Reverse Evolution and Gene Expression Studies on Populations of *Drosophila melanogaster* Selected for Rapid Pre-adult Development and Early Reproduction

A Thesis

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Doctor of Philosophy

By

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Dedicated to my

family and friends

CONTENTS

DECLARATION

I hereby declare that the work embodied in my thesis entitled "**REVERSE EVOLUTION AND GENE EXPRESSION STUDIES ON POPULATIONS OF *DROSOPHILA MELANOGASTER* SELECTED FOR RADID PRE-ADULT DEVELOPMENT AND EARLY REPRODUCTION**" has been carried out by me at Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Amitabh Joshi, and this work has not been submitted elsewhere for any degree or diploma.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the finding of other investigators. Any omission, which might have occurred by oversight or error of misjudgment, is regretted.

Place: Bangalore

Date: 6 May, 2010 Satish, K. M.

Evolutionary & Organismal Biology Unit

JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH

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6 May, 2010

CERTIFICATE

This is to certify that the work described in the thesis entitled "**REVERSE EVOLUTION AND GENE EXPRESSION STUDIES ON POPULATIONS OF *DROSOPHILA MELANOGASTER* SELECTED FOR RAPID PRE-ADULT DEVELOPMENT AND EARLY REPRODUCTION**" is the result of investigations carried out by Mr. K. M. Satish in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Amitabh Joshi, Ph.D.

Professor

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SUMMARY

The fruit fly *Drosophila melanogaster* has been used in studies of genetics since the early 1900s, and is a powerful model system for both experimental evolution studies based on laboratory selection and studies focussing on the genetic control of developmental processes. It is, thus, an ideal system with which to address questions pertaining to the developmental and molecular biological underpinnings of adaptive evolutionary change in life-history related traits, an approach often termed developmental evolutionary biology. Laboratory selection experiments also provide an opportunity to address the reversibility, or lack thereof, of microevolutionary trajectories. In this thesis, I present results from two lines of investigation I carried out on a set of replicate *D. melanogaster* populations subjected to selection for rapid pre-adult development and early reproduction for over 250 generations. One one hand, I studied the evolutionary trajectories of several life-history related traits in these populations when subjected to 54 generations of reverse selection. In a separate set of experiments, I examined the expression levels of certain developmentally important genes in specific life-stages or tissues, as well as genome-wide expression levels in larvae, pupae and young adults of the selected populations and their ancestral controls.

When I started my work, the four replicate selected populations of *D. melanogaster* (FEJ_{1-4}) had already undergone 250 generations of selection for faster pre-adult development and early reproduction, and had diverged substantially for a variety of traits from the four matched ancestral control populations (JB_{1-4}) that were maintained on a 21 day discrete generation cycle with no conscious selection on development time and early reproduction. Briefly, relative to the JBs, the FEJs showed reductions in the duration of all pre-adult life-stages, larval survivorship, body size and

dry weight, lipid and glycogen content, adult lifespan and starvation and dessication resistance, and early life as well as lifetime fecundity. The FEJs also showed significantly reduced larval feeding rate and growth rate, foraging path length, digging propensity, pupation height and urea tolerance. Relative to the JBs, the FEJs had higher fecundity per unit dry weight early in life and took a longer time from eclosion to first mating.

The reversibility of evolution has been debated extensively but rarely studied empirically except for a couple of studies on *D. melanogaster* and *E. coli* by M. R. Rose and R. E. Lenski, and colleagues, respectively. The reversibility of evolved phenotypes depends on different factors that could have changed during the course of forward selection, such as the availability of genetic variation, complexity and pattern of epistatic interactions, and accumulation of mutations. I derived four populations (RF_{1-4}) from the FEJs, returned them to the ancestral JB maintenance regime and studied the trajectories of several traits over 54 generations of reverse selection. I found that larval and egg-to-adult survivorship, egg duration and early-life and middle-life fecundity converged back to ancestral control levels, whereas larval, pupal and egg-to-adult duration and dry weight at eclosion did not converge completely. During the terminal few assays of the RFs, the correspondence between development time and dry weight at eclosion was parallel to that seen in the first 20 generations or so of forward selection in the FEJs, suggesting that despite incomplete convergence, the joint trajectory of these traits was similar under both forward and reverse selection. I also observed that the response to reverse selection with respect to durations of different pre-adult life stages was similar: the response was slow in the beginning up to generation 5 of reverse selection and hastened up thereafter and was fast till generation

25, and after that again slowed down. My observations on development time and dry weight at eclosion are consistent with those of M. R. Rose and colleagues who used flies from the same ancestry and subjected them to selection for rapid development in much the same way as us. However, in their study, fecundity did not converge back to ancestral levels, and this difference in our results is probably due to the "early reproduction" part of the selection protocol being very different between the two sets of studies. Overall, the degree and mode of convergence I observed for the traits studied suggests (a) no erosion of genetic variation for these traits over 250 generations of forward selection in the FEJs, and (b) that it is unlikely that novel patterns of epistasis or new mutations have accumulated in the FEJs over the course of forward selection. My results also suggest that the broad contours of reverse evolution trajectories may be quite repeatable across studies if the past selection history and starting genetic material have been similar.

Regulating gene expression is a key step by which an organism activates the information encoded in its genome to effect developmental changes, and differences in this regulation can cascade through development resulting in different morphological or physiological character states. Keeping this in view, I studied the gene expression through different methods in FEJs in comparison to the JB controls. *Drosophila* neuropeptide F (*dnpf*) is a homolog of mammalian *NPY* gene which is involved in food/foraging-related behaviors in mammals. *dnpf* is expressed in the central nervous system of *Drosophila* and plays a major role in the maintenance of foraging behavior. Its expression is high at foraging stage (early third instar) and low in the wandering stage (late third instar) in wild type larvae, and *dnpf* downregulation has been shown to act as a switch between foraging and pupation behavior in *Drosophila*. In a gene

expression study done through semi-quantitative RT-PCR method, I found that *dnpf* expression in JBs was as expected (*i.e.* high at early third instar and low at late third instar), whereas *dnpf* expression in FEJs was low right from early third instar larva and it did not change till late third instar. This change in temporal pattern of *dnpf* expression could be an important causal factor underlying the huge reduction in larval third instar duration observed in the FEJs.

Precise spatial and temporal expression of genes is important for proper pattern formation during development. In FEJs, some leg and wing malformations had been observed from about the 100^{th} generation of selection. Therefore, to check if there was any change in the expression pattern of developmentally important proteins, I studied the expression patterns of some such proteins in the embryos as well as in the wing discs of third instar larvae by antibody staining technique. I observed no significant difference in the spatial expression pattern of these proteins in FEJs compared to their JB counterparts, suggesting that the expression patterns of these developmentally important proteins have not changed in FEJs over the course of selection.

I also examined cell number and cell size in FEJs relative to the to JBs by staining wing discs of third instar larvae with antibody against the protein Armadillo, whereby one can mark the cell borders, count the cells and estimate their sizes. Using this technique, I found that FEJ wing discs had less number of bigger cells whereas JBs had more number of smaller cells. The reason for this is not clear at this time, but it may be that the FEJs have evolved a reduction in the number of cell divisions as part of a strategy to conserve energy.

I further subjected one replicate population each of the FEJs and JBs to microarray analysis to examine differences in genome-wide patterns of gene expression between selected and control larvae, pupae and young adult males and females. I found that expression level of a few hundred genes was changed in FEJs in different life-stages used for the analysis. These changes were in both the directions *i.e,* many genes were up-regulated and many were down-regulated in FEJs in comparison to JBs, and many genes were consistently differentially expressed in FEJs across all life-stages studied. Genes related to epigenetic control were up-regulated in all the stages studied suggesting that changes in expression of many genes are possibly mediated by epigenetic mechanisms in the FEJs. Further, gene ontology (GO) term enrichment analysis using DAVID online bioinformatics tool showed that among the up-regulated genes were many eclusters of genes related to translation, developmental processes, phagocytosis *etc.,* all of which are related to development. The down-regulated genes were related to glutathione metabolism which consist genes such as glutathione-S-transferase which is involved in oxidative stress mechanism. FEJs are less resistant to different stresses compared to JBs. This could be because of the down-regulation of the genes involved in glutathione metabolism. Further, it was observed that the genes involved in the insulin signaling pathway are down-regulated and that of ecdysone action were up-regulated in the FEJs. The final body size of *Drosophila* is known to be greatly affected by an antagonistic interaction of insulin signaling and ecdysone action, and these results suggest that the faster development of FEJS, and their smaller body size, could be mediated by the evolution of higher basal levels of ecdysone and reduced levels of insulin signalling. Though preliminary in nature, the gene expression results indicate several avenues of further research that are likely to enhance our understanding

of the molecular genetic and developmental underpinnings of the rapid development phenotype in the FEJs.

