Adaptation to cold stress: Exploring the evolution of life-history and reproductive traits using *Drosophila melanogaster* as model system

A thesis

Submitted for the degree of

Doctor of Philosophy

By

Karan Singh

Department of Biological Sciences

Indian Institute of Science Education and Research (IISER) Mohali

Mohali-140306 (India)

December 2015

Dedicated to

My Family and Teachers

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH MOHALI
Sector 81, SAS Nagar, Mohali
PO Manauli, Punjab 140306, India

DECLARATION

The work presented in this thesis 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 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 acknowledgment 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

.....

(Karan Singh)

Date:

Place:

In my capacity as supervisor of the candidate's doctoral thesis work, I certify that above statements by the candidate are true to the best of my knowledge.

.....

(Dr. N. G. Prasad)
Associate Professor
Department of Biological Sciences
Indian Institute of Science Education and Research Mohali

Date:

Place:

Acknowledgements

Foremost I owe my greatest debt and heartfelt gratitude to my PhD supervisor Dr. N. G. Prasad who has been the soul behind every effort that I have put forth in this thesis. His suggestions, extraordinary patience, understanding, encouraging words and confidence in me have shaped my personality to a large extent. I admire and respect him for his loving and caring behavior, scientific observation, valuable feedback and amount of freedom he renders to each of his students. His exceptional dedication, guidance and enthusiasm helped me to expand my knowledge and horizon throughout the journey of my thesis work. He has been a great mentor, always willing to extend help in all spheres of life. Thank you so much Sir!

After receiving Master of Philosophy in Microbiology, it never occurred to me that that I would be pursuing a doctoral degree, yet here I am writing my very own PhD thesis. Looking back at these five and half years of my life, I am surprised and at the same time highly grateful for all that I received and achieved. It certainly shaped me as a student as well as a person and led me where I am now. I feel evolved. I can appreciate maturity and understand the alphabets of science. It would have never been possible for me to write a doctoral thesis without the support and help of people around me. I would like to take this opportunity to express my deepest gratitude and regards for all of them who have aided me in this journey of mine.

I thank my doctoral committee members Dr. Lolitika Mandal and Dr. Kavita Babu for their helpful suggestions and timely review of the progress of my work.

I would also like to express my special thanks to Dr. Brent Sinclair for his insightful comments on the manuscript. I am grateful to Prof. Daniel Promislow (University of Washington) for providing the isofemale lines.

I am indebted to Prof. N. Sathyamurthy, Director, IISER Mohali for giving me the opportunity to work at this premier research institute and avail its excellent infrastructure to carry out my research work smoothly.

I express my immense gratitude to Prof. T. R. Rao (IISER Mohali), Dr. Manjari Jain (IISER Mohali), Prof. Amitabh Joshi (JNCASR, Bangalore) and Dr. V. K. Dua (NIMR, New Delhi) for their prudent comments and recommendations about the work. I would like to specially thank Professor Anand Kumar Bachhawat, Dr. Arunika Mukhopadhaya, Dr. Rhitoban Ray Choudhury, Dr. Vinayak Sinha

and their lab members for providing me access to their lab facilities and for many fruitful discussions during the course of my PhD work.

Thanks to my incredible lab mates- Bodhi, Vanika and Vinesh for being wonderful seniors, mentors and friends who taught me all the nitty-gritties of fly work. A big thanks to Manas, Megha, Vanika, Zeeshan and Saudamini for their great help with my experiments and proof reading of my thesis. I would like to place on record the help I received from Dr. Rochishnu, Lokesh, Sharmi, Tejinder, Radhika, Karan, Prakhar, Vabhavi, Ashima, Hari, Zulfi, Muskan, Anuj, Ekta, Saurabh, Chandrakala, Pranay, Ruchika, Athira, Aprajita, Komal and Neetika. Without their help my project would have not been possible.

I thank Dr. G. C. Prasad (MTCC, IMTECH, Chandigarh) and Dr. Stefanie Van Trappen (BCCM. LMG, Belgium) for their help in identifying the new strain of Staphylococcus succinus subspecies succinus PK-1.

My acknowledgment would be incomplete would the mention of my mentors Dr. P. K. Sharma, Dr. Ravi Dutta Sharma, Mr. Sudhir Kumar, Dr. Deepak Chand Sharma, Dr. Pramod Kumar, Mr. Anurag Sharma . They were the ones who in first place identified my research potential and thereby motivated and encouraged me to pursue research.

I extend my gratitude to Prasad's family, Jay Lakshmi Madam and Mrs. Jyothi, for making IISER a real home-like environment.

I thank Indian Institute of Science Education and Research Mohali, India for providing financial assistance in the form of Junior and Senior Research Fellowship.

I would like to thank Nature Publishing Group, Immunology Foundation, International Union of Biological sciences (IUBS), European Society for the Study of Evolutionary Biology (ESEB) International Centre for Theoretical Sciences (ICTS)-TIFR, Department of Biotechnology and Indian Institute of Science Education and Research Mohali for providing travel grants to presnet my work at national and international conferences.

I would like to express my thanks to my new lab members Dr. Rajbir Kaur, Alok, Divya Sharma, Renuka, Manisha, Yogesh and Thapasya for their help and making a wonderful environment in the lab. My special thanks to Dr. Rhitoban Rayuchoudhary for giving me new oppurtunity to persue my

scientific carrier and I am also thankful to Dr. Ruchira Sen for making IISER Mohali my another home.

I specially thank my parents, wife Megha Kothari, daughter Princi Labana, brothers, sisters and friends. Without your enduring love and constant support, traversing these many miles in my PhD journey would have been difficult. Your sacrifices have given me wonderful opportunities that my mind never conceived. I promise to keep up the hard work and make you proud in all my future endeavors. My appreciation for all of you is endless, and continuing to grow with each passing day.

Karan Singh

List of Publications

Paper published:

1. Singh K, Kochar E and Prasad NG (2015). Egg viability, male mating ability and mating frequency evolve in populations of *Drosophila melanogaster* selected for resistance to cold shock. *PloS One.* 10 (6): e0129992.
2. Singh K, Samant MA, Tom MT and Prasad NG (2016) Evolution of pre- and post-copulatory traits in male *Drosophila melanogaster* as correlated response to selection for increased resistant to cold stress. Accepted in *PLoS One*.

Manuscripts under review:

3. Singh K**,** Zulkifli M and Prasad NG (2016) Identification and characterization of novel natural pathogen of *Drosophila melanogaster* isolated from wild captured *Drosophila* spp. At *Microbes and Infection.*
4. Singh K and Prasad NG (2016). Evolution of pre- and post-copulatory traits in female *D. melanogaster* as correlated response to selection for increased resistant to cold stress. At *Journal of Insect Physiology*.

Manuscripts in prepration:

1. Singh K and Prasad NG. Evolution of cross-tolerance in populations *Drosophila melanogaster* selected for increase resistance to cold stress.
2. Singh K and Prasad NG. The life-history costs associated with increased resistance to cold stress.
3. Gupta V, Nandy B, Shenoi VN, Singh K and Prasad NG. Genetic architecture of immune response in *D. melanogaster*: A study using isofemale lines.

Gene sequences submitted to GenBank:

1. Singh K and Prasad NG (2013). *Staphylococcus succinus* subsp. *succinus* strain PK-1 16S rRNA gene sequence. *GenBank*: JQ988944.
2. Singh K, Zulkifli M and Prasad NG (2014). *Staphylococcus succinus* subsp. *Succinus* strain PK-1 heat shock protein 60 gene sequence. *GenBank*: KM516055.

Contents

Synopsis

Organisms face various types of environmental stress during their entire lifespan. Environmental stress is a major ecological driver and plays a significant role in driving the evolution of populations in nature. Environmental stress is often manifested by a change in the optimum conditions in the biotic as well as the abiotic components of the organism's habitat/surroundings. Temperature is a fundamental ecological factor (abiotic) of an organism's environment (especially ectotherms) and it is known to control various important life-history traits. Insects are ectothermic and their body temperature is determined by the ambient temperature. 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 ecology and evolution.

Multiple clinal and laboratory studies have investigated the effects of high and low temperatures on fitness related traits. In insects, stress due to increased temperature, i.e. heat stress (beyond the optimum temperature where absolute fitness is maximum) can reduce survivorship, female fecundity, ejaculate size and sperm competitive ability in males and may also increase male sterility. Evolution in response to heat stress in terms of reproductive traits and adult survival along with the mechanisms of increased heat stress resistance are well documented. Similarly cold stress (stress due reduced temperature) can also induce adult mortality and reduce gamete viability. Therefore adaptation to cold stress is likely to involve responses in reproductive trait along with other life-history related traits, which most previous studies have ignored.

In the present study, I used experimental evolution approach to select replicate populations of *Drosophila melanogaster* for resistance to cold shock. I used large outbred populations of *D. melanogaster* named Blue Ridge Baseline (BRB 1-5) as ancestral populations. I derived one selected (FSB) and one control population (FCB) from each of the five replicate BRB populations. Thus, my study consisted of ten populations in all - five selected populations (FSB 1-5) and five control populations (FCB 1-5). The selected populations (FSB 1-5) are maintained on a 13 day discrete generation cycle. On the 12th day post egg collection, flies are exposed to a non-lethal temperature shock (-5°C for one

hour). Following this cold shock, flies are allowed to recover and males and females are given 24 hours to interact at normal rearing temperature of 25°C. One day later (post cold shock), a fresh food plate is provided to collect eggs to the start next generation. The FCB populations are also maintained in conditions identical to those of the FSB populations except that the FCB populations are not subjected to cold shock (they are held at normal rearing temperature of 25°C throughout). Initial experiments suggested that this cold shock had negligible effect on adult mortality (about 5% at 24 hours post cold shock) but reduced egg viability (proportion of eggs that hatch) to about 40%. Therefore, the primary focus of selection in my study is likely to be egg viability. In this thesis, I present the results from experiments conducted over 20-71 generations of selection. I found that egg viability post cold shock increased rapidly in the FSB populations. I then used these populations to address the following questions:

a. What are the mechanisms underlying the evolved differences in egg viability post cold shock between selected (FSB) and control (FCB) populations?

b. Do pre- and post-copulatory traits evolve in selected males and females? Are there trade-offs between reproductive performance under stressful and benign conditions?

c. What are the life-history costs associated with increased resistance to cold shock?

d. What is the mechanism underlying increased resistance to cold shock?

e. Is the evolved resistance specific to cold shock or is it generic?

Between 20-30 generations of selection, I investigated egg viability and reproductive behavior along with other components of fitness such as fecundity, larva to adult survivorship and adult mortality, in the FSB and FCB populations. I found that egg viability immediately post cold shock was extremely low (2-3%) and was not significantly different between the FSB and FCB populations. However, by 24 hours post cold shock, egg viability in the FSB populations had improved to about 52% while egg viability in the FCB populations was about 38% and this difference was significant. The

faster recovery of egg viability in the FSB populations could be because the FSB females can protect sperm/fertilized eggs from cold damage better than the FCB females and use them later. However, when mated females were subjected to cold shock, and held without access to males, egg viability in both the FSB and FCB populations remained low and not significantly different from each other even 24 hours post cold shock indicating that the FSB females are not better at protecting gametes from cold shock. I found that the FSB populations had higher mating frequency relative to the FCB populations post cold shock. I also found that post cold shock, males from the FSB populations were significantly better at inducing non-virgin females to mate and were better at siring progeny compared to the FCB males. However, larval survivorship, adult mortality and fecundity post cold shock was not significantly different between the FSB and FCB populations.

Following the results from my previous experiments, I investigated reproductive traits at the individual level in males and females from both the FSB and FCB populations. To this end, I subjected males and females from the FSB and FCB populations to two treatments- cold shock and no cold shock. The flies from the cold shock treatment were then assayed for various reproductive traits such as mating latency, copulation duration, mating frequency and progeny production after different durations of recovery post cold shock. Cold shock increased mating latency in both the FCB and FSB populations compared to no cold shock flies. The FSB males had significantly lower mating latency after 4 and 12 hours of recovery compared to the FCB males. Greater fraction of the FSB males managed to mate with virgin females post cold shock compared to the FCB males. Additionally, The FSB males showed higher sperm offense ability post cold shock compared to the FCB males. Cold-shock induced infertility was lower in the FSB males compared to the FCB males.

In case of females, post cold shock, the FSB females had lower mating latency, higher mating success and higher progeny production compared to the FCB females. Taken together, these results indicate that the FSB males and females are better at recovering from the effects of cold shock in terms of their reproductive performance. These results are important in understanding the mechanisms that maintain variation in stress resistance through life-history trade-offs. Several studies have suggested that trade-offs in reproductive performance, especially of the males, across benign and stressful conditions can maintain variation in stress resistance. However, my studies clearly show that such

trade-offs need not necessarily occur. Even though the FSB populations were superior in terms of reproductive performance under stressful conditions, their performance under non-stressed conditions was not adversely affected. Thus, at least in the FSB and FCB populations, performance of the flies across stressed and non-stressed conditions did not indicate any trade-off.

My previous experiments clearly show that the FSB populations have evolved better ability to recover from cold shock compared to the FCB populations (in terms of egg viability and various reproductive traits as mentioned before). Therefore, it is reasonable to expect that increased ability to resist/recover from cold shock comes at a cost. If resources are limiting, then, this can potentially lead to trade-offs with other energy demanding traits. To address this possibility, in the next part of my study, I measured a number of important life-history traits i.e., longevity, fecundity, development time and body size in the FSB and FCB populations. I did not find any difference in fecundity and longevity of the FSB and FCB populations. The FSB populations had significantly longer pre-adult development time compared to the FCB populations. The FSB females weighed significantly more than the FCB females but no such difference was observed in the males. These results indicate that evolved stress resistance need not come at a cost of life-history traits such as fecundity and longevity. It is quite likely that the cost of increased cold stress resistance is paid in terms of reduced resistance to other stresses.

I have also tried to understand the genetic basis of adaptation to cold stress in these populations. I have tried doing this by quantifying the gene expression profile of male flies from the FSB and FCB populations with and without cold shock. Based on results from other studies, I selected a set of cold inducible genes (*hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa*, *hsp83* and *frost*). In males 4 and 12 hours post cold shock the expression of almost all the genes that were assayed was higher in both the FSB and FCB populations relative to under the no shock treatment condition in both the FSB and FCB populations. However, in both the cold shock and no shock treatments there was no significant difference in the expression levels of these genes between the FSB and FCB. This suggests that the expression patterns of these genes have not evolved in reponse to selection for cold shock resistance.

Evolution of resistance to a particular kind of stress can affect the ability of the organism

to resist other kinds of stresses either positively or negatively. If the mechanisms of resistance to a particular stress are specialized and costly, then, mechanisms of resistance to other stresses can be negatively affected. However, it is also possible that at least a part of the stress resistance mechanisms is generic. In such a situation, increased resistance to a particular stress can increase resistance to other kinds of stresses. To address these questions, I subjected the FSB and FCB populations to a variety of environmental stresses such as heat, starvation, desiccation and bacterial infection. I found that the FSB populations had higher resistance to heat stress in terms of adult survivorship and mating ability post heat shock. Desiccation resistance was higher in the FSB females compared to the FCB females but no such difference was found in males. The FSB populations had lower starvation resistance compared to the FCB populations. There was no difference between the FSB and FCB populations in their ability to survive bacterial infection (*Staphylococcus succinus* subsp. *succinus* strain PK-1 is a natural pathogen of *D. melanogaster*, which I isolated from wild captured flies). Therefore, my results indicate that correlations between cold shock resistance and resistance to other stresses are complex.

To summarize, in the present thesis, I selected replicate populations of *D. melanogaster* for resistance to cold shock with the focus of selection being egg viability post cold shock. I observed a rapid primary response to selection with the FSB populations evolving increased egg viability post cold shock. In addition to egg viability, the reproductive traits also rapidly evolved. The males and the females from the FSB populations were better at recovering in terms of their reproductive activity post cold shock. Investment in cold shock resistance did not trade-off with any of the major life-history traits (fecundity and longevity). I found that the correlation between cold stress and other stresses is quite variable. My results further our understanding of the role of reproductive behavior in adaptation to environmental stress as well as the genetic architecture of stress resistance.

Chapter 1

Introduction

Organisms encounter different kinds of environmental stress over their life span. Stress is defined as an environmental condition which has detrimental effects on the performance of organism, leading to impaired Darwinian fitness (Sibly and Calow 1989, Zhivotovsky 1997) or an "environmental factor causing a change in a biological system, which is potentially injurious" (Hoffmann and Parsons 1991). Environmental stress is a major factor that shapes an organism's physiology, behavior and life-history. Extreme temperature is one of the most important environmental stresses, 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. Temperature can greatly affect the fitness of an organism through its effects on physiology, life-history traits like survivorship, longevity, fecundity and other life-history related traits i.e., immunity (Norry et al. 2006, Lazzaro et al. 2008, Robinson et al. 2000). Temperature can also affect reproduction through its effects on mating behavior, fecundity and fertility (Krebs and Loeschcke 1994, Reeve et al. 2000, David et al. 2005). Therefore, insects typically show preference for an optimal temperature to which their physiological functions are well adapted (Angilletta et al. 2002, Chown and Terblanche, 2006). Therefore, temperature is one of the key factors that determine the distribution of ectotherms, especially insects.

In this thesis, my major goal is to explore the evolution of reproductive traits and other life-history traits in insect populations subjected to extreme cold shock.

Effect of low ambient temperature on insect physiology

Extreme temperatures are potentially injurious and even lethal for organisms. Even temperatures not reaching the lethal limits have major consequences on an organism's performance and 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, Anderson et al. 2005, MacMillan et al. 2009). Apart from mortality, cold stress can affect fitness through its effects on reproduction. Cold shock can decrease sperm count (Lacoume et al. 2007) as well as male mating ability (Rinehart et al. 2000, Lacoume et al. 2007). Lefevre and Jonsson (1962) show that after being subjected cold shock, male fruit flies had no motile sperm over a period of 24 hours. Cold stress also kills sperm 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. Several studies have addressed such adaptations by insects. Two of the important approaches have been clinal studies and laboratory studies.

Clinal studies for thermal adaptation

A large number of studies have explored the clinal variation in cold tolerance and other life-history traits. A brief summary of a few of such studies is presented below. Populations of *D. melanogaster* from higher latitudes show a greater resistance to cold stress relative to populations from lower latitudes. Multiple studies have shown that temperate populations of *D. melanogaster* tend to have higher cold tolerance compared to tropical and subtropical populations (Bubliy et al. 2002, Davidson 1990, Karan et al. 1998, Parsons 1980, Schmidt et al. 2005). Such clinal variation provides indirect indication for thermal adaptation within a species (Hoffmann et al. 2003a, Schmidt and Paaby 2008). Temperate populations have better survival post cold shock compared to tropical populations (Davidson 1990). Temperate Australian populations of *Drosophila* are less resistant to heat shock than tropical populations (Hoffmann and Watson 1993). Populations of *Drosophila* found at high latitudes (temperate climates) recover faster from chill coma than tropical populations (Ayrinhac et al. 2004). Similarly, Gibert et al. (2001) assayed *Drosophila* species from temperate and tropical regions and found that the a set of 26 species from temperate regions recovered faster from chill coma relative to the 48 tropical species. Thus, variation in cold tolerance is associated with the latitude (or altitude) both at the population and species level.

While there are multiple studies documenting clinal variation in cold tolerance, few studies have directly addressed genetic variation associated with cold tolerance (Overgaard et al. 2010). A species level study of cold resistance suggests that evolutionary responses in cold resistance in *Drosophila* are likely to be slow because of

considerable phylogenetic inertia (Kellermann 2012). Oakeshott et al. (1982) observed signatures of climatic adaptation in wild populations of *D. melanogaster* in Australia. One of their most striking results was the clinal pattern observed in the frequencies of different alleles of *Adh*, namely Adh^{s} *and* Adh^{F}. They showed that the frequency of the Adh^{s} allele decreases as one moves southwards. Lavington (2014) study shows that adaptation to climatic conditions is affected by changing the expression of genes related to metabolism.

Life-history traits (body size, fecundity and longevity) co-vary with cold tolerance along clines. For example, Fabian et al. (2015) show that body size increases along altitude and latitude. Sub-Saharan African populations show increased wing size and thorax length along the altitude (Pitchers et al. 2013). In *D. melanogaster* and *D. buzzatii*, fecundity increases along latitude and altitude (Fabian et al. 2015, Norry et al. 2006). Temperate populations of *D. melanogaster* mostly have higher fecundity compared to tropical populations (Bouletreau-Merle et al. 1982, Klepsatel et al. 2013). Similarly fecundity correlates positively with latitude of origin in Australian and Indian populations (Hoffmann et al. 2003a, Rajpurohit and Nedved, 2013). Lifespan decreases with latitude and longitude among Afro-tropical species (Fabian et al. 2015). Latitude significantly affects ovariole number, bristle number, thoracic pigmentation and thorax length in *Drosophila* (reviewed in Gibert et al. 2004).

Laboratory selection studies for thermal adaptation

Large number of studies have quantified the effects of high and low temperature shock on fitness. However most of studies have focussed on fitness consequences of temperature shock over a single generation and very few studies have investigated the evolution of resistance to cold shock. Most of the laboratory selection studies on resistance to cold stress have focused on survivorship post shock as the measure of fitness. Some of them have also assayed correlated evolution of traits such as longevity and fecundity. For example Tucic (1979) focused on the evolution of survivorship post cold stress at different life stages such as egg, larva, pupa and adult. Chen and Walker (1993) have addressed the evolution of adult survivorship post cold shock in a population selected for resistance to cold stress at adult stage. Anderson et al. (2005) selected populations of *D. melanogaster* for faster chill coma recovery and found increased survivorship in response to cold stress in the selected populations. These populations also had higher fecundity. Bubliy and Loeschcke (2005) selected *D. melanogaster* for resistance to cold or heat

stress and found that populations show high resistance to the stress that they were selected against. MacMillan et al. (2009) addressed the effects of cold shock on life-history traits (such as the survivorship, longevity) and cross resistance. They found reduced chill coma recovery, starvation resistance, longevity in female and increased survivorship post cold stress. While these laboratory studies have been extremely informative, there are a couple of limitations. (a) All the previous studies on cold stress tolerance have focussed on survivorship as the major component of fitness. Cold stress can have effects on fitness independent of its effects on mortality. Cold stress can affect a range of reproductive traits including male and female fertility, gamete viability, mating success etc (Novitski and Rush 1949, Lefevre and Jonsson 1962, Lacoume et al. 2007). These have received very little attention. (b) There are methodological improvements that are needed with respect to the previous studies. Chen and Walker (1993) used inbred lines for selection. Therefore, we decided to undertake an experimental evolution study focussing on the effects of cold stress on reproductive traits and other important life-history related traits to understand the genetic architecture of cold stress resistance in *D. mealnogaster* We also ensured that our study populations were large and amply replicated.

Organization of the Thesis:

Effects of temperature stress on reproductive fitness components in insects

High temperature influences the reproductive behavior in many arthropods including male behavior, male fertility, sperm number, ejaculate size, sperm competitive ability, female fertility and fecundity (Zeh et al. 2013, Dick et al. 2013; Rinehart et al. 2000; Arbogast 1981; Yenisetti et al. 2006; Lieshout et al. 2013). Just like heat stress, cold stress also profoundly affects reproductive fitness in ectotherms. Exposure of *D. melanogaster* to low temperatures significantly reduces mating success (Dolgin et al. 2006). Exposure of male *(Aphidius picipes)* wasps to cold (4^{o}C) for two week reduces mating success (Amice et al. 2008). In *D. melanogaster* and *D. simulans* ambient growth temperature affects male fertility (Chakir et al. 2002). Cold shock can reduce progeny production (Iyengar and Baker 1960) and male fertility (Lefevre and Jonsson 1962, Wedvik 1962, Kelty and Lee 1999, Chakir et al. 2002, David et al. 2005, Overgaard et al. 2007, David 2008, Mockett and Matsumoto 2014). Quality of ejaculates in insects is a crucial component of male fitness (Campbell et al. 1992, Perez-Crespo et al. 2008, Hansen 2009) and can be potentially affected by thermal stress (Lieshout et al. 2013).

However, most of the laboratory selection studies have completely ignored this aspect of reproduction.

In the third chapter of this thesis, I will be exploring the evolution of reproductive fitness in response to selection for cold shock. In chapter four, I will be specifically concentrating on the evolution of pre and post copulatory traits in males and females in response to selection.

Consequences of evolved resistance to cold stress on other important life-history traits (life-history trade-offs)

Life-history theory attempts to understand "how natural selection and other evolutionary forces shape organisms to optimize their survival and reproduction in the face of ecological challenges imposed by the environment" (Stearns 1992, Roff 1992, Stearns 2000). Environmental stress plays a vital role in shaping the life-history traits of an organism. Adaptation to extremes of temperature can involve changes at multiple levels-behavioral to molecular. Such changes might be costly in terms of resources. Life-history theory suggests that when resources are limiting, investment in a given trait can lead to trade-off with another trait. Thus, a number of adaptations in response to cold shock could potentially trade-off with other important life-history related traits. A prime example of the energetic costs is the production of heat shock proteins in response to temperature stress. Resources used up for production of heat shock proteins (Hsps) might be unavailable for progeny production or other body maintenance activities. Studies suggest that thermal stress tolerance may indeed trade-off with life-history traits. For example, tropical populations of *Drosophila* are known to be more resistant to heat shock and short lived compared to temperate populations (Davidson 1990, Hoffmann and Parsons 1991, Hoffmann et al. 2002, reviewed in Hoffmann et al. 2003b, Schmidt and Paaby 2008). Thus, cold stress resistance and life-history traits are very likely to be interlinked.

In chapter 5 of this thesis, I explore the evolution of life-history traits in populations selected for increased cold shock resistance.

Mechanisms of cold shock resistance

There are different mechanisms that can potentially increase resistance to cold stress. (a) Organisms can increase the concentration of certain metabolites that act as anti-freeze

agents. Chen and Walker (1994) showed a positive correlation between glycogen content and cold tolerance. Other metabolites such as trehalose, proline and triacylglycerol also act as antifreeze compounds and help resist cold stress in organisms **(**Hodkova and Hodek 2004). In addition, there are several changes in membrane composition and increased production of molecular chaperone proteins which help in cold stress tolerance (Rinehart et al. 2007). (b) Increase in expression of cold inducible genes during the recovery of organism post cold stress (Colinet et al. 2010, Bing et al. 2012). There are genes such as *hsps hsp22, hsp23, hsp26, hsp27, hsp40, hsp68, hsp70Aa* and *hsp83*, *stv* and frost that are up-regulated after cold shock. They are suggested to have a very important role in recovery from cold shock (Goto 2001, Sinclair et al. 2007, Colinet et al. 2010, Colinet and Hoffmann 2010). Additionally in insects, the expression of capa neuropeptides which are involved in neuroendocrine responses of the organism is upregulated (Terhzaz et al. 2015).

In chapter 6 of this thesis, I will be discussing the genetic mechanism of resistance to cold stress. I explore the expression of cold inducible *hsp* genes and *frost* gene in the selected and control populations with and without cold shock. I also attempt to address the possible differences in lipid storage as a consequence of adaptation to cold shock.

Are stress resistance mechanisms specific or general?

When organisms are subjected to a specific kind of stress, quite commonly, they evolve to resist that particular stress. Whether the evolved mechanisms of resistance are specific to a given stress has long been a question. In fact, it has been suggested that evolution under a particular stress can have positive, negative or no effect on the ability of the organism to face other kinds of stresses. If stress resistance mechanisms are sufficiently general or if there is a strong positive genetic correlation between resistance to two stresses, then, one would expect to see simultaneous increase in the ability to resist two stresses (when selection is only on one stress). On the other hand, mechanisms evolved against a particular stress might interfere with mechanisms against a second stress or there might be a strong negative genetic correlation between two stresses. Then, we would expect a decline in the ability to resist one stress as the ability to resist another stress increases.

Cold stress has been linked with several other stresses such as desiccation, starvation, heat and pathogenic infections (Bubliy and Loeschcke 2005, Ring and Danks 1994,

Bubliy and Loeschcke 2005, Sinclair et al. 2007a, Kristensen et al. 2007, Le Bourg et al. 2009, Zhang et al. 2011, reviewed in Sinclair et al. 2013). However, results from various studies regarding the genetic correlation between cold resistance and these other stresses are highly variable. For example, Bubliy and Loeschcke (2005) selected populations for either cold tolerance or desiccation resistance and found that resistance to both the stresses evolved in both the selection regimes. Contrary to this, Hoffmann et al. (2005b) and Sinclair et al. (2007a) found no correlation between cold and desiccation stress.

In chapter 7 of this thesis, I address the genetic correlation between resistance to cold shock and other stresses such as heat stress, starvation, desiccation and bacterial infection.

Isolation and characterization of novel natural pathogen of *D. melanogaster*

In chapter 8 of this thesis, I describe the details of identification and characterization of a novel natural pathogen of *D. melanogaster*. A review of *Drosophila* literature regarding immunity suggests that there are very few known natural bacterial pathogens of *Drosophila* and a large proportion of the research uses non-natural pathogens. Hence, I isolated and characterized a novel pathogen of *Drosophila*. This pathogen was then used to carry out experiments related to immunity in this thesis.

Chapter 2

Experimental system

D. melanogaster is a holometabolous insect which belongs to class insecta, order diptera and family drosophilidae. In nature they are found most of the time feeding on rotting fruits such as pineapple, grapes, banana, apple etc. *Drosophila* are polygamous species, females store sperm in a specialised organ known as spermatheca from multiple mating for future use (Lefevre and Jonsson 1962).

D. melanogaster is an excellent model system for the study of several topics such as adaptation to environmental stress, disease and immunity (Tucic 1979, Rose 1984, Watson and Hofmann 1991, Partridge and Barton 1993, Chippindale 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). *Drosophila*, which traces its ancestry to Africa has colonized different temperate continents, making it a good model to study adaptation to cold stress. *Drosophila* has been used for the study of laboratory experimental evolution along with studies on altitudinal and latitudinal clines (Tucic 1979, Partridge and Barton 1993, Chippindale et al. 1994, Chen and Walker 1993, Joshi and Mueller 1996, Watson and Hoffmann 1996, MacMillan et al 2009, Kellerman et al. 2012, Prasad et al. 2007, Nandy et al. 2013).

For my thesis, I have also used *D. melanogaster* (fruit fly) as a model organism. *D. melanogaster* complete their life cycle in about 9-10 days through different developmental stages from egg to larva, pupa and finally adult. In standard laboratory culture conditions (25^{o}C temperature, 50-60% relative humidity, 12hours:12hours ligh/dark cycle) *D. melanogaster* eggs hatch into first instar larvae in about 18-24 hours. Larval stage is the most important stage where they acquire maximum resources. Twenty-hours later first instar larvae molt into second instar larvae. Then in another 24 hours they molt again into third instars larvae. Third instar larvae move to a clean surface to pupate and then go through metamorphosis. They finally eclose on 9-10th day after egg collection. *D. melanogaster* adults take about 8-10 hours to become sexually mature once they emerged from puape. Females start laying eggs one day after eclosion. For a

summary of the life cycle of *D. melanogaster (*see figure 2.1). On an average the life span of adult *D. melanogaster* is about 45 to 50 days. Fecundity of females heavily depends upon quality of food they acquire (Prasad and Joshi 2001, Stewart et al. 2005, Nandy et al. 2012). At the usual conditions of the laboratory, i.e., under 25^{o}C temperature, 50-60% relative humidity, their metamorphosis follows the pattern described here in brief.

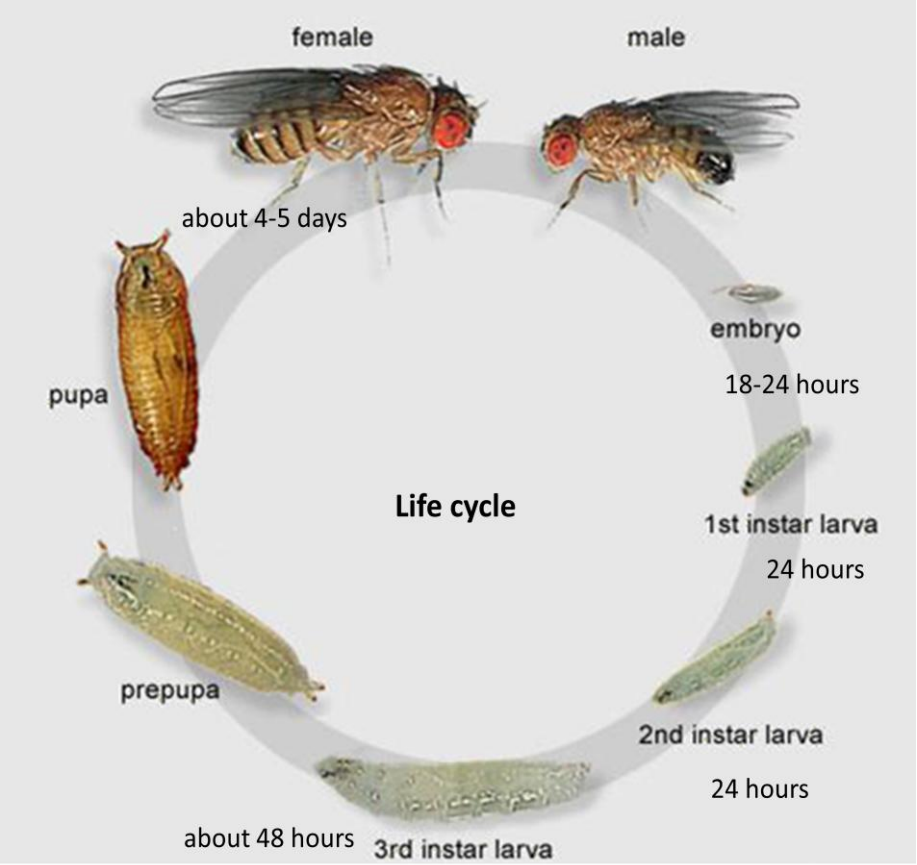

Figure 2.1: Life cycle of *D. melanogaster* (retrieved from http://flymove.uni-muenster.de/Media/FindMediaOutput.php?thema=Genetics).

Laboratory selection experiments

Laboratory selection experiment is a powerful experimental approach, where laboratory setting enables the experimenter to manipulate environmental parameters as per requirements and makes replication of experiments possible. The impact of individual environmental factors (for example, cold stress) on shaping life-history traits of populations can be studied unambiguously using this approach. Effective population size

is a crucial parameter which can influence the outcome of laboratory selection experiments. Because small population sizes can severely cause inbreeding depression and lead to incorrect interpretations of the effect of laboratory selection on the populations (Rose 1996) it is very important to minimise the effect of inbreeding by keeping effective population sizes high. The base-line populations (BRB) used in this thesis are maintained under sufficiently high breeding population sizes of N = 2800 individuals to minimise the effect of inbreeding and drift. The selected and control populations discussed in this thesis are also maintained under the sufficiently high breeding population sizes of N = 1400 individuals.

Experimental populations

Base line population

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

The five replicate BRB populations are maintained on a 14 day discrete generation cycle at 25°C temperature, 50-60% RH, 12:12 hours light-dark cycle on standard banana-yeast-jaggery food (Prasad et al. 2003). Eggs are collected from adult flies and dispensed into glass vials (25mm diameter × 90mm height) containing 6 ml of banana-yeast-jaggery food at a density of about 70 eggs per vial and incubated at standard laboratory conditions (see above). Forty such vials are set up for per population. On the 12^{th} day post egg collection (by which time almost all the adults eclose and mate), the adults are transferred to Plexiglas cages (25cm length × 20cm width × 15cm height) provided with a Petri plate containing standard banana-yeast-jaggery food supplemented with live yeast paste (ad-lib). Each cage contains approximately 2800 adults. On the 14^{th} day post egg collection, fresh food 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 under the above mentioned conditions for 35 generations before starting the present study.

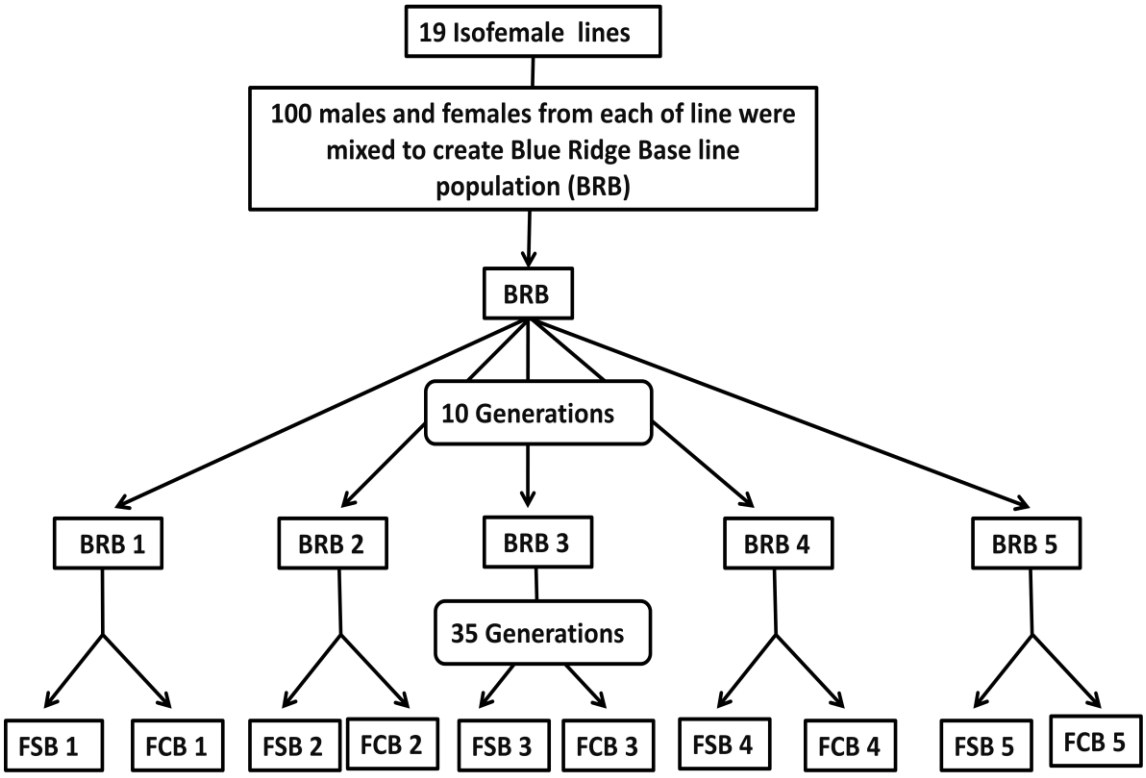

Figure 2.2: Derivation of the base line (BRB) populations by mixing of 19 isofemale lines, and creation of FSB (1-5) and FCB (1-5) populations from base line BRB (1-5) populations (Singh et al. 2015)

Derivation and maintenance of selected and control populations

Most of the work presented in this thesis was performed on these 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 o/f the mixing of the 19 isofemale lines. Following this, I derived one selected (FSB**;** selected for cold shock resistance**,** derived from BRB population) and one control population (FCB**;** cold shock resistance control, derived from BRB population) from each of the five BRB populations (Figure 2.2), yielding 10 populations in all (five selected populations FSB 1-5 and five control populations FCB 1-5). The selected and control populations that were derived from the same BRB

population were given the same numerical subscript as they were more closely related to each other and were treated as Blocks in statistical analysis. Hence, Blocks refer to ancestry in my selection regime. For example, FSB 1 and FCB 1 are derived from BRB 1 and are more closely related to each other (by ancestry) than to FSB 2. FSB 1 and FCB 1 constitute Block 1. Thus, the experiment consisted of five Blocks which were handled together during maintenance and experimentation. For example, FSB 1 and FCB 1 were always handled together during maintenance and experiments.

The details of the maintenance of the selection regime are presented in Singh et al. (2015). The selected populations (FSB 1-5) are maintained on a 13 day discrete generation cycle (Figure 2.3). Eggs are collected from adults and dispensed into vials (25mm diameter × 90mm height) containing about 6 ml 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 incubated at 25°C temperature, 50-60% relative humidity (RH) and 12 hour:12 hour light:dark cycle. The flies start eclosing by the 9^{th} day post egg collection with peak eclosion happening on the 10^{th} day post egg collection. On day 12 post egg collection (by which time all the adults eclose and are about 2-3 days old post eclosion), the adults from each of the 20 vials are transferred into 20 clean empty, dry glass vials (about 60 to 70 flies per vial). The cotton plug is inserted in vial such that the flies are confined to a small area at the bottom one third of the vial (25mm diameter × 30mm height). These vials are then placed into salt-water-ice slurry maintained at -5°C and held there for one hour. Care is taken to see that 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 (25cm length × 20cm width × 15cm height) containing a Petri plate of banana-yeast-jaggery food and maintained at 25°C temperature.

Twenty four hours after the cold shock treatment (i.e., the 13^{th} day post egg collection), the flies are provided with a fresh banana-yeast-jaggery food plate and are allowed to oviposit for 18 hours. Hence, during normal maintenance regime, eggs are collected to start the next generation in 18 hours window lasting between 24-42 hours post cold shock. These eggs are then dispensed into food vials to start the next generation. It is important to note that the cold shock treatment that we use causes low adult mortality (~3 to 9%). However, the viability of the eggs laid 24 hours post cold shock is reduced by about 30-40%. All else being equal, individuals with higher egg viability 24 hours post cold shock have a higher Darwinian fitness in the context of the selection regime.

Therefore, in our study, while selection is directly on differences in egg viability 24 hours post cold shock, all factors that affect egg viability at 24 hours post cold shock (such as sperm mortality, maternal effects, mating rates, etc.) are under indirect selection. 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. Thus there are about 1200 to 1400 adults per generation per population.

The control populations (FCB 1-5) are maintained under exactly the same conditions as the selected (FSB 1-5) (Figure 2.3) populations but for the following changes

1. On the 12th day post egg collection, the flies from the FCB population are transferred into empty vials and are held in a water bath maintained at 25°C for one hour (Figure 2.3).

2. The eggs are collected from the adults and dispensed into vials containing 6 ml of banana-yeast jaggery food at a density of 60-70 eggs per vial.

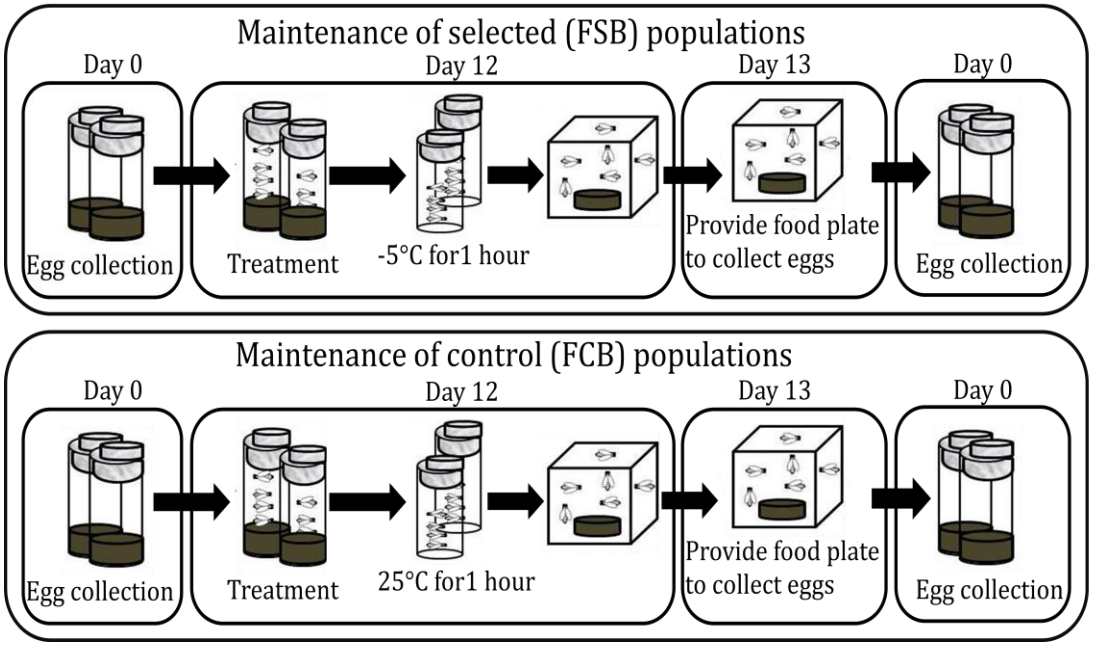

Figure 2.3: Maintenance regime of the FSB (1-5) and FCB (1-5) populations (Singh et al. 2015).

Standardization of flies

To account for differences between selected and control populations due to non-genetic parental effects (Rose 1984), all the populations (FSB 1-5 and FCB 1-5) were reared under laboratory conditions as described below for one generation before performing experiments. During this generation, the FSB populations were not subjected to selection. We call this process 'standardization' and the 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 6 ml of standard banana-yeast-jaggery food at a density of about 70 eggs per vial. Twenty such vials are set up per population. The vials are incubated at standard laboratory conditions (see above). On the 12^{th} day post egg collection, the adults from a given population are transferred into a Plexiglas cage provided with banana-yeast-jaggery food. These flies are called the standardized flies. On the 13^{th} day post egg collection, a fresh food plate is provided and the standardized flies are allowed to oviposit for 6 hours. Using moist camel-hair paint brushes, eggs are collected from these food plates and dispensed into vials containing 6 ml of standard banana-yeast-jaggery food at an exact density of 70 eggs per vial. Adults emerging from these vials (i.e., the progeny of the standardized flies) are used for further assays. Most of the experiments reported in the present study were conducted after 19 to 73 generations of selection.

LH population

This population is named LH after its founder Larry Harshman. The large outbred LH population was established in 1991 from around 400 wild caught inseminated females. The LH population has been maintained under laboratory conditions for more than two decades now (Chippindale and Rice 2001). The LH flies are maintained on a 14 day discrete generation cycle and reared on a standard cornmeal-mollasses-yeast diet. At all times the flies experience a temperature of 25^{o}C, relative humidity between 50% and 60% and alternate 12 hour long light and dark periods. Egg densities are controlled with each vial containing approximately 150 eggs in close to 6 ml food. The population has a total of 60 such vials, arranged in 6 bunches of 10 vials each. On the 12^{th} day post egg collection, by which time all flies emerge as adults from their pupae, the adult flies from each of the six bunches of 10 vials are transferred to a separate plastic flask. From each of the six plastic flasks ten sets of 16 males and 16 females each are sorted using light CO_2

anaesthesia. Each set of 16 males and 16 females is transferred to a vial containing close to 6 ml of food and additional 5-6 mg live yeast. The population consists of 60 vials each containing 16 males and 16 females. These vials are randomly divided into six bunches of ten vials each. Two days later, the flies are transferred to fresh vials containing around 6 ml foods. In order to start the next generation, they are allowed to oviposit for 18 hours and subsequently discarded. Egg densities in each vial are adjusted to around 150 per vial by removing excess eggs. The total census size for the LH population is ~1920 individuals.

LH_{st} population

LH_{st} is a large outbred population which was created from the LH base population by infixing a recessive- homozygous autosomal trait i.e., scarlet coloured eye ('st') in the LH genetic background by back crossing again and (Prasad et al. 2007). The maintenance of LH_{st} is similar to that of the LH population, except that the LH_{st} population comes from 30 vials (150eggs/vial) instead of 60 vials. To maintain genetic homogeneity the LHst population is periodically backcrossed with the LH base population.

PJB population

Each of the four Prasad Joshi Baseline (PJB 1-4) populations is derived from the corresponding Joshi Baseline (JB 1–4) population. The JB populations were originally derived from four of the five UU populations (see details in Joshi and Mueller 1996). The PJB populations are maintained at 25°C under constant light on a 21-day discrete generation cycle on a standard banana-jaggery-yeast food. Each generation, in order to collect eggs, fresh food plates are introduced into Plexiglas cages (25cm length × 20cm width × 15cm height) containing close to 2800 adults. Eighteen hours after the introduction of the plates eggs laid on the plates are transferred to fresh food vials at a density of 60-80 eggs per vial, with each vial containing close to 6 ml of food. For each of the four PJB populations 40 such vials are set up. By the 12^{th} day post infection, all flies emerge out of their pupae as adults. They are then transferred to Plexiglas cages (one cage per population) provisioned with a food plate. Every alternate day the food plate is replaced with a fresh one. On the 18^{th} day post egg collection, each population cage is provided a food plate smeared with yeast paste in order to boost fecundity. On the 20^{th} day post egg collection, a fresh food plate is introduced into each population cage and the flies are allowed to oviposit on it for 18 hours. These eggs are used to start the next

generation.The PJB populations are maintained at a total census size of around 2800 individuals each.

W1118 population

This is an inbred wild-type population. The population stock was obtained from Bloomington Drosophila stock centre (BDSC), Indiana University, USA. Ten vials of around 70 eggs/vials are set up with each vial having ~6 ml of banana-yeast-jaggery food. These vials are incubated at 25°C, 50-60% RH and are maintained on a 12:12 hour light-dark cycle on a standard banana-yeast-jaggery diet. On the 12^{th} day post egg collection, flies are pooled together and cultured into groups of 16 pairs (male and female) per vial. On the 14^{th} day, flies are transferred into fresh food vials and allowed to oviposit for 18 hours. Subsequently they are discarded and the egg density is adjusted at a density of 70 eggs/vial.

Canton-S population

This is an inbred wild-type population. The population stock was obtained from *Drosophila* stock centre based at University of Mysore, Mysore, India. Maintenance of this population is similar to W1118 as described above.

Cold shock protocol

On the 12^{th} day after egg collection (~2-3 days old as adults), flies are transferred to clean dry glass vials (25mm diameter × 90mm height) at a density of 50 individuals per vial. The cotton plug is inserted deep into the vial such that the flies are allowed to stay in the bottom one third of the vial which is roughly 25mm diameter × 30mm length of the vial. The vials are then placed in water-ice-salt slurry maintained at -5°C and allowed to stand for one hour, and proper care is taken to make sure that part of the vial conaining of flies is dipped in water-ice-salt slurry. Immediately after cold shock, the flies are then transferred into Plexiglas cages (14cm length × 16cm width × 13cm height) at a density of 100 pairs (male and female) per cage. The cage is provided with a Petri plate having standard banana-yeast-jaggery food and is maintained under standard laboratory conditions (25^{o}C temperature, 50-60% relative humidity, and 12hours-12hours light/dark cycle).

