grateful to my thesis committee members Professor T. R Rao and Dr. Rhitoban Ray Choudhury for their valuable suggestions on the thesis.

I would also like to express my special thanks to Professor Purnananda Guptasarma for providing me access to their lab facilities and his lab members Nitin Kishor, Shahanaz Nazar, Bhisham Thakur, Prince Tiwari, Pallavi Kaila and Santosh Kumari for helping me out with the handling of equipments as well as for the fun time I had even though it was only for a few days.

I express my immense gratitude to Karan Singh for teaching me the basic of fly handling and helping me whenever I asked irrespective of his busy schedules. It must have been a difficult decision for him to hand over the cold lines (FSB and FCB population of flies) which he has been taking care of like his own family for the past five years. But thanks to him, I could use them for my entire thesis.

Thank you Ruchika, for being understanding, forgiving and for helping me out in experiments. Next off, I would like to thank Vinesh for being like a brother to me by scolding at times when needed, giving me advices and taking care of me. I am grateful to Aparajita for helping me out (both in experimental and non experimental ways) and feeding me when I was hungry. I would also like to thank Zeeshan, TJ, Manas, Neetika, Komal, Radhika, Saudamini and Nagender for helping me out at different times. Without you people it would have been difficult to complete my thesis.

I would like to thank my friends for all the good and bad times together and teaching me how to cope up with different situations in life. Thank you Akhilrag, Anjali, Jopaul, Arsi, Deepu, Anu, Tess, John, Manu, Partha, James Nair, Abhishek , Sekhar, Sruthi, Aisu, Thapu, Ammu and Sreenath for all those wonderful times- the late night walks, birthday parties, fun trips, Onam celebrations, dance classes, gossiping, cooking sessions and the list goes on.. It will never be forgotten!

I thank Professor K. Vijay Raghavan (NCBS, Bangalore) for letting me get a hand on experience in a research lab for the first time using *Drosophila melanogaster* as a model organism.

Never in my dreams I thought of joining an institute like IISER M and I would like to thank my teacher Mr. Pyarilal for guiding me in my school years and making me believe that everything is possible.

I would like to thank Professor K. S Viswanathan, Dr. Mahak Sharma, Dr. Ram Yadav and Ms. Yogeet Brar for all the moral support in the last five years.

Last but not least I thank my parents for being supportive and loving all this years. I believe all the wonderful things happened to me was because of your blessings. Thank you for having me as your daughter. Also I would like to thank my brother Akhil for tolerating and supporting me all my life. Special thanks to you for coming here whenever I asked you to, irrespective of that tiring 3 days journey! I appreciate all your hard work and I promise you that I will make you all proud in my future endeavors.

List of figures

List of tables

Notations

1. FSB – Selected for cold shock resistance, derived from BRB population

2. FCB – Cold shock resistance control, derived from BRB population

3. BRB – Blue Ridge Base lines

4. ANOVA – Analysis of Variance

Contents

Title **Page No.**

Abstract

Organisms face various types of environmental stress during their entire lifespan. Environmental stress acts as a major ecological driver and plays a significant role in driving the evolution of populations in nature. According to Hoffmann and Parsons 1991, environmental stress is "environmental factors causing change in the biological system which is potentially injurious". A change in the optimum conditions in the biotic as well as the abiotic components of the organism's habitat can lead to environmental stress. Temperature is an important abiotic factor of an organism's environment which can control various important life history traits. Since most physiological and biochemical processes underlying behavioral patterns are dependent on temperature, it plays a crucial role in both the distribution and abundance of insect species as well as in their evolution.

Multiple studies conducted previously in insects showed that cold stress (stress due to reduced temperature) can induce adult mortality and reduce gamete viability. A recent study from our own lab showed that egg viability, mating frequency, and male mating ability evolve in populations of *Drosophila melanogaster* selected for resistance to cold shock (Singh et al. 2015).

Several different mechanisms have been reported in organisms that can potentially increase its resistance to cold stress. One among that is the increase in concentration of certain metabolites that acts as anti-freeze agents. Such metabolites include Glycogen, trehalose, proline, triacylglycerol etc. Chen and Walker (1994) showed a positive correlation between glycogen content and cold tolerance. Similarly studies conducted by Hodkova and Hodek in 2004 showed that trehalose acts as anti-freeze compound and helps to resist cold stress in organisms.

So here in this study we hypothesize that the amount of glycogen produced will be higher in the FSB (selected) populations compared to the FCB (control) populations since they are being selected for resistance to cold shock for the last 90 generations. Using Drosophila melanogaster as model organism we are also looking at how glycogen concentration varies within a population post cold shock.

In order to understand this, we designed an experiment where flies were collected at 4 different time points: immediately after eclosion, 4 hours post cold shock, 12 hours post cold shock and 24 hours post cold shock. 30mg of male flies were used in each treatment for conducting the assays.

Our results indicated that there is no significant difference in the amount of glycogen produced in the male flies of selected and control populations subjected to cold stress. Effect of period and selection regime appeared to be insignificant while period effect was evident.

Chapter 1

Introduction

Environments frequently undergo fluctuations that can place organisms under different kinds of stresses. Such conditions include exposure to toxins, disease, and adverse environmental conditions such as drought or cold. Sometimes the action of the organism itself, such as migration, reproduction or hatching can expose it to stress. Therefore stress is considered as a ubiquitous hazard, one that has recently come into greater prominence as an area of research.

In accordance with the different ecological specializations, the phenomenon of stress is viewed somewhat differently. Since stress plays an important role in the survival of organisms and thus helps in determining the fitness, evolutionary biologists keeps close attention of the studies being conducted on this topic.

Stress is defined as "any environmental change that acts to reduce the fitness of organisms" (Koehn and Bayne, 1989) or "an environmental factor causing a change in a biological system, which is potentially injurious" (Hoffmann and Parsons, 1991). It shapes an organism's physiology, behavior and life history. Out of all the different abiotic and biotic factors that can cause stresses, temperature is one of the most important especially for ectotherms.

Since insects are ectotherms and are generally very small in size, the body temperature of insects depends greatly on the environmental temperature. Extreme temperatures are potentially injurious and even lethal for these kinds of organisms. Even if the temperature is not up to the lethal limit, it still can influence an organism's performance and its Darwinian fitness (Dillon et al. 2009). Cold stress can affect an animal's survivorship at various life stages (Tucic 1979, Czajka and Lee 1990, Chen and Walker 1993, Chen and Walker 1994, Anderson et al. 2005, Macmillan et al. 2009).

A large number of studies conducted on *Drosophila melanogaster* have focused on fitness consequences of temperature shock over a single generation and very few studies have investigated the evolution of resistance to cold shock. Apart from mortality, cold stress can affect fitness through its effects on reproduction, can decrease sperm count (Lacoume et al. 2007) as well as male mating ability (Rinehart et al. 2000, Lacoume et al. 2007)and can even kill sperms stored in female sperm storage organ known as

spermatheca (Novitski and Rush 1949). Given that temperature shock has major consequences on fitness and that temperatures vary across time and space, it is expected that insects adapt to temperature fluctuations over temporal and spatial scales.

If these insects are getting adapted to cold temperatures, there can be different underlying mechanisms that help them to increase resistance to cold stress. One possible way is by increasing the concentration of certain metabolites that acts as anti- freezing agents. Studies have shown a positive correlation between glycogen content and cold tolerance (Chen and Walker, 1994). There are certain other metabolites such as trehalose, proline and triacylglycerol which can also act as anti freeze compounds (Hodkova and Hodek, 2004) and in addition to this, changes in membrane composition and increased production of molecular chaperone proteins can help in cold stress tolerance (Rinehart et al. 2007). Another possible way of increased tolerance to cold stress is by the increase in expression of cold inducible genes such as *hsps hsp22, hsp23, hsp26, hsp27, hsp40, hsp68, hsp70Aa, hsp83, stv and frost.* All these genes are found to be getting up-regulated after cold shock (Colinet et al. 2010, Bing et al. 2012).

In this study I tried to find out whether there is any significant difference in the amount of glycogen produced in the male flies of both the selected (FSB) and control (FCB) populations subjected to cold shock as well as how the glycogen concentration varies over the next 24 hours post cold shock within a population.