Chapter-I

Introduction

The fruit fly *Drosophila melanogaster* has been used as an experimental organism in studies of genetics since the early 1900s (Kohler, 1994). *D. melanogaster* is now widely used not only in classical and molecular genetics but also with many new biochemical, cell biological and physiological techniques, to research addressing problems requiring a multidisciplinary approach, such as those in developmental biology (White *et al*., 1999; Sucena and Stern, 2000; Reinke and White, 2002; Lebo *et al*., 2009). It has been utilized as a powerful biological system to address fundamental questions concerning neurological disorders in humans, since the related basic molecular components and signal transduction pathways in humans are mostly conserved in *Drosophila* (Koh, 2006). In addition, *Drosophila* offers great experimental advantages in genetics, behavioral analysis and cell and molecular biology.

Laboratory cultures of *Drosophila melanogaster* also constitute a powerful model system that has been and continues to be extensively used to study life history evolution and the various developmental and molecular correlates of evolved life history related traits (Rose *et al*., 2004). One of the important advantages of using this system as model is the ability of the experimenter to change the laboratory ecology as per the requirement and study its effects on the life history trait values. One can conduct long term selection experiments in the laboratory using *Drosophila* model system and study adaptations to different laboratory ecologies, as well as the physiological and genetic mechanisms underlying these evolved changes in the life history and related traits (Rose *et al.*, 1996; Joshi, 1997; Mueller, 1997; Zwaan, 1999; Prasad and Joshi, 2003).

The life history of an organism primarily refers to the timing and distribution of its reproductive output during the course of its life (Roff, 1992; Stearns, 1992). Evolutionary biologists have long been interested in understanding the forces that mould life histories because of their great variability in the nature. Moreover, life history traits like development time, pre-adult survival and number of offspring produced are closely connected to the fitness of an organism. An organism's life history may be looked upon as a resultant of three biological processes, namely, maintenance, growth and reproduction. These life history components in any organism compete for resources and time which are limiting factors (Gadgil and Bossert, 1970). Depending on the environmental conditions and/or selective forces acting upon them, organisms typically show trade-offs among various life history and life history related traits. For example, in populations of *Drosophila* selected for late life survival and reproduction, increased longevity was accompanied by declines in early life fecundity (Rose, 1984).

In insect species whose larvae inhabit ephemeral habitats like rotting fruits in the wild, two important selective pressures thought to operate are larval overcrowding and the necessity to complete pre-adult development relatively fast, before the food runs out or the habitat patch becomes otherwise inhospitable (Joshi *et al*., 2001). A number of laboratory selection experiments have been carried out involving *Drosophila* species as model systems (Rose, 1984; Service and Rose., 1985; Joshi *et al*., 1996). These studies have identified trade-offs among various components of fitness that seem to play a central role in the evolution of life-histories (reviewed by Prasad and Joshi, 2003), including trade-offs between developmental rate and adult weight at eclosion (Nunney, 1996, Chippindale *et al.*, 1997; Prasad *et al*., 2000).

In an ongoing study in our laboratory, we have successfully selected four replicate populations of *D. melanogaster* for rapid pre-adult development and early reproduction relative to controls. These populations are designated as FEJ_{1-4} (F, faster development; E, early reproduction; J, JB derived). Their ancestral control (JB) populations have been maintained on a three week discrete generation cycle with no conscious selection on development time. Both control and the selected populations are maintained under the same environmental conditions except for the selective pressures for faster development and early reproduction on selected FEJ populations.

Over the course of selection, the FEJ populations have undergone significant phenotypic changes in several behavioural, physiological and life history related traits.

1. The duration of all the pre-adult life stages in the FEJ populations have been reduced substantially compared to controls (Modak, 2009).

2. FEJ flies have evolved to become substantially smaller and lighter than the control JB flies at eclosion and also exhibit considerably reduced egg-to-adult viability (Prasad *et al.*, 2000, 2001; Modak, 2009).

3. Among larval traits, the FEJ populations have evolved significantly reduced feeding rate, foraging path length, digging propensity, pupation height and urea tolerance (Prasad *et al*., 2001; Joshi *et al*., 2001).

4. The adult FEJ flies have evolved significantly lower lipid content, fecundity and life span, and an increased time from eclosion till first mating (Prasad, 2004).

These selected FEJ populations have been thoroughly studied for different behavioral, physiological and life history related traits at the phenotypic level in our

laboratory (Prasad *et al.*, 2000, 2001; Prasad, 2004; Modak, 2009). However, no attempt had been made so far to understand the underlying developmental and molecular mechanisms involved in their impressively rapid development. I continued this selection experiment 250 generations onwards with an aim to address several evolutionary questions with a populational as well as a developmental focus, using this experimental system.

One issue I chose to study was reverse evolution in the laboratory. I derived four new populations from FEJ populations and released the directional selective pressures operating on them by returning them to their ancestral JB environment and designated them as RF (Relaxed FEJs). Using these sets of populations, I studied reverse evolution in the laboratory for 54 generations. In the next two chapters, I present the results of various assays performed over the generations of relaxed selection. Effect of relaxed selection on the pre-adult traits viz, pre-adult development time and viability is discussed in chapter II. In chapter III, I discuss the reverse evolution of adult life history traits in the RF populations.

Given the large (~ 60 hours; 30%) reduction in pre-adult development time in the FEJ populations, relative to controls, it is very interesting to probe the underlying molecular basis for the various direct and correlated responses observed in FEJ populations. The expression of different genes in a complex spatiotemporal pattern exerts a great ontogenetic influence control on the development of the final organismal phenotype. Therefore, I studied gene expression patterns during different stages of development in the FEJ and JB populations. These studies, along with some other experiments aimed at understanding some of the developmental underpinnings of the FEJ phenotype are detailed in the chapter IV, V and VI of this thesis.

The Experimental Populations

The studies reported in the chapter II and III were done on eight laboratory populations of *D. melanogaster*. Four populations served as ancestral controls (JB_{1-4}) and the other four were the reverse-selected populations derived from populations selected for faster development and early reproduction (FEJs) by releasing their selection pressure, the RF populations. The studies reported in chapter IV to VI were done on laboratory populations of *D. melanogaster* selected for faster pre-adult development and early reproduction (FEJ_{1-4}) and their control JB counterparts. All the populations are maintained at 25 ± 1°C temperature, around 90% relative humidity, under constant light, at moderate densities of approximately 60-80 larvae per 8-dram vial (9 cm high × 2.4 cm diameter) containing approximately 6 ml of banana-jaggery food medium (Table 1.1). The control populations employed here are the four populations (JB_{1-4}) first described by Sheeba *et al.* (1998). The JB populations are maintained in incubators on a 21 day discrete generation cycle. Every generation, adults of each JB population are allowed to oviposit for about 18 h on Petri dishes of fresh banana-jaggery food placed in a Plexiglas cage (25 × 20 × 15 cm^3). From the food in these Petri dishes, approximately 60-80 eggs are collected into each of 40 vials in which larvae then develop into adults. Adults eclosing from these vials are transferred to fresh food vials on day 12, 14 and 16 after egg lay. On day 18 after egg lay, adults are transferred into Plexiglas cages and supplied with banana-jaggery food supplemented with a live yeast and acetic acid paste for 2 days, after which eggs are collected to initiate next generation and the adults are discarded. The population typically consists of 1600-1800 flies at this stage.

The four JB populations are ultimately descended from a single population of *D. melanogaster*, the IV population described by Ives (1970). The immediate ancestors of JBs are the UU populations described by Joshi and Mueller (1996) that had been maintained for 170 generations on a 21 day discrete generation cycle at 25^{o}C and constant light. The four JB populations had, therefore, been independent evolutionary entities for over 600 generations and had been on a three week cycle for over 300 generations at the time the FEJ populations were initiated from them.

The four populations selected for faster pre-adult development and early reproduction were derived from the four JB populations and are designated as FEJ_{1-4} (F- faster development; E- early reproduction; J- JB derived) (Prasad *et al*. 2000). Each FEJ population was derived from one JB population; thus, selected and control populations bearing identical numerical subscripts are more closely related to each other, than to other populations with which they share a selection regime (JB_i and FEJ_i are more closely related than JB_i and JB_j or FEJ_i and FEJ_j; i, j = 1-4). Consequently, control and selected populations with identical subscripts were treated as random blocks in all statistical analyses.

The FEJ populations are maintained on a similar regime except that 120 vials of approximately 60-80 eggs are collected per population, and once the pupae have darkened the vials are closely monitored and only the first 25% or so of the eclosed flies per vial regardless of sex are transferred into the cages to constitute the pool of breeding adults. The flies in the cage are supplied with banana-jaggery food supplemented with live yeast and acetic acid paste for 2 days, and then allowed to oviposit for about one hour on fresh food. Thus, the differences between the JB and FEJ populations are:

- Only the first 25% or so of eclosing flies contribute to the next generation in FEJ populations, whereas in JB populations all flies eclosing on or before day 12 after egg collection contribute to the next generation.

- The egg-laying window is approximately 1 hour for FEJ populations and 18 hours for JB populations.

- FEJ eggs are collected on day 10 while those of JB are collected on day 21 after egg lay.