No shock protocol

The FSB and FCB flies for the control treatment (no shock treatment) are handled similar way to the cold shock treatment except that the vials containing male flies are placed in water bath for one hour maintained at 25°C instead of -5°C for one hour.

Heat shock protocol

Flies are handled in a similar way as described above for the cold shock treatment with the only exception that flies are subjected to 37.5°C in water bath for one hour (instead of being exposed to -5°C). After heat shock, flies are immediately transferred into Plexiglas cage provided with banana-yeast-jaggery food plate.

Bacterial stock

Staphylococcus succinus subsp. *succinus* PK-1 is cultured in Luria-Bertani (LB) medium, incubated at 37^{o}C and shaking at 170 rpm for overnight growth. The following day a sub-culture is started by inoculating 100 µl overnight culture in 10 ml of LB broth and incubated (at 37^{o}C and at 170 rpm) until optical density (OD_{600nm}) of 1 ± 0.1. Then 2 ml of culture is centrifuged (10000 rpm for 4 minutes) and the pellet is resuspended in1 ml of 10 mM $MgSO_4$ to yield bacterial slurry (Apidianakis et al. 2009).

Infection

Flies are lightly anesthetised using CO_2. They are infected by pricking the lateral thorax with a fine needle (Minutein pin 0.1 mm, Fine Science Tools, CA) dipped in bacterial slurry (bacteria suspended in 10 mM $MgSO_4$). Sham infected flies are handled the same way, except that the needle is dipped in sterile 10 mM MgSO4 solution (Apidianakis et al. 2009).

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

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

Table 2.2. The composition of 1 liter of regular cornmeal-molasses food.

Ingredient	Amount
Water (ml)	1100
Agar (g)	14.8
Cornmeal (g)	100
Molasses (ml)	100
Yeast (g)	41.2
p-Hydroxymethyl benzoate (g)	2.25
Ethanol (ml)	22.5
Propionic acid (ml)	8

Table 2.3. The composition of Luria Bertani Broth.

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	10
pH	7.5 ±0.2

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Chapter 3

Evolution of egg viability as a direct response to selection

INTRODUCTION

Environmental stress such as extreme temperatures, desiccation, crowding can have major consequences for fitness of organisms. In holometabolous insects like *D. melanogaster*, different life stages (which live in different environments), can experience different types of stresses. The ability to resist environmental stress is an important component of fitness. Using *Drosophila*, a large number of studies have investigated the evolution of resistance to various environmental stresses such as larval crowding (Joshi and Mueller 1996), increased urea and ammonia content in larval food (Borash et al. 2000), adult desiccation, starvation (Chippindale et al. 1996, 1998) and extreme temperatures (Tucic 1979, Chen and Walker 1993, Watson and Hoffmann 1996, Anderson et al. 2005, MacMillan et al. 2009).

Temperature is one of the most important ecological factors that affect the fitness of an organism (Kingsolver 2009). This is especially true of insects which are ectothermic (Chown and Nicolson 2004, Denlinger and Lee 2010). Effects of high temperature shock on fitness are well studied (Scott et al. 1997, Rinehart et al. 2000, Zizzari and Ellers 2011, Lieshout et al. 2013, Nguyen et al. 2013). High temperature shock (i.e., heat shock) can affect longevity, survivorship, fecundity and male fertility (Czajka and Lee 1990, Denlinger and Yocum 1998, Rohmer et al. 2004, Bubliy and Loeschcke 2005, Malmendal et al. 2006, reviewed by Hance et al. 2007, Schmidt and Paaby 2008, Zizzari and Ellers 2011, Lieshout et al. 2013, Nguyen et al. 2013). Just like heat shock, sudden exposure to sub-zero temperature (i.e., cold shock) can also potentially affect ectotherms.

Cold shock 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, Schmidt and Paaby 2008, MacMillan et al. 2009, Overgaard et al. 2007, Dillon et al. 2007, Marshall and Sinclair 2010, Mockett and Matsumoto 2014). Consequently, insects have evolved a number of mechanisms to deal with cold stress

(Sinclair et al. 2003). Some studies have addressed the evolution of resistance to cold shock (Overgaard et al. 2010) for detail account. A study across 95 species of *Drosophila* indicated that evolutionary responses in cold resistance are likely to be slow because of considerable phylogenetic inertia (Kellermann et al. 2012).

Other studies have found clinal variation in traits related to cold tolerance in populations of *Drosophila* (Hoffmann et al. 2002, Hoffmann et al. 2005, Hoffmann and Weeks 2007, Ayrinhac et al. 2004). Results from laboratory studies suggest that populations selected for resistance to cold shock evolve increased survivorship post cold shock (Tucic 1979, Chen and Walker 1993, MacMillan et al. 2009). Other studies show that selection for faster recovery from chill induced coma in *D. melanogaster* leads to a correlated increase in adult survivorship and fecundity post cold stress (Anderson et al. 2005). However, longevity of the selected populations was significantly lower than that of the controls (when measured in the absence of cold shock). Increased tolerance to cold shock has not been found to be correlated with resistance to other stresses such as heat shock (Anderson et al. 2005), starvation and desiccation (MacMillan et al. 2009).

Apart from affecting life-history traits such as fecundity and longevity, cold shock can also affect reproduction by decreasing sperm stock (Lacoume et al. 2007) as well as mating ability of males (Rinehart et al. 2000, Lacoume et al. 2007). In *D. melanogaster*, females are de-seminated by cold shock treatment (Novitski and Rush 1949). Previous study documented that cold shock kills sperm in the storage organs of female *D. melanogaster* (Lefevre and Jonsson 1962). The dead sperm are then ejected by the females. Their future fecundity is, therefore, dependent upon re-insemination. Cold shock also kills the mature sperm stored in the seminal vesicles of the male *D. melanogaster* (Lefevre and Jonsson 1962). The males do not show any motile sperm for a period of 24 hours after cold shock and fail to transfer any sperm to females during this period. Thus, the future fitness of cold-shocked males depends upon their ability to produce fertile sperm and successfully mate with females post cold shock (Lefevre and Jonsson 1962). Thus, it is very clear that cold shock can severely affect fitness of *D. melanogaster* through its effects on reproductive traits such as sperm production, storage and mating. Therefore, it is very likely that adaptation to cold shock will involve changes in reproductive physiology. Some studies have addressed the evolution of life-history traits in response to selection for resistance to cold shock (see references above). However, to

the best of my knowledge, no study has so far addressed the evolution of reproductive behavior in response to selection for resistance to cold shock.

In the present experiment, my major goal was to address the evolution of components of reproductive fitness such as mating frequency and mating ability in response to cold shock**.** I subjected adult flies to a non-lethal cold shock (-5°C for one hour) which reduced egg viability (defined as the proportion of the eggs that hatch) by nearly 40% but had relatively little effect on adult mortality (about 3-9%). Thus, in my study, selection was primarily on egg viability. The selection protocol I used was similar to that of several previous studies that have addressed the evolution of cold shock resistance (Tucic 1979, Chen and Walker 1993, Watson and Hoffmann 1996, MacMillan et al. 2009). The major focus of these previous studies has been the evolution of adult mortality and other life-history traits. In my study, I focused on the evolution of reproductive traits. The study consisted of 10 populations of *D. melanogaster* (5 selected populations and 5 control populations) and was conducted over 30 generations of selection. I specifically addressed the following questions:

(a) Does egg viability post cold shock evolve as the primary response to selection?

(b) Do fecundity, larval mortality and adult mortality post cold shock evolve as a correlated response to selection?

As noted before, cold shock can kill stored sperm in males and females (Novitski and Rush 1949, Lefevre and Jonsson 1962). Hence, I also tested whether –

(c) The ability of the females to protect the sperm from cold shock has evolved and

(d) The ability of the males to mate successfully and sire progeny post cold shock has evolved.

I found that larval survivorship, adult mortality and fecundity post cold shock did not evolve. However, egg viability and components of reproductive behavior had evolved in the selected populations.

METHODS

Base line population and experimental population: A detailed account of maintenance of the base line population and the derivation of selected (FSB) and control (FCB) populations are outlined in chapter 2.

Experimental protocol:

Experiment 1: Have egg viability, fecundity and mating frequency post cold shock evolved?

As noted before, cold shock that I use reduces egg viability by about 40% but causes about 3-9% mortality in the adults. Therefore, in my experiment, egg viability is primarily under selection. Hence, I first assayed the direct response to selection in terms of egg viability 24 hours post cold shock. Using the same experimental set up, I also collected data about fecundity, adult mortality and mating frequency of the selected and control populations. See details of the experimental design in the pictorial representation.

I specifically asked the following questions- are egg viability and fecundity affected by (a) treatment i.e., Cold shock vs. Neither-cold shock (b) selection history (c) time gap between cold shock and egg viability measurement and (d) access to healthy vs. cold shocked mates.

After 19 generations of selection, eggs were collected from standardized flies as described in chapter 2. Thirty nine vials were set up for each of the ten populations. For each of the FSB and FCB populations, on the 12^{th} day post egg collection (by which time all the flies had eclosed and mated) the vials were randomly assigned to one of the following three treatments:

a) **Both-shocked**: both males and females from a given population were subjected to cold shock (following the protocol mentioned in chapter 2) and were then transferred to a Plexiglas cage at a density of 100 pairs per cage.
b) **Female-shocked**: only females (non-virgin) from a given population were subjected to cold shock (following the protocol as described in chapter 2). Following the cold shock, they were transferred into Plexiglas cages at a density of 100 females per cage. I then added 50 non-shocked males from the corresponding BRB base population to the cage. For example, a cage containing 100 FSB 1 cold shocked females received 50 non-shocked BRB 1 males. Thus the

shocked females had access to healthy males for one day post cold shock. I deviated from 1:1 sex ratio under the assumption that cold shock imposes stress and/or physiological harm to the females (MacMillan et al. 2009). Given this, I wanted to minimize the confounding factor of physical mate harm imposed by males (e.g., unsuccessful courting) while making sure that there are enough males to inseminate all the females.

c) **Neither-shocked**: Neither the males nor the females were subjected to cold shock. Instead, the males and females from a given population were held in a water bath maintained at 25°C for one hour. They were then transferred to Plexiglas cages at a density of 100 pairs per cage.

I assayed fecundity and egg viability at two points - (a) 0 hours post cold shock and (b) 24 hours post cold shock. I chose these two time points because (a) measures of fecundity and egg viability at 0 hours post cold shock represents the immediate effect of cold shock and (b) 24 hours post cold shock is the time that eggs are collected from the flies to start the next generation in their normal maintenance regime and is hence directly relevant to the fitness of the flies. At the beginning of each time period, dead flies (if any) were aspirated out of the cage and counted. A fresh food plate was provided in the cage for the females to lay eggs for 6 hours. A sample of 200 eggs from the food plate were then moved to a Petri plate containing 1.2% agar and incubated at 25°C for 30 hours, after which the numbers of hatched eggs were counted to obtain an estimate of the viability of the eggs. The rest of the eggs in the food plate were counted to measure total fecundity. This value was divided by the number of females that were alive at the start of the 6-hour oviposition window to obtain fecundity per female. To assess the effect of cold shock on adult mortality, I used the adult mortality values from the Neither-shocked and Both-shocked treatments.

In blocks 1, 2, 4 and 5, I set up three cages per Selection × Treatment combination. However, in block 3, there were two cages per Selection × Treatment combination. Fecundity and egg viability were assayed from each cage as described above. I used the fecundity per female and egg viability values from each cage as the units of analysis.

I also quantified the total number of matings in the Both-shocked and Neither-shocked treatments. Once the flies were transferred into a cage, I observed them every half an hour

and noted the total number of mating pairs. These observations were carried out until 36 hours post cold shock. In the normal maintenance regime, eggs are collected from the flies to start the next generation in an 18 hour window between 24-42 hours post cold shock. In *Drosophila*, post mating, a majority of the females finish processing the sperm (transport of sperm to seminal receptacle, ejection of sperm from bursa etc.) in about 5 hours (Manier et al. 2010). Hence, matings that happen until about 36 hours post cold shock can, in principle, result in progeny. Hence I chose to observe mating until 36 hours post cold shock. I then summed the number of mating pairs across all the observations for a given cage to obtain an estimate of the total number of matings. The total number of mating pairs per cage was used as the unit of analysis.

Experiment 2: Has the larval survivorship post cold shock evolved in the selected populations?

I wanted to test whether my selected populations have evolved better larval survivorship post cold shock. After 30 generations of selection, I assessed the effect of cold shock on survivorship of larvae from the selected and control populations. Eggs were collected from standardized flies as described before. Ten such vials were set up per population. On 12th day post egg collection (by which time all adults had eclosed), the vials from each population were randomly assigned to one of the two treatments- (a) subjected to cold shock or (b) subjected to control (25°C) treatment. The flies were transferred to Plexiglas cages at a density of approximately 150 pairs per cage. Twenty four hours later, eggs were collected from the cages by providing them with fresh food plates for a four hour window. The food plates containing the eggs were then incubated at 25°C. Twenty four hours later, when the eggs had hatched and the first instar larvae had emerged, using a fine, moist paint brush, I transferred the first instar larvae into fresh food vials at a density of 30 larvae per vial. Ten such vials were set up per population. The vials were then incubated under standard laboratory conditions for 15 days and all the emerging adults were counted. Thus each vial yielded a value of larva to adult viability. These vial values were used as the units of analysis.

Experiment 3: Are females from the selected populations better at protecting eggs/stored sperm from cold shock?

Previous studies suggest that cold shock kills the sperm in female storage organs. Thus insemination post cold shock is necessary for the females to lay fertile eggs. However, it is possible that females from the selected populations are better able to protect sperm from cold shock. To assess this possibility, I carried out an experiment after 30 generations of selection. Eggs were collected from standardized flies as described before. Twenty four such vials were set up per population. On the 12th day post egg collection, the vials from each population were randomly assigned to one of the two treatments- (a) subjected to cold shock or (b) subjected to control (25°C) treatment. For both the treatments, females were separated from the males (under light carbon dioxide anesthesia) and were transferred into clean, dry glass vials. The males were discarded. The females were then subjected to their respective treatments (as described before) and transferred to Plexiglas cages at a density of 100 females per cage. Thus the females (from the cold shock treatment and the control treatment) had no further access to males. Twenty four hours later, I assayed fecundity and egg viability of the females from the two treatments as described before. I set up three cages per Selection × Block × Treatment combination. Fecundity and egg viability values from each cage were used as the units of analysis.

Experiment 4: Has the ability to sire progeny post cold shock evolved in males of my selected populations?

I wanted to test if the ability to sire progeny post cold shock had evolved in my selected populations. After 19 generations of selection, I assessed the ability of cold shocked males from the FSB and FCB populations to mate non-shocked, non-virgin females and sire progeny. Eggs were collected from standardized flies as described before. Ten such vials were established per population. On the 12th day post egg collection, males from each of the vials were separated from the females under light carbon dioxide anesthesia and subjected to cold shock as described before. One hundred males were then transferred into a Plexiglas cage that contained 100 non-shocked, non-virgin females from an unrelated base stock called LHst which contains a recessive scarlet eye color marker (Prasad et al. 2007).

The LHst females were generated by collecting eggs from the LHst base population at a density of 70 eggs per vial and incubating the vials containing standard cornmeal-

molasses-yeast food at standard laboratory conditions (Prasad et al. 2007). The flies started eclosing by the 9th day post egg collection with peak eclosion on the 10th day post egg collection. The LHst males and females continued to interact with each other in the same vials for a further two days (by which time all females are mated). On the 12th day post egg collection, females were isolated using light carbon dioxide anesthesia and transferred into Plexiglas cages just before the males from the FSB and FCB populations were introduced into the cages.

The FSB/FCB males were allowed to interact with the LHst females for 24 hours post shock after which time, 50 LHst females from each cage were randomly sampled using carbon dioxide anesthesia and transferred individually into test tubes (12mm diameter × 75mm length) containing standard banana-yeast-jaggery food. The females were allowed to oviposit for 18 hours after which the females were discarded and the test tubes were incubated under standard laboratory conditions. Thirteen days later, progeny emerging from each of these tubes were counted and their eye color was noted. The males from the FSB/FCB populations have a dominant red eye color while the females from the LHst populations have a recessive scarlet eye color. Hence, the progeny from the mating of LHst females with FSB/FCB males will have red eye color while the progeny from the previous matings of LHst females (with LHst males) will have scarlet eye color.

I noted the number of females that produced progeny with red eye color (since this indicates successful mating with FSB/FCB males at least once). I also calculated the proportion of red eyed progeny within the total progeny pool produced by the LHst females. Thus each cage yielded one value for proportion of LHst females mating with males of interest (i.e., FSB or FCB males) and one value for proportion of progeny sired by males of interest. These values were used as the units of analysis.

Statistical analysis

Since selected and control populations bearing the same numerical subscript were derived from the same BRB population, they are more closely related to each other than they are to any other population. For example, FSB 1 is more closely related to FCB 1 (since they both were derived from BRB 1) than to FSB 2. Hence they are treated as statistical blocks in all the analyses. For experiment 1, fecundity and egg viability were analyzed using a four factor, mixed model Analysis of Variance treating Selection regime (FCB vs. FSB),

Treatment (Neither-shocked, Both-shocked and Female-shocked) and Period (0 hours vs. 24 hours post cold shock) as fixed factors with Block (1-5) as random effect. Adult mortality data were analyzed using a four factor, mixed model Analysis of Variance treating Selection regime (FCB vs. FSB), Treatment (Neither-shocked and Both-shocked) and Sex (male and female) as fixed factors with Block (1-5) as random effect. Mating frequency data from Experiment 1 along with data from experiment 2 and 3 were analyzed using a three factor mixed model Analysis of Variance treating Selection regime and Treatment as fixed factors crossed with random Block. Data from experiment 4 (proportion of non-virgin females mated by FCB vs. FSB males after cold shock; proportion of progeny sired by FCB vs. FSB males after cold shock) were subjected to paired t tests (two tailed). Multiple comparisons were done using Tukey's HSD.

RESULTS

Experiment 1: Egg viability and mating frequency post cold shock have evolved in the selected populations. However, fecundity and adult mortality post cold shock have not evolved

In my experiment, egg viability responded to selection. I found a significant effect of selection, treatment and period (Table 3.1). I also found a significant three way interaction between selection, treatment and period (Table3. 1). Eggs from FCB and FSB flies from Neither-shocked treatment had viability greater than 90% and there was no significant difference between them. Cold shock significantly reduced egg viability (Figure 3.1). At 0 hours post cold shock, the viability of eggs was extremely low (about 2%) and was not significantly different between the FCB and FSB populations (Figure 3.1). At 24 hours post cold shock, the viability of the eggs had increased to about 45% (Figure. 3.1). Additionally, 24 hours after cold shock, the viability of eggs from FSB populations was significantly higher than that of the FCB populations (Figure 3.1) indicating that the FSB populations had evolved a greater rate of recovery of egg viability.

The comparison between Both-shocked and Female-shocked treatments yields interesting insights. Twenty four hours post shock, egg viability was not significantly different between Both-shocked and Female-shocked treatments (Figure 3.1). It is important to note that in the Female-shocked treatment, the females interacted with healthy, non-

shocked males, while in the Both-shocked treatment females interacted with males subjected to cold shock. Thus, my results indicate that recovery of egg viability is the same irrespective of whether the females interacted with healthy males or cold shocked males. This would indicate that at least in our populations, males can recover from cold shock and mate successfully within 24 hours of cold shock. This is in contrast to previous studies which found that males are effectively sterile for 24 hours post cold shock.

In contrast to egg viability, fecundity remained unresponsive to selection. I found no significant effects of selection, block or period (Table 3.2, Figure 3.2). The effect of treatment was marginally significant (Table 3.2). However, I found a significant treatment × period interaction. Multiple comparisons using Tukey's HSD indicated that at 0 hours post cold shock, individuals subjected to cold shock had significantly higher fecundity compared to Neither-shocked individuals. However, 24 hours post cold shock, this difference in fecundity had disappeared (Figure 3.2). Females in the Female-Shocked treatment interacted with healthy males post cold shock while females from the Both-shocked treatment interacted with cold shocked males. However, fecundity of females from these two treatments (i.e., Female-shocked and Both-shocked) was not significantly different indicating that the type of male that the females interact with does not affect fecundity.

To see if adult mortality post cold shock was different between the FSB and FCB populations, I used data from the Both-shocked and Neither-shocked treatments. I did not use the data from the Female-shocked treatment because in this treatment only females were shocked and combined with healthy males. Adult mortality did not differ significantly between the FSB and FCB populations (Table 3.3, Figure.3. 3). Treatment had a significant effect on mortality. I found that mortality of males and females that were not subjected to cold shock was low (about 2.5-3.5%) and cold shock increased the mortality of individuals. Mortality of females subjected to cold shock was about 7-9% while that of males was about 3-3.5%, leading to a significant Treatment by Sex interaction. Thus, while the overall mortality of adults due to cold shock was low, females died more often due to cold shock compared to males.

I found significant effect of Selection regime and Treatment on the number of mating pairs observed. Flies subjected to cold shock showed nearly twice as many mating pairs

as flies not subjected to cold shock (mean ± SE; Both-shocked = 65.66 ± 2.84; Neither-shocked = 32.83 ± 2.84 (Table 3. 4). Under both treatments, the FSB populations showed higher number of mating pairs compared to FCB populations (mean ± SE; Both-shocked treatment: FCB = 62.53 ± 2.53; FSB = 68.8 ± 2.53; Neither-shocked treatment: FCB = 29.46 ± 2.53; FSB = 36.2 ± 2.53). However, the selection × treatment interaction was not significant (Table 3. 4). Thus, my results indicate that the mating frequency of the FSB populations has evolved to be higher even under Neither-shocked conditions.

Experiment 2: Larval survivorship post cold shock has not evolved

I wanted to test whether cold shock can also affect larva to adult survivorship. Unlike egg viability, larval survivorship was not affected by treatment (Table 3. 5). Survivorship of larvae from the two treatments was high (>90%). Additionally, there was no significant difference between FCB and FSB populations in larval survivorship (mean percentage ± SE; Cold shock treatment: FCB = 96.400 ± 0.642; FSB = 95.800 ± 0.642; Control treatment: FCB = 93.333 ± 0.642 FSB = 95.667 ± 0.642).

Experiment 3: Selected females are not better at protecting eggs/stored sperm from cold shock

In this experiment I allowed females to interact with males until the time they were subjected to cold shock (or a control treatment at 25°C). However, post cold shock (or 25°C treatment), I denied the females access to males. I assayed egg viability and fecundity of these females 24 hours post cold shock. I found no significant effect of selection on egg viability or fecundity of the FSB and FCB regimes (Table 3.6). However, treatment had a significant effect on both egg viability and fecundity. When assayed without cold shock, egg viability and fecundity were high (Figure3. 4). However, when females were subjected to cold shock, there was a drastic decline in both egg viability and fecundity (Table 3.6, Figure 3.4). I found no significant Selection × Treatment interaction, indicating that the decline in egg viability and fecundity (due to cold shock treatment) was similar in the FCB and FSB populations (Table 3.6, Figure 3.4). Thus, when females were denied access to mates post cold shock, egg viability and fecundity remained extremely low even 24 hours post cold shock in both the FCB and FSB populations. This finding clearly indicates that mating post cold shock is necessary to restore egg viability and fecundity.

Experiment 4: Post cold shock, selected males are better at mating non-virgin females and siring progeny

The non-virgin females in this experiment came from a base population with a recessive eye color marker (scarlet). The females were previously mated to males from their own population. Thus any female that produced progeny with dominant eye color marker (red) would have mated with FCB or FSB males at least once. I found that the proportion of females that produced red eyed progeny was significantly more when the females were held with FSB males than when they were held with FCB males (mean ± SE; FSB = 0.219 ± 0.022; FCB = 0.065 ± 0.022; paired t test, t = 4.846, df = 4, p = 0.008).

Thus, after being subjected to cold shock, males from the FSB populations were successful to mate with more non-virgin females compared to males from the FCB populations. Consequently the FSB males also sired greater proportion of progeny compared to the males from the FCB populations (mean ± SE; FSB = 0.1211 ± 0.011; FCB = 0.0382 ± 0.011; paired t test, t = 5.484, df = 4, p =0.005).

Table 3.1. Effect of cold shock on egg viability (Experiment 1). Summary of results from a four-factor mixed model ANOVA on egg viability data using Selection (FCB and FSB), Treatment (Both-shocked, Female-shocked and Neither-shocked) and Period (0 and 24 hours post cold shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	742.697	742.697	1	5.190	183.743	**<0.001**
Treatment (Trt)	169973.464	84986.732	2	8.033	355.787	**<0.001**
Block (Blk)	999.989	249.997	4	4.282	0.779	0.590
Period (Per)	33932.819	33932.819	1	4.017	145.340	**<0.001**
Sel × Trt	494.448	247.224	2	8.298	9.073	**0.008**
Sel × Blk	14.493	3.623	4	4.311	0.118	0.969
Sel × Per	866.714	866.714	1	4.212	44.663	**0.002**
Trt × Blk	1947.861	243.483	8	8.919	1.679	0.229
Trt × Per	16246.977	8123.489	2	8.061	61.844	**<0.001**
Blk × Per	951.878	237.969	4	7.962	1.737	0.235
Sel × Trt × Blk	218.604	27.325	8	8.000	1.713	0.232
Sel × Trt × Per	528.914	264.457	2	8.514	16.411	**0.001**
Sel × Blk × Per	77.265	19.316	4	8.000	1.211	0.378
Trt × Blk × Per	1069.308	133.663	8	8.000	8.378	**0.003**
Sel × Trt × Blk × Per	127.633	15.954	8	108.000	0.677	0.711

SS: Numerator sum of squares, MS Num: Numerator mean square, DF Num: Numerator degrees of freedom, DF Den: Denominator degrees of freedom.

Table 3.2. Effect of cold shock on fecundity (Experiment 1). Summary of results from a four-factor mixed model ANOVA on fecundity (eggs per female) data using Selection (FCB and FSB), Treatment (Both-shocked, Female-shocked and Neither-shocked) and Period (0 and 24 hours post cold shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	85.190	85.190	1	4.026	1.968	0.232
Treatment (Trt)	127.288	63.644	2	8.167	4.514	**0.047**
Block (Blk)	92.112	23.028	4	5.630	0.127	0.966
Period (Per)	309.728	309.728	1	4.008	2.191	0.213
Sel × Trt	7.021	3.510	2	8.998	1.385	0.299
Sel × Blk	176.264	44.066	4	1.546	24.200	0.071
Sel × Per	5.250	5.250	1	4.724	2.897	0.153
Trt × Blk	114.001	14.250	8	7.880	0.689	0.694
Trt × Per	551.952	275.976	2	8.115	13.625	**0.003**
Blk × Per	576.802	144.200	4	7.330	7.238	0.011
Sel × Trt × Blk	19.524	2.440	8	8.000	1.050	0.473
Sel × Trt × Per	15.749	7.874	2	9.050	3.255	0.086
Sel × Blk × Per	6.811	1.702	4	8.000	0.733	0.594
Trt × Blk × Per	164.324	20.540	8	8.000	8.843	**0.003**
Sel × Trt × Blk × Per	18.580	2.322	8	108.000	0.335	0.950

Table 3.3. Effect of cold shock on adult mortality (Experiment 1). Summary of results from a four-factor mixed model ANOVA on adult mortality data using Selection (FCB and FSB), Treatment (Both-shocked and Neither-shocked) and Sex (male and female) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	9.818	9.818	1	4.166	1.394	0.301
Treatment (Trt)	177.341	177.341	1	4.139	21.143	**0.009**
Block (Blk)	217.679	54.420	4	0.436	7.503	0.481
Sex	3.341	3.341	1	4.108	0.311	0.606
Sel × Trt	13.364	13.364	1	4.123	1.417	0.298
Sel × Blk	28.190	7.048	4	3.152	0.557	0.712
Sel × Sex	0.121	0.121	1	4.131	0.014	0.913
Trt × Blk	33.690	8.423	4	2.490	0.833	0.595
Trt × Sex	86.735	86.735	1	4.183	13.545	**0.020**
Blk × Sex	43.357	10.839	4	2.373	1.133	0.501
Sel × Trt × Blk	37.964	9.491	4	4.000	1.644	0.321
Sel × Trt × Sex	7.758	7.758	1	4.203	1.339	0.309
Sel × Blk × Sex	35.774	8.943	4	4.000	1.549	0.341
Trt × Blk × Sex	25.583	6.396	4	4.000	1.108	0.462
Sel × Trt × Blk × Sex	23.095	5.774	4	72.000	0.854	0.496

Table 3.4. Effect of cold shock on number of mating pairs observed (Experiment 1). Summary of results from a three-factor mixed model ANOVA on the number of mating pairs observed using Selection (FCB and FSB) and Treatment (Both-shocked and Neither-shocked) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	576.136	576.136	1	4.477	21.235	**0.008**
Treatment (Trt)	14700.379	14700.379	1	4.055	66.760	**0.001**
Block (Blk)	12743.756	3185.939	4	1.794	19.700	0.062
Sel × Trt	0.742	0.742	1	4.140	0.008	0.931
Sel × Blk	104.756	26.189	4	4.000	0.298	0.866
Trt × Blk	893.565	223.391	4	4.000	2.543	0.194
Sel × Trt × Blk	351.423	87.856	4	36.000	1.235	0.313

Table 3.5. Effect of cold shock on larval survivorship (Experiment 2). Summary of results from a three-factor mixed model ANOVA on larval survivorship data using Selection (FCB and FSB) and Treatment (Cold shock and Control) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	37.556	37.556	1	4.000	2.449	0.193
Treatment (Trt)	128.000	128.000	1	4.000	3.578	0.132
Block (Blk)	1270.889	317.722	4	1.918	10.417	0.096
Sel × Trt	107.556	107.556	1	4.000	5.218	0.084
Sel × Blk	61.333	15.333	4	4.000	0.744	0.609
Trt × Blk	143.111	35.778	4	4.000	1.736	0.303
Sel × Trt × Blk	82.444	20.611	4	180.000	1.013	0.402

Table 3.6. Egg viability and fecundity post cold shock (Experiment 3). Summary of results from a three-factor mixed model ANOVA on (A) Egg viability and (B) Fecundity (number of eggs per female). In this experiment, females were held without access to males post cold shock. We modeled Selection (FCB and FSB) and Treatment (Cold shock and Control) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(A)	Selection (Sel)	29.570	29.570	1	4.000	5.847	0.073
Egg	Treatment (Trt)	112781.61	112781.61	1	4.000	8465.06	**0.001**
Viability	Block (Blk)	6.908	1.727	4	1.615	0.166	0.935
	Sel × Trt	15.777	15.777	1	4.000	1.973	0.233
	Sel × Blk	20.228	5.057	4	4.000	0.632	0.666
	Trt × Blk	53.293	13.323	4	4.000	1.666	0.317
	Sel × Trt × Blk	31.989	7.997	4	40.00	1.503	0.220
(B)	Selection (Sel)	13.599	13.599	1	4.000	4.497	0.101
Fecund-	Treatment (Trt)	298.787	298.787	1	4.000	23.915	**0.008**
dity	Block (Blk)	231.646	57.911	4	2.385	5.452	0.130
	Sel × Trt	3.693	3.693	1	4.000	0.754	0.434
	Sel × Blk	12.096	3.024	4	4.000	0.617	0.674
	Trt × Blk	49.975	12.494	4	4.000	2.552	0.193
	Sel × Trt × Blk	19.586	4.896	4	40.000	1.373	0.260

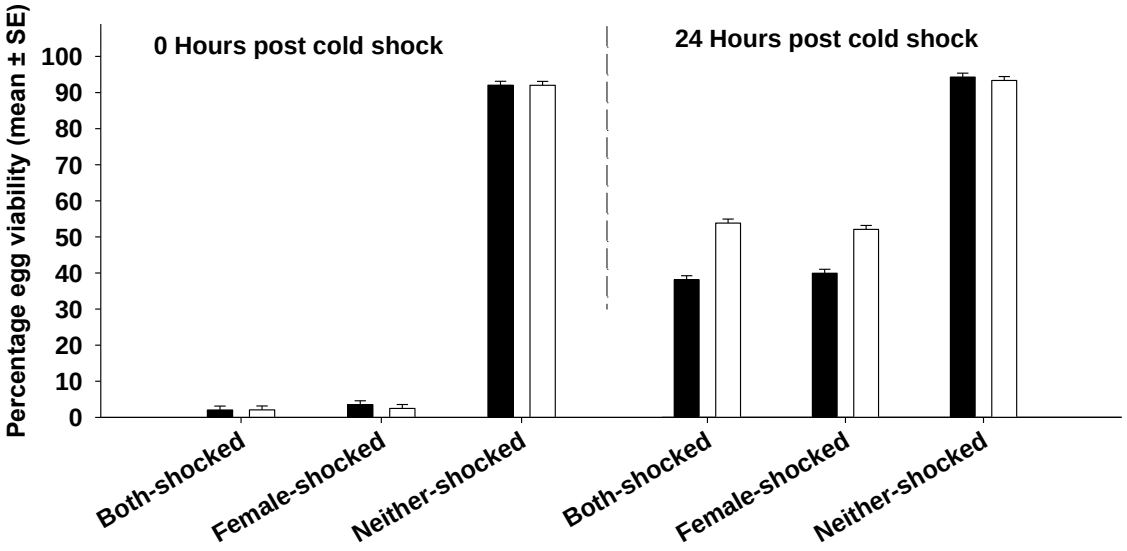

Figure 3.1: Effect of cold shock on egg viability. I assayed egg viability 0 and 24 hours post cold shock. Open bars represent FSB and closed bars represent FCB populations. Viability of eggs from Neither-shocked treatment was high and not different between FCB and FSB populations. At 0 hours post cold shock, viability of eggs from the Both-shocked and Female-shocked treatment was very low and not different between FCB and FSB populations. By 24 hours post cold shock, egg viability improved and the FSB populations had significantly higher egg viability than the FCB populations.

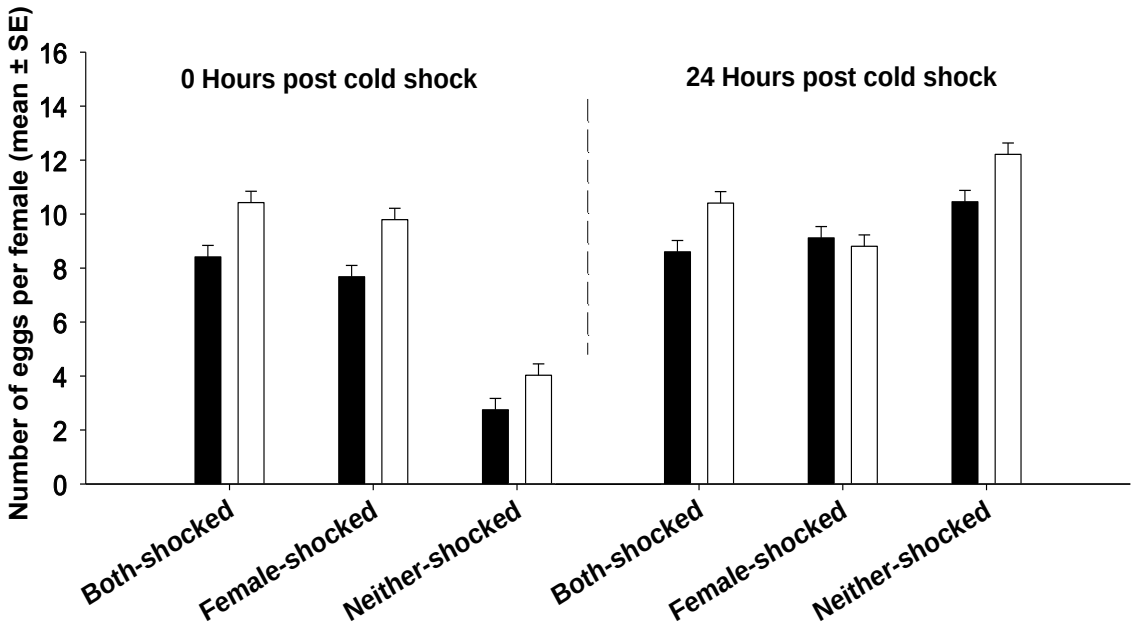

Figure 3.2: Effect of cold shock on fecundity. I assayed fecundity 0 and 24 hours post cold shock. Open bars represent FSB and closed bars represent FCB populations. At 0 hours after cold shock, fecundity of females subjected to cold shock (Both-shocked and Female-shocked treatments) was high compared to that of females not subjected to cold shock (Neither-shocked treatment). However, there was no such difference 24 hours after cold shock. None of the differences between FCB and FSB populations were significant.

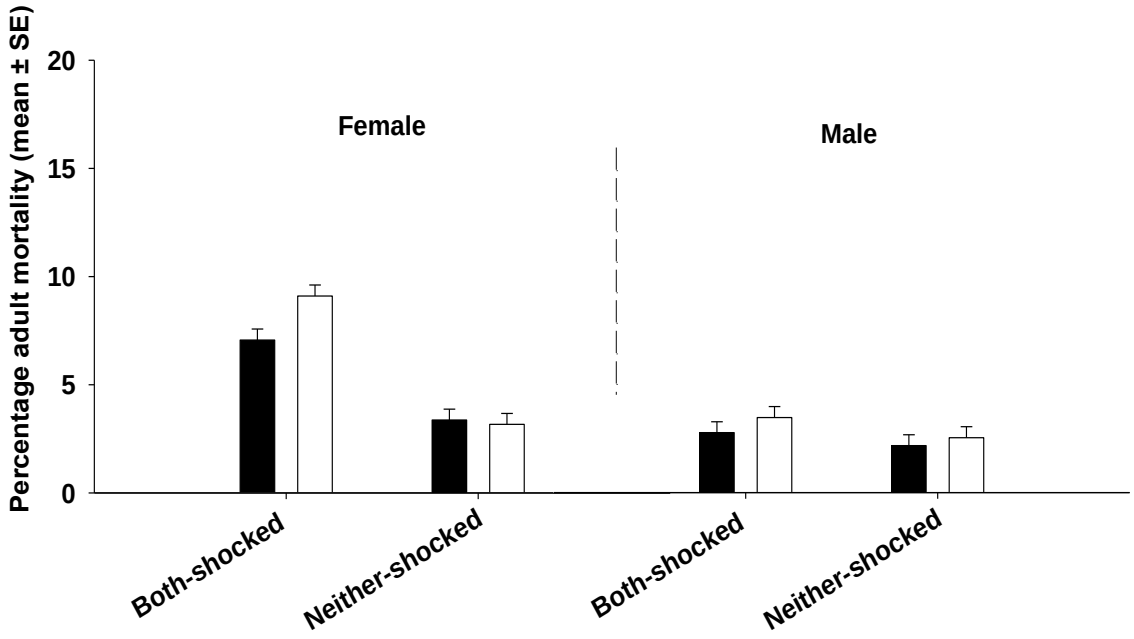

Figure 3.3: Effect of cold shock on adult mortality. Mortality of adults subjected to cold shock was significantly higher than the mortality of adults not subjected to cold shock. However, none of the differences between FSB (open bars) and FCB (closed bars) populations were significant.

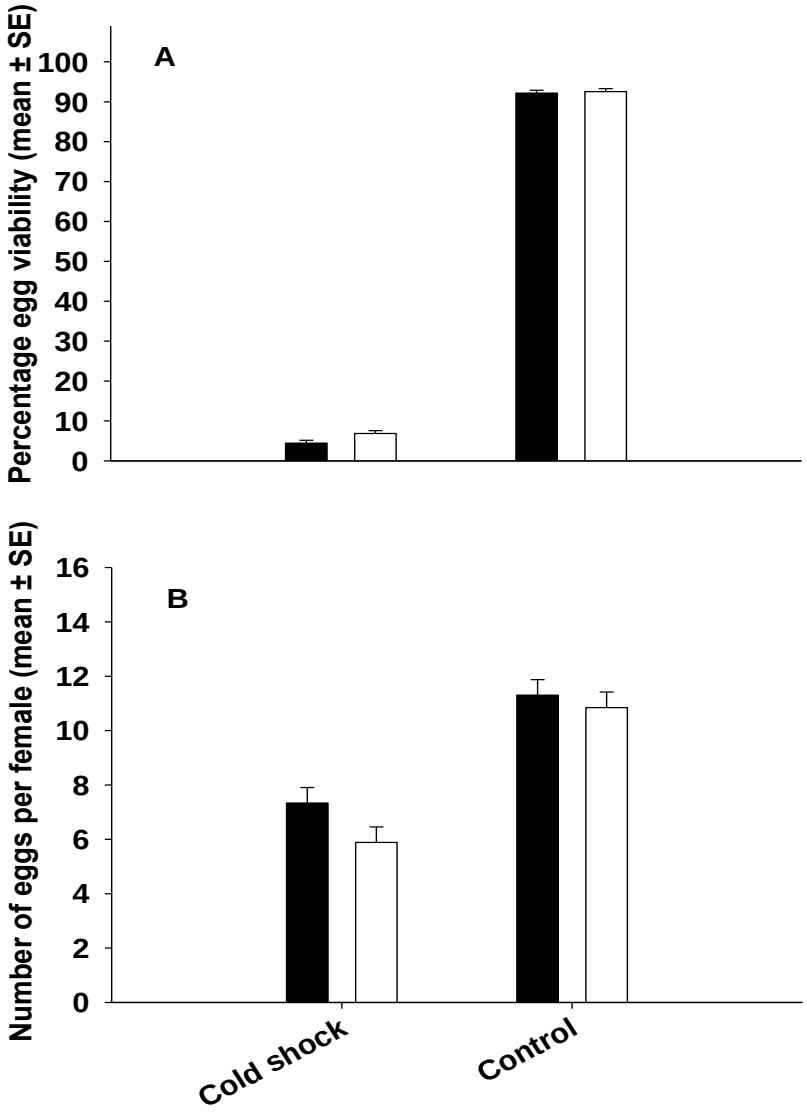

Figure 3.4: Effect of cold shock on egg viability and fecundity. To quantify egg viability (A) and fecundity (B) Females were held without access to males post cold shock. In this experiment, females were allowed to interact with males initially. However, after cold shock (or control treatment), females were not allowed to interact with males. I then assayed their egg viability (A) and fecundity (B) 24 hours later. Egg viability and fecundity of females subjected to cold shock and not allowed to interact with males thereafter was significantly lower. I found no significant differences in egg viability or fecundity between FSB (open bars) and FCB (closed bars) in either of the treatments. These results are in contrast to the results presented in Fig. 1 and 2 wherein females had access to males post cold shock. Thus, taken together, these results indicate that interaction with males post cold shock is necessary for egg viability and fecundity to improve.

DISCUSSION

Insect responses to cold stress have received considerable attention (Denlinger and Lee 2010). Recent studies have addressed the physiological, biochemical and genetic basis underlying the ability to survive cold stress among insects (Rinehart et al. 2000, Svetec et al. 2011, MacMillan et al. 2015, Alison et al. 2015). In the present experimental evolution study, I investigated the effect of cold shock on egg viability (percentage of eggs that hatch) and other important components of fitness- larval survivorship, adult mortality, fecundity and reproductive behavior. I used egg viability as an indicator of the ability of the flies to lay fertile eggs. I found that following a cold shock, egg viability and total number of matings were higher in the selected populations. The selected populations mated more even in the absence of a cold shock. The males from the selected populations were more successful in mating with non-virgin females and sired more progeny post cold shock. Adult mortality and fecundity post cold shock were unresponsive to selection.

In my study, during the normal maintenance regime, eggs were collected from the flies 24 hours post cold shock (see methods section) to start the next generation. Therefore, the ability to lay fertile eggs 24 hours post cold shock is an important component of fitness under this selection regime and is affected by the ability of the flies to protect the gametes from cold shock and/or ability to produce gametes and successfully mate post cold shock. I found that egg viability of FSB populations increases at a faster rate over the first 24 hours post cold shock compared to FCB populations. One possible reason for the increased egg viability in the selected populations could be that the females of the selected populations are better at protecting their eggs/stored sperm from damage due to cold shock. For example, studies on *D. pseudoobscura* by Collett and Jarman (2001) indicate that females in this species can store sperm through six months of cold weather. These sperm can then be used to fertilize eggs with the onset of warm weather. However this is unlikely to be the case in my populations. When FSB and FCB females are held without access to males post cold shock, viability of their eggs remains low (even 24 hours after cold shock) and is not significantly different from each other. Therefore, in my populations, improvement in egg viability happens only if the females have access to males post cold shock.

While males are necessary for egg viability to improve, the type of male that the females are held with (i.e., subjected to cold shock or non-shocked) does not matter. Egg viability

is likely to be limited by the availability of high-quality (i.e., not damaged by cold shock) eggs and sperm. Given that FCB females held with shocked FCB male or non-shocked base line males have similar egg viability (and the FSB females held with shocked FSB male or non-shocked base line males have similar egg viability), it follows that availability of high-quality sperm is not likely a limiting factor. The upper limit in egg viability within FCB and FSB populations is set primarily by the females. However, the difference in egg viability between FCB and FSB populations could be due to differences in the abilities of males and/or females of the two regimes to recover from cold shock.

Since my results suggest that access to males (and thereby mating) is necessary for increase in egg viability, it follows that egg viability post cold shock in a population is likely to increase as (a) more females in the population mate and/or (b) females are able to produce more high quality eggs. In contrast to previous studies - which found that cold shock reduced mating frequency (Rinehart et al. 2000, Lacoume et al. 2007) my results suggest that in *D. melanogaster*, when females are held with males post cold shock, the number of matings observed is significantly higher (compared to when non-shocked individuals are held together). This is consistent with the hypothesis that mating post cold shock is necessary to produce fertile eggs and can hence affect male and female fitness. I also observed that post cold shock, FSB populations mated more often compared to FCB populations (see results section). Thus the observed differences in egg viability between FSB and FCB populations post cold shock are at least partly due to the differences in their mating behavior. FSB populations also had significantly higher mating even without cold shock, clearly indicating that the basal mating frequency has evolved in these populations.

One possible reason for the observed results could be that copulation duration is higher in FSB populations. If this is true, then, it is quite possible that the same mating pairs are scored repeatedly, thereby artificially increasing the number of mating pairs in FSB populations. However, in a separate experiment, I quantified copulation duration in FSB and FCB populations with and without cold stress (data not shown). I found that the mean copulation duration did not differ between the selection regimes and the value varied between 15 to 17 minutes across the populations. In the present study, I checked for the number of mating pairs once every 30 minutes. Therefore, it is unlikely that copulation duration can explain the increased number of mating pairs in the FSB populations. While multiple studies have looked at the effect of high and low temperature on mating behavior

(Schnebel and Grossfield 1984, Chakir et al. 2002, David et al. 2005, Dolgin et al. 2006, David 2008, Zhang et al. 2013), very few studies have looked at the effect of cold shock on mating behavior. To the best of my knowledge, this is the first study to document the evolution of mating frequency in response to cold shock.

Increased mating rate (post cold shock) in the selected populations could be due to increased receptivity of the females or increased ability of the males to successfully mate or both. My results suggest that the selected males (post cold shock) mate more often with non-virgin females and sire a greater proportion of the progeny. In addition to differences in mating rate, the ability to sire progeny is likely to be affected by the ability of the males to transfer fertile sperm. Previous studies suggest that cold shock can reduce sperm content in males of some insects (Lacoume et al. 2007). In particular, *D. melanogaster* males have been shown to have no motile sperm up to 24 hours after cold shock (Lefevre and Jonsson 1962). Therefore, it is possible that males from the FSB populations sperm have also evolved the ability to either (a) protect their sperm during cold shock and/or (b) produce fertile sperm at a faster rate after cold shock. However, at this point of time I am unable to distinguish between these two possibilities.

Unlike egg viability, larval mortality and adult mortality did not evolve in my populations. These results are not surprising given that the cold shock treatment I use does not affect larval mortality and induces very low levels of adult mortality. This is in contrast to other studies which show an effect of cold stress on larval and adult survivorship. Evolution of cold tolerance in larvae of various *Drosophila* species is well studied (Strachan et al. 2011). Studies show that maintaining flies at 18°C reduces the viability of their eggs compared to those of flies maintained at 25°C (Dillon et al. 2007). Embryos and larvae are known to be more sensitive to cold stress than pupae or adults (Mockett and Matsumoto 2014). Similarly, a cold shock of -5°C for one hour has been shown to reduce adult survivorship by over 60% (Overgaard et al. 2007). When flies are maintained at 4°C, significant adult mortality is observed within 5 days while in flies maintained at 11°C, significant adult mortality occurs only after 10-12 weeks (Mockett and Matsumoto 2014). However, other studies find that the cold tolerance of adults and larvae from populations evolving at high (29°C) and low (16°C) temperatures are not different from each other (Gilchrist et al. 1997).

I also found that fecundity was unresponsive to selection. Females subjected to cold shock laid more eggs immediately post-shock compared to females that were not subjected to cold shock. Given that viability of the eggs laid immediately post cold shock is extremely low, the increased fecundity immediately post-shock could represent the females effort to discard damaged eggs. The effect of cold shock on fecundity/progeny production has been reported by multiple studies. In a study similar to my study (Overgaard et al. 2007) a cold shock at -5°C for one hour severely reduced the progeny production by female *D. melanogaster* over the first eight hours after cold shock. Other studies have subjected flies to prolonged periods of low temperature instead of an acute cold shock. Subjecting flies to 18°C temperature for 1-3 days reduces their progeny production during treatment. However, fecundity returns to normal levels after flies are returned to 25°C (Dillon et al. 2007). In another study, an exposure of 9 days at 4°C rendered the flies unable to produce any progeny when returned to 25°C. In addition, when adult flies were stored for prolonged periods of time (1 to 12 weeks) in low temperatures (4-11°C) and then returned to 25°C, they suffered a severe reduction in progeny production over the first two days of recovery (Mockett and Matsumoto 2014). Unlike a single bout of cold shock, repeated exposure to cold stress in *D. melanogaster* leads to a survival and reproduction trade-off (Marshall and Sinclair 2010). Flies subjected to multiple bouts of cold stress with periods of recovery in between show a greater survivorship compared to flies subjected to a single long bout of cold stress. The increased survivorship comes at the cost of decreased intrinsic growth rate (*r*). This study also found that a single 2-hour exposure does not reduce *r*. Thus the results about the effect of cold stress on fecundity have been variable. Similarly, evolution of fecundity in response to selection for resistance to cold shock has also been variable across studies, with selected populations evolving higher fecundity in some (Anderson et al. 2005) and lower fecundity in other (Watson and Hoffmann 1996) studies. Since these studies differ in many aspects including the base populations used, the details of the selection and the assay protocols, there could be multiple reasons for the variation in the results observed across these studies.