Studies have conducted on five selected (FSB) and five control (FCB) populations derived from each of the five BRB populations that were maintained in the laboratory conditions for 35 generations to allow for adaptation to laboratory conditions and for the decay of linkage disequilibrium.

Chapter 2

Experimental System

Drosophila melanogaster is a holometabolous dipteran insect which belongs to the family drosophilidae. They are about 3 mm long and generally known as 'common fruit fly' or 'vinegar fly' since they mostly feed on different kinds of fruits.

It was Charles W. Woodworth, who first proposed the idea of using this species as a model organism for biological research in studies of genetics, physiology, microbial pathogenesis, life history evolution etc. and ever since then Drosophila is so popular that it would be almost impossible to list the number of things that are being done with it.

Drosophila melanogaster can live in a wide range of habitats. Though its ancestry traces back to Africa, native habitats include those in the tropical regions of the world and in addition to that humans have helped to introduce them to temperate regions as well (Demerec 1950, Patterson and Stone 1952) thereby making it a good model to study adaptation to cold stress. Previous studies have also shown that *D. melanogaster* is an excellent model system to study several topics such as adaptation to different kinds of environmental stresses, evolution, disease and immunity (Tucic 1979, David et al. 1983, Rose 1984, Watson and Hoffman 1991, Partridge and Barton 1993, Chippendale et al. 1994, Joshi and Mueller 1996, Gibbs 1999, Zwaan 1999, Lazzaro and Clark 2003, Anderson et al. 2005, Wolf and Rockman 2008, MacMillan et al. 2009, Singh et al. 2015).

I have also used *D. melanogaster* as the model organism for my experimental studies. It has a life cycle consisting of four different stages – egg, larva, pupa and adult. Under usual laboratory conditions, i.e., under a temperature of 25^0C, 50-60% relative humidity, their metamorphosis follows the pattern described in (Figure 2.1). The *D. melanogaster* eggs which is about half a millimeter long hatch into first instar larva in about 18-24 hours. Maximal resource acquisition for these flies occurs during the larval stages where they dig into the food (the fly media in case of laboratory cultures) and actively feed upon the available food. 24 hours later first instars larvae molt into second instar larvae and then in another 24 hours they molt again into third instars larvae. After attaining a critical size the late third instar larvae stops feeding, comes out of the food and becomes stationary on some suitable substrate to pupate. It goes through metamorphosis and completes the development in about 4-5 days. The adult fly comes out of the pupal shell-

a process commonly referred to as 'eclosion' and this happens usually on 9-10th day. The adult males and females do not eclose as reproductively active individuals. They usually take 8-10 hours to become sexually mature. Females start laying eggs one day after eclosion and fecundity of the female heavily depends upon the equality of the food they acquire (Prasad and Joshi 2001, Stewart et al. 2005, Nandy et al. 2012).

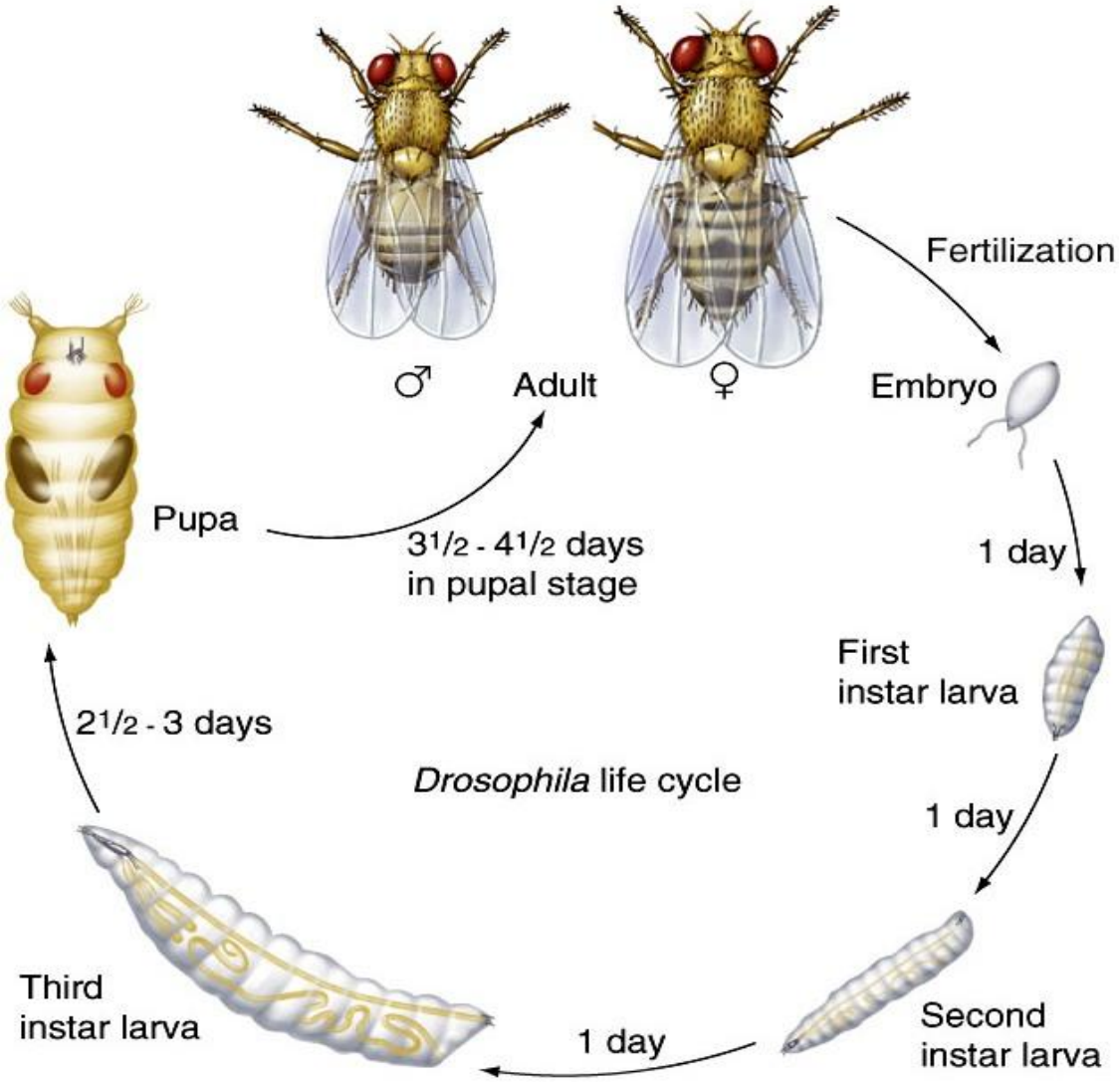


Figure 2.1- Life cycle of *Drosophila melanogaster*

Laboratory Selection Experiments

Laboratory selection experiments using *D.melanogaster* and other organisms are widely used in experimental biology. In particular, such experiments on *D.melanogaster* life history and stress- related traits have been instrumental in developing the emerging field of experimental evolution. However, similar selection experiments often produce inconsistent correlated responses to selection. Unfortunately, selection experiments are vulnerable to artifacts that are difficult to control. In spite of these problems, selection experiments are valuable research tool and can contribute to our understanding of evolution in natural populations. Laboratory setting enables the experimenter to manipulate environmental parameters as per requirements and makes replication of experiments possible. The impact of individual environmental factors to shape life history traits of populations can be studied unambiguously using this approach. When doing laboratory selection experiments effective size is one of the crucial parameters which can influence the end results. Since small population size can lead to inbreeding depression, it is essential to keep the effective population size high. The base-line population (BRB) that I had been working on was maintained under population sizes sufficiently high (N_e= 2800) enough to minimize the effect of inbreeding. The selected and control populations used were also maintained under high effective population size of N_e = 1400.

