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Evolution of Cold Shock Resistance Mechanisms in *Drosophila melanogaster*

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

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"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change."

-CHARLES DARWIN

Certificate of Examination

This is to certify that the dissertation titled **"Evolution of Cold Shock Resistance Mechanisms in *Drosophila melanogaster*"** submitted by **Ms. Akanksha Singh (Reg. No. MS12041)** 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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(Supervisor)

Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me with 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

Akanksha Singh
(Candidate)
Dated: April 21, 2017

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

Dr. N. G. Prasad
(Supervisor)

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Abbreviations

BRB	**B**lue **R**idge **B**aseline
FSB	**F**reeze-**S**hock **B**RB
FCB	**F**reeze-**C**ontrol **B**RB
D. melanogaster	***D****rosophila*-***melanogaster***
CHC	**C**uticular **H**ydro **C**arbons
RCH	**R**apid **C**old **H**ardening
GO	**G**lucose **O**xidase
nm	**n**ano **m**eter
NS	**N**o **S**hock
CS	**C**old **S**hock

Dedicated to my family

For their endless love, support and encouragement

Abstract

The ability of an insect to tolerate cold temperatures is different at both population and species level. To increase their chilling tolerance *D. melanogaster* adopt various methods like accumulation of carbohydrate cryoprotectants, membrane remodeling etc as a part of both evolutionary change and RCH. And due to chill injury flies' attractiveness can go down. Thus here we did two experiments first to check the effect of cold shock on the attractiveness of female *D. melanogaster* and second to quantify the levels of cryoprotectants. The first experiment was a mate choice experiment to see a difference in female *D. melanogaster* attractiveness after cold shock in selected (for cold shock) and control population. In this, we got the positive result that indeed post cold shock female's attractiveness goes down and females from selected populations are much better at recovering from cold shock than the females of control populations. The second experiment was Accumulation of Cryoprotectants which was done to quantify the levels of different cryoprotectants; namely Glucose, Glycogen and Trehalose; in *D. melanogaster*. BRB flies were given 4 types of treatments which were RCH, RCH with cold shock, Cold shock, no shock and were flash frozen at several time points $0, 4, 8, 12, 24, 36, 48, 60$ hours post treatments. We were successfully able to quantify the levels of glucose in *D. melanogaster* males but we did not see any statistically significant difference between the glucose levels of *D. melanogaster* post treatments. We still have to quantify levels of glycogen and trehalose.

Chapter 1

Introduction

As I quoted earlier *"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change"*, every organism on this planet has to encounter environmental stress of all kinds and if it wishes to continue; it has to learn to deal with different environmental stress. Stress is defined as an environmental condition which has detrimental effects on the performance of organism, leading to impaired Darwinian fitness [27], [28] or an "environmental factor causing a change in a biological system, which is potentially injurious". [29]

Low temperature is one of the major environmental stress that is faced by almost all organisms especially by the organisms which are smaller in size (thus have a large surface area to volume ratio) and are ectotherms or poikilotherms, insects are a perfect example of these types of organisms. Colonization by insects throughout the globe would not have been possible without them evolving various mechanisms, adapting different kinds of techniques to deal with cold stress. As *D. melanogaster* in itself is very cosmopolitan species, it can be found from tropics to temperate; desert to amazons (basically, all across the earth), therefore it must have developed some mechanisms; adopted some techniques to counter the negative effects associated with cold stress. And since every action has an equal and opposite reaction those techniques and mechanisms have an effect on its physiology; behavior and life history traits. [31], [30], [32]

Moreover, insect responses to low temperature can be broadly categorized into two groups: freeze-tolerant and freeze-intolerant. Freeze-tolerant insects respond to low-temperatures by allowing their body tissues to freeze, and damage is typically mitigated in this mechanism by limiting ice formation to the extra-cellular spaces. They accomplish this by increasing the amount of intra-cellular solutes and promoting ice formation in the extra-cellular spaces, which limits damage to membranes. Freeze intolerant insects suffer damage from cold at temperatures above the point at which body tissues freeze. These organisms prevent damage from low-temperature by increasing the concentrations of polyols in the hemolymph and repair the damage from low-temperature by the production of heat shock proteins. [33]

D. melanogaster is among the freeze-intolerant insects, it relies on various physiological and biochemical adaptations to survive low temperature exposure [36], [37], [38], [39], [40], [41], [42]. These include the accumulation of glycerol and trehalose, and other low molecular weight polyols and sugars; the synthesis of antifreeze proteins and ice-nucleating agents. In addition, there are several changes in membrane composition and increased production of molecular chaperone proteins which help in cold stress tolerance [43]. Also, a short exposure to a mild cold stress is sufficient to increase cold tolerance in many insects including *D. melanogaster*. This phenomenon, termed rapid cold hardening (RCH) expands the thermal interval that can be exploited by the insect [34]. The RCH response increases the cold tolerance of insects by protecting against non-freezing, cold-shock injury, it increases the cold tolerance of the adults at the organismal level [35]. Thus, I did an experiment to quantify different levels of various metabolites (glucose, glycogen and trehalose) in *D. melanogaster* after exposing them to different treatments (RCH, cold shock, RCH with cold shock, NO shock). Another experiment was a mate choice experiment done to check the differences between the attractiveness of females from selected (for cold shock) and control population post cold shock.

Chapter 2

Experimental System

2.1 Model System

I have used *Drosophila melanogaster* as the model system for my master's thesis work. Due to their short life-cycle and ease of handling and manipulation of their ecology in the laboratory, *D. melanogaster* have become popular for studies of life-history evolution [1].

The fruit flies are holometabolous insects that undergo complete metamorphosis. They have an adult life span of typically $35-40$ days [1]. The *D. melanogaster* life cycle consists of 4 stages: egg, larva, pupa and adult. Eggs hatch into 1st instar larvae around $18-24$ days after they are laid. The larvae molt into 2nd instar larvae after a day, and into 3rd instar larvae after another day. They remain at this stage for $2-3$ days, until they pupate. Pupal stage lasts for about 4-5 days after which flies eclose as adults. The flies attain sexual maturity after about $6-8$ hours post-eclosion [1].

All populations of flies used in this thesis have been reared in standard laboratory conditions of $25°C$, 55% relative humidity and $12h:12h$ light-dark cycle.