My experiments clearly show that reproductive traits such as rate of recovery of egg viability, mating rate and male mating ability evolve in response to cold shock. However, the mechanisms underlying the responses are as yet not clear. More importantly, it is very likely that the improved rate of recovery of egg viability, mating rate and male mating

ability of the FSB populations are likely to come at a cost. Such costs, if any, are yet to be explored.

To summarize, the present experimental evolution study looked at the evolution of important fitness components in response to selection for cold shock resistance. While larval survivorship, adult mortality and fecundity were unresponsive to selection, I found that egg viability and mating frequency evolved rapidly in the selected populations. Post cold shock, males from the selected populations had higher ability to mate and sire progeny compared to control males. Thus, clearly, reproductive behavior has evolved in my selected populations in response to selection for resistance to cold shock. My results clearly illustrate the role of environmental stress in shaping the evolution of reproductive behavior of organisms.

Note: This chapter has been adapted from the work published in PLoS One as a manuscript

"Singh K, Kochar E, Prasad NG. **Egg viability, mating Frequency and male mating ability evolve in populations of *Drosophila melanogaster* selected for resistance to cold shock**. PLoS One. 2015; 10: e0129992.

Chapter 4a

Evolution of pre- and post-copulatory traits in male

IINTRODUCTION

Extreme temperature stress causes a variety of detrimental effects on organisms, especially in insects that are sensitive to variation in the ambient temperature due to their small body size and ectothermic physiology (Deutsch et al. 2008). Exposure of organism to extreme temperatures can affect survival (Rinehart et al. 2000) at various life stages (Tucic 1979) due to its adverse effects on various aspects of cellular structure and physiology (Chapman 1998, Kostal et al. 2004b, Gullan and Cranston 2005, Overgaard et al. 2005).

Reproductive traits of the insects are more sensitive to thermal stress than other life history-traits (Zizzari and Ellers 2011). In promiscuous species like *Drosophila melanogaster*, male reproductive success is dependent on a large set of traits that can be broadly classified into pre- and post-copulatory traits. In order to successfully mate, the male has to perform a series of intricate courtship behaviors (Bastock and Manning 1955, reviewed in Sokolowski 2001). Additionally, since females in this species store sperm from multiple males, post-copulatory traits which may determine sperm competitive ability are crucial components of male fitness (reviewed in Snook 2005). Male fitness is affected by a number of sperm related traits such as numbers, quality, motility and morphology (Froman et al. 2002, Gage and Morrow 2003). The seminal fluid contains accessory gland proteins that can also influence female behaviour and there by affect sperm competition (Chapman 2001, Simmons 2001, reviewed in Gillott 2003, reviewed in Avila et al. 2011). It is well established that both sperm and accessory gland proteins (Acps) influence post copulatory sexual selection in insects (Simmons 2001, Simmons and Fitzpatrick 2012). Environmental stress can majorly affect the quality of ejaculates and can therefore affect male fitness (Campbell et al. 1992, Perez-Crespo et al. 2008, Hansen 2009).

Multiple previous studies have shown that temperature stress affects the pre- and post-copulatory reproductive traits of male insects. Temperature shock can adversely affect

several pre- copulatory traits such as male fertility (Chihrane and Lauge 1994, Krebs and Loeschcke 1994, Rinehart et al. 2000, Fischer et al. 2003, Zizzari and Ellers 2011), courtship behaviour, mating success (Schnebel and Grossfield 1984, Patton and Krebs 2001, Nguyen et al. 2013), copulation duration (Lieshout et al. 2013) and mating latency (Kvelland 1965a, Denlinger and Yocum 1998, Rinehart et al. 2000, Rohmer et al. 2004, Malmendal et al. 2006, Yenisetti et al. 2006, reviewed in Hance et al. 2007).

Specifically, cold shock is also known to affect male post-copulatory traits. When *D. melanogaster* males are subjected to cold shock, the sperm in seminal vesicle are known to become immobile (Lefevre and Jonsson 1962). Motile sperm are not detected in the vesicles for over twenty four hours. These immobile sperm need to be ejected out by the males in order to mate and ensure future fitness (Lefevre and Jonsson 1962). Given the effects of temperature on male reproductive traits, it is expected that adaptation to cold shock will incorporate changes in pre- and post-copulatory traits. Hence, in the present study, my primary aim was to investigate the evolution of the pre-and post-copulatory traits in populations of *D. melanogaster* selected for increased resistance to cold shock (Singh et al. 2015). I investigated the pre- and post-copulatory traits i.e., mating latency (time required to start mating), copulation duration (how long they remain in copula), mating success, male fertility, progeny production and sperm competitive ability with and without cold shock in populations of *D. melanogaster* selected for increased resistance to cold shock. These studies were carried out between 37-50 generations of selection. My findings indicate the rapid evolution of sperm competitive ability and other reproductive traits (i.e., mating latency, mating success, male fertility and progeny production) post cold shock in my selection lines.

METHODS

Generation of experimental flies

Experiments 1.1, 1.2, 1.3 and Experiment 2 that were performed after 37, 39, 40 and 45 generations of selection, respectively. For each of these experiments, eggs were collected from standardized flies (see chapter 2), for each of the total 10 populations (5 FSB and 5 FCB populations). Flies were grown under controlled egg density (70eggs/vial). For each of the FSB and FCB populations 16 vials (70 eggs/vial in 6 ml of banana-yeast-jaggery food per vial) were set up. Then these vials were incubated at standard culture conditions (25^{o}C, 50-60% relative humidity, and 12hours-12hours light/dark cycle). On 9-10th day

post egg-collection virgin male flies were collected at a very young stage (≤ 4 hours post eclosion) during their peak of eclosion using light CO_2 anaesthesia. Males were housed at a density of 10 per vial, in vials containing 2 ml of banana-yeast-jaggery food until the 12^{th} day post egg collection.

Generation of common females from ancestral BRB populations

In order to record the pre- and post-copulatory traits of males belonging to the two selection regimes, they were housed with females belonging to a common baseline population (BRB). The BRB flies were generated in a manner that was similar to that of generating males from the selection regimes. To collect eggs, banana food plates were given to the corresponding BRB population for 6 hours for oviposition. Then from these plates eggs were collected to rear flies under controlled egg density 70 eggs/vial containing 6 ml of banana-yeast-jaggery food. For each population, 28 vials were established and incubated at standard laboratory conditions as describe above. On $9\text{-}10^{th}$ day post egg collection, virgin females were collected using mild CO_2 anaesthesia as describe above. The virgin females were held in vials at a density of one female per vial provisioned with 2 ml of food. Vials containing flies were incubated at a standard laboratory culture condition as described above until the start of the mating trial assay (by the time of experiment, flies were ~2-3 old as adults).

Generation of common females and competitor males carrying a recessive genetic marker

To assess the fertilization success of the experimental males, I used flies from an outbred laboratory population – LH_{st} (Prasad et al. 2007). This population carries a recessive autosomal eye colour marker – scarlet eye – thereby allowing quantification of fertilization success (see below, Experiment 2 in this section). The LH_{st} flies were grown under similar conditions with a controlled egg density (70 eggs/vial in 10 ml of banana-yeast-jaggery food). The LH_{st} males (competitors) and females were collected as virgins, as described above, and held individually in vials for 2-3 days until the mating trials.

Experiment 1.1: The effect of cold shock on the pre- and post-copulatory traits

On the 12^{th} day post egg-collection virgin FSB and FCB male flies were transferred to clean, dry glass vials (25mm diameter × 90mm height) at a density of 50 individuals per

vial. The flies were given cold shcok as defined in the chapter 2. Quickly, post cold shock the flies were transferred into a Plexiglas cage (14cm length × 16cm width × 13cm height) containing a Petri plate of banana-yeast-jaggery food and maintained at a standard laboratory condition (see above). For each of the populations, one hour before the start of mating trials, experimental flies were aspirated out (to avoid CO_2 anesthesia) from the cages and placed singly in vials provisioned with 2 ml of banana-yeast-jaggery food.

Single pair experimental design was used to quantify the effects of cold shock on mating latency, copulation duration, fertility and progeny production. A virgin female (common base line BRB female) was combined with one of the cold shocked FSB or FCB male in a vial (25mm diameter × 90mm height) provisioned with 2 ml of banana-yeast-jaggery food at 4, 12 and 30 hours post cold shock. The cotton plug covering the vial was inserted deep into the vial such that the flies were restricted to the bottom one third of the vial (25mm diameter × 30mm height). The vial containing male and female were observed continuously for two hours for mating latency (the time taken to start copulation from introduction of both the male and the female into the vial) and copulation duration (the duration of the copulation or how long they remain in copula). For mating success, proportion of males that successfully mated with the base line females were computed (mating success = number of males that successfully mated with base line males/ total number of males assyed). For male fertility assay, after the single mating was over, females were immediately separated using mild CO_2 anesthesia and the males were discarded while the females were transferred individually into a fresh food vial to oviposit for 24 hours. Following this the female was discarded and the vial was held for another 24-30 hours to check egg hatchability. To consider a male to be fertile, at least one egg should hatch. After 4, 12 and 30 hours of recovery following cold shock 70, 60 and 60 males were used, respectively for each of the FSB and FCB populations. Proportion male fertility was the proportion of base-line females that produced at least one fertile egg after having mated with either a FSB or FCB male.

Experiment 1.2: The fitness of the ancestral females mated with cold shocked males

This experiment was performed after 39 generations of selection. To generate experimental flies, eggs were collected from standardized flies at a density 70 eggs per vial containing 6 ml of banana-yeast-jaggery food. Twelve such vials were established for each of the five FSB and FCB populations. Fitness of the ancestral females was assayed

using a protocol similar to the one described in the previous experiment (Experiment 1.1), except that, the effect of mating with cold shocked males (FSB or FCB) on female fitness was assayed only at two-time points namely, 4 and 12 hours post cold shock to males. After combining one male (cold shocked) from either FSB or FCB population with one baseline (BRB, not subjected to cold shock) female per vial, vials were kept undisturbed for two hours. Following this, females from all vials were quickly separated under light CO_2 anaesthesia and were held individually in test tubes containing banana-yeast-jaggery food to oviposit for 24 hours to measure 'day one' progeny production. Twenty hours later, the same female was transferred to another test tube containing 2 ml of fresh banana-yeast-jaggery food to oviposit for 24 hours in order to measure 'day two' progeny production. On the 13^{th} day post oviposition, the progeny emerging from these test tubes were counted, yielding a value of female fitness. Total number of progeny from each of the test tubes was used as the unit of analysis. Analyses were carried out in two ways. (a) For females that produced at least one offspring the total number of offspring produced by each female was used as the unit of analyses. (b) In addition to this, I also calculated the proportion of females that failed to produce any progeny. This proportion was used as the unit of analysis. The females with zero fitness could be the females that did not mate or mating happened but the female did not receive fertile sperm from its mate.

Experiment 1.3: The pre- and post-copulatory traits in males not subjected to cold shock

For no shock treatment, virgin males were collected as described above. On the 12^{th} day, 5 vials of males (10 males/vial) were transferred into single empty glass vial and the cotton plug was pushed deep into the vials (one third of vial) to ensure the flies stay in restricted space available (roughly 25 mm diameter × 30 mm height). Following this, each vial containing 50 flies was placed in a water bath maintained at 25°C for one hour. After this 300 males were immediately transferred into a Plexiglas cage (14 cm length × 16 cm width × 13 cm height) and provided a fresh banana-yeast-jaggery food plate. Three hours later 35 randomly chosen males were aspirated out (to avoid CO_2 anesthesia) and transferred individually to separate vials provisioned with 2 ml of banana-yeast-jaggery food. One hour later the each of these males was presented to a single virgin female from the BRB population in a vial having 2 ml of banana-yeast-jaggery food. The cotton plug was pushed down into the vial so that the space available to the flies was the bottom one third of the vial. The vials were observed for mating latency and copulation duration.

These vials were observed until a single mating was over. For mating success, proportion of males were successfully mated with the base line females was computed (mating success = number of males that successfully mated with base line males/ total number of males were assyed). To assess the ability of the FCB and FSB males to influence female progeny production, the male and the female from each vial were immediately sorted under light CO_2-anaesthesia and the male was discarded. Each female was individually transferred into a fresh food vial containing 6 ml of banana-yeast-jaggery food for oviposition for 24 hours, following which the female was discarded. Thirteen days later the progeny emerging from these vials were counted. Total number of progeny from each vial was used as the unit of analysis.

Experiment 2: Effect of selection regime on sperm offense (P2) ability

A virgin LH_{st} female and a LH_{st} male was combined in a vial (25mm diameter × 90mm height) provisioned with 2 ml of banana-yeast-jaggery food. The cotton plug was pushed deep into the vial to restrict flies the bottom one third (roughly 25mm × 30mm space) of the vial. The mating pair was observed for successful mating. Once a single mating was over, the male and the female were immediately sorted using mild CO_2 anaesthesia and the male was discarded. The female was transferred back into the vial and allowed to recover from the CO_2 anaesthesia for half an hour. After this, the female was combined with one of the experimental (FSB or FCB) males (which had been either cold shocked 12 hours before or not shocked). Vials were left undisturbed for 24 hours to let the experimental male and the LH_{st} female interact. Following this, the females were transferred individually into vial containing 6 ml of banana-yeast-jaggery-food vials to oviposit for 18 hours. After that, the females were discarded. Thirteen days later amongst the progeny, the numbers of red-eyed and scarlet-eyed flies were recorded. As the LHst flies are true breeding scarlet eyed flies (recessive character) while the experimental FSB and FCB flies are wild type (red eyed) flies, any progeny sired by the first male was scarlet eyed and those sired by the second (i.e., experimental) male was red eyed. For the P2 assay 80 males were used for the "cold shock" treatment and 50 males for the "no shock" treatment from each of the 10 populations (5 FSB and 5 FCB). To measure P2 from each vial, I used data from those vials that showed at least one red-eyed progeny (i.e., the second male had non-zero fitness). The proportion of red eyed progeny was calculated (Proportion of red eyed progeny = number of red eyed progeny produced by females/ total number of red and scartlet eyed progeny produced by female) and hence

each vial yielded a single value of P2 which was used as the unit of analysis. The final sample size for each of the population was roughly 29-60. The vials in which females failed to produce even a single red eyed progeny (i.e., second male had zero fitness) were analysed separately. For each population, I calculated the proportion of females that did not produce a single red eyed progeny and used this as the unit of analysis.

Statistical analysis

Since selected and control populations having the same numerical subscript originated from the same BRB ancestral population, they are more closely related to each other than they are to any other population. For example, FSB 1 is more closely related to FCB 1 (since they both were derived from BRB 1) than to FSB 2. Hence they are treated as statistical blocks in all the analyses. Data from Experiment 1.1, i.e., mating latency, copulation duration, mating success and male fertility were analysed using a three-factor mixed model analysis of variance (ANOVA) with selection regime (FCB vs. FSB) and period (4, 12 and 30 hours post cold shock) as fixed factors crossed with blocks as random factor. Multiple comparisons were performed using Tukey's HSD. Data from Experiment 1.2, i.e., the fitness of ancestral females mated with cold shocked males, were analysed using a four-factor mixed model analysis of variance (ANOVA) with Selection regime (FCB vs. FSB), Period (4 vs. 12 hours post cold shock) and Day (Day 1 vs. Day 2) as fixed factors crossed with Blocks (1-5) as random factor. I also analysed the proportion of females which did not produce progeny. Data on proportion of females that did not produce any progeny was analysed using a three-factor mixed model ANOVA selection regime (FSB and FCB), period (4 and 12 hours of recovery post cold shock) as fixed factors crossed with random blocks (1-5). Mating latency, copulation duration, mating success and male fertility data from Experiment 1.3 were analysed using a two-factor mixed model ANOVA with Selection regime (FCB vs. FSB) as fixed factor crossed with blocks as random factor. For Experiment 2, Proportion of red eyed progeny and proportion of females which produced no red eyed progeny were analysed using a three-factor mixed model ANOVA with selection regime (FCB vs. FSB) and Treatment (Cold shock vs. No shock) as fixed factors crossed with blocks as random factor. All the analyses were done using JMP 10 (SAS Institute, Cary, NC, USA).

RESULTS

Experiment 1.1: Effect of cold shock on the pre- and post-copulatory traits

(a) Mating latency

Mating latency has evolved in response to selection. I found a significant main effect of selection (FSB and FCB), period (4, 12 and 30 hours) and block (1-5) on mating latency (Table 4a.1a, Figure 4a.1A). Multiple comparisons employing Tukey's HSD indicated that males from the FSB populations took significantly less time to start mating compared to FCB populations (Figure 4a.1A). However, I did not find selection × period interaction and none of other interactions were significant (Table 4a.1a).

(b) Copulation duration

There was no significant effect of selection regime (FSB and FCB), period (4, 12 and 30 hours) and block (1-5) on copulation duration (Table 4a.1b, Figure 4a.2A). None of the other interactions were significantly different (Table 4a.1b).

(c) Mating success

I found a main effect of selection and period on mating success. The FSB males had significantly higher mating success relative to FCB males. Mating success increased with the time of recovery post cold shock. However, there was no significant effect of the block and none of the other two and three way interactions were significant (Table 4a.1c, Figure 4a.3A).

(d) Male fertility

I found a significant effect of selection and period on the male fertility. The FSB males were significantly more fertile than FCB males (Table 4a.1d, Figure 4a.4A). Male fertility increased with time of recovery post cold shock. However none of the interactions were significant (Table 4a.1d).

Experiment 1.2: Fitness of the ancestral females mated with cold shocked males

I found that selection and period had significant effect on female progeny production (Table 4a. 2a, Figure 4a.5A). Females mated with the FSB population males had a significantly higher progeny production compared to females mated with the FCB population males. Period had a significant effect on female progeny production. Progeny

production increased with time of recovery. Ancestral females mated to males that had recovered for 4 hours following cold shock had significantly lesser progeny production comparative to females mated to males that had recovered for 12 hours post cold shock. Females produced more progeny on day one compared to day two, but the difference was not significant. None of the interactions were significant (Table 4a.2a, Figure 4a.5A).

I calculated the proportion of females that had zero fitness after being exposed to FSB or FCB males post cold shock. Tukey's HSD indicated that lesser number of females produced zero progeny after interacting with cold shocked FSB males relative to females held with cold shocked FCB males. This finding clearly indicates that the ability of FSB males to sire more progeny post cold shock has evolved in FSB populations (Table 4a.2b, Figure 4a.5C).

Experiment 1.3: The pre- and post-copulatory traits in males not subjected to cold shock

I assayed- (a) mating latency, (b) copulation duration, (c) mating success (D) male fertility and (e) effect of selection regime male (not subjected to cold shock) on progeny production by ancestral females. I found that the FSB and FCB males did not differ significantly in any of these traits, indicating that there are no differences in the basal levels of these traits between the males of the two populations (FSB and FCB) (Table 4a.3a, b, c, d, e Figure 4a.1B, 2B, 3B, 4B, 5B)

Experiment 2: Effect of selection regime on sperm offense (P2) ability

I found a significant effect of selection and treatment on sperm offense ability. Post cold shock, the FSB males sired 12% more progeny compared to FCB males. Interestingly, even without cold shock, the FSB males sired 4% more progeny relative to FCB males. However this difference was not significant (Table 4a.4a, Figure 4a.6A). Cold shock reduced sperm offense in both the FCB and FSB populations. I also calculated the proportion of males that produced zero progeny post cold shock. I found that significantly greater fraction of the FCB males produced zero progeny compared to the FSB males (Table 4a.4b, Figure 4a.6B).

Table 4a.1. Effect of cold shock on the pre- and post-copulatory traits. Summary of results of a three-factor mixed model ANOVA considering selection regime (FSB and FCB) and period (recovery period 4, 12 and 30 hours after cold shock) as fixed factors crossed with blocks as random factor on the (a) mating latency, (b) copulation duration data. *p*-values in bold are statistically significant. Estimated denominator DF (Satterthwaite method) was very low. Hence *F* ratio and *p* values are unavailable for this effect.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	5914.46	5914.46	1	4.037	18.547	**0.012**
Mating	Period (Per)	25814.1	12907.1	2	8.052	25.368	**<0.001**
latency	Block (Blk)	11609	2902.25	4	6.897	4.44	**0.043**
	Sel×Per	805.36	402.679	2	8.152	2.279	0.164
	Sel×Blk	1277.86	319.466	4	8.017	1.811	0.220
	Per×Blk	4086.44	510.805	8	8.000	2.897	0.077
	Sel×Per×Blk	1410.61	176.326	8	1449	0.765	0.634
(b)	Selection (Sel)	118.852	118.852	1	4.022	1.520	0.285
Copulation	Period (Per)	278.046	139.023	2	8.041	1.497	0.280
duration	Block (Blk)	766.883	191.721	4	4.175	1.697	0.306
	Sel×Per	20.966	10.483	2	8.065	0.179	0.839
	Sel×Blk	313.955	78.489	4	8.007	1.336	0.336
	Per×Blk	746.312	93.289	8	8.000	1.587	0.264
	Sel×Per×Blk	470.204	58.775	8	1449	1.776	0.077

Table 4a.1. Effect of cold shock on the pre- and post-copulatory traits. Summary of results of a three-factor mixed model ANOVA considering selection regime (FSB and FCB) and period (recovery period 4, 12 and 30 hours after cold shock) as fixed factors crossed with block as random factor on the (c) mating success and (d) male fertility data. *p*-values in bold are statistically significant. Estimated denominator DF (Satterthwaite method) was very low. Hence, *F* ratio and *p* values are unavailable for this effect (Block).

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(c)	Selection (Sel)	0.051	0.051	1	4.000	17.154	**0.014**
Mating	Period (Per)	0.152	0.076	2	8.000	18.937	**0.001**
success	Block (Blk)	0.078	0.020	4	1.127	7.214	0.243
	Sel×Per	0.017	0.008	2	8.000	1.986	0.199
	Sel×Blk	0.012	0.003	4	8.000	0.696	0.616
	Per×Blk	0.032	0.004	8	8.000	0.937	0.535
	Sel× Per×Blk	0.034	0.004	8	.	.	.
(d)	Selection (Sel)	0.053	0.053	1	4.000	66.681	**0.001**
Male	Period (Per)	0.052	0.026	2	8.000	8.104	**0.012**
fertility	Block (Blk)	0.016	0.004	4	0.588	.	.
	Sel×Per	0.020	0.010	2	8.000	1.694	0.244
	Sel×Blk	0.003	0.001	4	8.000	0.136	0.965
	Per×Blk	0.026	0.003	8	8.000	0.551	0.792
	Sel× Per×Blk	0.047	0.006	8	.	.	.

Table 4a.2a. Fitness of the ancestral females mated with cold shocked males. Summary of results from a four-factor ANOVA using selection regime (FSB and FCB), period (recovery period 4 hours and 12 hours post cold shock) and day (progeny production on day1 and day 2) as fixed factors crossed with blocks (1-5) as random factor on the progeny production of females mated to the FSB or FCB males (number of progeny from each vial was used as unit of analysis). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selction (Sel)	1964.166	1964.166	1	4.125	8.251	**0.044**
Day	13025.704	13025.704	1	4.526	215.367	**<0.001**
Block (Blk)	295.721	73.930	4	4.727	0.196	0.930
Period (Per)	987.046	987.046	1	4.148	4.913	0.089
Sel×day	62.607	62.607	1	4.571	1.114	0.344
Sel×Blk	1005.593	251.398	4	5.591	3.051	0.115
Sel×Per	7.164	7.164	1	4.839	0.179	0.690
Day×Blk	243.893	60.973	4	6.211	0.568	0.696
Day×Per	0.181	0.181	1	4.501	0.003	0.960
Blk×Per	846.199	211.550	4	5.613	2.351	0.174
Sel×Day×Blk	225.466	56.367	4	4.000	4.375	0.091
Sel×Day×Per	35.311	35.311	1	6.777	2.258	0.178
Sel×Blk×Per	155.685	38.921	4	4.000	3.021	0.155
Day×Blk×Per	255.795	63.949	4	4.000	4.964	0.075
Sel×Day×Blk×Per	51.531	12.883	4	1155.000	0.240	0.916

Table 4a.2b. Proportion of ancestral females with zero fitness after mating with cold shocked males. Summary of results from a three-factor ANOVA on proportion of females that do not produce progeny (post mating with FSB or FCB males) using selection regime (FSB and FCB), period (recovery period 4 and 12 hours post cold shock) as fixed factors crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selction (Sel)	0.313	0.313	1	4.000	18.654	**0.012**
Block (Blk)	0.114	0.029	4	5.068	1.353	0.366
Period (Per)	0.168	0.168	1	4.000	21.964	**0.009**
Sel×Blk	0.067	0.017	4	4.000	5.047	0.073
Sel×Per	3×10^{-4}	3×10^{-4}	1	4.000	0.101	0.766
Blk×Per	0.031	0.008	4	4.000	2.294	0.221
Sel×Blk×Per	0.013	0.003	4	.	.	.

Table 4a.3. The pre- and post-copulatory traits in males not subjected to cold shock. Summary of the results from two-factor mixed model ANOVA on (a) mating latency (b) copulation duration (c) mating success and (d) effect of selection regime male on female fitness data considering selection regime (FSB and FCB) as the fixed factor crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(A)	Selection (Sel)	1.207	1.207	1	4.005	0.070	0.804
Mating	Block (Blk)	870.409	217.602	4	4	12.617	**0.015**
latency	Sel×Blk	68.988	17.247	4	322	1.102	0.356
(B)	Selection (Sel)	0.605	0.605	1	4.005	0.066	0.811
Copulation	Block (Blk)	154.364	38.591	4	4	4.182	0.097
duration	Sel×Blk	36.911	9.228	4	322	1.249	0.290
(C)	Selection (Sel)	3.3×10^{-4}	3.3×10^{-4}	1	4	2.268	0.207
Mating	Block (Blk)	1.4×10^{-3}	3.4×10^{-4}	4	4	2.385	0.210
success	Sel×Blk	5.7×10^{-4}	1.4×10^{-4}	4	.	.	.
(D)	Selection (Sel)	4.6×10^{-3}	4.6×10^{-3}	1	4	7.064	0.057
Male	Block (Blk)	3.1×10^{-3}	7.7×10^{-4}	4	4	1.189	0.435
fertility	Sel×Blk	2.6×10^{-3}	6.5×10^{-4}	4	.	.	.
(E)	Selection (Sel)	120.915	120.915	1	4.006	2.915	0.163
Progeny	Block (Blk)	8160.108	2040.027	4	4	49.195	**0.001**
production	Sel×Blk	165.873	41.468	4	325	0.692	0.598

Table 4a. 4. Effect of cold shock on sperm offense ability. Summary of results from a three-factor mixed model ANOVA treating selection regime (FSB and FCB) and treatment (cold shock and no shock) as fixed factors crossed with blocks (1-5) as random factor on (A) sperm offense ability and (B) proportion of females that produce zero red eyed progeny. *p*-values in bold are statistically significant.

Traits	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***P***
(A)	Selection (Sel)	1.254	1.254	1	4.020	21.538	**0.010**
Offense	Block (Blk)	2.602	0.650	4	2.548	5.451	0.121
ability	Treatment (Trt)	17.523	17.523	1	4.009	142.599	**<0.001**
	Sel×Blk	0.233	0.058	4	4.000	0.94	0.523
	Sel×Trt	0.316	0.316	1	4.018	5.099	0.087
	Blk×Trt	0.492	0.123	4	4.000	1.987	0.261
	Sel×Blk×Trt	0.248	0.062	4	737.000	0.908	0.459
(B)	Selection (Sel)	0.070	0.070	1	4.000	9.211	**0.039**
Proportion of	Block (Blk)	0.014	0.004	4	2.809	0.511	0.739
females that	Treatment (Trt)	0.540	0.540	1	4.000	266.796	**<0.001**
produced	Sel×Blk	0.031	0.008	4	4.000	2.861	0.167
zero red eyed	Sel×Trt	1×10^{-5}	1×10^{-5}	1	4.000	0.004	0.954
progeny	Blk×Trt	0.008	0.002	4	4.000	0.757	0.603
	Sel×Blk×Trt	0.011	0.003	4	.	.	.

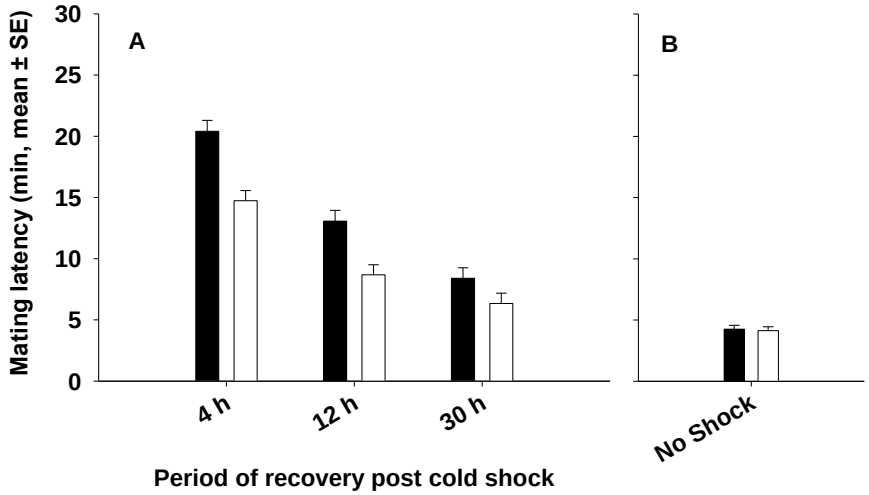

Figure 4a.1: Effect of cold shock (A) or no shock (B) on mating latency. (A) We assayed mating latency at 4 hours (h), 12 h and 30 h post cold shock. Closed bars represent FCB and open bars represent FSB populations. Selection and period had significant effects on mating latency. However, selection × period interaction was not significant. (B) Under no-shock treatment, there was no significant difference in mating latency between FSB and FCB males.

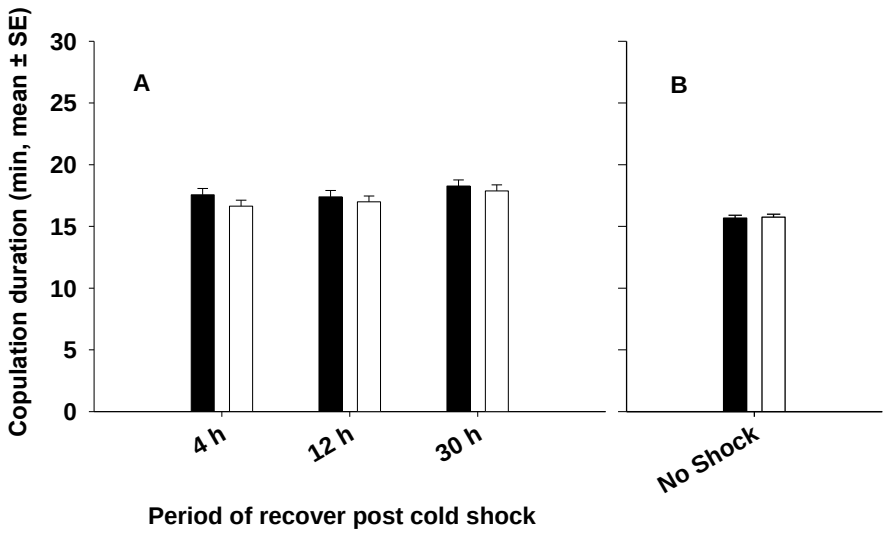

Figure 4a.2: Effect of cold shock (A) or no shock (B) on copulation duration. (A) We assayed copulation duration 4 h, 12 h and 30 h post cold shock. Closed bars represent FCB and open bars represent FSB populations. Selection, period and Selection × period had no significant effect on copulation duration. (B) Under no-shock treatment, there was no significant difference in copulation duration between FSB and FCB males.

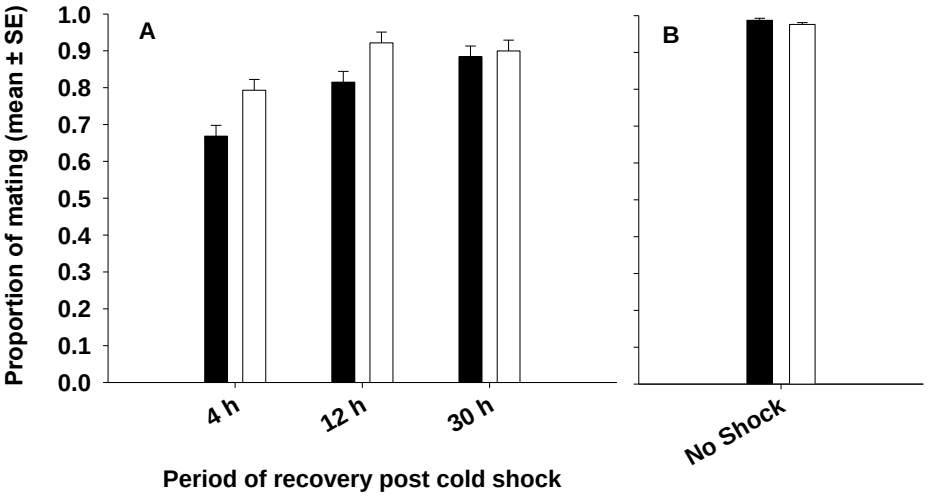

Figure 4a.3: Effect of cold shock (A) or no shock (B) on mating success. Closed bars represent FCB and open bars represent FSB populations. (A) Selection and period had significant effects on mating success. However, Selection × period interaction was not significant. (B) Under no-shock treatment, there was no significant difference in mating success between FSB and FCB males.

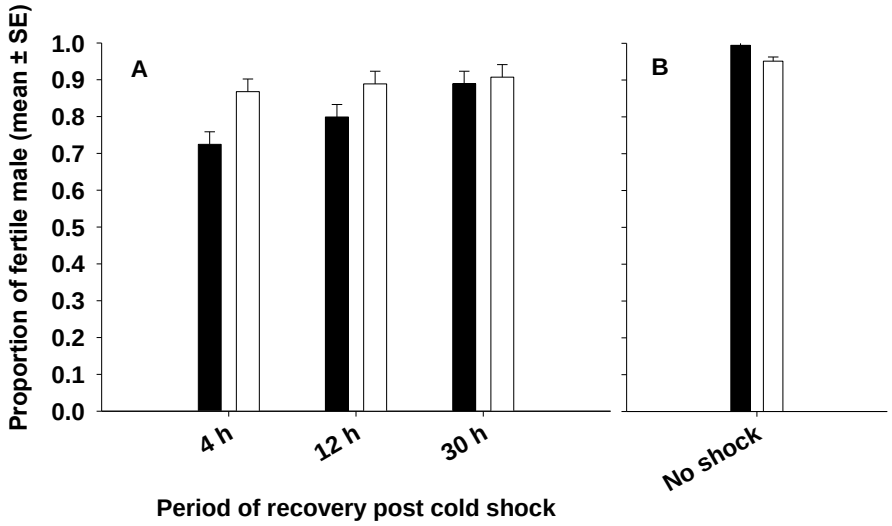

Figure 4a.4: Effect of cold shock (A) or no shock (B) on male fertility. Closed bars represent FCB populations and open bars represent FSB populations. If a female mated to a given male produced at least one egg that hatched, the male was considered to be fertile. (A) Selection and period had significant effect on male fertility. However, selection × period interaction was not significant. (B) Under no-shock treatment, there was no significant difference in male fertility between FSB and FCB males.

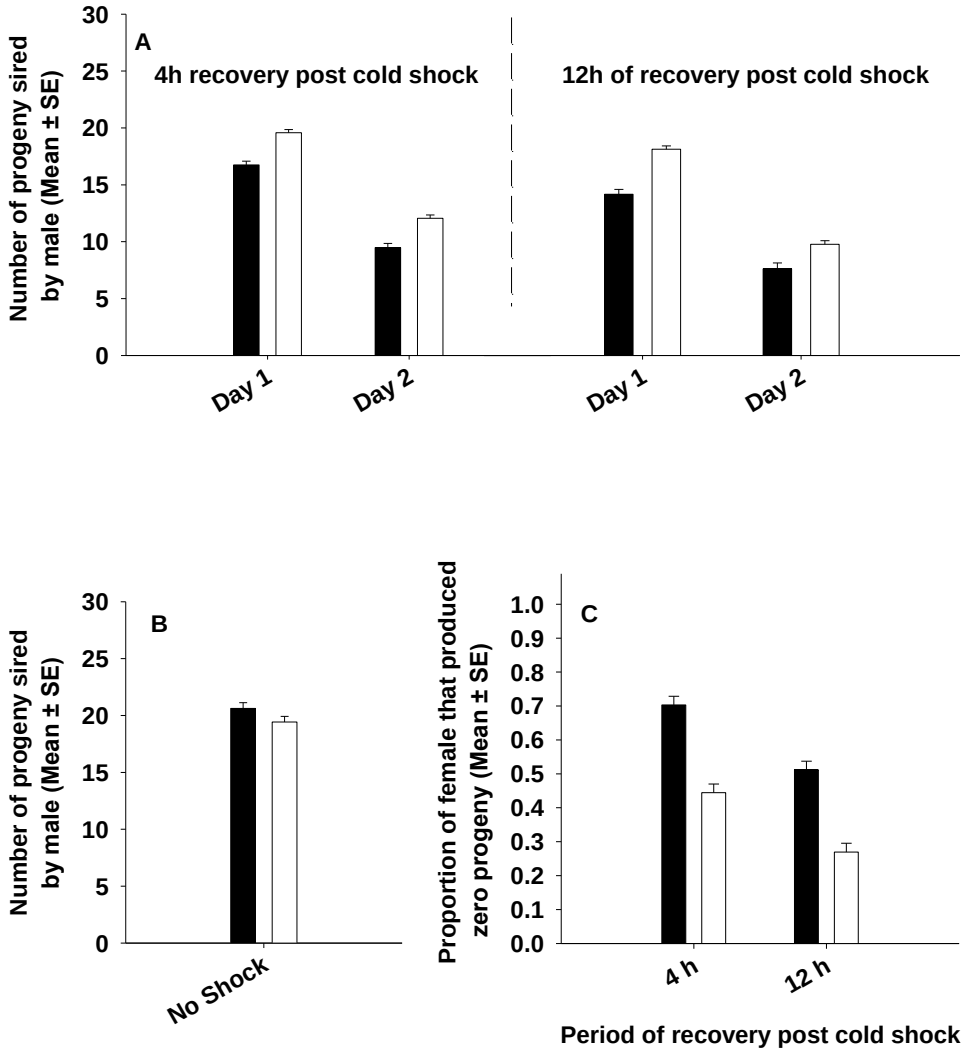

Figure 4a. 5: Effect of cold shock (A and B) or no shock (C) on progeny production. Closed bars represent FCB populations and open bars represent FSB populations. (A) Number of progeny produced by (ancestral) BRB females exposed to FSB or FCB males subjected to cold shock and allowed to recover for 4 or 12 hours. Selection and day had significant effect on the progeny production. (B) Under no shock treatment, there was no significant difference in the number of progeny sired by FSB and FCB males when exposed to ancestral BRB females. (C) The proportion of ancestral females which had produced zero progeny after being exposed to FSB or FCB males that were cold shocked and allowed to recover for 4 or 12 hours. Selection and period had significant effect on the proportion of females that produce zero progeny after being exposed to cold shocked male. However, two way interaction of selection × period was not significant.

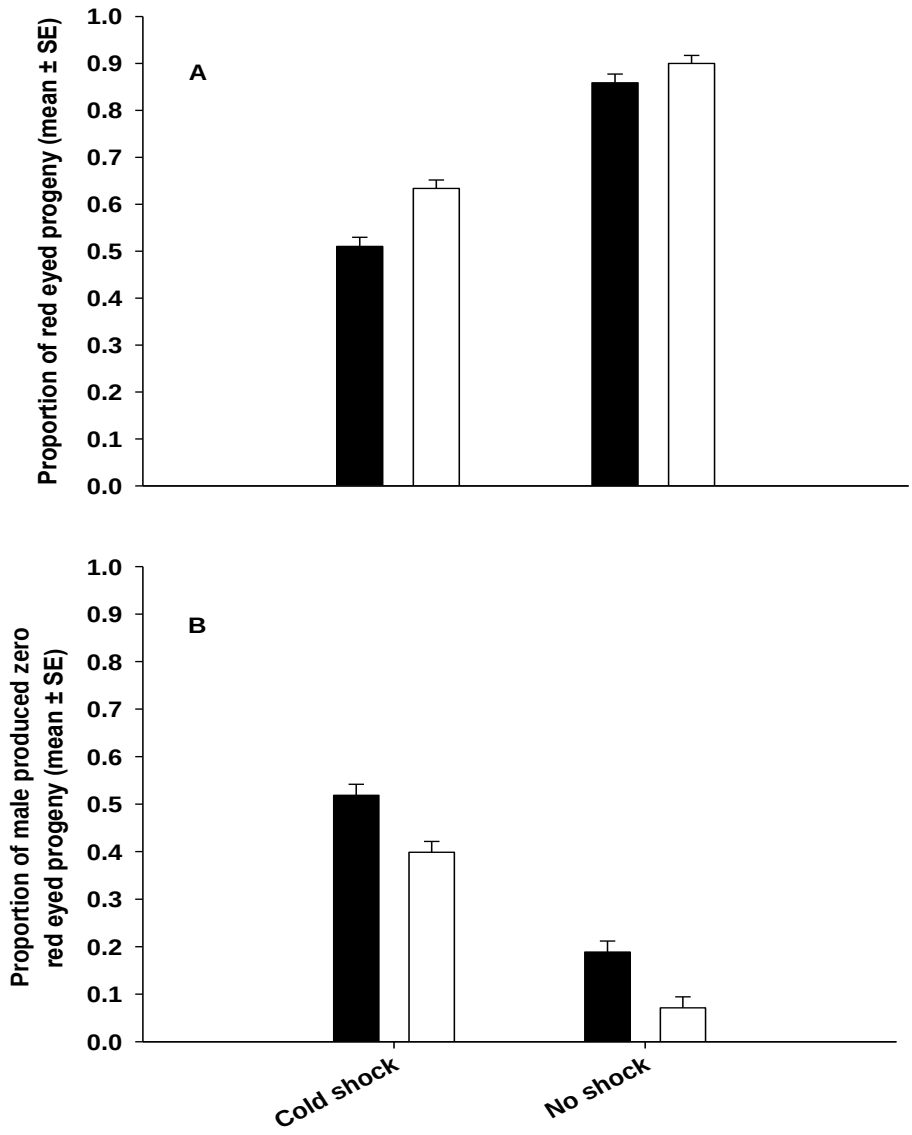

Figure 4a.6: Effect of cold shock on sperm offense ability (P2). Closed bars represent the FCB and open bars represent the FSB populations. Since the females and competitors males have recessive scarlet eye marker and the FSB and FCB males have dominant red eye marker, progeny sired by FSB and FCB males will show red eye color. Hence, in this experiment, the proportion of red eyed progeny is an indicator of sperm offense ability. (A) These data come from females that produced at least one progeny from the FSB or FCB male (that is non-zero sperm offense ability). Compared to FCB males, FSB males had higher sperm offense ability under cold shocked and non-shocked conditions. Selection and treatment had significant effect on P2. However, selection × treatment interaction was not significant. (B) Proportion of males that had zero-sperm offense ability (P2). Significantly less proportion of FSB males had zero sperm offense compared to FCB males. Selection and treatment effects were significant. However, selection × treatment interaction was not significant.

DISCUSSION

In the present study, I have assessed the evolution of the pre and post-copulatory traits of males in populations of *D. melanogaster* selected for cold shock resistance. My results clearly indicate that post cold shock, FSB males (when exposed to ancestral females) take lesser time to start mating, have a higher mating success, are more fertile and produce more progeny relative to FCB males. Interestingly, post cold shock, FSB males also had higher sperm competitive ability when compared to FCB males. However, unlike other traits, copulation duration was not different between FSB and FCB males. When the males were not subjected to cold shock, there was no difference in mating latency, copulation duration, mating success, or progeny production between FSB and FCB males.

Time to start mating (mating latency) and mating success are affected both by the ability of the male to induce the female to mate as well as the female's own eagerness to mate. In the present study, FSB and FCB males were provided with common, non-cold shocked, ancestral females. Hence, differences in mating latency and mating success would represent inherent differences in the FSB and FCB males' ability to successfully mate and/or females mating preference across these two types of males. Both high and low temperature treatments are known to affect mating latency (Yenisetti et al. 2006). Both heat stress and cold stress are known to reduce male mating success (Kvelland 1965a, Yenisetti et al. 2006). In agreement with these results, I found that cold shocked males (both FSB and FCB) show higher mating latency and lower mating success relative to males not subjected to cold shock. As males were allowed to recover from cold shock, mating latency decreased while mating success increased. However, post cold shock, FSB males took lesser time to start mating (lower mating latency) and were more successful at mating (higher mating success) compared to FCB males. Given that there were no differences in mating latency and mating success of FCB and FSB males under no shock conditions, there are two possible explanations (not mutually exclusive) for the observed results; (a) FSB males recover from cold-shock at a faster rate compared to FCB males. This is consistent with the observation that the FCB males move closer to the FSB males in terms of their mating latency and mating success values with increasing durations of recovery. (b) FSB males are better protected against injury from cold shock and hence suffer lesser damage due to cold shock. Populations of *D. melanogaster* selected for resistance to cold shock are known to have evolved increased levels of specific metabolites such as glycogen, trehalose and proline which are known to act as

cryoprotectants (Chen and Walker 1994). Similarly it is possible that FSB populations have evolved mechanisms to protect against cold shock induced damage. It is important to note that these possibilities are not mutually exclusive.

In many insects, including *D. melanogaster*, selection for resistance to certain kinds of stress leads to increase in body size (Chippindale et al. 1994, 1998). At least some studies show that in *Drosophila*, larger males have better mating success (Partridge et al. 1987a, b, Markow 1988, Santos et al. 1988, Markow and Ricker 1992, Markow and Sawka 1992). Thus, selection for increased stress resistance can increase mating success through its effects on body size. This, however, is not an explanation in my study since I found no difference between the male body size of the FSB and FCB populations (chapter 5). These findings are parallel to those reported by Dolgin et al. (2006) where *D. melanogaster* males had a higher mating success when exposed to their thermal environment in which they had adapted.

While copulation is necessary, it is not sufficient to ensure the fitness of a male. The male should be able to successfully transfer a functional ejaculate during copulation to the females. Copulation duration is often used as a measure of the amount of ejaculate transferred during copulation. It is known to be positively correlated with the amount of some components of ejaculate (Wigby 2005). I found no difference between the FSB and FCB males in copulation duration indicating that the amount of ejaculate transferred was probably not different. I further analysed the effectiveness of the males in transferring a functional ejaculate by assessing two traits - male fertility and progeny production.

Cold shock reduces male fertility by killing/immobilising the sperm or affecting the sperm quality (Lefevre and Jonsson 1962). When young *Drosophila* males are subjected to cold shock, progeny production is reduced, compared to males not subjected to cold shock (Iyengar and Baker 1960). My results also show that cold shock reduces male fertility and females mated to males subjected to cold shock suffer from reduced progeny production. However, I find that post cold-shock, male fertility and female progeny production are higher in FSB males relative to FCB males. It is important to note that under no shock treatment, there are no differences between the FSB and FCB males in their fertility or progeny production.

Male fertility and progeny production require that functional sperm are transferred to females during copulation. Previous studies indicate that when males are subjected to a cold shock of -5 ^{o}C for one hour, no motile sperm are found in their reproductive tracts for the next 24 hours (Lefevre and Jonsson 1962). Thus, in my study, at least some functional sperm are transferred by males (FSB and FCB) during copulation even within 4 hours post cold shock. The higher fertility and progeny production in FSB males post cold shock could be because (a) FSB males are better at protecting sperm from cold-induced damage (b) FSB males produce more sperm but are not necessarily better at protecting the sperm from cold-induced damage (c) FSB males can produce functional sperm at a faster rate post cold shock. Given that cold shocked males produce progeny even within 4 hours post cold shock and that sperm production is generally expected to be a lengthy process, it is unlikely that option (c) alone would account for the observed differences. It is very likely that FSB and FCB populations differ in the total number of sperm produced and/or the ability to protect sperm from cold-induced damage.

Post cold shock, FSB males show higher sperm offense ability compared to FCB males. This is consistent with the idea that the FSB populations probably produce more sperm and/or ejaculate quality. This result is also in agreement with our own previous studies where we found that post cold shock, FSB males mated with a higher number of non-virgin females and sired more progeny compared to FCB males (Singh et al. 2015). An un-explored aspect of the effect of cold shock on sperm competitive ability is the effect of cold shock on accessory gland proteins. Given that cold shock affects an array of proteins in the fly body, it is quite likely that it also affects the Acps. If true, then, it is quite likely that the FSB populations have also evolved with respect to their Acps. It is to be emphasised here that differences in mating rate post cold shock are enough to explain the differences in sperm offense ability (as measured in our study). Specifically, if FSB males mate more often with females post cold shock (compared to FCB males), then, given the strong last male sperm precedence in this species, FSB would have higher sperm offense ability compared to FCB males, even without any changes in sperm number, physiology or Acps. Since in this assay I did not observe the number times the flies mated with the second male, I cannot account for this possibility.

This study clearly shows that under no cold shock treatment, males from the FSB and FCB populations have identical reproductive behavior and fitness. But upon cold shock, males from the FSB populations are better at reproductive recovery with respect to pre- and post-copulatory traits i.e., mating latency, mating success, male fertility, progeny production and sperm offense ability. Central results of this study help us in understanding the evolution of reproductive traits in response to environmental stresses.

Note: Data from this chapter has been accepted for publication in PLoS One.

Singh K, Samant MA, Tom MT and Prasad NG (2016). **Evolution of pre- and post-copulatory traits in male *Drosophila melanogaster* as correlated response to selection for increased resistant to cold stress** (Accepted at PLoS One)

Chapter 4b

Evolution pre- and post-copulatory traits in female

INTRODUCTION

Cold shock has been shown to have major consequences for the fitness of organisms, especially insects. Apart from its effects on survival at various life stages (Tucic 1979), cold shock also affects reproductive traits in both males and females of several insect species (Colinet and Hance 2009, Schnebel and Grossfield 1984, Carriere and Boivin 1997, Fischer et al. 2003, Singh et al. 2015).