Base line population used for the experiment

In 2010 nineteen isofemale lines of *D.melanogaster* were established using wild inseminated females collected from Blue Ridge, Georgia, USA and maintained in the laboratory of Daniel Promislow at University of Georgia, USA. These lines were provided to us later in 2010, after which they were maintained in our laboratory for 6 generations. In 2011, from each of this 19 isofemale lines 100 males and females were taken and then combined to create a single large population of *D.melanogaster* called **B**lue **R**idge **B**ase line (BRB). This BRB population was maintained for ten generations under standard laboratory conditions after which it was split into 5 replicate populations called BRB 1 -5 (see the flow diagram in Figure 2.2)

The five BRB populations are maintained on a 14 day discrete generation cycle at 25^0C temperature, 50-60% relative humidity. 12:12 hours light- dark cycle on standard banana-yeast-jaggery food (Prasad et al. 2003). Eggs are collected from adult flies and are dispensed into glass vials (25mm diameter x 90mm height) containing 6 ml of banana-yeast- jaggery food at a density of about 70 eggs per vial and incubated under standard laboratory conditions. Forty such vials are set up per population. On 12^{th} day post egg collection the adults are then transferred to Plexiglas cages (25cm length x 20cm width x 15cm height) provided with a Petri plate containing standard banana- yeast- jaggery food. Each cage contains approximately 2800 adult flies. On the 13^{th} day post egg collection, fresh cut plates are provided in the cages for 18 hours and the eggs are collected from these plates to start the next generation. The BRB 1-5 populations were maintained in these conditions for 35 generations before deriving the selected and control populations.

Derivation and maintenance of selected and control populations

The BRB populations were maintained under standard laboratory conditions for 35 generations to allow for adaptation to laboratory conditions and for decay of linkage disequilibrium that might have arisen as a result of the mixing of 19 isofemale lines. Following this, one selected (FSB) and one control (FCB) population was derived from each of the five BRB populations which resulted into a total of 10 populations (five selected populations FSB 1-5 and five control populations FCB 1-5).

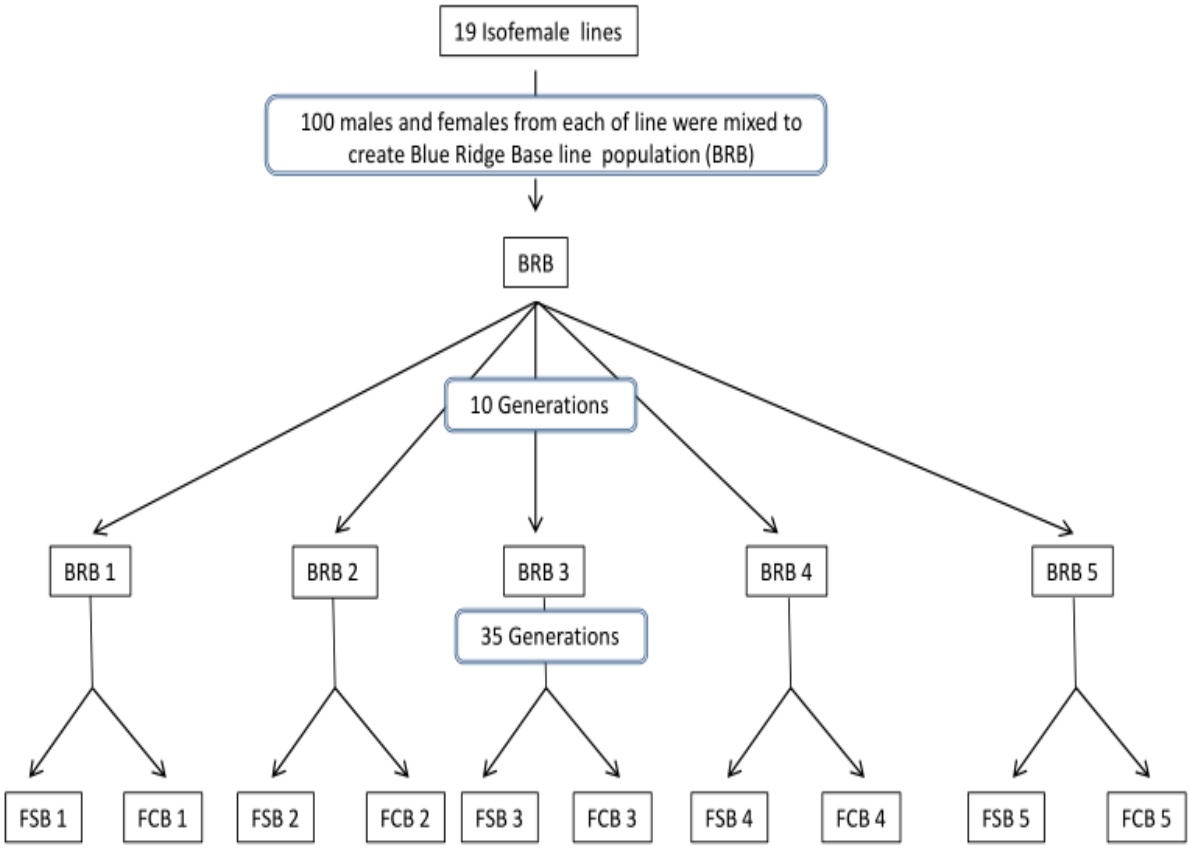


Figure 2.2: Derivation of base line BRB population from 19 isofemale lines and the creation of FSB (1-5) and FCB (1-5) populations from BRB (1-5) populations (Singh et al. 2015).

Same numerical subscript were given for the selected and control populations that were derived from the same BRB population as they were more closely related to each other and were treated as blocks. So here blocks refer to ancestry in the selection regime. For example, FSB 1 and FCB 1 are derived from BRB1 and are more closely related to each other than FSB 2. So the experiment consisted of five blocks which were handled together during maintenance and experimentation.

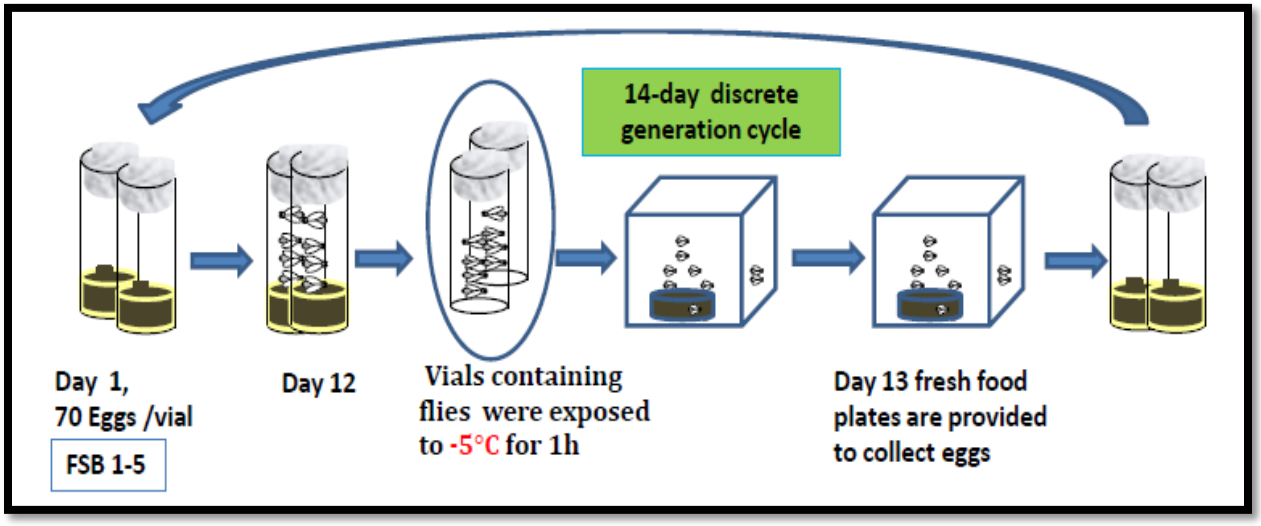
The details of the maintenance of the selection regime are presented in Singh et al. (2015). The selected populations are maintained on a 14 day generation cycle (Figure 2.3). Eggs are collected from adults and dispensed into vials(25mm diameter x 90mm height) containing about 6ml of standard banana- yeast- jaggery food at a density of about 100 per vial. Twenty such vials are set up per population. The vials are then incubated at 25^0C temperature, 50-60% RH and 12:12 light: dark cycle. The flies start eclosing by the 9^{th} day post egg collection with peak eclosion happening on the 10^{th} day. By day 12, all the adults eclose and are about 2 days old post eclosion and the adults from each of the 20 vials are then transferred into 20 dry, empty vials. The cotton plug is pushed in such a way that the flies are confined to a small area at the bottom one third of the vial. These vials are then placed into salt-water-ice slurry maintained at -5^0C and held there for one hour. The part of the vial containing the flies is completely immersed in the slurry. After one hour, the flies are transferred into a Plexiglas cage containing a Petri plate of banana- yeast-jaggery food and maintained at 25 ^{0}C.