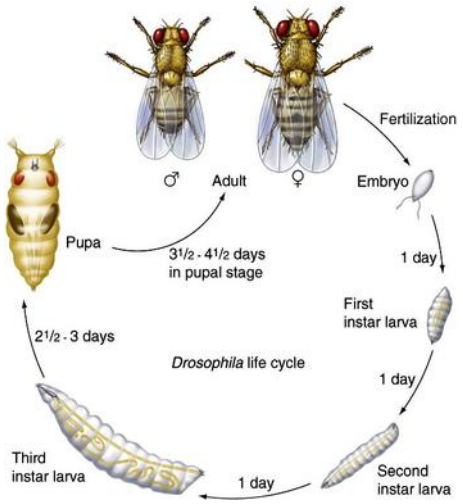

FIGURE 2.1: Life cycle of *D. melanogaster*

2.2 Experimental Population

For my thesis, I have used the following populations of *D. melanogaster* all of which are reared in banana jaggery food.

2.2.1 BRB

BRB is a large outbred population of *D. 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. The populations are maintained in cages on a 14-day discrete generation cycle. There are five replicate populations (BRB $1-5$) comprising 2500 adults per generation for every replicate [2].

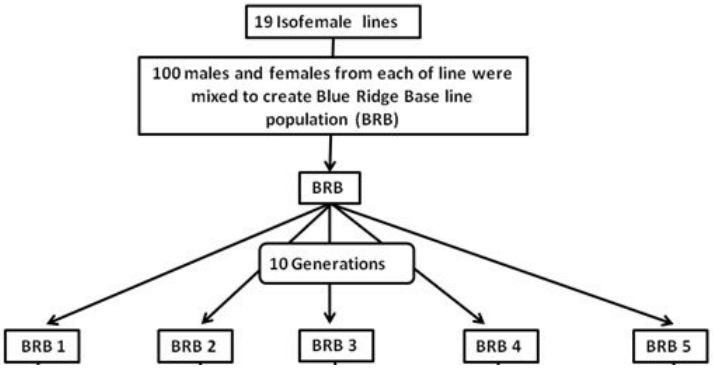

FIGURE 2.2: Derivation of BRB [44]

2.2.2 Cold Shock Resistance Lines (FSB and FCB)

FSB and **FCB** were derived from BRB populations. FSB is the selected population for cold shock whereas FCB is the control population. The populations are maintained in cages on a 13-day discrete generation cycle. There are five replicate populations (FSB $1-5$ and FCB $1-5$) comprising 1400 adults per generation for every replicate. FSB populations are exposed to -5°C for 1 hour every generation on 12th day post egg collection and FCB are kept at 25°C.

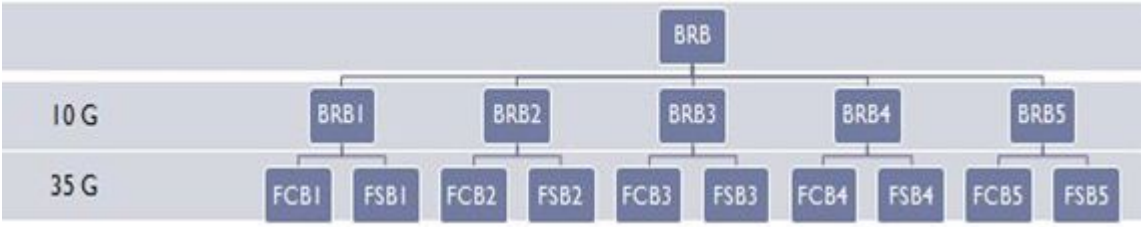

FIGURE 2.3: Derivation of FSB and FCB populations

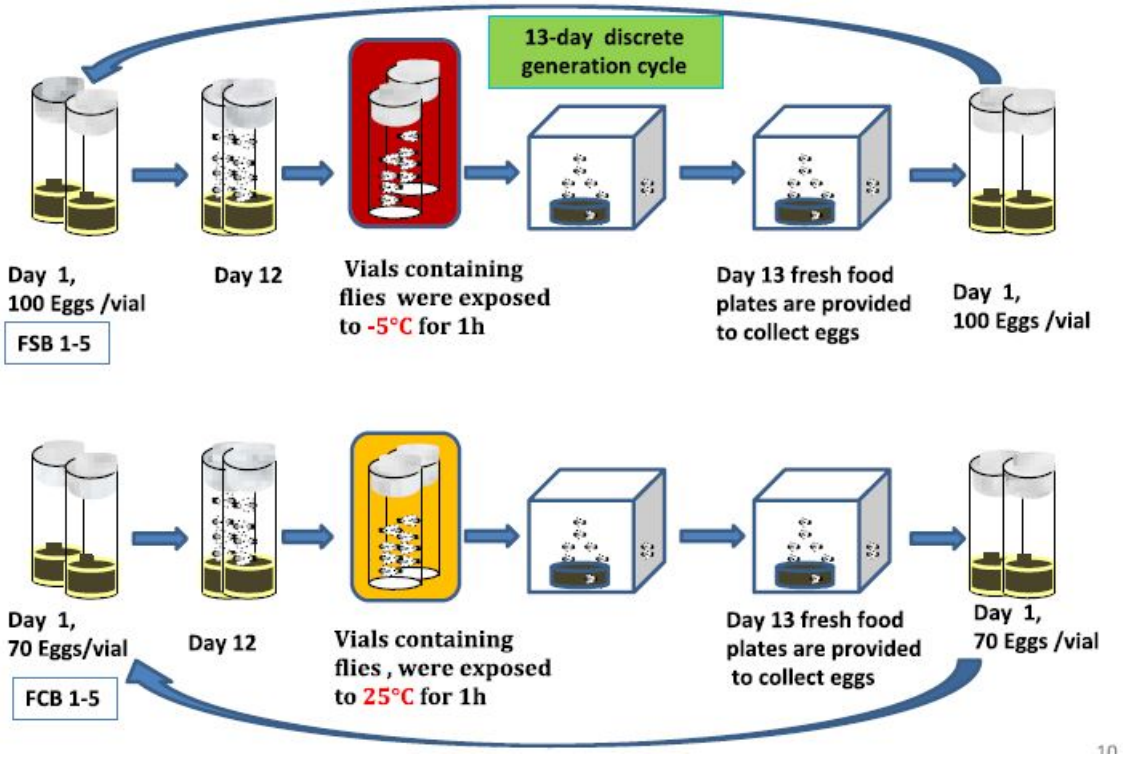

FIGURE 2.4: Maintainence of FSB and FCB populations [44]

2.3 Standardization

Fitness related-traits may be heavily affected by non-genetic parental effects. Therefore, for conducting experiments, flies are generated from parental flies that have been maintained under similar conditions for one generation. This process is called standardization [3]. Eggs are collected from stock populations at a density of 70 eggs per vial. On 12th day, flies are transferred to cages. For generating experimental flies, eggs are collected from these standardized flies.