In the present study, I focus on the effects of cold shock on the reproductive traits of female *D. melanogaster*. Exposure to cold shock can affect reproductive output of female *D. melanogaster* through its direct effects on the survivorship of stored sperm and eggs as well as its indirect effects on female mating propensity and sperm handling. In *D. melanogaster*, cold shock at sub-zero temperatures leads to the death of stored sperm in mated females (Novitski and Rush 1949, Lefevre and Jonsson 1962) and therefore, they fail to produce any progeny (Singh et al. 2015). In fact, cold shock has been used as an effective virginizing treatment in flies (Lefevre and Jonsson 1962). Post cold shock, females need to re-mate and gather sperm afresh in order to produce progeny (Singh et al. 2015). Therefore, mating post cold shock is extremely important for the female's fitness. Female remating frequency is affected by temperature in multiple insects (Kindle et al. 2006, Katsuki and Miyatake, 2009 and Best et al. 2012). Adaptation to lower temperatures increases female mating propensity in *D. pseudoobscura* with females from northern populations mating more frequently than females from the southern populations (Price et al., 2014). Thus, temperature can indirectly affect post-copulatory traits through female remating frequency. Post mating, *Drosophila* females need to process sperm and other ejaculate components before they can lay fertile eggs. Female nervous system is essential for this process. Females with masculinized nervous system or isolated female abdomens show abnormal sperm storage patterns (Arthur et al. 1998). Cold shock can lead to chill coma and serious damage to the nervous system (Garrity et al. 2010, Armstrong et al. 2012). Therefore, it is quite possible that cold shock interferes with the normal sperm processing abilities of females. In addition to this, cold stress affects the

survivorship and fecundity of females (Anderson et al. 2005, Watson and Hoffmann 1996), reduces offspring production (Jakobs 2014) and alters last male sperm precedence (Giraldo-Perez et al. 2015). Thus cold shock can have major effects on female reproductive traits.

In the present study, I assayed female reproductive traits (mating latency, copulation duration, mating success and progeny production) in populations of *D. melanogaster* selected for over 50 generations for resistance to cold shock. I found that the females from selected populations were better at recovery post cold shock in terms of mating latency, mating success and progeny production relative to the control populations.

METHODS

Base line population and Experimental population

Details of the base line maintenance and experimental populations are mentioned in chapter 2.

Generation of experimental flies

All the experimental flies were generated under controlled eggs density at standard laboratory culture conditions (25^{o}C temperature, 50-60% RH, 12hours:12hours light/dark cycle). For each of the FSB (1-5) and FCB (1-5) populations, 16 vials were set up at a density of 70 eggs per vial containing 6 ml of banana-yeast-jaggery food. On 9-10th day post egg collection, virgin females were collected for two treatments- (a) Cold shock treatment (b) No shock treatment during the peak of eclosion using light CO_2 anaesthesia in vials containing 2 ml of banana-yeast-jaggery food at a density of 10 females per vial. These flies were held in food vials until the 12th day post egg collection, by which time the females were roughly 2-3 days old as adults.

Generation of common males from ancestral populations (BRB)

To obtain common males, rearing vials were set up at a controlled egg density of 70 eggs per vial, each vial containing 6 ml of banana-yeast-jaggery food. Twenty eight vials were established for each of the five replicate populations of BRB. Virgin males were collected using light CO_2 anesthesia and were housed singly in vial contining 2 ml of banana-yeast-jaggery until mating trials were conducted (by the time of experiment males were ~2-3 days as adults).

Experiment 1: Effect of cold shock on the pre- and post-copulatory traits

On the 12th day post egg collection virgin females from FSB and FCB populations were exposed to the cold shock treatment (following the protocol described in chapter 2). Quickly, post cold shock females were transferred to Plexiglas cages (14cm length × 16cm width × 13cm height) at a density of 250 females per cage. Each cage was provided with a Petri plate containing standard banana-yeast-jaggery food and was maintained under standard laboratory conditions (see above). Then from each of FSB or FCB populations 70 and 60 females were randomly aspirated out from the Plexiglas cages 3 hours and 11 hours post cold shock, respectively. These females were placed singly in vials, each having 2 ml of banana-yeast-jaggery food. Four or twelve hour post cold shock, a female of FSB or FCB from the 'cold shock' treatment was combined with a baseline male in a vial containing 2 ml banana-yeast-jaggery food. The cotton plug of the vial was pushed deep into the vial to adjust the space available to the flies to roughly the bottom one-third of the vial (25mm diameter × 30mm height). Each vial was continuously observed for mating latency (time required to start copulation, from the time of combining the male and female) and copulation duration (time from beginning to end of copulation) until a single mating was over. The proportion of females that copulated was used as a measured of mating success (mating success = number of females that mated/ total number of females). Following this, each successfully mated female was separated using light CO_2 anaesthesia and transferred to a vial containing fresh 6 ml of banana-yeast-jaggery food and was allowed to oviposit for 24 hours. The eggs laid during this period were used as a measure of its fitness for 'day one'. Subsequently, the female was transferred to another fresh banana-yeast-jaggery food vial and allowed to oviposit for next 24 hours and the eggs laid in this period were used as a measure of the female's fitness for 'day two'. The number of adult flies emerging from 'day one' and 'day two' was used as the measure of female-fitness. This protocol was repeated at two time points (recovery time, see "cold shock treatment" above) post cold shock – 4 hours and 12 hours with sample sizes of 70 and 60 vials per selection regime per block respectively.

Experiment 2: The pre- and post-copulatory traits in females not subjected to cold shock

On the 12th day post egg collection, virgin females from the FSB and FCB populations were subjected no shock treatment as described in chapter 2. A single female from 'no

shock' treatment was combined with a base line male in a vial provisioned with banana-yeast-jaggery food. Mating latency and copulation duration of the flies were measured using the same protocol as in Experiment 1 (see above). After a single mating the males were discarded and the female from each vial was transferred to a fresh vial containing 6 ml of banana-yeast-jaggery food and allowed to oviposit for 24 hours. After this, the female was discarded. The adult flies which emerged from each of the vials were counted and used as a measure of the female's fitness. Sample size for this assay was 30-35 vials per selection regime per block.

Statistical analysis

Experiment 1: The pre- and post-copulatory traits in females subjected to cold shock

I measured the effect of cold shock on mating latency, copulation duration and mating success of females from the two selection regimes when combined with common baseline males. It was measured at two periods of recovery: 4 and 12 hours after the females were subjected to cold-shock. All statistical analyses on these data were done using a three-factor mixed model ANOVA, treating the selection regime (FSB and FCB) and period (4 hours and 12 hours post cold shock) as fixed factors crossed with blocks (1-5) as random factor. For female fitness, data were analyzed using a four-factor mixed model ANOVA with selection regime (FSB and FCB), period (4 and 12 hours post cold shock) and day ('day one' fitness and 'day two' fitness) as fixed factors crossed with random blocks (1-5). Progeny number from each vial was used as the unit of analysis.

Experiment 2: The pre- and post-copulatory traits in females not subjected to cold shock

Mating latency, copulation duration, mating success and female fitness were analyzed using a two-factor mixed model ANOVA with selection regime (FSB and FCB) as fixed factor crossed with random blocks (1-5). For mating latency and copulation duration, time per vial were used as units of analyses. Progeny number per vial was used as the unit of analysis. All the analyses were done using JMP 10 (SAS Institute, Cary, NC, USA). All multiple comparisons were done using Tukey's HSD.

RESULTS

Experiment 1: The pre- and post-copulatory traits in females subjected to cold shock-

(a) Mating latency

I found significant effect of selection regime (FSB and FCB), period (4 and 12 hours) and interaction between block (1-5) and period on the mating latency data (Table 4b.1a, Figure 4b.1A). Multiple comparison employing Tukey's HSD indicated that females from the FSB populations take significantly less time to start mating comparative to FCB populations (Figure 4b.1A) only at 4 hours post cold shock. With increasing time of recovery post cold shock, mating latency significantly decreased in both FSB and FCB populations. Females from the FSB or FCB populations that were allowed to recover for 4 hours post cold shock took significantly more time (~7 min more) to mate with virgin base line males compared to females from the FSB or FCB populations that were allowed to recover for 12 hours post cold shock (Table 4b.1a, Figure 4b.1A). None of the other interactions were significant (Table 4b.1a, Figure 4b.1A). We found a two way interaction between block × period (Table 4b.1a).

(b) Copulation duration

There was no significant effect of selection regime, period and block on copulation duration (Table 4b.1b, Figure 4b.2A). None of other interactions were significant. This suggests that cold-shock recovery in terms of mating latency, but not copulation duration has evolved in the selected populations.

(c) Mating success

Similar to mating latency, I found significant main effect of selection regime and an interaction between selection regime and period on mating success. Multiple comparisons using Tukey's HSD suggested that females from the FSB populations have significantly more mating success relative FCB populations at 4 hours after cold shock. This finding suggests that the FSB females have evolved to regain mating ability faster than the FCB females. Mating success increased in both selection regimes with the time of recovery post cold shock and after 12 hours, however, the two selection regimes were not different from each other (Table 4b.1c, Figure 4b.3A).

(d) Progeny production

I found a significant effect of selection regime on progeny production (Table 4b.1d, Figure 4b.4A). Multiple comparisons using Tukey's HSD indicated that post cold shock, FSB females mated with common ancestral males produced significantly more progeny compared to FCB females (Figure 4b.4A) across time points (4 and 12 hours) and days ('day one' and 'day two'). None of the other two, three and four way interactions were significant (Table 4b.1d).

Experiment 2: The pre- and post-copulatory traits in females not subjected to cold shock.

I measured- (a) Mating latency (b) copulation duration and (c) progeny production, when FSB and FCB females that were not subjected to cold shock were combined with males from common baseline (ancestral) populations. I found that the FSB and FCB females did not differ significantly in any of these traits, indicating that there are no differences in the basal levels of these traits between the two populations (FSB and FCB) (Table 4b.2a,b,c,d, Figure 4b.1B, 2B, 3B, 4B).

Table 4b.1. Summary of results from a three-factor mixed model ANOVA on (a) mating latency, (b) copulation duration and (c) mating success data using selection (FSB and FCB) and period (4 and12 hours after cold shock) as fixed factors crossed with random blocks (1-5). *p*-values in bold are statistically significant.

Traits	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	1081.45	1081.45	1	4.113	11.829	**0.025**
Mating	Block (Blk)	4155.88	1038.97	4	3.208	0.796	0.595
latency	Period (Per)	15651.1	15651.1	1	4.007	10.932	**0.030**
	Sel × Blk	362.255	90.564	4	4.000	0.407	0.798
	Sel × Per	455.098	455.098	1	4.046	2.041	0.226
	Blk × Per	5747.46	1436.86	4	4.000	6.451	**0.049**
	Sel × Blk × Per	890.953	222.738	4	996.0	0.785	0.535
(b)	Selection (Sel)	181.665	181.665	1	4.041	5.446	0.079
Copulation	Block (Blk)	417.096	104.274	4	3.565	0.516	0.732
duration	Period (Per)	208.678	208.678	1	4.007	1.008	0.372
	Sel × Blk	133.339	33.335	4	4.000	0.854	0.559
	Sel × Per	155.172	155.172	1	4.035	3.975	0.116
	Blk × Per	830.924	207.731	4	4.000	5.320	0.067
	Sel × Blk × Per	156.175	39.044	4	996.0	1.020	0.396
(c)	Selection (Sel)	0.037	0.037	1	4.000	9.835	**0.035**
Mating	Block (Blk)	0.052	0.013	4	5.907	1.457	0.325
success	Period (Per)	0.004	0.004	1	4.000	0.615	0.477
	Sel × Blk	0.015	0.004	4	4.000	3.566	0.123
	Sel × Per	0.009	0.009	1	4.000	8.280	**0.045**
	Blk × Per	0.025	0.006	4	4.000	5.926	0.057
	Sel × Blk × Per	0.004	0.001	4	.	.	.

Table 4b.1d. Summary of results from a four-factor mixed model ANOVA on progeny production (progeny per female) data using selection (FCB and FSB), period (4 and12 hours after cold shock) and day (day 1 and day 2) as fixed factors crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selction (Sel)	1489.497	1489.497	1	4.297	56.182	**0.001**
Period (Per)	10.800	10.800	1	4.088	0.125	0.741
Block (Blk)	6083.820	1520.955	4	3.737	2.087	0.256
Day	142.569	142.569	1	4.010	0.198	0.679
Sel × Per	91.826	91.826	1	4.221	2.611	0.178
Sel × Blk	104.457	26.114	4	0.590	0.486	0.798
Sel × Day	648.136	648.136	1	4.073	6.262	0.065
Per × blk	348.505	87.126	4	0.048	7.096	0.879
Per × day	335.058	335.058	1	4.121	5.328	0.080
Blk × Day	2929.509	732.377	4	1.205	8.927	0.201
Sel × Per × Blk	139.812	34.953	4	4.000	0.407	0.798
Sel × Per × Day	16.819	16.819	1	4.089	0.198	0.679
Sel × Blk × Day	418.871	104.718	4	4.000	1.219	0.426
Per × Blk × Day	252.990	63.247	4	4.000	0.736	0.613
Sel × Per × Blk × Day	343.686	85.921	4	1735.000	1.888	0.110

Table 4b.2. Summary of the results from a two-factor ANOVA on data from females that were not subjected to cold shock. Here, selection (FSB and FCB) is modelled as a fixed factor crossed with random blocks (1-5). (a) Mating latency, (b) copulation duration (c) mating success and (d) female fitness . *p*-values in bold are statistically significant.

Traits	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	52.319	52.319	1	4.004	3.259	0.145
Mating	Block (Blk)	692.760	173.190	4	4.000	10.793	0.020
latency	Sel × Blk	64.184	16.046	4	307.00	0.425	0.790
(b)	Selection (Sel)	3.867	3.867	1	4.002	0.519	0.511
Copulation	Block (Blk)	55.355	13.839	4	4.000	1.858	0.282
duration	Sel × Blk	29.792	7.448	4	307.00	0.894	0.468
(c)	Selection (Sel)	4.4×10^{-5}	4.4×10^{-5}	1	4.000	0.048	0.837
Mating	Block	0.002	3.8×10^{-4}	4	4.000	0.418	0.79
success	Sel × Blk	0.004	9.1×10^{-4}	4	.	.	.
(d)	Selection (Sel)	86.414	86.414	1	4.005	0.320	0.602
Progeny	Block (Blk)	1191.916	297.979	4	4.000	1.103	0.463
production	Sel × Blk	1080.462	270.115	4	303.00	1.947	0.103

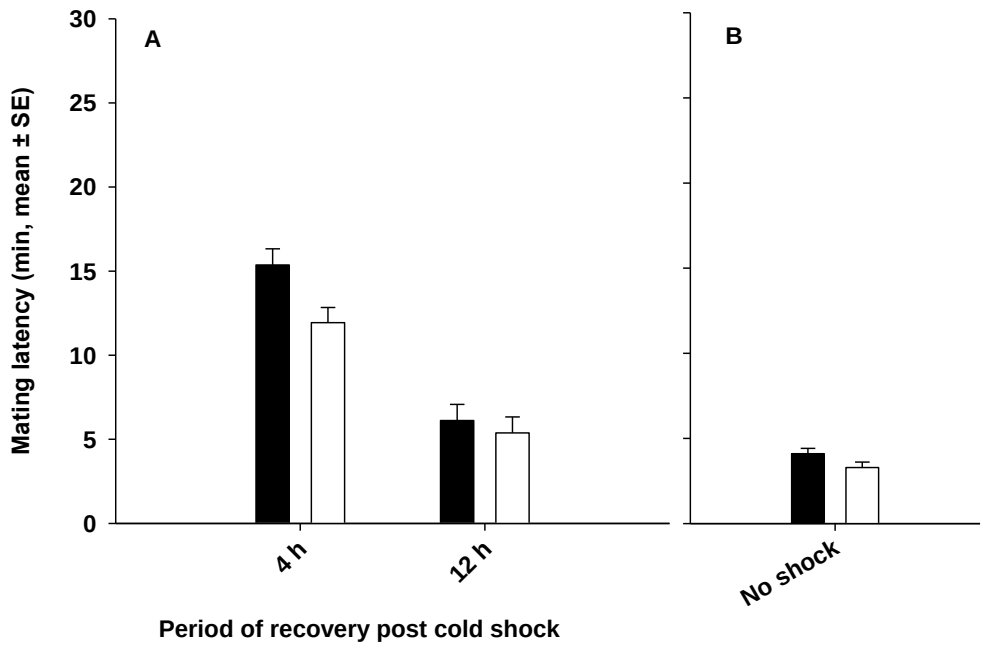

Figure 4b.1: Effect of cold shock (A) or no shock (B) on mating latency. (A) Effect of cold shock on mating latency in females. We assayed mating latency at two time points of recovery at 4 hours (h) and 12 hours post cold shock to females**.** Closed bars represent FCB and open Bars represent FSB populations. Selection and period had significant effect on mating latency. However, the interaction between selection and period was not significant. (B) Effect of selection regime on mating latency in absence of cold shock. Selection did not have any effect on mating latency.

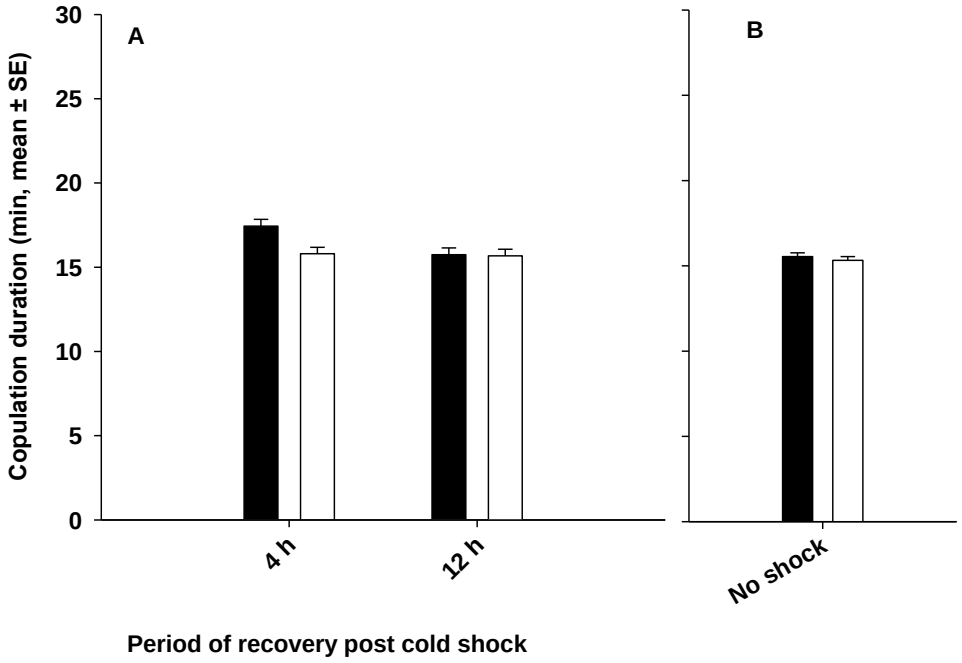

Figure 4b.2: Effect of cold shock (A) or no shock (B) on copulation duration. (A) Closed bars represent FCB and open bars represent FSB populations. Selection, period and selection × period had no significant effect on mating latency. (B) Effect of selection regime on copulation duration in absence of cold shock. Effect of selection on copulation duration was not significant.

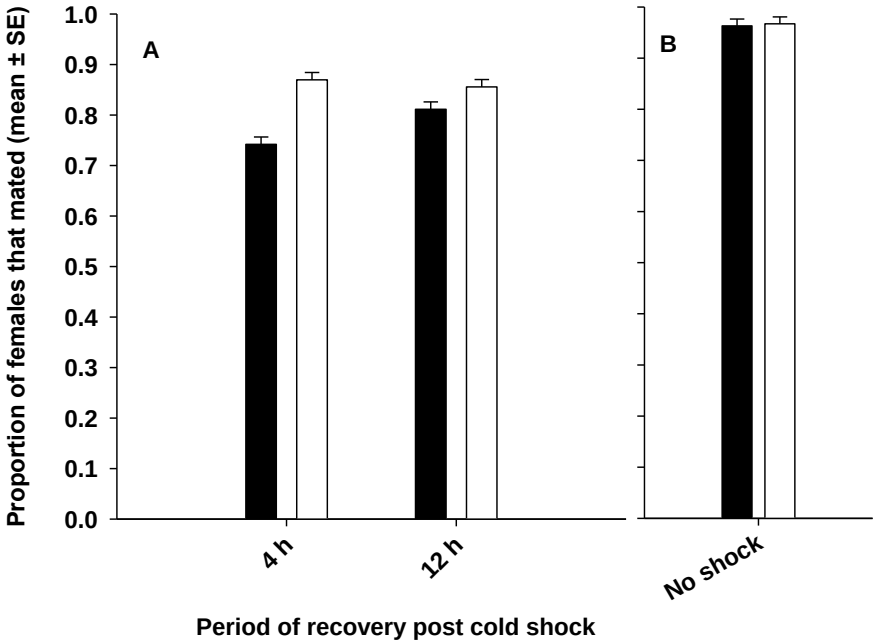

Figure 4b.3: Effect of cold shock (A) or no shock (B) on mating success. (A) Closed bars represent the FCB and open bars represent the FSB populations. Selection had significant effect on mating success. Interaction between selection and period was also significant indicating that at 4 hours proportion of mating was higher in FSB relative FCB populations. (B) Effect of selection regime on mating success in absence of cold shock. Selection did not have significant effect on mating success.

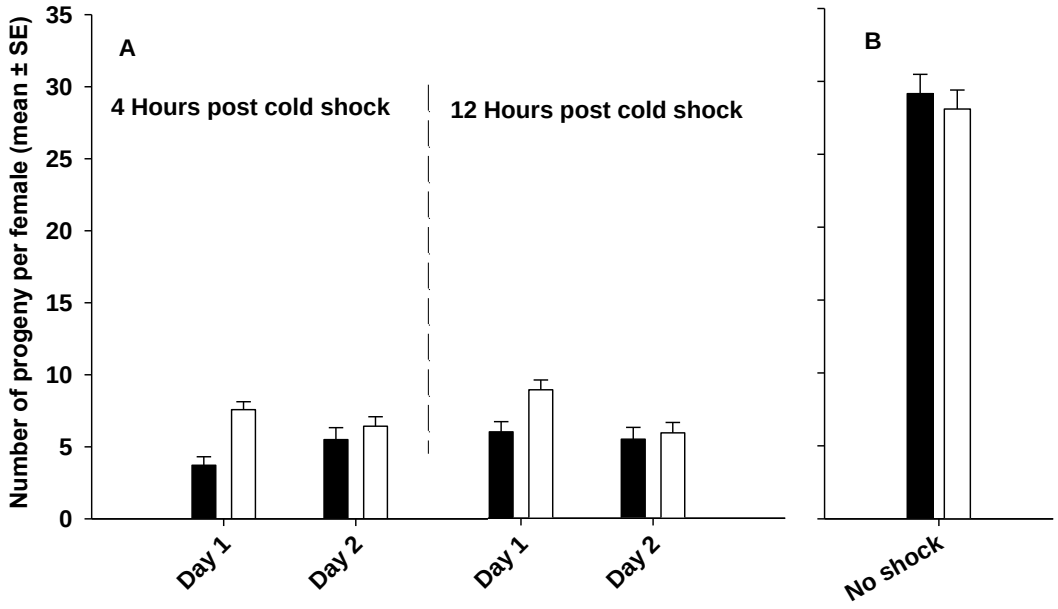

Figure 4a.4: Effect of cold shock (A) or no shock (B) on progeny production. (A) Closed bars represent the FCB and open bars represent the FSB populations. Selection had significant effect on the progeny production data. However, selection × period and selection × period × day interactions were not significant. (B) Effect of selection regime on progeny production in absence of cold shock. Effect of selection on progeny production was not significant.

DISCUSSION

Cold shock kills the sperm stored by females and hence reduces egg viability (Lefevre and Jonsson 1962). My previous study (Singh et al. 2015) found that populations selected for resistance to cold shock lay more viable eggs 24 hours post cold shock (compared to controls). They have also evolved higher mating rate. We speculated that the recovery of egg viability might be associated with selected females mating more/faster post cold shock and hence gaining access to healthy ejaculates. The present study confirms these suggestions. Over 50 generations of selection, FSB females have evolved to mate faster, mate more and produce more progeny post cold shock. The FSB and FCB females were not different in any of these traits when assayed under no-shock condition. Multiple studies have documented the negative effects of temperature shock on male reproductive traits (Rohmer et al. 2004, Zizzari and Ellers 2011, Lieshout et al. 2013, Nguyen et al. 2013 Rinehart et al. 2014, Singh et al. 2015,) and female fecundity (Irwin and Lee 2003, Marshall and Sinclair 2010, Rinehart et al. 2014). To the best of our knowledge, our study is the first to assess the evolution of female reproductive traits in response to cold shock.

Similar to the effect of extreme temperature shock in males (Kvelland 1965a, Denlinger and Yocum 1998, Rinehart et al. 2000, Rohmer et al. 2004, Malmendal et al. 2006, Yenisetti et al. 2006, reviewed in Hance et al. 2007), mating latency and mating success was lower in females exposed to cold shock compared to females not exposed to cold shock. Both the traits improved as females were allowed more time to recover from the shock. The shorter mating latency and greater mating success of FSB females compared to FCB females after 4 hours post shock can be because of two reasons: (a) FSB females may be better at resisting and/or recovering from cold induced damage than FCB females and therefore might be able to restart reproductive activity earlier than FCB females. (b) Base line males might prefer to court and mate with FSB females because they are weaker post cold shock and hence cannot resist male coercion. Thus lower mating latency and higher mating success might simply indicate that FSB females are poorer at resisting the effects of cold shock. If the second reason is true, then, FSB females should suffer more male induced harm than FCB females. Male induced harm can be quantified using the number of progeny produced. FSB females produce significantly more progeny post cold shock compared to FCB females, showing no signs of suffering from male induced harm. Thus it is likely that FSB females are better at resisting/recovering from cold induced harm and hence engage in reproductive activity quicker than FCB females.

Reduction in egg/progeny production in response to cold shock has been previously reported (Rinehart et al. 2000, Irwin and Lee 2003, Jakobs 2014, Mockett and Matsumoto 2014, Singh et al. 2015). My results are in agreement with these studies. As stated before, cold shock kills the sperm in the female's storage organs. The females need to eject the dead sperm (Lefevre and Jonsson 1962) and mate to restore fertility (Singh et al. 2015). Thus one way in which FSB females could have higher fitness than FCB females is by being better at ejecting the dead sperm.

Post mating, female *Drosophila* has to successfully process sperm and other ejaculate components in order to start producing fertile eggs (reviewed in Wolfner 1997). Both FSB and FCB females produced progeny after mating within four hours of cold shock. Thus, the females were able to process the ejaculate sufficiently even within four hours of cold shock. The increased progeny production of FSB females post cold shock could be due to: (a) increased ability of FSB females to resist cold induced harm to eggs/egg production mechanism. (b) Better ejaculate processing by FSB females post cold shock. This would include better/higher storage of sperm and processing of accessory gland proteins such as Ovulin that promote fecundity (reviewed in Wolfner 1997). (c) Increased investment of ejaculate by the common base line males in FSB vs. FCB females. While males are known to vary ejaculate investment based on their assessment of female fitness (Lupold et al. 2011), in my study, there was no difference in copulation duration- a measure of ejaculate investment- when males mated FSB or FCB females. Hence, difference in progeny production between FSB and FCB females is likely to represent intrinsic differences between these two types of females in their ability to resist/recover from cold shock.

This study shows that in absence of cold shock, females from the FSB and FCB populations have identical pre- and post-copulatory traits, but upon cold shock, FSB female are better at reproductive recovery in terms of mating latency, mating success and progeny production. These results indicate adaptive evolution of reproductive traits in females in response to selection for resistance to cold shock.

Chapter 5

Life-history costs associated with increased resistance to cold shock

INTRODUCTION

Various ecological factors, including temperature, are known to vary across time and space and as a result, various organisms face different kinds of unfavorable environmental conditions during their life span. These environmental stresses can be major drivers of evolution of life-history of organisms in nature (Hoffmann and Parsons 1991, reviewed in Parsons 2005).

Temperature is one of the fundamental ecological features of an organism's environment. Organisms can respond to extreme temperatures in various ways including changes in behavior, physiology or life-history (Hoffmann and Parsons 1991, Patton and Krebs 2001, Fasolo and Krebs 2004). Resources used for coping with stress are unavailable for other functions and under resource limited conditions, this can lead to trade-offs across important life-history traits such as somatic maintenance and reproduction (Stearns 1992). For example, one important way in which organisms cope with immediate changes in temperature (heat and cold shock) is by expressing heat shock proteins (Hsps). Expression of these proteins is extremely costly and is known to affect reproduction (Janowitz and Fischer 2012). Thus, temperature shock can affect various important life-history traits (reviewed in Bennett 1980, Huey and Berrigan 2001, Hochachka and Somero 2002, Sinclair et al. 2003, Angilletta 2009, Dell et al. 2011). Deviation from ambient temperature (where absolute fitness is maximum) drastically affects various life-history and related traits of insects (Parsons 1982, Lee and Denlinger 1991, Voituron et al. 2002 Hoffmann et al. 2003a) such as fecundity, male fertility, longevity (Denlinger and Yocum 1998, Bubliy and Loeschcke 2005, Rohmer et al. 2004, reviewed in Hence 2007, Lieshout et al. 2013, Nguyen et al. 2013), reproduction (Fischer et al. 2003), mating ability (Schnebel and Grossfield 1984, Singh et al. 2015), developmental time (Trotta et al. 2006, Austin and Moehring 2013) and motility (Gibert et al. 2001, Angilletta et al. 2002, Berwaerts and Dyck 2004).

Several studies have addressed the evolution of life-history traits in response to temperature variation. *D. melanogaster* being widely distributed offers a great model to study the evolution of life-history in response to temperature variation across latitudes and altitudes. In general, a number of traits vary progressively across populations inhabiting various latitudes. This pattern of results suggests that life-history evolution in populations of *Drosophila* is primarily being driven by environmental differences and that the populations are adapting to local environment, most probably, including temperature, which is an important component of the environment Latitudinal clines have been found in a number of life-history traits such as development time, survivorship, larval competitive ability, fecundity and body size (Stanley and Parsons 1981, Bouletreau-Merle et al. 1982, Davidson 1990, James and Partridge 1995, 1998, Guerra et al. 1997, Karan et al. 1998, Hallas 2002, reviewed in Hoffmann et al. 2003b, Hangartner et al. 2015).

Some experimental evolution studies have addressed the evolution of life-history traits in response to selection for cold stress tolerance (Tucic 1979, Chen and Walker 1993, Watson and Hoffmann 1996, Anderson et al. 2005, Bubliy and Loeschcke 2005, MacMillan et al. 2009). However, such studies have been few (Overgaard et al. 2010). Anderson et al. (2005) found increased female fecundity and decreased male longevity in populations of *D. melanogaster* selected for rapid chill coma recovery. MacMillan et al. (2009) also found reduced longevity in females (but not in males) in populations selected for increased resistance to freeze shock. However, Bubliy and Loeschcke (2005) did not find difference in longevity and development time in populations of *D. melanogaster* selected for increased resistance to cold stress. Thus the correlated evolution of life-history traits in response to cold stress has been fairly variable.

In the present study, I was interested in probing the life-history costs, if any, of increased resistance to cold stress. I assayed various life-history related traits such as life-span, life time fecundity, development time and dry body weight in the populations of *D. melanogaster* selected for increased resistance to cold stress. These experiments were performed over 24-33 generations of selection.

METHODS

Details of experimental populations used for this study have been described in chapter 2.

Experimen: 1.1: Longevity assay

The longevity assay was performed after 24 generations of selection. Eggs were collected from standardized flies (see chapter 2) at a controlled egg density of 70 eggs/vial provisioned with ~6 ml of banana-yeast-jaggery food. Twenty four vials were set up for each of the FSB (1-5) and FCB (1-5) populations. On the 12^{th} day post egg collection, flies were sorted (25 mating pairs per vial) under light CO_2 anesthesia. After sorting, flies were divided into two treatments: (a) Cold-shock (both male and female were given cold shock) and (b) No-shock (neither male and nor female were given cold-shock).

(a) Cold-shock: For each population, 12 vials containing flies (25 mating pairs) each were given cold-shock (-5^{o}C for one hour) as metioned in the chapter 2. Immediately, after cold shock, randaomly 12 vials were divided in to 3 sets. Each set having 4 vials of flies (100 mating pairs each) were transferred into a Plexiglas cage and provided afresh banana-yeast-jaggery food. Hence, each population (FSB 1-5 and FCB 1-5) had 3 replicates.

(b) No-shock: For each population, 12 vials containing flies (25 mating pairs) were subjected to no-shock treatment (25^{o}C for one hour). Post treatment, I randomly divided 12 vials into three set, each set having 4 vial containg flies were transferred into Plexiglas cages. Hence, each population (FSB 1-5 and FCB 1-5) had 3 replicates.

I set up three cages (replicate) per selection × block × treatment combination (Except block 1 of the FCB population which had 2 replicates for both-shock treatment, due to accidental death of one of the replicates during the assay). Food plate was changed every alternate day and dead flies were aspirated out and counted. Sex of the dead flies was determined under microscope on the basis of sex combs. Mortality was recorded until the last fly died. Using the mortality data, for each cage, I measured mean longevity of males and females from the selection regime (FSB and FCB), treatment and block. For the analysis of mean longevity, cage means were used as the unit of analysis.

Experiment 1.2: Life time fecundity assay

Fecundity assay was performed along with longevity assay, using the same flies. I measured fecundity at every sixth day along with longevity. To measure fecundity, fresh food plate was placed in the Plexiglas cage for 6 hours for oviposition. After that, total number of eggs on each plate was counted under the microscope. Following this, I calculated fecundity per female - number of eggs divided by total number of live females at that time point. Average fecundity of the eleven time points was calculated for each population and treatment. I also calculated median and maximum longevity for each cage. I analyzed the aging rates data using the Gompertz model (see below).

Experiment 2: Development time (first instar larva to eclosion)

Development time was assayed after 33 generations of selection. Following one generation of standardization (see details in chapter 2), 12 vials each were set up from FSB and FCB populations at a density of 70 eggs per vial. On the 12^{th} day post egg collection, vials were randomly divided into two sets for - (a) cold-shock (b) no-shock treatment. For both 'cold shock' and 'no shock' treatments, flies were transferred into empty glass vials and the cotton plug was pushed deep up to the bottom one-third of the vial. After that, the flies were subjected to cold shock or no shock treatments, following the protocol mentioned in chapter 2. Immediately after treatment, flies (200 males and 200 females) were transferred to Plexiglas cage and provided with a fresh banana-yeast-jaggery food plate. Twenty four hours post cold shock, fresh banana-yeast-jaggery food plates were given to each cage for one hour to lay stored eggs. After that another set of fresh banana-yeast-jaggery plates were given for four hours. The second set of plates containing eggs were then incubated at standard conditions for 18 hours to allow eggs to hatch and first instar larvae to emerge. The larvae were collected (using a moist brush) into vials with 6 ml of banana-yeast-jaggery food. For each population and treatment combination, 10 replicate vials each containing 30 larvae per in 6 ml of food were established. The vials were incubated at standard laboratory conditions. The positions of the vials were randomized and moved daily within the incubator. Once pupae formed, each vial was manually scanned every 2 hours. Freshly eclosed flies were transferred into empty glass vials, sexed and counted. The flies were then flash frozen using liquid nitrogen and then transferred to -80^{o}C for storage (and used to assess dry body weight- see below). Mean larva to eclosion development time was calculated for each vial and this vial mean time was used as the unit of analysis.

Experiment 3: Measurement of dry body weight of males and females

I used the flies from the development time assay (mentioned above) to measure dry body weights. Freshly eclosed flies were flash frozen using liquid nitrogen and stored at -80^{o}C until dry body weight measurement. Five flies of a given sex were grouped together, dried in a hot air oven at 65^{o}C for 48 hours and weighed in a high precision electronic balance (Sartorius CPA225D). For each population, treatment and sex combination, ten such sets were weighed. Thus, a total of 50 males and 50 females per population and treatment were measured for body weight. Body weight of each group of five flies was used as the unit of analysis.

Data analysis

Mean longevity, development time and dry body weight of males and females were analyzed using a three-factor mixed model ANOVA treating selection regime (FSB vs. FCB), treatment (Cold shock vs. No shock) as fixed factors crossed with random block (1-5). The sexes were analyzed separately. Fecundity per female was analyzed using a three-factor mixed model ANOVA treating selection regime (FSB vs. FCB) and treatment (Cold shock vs. no shock) and as fixed factors crossed with block as a random factor. All the analyses were done at α=0.05 level of significance using Statistica (for Windows, version 10, Statsoft). Multiple comparisons were done using Tukey's HSD.

Rates of aging

Gompertz model: Age dependent and age independent rate of aging

'Proportion survival' values were calculated using the raw survivorship data. Running average of the proportion survival data, r_x was then calculated.

$$r_x = (p_x + p_{x+2})/2 \qquad (1)$$

Where, p_x is the proportion of individuals surviving at a given age x. Since mortality was monitored every alternate day, x and $x+2$ are two successive age intervals observed. The hazard rate i.e., the probability of death per unit time, μ_x at age x was computed using the following equation:

$$\mu_x = (r_x - r_{x+2})/ r_x \qquad (2)$$

According to the Gompertz equation, the mortality rate at age x is given by,

$$\mu_x = ae^{bx} \qquad (3)$$

Where, *a* and *b* represent age-independent and age-dependent rate of aging respectively. Log-hazard rate was regressed against age intervals; the intercept and the least square slope gave the estimates of *Gompertz a* and *Gompertz b* respectively. The derived parameters were analyzed using three factor mixed model ANOVA with selection regime (FSB vs. FCB), treatment (cold shock vs. no shock) as fixed factor crossed with random blocks (1-5).

RESULTS

Experiment 1.1 Longevity assay for male and female

I quantified longevity in terms of mean, median and maximum longevity. Analyses revealed that the results were similar regardless of the measure used. Therefore, I am showing results from analyses using mean longevity only. After 24 generations of selection, I found, there was no significant main effect of selection, treatment or selection × treatment interaction on male or female mean longevity (Table 5.1a, b, Figure 5.1a, b). Interestingly, the absence of any significant effect of treatment indicates that flies subjected to cold shock treatment lived as well as the flies that were not subjected to cold shock. Thus cold shock had no direct effect on mean longevity.

I found a significant effect of treatment on the *Gompertz a* (age independent mortality rate) and b (age dependent mortality rate) parameters among males. FSB and FCB males subjected to cold shock had significantly higher age independent mortality but significantly lower age dependent mortality compared to the males not subjected to cold shock (Table 5.1.1). The net effect of these two factors was that the average (and median) lifespan of the males subjected to cold shock and those not subjected to cold shock was not different. There was no effect of selection or a selection × treatment interaction on the Gompertz parameters. Among the females, none of the factors affected the Gompertz parameters (Table 5.1.1). Thus, I found no evidence for any change in longevity or rates of aging as a correlated response to selection for increased resistance to cold stress.

Experiment 1.2 Life time fecundity

I calculated the mean number of eggs laid per female in a population (by averaging across the 11 time points of measurement) and used it as the unit of analysis. I did not find significant effects of selection, treatment or selection × treatment interaction on female fecundity (Table 5.1c, Figure 5.1c). Just like longevity, the absence of any significant effect of treatment on fecundity indicates that cold shock treatment had no direct effect on lifetime fecundity.

Experiment 3: Development time (first instar larva to adult eclosion)

Unlike longevity and fecundity, selection did affect mean development time. Mean development time of males showed a significant main effect of selection. (Table 5.2a, Figure 5.2a). Starting as first instar larvae, FSB male takes about 2-4 hours more time to eclose as adults compared to FCB males (Figure 5.2a). Female mean development time analysis shows that there was significant main effect of selection (Table 5.2b, Figure 5.2b). However, none of the other effects were significant (Table 5.2b). Just like the males, FSB females also take 3-6 hours more to eclose as adults compared to FCB females (Figure 5.2b). Again, the cold shock experienced by the parents had no effect on offspring development time (no significant treatment effect).

Experiment 3: Dry body weight

Male mean dry weight analysis revealed that there was no significant main effect of selection, treatment or selection × treatment interaction (Table 5.3a, Figure 5.3a). In case of female dry body weight, I found significant main effect of selection (Table 5.3b). However, there was no significant effect of treatment or selection × treatment interaction (Table 5.3b). Mean body weight of FSB females was about 0.01 mg higher than that of FCB females (Figure 5.3b).

Table 5.1a. Effect of cold shock on mean longevity of males (Experiment 1). Summary of results from a three-factor mixed model ANOVA on the male mean longevity using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5).

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	33.439	33.439	1	4.006	1.348	0.310
Treatment (Trt)	14.549	14.549	1	4.008	0.752	0.435
Block (Blk)	478.234	119.559	4	6.177	3.024	0.107
Sel × Trt	9.169	9.169	1	4.032	1.972	0.232
Sel × Blk	99.283	24.821	4	4.000	5.350	0.067
Trt × Blk	77.398	19.349	4	4.000	4.170	0.098
Sel × Trt × Blk	18.559	4.640	4	39.000	0.422	0.792

Table 5.1b. Effect of cold shock on mean longevity of females (Experiment 1). Summary of results from a three-factor mixed model ANOVA on the female mean longevity using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	56.044	56.044	1	4.007	2.756	0.172
Treatment (Trt)	1.581	1.581	1	4.004	0.047	0.839
Block (Blk)	91.967	22.992	4	7.012	0.443	0.775
Sel × Trt	0.160	0.160	1	4.071	0.084	0.786
Sel × Blk	81.399	20.350	4	4.000	10.751	**0.020**
Trt × Blk	133.984	33.496	4	4.000	17.697	**0.008**
Sel × Trt × Blk	7.571	1.893	4	39.000	0.192	0.941

Table 5.1.1. Effect of cold shock on age independent and age dependent mortality rates of males and females (Experiment 1). Summary of results from a three-factor mixed model ANOVA on (a) age independent and (b) age dependent mortality rate among males using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a) Male	Selection (Sel)	0.479	0.479	1	4.021	2.920	0.162
age	Treatment (Trt)	9.979	9.979	1	4.021	61.398	**0.001**
independent	Block (Blk)	8.791	2.198	4	0.008	126.988	0.960
mortality	Sel × Trt	0.127	0.127	1	4.011	0.410	0.557
	Sel × Blk	0.656	0.164	4	4.000	0.531	0.723
	Trt × Blk	0.649	0.162	4	4.000	0.525	0.726
	Sel × Trt × Blk	1.236	0.309	4	39.00	1.220	0.318
(b) Male	Selection (Sel)	3.9×10^{-5}	3.9×10^{-5}	1	4.017	0.340	0.591
age	Treatment (Trt)	7.3×10^{-3}	7.3×10^{-3}	1	4.030	112.215	**<0.001**
dependent	Block (Blk)	1.4×10^{-3}	3.5×10^{-4}	4	9×10^{-6}	3470.59	1.000
mortality	Sel × Trt	1.8×10^{-9}	1.8×10^{-9}	1	4.011	1×10^{-5}	0.998
	Sel × Blk	4.6×10^{-4}	1.1×10^{-4}	4	4.000	0.638	0.663
	Trt × Blk	2.6×10^{-4}	6.4×10^{-5}	4	4.000	0.362	0.825
	Sel × Trt × Blk	7.1×10^{-4}	1.8×10^{-4}	4	39.00	1.251	0.305

Table 5.1.1. Effect of cold shock on age independent and age dependent mortality rates of females (Experiment 1). Summary of results from a three-way mixed model ANOVA on (c) age independent and (d) age dependent mortality rate among females using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5).

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(c) Female	Selection (Sel)	0.005	0.005	1	4.008	0.014	0.913
age	Treatment (Trt)	0.240	0.240	1	4.007	0.597	0.483
independent	Block (Blk)	0.600	0.150	4	1.491	0.366	0.822
mortality	Sel × Trt	0.092	0.092	1	4.008	0.245	0.647
	Sel × Blk	1.534	0.384	4	4. 000	1.019	0.493
	Trt × Blk	1.611	0.403	4	4.000	1.07	0.475
	Sel × Trt × Blk	1.505	0.376	4	39.000	1.74	0.161
(d) Female	Selection (Sel)	1.4×10^{-4}	1.5×10^{-4}	1	4.008	0.904	0.395
age	Treatment (Trt)	5.2×10^{-4}	5.1×10^{-4}	1	4.012	4.646	0.097
dependent	Block (Blk)	6.9×10^{-4}	1.7×10^{-4}	4	1.536	1.204	0.532
mortality	Sel × Trt	1.3×10^{-4}	1.3×10^{-4}	1	4.010	1.038	0.366
	Sel × Blk	6.4×10^{-4}	1.6×10^{-4}	4	4.000	1.265	0.413
	Trt × Blk	4.4×10^{-4}	1.1×10^{-4}	4	4.000	0.871	0.552
	Sel × Trt × Blk	5.1×10^{-4}	1.3×10^{-4}	4	39 .000	1.355	0.267

Table 5.1c. Effect of cold shock on life time fecundity (Experiment 1.1). Summary of results from a three-factor mixed model ANOVA on the life time fecundity using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5).

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	0.807	0.807	1	4.002	0.934	0.389
Treatment (Trt)	3.078	3.078	1	4.003	5.782	0.074
Block (Blk)	7.899	1.975	4	5.661	1.610	0.292
Sel × Trt	0.134	0.134	1	4.010	0.778	0.428
Sel × Blk	3.464	0.866	4	4.000	5.030	0.073
Trt × Blk	2.132	0.533	4	4.000	3.097	0.150
Sel × Trt × Blk	0.689	0.172	4	39.000	1.418	0.246

Table 5.2a. Effect of cold shock on parents on male developmental time (larvae to adult eclosion) (Experiment 2). Summary of results from a three-factor mixed model ANOVA on the mean larva to adult development time of males using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	758.240	758.240	1	4.000	17.449	**0.014**
Treatment (Trt)	214.335	214.335	1	4.000	0.601	0.482
Block (Blk)	141.786	35.446	4	3.993	0.098	0.977
Sel × Trt	54.776	54.776	1	4.000	1.404	0.302
Sel × Blk	173.816	43.454	4	4.000	1.114	0.460
Trt × Blk	1427.513	356.878	4	4.000	9.150	**0.027**
Sel × Trt × Blk	156.012	39.003	4	180.000	1.111	0.353

Table 5.2b. Effect of cold shock on parent on female developmental time (larvae to adult eclosion) (Experiment 2). Summary of results from a three-factor mixed model ANOVA on mean larva to adult development time of females using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	907.888	907.888	1	4.000	8.374	**0.044**
Treatment (Trt)	206.835	206.835	1	4.000	0.453	0.538
Block (Blk)	950.196	237.549	4	1.019	0.855	0.658
Sel × Trt	204.729	204.729	1	4.000	0.712	0.446
Sel × Blk	433.683	108.421	4	4.000	0.377	0.816
Trt × Blk	1828.026	457.007	4	4.000	1.590	0.332
Sel × Trt × Blk	1149.930	287.483	4	180.000	1.840	0.123

Table 5.3a. Effect of cold shock on dry body weight of males (Experiment 3). Summary of results from a three-factor mixed model ANOVA on the mean dry body weight of males using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	8.2×10^{-5}	8.2×10^{-5}	1	4.000	0.178	0.694
Treatment (Trt)	7×10^{-4}	7×10^{-4}	1	4.000	0.701	0.450
Block (Blk)	6×10^{-3}	1.5×10^{-3}	4	6.553	1.050	0.450
Sel × Trt	1.2×10^{-5}	1.2×10^{-5}	1	4.000	0.240	0.650
Sel × Blk	1.8×10^{-3}	4.6×10^{-4}	4	4.000	9.550	**0.025**
Trt × Blk	4×10^{-3}	1×10^{-3}	4	4.000	20.974	**0.006**
Sel × Trt × Blk	2×10^{-4}	4.8×10^{-5}	4	180.000	0.145	0.965

Table 5.3b. Effect of cold shock on dry body weight of females (Experiment 3). Summary of results from a three-factor mixed model ANOVA on the mean dry body weight of females using Selection (FCB and FSB) and Treatment (Cold-shock and No-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	6.7×10^{-3}	6.7×10^{-3}	1	4.000	32.942	**0.005**
Treatment (Trt)	3×10^{-4}	3×10^{-4}	1	4.000	0.287	0.621
Block (Blk)	1.4×10^{-2}	3.4×10^{-3}	4	3.756	3.620	0.128
Sel × Trt	2×10^{-4}	2×10^{-4}	1	4.000	1.199	0.335
Sel × Blk	8×10^{-4}	2×10^{-4}	4	4.000	1.059	0.479
Trt × Blk	3.7×10^{-3}	9×10^{-4}	4	4.000	4.828	0.078
Sel × Trt × Blk	8×10^{-4}	2×10^{-5}	4	180.000	0.491	0.743

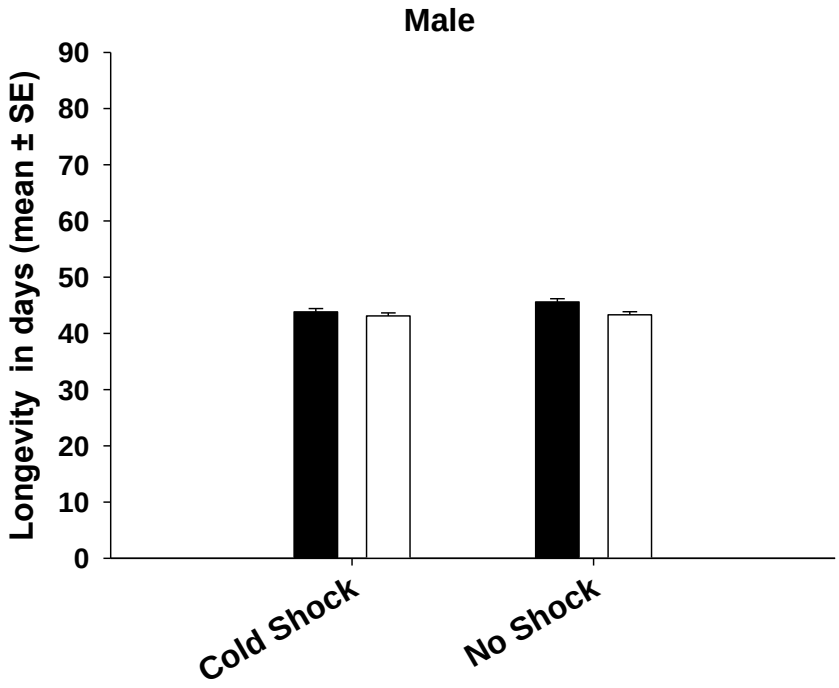

Figure 5.1a: Mean longevity of FSB and FCB males after being subjected to Cold shock or No shock treatment. Selection, treatment or selection × treatment interaction did not have significant effect on male mean longevity. Open bars represent FSB and closed bars represent FCB populations.