24 hours after the cold shock treatment (i.e. 13^{th} day post egg collection), the flies are provided with a fresh food plate and are allowed to oviposit for 18 hours. After 18 hours eggs are collected and then dispensed into food vials to start the next generation. The cold shock treatment that we use causes low adult mortality which can be up to 3-9% whereas the viability of the eggs laid 24 hours post cold chock is reduced by about 30-40%. For the FSB populations, we collect eggs at a density of about 100 per vial such that the numbers of emerging larvae and adults is approximately 60 – 70 per vial.

The control populations (FCB 1-5) are maintained under exactly the same conditions as the selected (FSB 1-5) populations with slight changes:

- On 12^{th} day post egg collection, the flies from the FCB population are transferred into empty vials and are kept at 25^0C for one hour (Figure 2.3)
- The eggs are collected from the adults and are dispensed into food vials at a density of 60 – 70 per vial.

Maintenance of selected (FSB) populations:



Maintenance of control (FCB) populations:

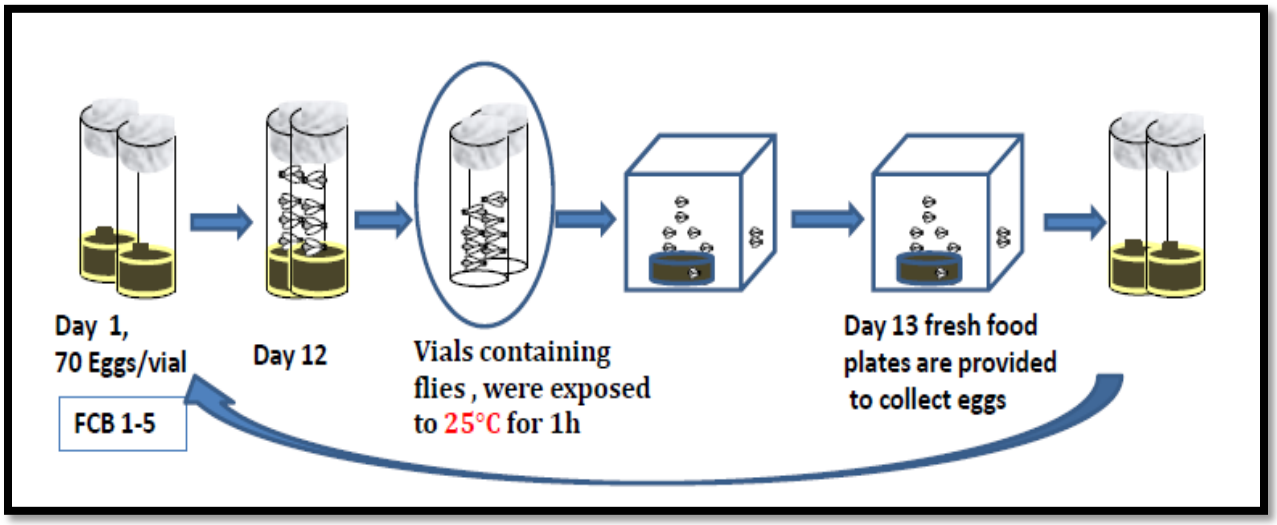


Figure 2.3: Maintenance regime of the FSB (1-5) and FCB (1-5) populations (Singh et al. 2015)

All the populations (FSB 1-5 and FCB 1-5) were reared under laboratory conditions as described above for one generation.FSB populations were not subjected to any selection during this generation and this process is called 'standardization' and we call those flies 'standardized flies'. For standardization eggs are collected from each of the FSB and FCB stock populations. Eggs from a given population are distributed into vials containing standard banana- yeast- jaggery food at a density of about 70 eggs per vial. Per population 20 such vials were set up. The vials are then incubated at standard laboratory conditions and on 12^{th} day post egg collection the adults from a given population are transferred into Plexiglas cage provided with banana- yeast- jaggery food. These flies are called the standardized flies. On the next day, a fresh food plate is provided and the standardized flies are allowed to oviposit for 6 hours. Eggs are then collected from these food plates and dispensed into vials containing approximately 6ml of standard banana-yeast- jaggery food at an exact density of 70 eggs per vial. For further assays, adults emerged from these flies are used (i.e. the progeny of the standardized flies). My experimental studies were conducted on 76^{th} to 90^{th} generation of flies.

Table 1: The composition of 1 liter of regular banana- yeast- jaggery food.

	Ingredient	**Amount**
1	Banana (g)	205
2	Barley flour (g)	25
3	Jaggery(unrefined cane sugar) (g)	35
4	Yeast (g)	36
5	Agar (g)	12.4
6	Ethanol (ml)	45
7	Water (ml)	180
8	p-Hydroxymethyl benzoate (g)	2.4

Preparation:

- Put the ingredients 1,2,3,4,6 and 7 in a mixer or grinder
- Grind and mix it very finely so that no yeast is visible and the mixture is a uniform thick liquid (Banana – Yeast – Jaggery mixture)
- Take water in a cooker and heat it up to greater than 50^0C
- When the water gets warm enough add agar and keep stirring it to prevent the formation of lumps
- When the agar gets completely mixed add the banana- yeast – jaggery mixture and stir it well
- Let the mixture boil and once it gets boiled bring down the temperature to below 60^0C
- Mix the ingredients 6 and 8 to make a solution and add this solution to the food
- Pour to vials /plates and cool them with proper precautions

Chapter 3

Mechanism of cold adaptation

Introduction

For ectotherm species, such as insects, temperature has long been recognized as a major environmental factor responsible for species abundance and geographic distribution (Andrewartha & Birch 1954; Precht, Christophersen & Hensel 1955; Cossins & Bowler 1987; Leather, Walters & Bale 1993). Ambient temperature varies according to daytime and season, so that natural populations are often exposed to heat or cold stress (Gibbs, Perkins & Markow 2003). The capacity to adapt to and tolerate such stresses is crucial for the persistence of populations (Hoffmann & Parsons 1991, 1997; Addo-Bediako, Chown & Gaston 2000; Chown, Addo-Bediako & Gaston 2002; Hoffmann, Sorensen & Loeschcke 2003; Klok & Chown 2003). In temperate countries species must tolerate cold conditions during winter and have developed a diversity of adaptive mechanisms to do so, including the occurrence of diapause and the production of antifreeze compounds (Leather *et al.* 1993; Graham, Walker & Davies 2000).

Drosophila melanogaster is a cosmopolitan species, and its broad geographical range is accompanied by genetic variation in a diversity of traits which in most cases, vary progressively with latitude (David & Capy 1988) and this pattern suggests that local climate and especially temperature is the selective factor. Previous studies have shown that exposure to very cold temperatures decreases the survivorship of adults, eggs and pupae and reduces fecundity (Tucic 1979, Czajka and Lee 1990, Chen and Walker 1993, Watson and Hoffmann 1996, Anderson et al. 2005, Schimidt and Paaby 2008, Macmillan et al. 2009, Overgaard et al. 2007, Dillon et al. 2007, Marshall and Sinclair 2010, Mockett and Matsumoto 2014). Over the time insects have evolved a number of mechanisms to deal with cold stress (Sinclair et al. 2003).

Studies conducted from our own lab have found that egg viability, mating frequency and male mating ability evolve in populations of *D. melanogaster* selected for resistance to cold shock (Singh et al. 2015). In this study, I am trying to find out the possible reasons for the adaptation of the selected lines to cold stress. My goal is to find out how they biochemically adapt to this kind of stresses by looking at the concentration of Glycogen

which is known as an anti freezing compound (Vladimır Kostal 2011) as well as one among the three major storage molecules representing the largest readily accessible energy sources available to the fly (Qiaofang Chen et al. 2002).