EXPERIMENTS

Mate Choice Experiment

Accumulation of Cryoprotectants

Chapter 3

Mate Choice Experiment

3.1 Introduction

This experiment was done to see the effect cold shock have on the attractiveness of females in *D. melanogaster*. Cold shock directly affects the attractiveness of *D. melanogaster* as it changes CHC profile, behavioral cues, survivorship, fecundity etc. CHC function as a pheromonal system that is involved in sexual communication in fruit flies [4]; [5]; [6]; [7]; [8]; [9]; [10]. Their expression is plastic, and individuals can rapidly alter CHC composition in relation to their social context in D. melanogaster [11]. CHC profile of Drosophila can change due to many reasons like dietary changes [12]; [13], Aging [14], Stressful Environment [15] etc. At such a low temperature, *D. melanogaster* adults loose their mobility and feeding capacity, exhibit a knockdown which is also called a chill coma. A number of studies have reported the evolution of survivorship in response to selection for temperature shock resistance [16]

3.2 Hypothesis

The attractiveness of females and their ability to acquire mates is dependent on many factors like body size, CHC profile, behavioral cues, survivorship, fecundity etc. The fitness of females is heavily dependent on their attractiveness. Cold Shock is known to affect almost all of the above factors, therefore, FSB females must have evolved to counter the negative effects of cold shock.

3.3 Experimental Setup

Egg collection was done from the BRB population and the standardized populations of FSB and FCB. Virgin males and females were collected on $8th, 9th$ and $10th$ day from BRB and FSB; FCB populations respectively. Females were of the following kinds whereas the male used was a common BRB male. The male was sperm depleted on 11th day by keeping each male with 3 virgin females for 12 to 13 hours and were given $2-3$ hours of a refractive time period before taking the courtship observations. The females were colored (pink and green) for easy identification of male choice. Reverse coloration was done to eliminate any biases that might occur due to coloring. On the 12th day females were cold shocked and were given 4 hours of recovery time period before taking the courtship observations [16].

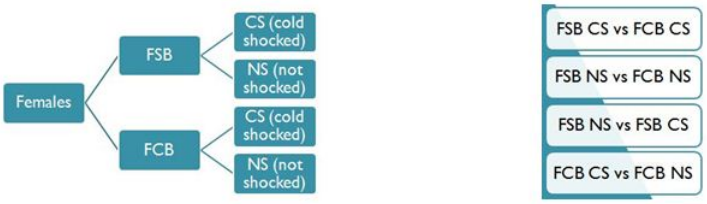

FIGURE 3.1: **3.1a** Kinds of Females **3.1b** Combinations

Courtship observations were taken. Each vial contained two females of different kind along with a BRB male and was observed for 30 minutes for every 30 second. Above four combinations were observed.

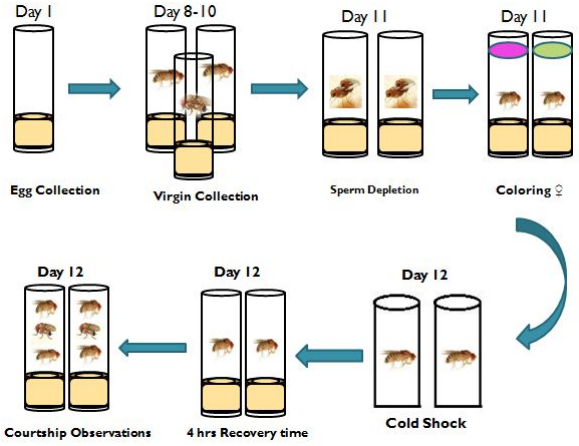

FIGURE 3.2: Experimental Design

Chapter 4

Accumulation of Cryoprotectants

4.1 Introduction

In insects, it is well known that overwintering individuals accumulate low-molecular-mass cryoprotectants, such as glycerol, sorbitol, inositol, or trehalose, which provide colligative depression of freezing and supercooling points and also stabilise the native state of proteins or membranes to prevent denaturation as a consequence of low temperature [17]; [18]; [19]. Cryoprotectant is a substance used to protect biological tissue from freezing damage, insects accumulate these in their bodies to minimize freezing damage when exposed to low temperatures. Insects most often use sugars or polyols as cryoprotectants. Cryoprotective molecules including glycerol, sugars such as glucose and trehalose, and amino acids such as proline have been implicated in maintaining cell function at low temperatures [20].

RCH is an example of phenotypic plasticity which takes place on a shorter time scale and is well-documented across numerous insect taxa including D. melanogaster [22]; [21]. When pre-exposed to a non-lethal low temperature, insects are better able to survive a subsequent cold event that would otherwise be lethal. While acclimation may take weeks or months to increase low temperature survival, RCH has been shown to enhance thermal tolerance in as little as 30 min [22].

Cryoprotectants which are being quantified in this experiment are Trehalose [24], Glycogen [24], Glucose [23].

4.2 Hypothesis

Since RCH is sufficient to increase cold tolerance and to expand the thermal interval that can be exploited by the insect, therefore, *D. melanogaster* exposed to RCH before cold shock must have higher cryoprotectants levels than those exposed directly to cold shock.

4.3 Experimental Setup

Egg collection was done from BRB population. On the 12th day eclosed flies were divided into 4 groups and flipped into empty vials. These 4 groups were given 4 different treatments namely No Shock; Cold Shock; RCH and RCH with Cold Shock.

In the No Shock treatment flies were kept at $25°C$ for 3 hours in empty vials. In the Cold Shock treatment, first flies were kept at $25°C$ for 2 hours then they were transferred to $-5°C$ for 1 hour. In the RCH treatment flies were kept at $0°C$ for 2 hours. And in RCH with Cold Shock, flies were first kept at $0°C$ for 2 hours and then at $-5°C$ for 1 hour.

After the treatments flies were transferred to food vials and were flash frozen with liquid nitrogen at different time points and were stored at $-80°C$. The time points are $0, 4, 8, 12, 24, 36, 48, 60, 72, 84$ hours post treatments.