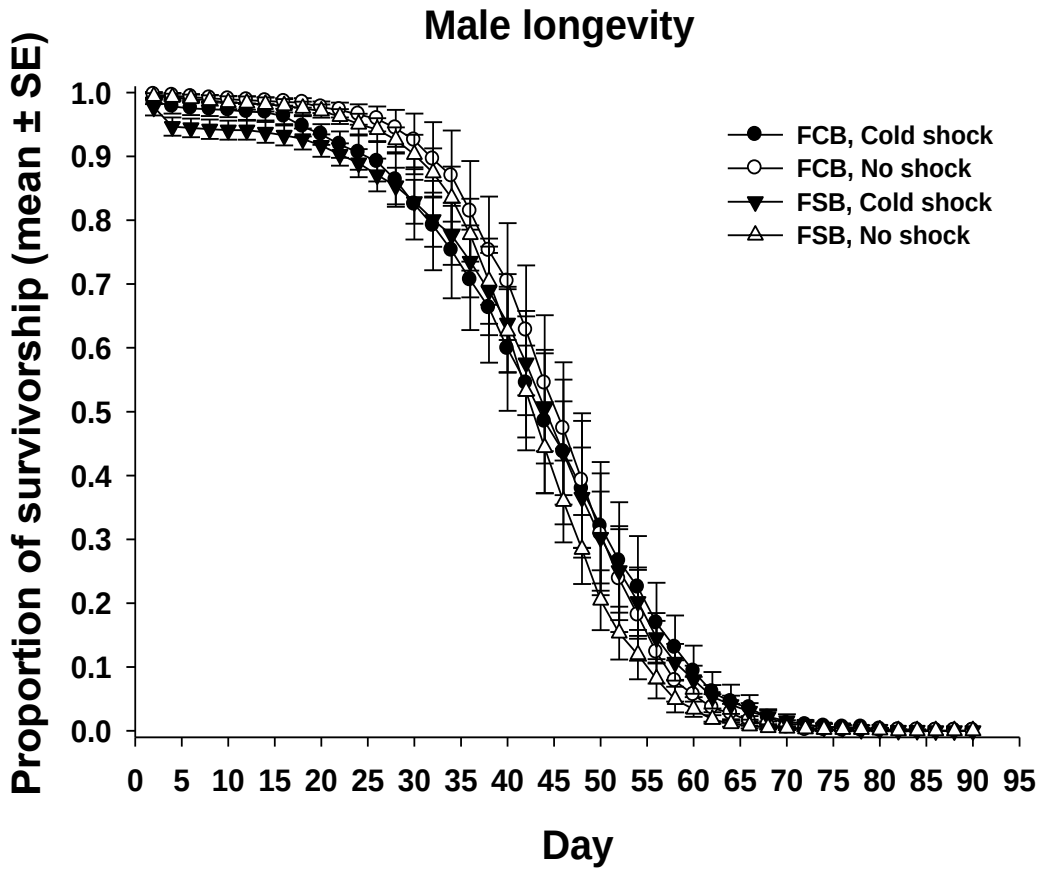

Figure 5.1a.1: Male survivorship across ages. Longevity was assayed after adult flies were subjected to Cold shock or No shock treatment. There was no difference in the mean, median and maximum longevity of FSB and FCB males. There was no significant difference in the Gompertz parameters between FSB and FCB populations.

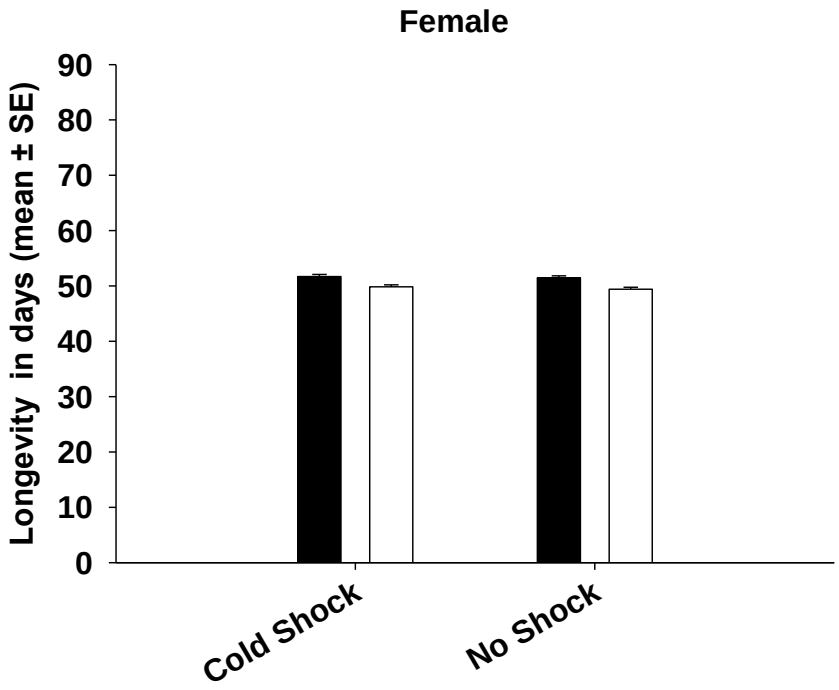

Figure 5.1b: Mean longevity of FSB and FCB females after being subjected to Cold shock or No shock treatment. Selection, treatment or selection × treatment interaction did not have significant effect on female mean longevity. Open bars represent FSB and closed bars represent FCB populations.

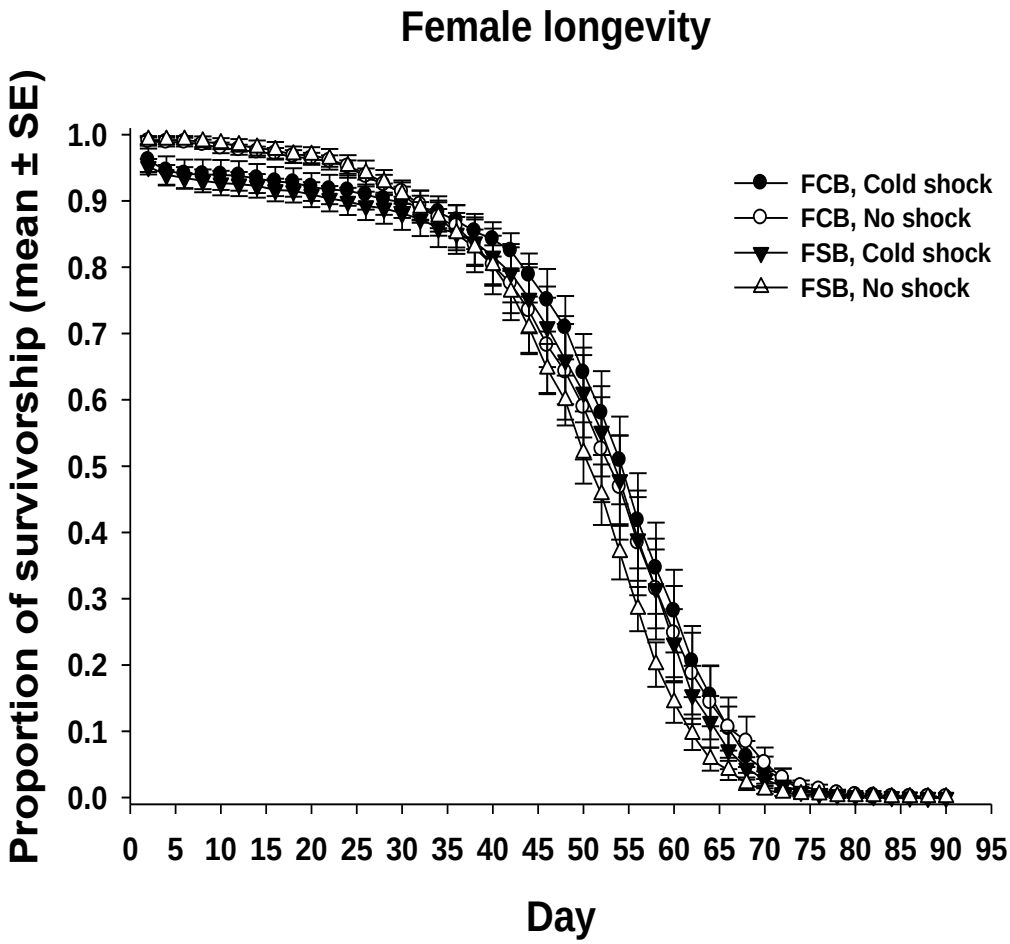

Figure 5.1b.1: Female survivorship across ages. Longevity was assayed after adult flies were subjected to Cold shock or No shock treatment. There was no difference in the mean, median and maximum longevity of FSB and FCB females. There was no significant difference in the Gompertz parameters between FSB and FCB populations.

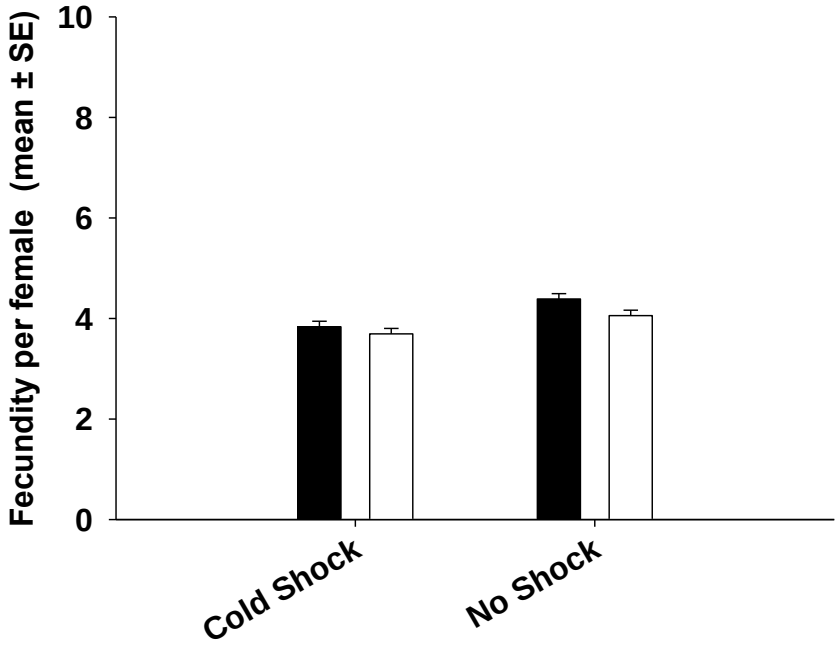

Figure 5.1c: Mean life time fecundity per female. Fecundity was measured at eleven time points once in every 6 days, and mean of eleven time points for fecundity was calculated. Selection, treatment or selection × treatment interaction did not have significant effect on fecundity. Open bars represent FSB populations and closed bars represent FCB populations.

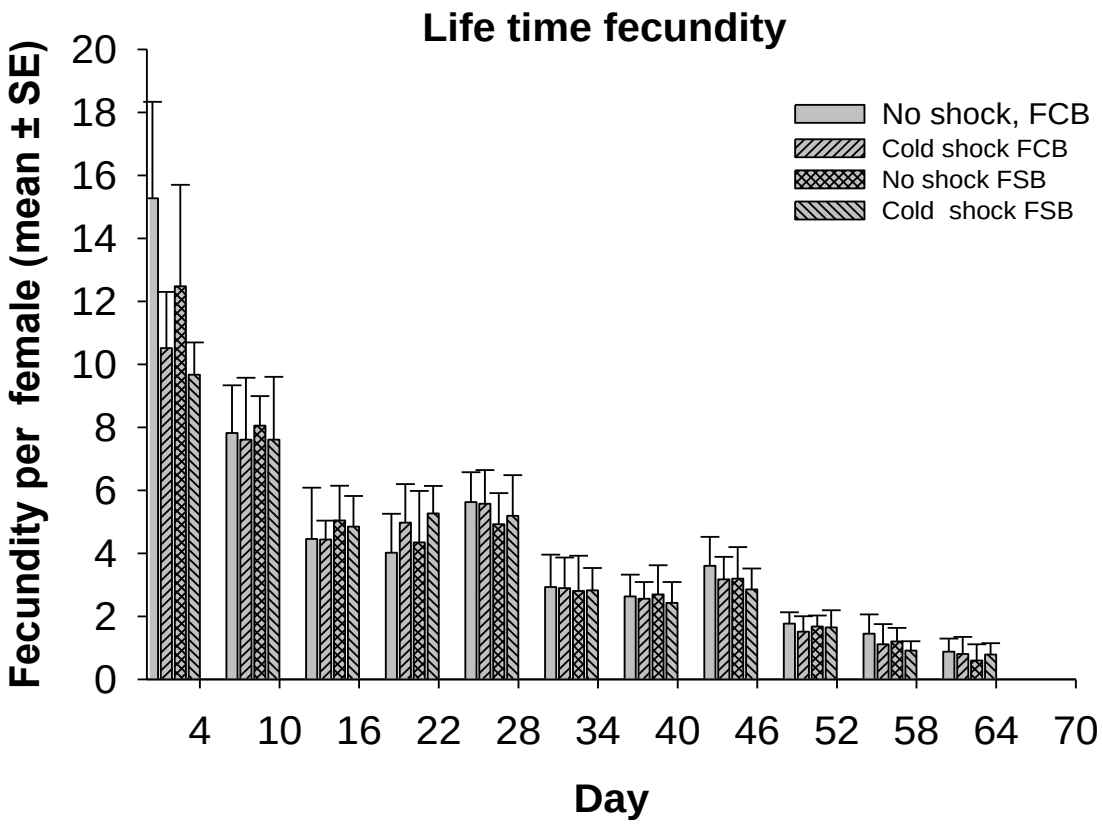

Figure 5.1d: Life time fecundity per female. Fecundity was measured at eleven time points once in every 6 days. Mean fecundity per female for each population and treatment was computed for eleven time points. Results indicate that fecundity reduces with age. However, none of the other effects were significant.

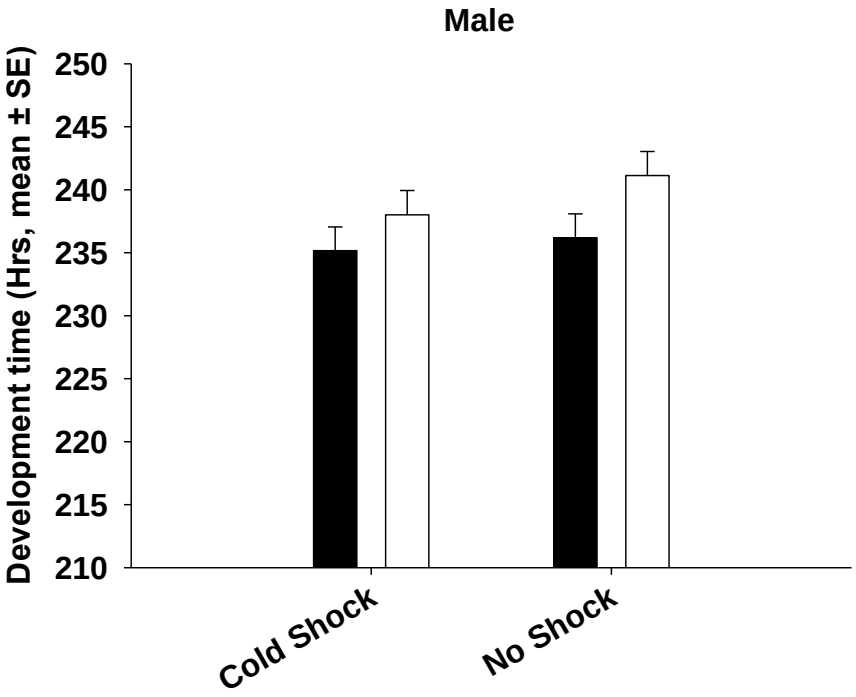

Figure 5.2a: Mean development time (larva to adult) of FSB and FCB males when their parents were subjected Cold shock or No shock treatments. I found a significant effect of Selection regime with FSB males developing 3-4 hours slower than FCB males. Treatment had no significant effect. Open bars represent FSB populations and closed bars represent FCB populations.

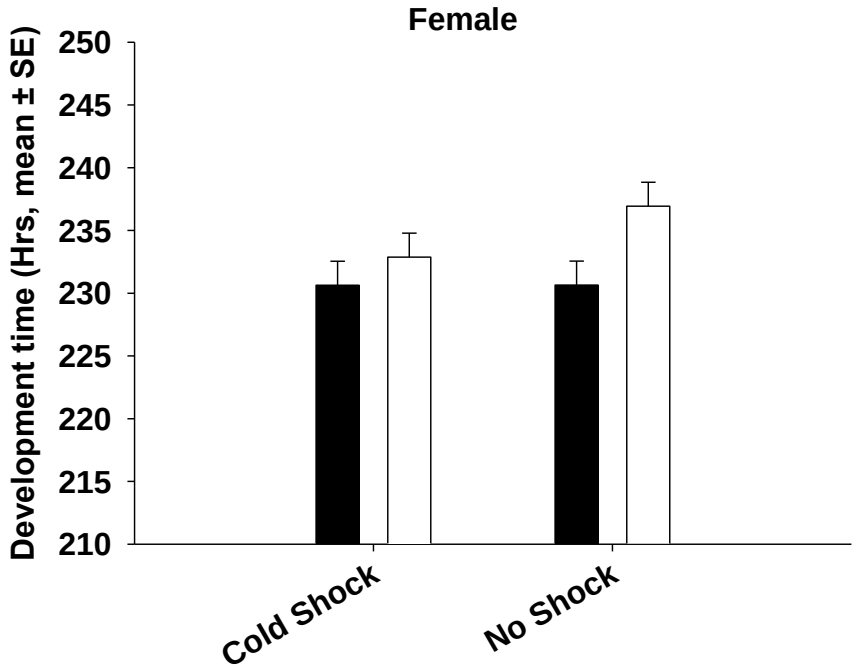

Figure 5.2b: Mean development time (larva to adult) of FSB and FCB females when their parents were subjected Cold shock or No shock treatments. I found a significant effect of Selection regime with FSB females developing 3-4 hours slower than FCB females. Treatment had no significant effect. Open bars represent FSB populations and closed bars represent FCB populations.

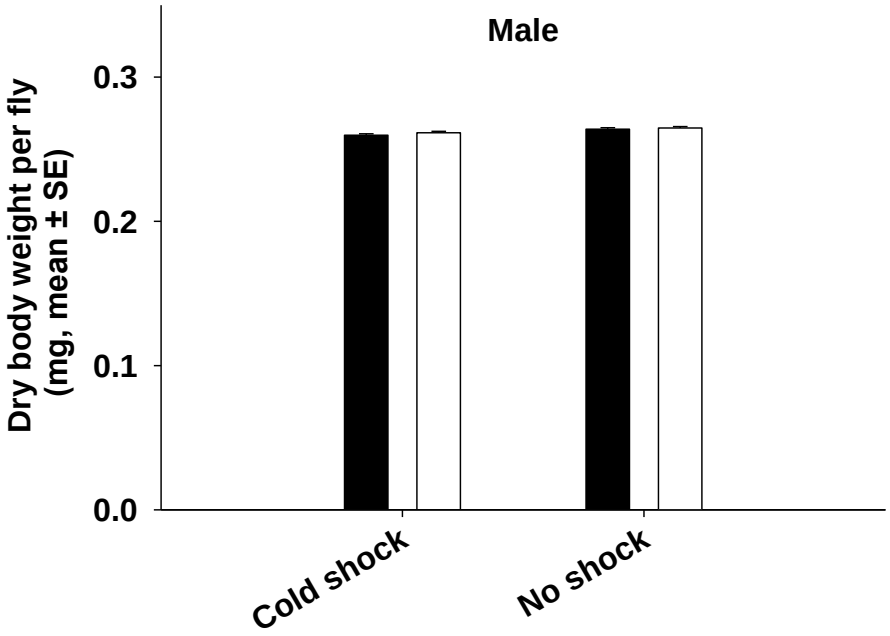

Figure 5.3a: Dry weight at eclosion of males from the FSB and FCB populations. Selection, treatment or selection × treatment interaction did not have significant effect on mean dry body weight. Open bars represent FSB and closed bar represent FCB populations.

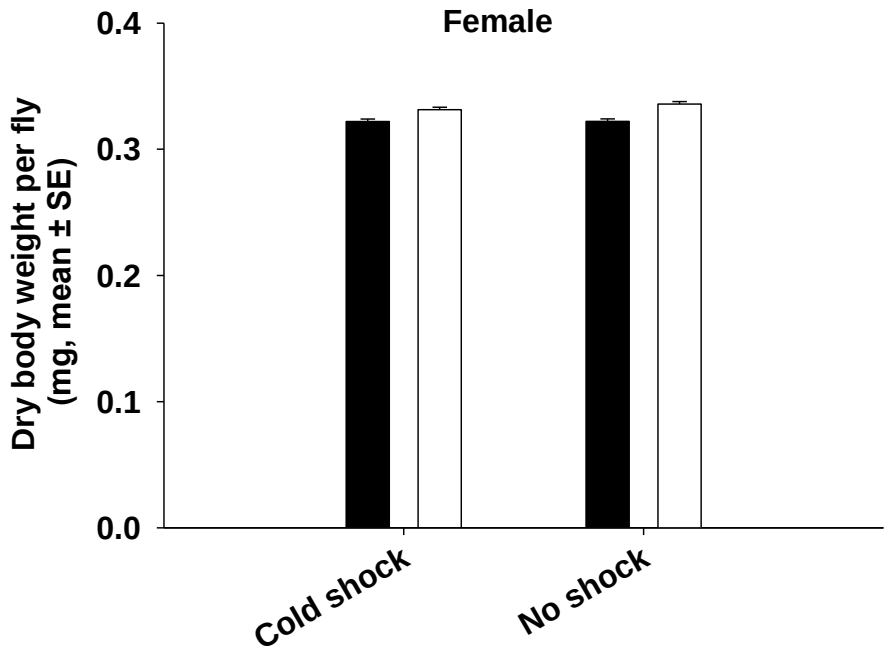

Figure 5.3b: Dry weight at eclosion of females from the FSB and FCB populations. Selection had significant effect on mean dry body weight. However, treatment or selection × treatment did not have significant effects on mean dry body weight. Open bars represent FSB and closed bar represent FCB populations.

DISCUSSION

In this study, I have quantified mean longevity, rates of aging, developmental time and dry body weight in FSB and FCB populations with and without cold shock. Neither longevity nor fecundity was different between FSB and FCB populations. I found that males and females from FSB populations take more time to develop (from first instar larvae to adult). Females from FSB populations were heavier than females from FCB populations. However there was no difference in male body size between FSB and FCB populations. Taken together, I find no evidence for a tradeoff between the ability to resist cold stress and important life-history traits.

The correlation between cold shock resistance and longevity is variable across studies. MacMillan et al. (2009), using a selection protocol very similar to mine found that females of the cold shock selected populations had decreased longevity compared to controls whereas no such difference was visible in the males. Populations selected for faster chill coma recovery have reduced lifespan compared to controls (Anderson et al. 2005). As opposed to these, Norry and Loeschcke (2002) found that cold adapted populations lived longer at 14°C and shorter at 25°C compared to control populations. Bubliy and Loeschcke (2005) found no change in female longevity between populations selected for cold resistance and their controls. In populations directly selected for increased lifespan, increased cold resistance evolves as a correlated response in adults and pupae of *D. melanogaster* (Luckinbill 1998). In contrast to all these studies, I found that selection for resistance to cold shock has no effect on lifespan or rates of aging. There are several differences (including the base population used for selection, the definition of 'cold stress', the assay protocols, etc.) between these studies that preclude a direct comparison of results. More importantly, other studies, typically select for increased survivorship post cold shock. However, in my study there is very little cold induced mortality. This is further strengthened by the fact the lifespan of FSB and FCB populations that were subjected to cold shock was not different from the longevity of those populations not subjected to cold shock. Thus it is not surprising that longevity did not evolve in my populations.

In several previous studies, fecundity has responded to selection for cold resistance. Anderson et al. (2005) found that at least two of the three replicates in their selection regime evolved lower fecundity. Watson and Hoffmann (1996) found that cold selected

populations had lower fecundity. However, I find no difference in the life time fecundity of FSB and FCB populations. This is in agreement with my earlier, short-term measurement of fecundity in these two populations (see chapter 3). Thus I find no evidence of a trade-off between evolved cold stress resistance and fecundity.

Increased development time can potentially be a cost in organisms like *Drosophila* that inhabit ephemeral habitats and have to complete their development before the habitat disappears. I did find that the FSB males and females have increased development time. However, the magnitude of the increase is very small (3-4 hours) and hence I am not sure that this represents a cost. Increased development time could also represent an adaptation to increase resource storage that might help in resisting stressful conditions. During the late third larval instar stage, *Drosophila* larvae feed rapidly and increase their weight exponentially (Prasad and Joshi 2003). An increase of 3-4 hours of feeding time during this period could drastically increase the amount of resources stored by the larvae. Accordingly, populations of *Drosophila* selected for increased starvation and desiccation stress resistance are known to show increased development time and increased body size (Chippindale et al. 1996, 1998). In my study, therefore, increased development time could represent an adaptation to acquire necessary resources to face cold stress.

Body weight at eclosion is often used as a proxy for the amount of resources stored by the larva. Anderson et al. (2005) and Watson and Hoffmann (1996) found no difference in body size of flies selected for increased cold resistance. In this study, FSB females were heavier at eclosion compared to FCB females. This might indicate that FSB females are storing extra/specific nutrients to survive cold shock. However, body weight was not different between FSB and FCB males. Taken together, this indicates that at least in females, increased development time is likely to be beneficial in terms of increased resource acquisition. It is also to be noted that in my study, females suffer more mortality post cold shock relative to males (see chapter 3).

Absence of any change in lifespan and fecundity of FSB populations can be because of many reasons. Firstly, the evolved cold shock resistance ability of FSB populations might be very cheap. Thus the resources required to combat the effects of cold stress in my selection regime might be very low. This, however, is unlikely to be the case. I already know that the flies in my population need to produce active gametes and mate in order to

increase egg viability post cold shock. Accordingly, FSB populations mate more often than FCB populations post cold shock. Courtship and mating carry a substantial cost to both males and females. Thus the costs of evolved cold shock resistance are expected to be substantial in my selection regime. A second alternative is that the resources are abundant and the FSB populations are able to acquire them as adults. The banana-yeast-jaggery food used in my selection regime is indeed rich. The larval and adult densities are low. Therefore, it is possible that my flies do inhabit resource-rich environment. If this is true, then assays under resource depleted condition should lead to different results. Finally, it is quite possible that the cost of increased cold resistance is paid in a different currency. While I did not find any difference in adult longevity or fecundity, other traits that I have not measured here might have been reduced in FSB populations. The possible set of such traits include starvation and desiccation resistance.

Chapter 6

Mechanisms of cold shock resistance

INTRODUCTION

Insects can be found in almost all habitats ranging from hot springs to cold deserts. Insects are ectothermic organisms and therefore, cannot regulate internal temperature when temperature changes in external environment. Therefore, insects have evolved a number of mechanisms to protect themselves when subjected to external environmental fluctuations. Thermal stress (hot or cold) is reported to cause damage to organisms at both cellular and physiological level (reviewed in Sinclair et al. 2013). Multile studies suggest that damage is caused to various molecules like proteins and lipids along with damage to cell membranes, protein transport machinery, etc. (Chapman 1998, reviewed by Bale 2002, Gullan and Cranston 2005, Gulevsky and Relina 2013). Insects are known to have evolved a range of mechanisms to mitigate and prevent the damage caused due to stress. Change in lipid composition imparting more fluidity to cell membranes is one of the major changes that is often employed (Ronges et al. 2012).

A number of proteins that are involved in repairing protein damage (mis-folding, aggregation, denaturation, etc.) known as heat shock proteins play a crucial role. Heat shock proteins (Hsps) help mitigate the effects of heat stress by protecting proteins and enzymes and facilitating their proper functioning under stressful conditions (reviewed in Feder et al. 1999, Nadeau et al. 2001). Also, an increase in synthesis of a number of other metabolites like glycerol (Chen and Walker 1994), glycogen and cryoprotectants like trehalose, proline, etc (Hodkova and Hodek 2004) is also reported. These metabolites help insects to survive under thermal stress.

Various insects like flesh fly (Teets et al. 2012), stick insects (Dennis et al. 2015), field crickets (MacMillan and Sinclair 2011b) and corn borer (Shang et al. 2015) amongst others, have been used to study the mechanisms that are involved in providing protection against environmental stress. These insects employ a number of mechanisms which could be distinct yet overlapping. *D. melanogaster* has been a favourite model to address questions pertaining to underlying mechanisms of cold tolerance. Results from these

studies suggest that *Drosophila* also employs mechanisms like lipid modification, increase in expression of heat shock proteins, synthesis of other metabolites like proline, glycogen, etc. to survive thermal stress (Chen and Walker 1994, Chen and Walker 1994, reviewed by Bale 2002, Kostal et al. 2011a. Gehrken and Southon 1997).

To understand the underlying mechanisms of evolved cold tolerance, lipid content and mRNA expression of a number of stress response genes was measured in the FSB and FCB populations. These experiments were performed after 63 generations of selection.

METHODS

Maintenance details of the experimental populations (FSB and FCB) are provided in Chapter 2 of this thesis. Experimental flies were generated from standardized flies as explained in Chapter 2. Briefly, flies from both the FSB and FCB regime were not subjected to any selection for one generation. Eggs were collected from these standardized flies at a density of 70 eggs per vial. Eighteen such vials were established for each FSB and FCB population. Flies that emerged out from these vials were used for the experiments. Due to various logistical constraints, gene expression was measured only in males. .

Experiment 1:

Fly rearing for Lipid estimation

Eggs were collected from each of the FSB and FCB populations from the one generation of standardized flies at density of 70 eggs/ per vial provisioned ~ 6 ml of banana-yeast-jaggery food. On the 12^{th} day post egg collection, by which time the flies were roughly 2-3 days old as adults were randomly divided into two treatments – (a) cold shock and (b) no shock treatment. For both cold shock and no shock treatments, flies were transferred to empty glass vials and the cotton plugs were pushed deep in to the bottom leaving one third of the vial space for flies. After that, flies were subjected to either of the two treatments following the protocol mentioned in chapter 2 of the thesis. For each treatment four vials containing 50 males and 50 females (200 males and 200 females in all) were subjected to the treatment. Immediately after treatment, flies were transferred to a Plexiglas cage and a fresh food plate was provided. 24 hours post cold shock, a fresh food plate was provided for one hour. It is expected that most of the stored eggs would be laid in this one hour. After this, another fresh food plate was provided for four hours. These

plates were incubated for 18 hours to allow eggs to hatch. From this plate, 30 first instar larvae were collected into vials each having 6 ml of banana-yeast jaggery food. For a given population and treatment, 10 such vials were set up. These vials were carefully monitored for eclosion. Flies were collected within two hours of eclosion and were immediately flash frozen using liquid nitrogen. Fifty males and 50 females per treatment per population were frozen. These flies were stored at -80°C until assayed for lipid content.

Lipid estimation

Method described in Zwaan et al. (1991) with minor modifications was used to measure lipid content. Single sex groups of five flies were set up. A group of five flies was transferred to a clean 2 ml microcentrifuge tube. Ten such replicates for a given treatment and populations were set up. These tubes were incubated at 65°C for 48 hours. After 48 hours, body weight for each group of five flies was recorded using a fine micro balance. These flies were transferred back to a micro centrifuge tube. Following this, 1.5 ml of diethyl ether was added to each of the tubes. These tubes were placed on a shaker for mild agitation for 24 hours at 25°C. The lipids are extracted out into the ether. After 24 hours, Diethyl ether was discarded, and flies were again dried at 70°C for 12 hours. Body weight was measured after lipid extraction using fine micro balance. Absolute lipid content was calculated as the difference between body weight before and after lipid extraction. Lipid content per fly was calculated by dividing this difference by a number of flies per sample. Fractional lipid content was measured by dividing the lipid content with the body weight of the given sample (Bharathi et al. 2003).

Fractional lipid content was analysed using a three-factor mixed model analysis of variance (ANOVA) with selection regime (FSB vs. FCB) and treatment (Cold shock vs. no shock) as fixed factors crossed with blocks (1-5) as random factors. Multiple comparisons using Tukey's HSD were performed. All the analyses were done at α=0.05 level of significance using Statistica (for Windows, version 10, Statsoft).

Experiment 2:

Fly rearing for gene expression assays

On 9-10th day post-egg collection, males were collected as virgins and were held in groups of ten flies per vial containing 2 ml of banana-yeast-jaggery food, 40 vials per populations were collected. Two days later, i.e., on the 12th day post egg collection, the vials from each population were randomly divided into two treatment groups – Cold Shock and No-Shock. Thus each population × treatment combination now had 20 vials. The protocol described in Chapter 2 of the thesis was used for both the treatments (Cold shocked and Non-shocked). After the treatment, flies from each population × treatment combination were transferred to a Plexiglas cage at a density of 10 vials per cage. Gene expression was measured at two time points – 4 hours and 12 hours post treatment. After subjecting males to a recovery period of 4 (or 12) hours post treatment, flies were flash frozen using liquid nitrogen. Flies were stored at -80°C until RNA extraction.

RNA extraction

For each of the FSB and FCB populations, period (4 and 12 hours) and block (1-5), group of 20 male flies were homogenized in TRI reagent (Sigma-Aldrich) using a motorized pestle. Manufacturer's protocol was followed to extract RNA. Extracted RNA was suspended in 30 µL of diethylpyrocarbonate (DEPC) treated water. Genomic DNA contamination was removed from the sample using RNase-Free DNase digestion column. Following this, RNA quality and quantity was checked using NanoDrop 2000 Spectrophotometer (Thermo Scientific). Absorbance was measured at 260 nm and 280 nm. Absorbance$_{260/280}$ ($A_{260/280}$) was calculated, and samples with $A_{260/280}$ > 1.9 were further used. An aliquot of the RNA sample was taken and first strand cDNA was synthesized using M-MuLV reverse transcriptase enzyme and random hexamers (Thermo Scientific Maxima First-Strand Synthesis kit #K1641). The obtained cDNA was diluted by ten-fold and was later used for measuring the gene expression.

Quantitative Real-Time PCR (qRT-PCR)

A number of genes involved in the stress response were measured. Gene expression of seven genes *viz. hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa (hsp70)*, *hsp83* and *frost* was measured. Expression of a housekeeping gene *rps20* was used as an internal control. Primer sequences for all the genes were obtained from the previous studies (Sinclair et al. 2007a and Colinet et al. 2010). More details of the primer sequences have been given in

Table 6.2. Gene expression was measured using Maxima R green (Thermo Scientific kit # K0221) on Eppendorf Mastercycler. All the samples were run in duplicates. The cycle threshold (C_t) values were obtained and expression of each gene of interest was normalized using the expression of internal control. The following calculations were done:

$$\Delta C_T \text{ (Normalized gene expression)} = C_T\text{(Gene of Interest)} - C_T\text{(Housekeeping gene)}$$

ΔC_T values under shocked and non-shocked conditions were obtained for both FSB and FCB. These ΔC_T values were further used to derive gene expression difference between FSB and FCB under either shocked or non-shocked condition. Therefore, for all the genes, shocked FSB was compared with shocked FCB and non-shocked FSB was compared with non-shocked FCB. This was denoted as $\Delta\Delta C_T$ following:

$$\Delta\Delta C_T = \Delta C_T\text{(FSB)} - \Delta C_T\text{(FCB)}$$

The difference in normalized gene expression between FSB and FCB ($\Delta\Delta C_T$) was used to calculate the fold change in the expression of genes (Pfaffl 2001).

$$\text{Fold Change} = 2\text{^-(}\ \Delta\Delta C_T\text{)}$$

A fold change value of 1 would indicate no difference in expression for the given gene between FSB and FCB. For the analysis, mean fold change across five blocks was calculated and a 95% confidence interval (CI) was constructed around it. If CI overlaps with 1, that indicates no difference in expression between the FSB and FCB.

RESULTS

Experiment 1: Lipid Content

I did not find a significant effect of selection regime on fractional lipid content in both males (Table 6.1a, Figure 6.1a) and females (Table 6.1b, Figure 6.1b). A significant effect of treatment (Cold-shock vs. No-shock) was found in females (p = 0.038). Lower fractional lipid content under No-shock condition was observed in females. None of the interactions were found to be significant.

Experiment 2: Quantitation of gene expression

Expression of almost all the genes that we assayed was higher under Cold-shock condition compared to No-shock condition in both the FSB and FCB populations (data not shown) indicating that these genes are associated with cold shock response. However, my interest was to compare the expression of these genes in the FSB populations relative to FCB populations to address questions about evolution of gene expression patterns. Gene expression was quantified under the Cold-shock (Figure 6.2a) and No-shock (Figure 6.2b) conditions. The confidence interval around fold change in expression (of FSB) for all the genes overlapped with 1 under both Cold-shock and No-shock treatments. The pattern was similar across the two time points of recovery. These results indicate that there is no substantial difference in the expression of these genes between FSB and FCB populations.

Table 6.1. Summary of the results from a three-factor mixed model ANOVA on fractional lipid content in (a) male and (b) female with selection regime (FSB and FCB) and treatment (cold shock and no shock) as fixed factors crossed with blocks (1-5) as random factor. *p*-values in the bold case are statistically significant. Estimated denominator DF (Satterthwaite method) was very low. Hence, for some of the effect (Block) *F* ratio and *p* values are unavailable for this effect.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	0.016	0.016	1	4.000	4.018	0.116
Male	Treatment (Trt)	0.002	0.002	1	4.000	0.350	0.586
fractional	Block (Blk)	0.043	0.011	4	3.526	1.526	0.360
lipid	Sel × Trt	3×10^{-4}	3×10^{-4}	1	4.000	0.120	0.746
content	Sel × Blk	0.016	0.004	4	4.000	1.465	0.360
	Trt × Blk	0.023	0.006	4	4.000	2.158	0.237
	Sel × Trt × Blk	0.011	0.003	4	180.00	0.845	0.498
(b)	Selection (Sel)	0.012	0.012	1	4.000	3.612	0.130
Female	Treatment (Trt)	0.004	0.004	1	4.000	9.272	**0.038**
fractional	Block (Blk)	0.009	0.002	4	0.300	.	.
lipid	Sel × Trt	0.002	0.002	1	4.000	0.399	0.562
content	Sel × Blk	0.014	0.003	4	4.000	0.604	0.681
	Trt × Blk	0.002	4×10^{-4}	4	4.000	0.075	0.986
	Sel × Trt × Blk	0.022	0.006	4	180.00	2.814	**0.027**

Table 6.2. Primer sequences for genes

Gene	Primer	Primer sequence (5' - 3')	Fragment length (bp)
hsp22	Forward	GCCTCTCCTCGCCCTTTCAC	66
	Reverse	TCCTCGGTAGCGCCACACTC	
hsp23	Forward	GGTGCCCTTCTATGAGCCCTACTAC	153
	Reverse	CCATCCTTTCCGATTTTCGACAC	
hsp40	Forward	GAGATCATCAAGCCCACCACAAC	112
	Reverse	CGGGAAACTTAATGTCGAAGGAGAC	
hsp68	Forward	GAAGGCACTCAAGGACGCTAAAATG	88
	Reverse	CTGAACCTTGGGAATACGAGTG	
hsp70Aa	Forward	TCGATGGTACTGACCAAGATGAAGG	98
	Reverse	GAGTCGTTGAAGTAGGCTGGAACTG	
hsp83	Forward	GGACAAGGATGCCAAGAAGAAGAAG	150
	Reverse	CAGTCGTTGGTCAGGGATTTGTAG	
frost	Forward	CGATTCTTCAGCGGTCTAGG	92
	Reverse	CTCGGAAACGCCAAATTTTA	
RpS20	Forward	CCGCATCACCCTGACATCC	134
	Reverse	TGGTGATGCGAAGGGTCTTG	

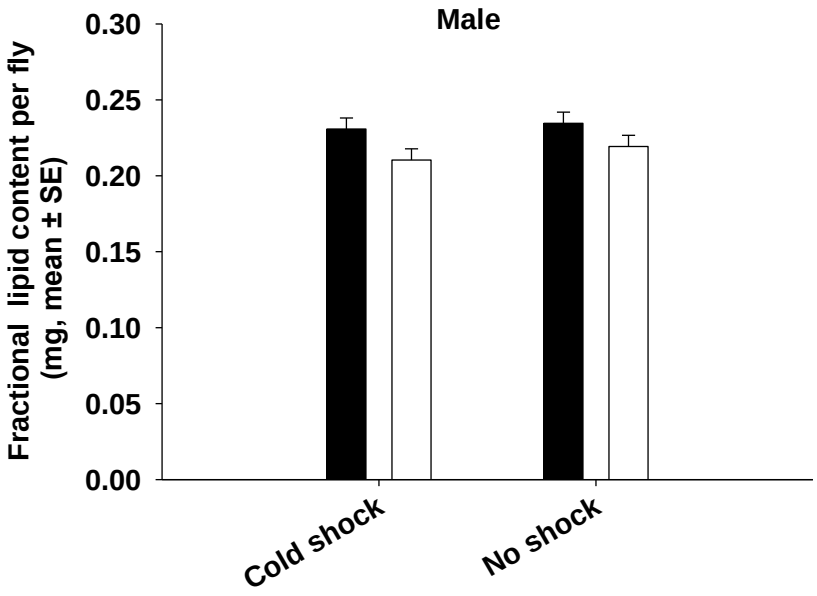

Figure 6.1a. Fractional lipid content in the males. I did not find significant effects of selection, treatment or selection × treatment interaction. Open bars represent FSB and closed bars represent FCB populations.

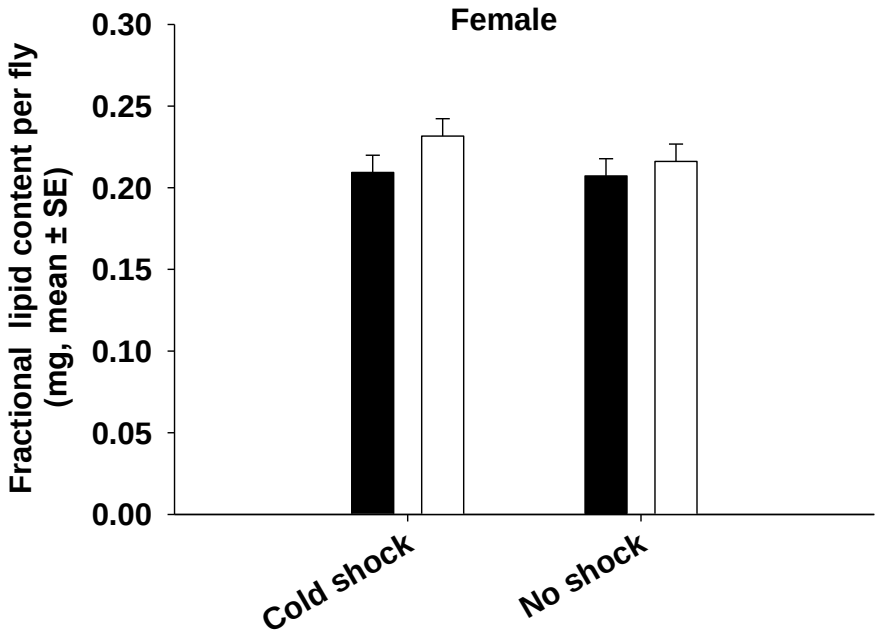

Figure 6.1b. Fractional lipid content in the females. I did not find significant effects of selection or selection × treatment interaction. Open bars represent FSB, and closed bars represent FCB populations.

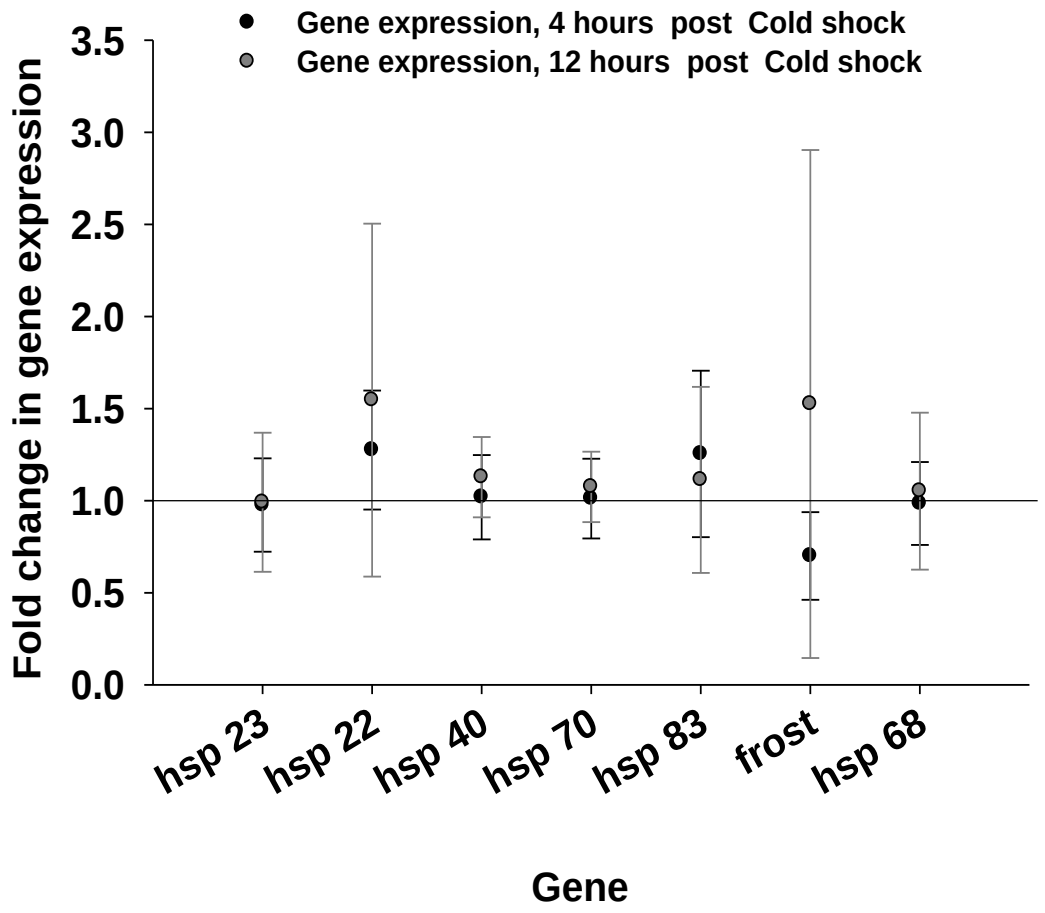

Figure 6.2a: Difference in expression level of genes between FSB and FCB populations under cold shocked condition. I did not find significant expression differences in any of the genes assayed at either 4 hours of recovery or 12 hours of recovery. For each of the genes, expression levels in the FSB populations were relativised to expression level in the FCB populations. Comparisons were within a treatment and time point. For details, see Methods section of this chapter.

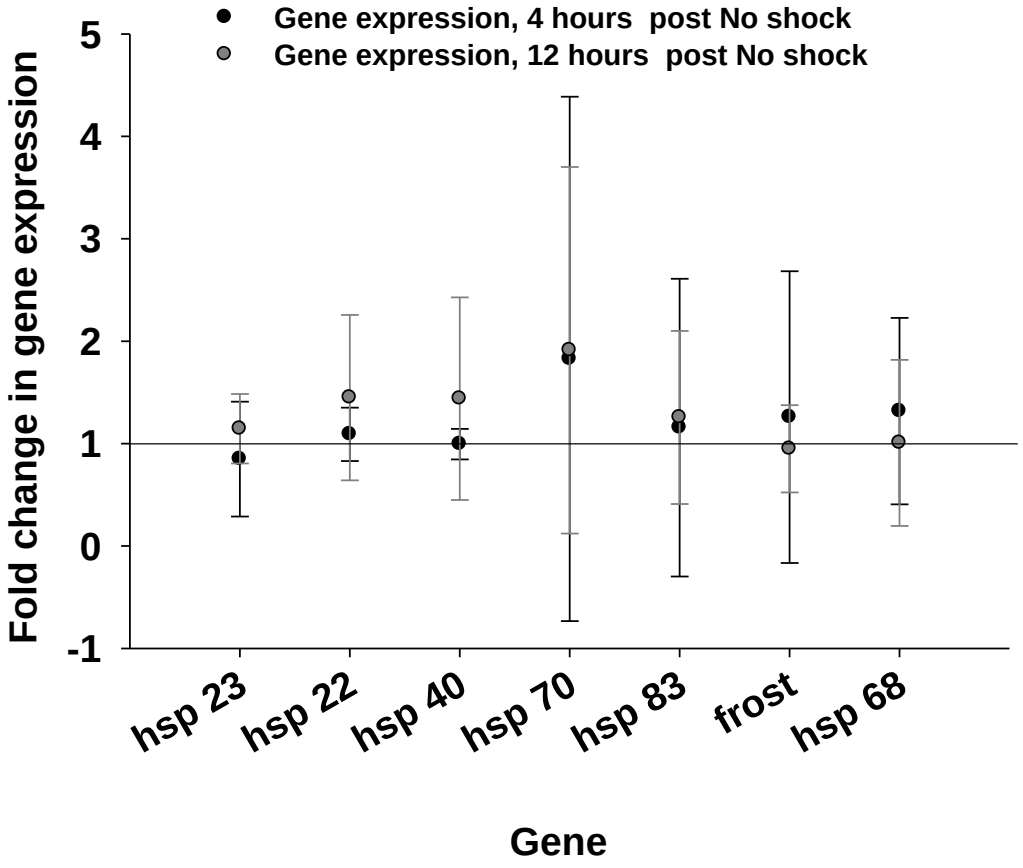

Figure 6.2b: Difference in expression level of genes between FSB and FCB populations under no shocked condition. Under no shock condition, I did not find significant expression differences in any of the genes assayed at either 4 hours or 12 hours. For each of the genes, expression levels in the FSB populations were relativised to expression level in the FCB populations. Comparisons were within a treatment and time point. For details, see Methods section of this chapter.