Four new populations were subsequently derived from FEJ populations after 286 generations of FEJ selection, and returned to their ancestral, control JB environmental conditions. These new populations were designated as RF_{1-4} (Relaxed FEJs). Each RF population was derived from one FEJ population; thus RF and JB populations bearing identical numerical subscripts are more closely related to each other than to other populations with which they share a selection regime (JB_i and RF_i are more closely related than JB_i and JB_j or RF_i and RF_j; i, j=1-4). Consequently, control and selected populations with identical subscripts were treated as random blocks in all statistical analysis.

RF populations are maintained under conditions similar to the JB populations, except that for the first five generations of the reverse evolution experiment, 60 vials containing 60-80 eggs were collected per population instead of 40 vials. This was done to maintain a breeding population of 1600-1800 adults, allowing for the lower egg-to-adult survivorship of FEJ populations. Moreover, during the first five generations of relaxed selection, the eclosed adult flies were transferred into fresh food vials on 8^{th}, 10^{th} and 12^{th} day after egg collection, and on the 14^{th} day after egg collection all the

flies were collected into Plexiglas cages containing Petri plates of food to which a generous smear of yeast-acetic acid paste had been applied. From the sixth to twelfth generation of relaxed selection, eclosed flies were transferred to fresh food vials on the 10^{th}, 12^{th} and 14^{th} day after egg collection, and on the 16^{th} day after egg collection, all the eclosed flies were transferred into Plexiglas cages containing a Petri plate of food with yeast-acetic acid paste. From the 12^{th} generation onwards, the RFs were maintained under conditions exactly similar to the JBs. The discrepancy during the first twelve generations of RF selection was due to the much shorter (~ 60 hours) development time of the RF populations. Thus, if the flies were left in the larval vials till the 12^{th} day after egg collection, as in the JBs, they would be likely to suffer high mortality due to the used food medium which can be very fluid.

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

Table 1.1: The composition of 1 Litre of regular banana-jaggery food medium used in the maintenance of the experimental populations.

Collection of flies for assays

Imposition of different maintenance regimes in laboratory selection experiments can induce non-genetic parental effects. Consequently, all selected and control populations were maintained under common rearing conditions for one complete generation prior to assaying to eliminate such non-genetic parental effects. Eggs were collected from running cultures and dispensed into vials with about 6 mL food at a density of 60-80 eggs per vial. All the eclosed flies from these vials were collected into Plexiglas cages with abundant food. The adult numbers were 1500-1800 per population. They were supplied with live yeast-acetic acid paste along with normal banana-jaggery food for 3 days prior to egg collection for assays. The progeny of these flies, hereafter referred to as standardized flies, were then used for the various assays. For assays involving adult flies, eclosion of the assay flies from control and relaxed selected populations (RFs) was synchronized by staggering the egg collection from the standardized flies according to the differences in the egg-to-adult development time of the control and selected populations.

Chapter-II

Evolution of Pre-adult Traits in RF populations

INTRODUCTION

The reversibility or irreversibility of evolutionary changes has continued to be an important theme in evolutionary biology. In more recent years, there has been a shift in focus to studying reverse evolution within the framework of relatively short term laboratory selection experiments in which ancestral populations are available for comparison, there is good knowledge of the ancestral selective environment and some hope of identifying the underlying genetic mechanisms involved in facilitating or constraining reverse evolution (Teotonio and Rose, 2001). Moreover, it is unlikely that longer term evolution would be reversible at all levels of biological organization, because of the difficulty of retracing numerous evolutionary events over long periods of time, rendering the prospects for studying reverse evolution in nature somewhat bleak.

Reverse evolution can be defined as "the reacquisition of the same character states including fitness by the derived populations as those of their ancestor populations" (Bull and Charnov, 1985). Similarly, reverse selection refers to the re-imposition of the same selective pressures on derived populations as those of recent ancestor populations. Hence, the process of reverse evolution need not always occur due to reverse selection. In a laboratory selection experiment, when the reproducing individuals are no longer selected by the experimenter before each generation, the process is often termed 'relaxed selection' (Falconer and Mackay, 1996). Therefore, reverse evolution can also occur with relaxed selection. Whatever may be the mode of selection, whether reverse or relaxed, the experimenter must know the phenotypic states and selective conditions of the ancestral populations to study reverse evolution because reverse evolution is detectable only when convergence to an ancestral phenotypic state can be measured. Therefore, an ancestral population must be present

for comparison with the derived populations to infer convergence to the ancestral phenotype.

Irreversible evolution can be viewed as an extreme type of evolutionary restriction (Bull and Charnov, 1985) and one of the main questions to be addressed is the degree to which evolutionary history constrains reverse evolution. The other interesting questions are, whether the diverged populations, when their selection pressures are relaxed, will converge to their ancestral trait values? And if they converge, will they follow the same evolutionary trajectory going back to their ancestral phenotypic states as they had followed during their original divergence?

Teotonio and Rose (2000) studied reverse evolution by returning 20 populations of *D. melanogaster* with heterogeneous evolutionary histories to their common ancestral environment for 50 generations. They found that some of the traits studied, such as development time in populations earlier selected for reproduction late in life, converged completely to their ancestral trait values. Other traits, such as fecundity at high density in populations selected for reproduction late in life, converged towards the ancestral value but the convergence was not complete by 50 generations of reverse selection. Some other traits showed rapid convergence initially and then stalled at some point without full convergence to ancestral values e.g. starvation resistance in populations selected for survival under conditions of complete starvation. On the other hand, fecundity at high density in populations selected for reproduction late in life showed no significant change during 50 generations of selection. These results show that the evolutionary history of the selected populations, although is only a few hundred generations in duration, can play a significant role in mediating the pattern, rate and extent of reversion to ancestral phenotypes during reverse evolution, even though

fitness appears to revert fully to ancestral levels (Teotonio and Rose, 2000; Teotonio *et al*., 2002). The studies by Teotonio and Rose (2000) and Teotonio *et al*. (2002) used populations selected for different age of reproduction, starvation resistance and accelerated pre-adult development, and these studies along with a reverse evolution study of larval feeding rate and pupation height in populations adapted to larval crowding (Joshi *et al*., 2003) are the only such studies done on *Drosophila* to my knowledge.

A general observation in multiple studies of experimental evolution over the past few decades has been that often very similar selection regimes yield fairly different patterns of correlated responses of fitness-related traits to selection, even when the populations used in the different studies are from related lineages descended from common ancestors nor more than a few hundred generation before (Prasad and Joshi, 2003; Rose *et al*., 2005; Archana, 2009). This raises the question of how generalizable are the results of reverse selection experiments done on populations with different forward selection histories. One set of populations (ACO 1-5) studied by Teotonio and Rose (2000) and Teotonio *et al*. (2002) had been selected for about 190 generations for rapid pre-adult development prior to the reverse selection study. Thus, studying the response of our FEJ populations to reverse selection offers the opportunity to investigate the consistency of observation of reverse evolution of such populations. The comparison is facilitated by the fact that the FEJ and ACO populations ultimately share a common ancestry in the B populations of Rose (1984). The FEJ and ACO populations were subjected to very similar selection pressures, except that the selection for early reproduction was much stronger in the ACO populationss: this led to some differences in correlated responses to selection, discussed in Prasad *et al*. (2000; 2001).

The populations selected for decreased development time were returned back to their ancestral environmental conditions by Teotonio and Rose (2000) after selecting for 190 generations. In the present study, I have returned the FEJ populations to their ancestral environmental condition after forward selecting for more than 285 generations. The questions that I asked were whether,

a) FEJ populations return back to their ancestral trait values quickly or not, since they have diverged for a longer duration (for ~285 generations).

b) FEJ populations completely converge back to their ancestral phenotypic states or not.

c) The very long-term selection on FEJ populations constrains their reversal to ancestral state.

d) The general patterns of change in different traits under reverse selection are at least qualitatively similar in the ACO and FEJ populations.

MATERIALS AND METHODS

Development time assay

Total egg-to-adult development time including pupal duration was assayed at 1, 5, 10, 14, 25, 49 and 54 generations of RF selection. Standardised flies of each replicate RF and JB population were provided with a fresh food plate for 1 h. This plate was then replaced by a second plate on which the flies were allowed to lay eggs for 1 h. After the end of this period, the second plate was removed from the cage and the eggs were

collected off the plate with a moistened brush. The eggs were then placed into vials containing 6 mL of banana-jaggery food at a density of exactly 30 eggs per vial and incubated at 25^{o}C. Ten such vials were set up per replicate population. The larvae grew in these vials and once they reached the wandering stage, the vials were closely monitored for the formation of pupae. After the formation of the first pupa, the vials were checked every 4 h in the assays done in between 1 and 40 generations of relaxed selection; thereafter, the vials were checked every 2 h and new pupae formed were marked with colour pens on the outer walls of the vials and counted. These observations were continued till no new pupae formed in the assay vials for two consecutive days. Once the pupae had darkened, the vials were checked for the first eclosion and thereafter monitored every 4 h (generations 1-40) or 2 h (after generation 40) for new eclosions. The eclosed flies were removed after every check, frozen, and their sex determined under the microscope. Time of egg collection (midpoint of the egg collection window) was subtracted from the time of pupation to obtain the total larval duration, whereas the difference between the time of eclosion of male and female flies from the time of egg collection gave the sex-specific egg-to-adult development time. Pupal duration was obtained by subtracting the mean larval duration from the mean egg-to-adult development time averaged across the two sexes for each vial. Mean development time for each vial was calculated for all the traits and the vial means were averaged to calculate the replicate means.