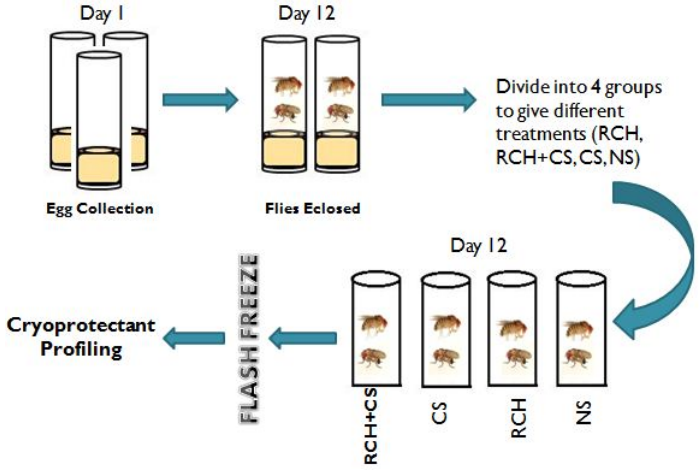

FIGURE 4.1: Experimental Design

4.4 Cryoprotectants Profiling

All the procedure used are enzymatic based assays. I quantified the cryoprotectants of the flies frozen at given 8 time points $0, 4, 8, 12, 24, 36, 48, 60$ hours post treatments with three replicates of each time point. So there were in total 96 samples of which cryoprotectants were to be quantified.

4.4.1 Preparing the samples

For making the samples only the male adult *D. melanogaster* were used by the following steps [25].

- Collect 5 male adult flies from the earlier stored flies.
- Transfer adult flies to a 1.5 ml microfuge tube.
- Rapidly homogenize animals in (1 X)PBS, a motor can be used to facilitate homogenization. Frozen samples should be kept on dry ice until addition of PBS. If samples are not kept cold, glycogen and trehalose will be enzymatically degraded into free glucose by endogenous enzymes and skew the final analysis.
- Heat supernatant for 10 minutes at $70°C$, then centrifuge for 3 minutes at $17,000$ rpm in a refrigerated tabletop centrifuge that has been prechilled to $4°C$.
- Pipette the resulting supernatant into a new 1.5 ml microfuge tube. At this time, the heat-inactivated sample can be frozen and stored at $-80°C$, if desired.

4.4.2 Preparing Standard Solutions

Before starting the enzymatic assays of Glucose, Glycogen and Trehalose, standard solutions of all three should be prepared as the following [25].
Dilute 16μl of 1 mg/ml glucose/glycogen/trehalose with 84μl PBS (1X) (100 final volume) for 0.16 mg/ml standard. Do four 2-fold serial dilutions into PBS (50μl 0.16 mg/ml + 50μl PBS for 0.08 mg/ml standard, etc.) to generate $0.01, 0.02, 0.04, 0.08$

and 0.16 mg/ml glucose standards. These assays are linear from 0.01 to 0.16 mg/ml glucose/glycogen/trehalose. All stock solutions can be stored at $-20°C$ or $4°C$.

4.4.3 Glucose Enzymatic Assay

For quantifying the amount of glucose in flies I used GO kit (Amplex Red Glucose/Glucose Oxidase Assay Kit; Catalog number: A22189). The steps for conducting the assay are given in kit's manual and can be found in Apendix B.

4.4.4 Glycogen Enzymatic Assay

Most carbohydrate in the fly is stored in the form of glycogen, which provides a large and accessible energy source during times of fasting and intense activity [25]. To determine the glycogen content, first it must be broken down into glucose by enzymatic degradation and then the total amount of glucose can be measured by using the GO kit. Then subtracting the free glucose amount from the total glucose amount gives the Glycogen content. In this experiment Amyloglucosidase enzyme was used to break glycogen into glucose. The procedure is as follows [25].

- Set up glucose and glycogen standards.
- Add 30μl of each glycogen standard (including PBS blank) to the top row of a clear-bottom 96 well plate. Similarly, add 30μl of each glucose standard in the next row down.
- Dilute heat-treated fly samples for glycogen measurements 1 : 5 in PBS (the required dilution may range from 1 : 5 to 1 : 20 depending on experimental conditions) and load 30μl into each well. Samples should be loaded in duplicate rows such that one row will be used to measure glycogen + glucose (treated with amyloglucosidase) and the adjacent row will be used to measure glucose alone (no amyloglucosidase).
- Prepare the working solution(GO kit's working solution) for your glycogen measurements by adding 1μl amyloglucosidase (Sigma A1602; 25 mg) per 1 ml of working solution.

- Using a pipette, add 100μl of working solution + amyloglucosidase to the glycogen standards and the first row of each set of duplicate samples. Then add 100μl of working solution alone (without amyloglucosidase) to the glucose standards and the remaining samples.
- Seal the wells with parafilm to prevent evaporation and incubate the plate at 37°C for 30 to 60 minutes.
- Measure absorbance at 540 nm using a plate reader.
- Free glucose concentration in each sample is calculated based on the glucose standard curve. For glycogen measurements, first subtract the absorbance measured for free glucose in the untreated samples from the absorbance of the samples that have been digested with amyloglucosidase. The glycogen content in each sample is then calculated based on the glycogen standard curve. This method is linear from $0.01 to 0.16$ mg/ml glycogen.

This protocol is under standardization.

4.4.5 Trehalose Enzymatic assay

To determine the trehalose content, first it must be broken down into glucose by enzymatic degradation and then the total amount of glucose can be measured by using the GO kit. Then subtracting the free glucose amount from the total glucose amount gives the Trehalose content. In this experiment Trehalase enzyme can be used to break trehalose into glucose [25].

The procedure for trehalose still needs to be standardized.

Chapter 5

Results and Discussion

5.1 Mate Choice Experiment

In this experiment, the attractiveness of females in *D. melanogaster* responded to selection(Figure 5.1). I found a significant effect of selection. Flies from FCB and FSB flies from neither-shocked treatment (FSB NS vs FCB NS) had an equal level of attractiveness and there was no significant difference between them. There was also no significant difference between the FSB cold shocked female and not shocked female(FSB NS vs FSB CS). Cold shock significantly reduced the attractiveness of females in the case of FCB populations, there was a significant difference in their attractiveness in the both shocked treatment(FSB CS vs FCB CS) and in the one shocked treatment(FCB NS vs FCB CS) (Figure 5.1). From all these results we can conclude that females from FSB populations were significantly more attractive than that of the FCB populations post cold shock indicating that the FSB populations had evolved a greater rate of recovery.