In order to do the experiment, I subjected adult flies to a non lethal cold shock (-5^0C for one hour) which had relatively little effect on adult mortality (about 3-9%). The protocol I used for the glycogen assay was similar to that of several previous studies (Clark and Keith 1988, Minou Djawdan 1997).

METHODS AND MATERIALS

Baseline population and experimental population

Maintenance of base line and the derivation of selected (FSB) and control (FCB) populations are given in Chapter 2 in detail.

Experimental protocol:

In order to generate experimental flies eggs were collected from one generation of standardized flies at a density of 70 eggs per vial, each vial containing approximately 6ml banana-yeast-jaggery food. I collected 100 such vials from each block. Flies were collected at four different time periods as described below:

1. **Soon after eclosion:** male flies were collected from both the selected (FSB) and control (FCB) lines immediately after eclosion (i.e. on 9^{th} or 10^{th} day). Soon after eclosion flies were flash frozen using liquid nitrogen.
2. **4 hours post cold shock:** on 12^{th} day after egg collection, male flies of the selected (FSB) and control populations were subjected to cold shock as mentioned in chapter 2 of this thesis. Post cold shock male flies were allowed to recover for 4 hours and following this flies were flash frozen using liquid nitrogen.

3. **12 hours post cold shock:** on 12th day after egg collection, male flies of the selected (FSB) and control populations were subjected to cold shock. Post cold shock male flies were allowed to recover for 12 hours and following this flies were flash frozen using liquid nitrogen.
4. **24 hours post cold shock:** male flies of the selected (FSB) and control populations were subjected to cold shock on 12th day after egg collection,. Post cold shock male flies were allowed to recover for 24 hours and following this flies were flash frozen using liquid nitrogen

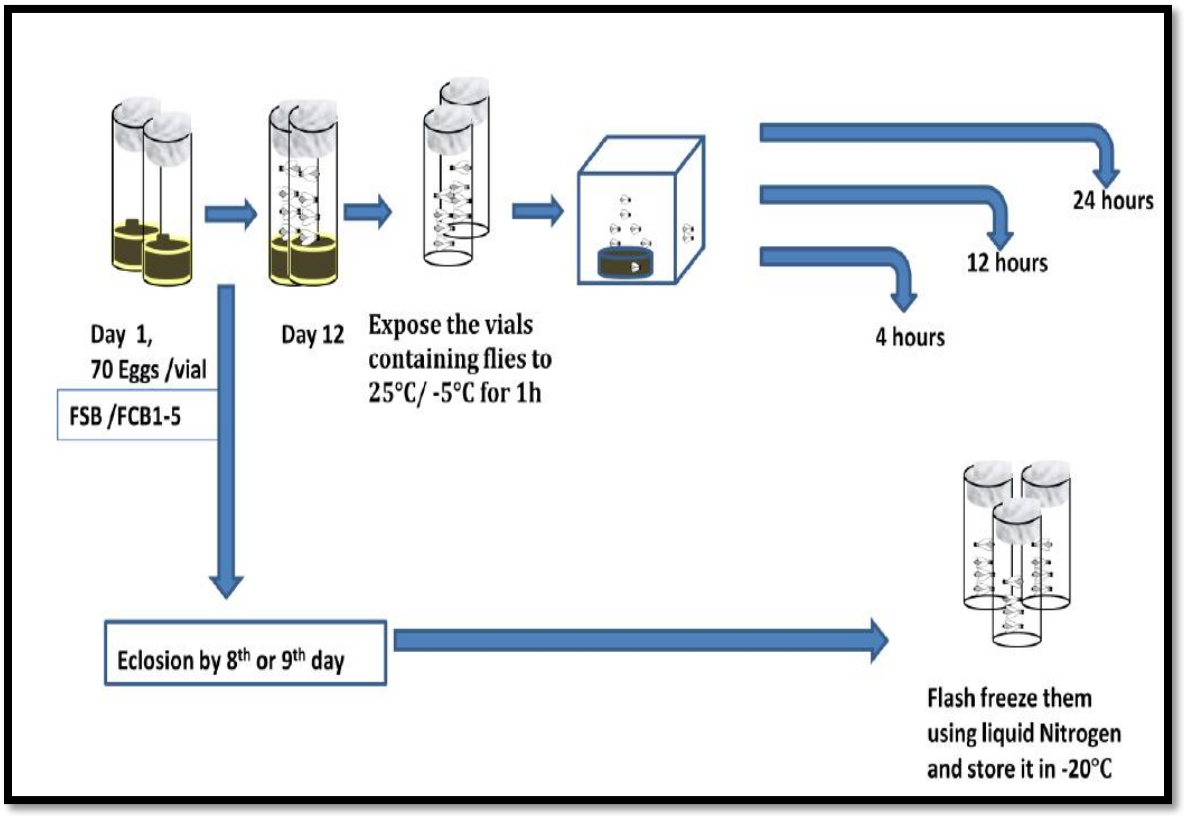


Figure 3.1: Collection of male flies from the selected (FSB) and control (FCB) lines for the glycogen assay.

Glycogen measurement:

- **a.** Weigh 30mg of male flies (approximately 40 male flies in number)
- **b.** Homogenize these flies in a 4^0C cold room temperature using a motorized glass/Teflon homogenizer and 1.2ml of homogenization buffer (Buffer-0.01M KH_2PO_4**,** 1mM EDTA pH-7.4)
- **c.** Centrifuge the homogenate at 1500 rpm for two minutes
- d. Cellular and cuticular debris gets pellet down and then add aliquots of 10µl of homogenate to 1.5ml of reagent (the test reagent contained 0.1 U/ml amyloglucosidase (1,4-α-D-glucan glucohydrolase EC 3.2.1.3), 5 U/ml glucose oxidase, 1 U/ml peroxidase, 0.04 mg/ml o-dianisidine dihydrochloride. The reagent is buffered by salts contained in the Sigma preparation of glucose oxidase and peroxidase (PGO enzyme, catalog no. 510-6)
- **e.** Incubate at 37^0C for 30 minutes
- **f.** Read OD at 450nm and determine the concentration of glycogen

Standard Curve Preparation:

In this experiment, a standard curve for the analysis of Glycogen will be constructed. In order to do this a number of solutions of known concentrations (as standards) will be used and the absorbance of each will be measured at 450 nm using a spectrophotometer.

After all the readings are obtained, each of it will be plotted against its corresponding concentration. If the procedure follows the Beer's Law, the points plotted will generally lie in such a way that a straight line can be drawn through them. The concentration of controls and other unknowns can be determined by locating their absorbance reading on the line and then dropping an imaginary line from that point to intersect the concentration axis.

Statistical Analysis

I analyzed the results using a three-way mixed model ANOVA treating selection and period as fixed factors crossed with random blocks. A two way mixed model ANOVA was used for the analysis of period effect and block effect. All analysis was performed using statistical software JMP (version 9.0).

Results

Amount of glycogen produced in the selected (FSB) and control (FCB) populations post cold shock was calculated with the help of a glycogen standard curve (Figure 3.2). I did not find a significant effect of selection or period on the amount of glycogen produced in the male flies. But a significant effect of blocks was found ($p = 0.031$). Selection x Block also had a significant effect. None of the other interactions were found to be significant.