Survivorship assay

Survivorship through the larval and pupal stages and overall egg-to-adult survivorship were assayed at 1, 5, 10, 14, 25, 49 and 54 generation of RF selection. Data from the development time assay were used to calculate survivorship during different developmental stages. The number of flies eclosed in each vial divided by the number of eggs collected yielded the mean egg-to-adult survivorship for each vial. Larval survivorship was calculated by dividing the number of pupae by 30. Number of eclosed adults divided by the number of pupae formed in each vial yielded the mean pupal survivorship for that vial. Eggs were collected similarly at a density of 30 eggs per vial and ten such vials were set up for each replicate population. The number of pupae formed and the eclosed male and female flies were counted for each vial. From these values, the larval, pupal and egg-to-adult survivorship was calculated.

Egg duration and hatchability assays

Egg duration and egg hatchability assays were conducted at generation 10 and 54 of RF selection. For assessing egg duration (time from egg lay to egg hatch), 10 vials were set up for each replicate population. 30 eggs were arranged on a small square piece of plain agar medium in a manner (eggs arranged in 6 rows each containing 5 eggs) such that it was possible to monitor each individual egg under a microscope. Each agar piece containing 30 eggs was placed into a vial containing 6 mL food and incubated at 25^{o}C. After 15 h from the mid-point of the egg collection window, each vial was monitored for egg hatching. Hatched *Drosophila* eggs appear translucent and crumpled, whereas unhatched ones are opaque and swollen in appearance. The number

of hatched eggs was counted at 1 h intervals and noted down. From these data, mean egg duration was calculated.

Statistical analysis

Analysis of variance (ANOVA) was performed on the replicate population means for each trait studied and the data from different generations were analyzed separately. Egg-to-eclosion development time data were subjected to three-way ANOVA with selection and sex being treated as fixed factors crossed with random blocks. Larval and pupal developmental times were subjected to separate two-way ANOVAs with fixed factor selection crossed with block. Similar analyses were done for hatching time and hatchability. Since the survivorship and hatchability data obtained from each vial were fractional, these data were subjected to arcsine square-root transformation to meet the normality assumption of ANOVA. Replicate population means of the transformed data were used for analysis. Survivorship data obtained from different stages were analyzed separately. All statistical analyses were performed using STATISTICA™ for Windows Release 5.0 B (StatSoft Inc., 1995).

RESULTS

Development time

I observed a strong and consistent response to reverse selection in mean egg-to-adult development time, with the mean difference between RF and JB populations decreasing from ~57 h at generation 1 to ~14 h at generation 54 of RF selection (figure 2.2). At the beginning of RF selection, the mean egg-to-adult development time in RF populations was 26% less that the JB populations. This percentage difference in mean egg-to-adult development time decreased from 26% to 24% at generation 5, 20% at generation 10, about 18% at generation 14, about 12% at generation 25 and about 7% by 54 generations of RF selection. Males took significantly longer to develop than females throughout the 54 generations of RF selection (figure 2.1), and the difference in mean development time between males and females in both RFs and JBs remained almost constant at all the generations assayed. ANOVA done at different generations revealed significant effects of selection regime and sex (males took longer to develop than females) (table 2.1).

Larval and Pupal duration

Separate ANOVAs done at different generations of RF selection, on both mean larval and pupal duration consistently revealed a significant effect on selection regime (table 2.2 and 2.3), with the mean larval and pupal duration in RF populations being less than the JB populations at all the generations assayed. At the beginning of RF selection, the mean larval duration of the RF populations was ~33 h less than the JB populations, accounting for ~58% of the difference in egg-to-adult development time (figure 2.3). The remaining 42% (~24 h) reduction was contributed by the reduced

pupal duration of the RF populations (figure 2.4). Overall, on an average, the JB-RF difference in larval duration accounted for ~56% of the egg-to-adult development time difference, all through the generations of reverse selection, while the remaining ~44% was contributed by the difference pupal duration between the JB and RF populations, a difference of about ~25% at the start of RF selection. This percent difference decreased to around 24, 20, 19, 13 and 7 percent by 5, 10, 14, 25 and 49 generations of RF selection respectively. During the last 5 generations of RF selection, mere 0.08% decrease in the difference was observed (6.57% at generation-49 and 6.49% at generation-54).

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	13028.6	5594.18	<0.001
	Sex	1	33.7634	120.672	<0.001
	Selection x Sex	1	0.4611	0.79444	0.4384
Gen 5	Selection	1	11964.6	6660.28	<0.001
	Sex	1	20.9073	228.913	<0.001
	Selection x Sex	1	0.00413	0.02594	0.8823
Gen 10	Selection	1	7457.71	100.077	<0.001
	Sex	1	34.392	266.601	<0.001
	Selection x Sex	1	1.17512	17.3339	0.0252
Gen 14	Selection	1	7091.82	66.7843	<0.001
	Sex	1	32.7549	133.561	<0.001
	Selection x Sex	1	0.09822	0.22342	0.6687
Gen 25	Selection	1	3424.89	53.0388	<0.001
	Sex	1	32.989	143.112	<0.001
	Selection x Sex	1	0.24739	1.33461	0.3316
Gen 49	Selection	1	789.006	73.332	<0.001
	Sex	1	46.5244	64.7403	<0.001
	Selection x Sex	1	3.40128	7.44324	0.0720
Gen 54	Selection	1	878.96	104.503	<0.001
	Sex	1	39.0667	122.687	<0.001
	Selection x Sex	1	2.29978	8.77166	0.0595

Table 2.1: Summary of results of separate ANOVAs done on mean egg-to-adult development time of RFs and JBs at different generations of RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

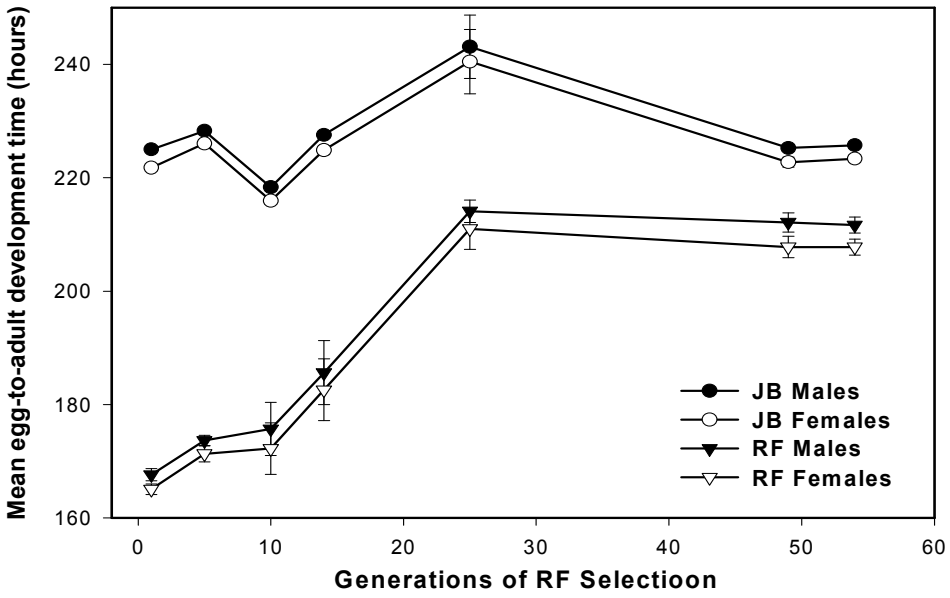


Figure 2.1: Mean (± s.e.) egg to eclosion development time of males and females from the RF and JB populations over the course of 54 generations of RF selection.

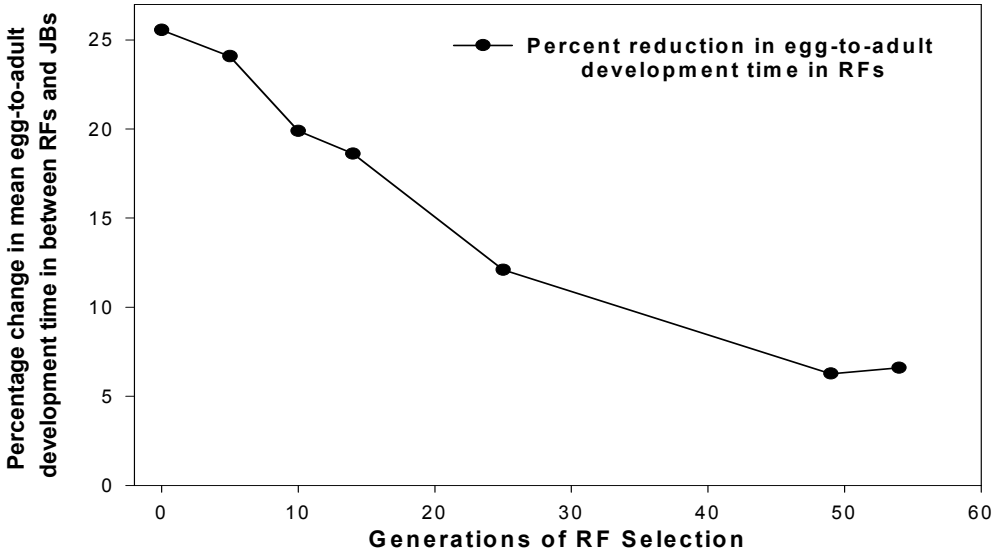


Figure 2.2: Percent reduction in mean egg-to-adult development time of RF populations compared to the control JB populations.