COMBINATIONS	t Statistics	p Value
FSB NS vs FSB CS	1.5362	0.1299
FSB NS vs FCB NS	0.2944	0.769
FSB CS vs FCB CS	3.0722	**0.0028**
FCB NS vs FCB CS	14.001	**0.0001**

FIGURE 5.1: Statistical Analysis (t test)

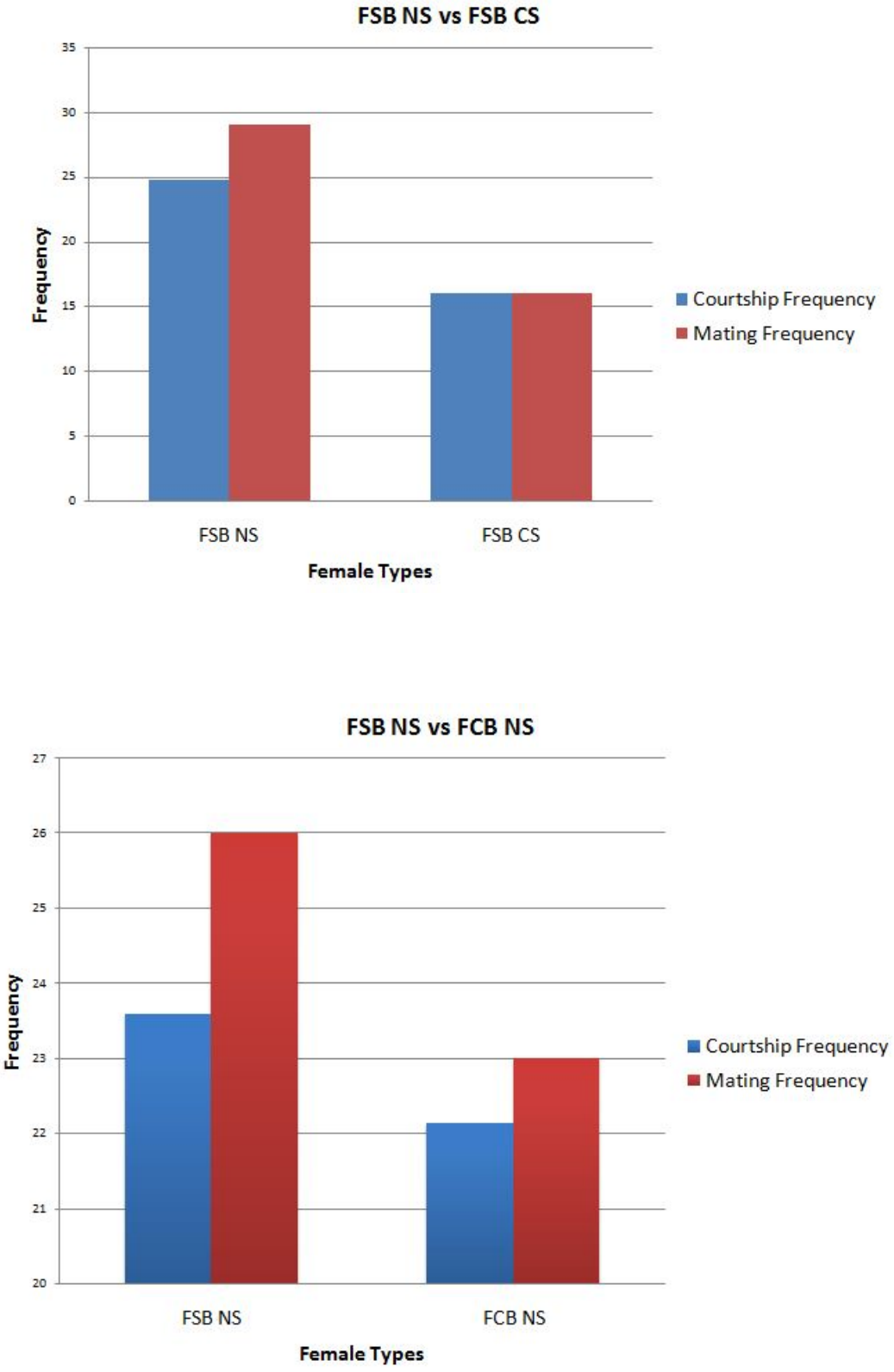

FIGURE 5.2: Graphs showing mating frequency and courtship frequency of different combinations