DISCUSSION

In the current study, I investigated the underlying mechanisms for evolved cold tolerance in FSB populations. As a measure of these mechanisms, I studied (a) fractional lipid content and (b) expression of several genes (*hsp*; heat shock proteins and *frost*) involved in stress response. I did not find any significant difference in either fractional lipid content or gene expression between FSB and FCB populations. A number of physiological and biochemical changes are expected to occur when organisms experience stress whether biotic or abiotic. Different kinds of stresses can elicit different responses. Cold stress often causes damage to proteins, lipids, etc. in the cellular machinery. Ohtsu et al. (1998) showed that lipids play a significant role in maintaining cold tolerance in different species of *Drosophila*. In this study, I did not find a difference in total fractional lipid content between selected and control populations. However, I did not investigate the composition of the lipid. Most of the previous studies suggested that an increase in the unsaturated fatty acid content provides protection against cold stress (Ohtsu et al. 1998) Therefore, there is a possibility that lipid composition might have evolved in the FSB populations without a significant change in fractional lipid content.

Heat shock proteins play an important role in mitigating the damage. I looked at the expression of a number of heat shock proteins (*hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa*, *hsp83)*. In addition to Hsps, I also looked at the expression of *frost*. *frost* is known to be linked with cold adaptation even though its molecular function is yet to be determined (Sinclair et al. 2007a, Colinet et al. 2010). The expression of all the genes that I assayed was higher under cold shocked condition compared to non shocked condition suggesting that these genes are actually involved in the cold shock response. This finding is in agreement with the results of previous studies (Sinclair et al. 2007a, Colinet et al. 2010).

However, I did not find a change in expression of either *hsps* or *frost* in the FSB populations relative to the FCB populations. These results are contrary to the previous studies (Qin et al. 2005, Colinet et al. 2010). There could be various reasons for the observed results. Firstly, my selection maintenance protocol is different from Colinet et al. (2010). Secondly, the time (post recovery) at which I measured the expression of these genes. Expression of Hsps is shown to be temporally regulated (Qin et al. 2005, Sinclair et al. 2007a, Colinet et al. 2010). Colinet et al. (2010) found differences in gene expression when measured after 2 or 4 hours of recovery. I chose the time points of 4 and

12 hours of recovery since I found clear differences in behavioural and fitness related traits between FSB and FCB populations at these time points. Therefore, there is a possibility that measurement of gene expression at different time points during and post treatment might show differences in the gene expression. An additional point of caution is that I assayed only the levels of mRNA, not the functional proteins. It is quite possible that the levels of the RNA products are higher in the FSB populations even when the levels of the RNA itself are not different between FSB and FCB populations.

There are a number of ways using which organisms' combat stress. A further investigation into these mechanisms would provide a better answer to mechanistic basis of evolved cold tolerance in FSB populations. Future research into lipid composition (Saturated vs. unsaturated lipids), other metabolites like glycogen, glycerol, proline, etc. (Kostal et al. 2011, Chen and Walker 1994) could help me find a clear answer. Microarray profiles (Zhang et al. 2011, Telonis-Scott et al. 2009, Qin et al. 2005) of other cold adapted lines in *D. melanogaster* have found a large number of other genes to be involved in cold stress. I also propose future investigation in to these genes to understand the mechanistic basis of the evolved response.

Chapter 7

Evolution of cross-tolerance

INTRODUCTION

Evolution of resistance to a specific type of environmental stress may confer an advantage or a disadvantage with respect to resistance to other types of environmental stresses**.** Multiple earlier studies have suggested that resistance to multiple stresses (such as desiccation and starvation, high and low temperature, starvation and cold temperature) might be correlated (Nghiem et al. 2000, reviewed in Hoffmann et al. 2003b, Bubliy and Loeschcke 2005). Other studies have asked if there are certain universal mechanisms that allow organisms to simultaneously increase resistance to multiple environmental stresses (Hoffmann and Parsons 1991).

A number of studies have documented increased cross-tolerance in insects indicating that either the underlying mechanisms of resistance to these stresses are common or that there are strong genetic correlations between resistance traits. Such a positive correlation has been found between resistance to cold and desiccation as well as between resistance to heat and desiccation (Bayley et al. 2001, Wu et al. 2002, Phelan et al. 2003, Bubliy and Loeschcke 2005, Vermeulen and Loeschcke 2007). Cross tolerance with respect to high and low temperature stress has also been documented. For example, exposure to mild desiccation can increase cold tolerance in the springtail, *F. candida* (Bayley et al. 2001). House flies subjected to anoxic conditions at 27°C show greater tolerance when exposed to -7°C (Coulson and Bale 1992). Positive correlated responses in stress resistance traits have also been observed in laboratory selection studies. For example, Bubliy and Loeschcke (2005) observed increased cold stress resistance in lines selected for resistance to heat stress or desiccation stress. They also observed increased desiccation resistance in lines selected for resistance to heat knock down. Chill coma recovery, cold resistance and desiccation are known to be positively correlated (Sinclair et al. 2007a, Anderson et al. 2005).

Alternatively, mechanisms required to adapt to a specific stress might conflict with mechanisms required to adapt to other kinds of stress, thereby leading to trade-offs across

stress resistance traits (Kellett et al. 2005, Overgaard et al. 2006). For example, Hoffmann et al. (2005a) show that flies selected for starvation resistance have reduced resistance to cold, whereas those selected for increased cold resistance show decreased starvation resistance. Quite often though, the relationship between resistance to various stresses seems to be complex. Bubliy and Loeschcke (2005) found a positive correlation between resistance to cold stress and desiccation. However, Sinclair et al. (2007b), found no change in cold resistance in populations of *D. melanogaster* selected for desiccation resistance. Though it has been suggested that resistance to the multiple stresses that insects commonly encounter in nature (such as temperature extremes, desiccation, etc.) should be positively correlated, the evidence for such correlations is variable (Ring and Danks 1994).

In this study, my major goal was to assess if increased resistance to cold shock leads to any correlated response in resistance to other environmental stresses such as resistance to desiccation, starvation and heat shock, bacterial infection. My study was carried out on 10 populations of *D. melanogaster* (5 selected populations and 5 control populations), and experiments were conducted over 57-71 generations of selection.

I specifically addressed the following questions:

(a) Does egg viability and reproductive behavior post heat shock evolve as a correlated response to increased resistance to cold shock?

(b) Does adult survival under starvation, desiccation, heat shock, cold shock and pathogenic infection evolve in the selected populations of *D. melanogaster*?

METHODS

Experimental population: Details of the experimental populations are described in chapter 2.

Experimental protocol:

Experiment 1: Effect of heat or cold stress on mating ability and egg viability

From my previous study I know that both mating behavior and egg viability are influenced by cold shock and that the populations selected for cold shock show higher mating frequency and egg viability relative to control populations post cold shock (Singh et al. 2015). In this experiment, I wanted to know whether egg viability and mating frequency show a correlated response with heat stress also. After 60 generations of selection, experimental flies were generated after one generation of standardization (as described in chapter 2) above. Twelve vials (with a density of 70 eggs per vial) were established for each FSB and FCB populations from the respective standardized populations. On the 12th day (by which time the flies were 2-3 days old as adults) after egg collection, vials containing flies were randomly assigned to one of the following three treatments:

(a) Cold-shock: both males and females from a given population were imposed to cold shock (as described in Chapter 2 of this thesis) and following this, flies were immediately transferred into a Plexiglas cage at a density of 100 mating pairs per cage.

(b) Heat-shock: both males and females from each FSB and FCB populations were subjected to (heat shock as described in Chapter 2 of this thesise) and after this flies were quickly transferred to the Plexiglass cage at a density of 100 mating pairs per cage.

(c) No-shock: both males and females from each FSB and FCB populations were subjected to a temperature of 25ºC for one hour (as described in Chapter 2) and subsequently transferred to a Plexiglas cage at a density of 100 mating pairs per cage.

Post treatment, I assayed the egg hatchability at two points -

(a) 0-hour post cold shock/heat shock/no shock

(b) 24 hours post cold shock/heat shock/no shock

I selected these two time points for the following reasonsEgg viability measurement at 0 hours post shock would show the immediate effect of treatment (cold shock/heat shock/no shock), also, in their normal maintenance regime eggs are collected from the flies 24 hours after shock to start the next generation and therefore the 24 hour time point it is directly important to the fitness of the flies. Immediately, post treatment (cold shock/heat shock/no shock) flies of each populations were transferred in cages. Fresh food plates were provided in the cages for the females to lay eggs for 6 hours. A sample of 200 eggs was moved to a Petri plates containing 1.24 % agar from the food plates. Thess plates were incubated at standard laboratory conditions (as described in chapter 2) for 48 hours, after which, the numbers of hatched eggs were counted and percentage egg hatchability was computed to estimate the egg viability. Twenty fours after the treatment (cold shock/heat shock/no shock), I remeasured egg viability, following the protocol as describerd above.

I also measured the total number of mating for all the three treatments. Once the flies were transferred to Plexiglas cages, I observed the cages every half an hour and recorded the total number of mating pairs (I chosed 30 minutes time intervals to observe matings because in my previous experiment I found that the mean copulation duration did not differ between the selected and control populations, and the value varied between 15 to 17 minutes across the populations as described in chapter 4a). These observations were carried out until 36 hours post treatment. In the normal maintenance regime, eggs are collected to initiate the next generation in an 18 hour period between 24-42 hours after the cold shock. Since any mating that occurred until about 36 hours after the cold shock can, contribute to progeny production, I decided to measure the number of mating pairs until 36 hours after treatment. I then summed the number of mating pairs from all the observations for period of 0-36 hours for each cage to obtain an estimate of the total number of mating pairs. The total number of mating pairs per cage was used as the unit of analysis.

Experiment 2: Effect of heat or cold stress on adult survivorship

I wanted to assess whether the adult survivorship has changed in the selected populations (FSB) after 63 generations of selection. I also wanted to know whether the population selected for increased resistance to cold shock could show cross-tolerance to other stress i.e., heat shock. I already knew from previous studies that both cold shock and heat shock

influence adult survivorship (Tucic 1979, Chen and Walker 1993, Rohmer et al. 2004, MacMillan et al. 2009).

I collected eggs to generate experimental flies after 63 generations of selection followed by one generation of standardization (see details in chapter 2). Twenty five vials were set up for each of the FSB and FCB population. Virgin males and females were collected on 9-10th day from the peak of eclosion under mild CO_2 anesthesia and were housed separately in single sex vials at a density of 10 flies/vial. On the 12th day post egg collection, flies were transferred into an empty, dry, glass vial and cotton plug was inserted down into the bottom one third of vial to allow flies to stay in a restricted space. After that these vials were randomly assigned one of the following treatments:

(a) Cold shock: Vials containing female flies were exposed to -5°C in water-ice-salt slurry for one hour (as described in Chapter 2). Male flies were handled as described in chapter 2 except that they were exposed to -5.6°C (I used -5.6°C to get at least 50% mortality post cold shock) instead of -5°C for one hour in water-ice-salt slurry.

(b) Heat shock: both male and female flies were handled as described in chapter 2 for heat shock treatment in this experiment except that the temperature was different in the following manner:
(1) Vials containing male flies were exposed to 38.9°C temperature for 1 hour in water-bath.
(2) Vials containing female flies were subjected to 39.2°C temperature for 1 hour in water-bath. I used different temperatures to get about 50% mortality.

Three replicate Plexiglas cages of 100 flies per cage were set up for per treatment, per population, per block and sex. I measured adult survivorship at 24 hours post shock. I selected this time point because 24 hours post cold shock is the time that eggs are collected from the flies to start the next generation in their normal maintenance regime and it is hence directly relevant to the fitness of the flies. Twenty four hours post cold shock, dead flies (if any) were aspirated out of the cage and counted. Mean percentage mortality of each cage was used as the unit of analysis.

Experiment 3: Starvation Resistance

Sex specific starvation resistance assay was carried out after 57 generations of selection. Experimental flies were generated from standardized flies (see chapter 2). Assay was carried out using the method described in Kwan et al. (2008) but with minor modifications. Ten vials with density of 70 eggs/vial were established for each of the FSB and FCB populations. Virgin flies were collected on 9-10th day during peak of eclosion, using light CO_2 anesthesia. Males and females were held separately at a density of 10 flies per vial containing 2 ml of fresh banana-yeast-jaggery food. On the 12^{th} day, flies were transferred from food vials to 1.24% agar vials (Kwan et al. 2008). Seven replicate vials were set up for each sex per populations (FSB and FCB). Flies were transferred into a fresh agar vial (1.24%) every alternate day until the last fly in a given vial died. Mortality was recorded every four hours. Mean time of mortality was computed for each vial and was used as the unit of analysis.

Experiment 4: Desiccation Resistance

Sex-specific desiccation resistance assay was performed for each of the FSB (1-5) and FCB (1-5) populations. After 57 generations of selection, experimental flies were raised following one generation of standardization for both the FSB and FCB populations. Ten vials containing eggs at a density of 70 eggs/vial were set up for each of the FSB and FCB populations. On 9-10^{th} day post egg collection, virgin flies were collected using light CO_2 anesthesia and were kept in vials provisioned with 2 ml of banana-yeast-jaggery food at a density of 10 virgin males or females in a vial. On the 12^{th} day the flies were transferred from food vial to food-less glass vial and thin film of cotton was inserted into the vial to confine flies to stay in one third of vials and 6 g of silica gel (desiccant) was added into each vials.The flies were separated from the silica gel by a thin layer of cotton. The open end of each vial was sealed with Parafilm (Kwan et al. 2008). Seven such replicate vials were set up at a density of 10 flies per vial for each of the FSB and FCB populations. Mortality was recorded every half an hour until the last fly died. Mean time to death was computed for each vial and was used as the unit of analysis.

Experiment 5: Resistance to bacterial infection

I investigated whether flies selected for resistance to cold stress have also evolved resistance to bacterial infection as a correlated response after 70 generations of selection. To generate the experimental flies, eggs were collected from standardized flies (see

chapter 2) at a density of 70 eggs/vial containing 6 ml of banana-yeast-jaggery food. Five vials were set up for each of the FSB and FCB populations. On 9-10th day post egg collection, virgin males and females were sorted using light CO_2 anesthesia at very young stage (approximately 4 hours post eclosion) and housed in vials provisioned with 2 ml of banana-yeast-jaggery food at a density of 10 individuals per vial. On the 12th day post egg collection, flies of known age (2-3 days old as adult), population regime and sex (see below for details) were lightly anaesthetized using CO_2. Fifty five to sixty flies of each sex for each of the FSB and FCB populations were infected by pricking the lateral thorax with a Minutien pin (0.1 mm, fine Science Tools, Foster City, CA, USA) dipped in the bacterial slurry (*Staphylococcus succinus* subsp. *succinus* strain PK-1 is a natural pathogen of *D. melanogaster*, which I isolated from wild captured *Drosophila*) of OD 2 as measured at600nm (Vanessa Corby-Harris et al. 2008). For sham infection, the pin was dipped in 10 mM $MgSO_4$ prior to pricking the thorax of the flies. The number of dead flies in each vial was tracked at 3 hour intervals till 30 hrs post infection. After this period, vials were checked every hour till 80 hrs post infection. Proportion of flies that survived the infection was calculated for each population and was used as the unit of analysis.

Statistical analysis

Egg viability data was analyzed using a four-factor mixed model analysis of variance (ANOVA) with selection regime (FSB vs. FCB), treatment (Cold shock/ no shock/ heat shock) and period (0 hour vs. 24hours) as fixed factors crossed with blocks (1-5) as random factor. All multiple comparisons were performed employing Tukey's HSD. Mating number data was analyzed using a three-factor mixed model ANOVA with selection regime (FSB vs. FCB) and treatment (Cold shock vs. no shock/ heat shock) as fixed factors crossed with blocks (1-5) as random factor. All multiple comparisons were performed using Tukey's HSD. For resistance to heat or cold stress, percentage of male and female mortality was calculated. These data was analyzed with a two-factor mixed model ANOVA where selection regime (FSB vs. FCB) was taken as a fixed factor crossed with random blocks (1-5). Starvation resistance and desiccation resistance data from males and females were analyzed separately using a two-factor mixed model ANOVA with selection regime (FSB vs. FCB) as fixed factor crossed with random blocks (1-5). Data from mortality of male and female post bacterial infection was analyzed with a two factor mixed model ANOVA with selection regime (FSB vs. FCB) as fixed factor

crossed with random blocks (1-5). I also analyzed the mortality post infection data using Kaplan-Meier estimator. All the analyses were done at $\alpha = 0.05$ level of significance using Statistica (for Windows, version 10, StatSoft).

RESULTS

Experiment 1: Egg viability and mating ability post heat shock or cold shock

My results indicate that egg viability has evolved in response to selection. I found significant main effect of selection and treatment on the egg viability (Table 7.1). I also found a two way interaction between selection (FCB and FSB) and treatment (Cold shock, heat shock and no shock) (Table 7.1). Multiple comparisons employing Tukey's HSD suggested that egg viability in no shocked treatment was more than 90% and there was no significant difference between the FCB and FSB populations (Figure 7.1). Cold shock and heat shock treatment significantly reduced egg viability (Figure 7.1). At 0^{th} hour post cold shock, egg viability was found to be drastically low (approximately 2-3%) and post heat shock egg viability was also severely reduced (approximately 5-10%). However, difference between FSB and FCB population was not significant at this time point (Table 7.1). However, 24 hours later multiple comparisons using Tukey's HSD suggested that FSB population had significantly greater egg viability when compared to FCB population 24 hours after cold shock (difference ~ 41%). Twenty four hours post heat stress FSB populations had ~ 7% greater egg viability relative to FCB populations, although this difference was not significant (Figure 7.1, Table 7.1). However in case of no shock treatment egg viability remained same and I did not find significant difference between FSB and FCB populations (Figure 7.1).

I found significant main effect of selection and treatment on the number of mating pairs. I also found a statistically significant two way selection × treatment interaction (Table 7.2). Multiple comparisons using Tukey's HSD indicated that flies subjected to cold shock treatment show nearly twice as many mating pairs when compared to flies subjected to heat shock or no shock treatment (Figure 7.2). FSB populations had approximately twice as many mating pairs post cold shock or heat shock when compared to FCB populations (Figure 7.2). However, in case of neither shock treatment FSB populations had about 7% more mating pairs compared to FCB populations (Figure 7.2).

Experiment 2: Mortality post cold or heat shock

I quantified the effect of selection on virgin male and female mortality post cold/heat shock. I found a significant effect of selection on male and female mortality post cold shock (Table 7. 3a, 3b, Figure 7.3a, 3b). In case of males, 24 hours post cold shock FSB populations had about 35% lower mortality compared to the FCB populations (Figure 7.3a). In case of females, 24 hours after receiving cold shock, FSB populations had approximately 29% lower mortality than FCB population (Figure 7.3b). These results indicate that post cold stress both males and females of the FSB populations have evolved in terms of significantly higher survivorship relative to FCB populations.

Twenty four hours post heat shock in males, I found significant effect of selection and block on mortality (Table 7.3c). A significant effect of selection suggested that FSB populations had lower mortality (about 15%) compared to FCB populations (Figure 7.3c). For females, I found a significant effect of selection on mortality post heat shock (Table 7.3d). A significant effect of selection indicated that FSB population had approximately 11% lower mortality compared to FCB population (Figure 7.3d).

Experiment 3: Evolution of starvation resistance

I found that starvation resistance was negatively correlated with resistance to cold stress. Starvation resistance was significantly lower in the FSB populations compared to FCB populations (Table 7.4a, 4b, Figure. 7.4A, B). I found a significant main effect of selection and block on starvation resistance in males (Table 7.4a) and in females (Table 7.4b). Compared to FCB populations, resistance to starvation (in terms of mean time to death) in FSB males is lower by approximately 15 hours and in FSB females by about 12 hours (Figure 7.4A, B).

Experiment 4: Desiccation resistance

I found significant main effect of selection on female desiccation resistance (Table 7.5b). FSB females have higher resistance to desiccation (in terms of mean time to death) by about one hour ten minutes as compared to FCB populations (Figure 7.5B). However, I did not find an effect of block or any two way interaction (selection × block). In case of males, I did not find any effect of selection or block on desiccation resistance (Table 7.5a, Figure 7.5A).

Experiment 5: Resistance to bacterial infection

I did not find any significant main effect of selection on male or female survivorship post bacterial (*Staphylococcus succinus* subsp. *succinus* strain PK-1) infection (Table 7.6a, 6b). Survivorship in case of males in both FSB and FCB population is about 58-63% (Figure 7.6A) and in case of females survivorship was about 62-65% (Figure 7.6B). The results were similar even if the data were analyzed using Kaplan-Meier method.

Table 7.1. Summary of results of a four-factor mixed model ANOVA on egg viability with Selection regime (FSB and FCB), period (0 hour and 24 hours) and Treatment (Cold Shock, Heat Shock, No Shock) as the fixed factors crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	809.927	809.927	1	4.0	32.281	**0.005**
Period (Per)	10548.740	10548.740	1	4.0	85.652	**<0.001**
Block (Blk)	372.991	93.248	4	0.8	1.299	0.603
Treatment (Trt)	68439.490	34219.740	2	8.0	480.344	**<0.001**
Sel × Per	1152.285	1152.285	1	4.0	21.762	**<0.010**
Sel× Blk	100.358	25.090	4	4.0	0.383	0.812
Sel× Trt	1337.526	668.763	2	8.0	15.001	**0.002**
Per× Blk	492.633	123.158	4	6.4	1.194	0.398
Per× Trt	6370.580	3185.290	2	8.0	38.732	**<0.001**
Blk× Trt	569.920	71.240	8	7.4	0.752	0.653
Sel× Per× Blk	211.798	52.950	4	8.0	1.653	0.253
Sel× Per× Trt	1107.268	553.634	2	8.0	17.280	**0.001**
Sel× Blk× Trt	356.647	44.581	8	8.0	1.391	0.326
Per× Blk× Trt	657.908	82.238	8	8.0	2.567	0.102
Sel× Per× Blk× Trt	256.309	32.039	8	.	.	.

Table 7.2. Summary of the results of a three-factor mixed model ANOVA on mating number with selection regime (FSB and FCB) and treatment (cold shock, heat shock or no shock) as fixed factors crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	6424.033	6424.033	1	4.000	36.134	**0.004**
Block (Blk)	3151.533	787.883	4	7.545	1.392	0.323
Treatment (Trt)	6744.800	3372.400	2	8.000	6.597	**0.020**
Sel×Blk	711.133	177.783	4	8.000	1.446	0.304
Sel×Trt	3819.467	1909.733	2	8.000	15.528	**0.002**
Blk×Trt	4089.867	511.233	8	8.000	4.157	**0.030**
Sel×Blk×Trt	983.867	122.983	8	.	.	.

Table 7.3. Summary of the results of a two-factor mixed model ANOVA on mortality in male (a) and in female (b) post cold shock, and on mortality in male (c) and in female (d) post heat shock with selection regime (FSB and FCB) as the fixed factor crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	0.566	0.566	1	4	40.209	**0.003**
Male	Block (Blk)	0.296	0.074	4	4	5.245	0.069
cold shock	Sel×Blk	0.056	0.014	4	20	2.138	0.114
(b)	Selection (Sel)	0.637	0.637	1	4	52.076	**0.002**
Female	Block (Blk)	0.268	0.067	4	4	5.486	0.064
cold shock	Sel×Blk	0.049	0.012	4	20	2.240	0.101
(c)	Selection (Sel)	0.154	0.154	1	4	69.272	**0.001**
Male	Block (Blk)	0.227	0.057	4	4	25.460	**0.004**
heat shock	Sel×Blk	0.009	0.002	4	20	0.206	0.932
(d)	Selection (Sel)	0.105	0.105	1	4	60.146	**0.001**
female	Block (Blk)	0.026	0.006	4	4	3.737	0.115
heat shock	Sel×Blk	0.007	0.002	4	20	0.630	0.647

Table 7.4. Summary of the results from a two-factor mixed model ANOVA on resistance to starvation in male (a) and female (b) with selection regime (FSB and FCB) as the fixed factor crossed with random blocks (1-5). Mean time to death in hours for each vial was used as the unit of analysis. *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	3895.987	3895.987	1	4	9.621	**0.036**
Male	Block (Blk)	27014.640	6753.661	4	4	16.678	**0.009**
starvation	Sel×Blk	1619.819	404.955	4	60	2.515	0.051
(b)	Selection (Sel)	2615.800	2615.800	1	4	8.713	**0.042**
Female	Block (Blk)	8144.076	2036.019	4	4	6.782	**0.045**
starvation	Sel×Blk	1200.860	300.215	4	60	0.799	0.530

Table 7.5. Summary of results from a two-factor mixed model ANOVA on resistance to desiccation in male (a) and female (b) using Selection regime (FCB and FSB) as fixed factor crossed with random Blocks (1-5). Mean time to death in hours for each vial was used as the unit of analysis. *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	18812.010	18812.010	1	4	1.106	0.352
Male	Block (Blk)	248600.700	62150.180	4	4	3.654	0.119
desiccation	Sel×Blk	68032.720	17008.180	4	60	6.778	**<0.001**
(b)	Selection (Sel)	108723.600	108723.600	1	4	16.430	**0.015**
Female	Block (Blk)	57620.890	14405.220	4	4	2.177	0.235
desiccation	Sel×Blk	26469.110	6617.276	4	60	0.757	0.558

Table 7.6: Summary of results of a two-factor mixed model ANOVA on proportion of survivorship post bacterial infection in male (a) and female (b) with selection regime (FSB and FCB) as the fixed factor crossed with blocks (1-5) as random factor. *p*-values in bold are statistically significant.

Traits	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	0.001	0.001	1	4	2.107	0.220
Male	Block (Blk)	0.011	0.003	4	4	3.963	0.105
survivorship	Sel×Blk	0.003	0.001	4	.	.	.
(b)	Selection (Sel)	0.003	0.003	1	4	3.114	0.152
Female	Block (Blk)	0.006	0.002	4	4	1.862	0.281
survivorship	Sel×Blk	0.003	0.001	4	.	.	.

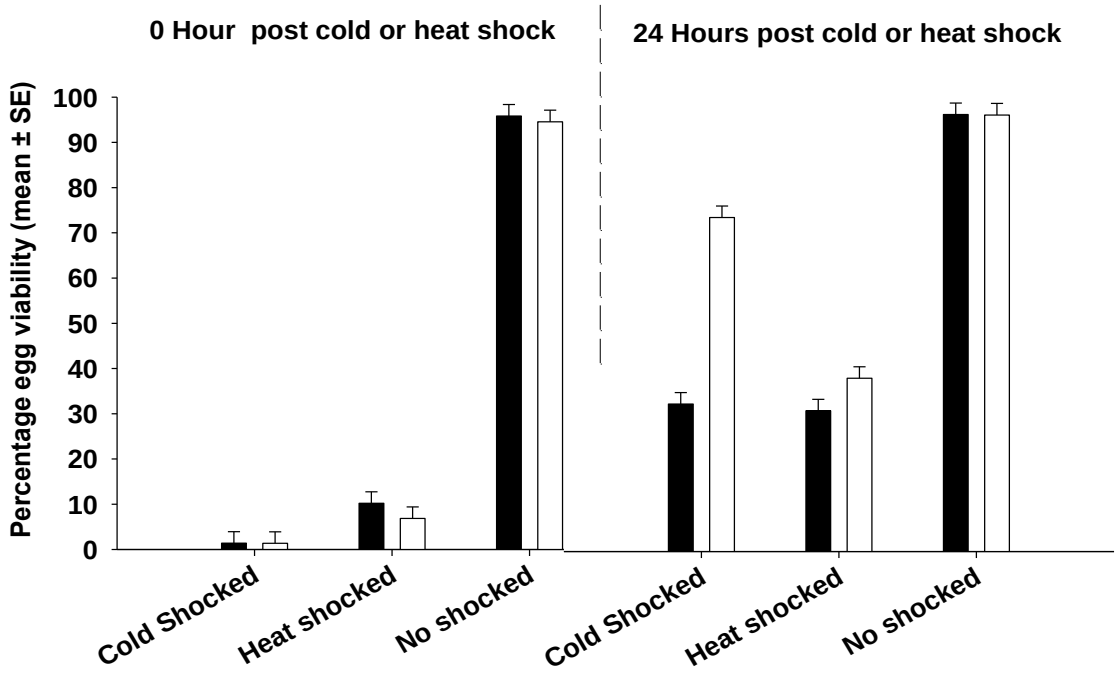

Figure 7.1: Effect of cold shock or heat shock on egg viability. I measured egg viability at 0 and 24 hours post heat/cold shock. Open bars represent FSB and closed bars represent FCB populations. Viability of eggs from No-shock treatment was high with no difference between FCB and FSB populations. At 0 hours post cold shock, viability of eggs from the cold-shock and heat-shock treatment was very low and not different between FCB and FSB populations. However, 24 hours post cold shock, egg viability improved and the FSB populations had significantly higher egg viability than the FCB populations.

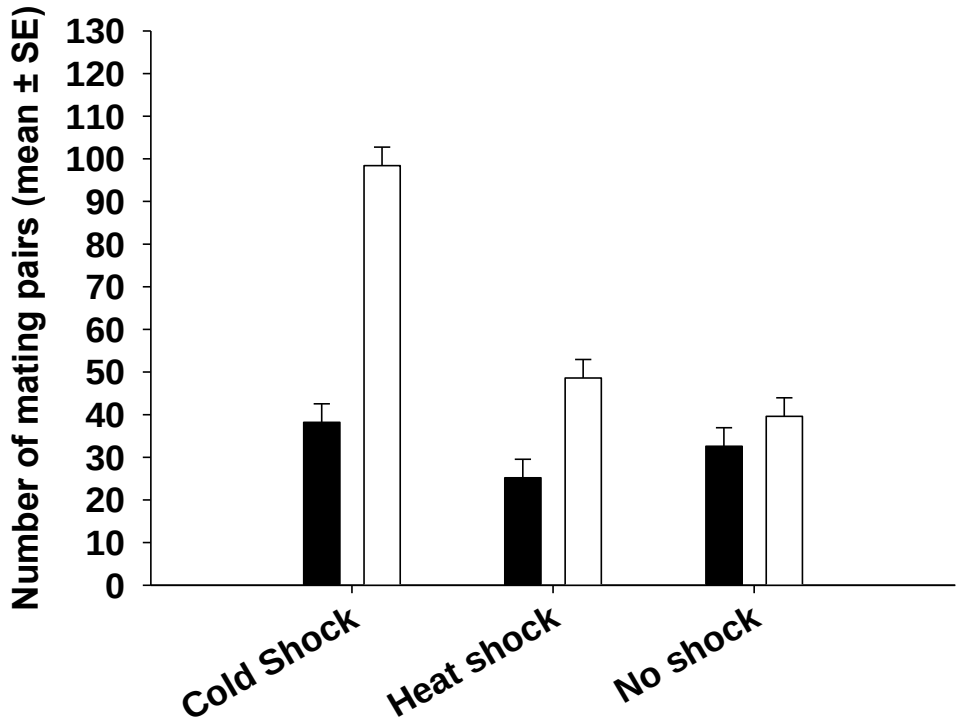

Figure 7.2: Effect of cold shock or heat shock on mating. I assayed mating frequency post heat or cold shock (0-36 hours). Open bars represent FSB and closed bars represent FCB populations. The number of mating pairs observed in FSB flies from cold-shock and heat shock treatment was significantly higher relative to FCB populations.

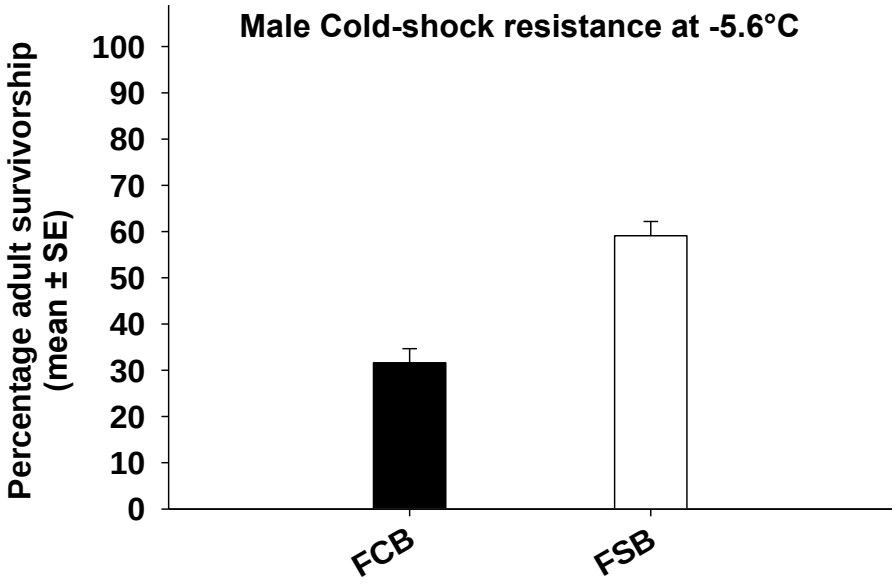

Figure 7.3a: Effect of cold shock on survivorship of virgin males. FSB populations had significantly higher survivorship relative to FCB populations.

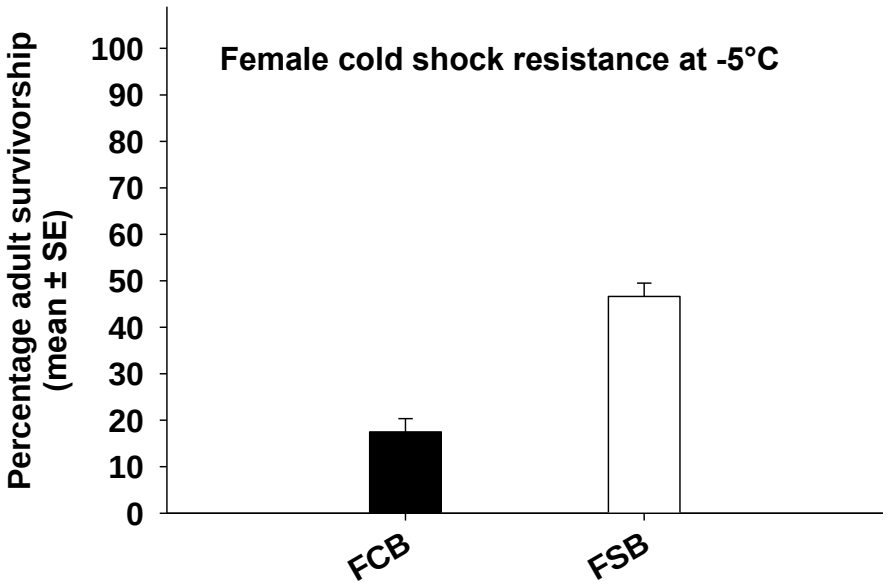

Figure 7.3b: Effect of cold shock on survivorship of virgin females. FSB populations had higher survivorship relative to FCB populations.

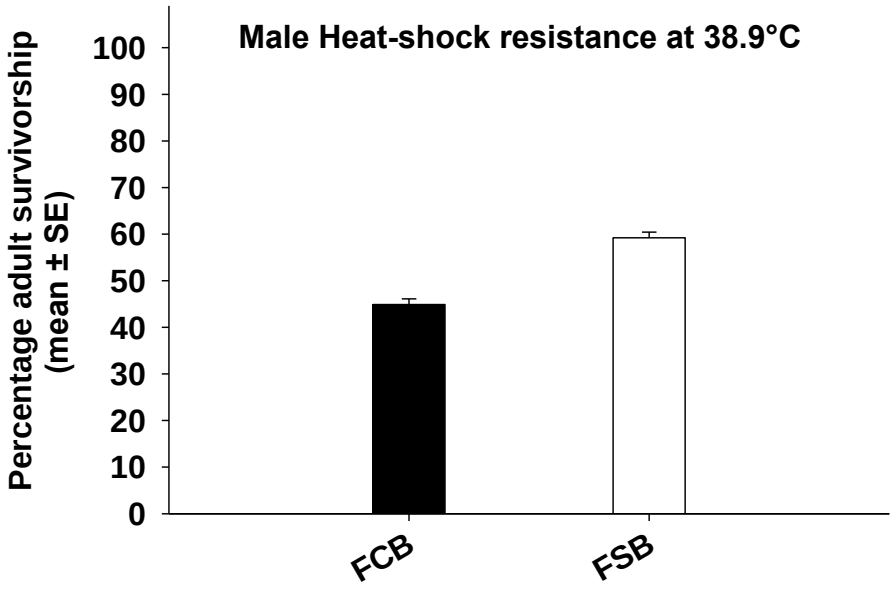

Figure 7.3c: Effect of heat shock on survivorship of virgin males. FSB populations had higher survivorship relative to FCB populations.

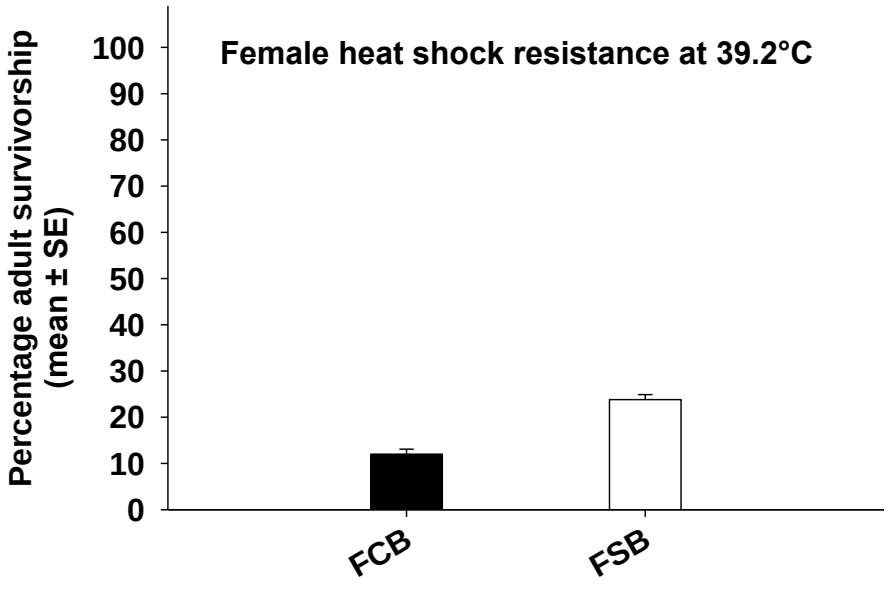

Figure 7.3d: Effect of heat shock on survivorship of virgin female FSB populations had higher survivorship relative to FCB populations.

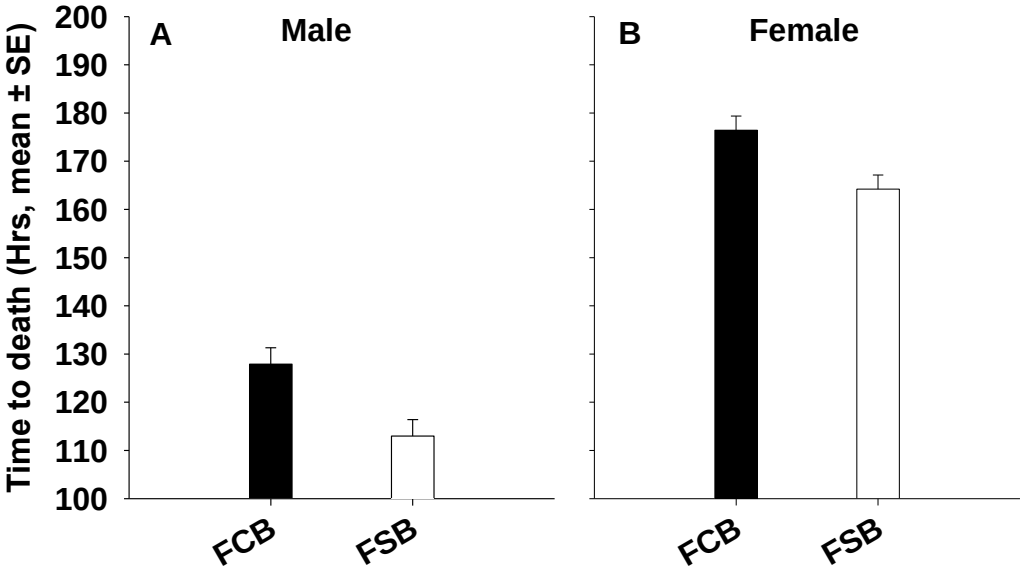

Figure 7.4: Starvation resistance in male (A) and (B) female. (A) Male from FSB populations had lower starvation resistance relative to male from FCB populations. (B) Female from FSB populations had lower starvation resistance relative to female from FCB populations.

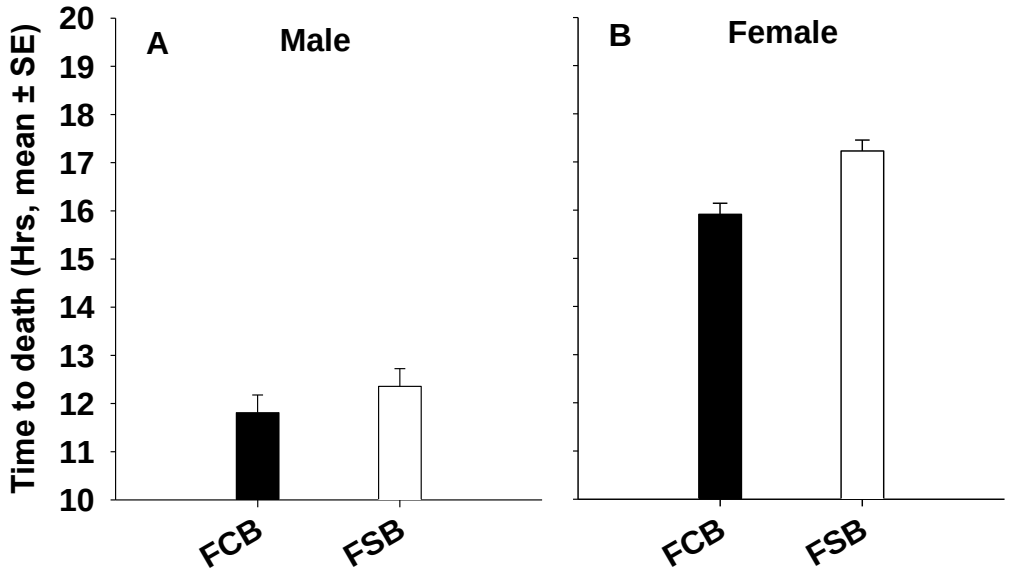

Figure 7.5: Desiccation resistance in male (A) and (B) female. (A) I did not find any significant main effect of selection on male desiccation resistance. (B) I found significant main effect of selection on female desiccation resistance, indicating that female from FSB populations had higher desiccation resistance relative to female from FCB populations.

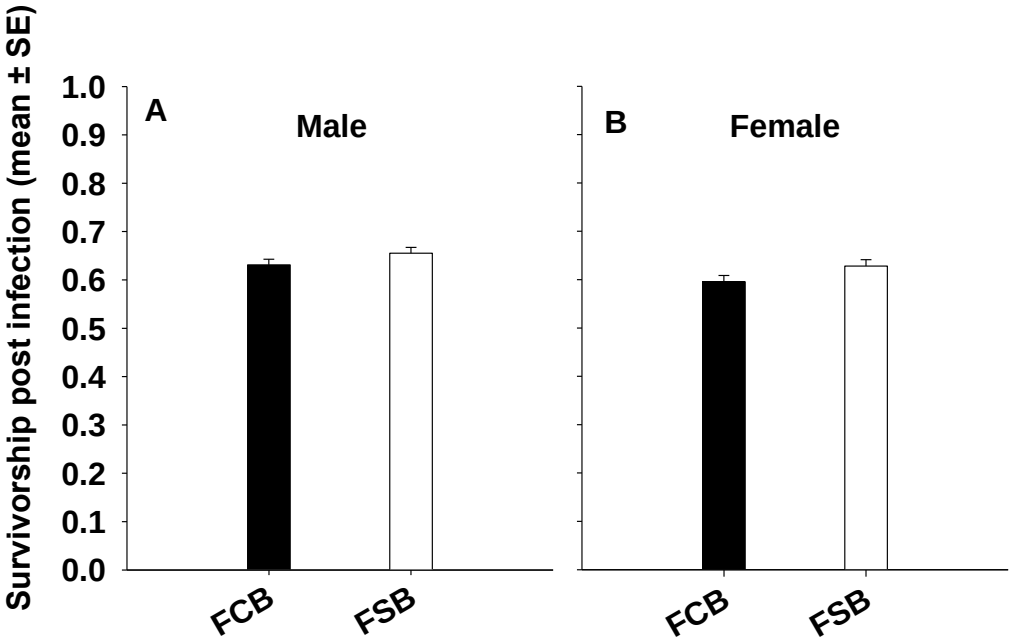

Figure 7.6: Male (A) and female (B) survivorship post infection. (A) There was no difference between FSB and FCB males in their survivorship post infection. (B) FSB and FCB females did not differ in their survivorship post infection.

DISCUSSION

In my present experimental evolution study, I measured resistance to starvation, desiccation, heat shock and cold shock in populations selected for resistance to cold shock. I found higher mating frequency and adult survivorship in the selected populations relative to control populations post heat and cold shock. I found greater egg viability post cold shock in FSB populations relative to FCB populations. I also found higher desiccation resistance in females of selected population indicating that selection for one kind of environmental stress can improve resistance towards other stress also. However, in case of starvation resistance, I found that the selected populations had lower starvation resistance relative to control populations suggesting that resistance to cold shock is negatively correlated with starvation resistance. I discuss each of these findings below in greater detail.

At 0 hours post cold shock, I found approximately 3-5% egg viability. This could be because sub-zero temperatures cause sperm mortality in male seminal vesicle, female seminal receptacle and spermathecae (Lefevre and Jonson 1962, Novitski and Rush 1949). This result is in line with several other studies that have found reduced egg viability and sterility in insects upon exposure to extremes of temperature (Arbogast 1981, Coulson and Bale 1992, Saxena et al. 1992). However, I found that egg viability has evolved to be higher in the FSB population compared to FCB populations 24 hours after cold or heat stress. There are multiple possible explanations for increased egg viability 24 hours post heat or cold shock. (a) The selected populations could be better at protecting their stored sperm/eggs from damage caused by heat or cold shock. For instance, Collett and Jarman (2001) have shown that *D. pseudoobscura* females can store the sperm up to six months during cold weather. These stored sperm can be used to fertilize ova in warm weather. (b) The selected populations mate more after heat or cold shock to enhance egg viability (Singh et al. 2015).

Previous studies have shown that high and low temperatures affect mating behavior (Schnebel and Grossfield 1984, Chakir et al. 2002, David et al. 2005, Dolgin et al. 2006 David 2008, Zhang et al. 2013). However, very few studies have addressed the effect of cold shock on mating behavior. In FSB populations the frequency of mating has increased post heat or cold shock compared to FCB populations. Hence, it is likely that increased mating post heat or cold shock is majorly responsible for increase in egg viability. While

the pattern of increased mating correlated with increased egg viability post cold shock had been observed in my populations earlier, it is interesting that this pattern is seen even under heat stress. This indicates that probably some of the mechanisms underlying resistance to heat and cold stress might be common (such as expression of heat shock proteins). This also forms an example of positive correlation between resistances to two stressors.

I found that FSB populations have lower mortality relative to FCB populations over 24 hours post cold shock. It is important to note that during regular maintenance regime, adult mortality due to cold shock is negligible. My results indicate that FSB population have evolved the ability to withstand colder temperatures in terms of reduced adult mortality along with their ability to maintain higher egg viability after shock at a temperature of -5°C. Multiple laboratory selection studies show increased adult survivorship as a correlated response to selection for cold tolerance (Anderson et al. 2005, MacMillan et al. 2009, Tucic 1979, Chen and Walker 1993).

In our previous study (Singh et al. 2015, chapter 3 of this thesis) I found that mortality post cold shock was negligible. However, in the present study, mortality post cold shock is substantial. These results seem quite contradictory. There are several possible explanations. First, the populations have evolved for first, number of generations between these two experiments. Second, in the current study, the flies were virgins when subjected to cold shock where as in the previous study, the flies had already mated by the time they were subjected to cold shock. Third, in the present study, the flies were moved into a fresh food vial soon after eclosion while in the previous study, the flies remained in the culture vials (with old, spent food) for two days after eclosion. I did a small experiment (data not shown) to differentiate between possibilities two and three. I used a factorial combination of mating status and food type to dissect out the effects. The experimental design was as follows-

	Old Food	New Food
Mated	X	X
Virgin	X	X

The results from this experiment indicate that flies maintained on new food soon after eclosion have higher mortality than flies maintained on old food soon after eclosion. Because files kept on old can experience environmental stresses such as urea and ammonia and even microbes. Or it is possible that some pathways involved in resistance to these stresses.

More interestingly, the FSB populations also showed lower mortality post heat shock compared to FCB populations. In the literature, there is some disagreement with regards to cross-resistance between cold and heat stress (reviewed in Hoffmann et al. 2003b). Anderson et al. (2005) and MacMillan et al. (2009) did not find correlated increase in heat shock resistance in populations of *D. melanogaster* selected for faster chill coma recovery or freeze resistance respectively. My results are in agreement with those of Kristensen et al. (2007) who show that cold selected lines of *D. melanogaster* were more heat tolerant and vice versa. Previous studies in *Drosophila* along latitudinal clines suggest that there is a trade-off between heat and cold tolerance (Hoffmann et al. 2002). My results suggest that heat and cold tolerance might be positively correlated in *Drosophila*. There could be multiple explanations for the superior survivorship of FSB populations post cold shock. (a) Chen and Walker (1994) report that cold selected lines have higher glycogen and total proteins relative to controls lines. Insects are known to store various sugars in order to tolerate cold temperatures (Ring and Danks 1994, Block 1996, Ring and Danks 1998). It is possible that the FSB populations have similarly altered resource storage in terms of carbohydrates, proteins or lipids. (b) Several studies have shown that there are several heat shock proteins that are expressed both during heat and cold stress. It is quite possible that at least some of these genes are expressed at a higher level in my populations. However, these genes are certainly not among the set that I analyzed for expression differences (see Chapter 6).