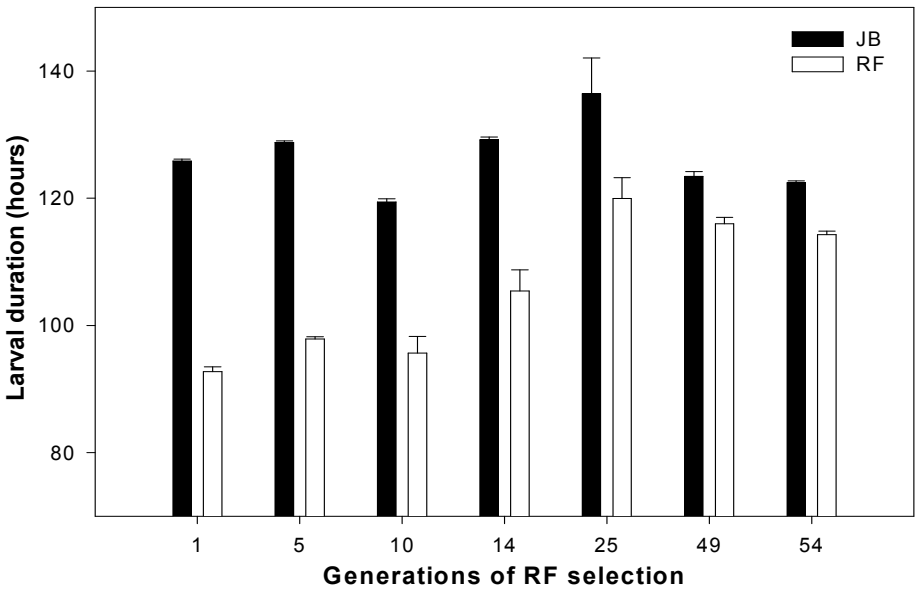


Figure 2.3: Mean (± s.e.) larval duration of the RF and JB populations over the course of 54 generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	2188.60	3305.45	<0.001
Gen 5	Selection	1	1907.97	5473.97	<0.001
Gen 10	Selection	1	1128.49	91.90	0.0024
Gen 14	Selection	1	1132.42	56.74	0.0048
Gen 25	Selection	1	540.91	25.97	0.0146
Gen 49	Selection	1	110.35	78.91	0.0030
Gen 54	Selection	1	134.20	122.41	0.0015

Table 2.2: Summary of results of separate ANOVAs done on mean larval duration of RFs and JBs at different generations of RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

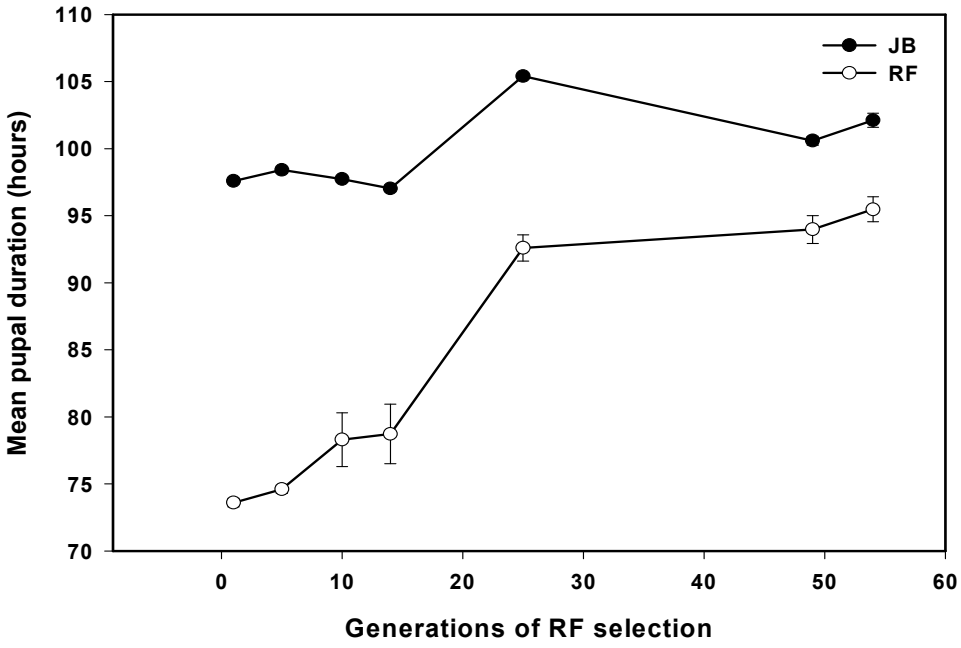


Figure 2.4: Mean (± s.e.) pupal duration of the RF and JB populations over the course of 54 generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	1151.16	5024.53	<0.001
Gen 5	Selection	1	1133.33	4888.24	<0.001
Gen 10	Selection	1	754.67	110.48	0.0018
Gen 14	Selection	1	670.60	82.20	0.0028
Gen 25	Selection	1	328.48	148.54	0.0011
Gen 49	Selection	1	87.55	65.28	0.0039
Gen 54	Selection	1	87.96	81.77	0.0028

Table 2.3: Summary of results of separate ANOVAs done on mean pupal duration of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

Pre-adult survivorship

Till generation 14 of RF selection, a significant difference in egg-to-adult survivorship was observed in between RF and JB populations, except at the start of RF selection. The RF populations consistently had lower survivorship than the JB populations at all the generations assayed till 14. This survivorship cost to rapid development was apparent in the RF populations till 14 generations, at which point the RF populations were still 19% faster in development than the JB controls. Generation 14 onwards, this cost in survivorship was not seen: there was no significant difference in egg-to-adult survivorship between the RF and JB populations, although egg-to-adult survivorship in the RF populations was consistently lower than controls by 0.01 to 0.04 at the various generations assayed (figure 2.5).

There was a significant difference in the mean larval survivorship between RF and JB populations till generation 14 of RF selection, except at generation 5 due to large variation in mean survivorship between different blocks (table 2.5). Similar to egg-to-adult survivorship, generation 14 onwards, significant difference in larval survivorship was not observed in between the RF and JB populations (figure 2.6).

No significant difference in mean pupal survivorship was observed between RF and JB populations at any of the generations assayed (table 2.6). Mean pupal survivorship was marginally lower in RF populations till generation 14, whereas at generation 49 and 54, RF populations showed marginally higher mean pupal survivorship than controls (figure 2.7).

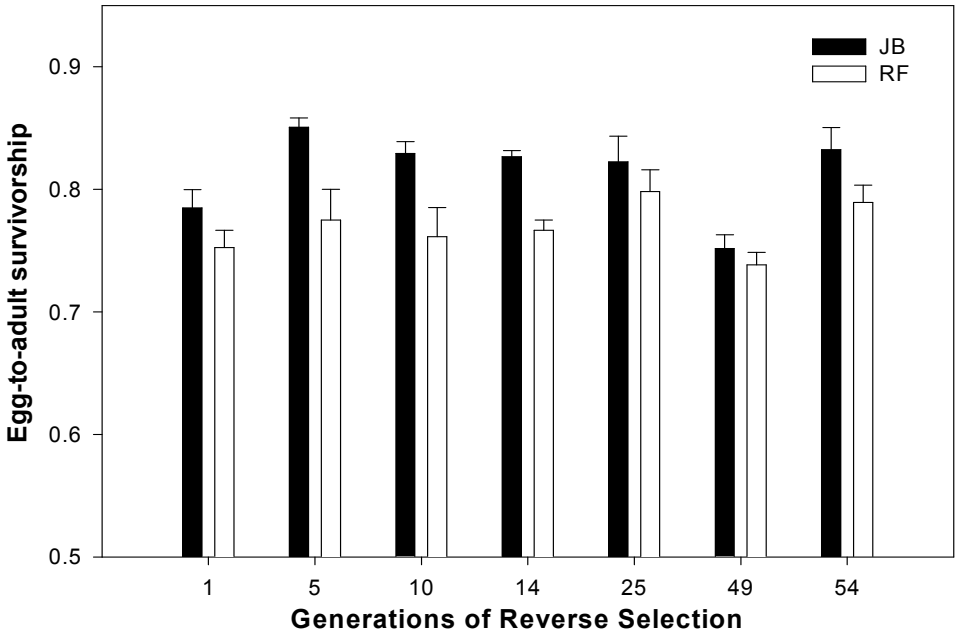


Figure 2.5: Mean (± s.e.) egg to adult survivorship of the RF and JB populations over the course of 54 generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	0.04567	3.94075	0.14135
Gen 5	Selection	1	0.19419	12.2475	0.03949
Gen 10	Selection	1	0.16948	10.5495	0.04756
Gen 14	Selection	1	0.1221	26.8885	0.01393
Gen 25	Selection	1	0.03938	0.66663	0.47403
Gen 49	Selection	1	0.00381	0.44552	0.55222
Gen 54	Selection	1	0.10784	9.61564	0.05326