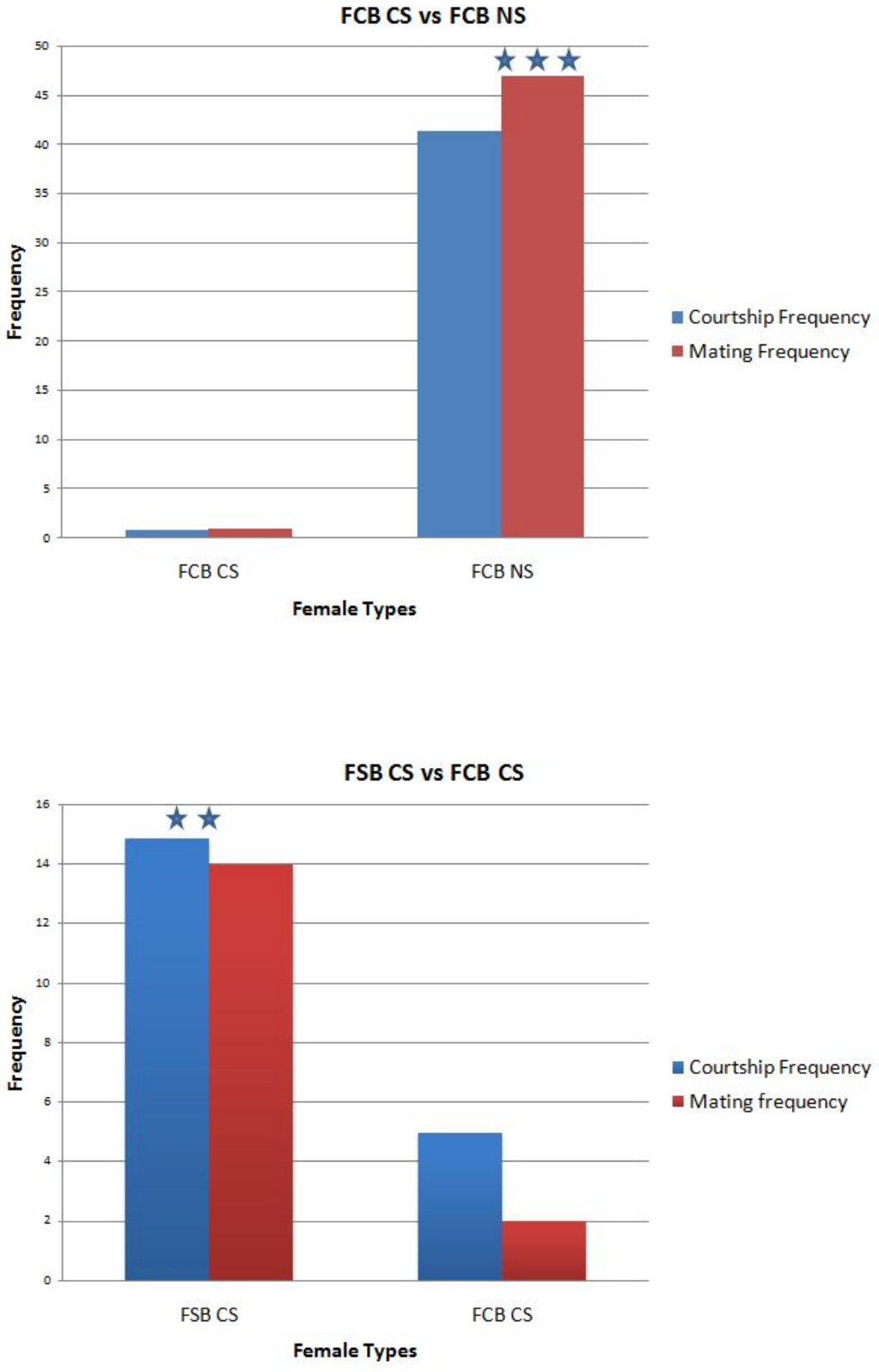

FIGURE 5.3: Graphs showing mating frequency and courtship frequency of different combinations

5.2 Accumulation of Cryoprotectants

5.2.1 Glucose Assay

In this experiment, I quantified glucose for above prepared samples. But after statistical analysis, no significant difference was seen in the amount of glucose in flies post different treatments. However, a trend was seen in glucose values of the different treatments(Figure 5.6).

GLUCOSE	0hr	4hr	8hr	12hr	24hr	36hr	48hr	60hr
	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.	Abs.
No Shock	0.734	0.815	0.785	0.785	0.661	0.712	0.659	0.748
	0.823	na	1.023	0.872	0.738	0.627	0.784	0.842
	0.81	1.161	1.281	0.874	0.671	0.683	0.56	0.828
mean	**0.789**	**0.988**	**1.0296667**	**0.8436667**	**0.69**	**0.674**	**0.6676667**	**0.806**
Cold Shock	0.765	0.637	0.761	0.733	0.65	0.674	0.674	0.74
	0.763	0.355	0.613	0.807	0.58	0.731	0.722	0.81
	0.821	0.732	0.595	0.797	na	0.736	0.726	0.748
mean	**0.783**	**0.5746667**	**0.6563333**	**0.779**	**0.615**	**0.71366667**	**0.7073333**	**0.766**
RCH	0.71	0.653	0.809	0.578	0.569	0.671	0.777	0.648
	0.796	0.802	0.713	0.758	0.746	0.587	0.661	0.699
	0.75	0.797	0.692	0.818	0.842	na	0.756	0.785
mean	**0.752**	**0.7506667**	**0.738**	**0.718**	**0.719**	**0.629**	**0.7313333**	**0.7106667**
RCH+ Cold Shock	0.791	0.823	0.573	0.544	0.662	1.163	0.793	0.779
	0.793	na	0.625	0.835	0.554	1.07	0.769	1.045
	0.772	0.569	0.592	0.71	0.868	0.777	0.737	0.759
mean	**0.7853333**	**0.696**	**0.5966667**	**0.6963333**	**0.69466667**	**1.00333333**	**0.7663333**	**0.861**

FIGURE 5.4: Absorbance Values

GLUCOSE	0hr	4hr	8hr	12hr	24hr	36hr	48hr	60hr
	Conc. (uM)	Conc. (uM)	Conc. (uM)	Conc. (uM)	Conc. (uM)	Conc. (uM)	Conc. (uM)	Conc. (uM)
No Shock	716	797	767	767	643	694	641	730
	805	na	1005	854	720	609	766	824
	792	1143	1263	856	653	665	542	810
mean	**771**	970	1011.6667	825.66667	672	656	649.66667	788
Cold Shock	747	619	743	715	632	656	656	722
	745	337	595	789	562	713	704	792
	803	714	577	779	na	718	708	730
mean	**765**	556.66667	**638.33333**	**761**	**597**	695.666667	689.33333	748
RCH	692	635	791	560	551	653	759	630
	778	784	695	740	728	569	643	681
	732	779	674	800	824	na	738	767
mean	**734**	732.66667	**720**	**700**	**701**	**611**	713.33333	692.66667
RCH+ Cold Shock	773	805	555	526	644	1145	775	761
	775	na	607	817	536	1052	751	1027
	754	551	574	692	850	759	719	741
mean	**767.33333**	**678**	**578.66667**	**678.33333**	**676.666667**	**985.333333**	**748.33333**	**843**