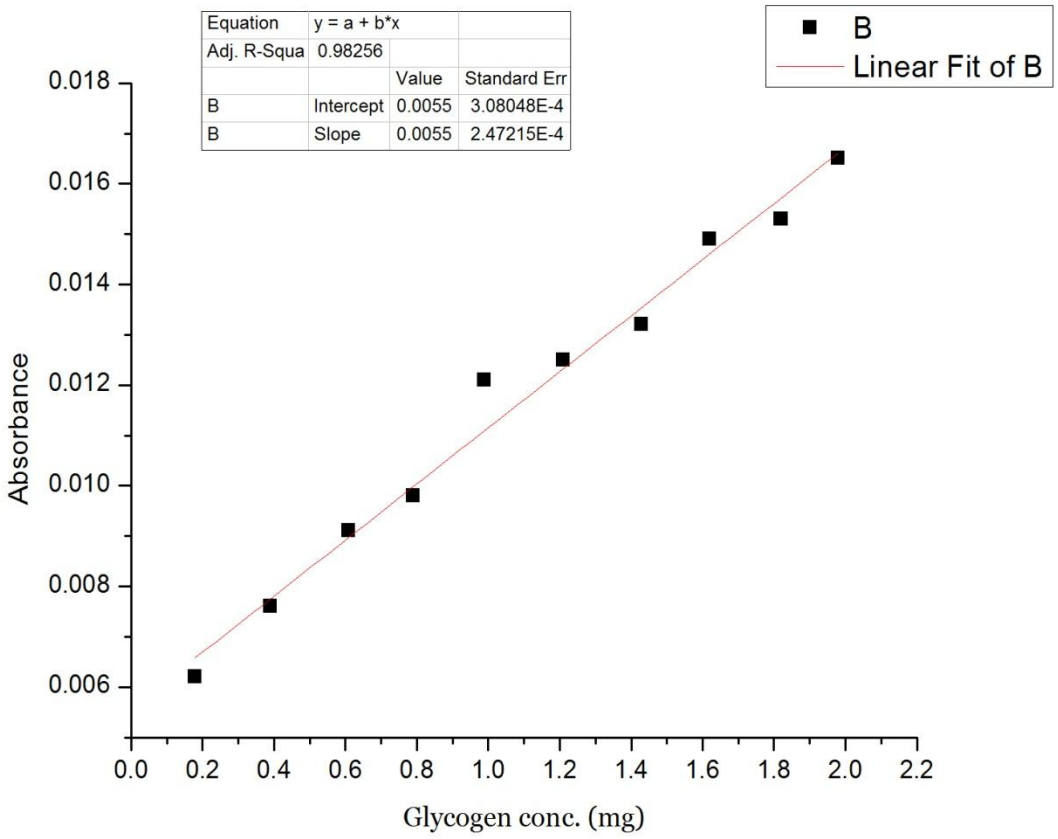


Figure 3.2: Glycogen standard curve

Glycogen content in males post cold shock

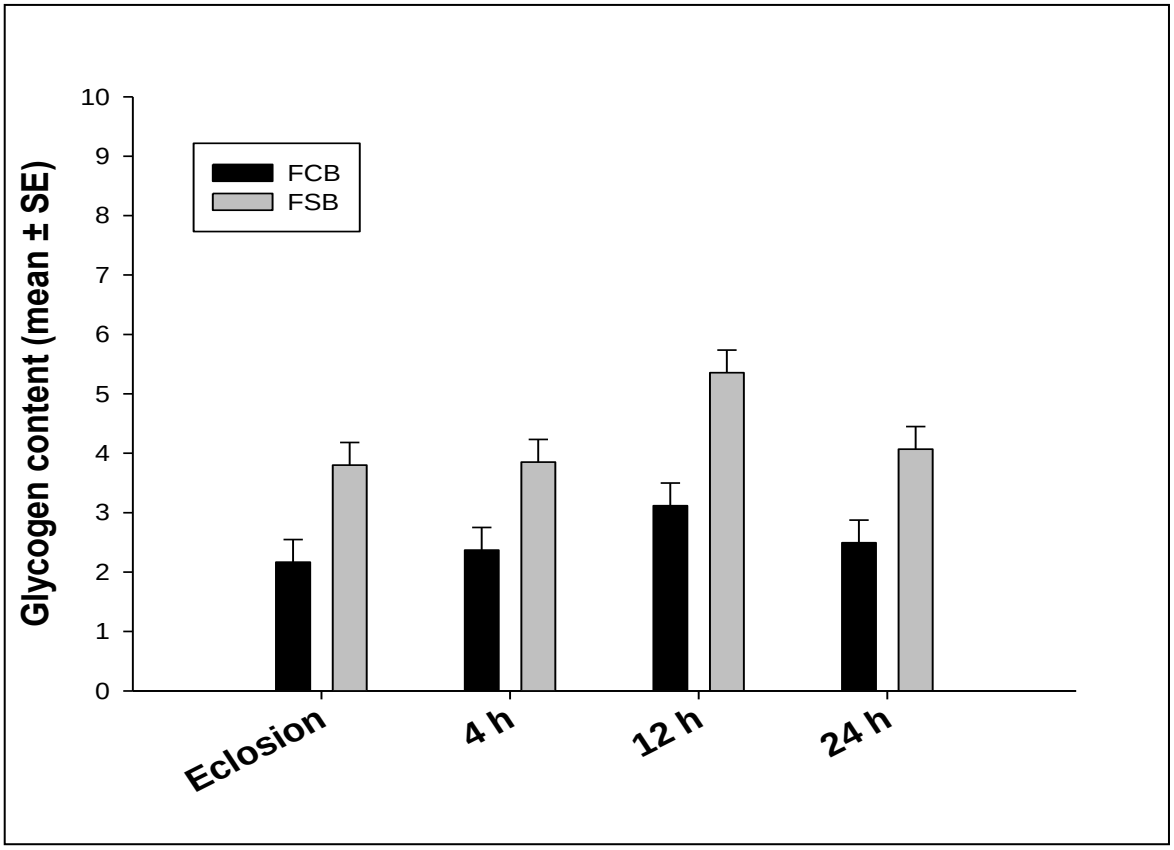


Figure 3.3: Result showing the amount of glycogen produced in the male flies of selected (FSB) and control (FCB) populations post cold shock. Eclosion; Freshly eclosed flies, 4 h; 4 hours post cold shock, 12 h; 12 hours post cold shock, 24 h; 24 hours post cold shock.

Table 3.1: Three way mixed model ANOVA using selection and period as fixed factors crossed with random blocks. p values in bold indicates that they are significant.

Effect	SS	MS Num	DF Num	DF Den	F Ratio	Prob > F
Selection	30.0108377	30.0108377	1	4	2.9855052	0.15907999
Period	9.7310962	3.24369873	3	12	3.36921219	0.05474673
Block	326.931784	81.7329459	4	4.17	7.94134565	**0.03182757**
Selection*Period	0.8823557	0.29411857	3	12	0.40688743	0.75086446
Selection*Block	40.2087226	10.0521807	4	12	13.9063167	**0.0001853**
Period*Block	11.5529633	0.96274694	12	12	1.33187657	0.31371276
Selection*Period*Block	8.67419967	0.72284997	12	.	.	.

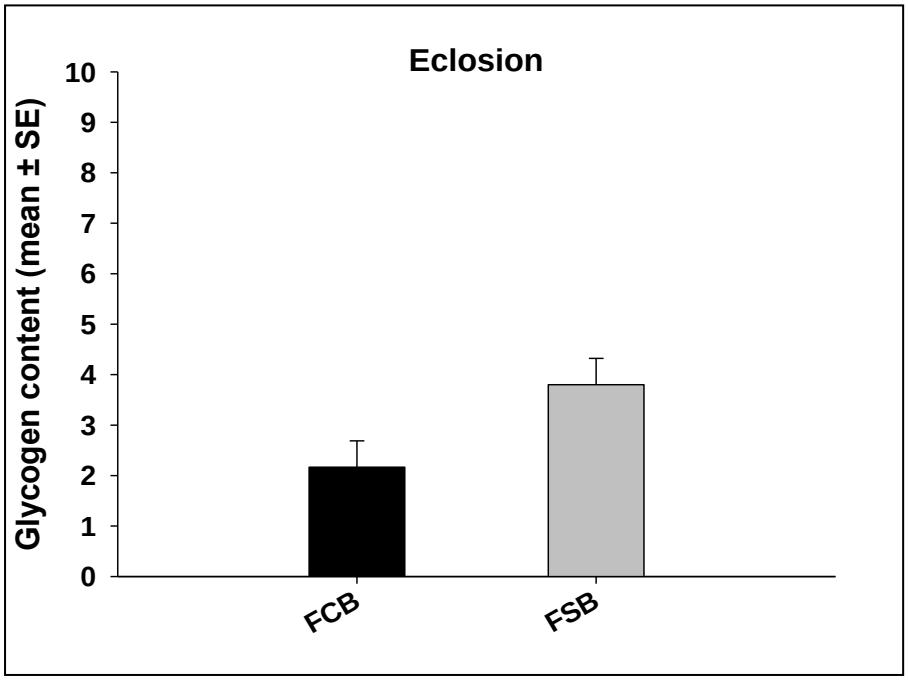


Figure 3.4 a: Effect of selection regime on the glycogen content of male flies soon after eclosion. I did not find any significant difference in glycogen content between the FSB and FCB male.