Table 2.4: Summary of results of separate ANOVAs done on mean egg-to-adult survivorship of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

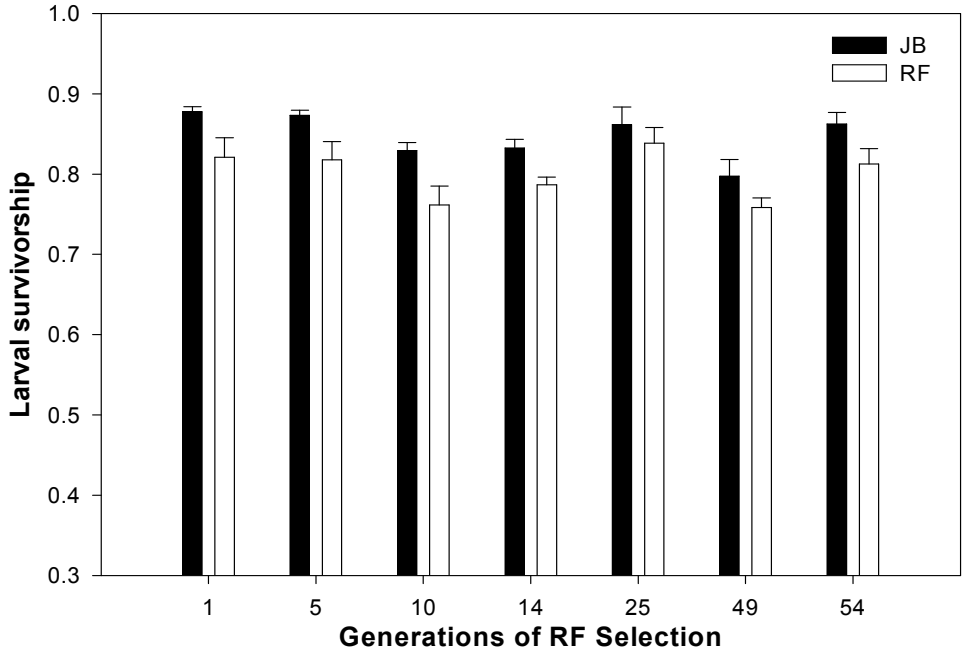


Figure 2.6: Mean (± s.e.) larval survivorship of the RF and JB populations over the course of 54 generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	0.05225	32.15	0.0108
Gen 5	Selection	1	0.12325	5.38	0.1030
Gen 10	Selection	1	0.16948	10.54	0.0475
Gen 14	Selection	1	0.09249	52.12	0.0054
Gen 25	Selection	1	0.03558	0.431	0.5583
Gen 49	Selection	1	0.04496	2.79	0.1929
Gen 54	Selection	1	0.14927	16.91	0.0261

Table 2.5: Summary of results of separate ANOVAs done on mean larval survivorship of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

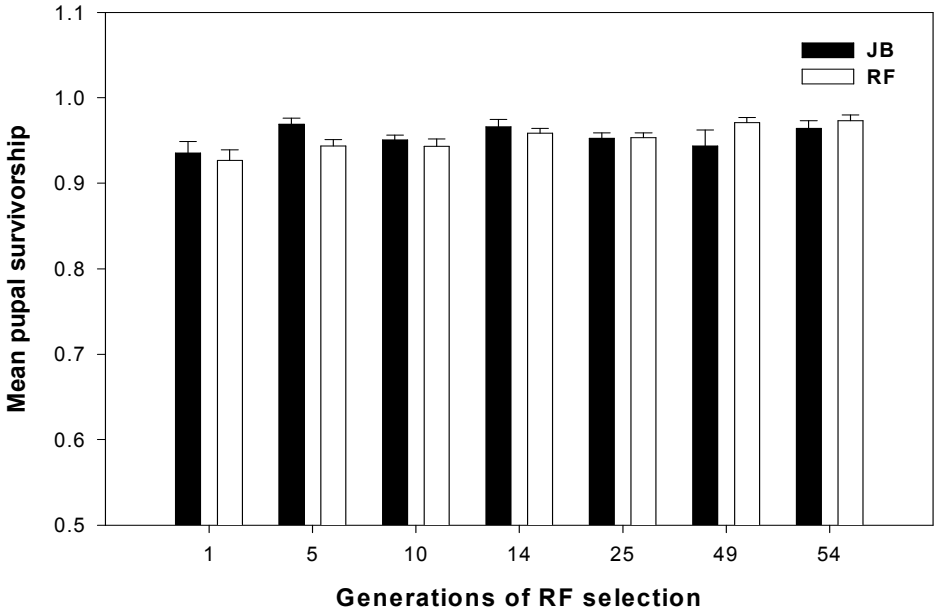


Figure 2.7: Mean (± s.e.) pupal survivorship of the RF and JB populations over the course of 54 generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	0.01541	0.33135	0.60521
Gen 5	Selection	1	0.1459	7.51869	0.07122
Gen 10	Selection	1	0.01859	2.01116	0.25118
Gen 14	Selection	1	0.01346	0.78296	0.44141
Gen 25	Selection	1	0.00001	0.00038	0.98572
Gen 49	Selection	1	0.08655	2.4643	0.21448
Gen 54	Selection	1	0.00718	0.2336	0.66195

Table 2.6: Summary of results of separate ANOVAs done on mean pupal survivorship of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

Egg duration and hatchability

RF populations had a significantly lower mean egg duration than JB populations before the start of this experiment and also at generation 10 of RF selection. However, no significant difference was observed in mean egg duration at generation 54 of RF selection (table 2.7 and figure 2.8). The difference in mean egg duration between the FEJ and JB populations at generation 263 of FEJ selection was 1.4 h (6.5% reduction, relative to controls) (Modak, 2009), which decreased to 0.85 h (4% reduction) after 10 generations of reverse selection and to 0.36 h at generation 54 (~1.5% reduction relative to controls).

Significantly lower egg hatchability (~5% reduction relative to controls) was observed in RF populations at generation 10 of reverse selection (figure 2.9 and table 2.8). At generation 54, RF populations and the JB controls showed no significant difference in egg hatchability, although the RF egg hatchability was marginally higher than controls (~1% increase relative to controls).

Generation	Effect	*df*	MS	*F*	*P*
Gen 10	Selection	1	1.476	231.8	0.00061
Gen 54	Selection	1	2.668	2.1	0.24259

Table 2.7: Summary of results of separate two-way ANOVAs done on mean egg duration of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

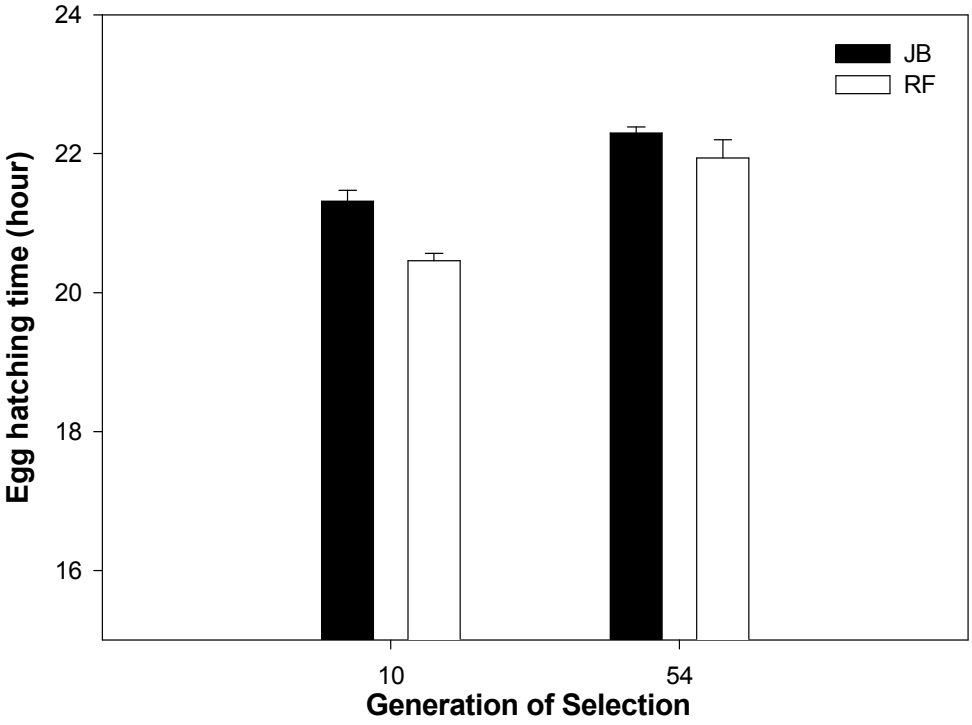


Figure 2.8: Mean (± s.e.) egg duration in RF and JB populations at different generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 10	Selection	1	0.10082	26.4728	0.0142
Gen 54	Selection	1	0.00642	0.28303	0.6316