FIGURE 5.5: Concentration Values

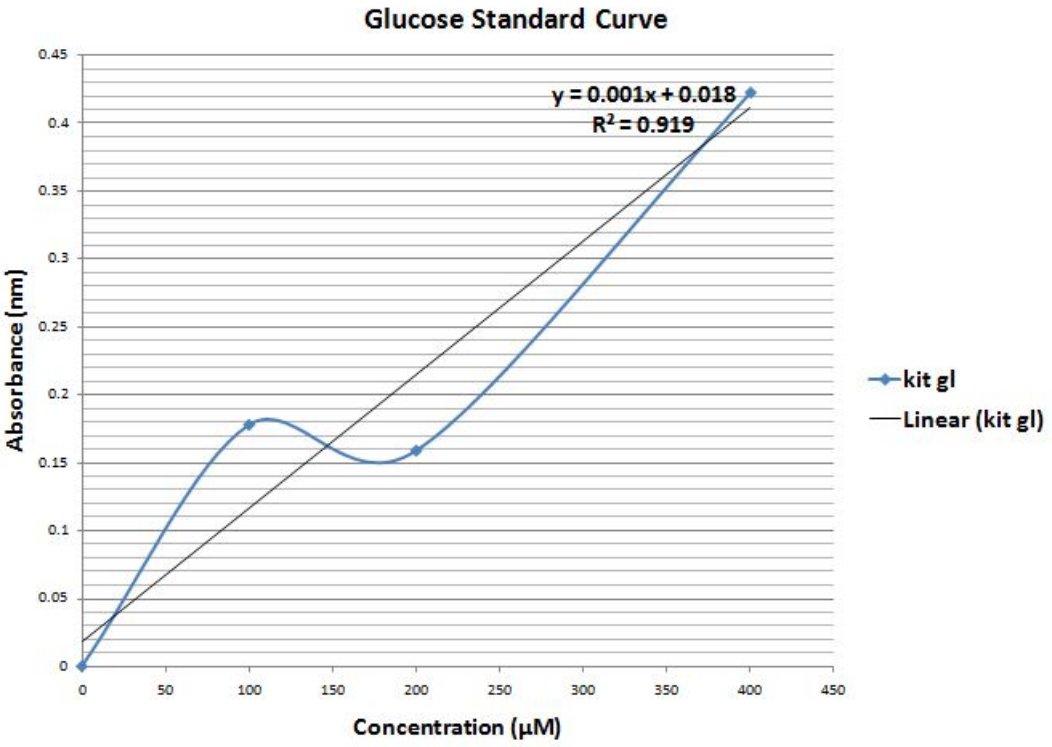

FIGURE 5.6: Glucose Standard Curve

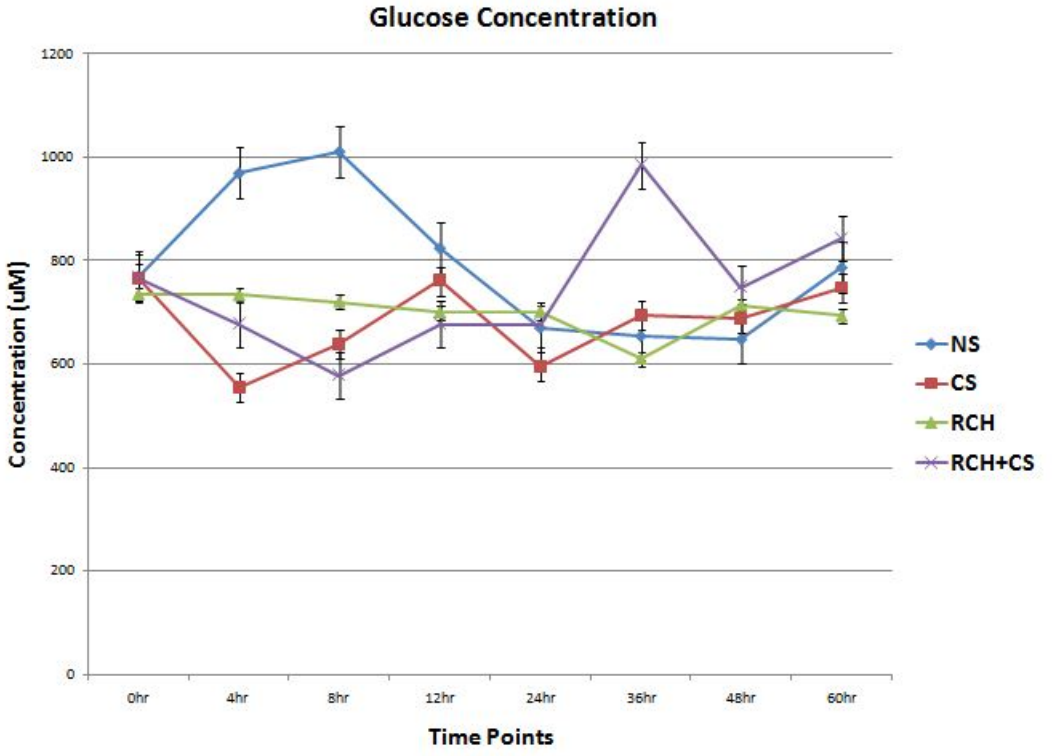

FIGURE 5.7: Graph showing Glucose Concentrations post treatments

5.2.2 Glycogen Assay

This assay is in the middle of standardization.

5.2.3 Trehalose Assay

This assay still needs to be standardized.

References

[1] Prasad, N.G., Joshi, A. J Genet, *Journal of Genetics* **82**, 45-76, (2003).

[2] Vanika Gupta, Zeeshan S Ali and Nagaraj G Prasad *BMC Evolutionary Biology* **13**, 185, (2013).

[3] Michael R. Rose, *Evolution* **38**, 1004-1010, (1984).

[4] Jean-Marc Jallon, *Behavior Genetics* **14**, 441-478, (1984).

[5] Therese Ann Markow, Eric C. Toolson, *Monographs in Evolutionary Biology* , 315-331, (1990).

[6] Jean-Franois Ferveur, *Bio Essays* **19**, 353-358, (1997).

[7] Mark W. Blows, Rachel A. Allan *The American Naturalist* **158**, 826-837, (1998).

[8] WJ Etges, MA Ahrens, *The American Naturalist* **158**, 585-595, (2001).

[9] Donna Petfield, Stephen F. Chenoweth, Howard D. Rundle, and Mark W. Blows, *PNAS* **102**, 60456050, (2005).

[10] Micheline Grillet, Laurence Dartevelle, Jean-Franois Ferveur, *Proceedings of the Royal Society B* **273**, 3332, (2006).

[11] Joshua J. Krupp et. al., *Current Biology* **18**, 1373-1383, (2008).

[12] Tatyana Y. Fedina et. al., *Plos Genetics* (2012).

[13] Tatyana Y. Fedina et. al., *Plos one* (2012).

[14] Tatyana Y. Fedina et. al., *Journal of Experimental Biology* **215**, 814-821, (2012).

[15] L Kwan, HD Rundle, *Evolution* **64**, 710-723, (2009).

[16] Karan Singh, Manas Arun Samant, Megha Treesa Tom, Nagaraj Guru Prasad, *Plos One* (2016).

[17] RE Lee, RA Ring, JG Baust, *Cryo Letters* **7**, 113-126, (1986).

[18] Richard E. LeeJr., *Chapter: Insects at Low Temperature* 17-46, (1991).

[19] KB Storey, JM Storey, *Chapter: Insects at Low Temperature* 64-93, (1991).

[20] J. S. Bale, *Philosophical Transactions of the Royal Society B* **357**, 1074, (2002).

[21] MC Czajka, RE Lee, *Journal of Experimental Biology* **148**, 245-254, (1990).