Table 3.2 a: Summary of results from two way mixed model ANOVA for the effect of selection regime on the glycogen content of male flies soon after eclosion. Though there was no significant effect of selection was observed, block effect was found to be significant.

Effect	SS	MS Num	DF Num	DF Den	F Ratio	Prob > F
Selection	6.67637553	6.676376	1	4	4.88643	0.0915
Block	66.4253699	16.60634	4	4	12.15416	**0.0164**
Selection*Block	5.46523769	1.366309	4	.	.	.

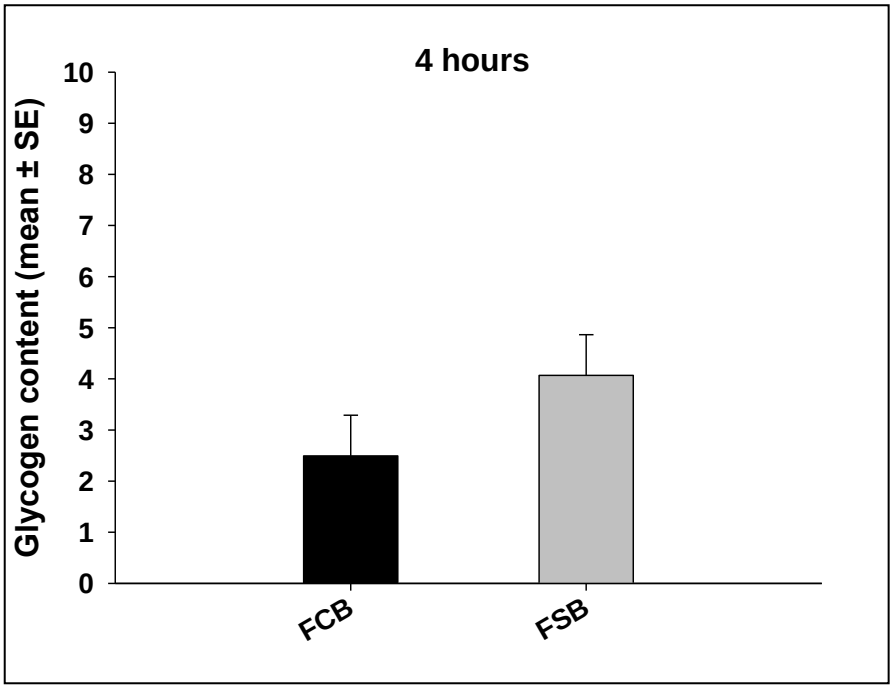


Figure 3.4 b: Effect of selection regime on the glycogen content of male flies 4 hours post cold shock. No significant difference was found in glycogen content between the FSB and FCB male.

Table 3.2 b: Results from two way mixed model ANOVA for the effect of selection regime on the glycogen content of male flies 4 hours post cold shock

Effect	SS	MS Num	DF Num	DF Den	F Ratio	Prob > F
Selection	6.20371	6.20371	1	4	1.96629	0.2334802
Block	70.99154	17.74789	4	4	5.625262	0.061469
Selection*Block	12.62013	3.155033	4	.	.	.

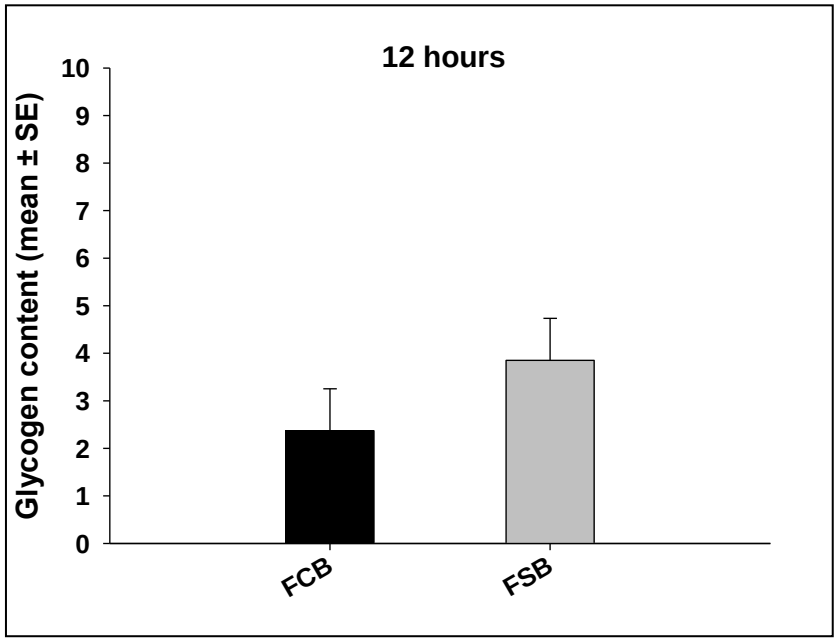


Figure 3.4 c: Effect of selection regime on the glycogen content of male flies 12 hours post cold shock. Significant difference was not found in the glycogen content between FSB and FCB male.

Table 3.2 c: Results from two way mixed model ANOVA for the effect of selection regime on the glycogen content of male flies 12 hours post cold shock

Effect	SS	MS Num	DF Num	DF Den	F Ratio	Prob > F
Selection	5.489463	5.489463	1	4	1.407808	0.30107665
Block	74.43706	18.60926	4	4	4.772466	0.07963443
Selection*Block	15.59719	3.899298	4	.	.	.

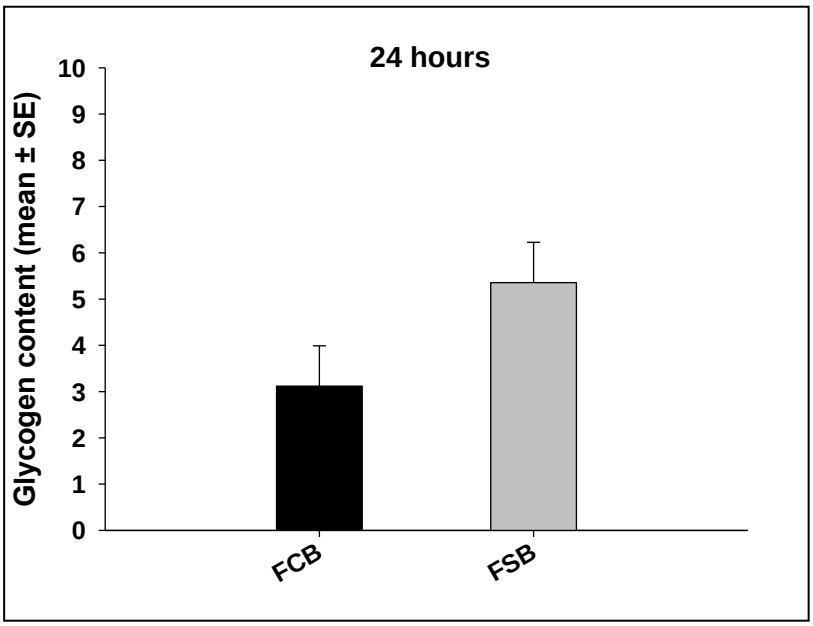
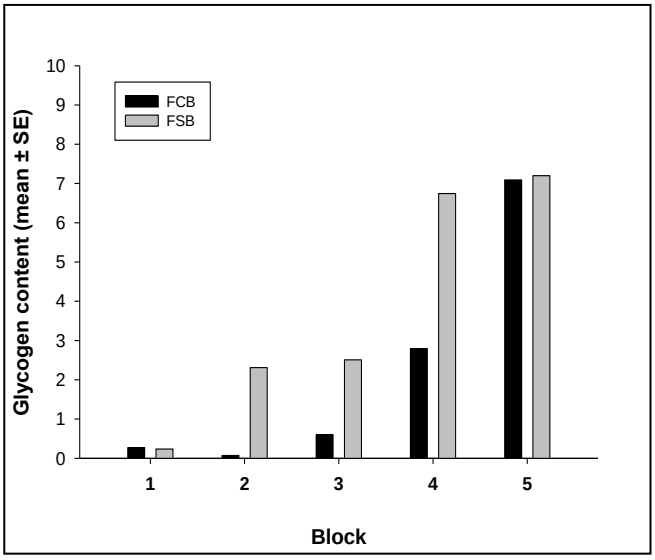