Table 2.8: Summary of results of separate two-way ANOVAs done on mean hatchability of RFs and JBs at different generations RF selection. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

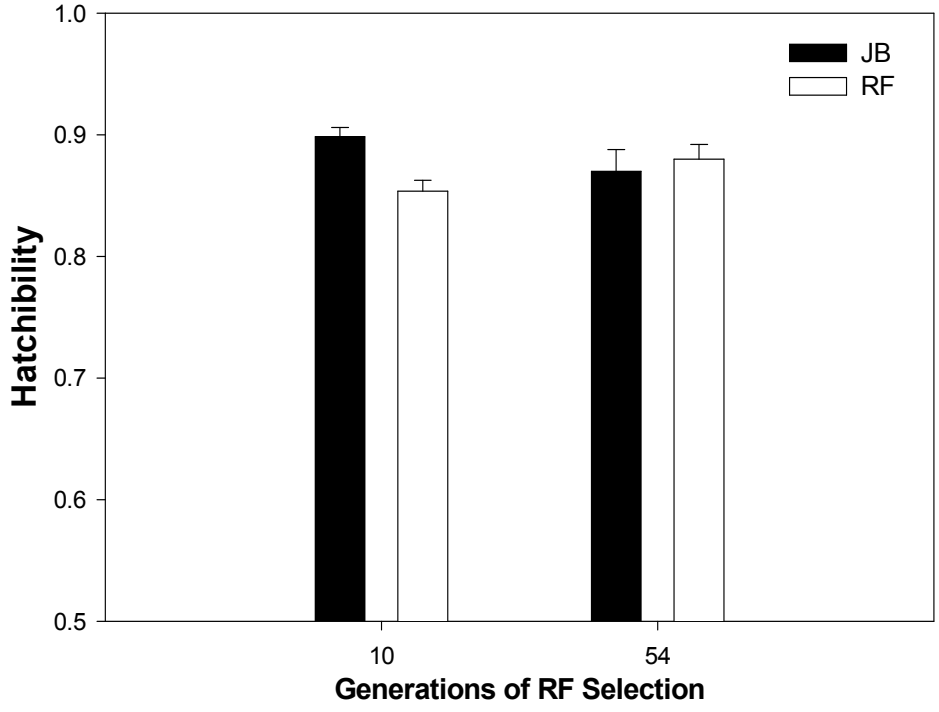


Figure 2.9: Mean (± s.e.) egg hatchability in RF and JB populations at different generations of RF selection.

DISCUSSION

On the whole, my results are similar to those of Teotonio and Rose (2000) and Teotonio *et al*. (2002), suggesting that the pattern of reverse evolution observed for populations forward selected for rapid egg-to-adult development is robust. In the RF populations that had first undergone forward selection (as FEJ populations) for over 280 generations, considerably longer than the ACO populations in the studies by Teotonio and Rose (2000) and Teotonio *et al*. (2002), mean egg-to-adult development time converged towards the ancestral value on a similar time scale (54 versus 50 generations) and to a similar degree (~75% of ancestral value) as that seen by Teotonio and Rose (2000) and Teotonio *et al*. (2002). Paralleling their observations of a full convergence of male and female adult fitness, and population viability (Teotonio *et al*., 2002), I found that egg-to-adult survivorship converged to ancestral values in about 25 generations of reverse selection. The congruence of my data with those from the first 40 generations of FEJ forward selection (Prasad *et al*., 2000), in which development time reduced by about 20 h relative to controls, whereas egg-to-adult survivorship did not significantly decline, suggests that the reverse evolution of pre-adult development time and survivorship may have followed a joint trajectory similar to that traced during forward selection.

The initial rate of convergence of RF populations to their ancestral egg-to-adult development time was slow till generation 5, and then increased till generation 25 after which it slowed down again and finally reached a plateau between generation 49 and 54. While the cause for the incomplete convergence of development time in the ACO (Teotonio and Rose, 2000) and RF populations is not clear, the dynamics of the reversal of mean trait value are consistent with a large additive component to genetic variance

for development time (Nunney 1996; Modak 2009) and the standard expectation of higher rates of change at intermediate allele frequencies (Falconer and Mackay, 1996). Clearly, the additional 100 generations or so of FEJ selection, compared to the ACO populations studied by Teotonio and Rose (2000) has not led to any significant amelioration of genetic variation or the development of patterns of epistasis or genotype-by-environment interaction that could render the reverse evolution trajectories very different.

Larval and pupal duration both showed a similar percentage reduction pattern as that of egg-to-adult development time in RF populations compared to JB controls, although the contribution from reduction in mean larval duration was comparatively larger (~56%) than the contribution from pupal duration (~44%). The percentage contributions by larval and pupal stages to the overall reduction in pre-adult development time did not differ much over the generations of RF selection.

There was a survivorship cost apparent in the RF populations from the beginning of the reverse selection till generation 14. At the same time, there was no significant difference in the pupal survivorship at any of the generations assayed. Thse observations suggest that the survivorship cost in RF populations was solely due to larval mortality, as also earlier observed in the FEJ populations at around 50 generations of forward selection (Prasad *et al*., 2001). Unlike egg-to-adult development duration that is presumably not a major determinant of fitness in the RF regime, larval and egg-to-adult survivorship, traits strongly correlated with fitness in all regimes, returned to their ancestral value within 25 generations of relaxed selection.

Mean egg duration and egg survivorship in the RF populations was significantly lower than their controls generation 10, and became almost similar to the JB control values by the end of 54 generations of RF selection. Reduction in egg duration probably carries a heavy fitness cost, as this trait evolved only very late in FEJ forward selection (Modak, 2009) and, therefore, these traits also converged to ancestral values.

Overall, the results from this study of pre-adult traits in the RF populations suggests that, as also seen by Teotonio and Rose (2000) and Teotonio *et al*. (2002), traits more closely related to fitness in both the forward and reverse selective environments have a higher chance of fully reverting to ancestral values. The incomplete reversion of pre-adult development time in both studies suggests some as yet unidentified constraint that either prevents or greatly retards the attainment of ancestral value for this trait in populations selected for extremely rapid development.

Chapter-III

Evolution of Adult Traits in RF Populations

INTRODUCTION

In life-history evolutionary studies, especially through laboratory selection experiments, a trade-off usually refers to a negative additive genetic correlation between the various life history and life history related traits (Prasad and Joshi, 2003). These traits are likely to have a common underlying physiological and metabolic network and in many cases they compete for time and resources that are limiting (Gadgil and Bossert, 1970). Although many of these traits also show negative correlations at the phenotypic level, ultimately it is the underlying additive genetic correlations that affect joint responses to selection and these can be different in both magnitude and sign from phenotypic correlations (Falconer and Mackay, 1996). Selection experiments, therefore, offer an opportunity to assess the relative magnitude and sign of additive genetic correlations among traits by examining correlated responses to selection (Falconer and Mackay, 1996).

A large number of life-history related trade-offs have been identified (Stearns, 1992) and most of them concern present reproduction versus future reproduction or survival. In *D. melanogaster*, trade-offs can be observed at different life stages. For example, many studies on the evolution of lifespan have reported a trade-off between lifespan and early life fecundity (Rose, 1984; Luckinbill and Clare, 1985). There is also evidence for a cost of reproduction early in life in terms of increased mortality and decreased lifespan (Rose, 1984; Partridge *et al.,* 1987, 1999; Chippindale *et al.,* 1993, 1997; Chapman, 2001).

An organism invariably has to survive till it attains reproductive maturity if it is to contribute genetically to the next generation. On the other hand, short pre-adult

duration, which is the time spent prior to the attainment of reproductive maturity, is associated with greater fitness in overlapping generation systems because it results in a greater turnover rate of generations. Hence, both pre-adult duration and survival are potentially important life-history traits (Stearns, 1992) and are known to trade-off with adult life history and life history related traits in *D. melanogaster*. For example, developmental rate (inverse of pre-adult development time) appears to trade-off with adult lifespan (Partridge and Fowler, 1992; Chippindale *et al.*, 2004; Prasad 2004; Modak, 2009). In holometabolous insects like *Drosophila*, adult size is largely determined by the larval resource acquisition. When populations are selected for faster pre-adult development, it has invariably led to a correlated decrease in adult size (Zwaan *et al.*, 1995, Nunney, 1996; Chippindale *et al*., 1997; Prasad *et al*., 2000). In some studies on *Drosophila*, adult size has also been found to be positively correlated with adult fitness. Larger flies found to have greater early fecundity (Hillesheim and Stearns, 1992) and they mate more often in the wild than smaller flies (Santos *et al.,* 1988). Thus, developmental rate can trade-off with adult fitness through size-mediated effects on fecundity and male mating success.

In our FEJ populations that have been selected for rapid pre-adult development and reproduction early in life for more than 280 generation, many such trade-offs have been observed and have been discussed thoroughly elsewhere (Prasad, 2004; Shakarad *et al*., 2005; Modak, 2009). These populations were returned back to their ancestral environmental condition in an attempt to study reverse evolution of these populations in the laboratory with an intent to inquire whether,

(a) Similar genetic correlations are seen during forward and reverse evolution.