[22] RE Lee, C Chen, DL Denlinger, *Science* **238**, 1415-1417, (1987).

[23] Heath A. MacMillan, Christopher G. Guglielmo, Brent J. Sinclair, *Journal of Insect Physiology* **55**, 243-249, (2008).

[24] M. T. Kimura, T. Awasaki, T. Ohtsij and K. Shimada, *Journal of Insect Physiology* **38**, 871-875, (1992).

[25] Jason M. Tennessen, William E. Barry, James Cox, Carl S. Thummel, *Methods* **68**, 105-115, (2014).

[26] Amplex Red Glucose/Glucose Oxidase Assay Kit Manual *Invitrogen, Molecular Probes*.

[27] Sibly RM, Calow PA, *Biol J Linn Soc* **37**, 101-116, (1989).

[28] Zhivotovsky LA., *EXS* **83**, 241-254, (1997).

[29] Hoffmann AA, Parsons A., *Oxford: Oxford University Press* (1991).

[30] Lazzaro BP, Flores HA, Lorigan JG, Yourth CP *PLoS Pathog* (2008).

[31] Norry FM, Sambucetti P, Scannapieco AC, Loeschcke V., *Genetica* **128**, 81-93, (2006).

[32] Robinson SJ, Zwaan B, Partridge L., *Evolution* **54**, 1819-1824, (2000).

[33] Michael R. Michaud, *PhD thesis, The Ohio State University* (2007).

[34] Johannes Overgaard, *Journal of Insect Physiology* **53**, 12181232, (2007).

[35] Shu-Xia Yi, Clifford W. Moore, Richard E. Lee Jr., *Apoptosis* **12**, 11831193, (2007).

[36] KB Storey, JM Storey *Physiological Reviews* **68**, 27-84, (1988).

[37] Lee, *BioScience* **39**, 308313, (1989).

[38] Baust and Rojas, *Journal of Insect Physiology* **31**, 755759, (1985).

[39] R. J. C Cannon and W. Block, *Biological Reviews* **63**, 23-77, (1988).

[40] J. Duman and K. Horwath, *Annual Review of Physiology* **45**, 261-270, (1983).

[41] Ring, R. A., and D. Tesar, *Cryobiology* **18**, 199-211, (1981).

[42] K. E. Zachariassen, *Physiological Reviews* **65**, 799-832, (1985).

[43] Joseph P. Rinehart, Aiqing Li, George D. Yocum, Rebecca M. Robich, Scott A. L. Hayward, and David L. Denlinger , *PNAS* **104**, 1113011137, (2007).

[44] Karan Singh, *PhD thesis, IISER Mohali* (2015).

Appendix A

Banana Jaggery Food

Composition of 1 of regular Banana-Jaggery Food is given below.

Ingredient	**Amount**
Banana (g)	205
Barley flour (g)	25
Jaggery (unrefined cane sugar) (g)	35
Yeast (g)	36
Agar (g)	12.4
Ethanol (ml)	45
Water (ml)	180
p-Hydroxymethyl benzoate (g)	2.4

FIGURE A.1: Composition of Banana Jaggery food (1L)

Appendix B

Glucose Assay (Kit's Procedure)

The following procedure has been taken from [26].

B.1 Preparing the Stock Solutions

B.1.1 Prepare a 10 mM stock solution of Amplex Red reagent

Allow one vial of Amplex Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent in 60μL of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 100μL per assay.

B.1.2 Prepare 1X Reaction Buffer

Add 4 mL of 5X Reaction Buffer (Component C, white cap) to 16 mL of deionized water (dH_2O). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100μL each with a 10 mL excess for making stock solutions.

B.1.3 Prepare a 10 U/mL stock solution of horseradish peroxidase (HRP)

Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1 mL of 1X Reaction Buffer. After the assay, any remaining unused solution should be divided into single-use aliquots and stored frozen at $20°C$.

B.1.4 Prepare a 100 U/mL glucose oxidase stock solution

Dissolve the contents of the vial of glucose oxidase (Component E, orange cap) in 1.0 mL of 1X Reaction Buffer. This stock solution should be stored frozen at $20°C$.

B.1.5 Prepare a 400 mM (72 mg/mL) glucose stock solution

Weigh out a portion of glucose (Component F, black cap), and dissolve it in the appropriate amount of 1X Reaction Buffer.

B.1.6 Prepare a 20 mM H_2 O_2 working solution

Dilute the 3% H_2 O_2 stock solution (Component G, red cap) into the appropriate volume of 1X Reaction Buffer. The actual concentration of H_2 O_2 is indicated on the label. For instance, a 20 mM H_2 O_2 working solution can be prepared from a 3.0% (0.88 M) H_2 O_2 stock solution by diluting 22.7μL of 3.0% H_2 O_2 into 977μL of 1X Reaction Buffer.

B.2 Assay Protocol

B.2.1 Prepare a glucose standard curve

Dilute the appropriate amount of the 400 mM glucose stock solution into 1X Reaction Buffer to produce glucose concentrations of 0 to 200μM, each in a volume of 50μL. Be sure to include a no-glucose control.

B.2.2 Dilute the glucose-containing samples in 1X Reaction Buffer

A volume of 50μL will be used for each reaction. A variable dilution will be required depending on the total glucose present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for the assay.

B.2.3 Load the samples

Pipet 50μL of the standard curve samples, controls, and experimental samples into individual wells of a microplate.

B.2.4 Working Solution

Prepare a working solution of 100μM Amplex Red reagent, 0.2 U/mL HRP and 2 U/mL glucose oxidase Mix the following:

- 50μL of 10 mM Amplex Red reagent stock solution.
- 100μL of 10 U/mL HRP stock solution.
- 100μL of 100 U/mL glucose oxidase stock solution.
- 4.75 mL of 1X Reaction Buffer

B.2.5 Begin the reactions

Add 50μL of the (Amplex Red reagent/HRP/glucose oxidase) working solution to each microplate well containing the standards, controls, and samples.

B.2.6 Incubate the reactions

Incubate at room temperature for 30 minutes, protected from light. Because the assay is continuous (not terminated), absorbance may be measured at multiple time points to follow the kinetics of the reactions.

B.2.7 Measure the absorbance

Use a microplate reader equipped for excitation in the range of 530560 nm and fluorescence emission detection at 590 nm, or for absorbance at 560 nm.

B.2.8 Correct for background absorbance

For each point, subtract the value derived from the no-glucose control.

"What if I fall?"

Oh, but my Darling

What if you fly?

-Erin Hanson