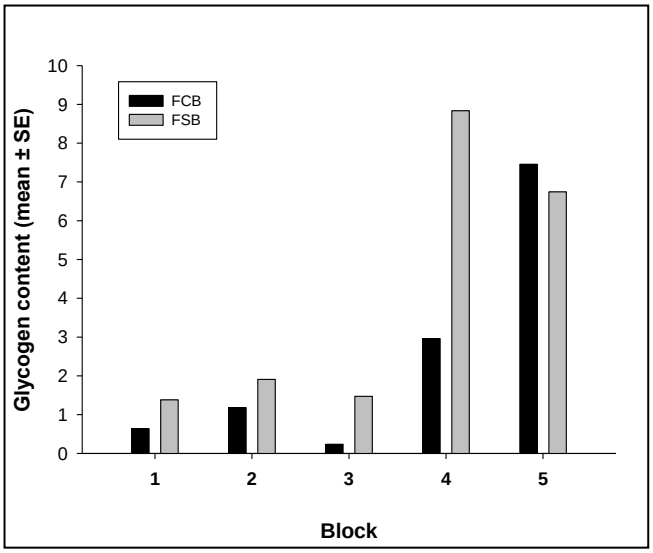
Figure 3.4 d: Effect of selection regime on the glycogen content of male flies 24 hours post cold shock. I did not find any significant difference in the glycogen content between FSB and FCB male.

Table 3.2 d: Results from two way mixed model ANOVA for the effect of selection regime on the glycogen content of male flies 24 hours post cold shock. Block effect was observed to be significant but no significant effect was found on selection regime

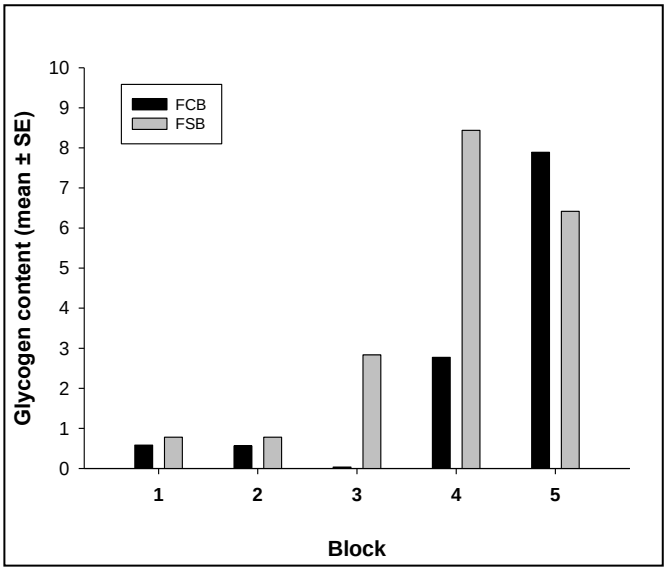
Effect	SS	MS Num	DF Num	DF Den	F Ratio	Prob > F
Selection	12.52364	12.52364	1	4	3.295617	0.143646
Block	126.6308	31.65769	4	4	8.330773	**0.031996**
Selection*Block	15.20036	3.800091	4	.	.	.



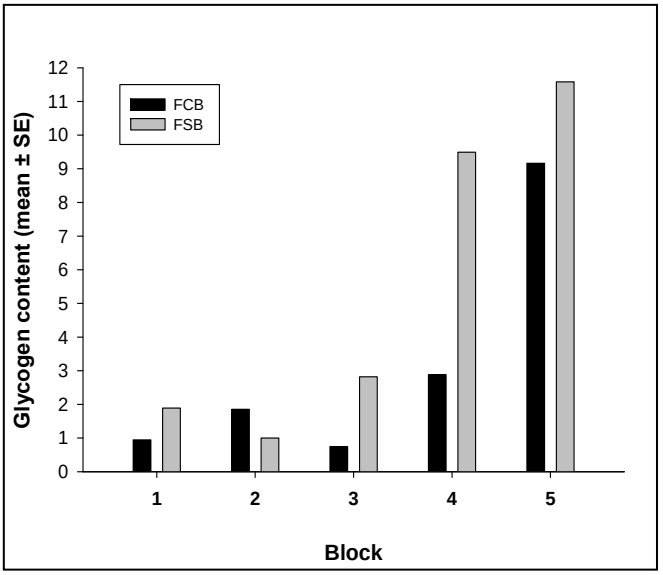
Soon after eclosion



4 hours post cold shock



12 hours post cold shock



24 hours post cold shock

Figure 3.3: Block wise analysis of the glycogen content in male flies at different periods

Chapter 5

Discussion

This present study was carried out in order to investigate the underlying mechanisms for evolved cold tolerance in the selected (FSB) populations. As a measure of this mechanism I studied about the glycogen content which is produced in the organisms in response to cold stress.

Stresses caused by different abiotic and biotic factors elicit different responses in organisms. It is known that cold stress often causes damage to cellular components such as proteins, lipids etc. Ohker et al. (1993) showed that lipids play a significant role in maintaining cold tolerance in different species of *Drosophila*. Chen and Walker in 1994 conducted experiments on *Drosophila* and showed that it is not just lipids but in lower temperatures a higher content of glycogen and other proteins are also observed in the cold selected lines than in the control lines. The highest concentrations were observed at the acclimation time with the highest Tcs (increase in the cold shock tolerance).

However in this study I did not find any significant difference in the amount of glycogen produced in the male flies of selected (FSB) and control (FCB) populations. Selection effect and period effect were turned out to be statistically insignificant while a significant effect of blocks and selection x block was observed. There is a possibility that the exposure to chilling temperatures eventually depleted all the energy resources where glycogen is one among the major three readily accessible energy sources for the flies when facing a stress condition.

Not just glycogen, but there are other metabolites as well which helps the organism to combat stress using different mechanisms. A further investigation into these mechanisms would provide a better understanding about the systemic regulation in these energetic reserves that helps for the cold hardening mechanism to cope with the fluctuations of low temperatures causing cold shock or chill injury. Microarray profiles of other cold adapted lines in Drosophila melanogaster have found a large number of other genes too which are involved in cold stress.

Conclusion

Various laboratory studies conducted over the last two decades have shown the importance of stress related mechanisms in different organism like bacteria, fungi, insects etc. In order to cope up with the stress caused by various environmental factors organisms are evolving by making use of different cellular components so that they could tolerate the unfavorable situations at any time.

For all the organisms stress is a hazard because it can cause detrimental effects on the performance and the Darwinian fitness of the organism. For ectotherms like insects, temperature is one of the crucial factor and studies have conducted on heat stress as well as cold stress.

Studies conducted previously found out the positive correlation between the glycogen content and cold tolerance. Metabolites such as Proline,and trehalose were also found to be helpful for cold tolerance. Gene expression patterns were also assessed during and after cold stress (Colinet et al. 2010).

In my study I attempted to understand the mechanism of adaptation to cold stress in the FSB (selected) populations. I did this by designing an experiment to find out the amount of glycogen content in the male flies from FSB and FCB populations with and without cold shock. We were expecting a significant difference in the glycogen content between the male flies of selected and control lines. However in my study even though I observed a difference in the glycogen content between the selection regimes i.e. between the FSB and FCB populations, they were not statistically significant to prove that more glycogen is produced in FSBs post cold shock.

Selection effect and period effect turned out to be non significant while block effect appeared to be significant. Since I could not find any effect of selection regime on the glycogen content it is possible that glycogen might not be the factor that plays a prominent role in the selected lines for its increased tolerance to cold stress compared to the control. It could be some other metabolites such as trehalose, proline or lipids acting as the key factor.

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