Starvation resistance has decreased in populations selected for increased resistance to cold shock relative control populations. My findings are similar to those of MacMillan et al. (2009) and Anderson et al. (2005) who found lower starvation resistance in populations of *D. melanogaster* selected for increased resistance to cold shock. Interestingly, Bubliy and Loeschcke (2005) found decreased cold stress tolerance in populations of *D. melanogaster* selected for increased starvation resistance. Thus across

multiple studies, the correlation between starvation resistance and cold stress tolerance seems to be robust.

I found that desiccation resistance increased in females of the selected populations. My findings are in line with results from other studies (Bubliy and Loeschcke 2005, Sinclair et. al. 2007a) which show that increased resistance to cold shock may lead to increased desiccation resistance as a correlated response. However populations selected for desiccation resistance do not show increased cold tolerance (Sinclair et al. 2007b). There is at least one common factor between cold and desiccation resistance that might explain their correlated evolution. Glycogen is known to act as cryoprotectant (Ramløv and Lee 2000, Holmstrup et al. 2002). Chippindale et al. (1998) showed that selection for increased desiccation resistance leads to increased glycogen content. Thus, increases in glycogen through selection on cold shock resistance could in principle lead to evolution of increased desiccation resistance. However, such increase, if any, is likely to be sex specific since I found no change in the desiccation resistance of FSB and FCB males.

In insects, cold stress can cause physical injury to the gut and malphigian tubules. This can open up a way for the gut flora to enter the haemocoel and thereby cause an infection (Yi and Lee 2003, MacMillan and Sinclair 2011, Marshall and Sinclair 2011, reviewed in Sinclair et al. 2013). Therefore in my selected populations, immune activity can potentially evolve. However, I did not find significant difference in survivorship after infection with *Staphylococcus succinus* subsp. *succinus* PK-1 between FSB and FCB populations.. One possibility is that the immune response is elicited only in response to the gut flora. In *Drosophila*, evolution against a pathogen can be fairly specific and the host might not have increased immunity against other pathogens (Roxstrom-Lindquist et al. 2004, Pham et al. 2007, Mikonranta et al. 2014). Thus in the present assay, where I use PK-1 as the pathogen, the appropriate immune response might not have been elicited.

To summarise, I found that cold shock resistance was positively correlated with heat shock resistance, negatively correlated with starvation resistance and not correlated with pathogen resistance. More interestingly, cold shock was positively correlated with desiccation resistance only in the females. Thus, genetic correlations across traits, at least to some extent seem to be independent of each other and might even be sex-specific.

Chapter 8

Isolation and characterization of a novel natural pathogen of *Drosophila melanogaster*

INTRODUCTION

Innate immunity is an important component of the antimicrobial defense mechanism of organisms and it is evolutionarily conserved across taxa to a large extent (Kimbrell et al. 2001). *Drosophila* has emerged as a good model to study innate immunity because, apart from other practical advantages, signalling pathways and other cellular and humoral components of the innate immune system are shared with humans (Hultmark 2003, Lemaitre et al. 2007). In any study of the evolutionary ecology of immunity, it is desirable to use natural pathogens of the host. Very few natural pathogens of adult *Drosophila* are known (for example *Pseudomonas entomophila, Lactococcus lactis, Providencia rettgeri, Providencia alcalifaciens, Enterococcus faecalis, Providencia burhodogranaria D, Providencia sneebia* (Short and Lazzaro 2010, Galac and Lazzaro 2011). However, a major limitation of using *Drosophila* for studies of innate immunity is the paucity of bacterial pathogens. Hence a large number of studies use non-pathogenic bacteria like *Escherichia coli* for the study of innate immunity. Additionally most of the bacteria that are known to cause mortality in adult *Drosophila* (and are hence used in studies) are not natural pathogens.

In this study, my aim was to isolate a natural pathogen of adult *D. melanogaster,* effects of initial pathogen dosage on the survivorships of the host post infection and effect of the mating status on the host survivorships post infection. Towards this end, I cultured microbes from wild caught flies and screened them for pathogenicity. Out of the ~200 isolates screened, I was successful in isolating a new strain (*Staphylococcus succinus* subsp. *succinus* PK-1) of bacteria that was pathogenic to adult *D. melanogaster*.

METHODS

Experiment Set 1: Isolation of natural pathogen of adult *D. melanogaster* from wild caught *Drosophila spp*.

Fly collection

Flies were collected during the months of January-March, 2011 from a fruit market in Chandigarh, Punjab, India (Longitude 76^0 47' 14E and Latitude 30^0 44' 14N) using fly traps baited with banana- yeast paste. Traps were harvested daily and the flies were processed immediately.

Isolation of bacteria

Individual flies were rinsed in 70% ethanol for surface sterilization (Cox et al. 2007), transferred to micro centrifuge tubes and homogenized in 200 µl of 10 mM $MgSO_4$. Homogenates were serially diluted up to 10^{-5}, then 100 µl of each dilution was spread on Luria Bertani (LB) agar plate of pH 7 and 10, and incubated at 25ºC or 37ºC for 48 hours. Each colony was preserved in 20% glycerol in - 80ºC and notionally identified based on the colonial and morphological characteristics.

Generation of experimental flies to screen whether isolates are pathogenic or not

I used three outbred (PJB, BRB and LH) and two inbred (Canton-S, W1118) populations to screen pathogenicity. Maintanence of these popolations have been detailed in chapter 2. The outbred populations were unrelated to each other. All flies used for assay were reared at a density of 50 eggs per vial (for two outbred populations- JB and BRB) and two inbred lines- Canton-S, W1118). For the LH population, the rearing density was 150 eggs/vial. 20 vials were set up for each population and reared at 25ºC temperature and 50-60% relative humidity. On 9-10^{th} day post egg collection, virgin males or females were collected using light CO_2 anaesthesia at very young stage (approximately 4 hours post eclosion). Five flies were housed in each vial (single sex groups) provisioned with 2 ml food. Every alternate day flies were transferred in to fresh food vials.

Screening of isolates and infection protocol

A single colony of each isolate was inoculated in LB broth, incubated at 37ºC and shaking at 170 rpm for overnight growth. The following day a sub-culture was started by inoculating 100 µl overnight culture in 10 ml of LB broth and incubated (at 37ºC and at

170 rpm) until optical density (OD_{600nm}) reached 1 ± 0.1. Then 1 ml of culture was centrifuged (10000 rpm for 4 minutes) and the pellet was resuspended in1ml of 10 mM $MgSO_4$ to yield bacterial suspension (Apidianakis et al. 2009). For screening isolates are pathogenic or not, flies of known age (4-5 days old as adult), type and sex (see below for details) were mildly anaesthetized using CO_2. Flies were infected by poking the lateral thorax with a minutien pin (0.1 mm, fine Science Tools, Foster City, CA, USA) dipped in the bacterial suspension (Vanessa Corby-Harris et al. 2008). For sham infection, pin was dipped in 10 mM $MgSO_4$ prior to poking the lateral thorax of the flies. All infections were done at the mid-point of the light phase of the 12:12 LD cycle of the flies.

Survivorship assay post infection

Forty to fifty males and females flies were infected separately for each of the PJB, BRB, LH, Canton-S and W1118 population to screen whether the isolates were pathogenic or not. Same numbers of flies were used for sham infection. The number of dead flies in each vial was recorded at 3 hour intervals. All populations were infected between 2-3 PM (Indian standard time). These experiments were repeated twice (Data not shown).

Growth of pathogen in flies

We carried out an assay to understand whether this pathogen (PK-1) can grow within the body of host and establish a sustained infection. Eighty male and female flies (4-5 day old adults) were infected with PK-1 (OD_{600nm} = 2.5). Immediately after infection, flies were randomly divided into two groups (40 males and females in each group). Flies from the first group were frozen soon after infection. Flies from the second group were maintained under standard laboratory conditions for 24 hours post infection after which they were frozen. These two sets of flies were used to estimate the number of CFUs at 0 hour and 24 hours post infection. Flies were surface sterilized with 70% ethanol using protocol as described previously (Cox et al. 2007) with minor modifications. Following this, set of 3 flies were homogenized in 100 µl of 10 mM MgSO4. There were 10 sets per group for each sex. For 0 hour post infection group, homogenate was diluted 10 times in 10 mM MgSO4. In the 24 hours post infection group, homogenate was diluted 1000 times in 10 mM MgSO4. After that, 100 µl of the homogenate was plated on Petri plate of LB agar medium and incubated at 37^{o}C for 24 hours. Following this, the number of colonies in each plate was counted. These counts were log_{10} transformed and used as units of analysis.

Experiment Set 2: Identification of the new isolate

After screening more than 200 isolates, I found one isolate that was pathogenic to our *Drosophila* populations. Hence I wanted to identify and further characterise this isolate. Morphological, biochemical, Fatty acid methyl esters (FAMES) and 16S-rRNA characterisation of this isolate of interest was carried out by Microbial Type Culture Collection and Gene Bank at Institute of Microbial Technology (Chandigarh, India). The isolate was initially identified based on these characters. The isolate was further characterised as described below.

DNA-DNA Hybridizations

Genomic DNA of the pathogenic bacteria was isolated accordingly to modified procedure of Gevers et al. (2001). DNA-DNA hybridizations were performed among strain PK-1 and *S. succinus* subsp. *succinus* (LMG 22185), *S. succinus* subsp. *casei* (LMG 22186), *S. xylosus* (LMG 20217) and *S. saprophyticus* subsp. *saprophyticus* (LMG 13350). DNA-DNA hybridizations were performed by BCCM/LMG Bacteria Collection at University of Gent, Belgium. Hybridizations were carried out in the presence of 50% formamide at 34^{o}C, a modification (Gories et al. 1998; Cleenwerck et al. 2002) of the method described by Ezaki et al. (1989).

G+C content

G+C content was determined by BCCM/LMG Bacteria Collection at University of Gent, Belgium using HPLC technique (Mesbah et al. 1989). The value that is reported is the mean of three independent analyses of the same DNA sample.

Isolation of Genomic DNA for the *hsp60* gene sequencing

This was done to determine the identity of the pathogenic bacteria (Strain PK-1) at the subspecies level. Genomic DNA of the pathogenic bacteria was isolated using the protocol described by (Place et. al. (2002) with minor modifications. Bacterial strain was cultured in LB broth until OD_{600nm} = 3. Culture (10 ml) of PK-1 strain was harvested centrifuged (6000 rpm/5 min/4^{o}C), washed with H_2O and pellet was resuspended in 250 µl H_2O. 125 µl of 10% sodium dodecyl sulphate was added and mixed well. Then the tubes were incubated at 45^{o}C for 30 min in water-bath. After that 200 µl glass beads were added to the tube, vortexed 5 times for 30 s each and centrifuged. Supernatant was transferred in to fresh tube having 500 µl PCI (Phenol: Chloroform: Isoamyl Alcohol

25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) solution was added and inverted 5 times and centrifuged at 13000 rpm for 10 min. Upper layer was collected in a fresh tube, isopropanol (twice the volume of the upper layer) was added to it and the tube was incubated at -20^{o}C for 30min. After that it was centrifuged at 13000 rpm for 10 min. Pellet was washed with 500 µl of 70% ethanol and dried at 37^{o}C for overnight. Finally it was dissolved in 30 µl TERNase.

PCR amplification

The *hsp60* gene was amplified by PCR (Bio-Rad Thermal cycler) using Vent DNA polymerase (New England Biolab), 50µl of reaction mixture was prepared containing 100 ng DNA templates. Thermal cycler condition for the amplification of *hsp60* genewas: 95^{o}C for 5 min for one cycle, after that 30 cycles at 95 ^{o}C for 45 seconds(s), 55^{o}C for 45 s, 72^{o}C for 1 min, finally at 72^{o}C for 10 min, 4^{o}C (infinite hold). Amplified DNA fragment was separated on 1% agarose gel and eluted from gel using a gel extraction kit (Thermo scientific). The *hsp60* gene sequencing was performed by Eurofins Genomic (Banglore, India) using the dideoxy chain-termination method with the Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems). The *hsp 60* gene sequence of strain (PK-1) generated in this work (900 bases) was aligned against *Staphylococcus sp*. A sequence similarity search was performed with GenBank BLASTN (Altschul et al. 1997) using default parameters.

Experiment Set 3: Characterisation of the new isolate (PK-1) as a new model pathogen

Based on the tests described above, the new isolate was identified as a new strain of the bacteria *Staphylococcus succinus* sub species *succinus*. The new strain was named as PK-1 and deposited in culture collections (see below). I then addressed three major factors that can potentially affect host-pathogen interaction. These were-

(a) Does initial dosage of the pathogen affect post infection survival of the host?

(b) Does mating status of the host affect its ability to survive post infection?

(c) Does the mode of infection affect the survival of the host?

Effect of initial dosage of pathogen PK-1 on post infection mortality

Single colony of PK-1 was inoculated into 10ml LB broth and incubated at 37^{o}C for overnight growth. The next day it was sub-cultured in multiple batches of 10ml LB broth

till the culture attained OD_{600nm} = 1. To obtain cultures with OD_{600nm} = 2 and 3, multiple cultures with OD_{600nm} = 1 were combined and spun down till the desired OD was obtained. Flies were then infected (or sham infected) as described before. I used virgin males and females from various host populations: PJB, LH, BRB, Canton-S and W1118 for this assay (N = 45-50 for each sex by treatment by population combination). Flies were 4 to 5days old at the time of infection.

Effect of mating status of host on post infection mortality

Flies from PJB, LH and BRB populations were isolated as virgins and randomly divided into two groups- virgin and mated. Males and females from the mated group, were combined on the 12^{th} day post egg collection (by this time flies were ~2-3 days old as adults) and allowed to interact for two days prior to infection. Flies in the virgin treatment were held as single sex groups till infection. Infections were carried out as described before on the 14^{th} day post egg collection.

Can flies be infected with PK-1 through oral route?

Single colony of PK-1 was inoculated into LB broth, Incubated (at 37^{o}C and 170 rpm) until OD_{600nm} = 1. Subsequently, 1.6 ml of the resultant culture was centrifuged and the pellet of bacteria was resuspended in 170 µl of 5% sterile sucrose solution. This slurry of bacteria was added on 2.3 cm Whatman filter paper disc and it was placed in a vial containing 5 ml of 1.2% agar. For controls only 170 µl of 5% sterile sucrose solution was transferred onto the Whatman filter paper discs. Virgin males and females from the W1118 population were transferred to the vials containing bacterial slurry (or only sucrose solution) at a density of 5 flies per vial. Ten such vials were set up per treatment by sex combination. The flies were allowed to feed for 5 days. Mortality was recorded at every 6 hours intervals.

Effect of feeding of pathogen PK-1 on larval survivorship

For the treatment group, 25 ml of OD_{600nm} = 1 of PK-1 suspension was centrifuged (7000 rpm for 5 min at 4^{o}C). Pellet was diluted in sterile yeast suspension (2 g yeast in 100 ml water) and vortexed well. Following this 2 ml of this solution was transferred in Petri plate containing 1.2% agar. Immediately following this, 100 second instar larvae were transferred into the Petri plate and allowed to feed. The plates were then monitored for

mortality of larvae and eclosion of adults. For the control group, I followed a similar protocol but I did not add bacteria in yeast suspension.

RESULTS

Isolation of bacteria I obtained 200 different isolates of bacteria from *Drosophila* spp. on the basis of colonial and morphological characteristics. All of them were screened for pathogenicity. I found only one strain of bacteria that was pathogenic to multiple populations of *D. melanogaster.*

Growth of pathogen in flies

To confirm whether a bacterium (PK-1) does grow within the fly body post infection, we assayed bacterial load 0 hour and 24 hours post infection. We found that males and females received about 330 bacterial cells at infection (Figure 8.6). Twenty two hours post infection, number of colony forming units was significantly higher in both sexes relative to 0 hour post infection. We did not find sex × time interaction indicates that both males and females having same bacterial load 24 hours post infection (Table 8.6, Figure 8.5).

Identification of strain PK-1 on the basis of morphological and biochemical test

I found that PK-1 strain is a gram positive, coccus, non-motile bacteria with average cell size of 5μm. Colonies which appear after 18 hours on Luria Bertani agar plates at 37°C are raised, opaque, smooth, circular, have an off white pigment and aerobic respiration. Growth occurs between 4 and 42°C. Optimal growth is obtained at 30°C. PK-1 strain was different from both *Staphylococcus succinus* subsp. *succinus* (AMG-D1) and with *Staphylococcus succinus* subsp. *casei* (SB72) on the basis of following tests- hydrolysis of arginine, lactose and galactose fermentation, tween 40 and tween 80 hydrolysis (Table 8.1).

Identification on the basis of 16S rRNA gene sequence similarity

The results obtained by sequencing of 16S rRNA gene (accession number JQ988944) shows that PK-1 strain belongs to genus *Staphylococcus*. It shares 99.7% sequence similarity with *Staphylococcus succinus* subsp. *succinus*, 99.57% sequence similarity with

Staphylococcus succinus subsp. *casei*, 99.2% sequence similarity with *Staphylococcus xylosus*, 99.01% with *Staphylococcus saprophyticus* subsp. *saprophyticus* and less than 99% with other species of *Staphylococcus*.

Identification on the basis of DNA relatedness

DNA-DNA hybridization studies were carried out among strains, PK-1 and near relatives (Table 8.2). The DNA relatedness of PK-1 strain with *Staphylococcus succinus* subsp. *succinus was* 85% and 75% with *Staphylococcus succinus* subsp. *casei*, 35% with *Staphylococcus xylosus* and 27% with *Staphylococcus saprophyticus* subsp. *saprophyticus.* Whole genome DNA-DNA relatedness assay shows more than 70% similarity with other species, which is generally accepted as a limit of species delineation (Wayne et al. 1987). Hence, PK-1 is either closely related to *Staphylococcus succinus* subsp. *succinus* or with *Staphylococcus succinus* subsp. *casei.*

G+C content of PK-1 strain was 33 mol % of genomic DNA. This is less than 3% different from both *Staphylococcus succinus* subsp. *succinus* or with *Staphylococcus succinus* subsp. *Casei* (Lambert et al. 1998, Place et al. 2002). Generally less than 3% difference in G+C content is the accepted range within well-defined species (Vandamme et al. 1996). The G+C content of the DNA of strain PK-1 strain was 33 mol %, in accordance with the overall content of the genus (Kloos et al. 1991).

Identification on the basis of Fatty acid methyl esters (FAMES)

FAMES results show that PK-1 strain contains tuberculostearic acid (TBSA 10Me18:0) (Table 8.3). It is the characteristic of coryneform bacteria but not previously described for the genus *Staphylococcus* sp. other than *Staphylococcus succinus* subsp. *succinus* ATCC 700337 and *Staphylococcus succinus* subsp. *casei* SB72 (Lambert et al. 1998, Place et al.2003). PK-1 strain is much closer to *Staphylococcus succinus* subsp. *succinus* on the basis of 13:0 iso, 13:0 anteiso, 15:0 anteiso, 16:0, Iso 17:1ω10c, 17:0 iso relative to *Staphylococcus succinus* subsp. *casei*. However in comparison with *Staphylococcus succinus* subsp. *succinus,* PK-1 strain contains more 11:0 anteiso, 13:0 antesio, 15:0 anteiso, 17:0 anteiso but less, 11:0 iso, 12:0 iso, 13:0 iso, 14:0, 15:0 iso, Iso 17:1ω10c, 17:0 iso, TBSA10Me 18:0, 19:0 iso (Table 8.3).

Identification on the basis of *hsp60* gene sequence similarity

Subspecies have a sequence similarity range of 91–98% (Kwok et al.1999). However, I found that PK-1 strain shares 98.5% *hsp* gene sequence similarity with *Staphylococcus succinus subsp. succinus* and 97.6% sequence similarity with *Staphylococcus succinus* subsp. *casei.* My findings strongly suggest that PK-1 belongs to *Staphylococcus succinus* subsp. *succinus*.

Effect of initial dosage of PK-1 pathogen on host survivorship post infection

I found that, survivorship post infection varied across several laboratory populations. In case of males I found significant effect of initial dosage of pathogen on post infection survivorship of various populations. In general, with increasing dosage, total death and mortality rate of the males increased in the host populations (Table 8.4A, Figure 8.1). However, among the females, initial dosage effects were much more variable with LH, PJB and CS females showing declining survivorship with increasing dosage while BRB females showed no such effect (Table 8.4B, Figure 8.2). I did not find any death in the sham infected controls.

Effect of mating status on post infection survivorship

Mating activity does not change the males' and females' survivorship in several populations of the *D. melanogaster* when infected with PK-1 strain. The Survivorship data of mating status was analysed using Cox proportion regression model and Kaplan-Meier estimator indicated that there was no significant difference between virgin and mated males and females (Figure 8.3). This result was consistent across the three outbred and two inbred populations (Table 8.5). I did not find any death in the sham infected controls.

Oral feeding assay

When PK-1 bacterial suspension was administered orally, I did not find any death in adult flies. Treated flies survived similar to control flies indicating that the bacteria fails to establish a sustained infection if administered orally. It could also be possible that oral infection is not good enough to cause pathogenecity relative pricking infection. But when PK-1 bacterial suspension was administered orally at larval level, the treated larvae looked healthier than the controls (Figure 8.4).

Table 8.1. Biochemical characteristics of PK-1 and other *Staphylococcus* species. Summary of biochemical results of PK-1 strain was compared with previously known results from other studies study (Lambert et al. 1998, Place et al. 2002). +, indicates more than 90% of the strain is positive; - denotes more than 90% of the strain is negative; +/- showing between 25 and 75% strain are positive according to Lambert et al. (1998)., (+) weak positive, ND not determined, W suggests weak and not easily reproducible results (Place et al. 2002). Data from [a] Lambert et al. (1998), [b] Place et al. (2002). AMG-D1 is a strain of *S. succinus* subsp. *succinus*, SB 72 is a strain *S. succinus* subsp. *casei*.

Biochemical assay	**PK-1**	**AMG-D1[a]**	**SB72[a]**	***S. xylosus*[a]**	***S. saprophyticus*[b]**
Nitrate reduction	(+)	-	+	+	-
Vogus-proskauer test	-	-	ND	+/-	+
Arginindihydrolase	+	-	-	-	-
Urease	+	+	+	+	+
Catalase	+	+	ND	+	+
Oxidase	-	-	-	-	-
Fermentation of :					
Raffinose	-	-	-	-	-
Lactose	-	+	+	+/-	+/-
Trehalose	+	+	+	+	-
Galactose	-	W	+	W	ND
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	ND
Maltose	(+)	+	+	+	ND
Tween 40	-	W	+	W	ND
Tween 80	-	W	+	W	ND
Esculin hydrolysis	(+)	+	+	-	ND
Antibiotic sensitivity:					
Novobiocin resistance	+	+	+	+	+
Bacitracin resistance	+	+	ND	+	+

Table 8.2. DNA-DNA hybridization among strain PK-1 and other *Staphylococcus* species. *S. succinus* subsp. *succinus* (LMG 22185), *S. succinus* subsp. *casei* (LMG 22186), *S. xylosus* (LMG 20217) and *S. saprophyticus* subsp. *saprophyticus* (LMG 13350).

Isolate identification	**Mean G+C content (mol %)**	**Similarity (%)**			
		LMG 22185	LMG 22185	LMG 20217	LMG 13350
PK-1	33	82	75	31	27

Table 8.3. Cellular fatty acid composition in percentage of strain PK-1 compared with other *Staphylococcus* species. Data from [a] Lambert et al.1998, [b]Place et al. 2002. AMG-D1 is a strain of *S. succinus* subsp. *succinus*, SB 72 is a strain *S. succinus* subsp. *casei*, ND means not determined.

Fatty Acid	**PK-1**	**AMG-D1[a]**	**SB72[b]**	***S.xylosus*[a]**	***S. saprophyticus*[a]**
11:0 iso	0.49	0.61	ND	0.00	0.00
11:0 anteiso	0.40	0.23	ND	0.00	0.00
12:0 iso	0.45	0.52	ND	0.00	0.00
12:00	ND	0.50	ND	0.00	0.00
13:0 iso	19.48	28.38	1.51	1.44	0.55
13:0 antesio	17.85	12.73	0.51	0.00	0.00
14:0 iso	ND	1.39	0.51	2.40	1.88
14:00	1.02	1.50	ND	1.13	1.10
15:0 iso	11.57	18.24	17.09	23.73	21.19
15:0 anteiso	28.86	18.12	41.69	39.24	41.55
16:0 iso	0.78	ND	ND	ND	ND
16:00	1.01	0.97	0.47	0.97	1.94
16:1ω11c	ND	0.21	ND	0.00	0.00
Iso 17:1ω10c	1.10	1.53	0.40	0.00	0.00
17:0 iso	5.40	6.02	15.10	8.69	6.56
17:0 anteiso	7.70	2.29	8.00	4.48	4.66
18:1ω9c	ND	0.00	0.70	0.00	0.00
18:0 iso	ND	0.00	ND	0.00	0.00
18:00	ND	1.65	2.63	5.66	5.52
TBSA10Me 18:0	1.10	1.45	1.19	0.00	0.00
19:0 iso	0.62	0.79	5.09	2.75	1.92
19:0 anteiso	ND	0.52	1.67	0.00	0.00
20:1ω9c	ND	1.07	0.56	0.00	0.00
20:00	ND	0.49	1.52	5.47	9.12

Table 8.4. Effect of bacterial dosage of infection on the survivorship of the males (A) and females (B) host. Summary of Kaplan-Meier analysis of survivorship data of males (A) and females (B) infected with PK-1. *p*-values in bold indicate significant differences.

Traits	**Dosage OD_{600nm}**	**Host populations**	**Median time to death**	**% of death**	***p***
(A)	1		---	28.889	
Males	2	PJB	---	46.939	**<0.001**
	3		41	60.417	
	1		---	14	
	2	LH	---	25	**0.017**
	3		---	37.5	
	1		---	23.404	
	2	BRB	---	36.735	**0.001**
	3		47	55.319	
	1		---	14	
	2	Canton-S	---	26	**<0.001**
	3		44	56.863	
	1		---	25	
	2	W1118	50	51.219	**<0.001**
	3		40	78.378	
(B)	1		---	39.583	
Females	2	JB	52	55.102	**0.043**
	3		53	59.574	
	1		---	14.286	
	2	LH		30.612	**0.006**
	3		48	41.667	
	1		---	37.5	
	2	BRB	---	45.833	0.899
	3		---	40.816	
	1		---	26	
	2	Canton-S	57	58	**<0.001**
	3		47	72.549	
	1		---	22.5	
	2	W1118	---	35	**<0.001**
	3		47	67.5	

Table 8.5. Effect of mating status on post infection survivorship of males (A) and females (B) flies. Summary of Kaplan-Meier analysis of survivorship data of virgin and mated flies (male and female) infected with PK-1 (all $p > 0.05$, Cox proportion regression analysis).

Sex	**Host population**	**Mating status**	**Median time to death**	**% of dead flies**	***p***
(A) Males	PJB	Mated	51	58.333	0.056
		Virgin	44	70.213	
	LH	Mated	---	29.787	0.094
		Virgin	67	51.219	
	Canton-s	Mated	---	50	0.846
		Virgin	---	42.553	
(B) Females	PJB	Mated	---	47.059	0.47
		Virgin	---	46.938	
	LH	Mated	---	43.75	0.981
		Virgin	---	44.897	
	Canton-s	Mated	51	69.387	0.288
		Virgin	49	77.551	

Table 8.6. Summary of a two-factor mixed model Analysis of Variance (ANOVA) on the colony forming units data. *p*-values in bold indicate significant differences.

Effect	**Df**	**Sum of Squares**	**F ratio**	***p***
Sex	1	0.099	0.948	0.333
Time	1	288.278	2767.295	**<0001**
Sex × Time	1	0.032	0.303	0.583

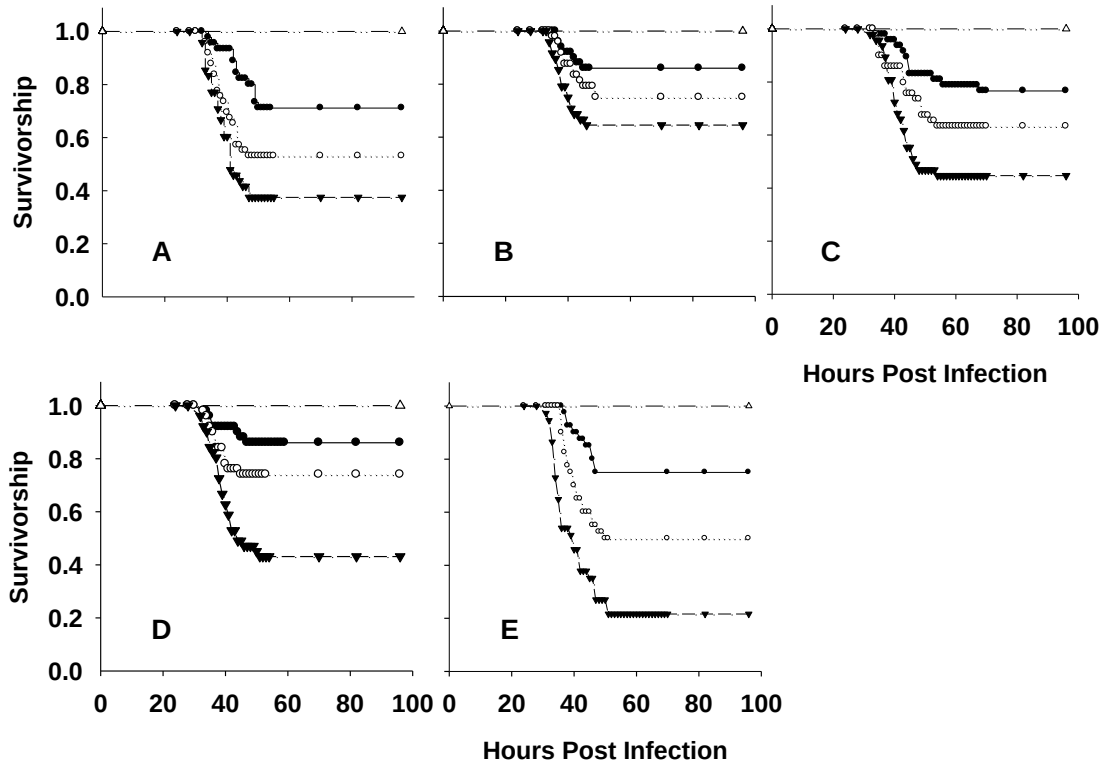

Figure 8.1: Effect of initial dosage on male survivorship after infection with PK-1. Kaplan-Meier plots of survivorship. Initial dosage of OD (optical density) 1 (●), 2 (○) and 3 (▼). (A) PJB, (B) LH, (C) BRB, (D) Canton-S and (E) W1118 populations. For the experiment, 45-50 male flies were infected for each population and for each OD. Survivorship significantly declines in all population (all $p < 0.05$, Cox proportion regression analysis). I did not find any death in case of sham infected flies (△) over the time of the observation.

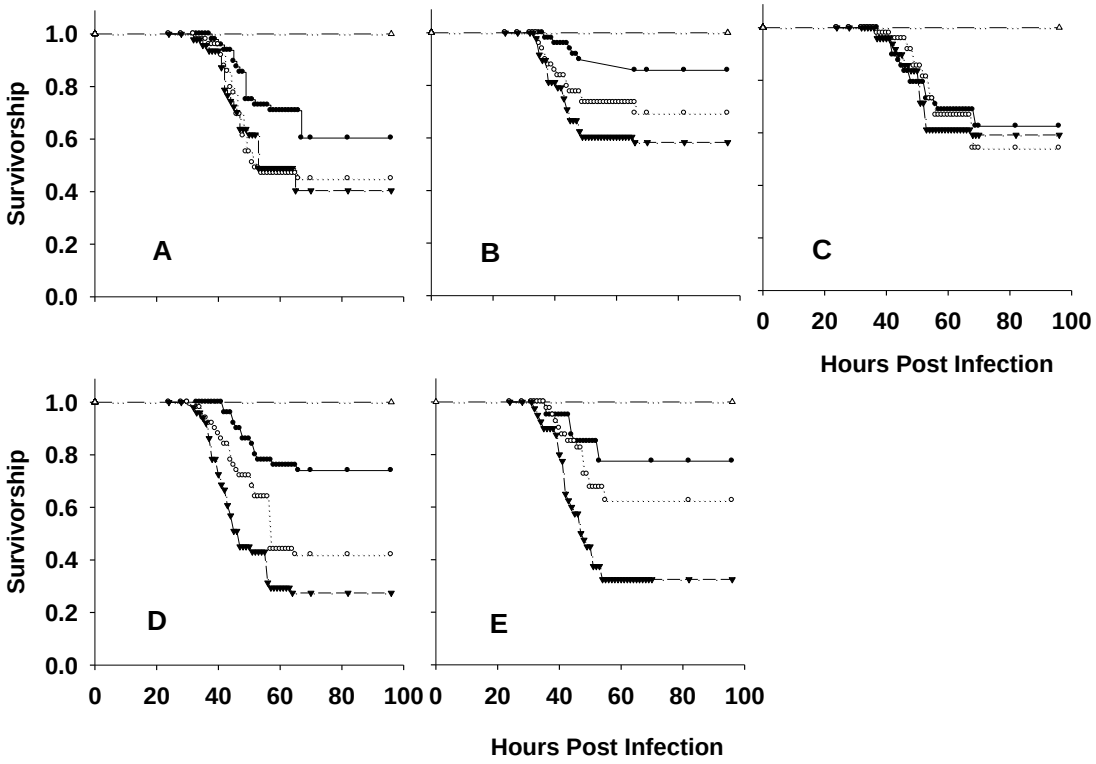

Figure 8.2: Effect of initial dosage on female survivorship after infection with PK-1. Kaplan-Meier plots of survivorship. Initial dosage of OD (optical density) 1 (●), 2 (○) and 3 (▼). (A) PJB, (B) LH, (C) BRB, (D) Canton-S and (E) W1118 populations. For the experiment, 45-50 female flies were infected for each population and for each OD. Survivorship significantly declines in all populations with increasing OD (all $p < 0.05$, Cox proportion regression analysis) except in BRB population where initial dosage had no significant effect on mortality. We did not find any death in case of sham infected flies (△) over the time of observation.

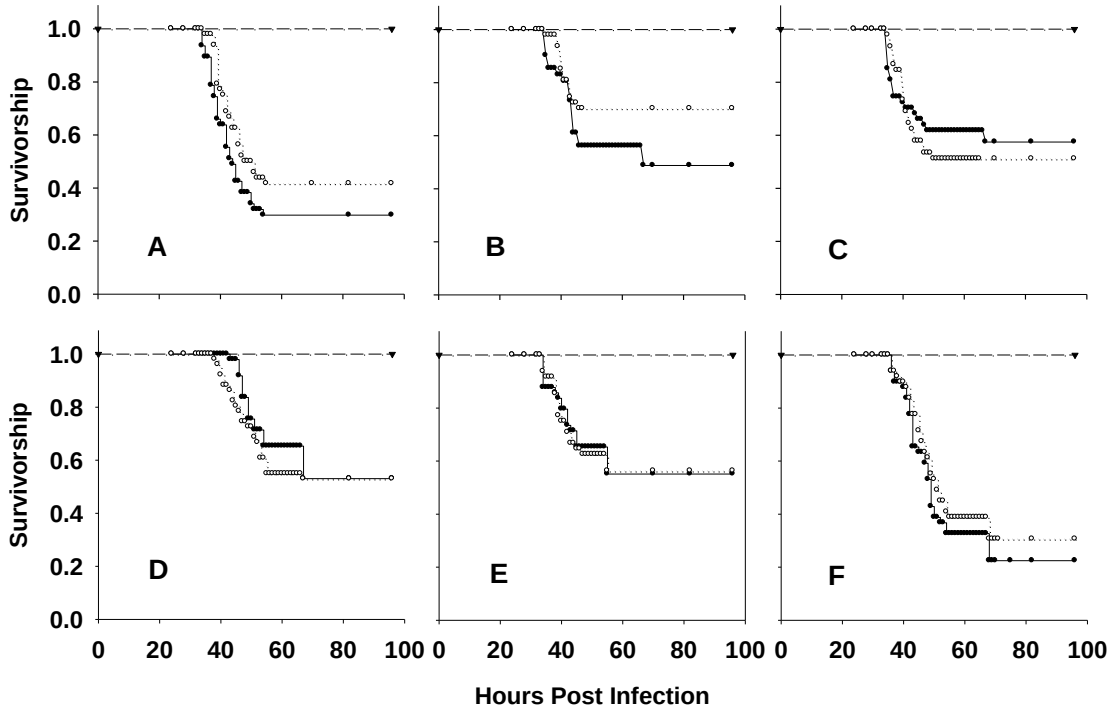

Figure 8.3: Effect of mating status on survivorship post infection with PK-1. Kaplan-Meier plots of survivorship of mated males (○) and virgin males (●) from PJB (A), LH (B) and Canton-S (C) populations. There was no significant difference between sexually active and virgin males (all $p > 0.05$, Cox proportion regression analysis). We did not find any death in the case of sham infected flies (▼) over the time of the observation

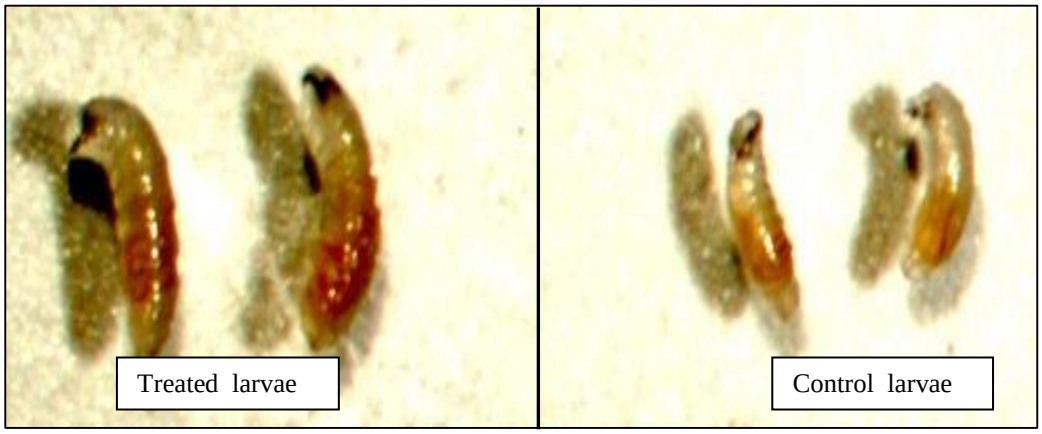

Figure 8.4: Effect of oral feeding of PK-1 on larval size. Larvae that were fed on PK-1 were larger than control larvae suggesting that the bacterium was probably used as an additional source of nutrition.

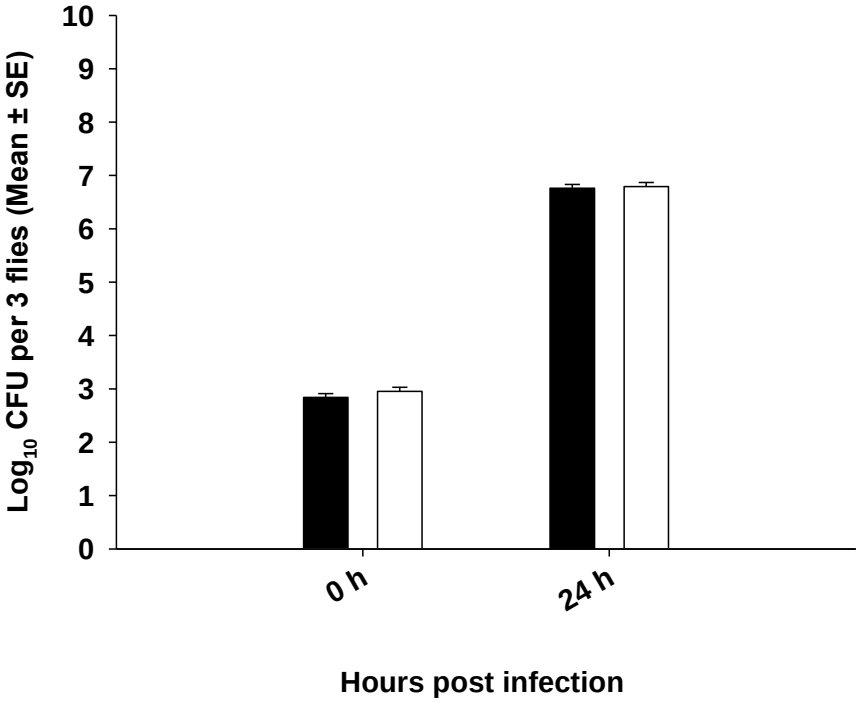

Figure 8.5: Number of colony forming units (CFUs) in males and females. Black bars represent males and open bars represent females. Soon after infection (0 hour) and 24 hours post infection, mean CFU was not different between males and females. Twenty four hours post infection, we found significantly higher CFU in both sexes relative to 0 hour post infection.

DISCUSSION

Drosophila has emerged as an important model in the studies of the evolutionary ecology of immune response. However, there seems to be few bacterial pathogens that are capable of causing mortality in adult flies *(Providencia rettgeri, Providencia alcalifaciens, Enterococcus faecalis, Providencia burhodogranaria, Providencia sneebia*; Galac and Lazzaro 2011, Short and Lazzaro 2010). My major aim in this study was to isolate bacterial strains from wild caught flies that could potentially induce mortality in adult flies. I screened a large number of bacterial isolates from wild caught flies and found one of them was pathogenic to flies. The bacteria belonged to *Staphylococcus succinus* sub species *succinus*. The strain was new and was named as PK-1. This strain was pathogenic across different host populations of *D. melanogaster* and the induced mortality in various populations was between 36% to 62.5% for males and 40.2 to 59.8% for females. It is important to note that several of the populations that I have used are maintained as large, outbred populations that are expected to harbour substantial genetic variation and are hence expected to be vigorous. Given that the new bacterial isolate was pathogenic to both outbred and inbred populations it is likely to be a good model pathogen for future studies. This new strain establishes sustained infection in flies by growing from ~320 cells to ~2.5×10^6 cells over a period of 24 hours post infection.

At present only two subspecies of *Staphylococcus succinus* is known, First *Staphylococcus succinus* subsp. *Succinus* (Lambert et al. 1998) and second *Staphylococcus succinus* subsp. *casei* (Place et al. 2002). According to the 16S rRNA gene sequence, new isolate (PK-1) belong to genus *Staphylococcus*, sharing 99.70% sequence similarity with *Staphylococcus succinus* subsp. *succinus* (AMG-D1) and 99.57% sequence similarity with *Staphylococcus succinus* subsp. *casei* (SB72).

DNA-DNA hybridization assay also indicates that the new isolate belongs to either *Staphylococcus succinus* subsp. *succinus* or *Staphylococcus succinus* subsp. *casei.* Whole genome DNA-DNA relatedness shows more than 70% similarity which generally accepted as a limit of species delineation a species (Wayne et al. 1987).

In FAMES analysis, presence of the fatty acid 11:0 iso, 11:0 anteiso, 12:0 iso, 13:0 antesio, Iso 17:1ω10c, TBSA10Me18:0 (Table 8.3) strongly suggested that the new

isolate belongs to either *Staphylococcus succinus* subsp. *succinus* or *Staphylococcus succinus* subsp. *casei. Because* TBSA10Me18:0 is reported in only one species (*Staphylococcus succinus*) in genus *Staphylococcus.* However, *hsp60* gene sequence similarty shows that the new isolateshares 98.6 % sequence similarty with *Staphylococcus succinus subsp. succinus* and 97.6 % with *Staphylococcus succinus subsp. casei*. Strongly suggesting that the new isolate is a new strain of *Staphylococcus succinus subsp. succinus.* The new isolate, named PK-1 has been deposited in two culture collections (MTCC, Chandigarh India and BCCM.LMG, Belgium).

My studies also suggest that the mode of infection and initial dosage are important in determining the mortality induced by PK-1. The new isolate is pathogenic only when injected into the thorax and shows no detectable effects on survivorship when fed to the flies. It is possible that the oral infection is not strong enough to show virulence relative to pricking infection. When PK-1 bacterial suspension was administered orally at larval stage, the treated larvae looked healthier than the controls. It is possible that PK-1 is involved in food metabolism. Alternatively, the bacterium can cause sterility and alter the provisioning of resources from reproduction to body maintenance. For example (Bond 2006) documented that a pathogen causes sterility redirecting the investment of resources from reproduction to maintainance of body which can cause gigantism. Additionally, the effect of initial dosage was sex and population specific with the males in all the populations showing increased mortality with increased initial dosage while this was true of females from only some of the populations.

Several previous study have suggested that mating activity can affect the antibacterial immunity of flies (McKean and Nunney 2008; Fedorka et al. 2007; Short and Lazzaro 2010). Using the new isolate, I found no significant effect of mating status on post infection survival in either males or females. My results are in agreement with several previous studies from our lab (Khan and Prasad 2013) which found no effect of mating status on anti-bacterial immunity of males or females. Thus, my results seem to indicate that the mating cost of immunity, if any, depends on the population and the pathogens used (Short and Lazzaro 2010).

To summarize, based on the results obtained from morphological, biochemical, 16S rDNA sequencing, DNA-DNA hybridization and FAMES and *hsp60* sequencing, sthe

new isolate is a new strain (PK-1) of *S. succinus* subsp. *succinus.* PK-1 strain significantly influences the adult survivorship across several laboratory populations of *D. melanogaster*. Effect of initial dosage of PK-1 strain on adult survivorship is sex specific. This new isolate is potentially useful for the study of evolutionary ecology of immunity.

Chapter 9

Conclusions

Temperature stress has profound impacts on the distribution, abundance, physiology and survivorship of ectotherms (Hoffmann and Parsons 1991, Huey and Berrigan 2001, Angilletta 2009, Kingsolver 2009). It is known that heat stress can affect a number of traits- survivorship, reproductive traits (i.e., mating latency, mating success, copulation duration, progeny production, sperm competation) and various other physiological functions of the organisms (Czajka and Lee 1990, Denlinger and Yocum 1998, Rohmer et al. 2004, Bubliy and Loeschcke 2005, Malmendal et al. 2006, reviewed by Hance et al. 2007, Schmidt and Paaby 2008, Zizzari and Ellers 2011, Lieshout et al. 2013, Nguyen et al. 2013). Similar to heat stress, cold stress also potentially reduces gamete viability (Novatski and Rush 1949, Lefevre and Jonsson 1962). In addition to this cold stress also influences adult survivorship, reproductive fitness traits, immunity (Yi and Lee 2003, MacMillan and Sinclair 2011, Marshall and Sinclair 2011, reviewed in Sinclair et al. 2013) and various physiological functions in *D. melanogaster*. Therefore, response to cold stress in principle can involve changes in the reproductive and various other crucial life-history traits. Hence, in this thesis, I have probed the evolution of reproductive traits along with other important life-history traits in large replicate populations of *D. melanogaster* that had been selected for increased resistance to cold stress for 71 generations. Multiple previous studies, both from the field and laboratory, have majorly focused on life-history and related traits such as survivorship, longevity, cross-tolerance, chill coma recovery time and fecundity (Tucic 1979, Chen and Walker 1993, Watson and Hoffmann 1996, Anderson et al. 2005, Bubliy and Loeschcke 2005, MacMillan et al. 2009). However, these studies did not focus on the evolution of reproductive traits in response to selection for tolerance to cold stress.

Using the laboratory experimental evolution approach, I successfully, selected the large replicate populations of *D. melanogaster* for increased resistance to cold shock for over 71 generations with the focus of selection being on egg viability. I then assayed various

reproductive and life-history traits over 19-71 generations of selection. In this chapter, I present the major findings of this thesis.

Evolution of the egg viability as a direct response to selection

The protocol for cold shock used by me in this thesis was such that while causing very low levels of mortality it dramatically reduced egg viability. Therefore, the focus of selection was on egg viability as opposed to post cold shock survivorship. I found that after 19 generations of selection the FSB populations had a significantly higher egg viability post cold shock relative to the FCB populations, suggesting that the FSB populations had responded to selection. I further probed the following possible mechanisms behind higher egg viability in the FSB populations. (a) FSB females have evolved to protect stored fertilized ova or sperm from cold shock and use them later on life and (b) FSB males and females have evolved to mount a faster recovery in terms of reproductive traits post cold shock. Collinett and Jarman (2001) documented that *D. pseudoobscura* female can store sperm during the colder weather and start utilizing the stored sperm upon the onset of favourable conditions. However, I found that this is not the case in FSB and FCB populations. When mated (non virgin) FSB and FCB females were subjected to cold shock and were held without males subsequently, the egg viability post cold shock remained very low and comparable. This finding indicates that FSB females have not evolved to protect stored sperm or fertilized ova from cold shock.

I found considerable evidence to suggest that the FSB populations have evolved to mount a faster recovery post cold shock in terms of the reproductive traits. I was able to show that the FSB populations had a significantly greater mating frequency over the period of 36 hours post cold shock relative to the FCB populations. Increment in mating success can partly answer the higher egg viability in the selected populations relative to the control populations. Greater mating success can in princlple either transfer the afresh sperm or Acps which can change post mating female reproductive physiology- such as Ovulin promoting ova production (reviewed in Wolfner et al. 1997).

Evolution of pre- and post- copulatory traits in males and females

I assayed pre-and post-copulatory traits such as mating latency, copulation duration, male fertility, progeny production, sperm competitive ability in males post cold stress. FSB males recovered faster from cold shock in terms of increased mating success, male

fertility, progeny production and decreased mating latency and have higher sperm offense abilities (see chapter 4a). Similarly, post cold shock, FSB females recovered faster in terms of reduced mating latency, higher mating success and progeny production (see chapter 4b). These results are in agreement with the findings of Price at al. (2014). Who found that females from northern latitudes had more mating relative female from southern regions. These findings suggest the evolution reproductive traits are correlated with increased reistance to cold stress resistance.

The higher fitness of FSB males and females post cold shock could be attributed to the following mechanisms: (a) FSB flies have an altered physiology. (b) FSB flies store specific metabolites for protection from cold. Metabolites such as glycogen, triacylglycerols, proline and certain proteins can act as an energy source and as antifreeze agents (Chen and Walker 1994, Kostal et al. 2011a). (c) FSB flies can produce more/better quality gametes post cold shock. However, at this point of time, we do not know if any or all of these factors contribute to the differences between FSB and FCB populations.

The life-history costs associated with increased resistance to cold shock

Trade-offs are central to the life-history theory (Stearns 1992, Travers et al. 2015). The trade-off between survival and reproduction is considered as a fundamental trade-off. In case of *D. melanogaster*, previous studies show that increased resistance to cold stress leads to decreased longevity in female (MacMillan et al. 2009). In my study, the FSB populations have evolved a range of reproductive traits (including, mating latency, mating rate progeny production, sperm competitive ability, etc.) which are likely to be costly. Therefore, I expected that this might lead to trade-offs with other important life-history traits such as longevity, life time fecundity and development time. I found no evidence of changes in longevity and life time fecundity of the FSB populations relative to the FCB populations. However, I found delayed first-instar larva to adult development (on an average 2-6 hours) in the FSB populations relative to the FCB populations. This can potentially be a life-history trade-off. However, it is known that by delaying development time, in principle, larvae can acquire more resources so that they can contribute energy resources to other stages of life. For example, larvae can acquire higher lipid and glycogen in order increase adult environmental stress resistance (Chippindale et al. 1994, 1998). Similary, it is possible that the delayed development time in FSB populations

helps them to acquire more resources to perform better under stressful condition. While I found an increase in female body size at eclosion in FSBs, this was not true of males. My findings of the female longevity are in line with the results of Bubliy and Loeschcke (2005) who found no significant change in longevity in the line selected for cold stress resistance. However, my results are in contrast with MacMillan et al. (2009) who documented reduced female longevity in populations selected for cold resistance relative to the control populations. Similarly, Anderson et al. (2005) also found decreased longevity in populations selected for faster chill coma recovery. These differences could be explained in terms of differences in the ancestry of experimental populations and the details of the selection protocols. Relationships among the life-history traits can be more complex than predicted by the simple Y model (Harshman and Zera 2001). It is also quite possible that trade-offs, if any, may involve traits other than life time fecundity and longevity; for example, resistance to other stresses.

Mechanisms of cold shock resistance

In order to understand the genetic basis of adaptation, I attempted to quantify the gene expression profile in the FSB and FCB populations. Many previous laboratory studies have assessed the gene expression patterns in *D. melanogaster* during and after (recovery phase) challenge with cold stress. For example, Colinet and Hoffmann (2010) found that *stv* is up regulated during cold shock and during the recovery phase. Similarly they also reported up regulation of *hsp70* gene during the recovery phase post cold stress. Further, Colinet et al. (2010) found that the expression of *hsp22, hsp23, hsp26, hsp27, hsp40, hsp68, hsp70Aa* and *hsp83* genes is up regulated during the recovery period after exposure of cold stress. Sinclair et al (2007a) reported higher expression levels of the *frost* (*fst*) gene during the recovery phase after exposure of *D. mealnogaster* to cold stress. These findings suggest that the higher expression of these genes may help in repairing the damage caused by cold stress.

In my study, I attempted to understand the genetic basis of adaptation to cold stress in the FSB populations. I did this by quantifying the gene expression profile of male flies from the FSB and FCB populations with and without exposure of cold shock. Based on previous work, I selected *hsp22, hsp23, hsp40, hsp68, hsp70Aa, hsp83 and fst* genes for study. I found that upon cold shock both the FSB and FCB males had significantly higher expression of these genes relative to no shock condition. However, the differences in

expression between FSB and FCB populations were very low (0.5 to 2.0 fold changes) and this difference was statistically not significant. This finding suggests that these genes are not majorly involved in the evolution of better response to cold stress in FSB populations. Other studies have found elevated levels of glycogen, triacylglycerols, proline and total proteins in response to cold stress (Misener et al. 2001, Shimada and Riihimaa 1990, Fields et al. 1998, Ramlov 1999) indicating that these metabolites might be important in resistance to cold stress. I investigated whether FSB populations have undergone a change in their metabolites. I found that the fractional lipid content of FSB and FCB populations are comparable. It remains a distinct possibility that the profile of various kinds of lipids may have changed in the FSB populations relative to FCB populations without any change in the total lipid content. For example, Kostal et al. (2011a) showed that cold acclimated larvae of *D. melanogaster* had restructured glycerophospholipid of cell membranes. In addition to this there may be other metabolites involved in higher cold tolerance in the selected populations. Chen and Walker (1994) reported higher amount of glycogen and total proteins in populations *D. mealnogaster* selected for cold shock tolerance relative to control populations.

Evolution of cross-tolerance

I investigated whether selection for resistance to cold stress in FSB populations resulted in a response that was generic or specific to cold stress. It is known from multiple laboratory studies that the evolution of the ability to resist a certain kind of environmental stress may show specific advantage or disadvantage with respect to resistance to other types of environmental stress (Bubliy and Loeschcke 2005, Kristensen et al. 2007, MacMillan et al. 2009)**.** I looked at various stresses such as resistance to starvation, desiccation, heat, and bacterial infection. FSB populations had reduced starvation resistance relative to FCB populations, indicating a probable life-history cost. This finding agrees with previous findings by MacMillan et al. (2009) who observed a decrease in starvation resistance in populations of *D. melanogaster* selected for increased resistance to cold stress. This result indicates that starvation resistance is negatively correlated with cold stress resistance. I found increased resistance to desiccation in females of the FSB populations compared to the FCB populations. FSB populations also had higher resistance to heat stress relative to FCB populations. Thus it is possible that resistance to multiple stresses might involve shared pathways. My findings are in agreement with Kristensen et al. (2007) and contrary to other studies (Anderson et al.

2005, Bubliy and Loeschcke 2005, MacMillan et al. 2009). Hence resistance to environmental stress is complex.

Isolation and characterization of a novel natural pathogen of *Drosophila melanogaster*

I isolated a novel bacterial pathogen (*Staphylococcus succinus* subsp. *succinus* strain (PK-1) of *D. melanogaster* from wild captured *Drosophila.* This pathogen can establish a sustained infection across various outbred and inbred populations of *D. melanogaster*. This novel pathogen has crucial applications especially in understanding the evolutionary ecology of immunity.

To summarize, the findings of this thesis further our understanding of the evolution of reproductive behavior and other life-history traits in response to environmental stress.

References

Alison R. Gerken AR, Eller OC, Daniel A. Hahn DA, Morgan TJ. Constraints, independence, and evolution of thermal plasticity: Probing genetic architecture of long- and short-term thermal acclimation. PNAS. 2015; 112: 4399-4404.

Amice G, Vernon P, Outreman Y, van Alphen JJM, van Baaren J. Variability in responses to thermal stress in parasitoids. Ecol Entomol. 2008; 33: 701-708.

Andersen JL, MacMillan HA, Overgaard J. Muscle membrane potential and insect chill coma. J Exp Biol. 2015; 218: 2492-2495.

Anderson AR, Hoffmann AA, McKechnie SW. Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: Physiology and life-history traits. Genet Res. 2005; 85: 15-22.

Andrewartha HG. Diapause in relation to the ecology of insects. Biol Rev. 1952; 27: 50-107.

Andrewartha HG. In: Introduction to the Study of Animal Populations. Chicago: University of Chicago Press; 1970.

Angilletta MJ Jr, Niewiarowski PH, Navas CA. The evolution of thermal physiology in ectotherms. J Therm Biol. 2002; 27: 249-268.

Angilletta MJ Jr. Thermal adaptation: A theoretical and empirical synthesis. New York: Oxford University Press; 2009.

Apidianakis Y, Rahme LG. *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. Nat Protoc. 2009; 4: 1285-1294.

Arbogast RT. Mortality and reproduction of *Ephestia cautella* and *Plodia interpunctella* exposed as pupae to high temperatures. Environ Entomol. 1981; 10: 708-710.

Armstrong GA, Rodríguez EC, Robertson RM. Cold hardening modulates K^+ homeostasis in the brain of *Drosophila melanogaster* during chill coma. J Insect Physiol. 2012; 58: 1511-1516.

Arthur B, Hauschteck-Jungen E, Nöthiger R, Ward PI. A female nervous system is necessary for normal sperm storage in *Drosophila melanogaster*: a masculinized nervous system is as good as none. P Roy Soc Lond B Bio. 1998; 265: 1749-1753.

Arya R, Mallik M, Lakhotia SC. Heat shock genes-integrating cell survival and death. J Biosci. 2007; 32: 595-610.

Austin CJ, Moehring AJ. Optimal temperature range of a plastic species, *Drosophila simulans*. J Anim Ecol. 2013; 82: 663-672.

Avila FW, Sirot LK, LaFlamme BA, Rubinstein CD, Wolfner MF. Insect seminal fluid proteins: identification and function. Annu Rev Entomol. 2011; 56: 21-40.

Ayrinhac A, Debat V, Gibert P, Kister AG, Legout H, Moreteau B, et al. Cold adaptation in geographical populations of *D. melanogaster*: phenotypic plasticity is more important than genetic variability. Func Ecol. 2004; 18: 700-706.

Bale JS. Insects and low temperatures: From Molecular Biology to Distribution and Abundance. Philos T Roy Soc B. 2002; 357: 849-862.

Bastock M, Manning A. The courtship of *Drosophila melanogaster*. Behaviour 1955; 8: 85-111.

Bayley M, Petersen SO, Knigge T, Kohler HR, Holmstrup M. Drought acclimation confers cold tolerance in the soil collembolan *Rolsomia candida*. J Insect Physiol. 2001; 47: 1197-1204.

Bennett AF. The thermal dependence of lizard behaviour. Anim Behav. 1980; 28: 752-762.

Berwaerts K, Dyck HV. Take-off performance under optimal and suboptimal thermal conditions in the butterfly *Pararge aegeria*. Oecologia. 2004; 141: 536-545.

Best AR, Lewis Z, Hurst GDD, Lize A. Thermal environment during and outside courtship jointly determine female re-mating rate in *Drosophila melanogaster*. Anim Behav. 2012; 83: 1483–1490.

Bharathi NS, Prasad NG, Shakarad M, Joshi A. Variation in adult life-history and stress resistance across five species *of Drosophila*. J Genet. 2003; 82: 191-205.

Bing X, Zhang J, Sinclair BJ. A comparison of Frost expression among species and life stages of*Drosophila*. Insect Mol Biol. 2012; 21: 31-39.

Block W, Webb NR, Coulson S, Hodkinson ID, Worland MR. Thermal adaptationn in the arctic collembolan *Onychiurus arcticus* (Tullberg). J Insect Physiol. 1994; 40: 715-722.

Bonds MH. Host life-history strategy explains pathogen-induced sterility. The American Naturalist, 2006; 168: 281-293.

Borash DJ, Pierce VA, Gibbs AG, Mueller LD. Evolution of ammonia and urea tolerance in *Drosophila melanogaster*: resistance and cross-tolerance. J Insect Physiol. 2000; 46: 763-769.

Bouletreau-Merle J, Allemand R, Cohet Y, David JR. Reproductive strategy in *Drosophila melanogaster:* significance a genetic divergence between temperate and tropical populations. Oecologia. 1982; 53: 323-329.

Bubliy OA, Loeschcke V. Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. J Evol Biol. 2005; 18: 789-803.

Bubliy OA, Riihimaa A, Norry FM, Loeschcke V. Variation in resistance and acclimation to low-temperature stress among three geographical strains of *Drosophila melanogaster*. J Therm Biol. 2002; 27: 337-344.

Campbell PM, Pottinger TG, Sumpter JP. Stress reduces the quality of gametes produced by rainbow trout. Biol Reprod. 1992; 47: 1140-1150.

Carriere Y, Boivin G. Evolution of thermal sensitivity of parasitization capacity in egg parasitoids. Evolution. 1997; 51: 2028-2032.

Chakir M, Chafik A, Moreteau B, Gibert P, David JR. Male sterility thermal thresholds in *Drosophila*: D. simulans appears more cold-adapted than its sibling *D. melanogaster*. Genetica. 2002; 114: 195-205.

Chapman RF. The insects: structure and function, 4th ed. Cambridge: Cambridge University Press; 1998.

Chapman T. Seminal fluid-mediated fitness traits in *Drosophila*. Heredity. 2001; 87: 511-521.

Chen CP, Walker VK. Cold-shock and chilling tolerance in *Drosophila.* J Insect Physiol. 1994; 40: 661-669.

Chen CP, Walker VK. Increase in cold-shock tolerance by selection of cold resistance lines in *Drosophila melanogaster*. Ecol Entomol. 1993; 18: 184-190.

Chihrane J, Lauge G. Effects of high temperature shocks on male germinal cells of *Trichogrammabrassicae* (Hymenoptera,Trichogrammatidae). Entomophaga. 19 94; 39: 11-20.

Chippindale AK, Chu TJF, Rose MR. Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. Evolution. 1996; 50: 753-766.

Chippindale AK, Gibbs AG, Sheik M, Yee KJ, Djawdan M, Bradley TJ, et al. Resource acquisition and the evolution of stress resistance in *Drosophila melanogaster*. Evolution. 1998; 52: 1342-1352.

Chippindale AK, Hoang DT, Service PM, Rose MR. The evolution of development in *Drosophila melanogaster* selected for postponed senescence. Evolution. 1994; 48: 1880--1899.

Chippindale AK, Rice WR. Y chromosome polymorphism is a strong determinant of male fitness in *Drosophila melanogaster*. Proc Natl Acad Sci USA. 2001; 98: 5677-5682.

Chown SL, Nicolson SW. Insect physiological ecology: Mechanisms and patterns. Oxford University Press; 2004.

Chown SL, Terblanche JS. Physiological diversity in insects: ecological and evolutionary contexts. Adv Insect Physiol. 2006; 33: 50-152.

Christopher R, Christopher RC, Michael SG. Native Microbial Colonization of *Drosophila melanogaster* and Its Use as a Model of *Enterococcus faecalis* Pathogenesis. Infect Immunity. 2007; 5: 1565-1576.

Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J. Re-examination of the geneus *Acetobacter* with descriptions of *Acetobacter cerevisiae* sp.nov. and *Acetobacter malorum* sp. Nov. Int J Syst Evol Microbiol. 2002; 52: 1551-1558.

Colinet H, Hance T. Male reproductive potential of *Aphidius colemani* (Hymenoptera: Aphidiinae) exposed to constant or fluctuating thermal regimens. Environ Entomol. 2009; 38: 242-249.

Colinet H, Larvor V, Laparie M, Renault D. Exploring the plastic response to cold acclimation through metabolomics. Funct Ecol. 2012; 26:711-722.

Colinet H, Lee SF, Hoffmann A. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. FEBS J. 2010; 277: 174-185.

Collett JI, Jarman MG. Adult female *Drosophila pseudoobscura* survive and carry fertile sperm through long periods in the cold: populations are unlikely to suffer substantial bottlenecks in overwintering. Evolution. 2001; 55: 840-845.

Corby-Harris V, Promislow DEL. Host ecology shapes geographic variation for resistance to bacterial infection in *Drosophila melanogaster*. J Anim Ecol. 2008; 77: 768-76.

Cossin AR, Bowler K. In: Temperature Biology of Animals. New York: Chapman and Hall; 1987.

Coulson SC, Bale JS. Effect of rapid cold hardening on reproduction and survival of offspring in the house fly *Musca domestica*. J Insect Physiol. 1992; 38: 421-424.

Czajka MC, Lee RE. A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. J Exp Biol. 1990; 148: 245-254

Dennis AB, Dunning LT, Sinclair BJ, Buckley TR. Parallel molecular routes to cold adaptation in eight genera of New Zealand stick insects. Sci Rep. 2015; 5: 13965.

Danks HV Diversity and integration of life-cycle controls in insects. In: Danks HV (ed) Insect Life-Cycle Polymorphism: Theory, Evolution and Ecological Consequences for Seasonality and Diapause Control. Kluwer Academic Publishers, Dordrechrt; 1994. pp 5-40.

David JR, Araripe LO, Chakir M, Legout H, Lemos B, Petavy G, et al. Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. J Evol Biol. 2005; 18: 838-46.

David JR. Male sterility at high and low temperatures in *Drosophila*. J Soc Biol. 2008; 202: 113-117.

David RJ, Gibert P, Pla E, Petavy G, Karan D, Moreteau B. Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. J Therm Biol. 1998; 23: 291-299.

Davidson JK. Non parallel geographic patterns for tolerance to cold and desiccation in *Drosophila melanogaster* and *Drosophila simulans*. Aust J Zool. 1990; 38: 155-161.

Dell AI, Pawar S, Savage VM. Systematic variation in the temperature dependence of physiological and ecological traits. PNAS. 2011; 108: 10591-10596.

Denlinger DL, Lee RE. Low temperature biology of insects. Cambridge: Cambridge University Press; 2010.

Denlinger DL, Yocum GD. Physiology of heat sensitivity. In: Temperature sensitivity in insects and applications for integrated pest management. West view Press, Boulder; 1998.

Denlinger DL. Relationship between cold hardiness and diapause. In: Lee RE, Denlinger DL, editors. Insects at Low Temperature. New York: Chapman and Hall; 1991. pp. 174-198.

Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin P R. Impacts of climate warming on terrestrial ectotherms across latitude. PNAS. 2008; 105: 6668-6672.

Dick CA, Rank NE, McCarthy M, McWeeney S, Hollis D, Dahlhoff EP. Effects of temperature variation on male behavior and mating success in a montane beetle. Physiol Biochem Zool. 2013; 86: 432-440.

Dillon ME, Cahn LRY, Huey RB. Life history consequences of temperature transients in *Drosophila melanogaster*. J Exp Biol. 2007; 210: 2897-2904.

Dillon ME, Wang G, Garrity PA, Huey RB. Thermal preference in *Drosophila*. J Therm Biol. 2009; 34: 109-119.

Dolgin ES, Whitlock MC, Agrawal AF. Male *Drosophila melanogaster* have higher mating success when adapted to their thermal environment. J Evol Biol. 2006; 19: 1894-1900.

Ezaki T, Hashimoto Y, Yabuuchi E. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microcondition wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. J Syst Evol Microbiol. 1989; 39: 224-229.

Fabian DK, Lack JB, Mathur V, Schlötterer C, Schmidt PS, Pool JE, Flatt T. Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa. J Evol Biol. 2015; 28: 826-840.

Fasolo AG and Krebs RA. A comparison of behavioural change in *Drosophila* during exposure to thermal stress. Biol J Linn Soc. 2004; 83:197-205.

Fedorka K, Linder J, Promislow DEL. Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*. Proc R Soc Series B. 2007; 274: 1211-1217.

Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 1999; 6: 243-282.

Fischer K, Brakefield PM, Zwaan BJ. Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology. 2003; 84: 3138-3147.

Froman DP, Pizzari T, Feltmann AJ, Castillo-Juarez H, Birkhead TR. Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus*. Proc Biol Sci. 2002; 269: 607-612.

Gage MJG, Morrow EH. Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. Curr Biol. 2003; 13: 754-757.

Galac M, Lazzaro BP. Comparative pathology of bacteria in the genus *Providencia* to a natural host, *Drosophila melanogaster*. Microb Infect. 2011; 13: 673-683.

Garrity PA, Goodman MB, Samuel AD, Sengupta P. Running hot and cold: behavioral strategies, neural circuits, and the molecular machinery for thermo taxis in *C. elegans* and *Drosophila*. Genes Dev. 2010; 24: 2365-2382.

Gehrken U**,** Southon TE. Effect of temperature on cold-hardiness and tissue ice formation in adult chrysome-lid beetle Melasoma collaris L. J insect Physiol. 1997; 43: 587-593.

Gevers D, Huys G, Swings J. Application of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbio Lett. 2001; 205: 31-36.

Gibbs AG. Laboratory selection for the comparative physiologist. J Exp Biol. 1999; 202: 2709-2718.

Gibert P, Capy P, Imasheva A, Moreteau B, Morin JP, Pétavy G, David JR. Comparative analysis of morphological traits among *Drosophila melanogaster* and *D. simulans*: genetic variability, clines and phenotypic plasticity. In *Drosophila melanogaster*, *Drosophila simulans*: So Similar, So Different. 2004. pp. 165-179.

Gibert P, Moreteau B, Petavy G, Karan D, David JR. Chill coma tolerance, a major climatic adaptation among *Drosophila* species. Evolution 2001; 51: 1063-1068.

Gilchrist GW, Huey RB, Partridge L. Thermal sensitivity of *Drosophila melanogaster*: evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. Physiol Zool. 1997; 70: 403-414.

Gillott C. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu Rev Entomol. 2003; 48: 163-184.

Goto SG, Kimura MT. Heat- and cold-shock responses and temperature adaptations in subtropical and temperate species of *Drosophila*. J. Insect Physiol.1998; 44: 1233-1239.

Goto SG, Udaka H, Ueda C, Katagiri C. Fatty acids of membrane phospholipids in *Drosophila melanogaster* lines showing rapid and slow recovery from chill coma. Biochem Biophys Res Commun.2010; 391: 1251-1254.

Goto SG. A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster*. Gene. 2001; 270: 259-264.

Guerra D, Cavicchi S, Krebs RA, Loeschcke V. Resistance to heat and cold stress in *Drosophila melanogaster*: intra and inter population variation in relation to climate. Genet Sel Evol. 1997; 29: 497-510.

Gulevsky AK, Relina LI. Molecular and genetic aspects of protein cold denaturation. Cryo Letters. 2013; 34: 62-82.

Gullan PJ, Cranston PS, In: The Insects: An Outline of Entomology third ed. Davis, USA, Blackwell Publishing Ltd; 2005.

Hangartner, SB, Hoffmann AA, Smith A, Griffin PC. A collection of Australian Drosophila datasets on climate adaptation and species distributions. Scientific Data; 2, 2015.

Hallman GJ, Denlinger DL. Introduction: temperature sensitivity and integrated pest management. In: Temperature Sensitivity in Insects and Application in Integrated Pest Management. Ed. Hallman GJ, Denlinger DL. West view Press, Boulder; 1998. pp. 1-5.

Hance T, Van Baaren J, Vernon P, Boivin G. Impact of extreme temperatures on parasitoids in a climate change perspective. Annu Rev Entomol. 2007; 52: 107-126.

Hansen PJ. Effects of heat stress on mammalian reproduction. Philos Trans R Soc Lond B: Biol Sci. 2009; 364: 3341–3350.

Harshman LG, Hoffmann AA. Laboratory selection experiments using *Drosophila*: what do they really tell us? Trends Ecol Evol. 2000; 15; 32-36.

Harshman LG, Zera AJ. The cost of reproduction: the devil in the details. Trends Ecol Evol. 2007; 22: 80-86.

Hazel J. Thermal adaptation in biological membranes: is home viscous adaptation the explanation? Annu Rev Physiol. 1995; 57: 19-42.

Hochachka P, Somero G. Temperature. Biochemical adaptation: Mechanism and Process in Physiological Evolution, Oxford Uni-versity press; 2002, pp. 290-438.

Hodkova M, Hodek I. Photoperiod, diapause and cold-hardiness. Eur J Exp Biol. 2004; 101: 445-458.

Hoffmann AA, Anderson A, Hallas R. Opposing clines for high and low temperature resistance in *Drosophila Melanogaster*. Ecol Lett. 2002; 5: 614-618.

Hoffmann AA, Parsons A. Evolutionary Genetics and Environmental Stress. Oxford: Oxford University Press; 1991.

Hoffmann AA, Scott M, Partridge L, Hallas R. Overwintering in *Drosophila melanogaster:* outdoor field cage experiments on clinal and laboratory selected populations help to elucidate traits under selection. J Evol Biol. 2003a; 16: 614-623.

Hoffmann AA, Shirriffs J, Scott M. Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. Func Ecol. 2005b; 19: 222-227.

Hoffmann AA, Sorensen JG, Loeschcke V. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol. 2003b; 28: 175-216.

Hoffmann AA Watson M. Geographical variation in responses of *Drosophila* to climatic extremes. Am Nat. 19993; 142: S93-S113.

Hoffmann AA, Weeks AR. Climatic selection on genes and traits after a 100 year-old invasion: A critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. Genetica. 2007; 129: 133-147.

Hollingsworth MJ, Bowler K. The decline in ability to withstand high temperature with increase in age in *Drosophila subobscura*. Exp Geront. 1966; 1: 251-257.

Holmstrup M, Bayley M, Ramløv H. Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable Arctic invertebrates. PNAS. 2002; 99: 5716-5720.

Huey RB, Berrigan D. Temperature, demography, and ectotherm fitness. Am Nat. 2001; 158: 204-210.

Irwin, JT, Lee REJ. Cold wintr microenvironments conserve energy and improeve overwintering survival and potential fecundity of the Goldenrod Gall Fly, *Eurosta solidaginis*. Oikos. 2003; 100: 71-78.

Iyengars V, Bakerr M. The influence of temperature on the pattern of insemination by *Drosophila melanogaster* males. Genetics. 1962; 47: 963-964.

Jakobs R. Low temperature tolerance of adult *Drosophila suzukii* (Diptera: Drosophilidae) (2014). Electronic Thesis and Dissertation Repository. Paper 2532.

James A, Partridge L. Geographic variation in competitive ability in *Drosophila melanogaster*. Am Nat. 1998; 151: 530-537.

James AC, Partridge L. Thermal evolution of rate of larval development in *Drosophila melanogaster* in laboratory and field populations. J Evol Biol. 1995; 8: 315-330.

Janowitz SA, Fischer K. Polyandry in *Bicyclus anynana* butterflies results from sexual conflict over mating. Ethology. 2012; 118: 1140-1148.

Joshi A and Mueller LD Density-dependent natural selection in *Drosophila*: trade-offs between larval food acquisition and utilization. Evol Ecol 1996; 10: 463-474.

Karan D, Dahiya N, Munjal AK, Gibert P, Moreteau B, Parkash R, David JR. Desiccation and starvation tolerance of adult *Drosophila*: opposite latitudinal clines in natural populations of three different species. Evolution. 1998; 52: 825-831.

Katsuki M, Miyatake T. Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*. J. of Insect Physiol. 2009; 55: 113-116.

Kellermann V, Loeschcke V, Hoffmann AA, Kristensen TN, Fløjgaard C, David J et al. Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila*. Evolution. 2012; 66: 3377-3389.

Kellett M, Hoffmann AA, McKechnie SW. Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermo tolerance. Funct Ecol. 2005; 19: 853-858.

Kelty JD, Lee RE. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. J Insect Physiol. 1999; 45: 719-726.

Khan I, Prasad NG. The aging of the immune response in *Drosophila melanogaster*. J Gerontol A Biol Sci Med Sci. 2013; *68:* 129-135.

Kindle TK, Johnson KM, Ivy TM, Weddle CB, Sakaluk SK. Female mating frequency increases with temperature in two cricket species, *Gryllodes sigillatus* and *Acheta domesticus* (Orthoptera: *Gryllidae*). Can J Zool. 2006; 84: 1345-1450.

Kingsolver JG, Watt WB. Thermoregulatory strategies in Colias butterflies: Thermal stress and the limits to adaptation in temporally varying environments. Am Nat. 1983; 121: 32-55.

Kingsolver JG. The well-temperatured biologist. The Am Nat. 2009; 174: 755-768.

Klepsatel P, Galikova M, De Maio N, Huber CD, Schleotterer C, Flatt T. Variation in thermal performance and reaction norms among populations of *Drosophila melanogaster*. Evolution. 2013; 67: 3573-3587.

Kloos WE, Schleifer KH, Gotz F. The Genus *Staphylococcu*s, pp. 1369–1420. In: The Prokaryotes. A handbook on the biology of bacteria: ecophysics, isolation, identification, applications (Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH. eds.) 2nd ed., New York, Springer-Verlag; 1991.

Kostal V, Korbelova J, Rozsypal J, Zahradncikova H, Cimlova J, Tomcala A, Simek P. Long-term cold acclimation extends survival time at 0 degrees C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. PLoS One. 2011; 6: e25025.

Kostal V, Tamura M, Tolarova M, Zahradnıckova H. Enzymatic capacity for accumulation of polyol cryoprotectants changes during diapause development in the adult red firebug, *Pyrrhocoris apterus*. Physiol Entomol. 2004a; 29: 344-355.

Kostal V, Vambera J, Bast J. On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. J Exp Biol. 2004b; 207: 1509-1521.

Krebs RA, Loeschcke V. Effects of exposure to short-term heat stress on fitness components in *Drosophila melanogaster*. J Evol Biol.1994a; 7: 39-49.

Kristensen TN, Loeschcke V, Hoffmann AA. Can artificially selected phenotypes influence a component of field fitness? Thermal selection and fly performance under thermal extremes. Proc R Soc B. 2007; 274: 771-778.

Kvelland I. Mating and fertility pattern of *Drosophila melanogaster* males exposed to different temparatures. Hereditas. 1965a; 54: 88-100.

Kvelland I. Some observations on the mating activity and fertility of *Drosophila melanogaster* males. Hereditas. 1965b; 53: 281-306.

Kwan L, Bedhomme S, Prasad NG, Chippindale AK. Sexual conflict and environmental change: trade-offs within and between the sexes during the evolution of desiccation resistance. J Genet. 2008; 87: 383-394.

Kwok AY, Su SC, Reynolds RP, Bay SJ, Av-Gay Y, Dovichi NJ, Chow AW. Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcu*s. Int J Syst Bacteriol. 1999; 49: 1181-1192.

Lacoume S, Bressac C, Chevrier C. Sperm production and mating potential of males after a cold shock on pupae of the parasitoid wasp *Dinarmus basalis* (Hymenoptera: Pteromalidae). J Insect Physiol. 2007; 53: 1008-1015.

Lambert LH, Cox T, Mitchell K, Rossello MRA, Del Cueto C, Dodge DE, Orkand P, Cano RJ. *Staphylococcus succinus* sp. nov., isolated from *Dominican amber*. Int J Syst Bacteriol. 1998; 48: 511-518.

Lavington E, Cogni R, Kuczynski C, Koury S, Behrman EL, O'Brien KR, Eanes WF. A small system—high-resolution study of metabolic adaptation in the central metabolic pathway to temperate climates in *Drosophila melanogaster*. Mol Biol Evol. 2014; msu146.

Lazzaro BP and Clark AG. Molecular population genetics of inducible antibacterial peptide genes in *Drosophila melanogaster*. Mol Biol Evol. 2003; 20:914-923.

Lazzaro BP, Flores HA, Lorigan JG, Yourth CP. Genotype by environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. PLoS Pathog.2008; 4: e1000025.

Le Bourg E, Massou I, Gobert V .Cold stress increases resistance to fungal infection throughout life in *Drosophila melanogaster*. Biogerontology. 2009; 10: 613-625.

Lee R, Denlinger D. Insects At Low Temperature. New York: Chapman and Hall; 1991.

Lefevre GJ, Jonsson UB. The effect of cold shock on *D. melanogaster* sperm. Drosoph Inf Serv. 1962; 36: 86-87.

Lieshout E, Tomkins JL, Simmons LW. Heat stress but not inbreeding affects offensive sperm competitiveness in *Callosobruchus maculatus*. Ecol Evol. 2013; 3: 2859-2866.

Lupold S, Manier MK, Ala-Honkola O, Belote JM, Pitnick S. Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity, and age. Behav Ecol. 2011; 22: 184-191.

MacMillan H A, Walsh JP, Sinclair BJ. The effects of selection for cold tolerance on cross-tolerance to other environmental stressors in *Drosophila melanogaster*. J Insect Sci. 2009; 16: 263-276.

MacMillan HA, Ferguson LV, Nicolai A, Donini A, Staples JF, Sinclair BJ. Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. J Exp Biol. 2015a. 218: 423-432.

MacMillan HA, Findsen A, Pedersen TH, Overgaard J. Cold-induced depolarization of insect muscle: differing roles of extracellular K^+ during acute and chronic chilling. J Exp Biol. 2014; 217: 2930-2938.

MacMillan HA, Sinclair BJ. Mechanisms underlying insect chill-coma. J Insect Physiol. 2011; 57; 12-20.

MacMillan HA, Sinclair BJ. The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. J Exp Biol .2011b; 214: 726-734.

MacMillan HA, Williams CM, Staples JF, Sinclair BJ. Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. PNAS. 2012; 109: 20750-20755.

MacMillan HA, Baatrup E, Overgaard J. Concurrent effects of cold and hyperkalaemia cause insect chilling injury. Proc R Soc B. 2015b. 282; 20151483.

Malmendal A, Overgaard J, Bundy JG, Sorensen JG, Nielsen NC, Loeschcke V, et al. Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. Am J Physiol Regul Integr Comp Physiol. 2006; 291: R205-212.

Manier MK, Belote JM, Berben KS, Novikov D, Stuart WT, et al. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science. 2010; 328: 354-357.

Markow TA, Ricker JP. Male size, developmental stability, and mating success in natural populations of three *Drosophila* species. Heredity 1992; 69: 122-127.

Markow TA, Sawka S. Dynamics of mating success in experimental groups of *Drosophila melanogaster* (Diptera, Drosophilidae). J Insect Behav. 1992. 5, 375-383.

Marshall KE, Sinclair BJ. Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. Proc R Soc B. 2010; 277: 963-969.

Marshall KE, Sinclair BJ. The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. J Exp Biol. 2011; 214: 1205-1212.

Maynard Smith J. Temperature tolerance and acclimatization in *Drosophila subobscura*. J Exp Biol. 1956; 34: 85-96.

McKean KA, Nunney L. Sexual selection and immune function in *Drosophila melanogaster*. Evolution. 2008; 62: 386-400.

Mesbah M, Premachandran U, Whitman WB. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol. 1989; 39: 159-167.

Mikonranta L, Mappes J, Kaukoniitty M, Freitak D. Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection. Front Zool, 2014; 11: 23.

Mockett RJ, Matsumoto Y. Effect of prolonged coldness on survival and fertility of *Drosophila melanogaster*. PLoS One. 2014; 9: e92228.

Mori N, Kimura MT. Selection for rapid and slow recovery from chill- and heat-coma in *Drosophila melanogaster*. Biol J Linnean Soc. 2008; 95:72-80.

Nandy B, Chakraborty P, Ali ZS. Prasad NG. Sperm competitive ability evolves in response to altered operational sex ratio. Evolution. 2013; 67: 2133-2141.

Nadeau D, Corneau S, Plante I, Morrow G, Tanguay RM. Evaluation for Hsp70 as a biomarker of effect of pollutants on the earthworm *Lumbricus terrestris*. Cell stress chaperon. 2001; 6: 153.

Nghiem D, Gibbs AG, Rose MR, Bradley TJ. Postponed aging and desiccation resistance in *Drosophila melanogaster*. Exp Geront. 2000; 35: 957-969.

Nguyen TM, Bressac C, Chevrier C. Heat stress affects male reproduction in a parasitoid wasp. J Insect Physiol. 2013; 59: 248-254.

Nielsen MM, Overgaard J, Sørensen JG, Holmstrup M, Justesen J, Loeschcke V. Role of HSF activation for resistance to heat, cold and high temperature knock-down. J Insect Physiol. 2005; 51:1320-1329.

Norry FM, Loeschcke V. Temperature induced shifts in associations of longevity with body size in *Drosophila melanogaster*. Evolution. 2002; 56: 299-306.

Norry FM, Sambucetti P, Scannapieco AC, Loeschcke V. Altitudinal patterns for longevity, fecundity and senescence in *Drosophila buzzatii*. Genetica. 2006; 128: 81-93.

Novakova D, Sedlacek I, Pantucek R, Stetina V, Svec P, Petras Petr. *Staphylococcus equorum* and *Staphylococcus succinus* isolated from human clinical specimens. J Med Microbiol. 2006; 55: 523-528.

Novitski E, Rush G. Viability and fertility of *Drosophila* exposed to sub-zero temperatures. Biol Bull. 1949; 97: 150-157.

Oakeshott JG, Gibson JB, Anderson PR, Knibb WR, Anderson DG, Chambers GK. Alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase clines in *Drosophila melanogaster* on different continents. Evolution 1982; 39: 86-96.

Ohtsu T, Katagiri C, Kimura M T, Hori SH. Cold adaptations in *Drosophila*. Qualitative changes of triacylglycerols with relation to overwintering. J Biol Chem, 1993; *268:* 1830-1834.

Overgaard J, Sorensen JG, Petersen SO, Loeschcke V, Holmstrup M. Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. J Insect Physiol. 2005; 51: 1173-1182.

Overgaard J, Kearney MR, Hoffmann AA. Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. Glob Chang Biol. 2014; 20: 1738-1750.

Overgaard J, Malmendal A, Sørensen JG, Bundy JG, Loeschcke V, et al. Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. J Insect Physiol. 2007; 53: 1218-1232.

Overgaard J, Sørensen JG, Loeschcke V. Genetic variability and evolution of cold tolerance. In low temperature biology of insects. Ed. Denlinger D and Lee RE. Cambridge University Press; 2010. pp. 276-296.

Ozgul A, Tuljapurkar S, Benton TG, Pemberton JM, Clutton-Brock TH, Coulson T. The dynamics of phenotypic change and the shrinking sheep of St. Kilda. Science. 2009; 325: 464-467.

Parsell DA, Lindquist S. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet. 1993; 27: 437-496.

Parsons P A. Evolutionary ecology of Australian Drosophila: a species analysis. Evol Biol. 1982; 4: 297-350.

Parsons PA. Environments and evolution: interactions between stress, resource inadequacy and energetic efficiency. Biol Rev. 2005; 80: 589-610.

Parsons PA. Parallel climatic races for tolerances to high-temperature desiccation stress in two *Drosophila* species. J Biogeogr. 1980; 7: 97-101.

Partridge L, Barton NH. Optimality mutation and the evolution of ageing. Nature-London, 1993; 362; 305-305.

Partridge L, Ewing E, Chandler A. Male size and mating success in *Drosophila melanogaster*. The role of female and male behavior. Anim Behav. 1987b: 35; 555-562.

Partridge L, Hoffman A, Jones JS. Male size and mating success in *Drosophila melanogaster* and *D. pseudoobscura* under field conditions. Anim Behav. 1987a; 35: 468-476.

Patton ZJ, Krebs RA. The effect of thermal stress on the mating behavior of three *Drosophila* species. Physiol Biochem Zool. 2001; 74: 783-788.

Pérez-Crespo M, Pintado B, Gutiérrez-Adán A. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. Mol Reprod Dev. 2008; 75: 40-47.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: 2002-2007.

Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. A Specific Primed Immune Response in *Drosophila* Is Dependent on Phagocytes. PLoS Pathog. 2007; 3: e26.

Phelan JP, Archer MA, Beckman KA, Chippindale AK, Nusbaum TJ, Rose MR. Breakdown in correlations during laboratory evolution. I. Comparative analyses of *Drosophila* populations. Evolution. 2003; 57: 527-535.

Pitchers W, Pool JE, Dworkin, I. Altitudinal clinal variation in wing size and shape in African Drosophila melanogaster: one cline or many? Evolution. 2013; 67: 438-452.

Place RB, Hiestand D, Burri S, Teuber M. *Staphylococcus succinus* subsp. *casei* subsp. nov., a dominant isolate from a surface ripened cheese. Syst Appl Microbiol. 2002; 25: 353--359.

Prasad NG, Bedhomme S, Day T, Chippindale AK. An evolutionary cost of separate genders revealed by male-limited evolution. Am Nat. 2007; 169: 29-37.

Prasad NG, Shakarad M, Rajamani M, Joshi A. Interaction between the effects of maternal and larval nutritional levels on pre-adult survival in *Drosophila melanogaster*. Evol Ecol Res. 2003; 5: 903-911.

Price TAR, Bretman A, Gradilla AC, Reger J, Taylor ML, Giraldo-Perez P, Campbell A, Hurst GD, Wedell N. Does polyandry control population sex ratio via regulation of a selfish gene? Proc R Soc B. 2014; 281:20133259.

Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK (2005) Cold hardening and transcriptional change in *Drosophila melanogaster*. Insect Mol Biol 14, 607-613.

Quinn PJ. A lipid-phase separation model of low-temperature damage to biological membranes. Cryobiology. 1985; 22: 128-146.

Rajpurohit S, Nedved O. Clinal variation in fitness related traits in tropical drosophilids of the Indian subcontinent. J Thermal Biol. 2013; 38: 345-354.

Ramløv H, Lee, RE Jr, Extreme resistance to desiccation in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). J Exp Biol. 2000; 203: 783-789.

Reeve MW, Fowler K, Partridge L. Increased body size confers greater fitness at lower experimental temperature in male *Drosophila melanogaster*. J Evol Biol. 2000; 13: 836-844.

Rinehart JP, Yocum GD, Denlinger DL. Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, Sarcophaga crassipalpis. Physiol Entomol. 2000; 25: 330-336.

Ring RA, Danks H. The role of trehalose in cold-hardiness and desiccation. Cryo Lett. 1998; 19: 275-282.

Ring RA, Danks HV. Desiccation and cryoprotection: Overlapping adaptations. Cryo Lett. 1994; 15: 181-190.

Robinson SJ, Zwaan B, Partridge L. Starvation resistance and adult body composition in a latitudinal cline of *Drosophila melanogaster*. Evolution, 2000; 54: 1819-1824.

Roff DA. The Evolution of Life Histories: Theory and Analysis New York: Chapman & Hall; 1992.

Rohmer C, David J R, Moreteau B, Joly D. Heat induced male sterility in *Drosophila melanogaster:* adaptive genetic variations among geographic populations and role of the Y chromosome. J Exp Biol. 2004; 207: 2735-2743.

Ronges D, Walsh JP, Sinclair BJ, Stillman JH. Changes in extreme cold tolerance, membrane composition and cardiac transcriptome during the first day of thermal acclimation in the porcelain crab *Petrolisthes cinctipes*. J Exp Biol. 2012; 215: 1824-1836.

Rose MR. Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution. 1984; 38: 1004-1010.

Rose MR, Nusbaum TJ, Chippindale AK. Laboratory evolution: the experimental wonderland and the Cheshire cat syndrome. In: Adaptation, edited by Rose MR and Lauder GV. San Diego, CA: Academic, 1996. pp. 221-241.

Roxstrom-Lindquist K, Terenius O, Faye I. Parasite specific immune response in adult *Drosophila melanogaster*: a genomic study. EMBO reports. 2004; 5: 207-212.

Ruel JJ, Ayres MP. Jensen's inequality predicts effects of environmental variation. Trends Ecol. Evol. 1999; 14: 361-366.

Shang Q, Pan Y, Peng T, Yang S, Lu X, Wang Z. et al. Proteomics analysis of overexpressed plasma proteins in response to cold acclimation in *Ostrinia furnacalis*. Arch Insect Biochem Physiol. 2015; 90: 195-208.

Santos M, Ruiz A, Barbadilla A, Quezada-Dí az JE, Hasson E, Fontdevila A. The evolutionary history of *Drosophila buzzatii*. XVI. Larger flies mate more often in nature. Heredity. 1988; 61: 255-262.

Saxena BP, Sharma PR, Thappa RK, Tikku K. Temperature induced sterilization for control of three stored grain beetles. J Stored Prod Res.1992; 28: 67-70.

Schmid PS, Paaby AB. Reproductive diapause and life history clines in north american populations of *Drosophila melanogaster*. Evolution. 2008; 62: 1204-1215.

Schmidt PS, Paaby AB, Heschel MS. Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. Evolution. 2005; 59: 2616-2625.

Schnebel EM, Grossfield J. Mating-temperature range in *Drosophila*. Evolution. 1984; 38: 1296-1307.

Scott M, Berringan D, Hoffmann AA. Costs and benefits of acclimatation to elevated temperature in *Trichogramma carverae*. Entomol Exp Appl. 1997; 85: 211-219.

Short SM, Lazzaro BP. Female and male genetic contributions to female post-mating susceptibility to infection in *Drosophila melanogaster*. Proc R Soc Series B (London). 2010; 277, 3649-3657.

Sibly RM, Calow PA. Life-cycle theory of responses to stress. Biol J Linn Soc.1989; 37: 101-116.

Simmons LW, Fitzpatrick JL. Sperm wars and the evolution of male fertility. Reproduction. 2012; 144: 519-534.

Simmons LW. Sperm competition and its evolutionary consequences in the insects. Princeton, New Jersey: Princeton University Press; 2001.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. Integr Comp Biol. 2013; 53: 545-56.

Sinclair BJ, Gibbs AG, Roberts SP, Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. Insect Mol Biol. 2007a; 16: 435-443.

Sinclair BJ, Nelson S, Nilson T, Roberts SP, Gibbs AG. The effect of selection for dessication resistance on cold tolerance of *Drosophila melanogaster*. Physiol Entomol. 2007b; 32: 322-327.

Sinclair BJ, Vernon P, Klok CJ, Chown SL. Insects at low temperatures: an ecological perspective. Trends Ecol Evol. 2003; 18: 257-262

Sinclair BJ, Williams CM, Terblanche JS. Variation in thermal performance among insect populations. Physiol Biochem Zool. 2012; 85: 594-606.

Sinclair BJ. Field ecology of freeze tolerance: inter annual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinquemaculata*. Oikos. 2001; 93: 286-293.

Singh K, Kochar E, Prasad NG. Egg viability, mating frequency and male mating ability evolve in populations of *Drosophila melanogaster* selected for resistance to cold shock. PLoS One. 2015; 10: e0129992.

Snook RR. Sperm in competition: not playing by the numbers. Trends Ecol Evol. 2005; 20: 46-53.

Sokolowski MB. *Drosophila*: genetics meets behaviour. Nature Reviews Genetics, 2001; 2: 879-890.

Sørensen JG, Kristensen GT, Loeschcke V. The evolutionary and ecological role of heat shock proteins. Ecol Lett. 2003; 6: 1025-1037.

Stanley SM, Parsons PA. The response of the cosmopolitan species, *Drosophila melanogaster*, to ecological gradients. Proceedings of the Ecological Society of Australia 1981; 11: 121-130.

Stearns SC: The Evolution of Life Histories Oxford: Oxford University Press; 1992.

Strachan LA, Tarnowski-Garner HE, Marshall KE, Sinclair BJ. The evolution of cold tolerance in *Drosophila* larvae. Physiol Biochem Zool. 2011; 84: 43-53.

Stratman R, Markow TAResistance to thermal stress in desert *Drosophila*. Funct Ecol. 1998; 12: 965-970.

Svetec N, Werzner A, Wilches R, Pavlidis P, Alvarez-Castro JM, Broman KW, et al. Identification of X-linked quantitative trait loci affecting cold tolerance in *Drosophila melanogaster* and fine mapping by selective sweep analysis. Mol Ecol. 2011; 20: 530-544.

Tauber MJ, Tauber CA, Masaki S Seasonal adaptations of insects. New York: Oxford University Press; 1986.

Teets NM, Denlinger DL. Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol Entomol. 2013; 38: 105–116.

Teets, NM, Peyton JT, Ragland GJ, Colinet H, Renault D, Hahn DA et al. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiological genomics. 2012; 44: 764-777.

Telonis-Scott M, Hallas R, McKechnie SW, Wee CW, Hoffmann AA. Selection for cold resistance alters gene transcript levels in *Drosophila melanogaster*. J Insect Physiol. 2009; *55*: 549-555.

Terhzaz S, Teets NM, Cabrero P, Henderson L, Ritchie MG, Nachman RJ, Davies SA. Insect capa neuropeptides impact desiccation and cold tolerance. PNAS. 2015; 112: 2882-2887.

Trotta V, Calboli FC, Ziosi M, Guerra D, Pezzoli MC, David JR et al. Thermal plasticity in *Drosophila melanogaster*: a comparison of geographic populations. BMC Evol Biol. 2006; 6: 67.

Tucic N. Genetic capacity for adaptation to cold resistance at different developmental stages of *Drosophila melanogaster*. Evolution. 1979; 33: 350-358.

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J. Polyphasic taxonomy. A consensus approach to bacterial systematic. Microbiol Rev. 1996; 60: 647-438.

Voituron Y, Mouquet N, de Mazancourt C, Clobert J. To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. Am Nat. 2002; 160: 255-270.

Watson MJO, Hoffmann AA. Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. Evolution. 1996; 50: 1182-1192.

Wayne LH, Moore WEC, Murray RGE. et al. International committee on Systematics Bcateriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematic. Int J Bacteriol. 1987; 37: 463-464.

Wedvik H. The effect of low temperature on the fertility of *Drosophila melanogaster* males. Drosoph Inf Serv. 1962; 36: 127.

Wigby S, Chapman T. Sex peptide causes mating costs in female *Drosophila melanogaster*. Curr Biol. 2005; 15: 316-321.

Wolf MJ, Rockman HA. *Drosophila melanogaster* as a model system for genetics of postnatal cardiac function. Drug Dis Today Dis Models, 2008; 5: 117-123

Wolfner MF. Tokens of love: functions and regulation of *Drosophila* male accessory gland products, Insect Biochem. Mol. Biol.1997; 27: 179-192.

Wu BS, Lee, JK, Thompson, KM, Walker, VK, Moyes, CD, Robertson, RM. Anoxia induces thermo tolerance in the locust flight system. J Exp Biol. 2002; 205: 815-827.

Yenisetti SC, Hegde SN, Venkateswarlu, Mididoddi, Krishna MS. Phenotypic plasticity of sexual behavior at different temperatures in a drosophilid: *Phorticella striata*. Korean J Genet. 2006; 28: 395-401.

Yi SX, Lee RE. Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. J Insect Physiol. 2003; 49: 999-1004.

Yocum GD. Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. J Insect Physiol. 2001; 47:1139-1145.

Zeh JA, Bonilla MM, Su EJ, Padua MV, Anderson RV, et al. Degrees of disruption: projected temperature increase has catastrophic consequences for reproduction in a tropical ectotherm. Global Change Biol. 2012; 18: 1833-1842.

Zhang W, Zhao F, Hoffmann AA, Ma CS. A single hot event that does not affect survival but decreases reproduction in the Diamondback moth, *Plutella xylostella*. PLoS One. 2013; 8: e75923.

Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ. Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. J Exp Biol. 2011; 214: 4021-4029.

Zhivotovsky LA. Environmental stress and evolution: a theoretical study. EXS. 1997; 83: 241-254.

Zizzari ZV, Ellers J. Effects of exposure to short-term heat stress on male reproductive fitness in a soil arthropod. J Insect Physiol. 2011; 57: 421-426.

Zwaan BJ, Bijlsma R, Hoekstra RF. On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult breeding conditions. Heredity. 1991; 66: 29-39.

Zwaan BJ. The evolutionary genetics of ageing and longevity. Heredity. 1999; 82: 589-597