(b) The reverse selected populations regain their ancestral adult phenotypes and, if so, how does the degree and rate of convergence compare to an earlier similar study (Teotonio and Rose, 2000; Teotonio *et al*., 2002).

MATERIALS AND METHODS

Dry weight at eclosion

Dry weight assays were conducted at generations 5, 10, 14, 25, 41 (only females), 49 and 54 of RF selection. Freshly eclosed adults (< 2 h post-eclosion) originating from eggs laid by standardized flies were collected, killed by deep freezing, dried for 36 h at approximately 70^{o} C and weighed in batches of 5 males or 5 females. The flies collected for the assay were reared at a density of 30 eggs per vial. From the frozen flies, eight batches each of males and females were chosen haphazardly and weighed.

Fecundity

Fecundity was measured by introducing one male and one female adult fly in each of 20 vials assayed per population. These flies were allowed to lay eggs for 24 h, after which the flies from each vial were transferred to a fresh vial with approximately 1 ml banana-jaggery food medium and the eggs laid in the earlier vial were counted and registered. The ages at which fecundity was assayed were chosen to match the age of the flies when eggs are collected from the RF and JB flies during their respective maintenance protocol. The flies were maintained as mixed sex groups in the plexiglass cages provided with petri-plate containing regular banana-jaggery food till the day of

assay set up. The fecundity measured for days 2, 3 and 4 was averaged and taken as early life fecundity, while the fecundity measured for days 10, 11 and 12 was averaged and taken as middle life fecundity. The fecundity assays were conducted at generation 1, 5, 10, 14, 25, 41, 49 and 54 of RF selection.

Statistical analysis

The data for dry weight from each generation assayed were analyzed using separate three-way ANOVAs, treating selection regime and sex as fixed factors crossed amongst themselves and also crossed with the random factor, block. Fecundity data from each generation assayed were analyzed using separate two-way ANOVAs with selection regime as a fixed factor crossed with random blocks. All statistical analyses were implemented using STATISTICA^{TM} for windows release 5.0 B (StatSoft Inc., 1995).

RESULTS

Dry weight at eclosion assay

Separate ANOVAs performed with data on mean dry weight at eclosion from assays carried out at different generations of RF selection revealed significant effects of selection regime and sex, whereas the interaction between selection regime and sex showed no significant effect of dry weight after generation 14 of RF selection (table 3.1). Both male and female flies of RF populations were significantly lighter than JB populations, and in both RF and JB populations, males were significantly lighter than females at all the generations assayed (figure 3.1). Before starting reverse evolution experiment, both male and female FEJ flies were ~59% lighter than JB flies at eclosion (Modak, 2009). After 5 generations of reverse selection, the mean dry weight of both male and female RF flies increased. After 5 generations of RF selection, the male and female flies were ~52% and 55% lighter than JBs at eclosion respectively. This percentage difference in mean dry weight between RF and JB flies consistently decreased at every generation assayed and at generation 54, both male and female flies of RF populations were observed to be ~14% lighter than the JBs.

Fecundity assay

Mean early life fecundity in RF populations was significantly lower (~67%) than their JB counterparts at the beginning of the reverse evolution experiment (figure 3.2). Separate ANOVAs done with data obtained from fecundity assays done at different generations of RF selection, revealed a significant effect of selection till generation 14; the results are summarised in table 3.2. The difference in mean early life fecundity between RF and JB which was ~67% at the beginning of the experiment

came down to ~5% by generation 49 of RF selection, though the decrease was not consistent as the selection progressed. At the assay done at generation 54, a slight raise in the difference in mean early life fecundity was observed and it was ~13% between the RF and JB populations, although the difference was not statistically significant.

Similar results were observed for middle life fecundity in RFs over the generations of selection. RF flies had ~58% lower middle life fecundity at generation 5 of RF selection and this difference in mean middle life fecundity between RF and JB populations dropped down to ~29% by the end of generation 49. Whereas, at generation 54 RF flies showed 3% higher mean middle life fecundity than JB flies (figure 3.3), although it was not significantly different as revealed by results of ANOVA performed at that generation. Separate ANOVAs performed with middle life fecundity at each generation assayed revealed a significant effect of selection regime at all the generations assayed except generation 54 (table 3.3).

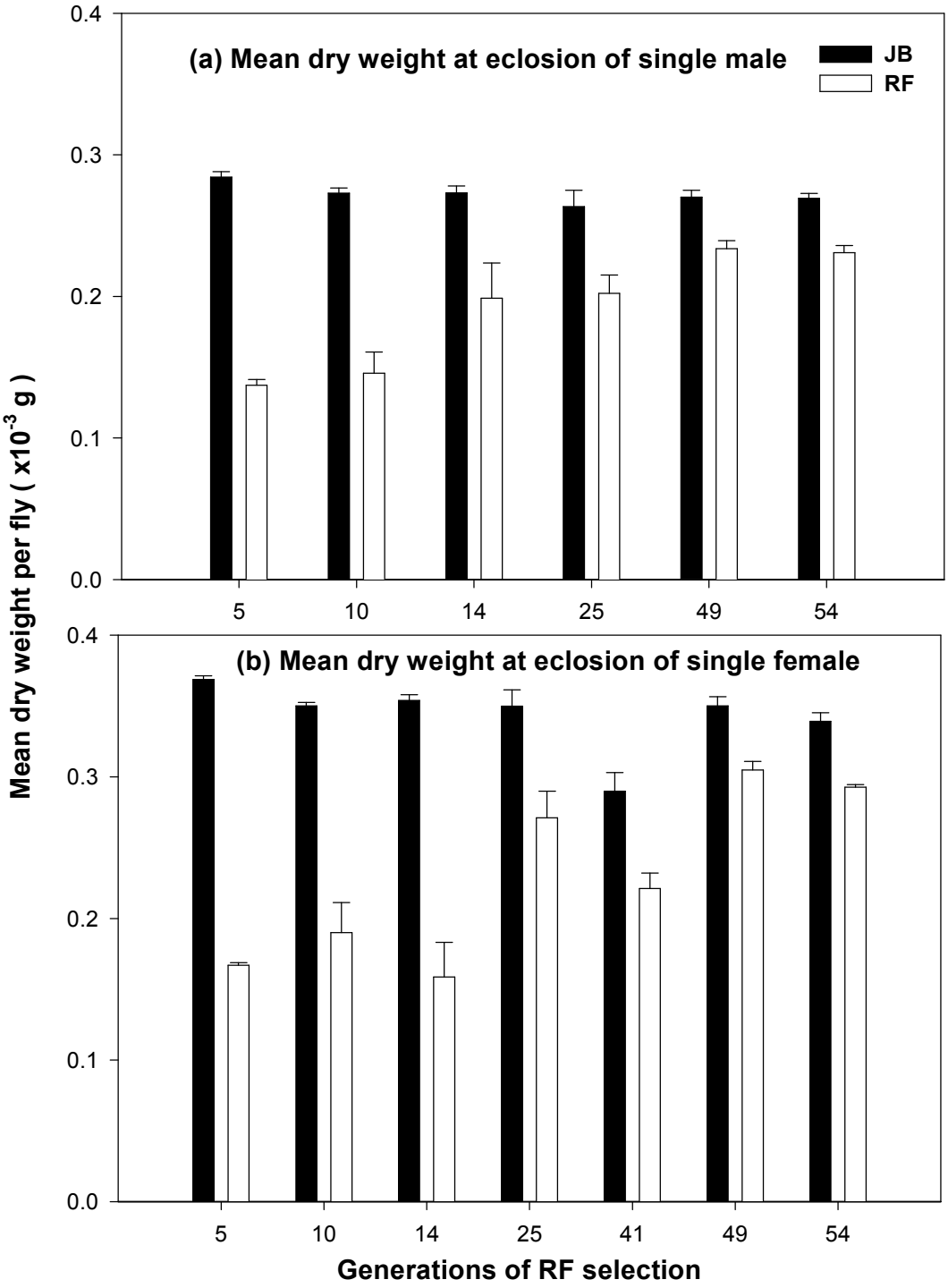


Figure 3.1: Mean (± s.e.) dry weight of the RF and JB populations over the generations of RF selection (a) dry weights of males (b) dry weights of females.

Generation	Effect	df	MS	F	P
Gen 5	Selection	1	3.038	1431.72	< 0.001
	Sex	1	0.324	219.88	< 0.001
	Selection x Sex	1	0.075	209.52	< 0.001
Gen 10	Selection	1	2.065	60.52	0.004
	Sex	1	0.368	155.51	0.001
	Selection x Sex	1	0.026	16.82	0.026
Gen 14	Selection	1	1.799	41.45	0.007
	Sex	1	0.355	228.21	< 0.001
	Selection x Sex	1	0.044	183.24	< 0.001
Gen 25	Selection	1	0.490	15.39	0.0294
	Sex	1	0.603	358.06	< 0.001
	Selection x Sex	1	0.007	6.72	0.081
Gen 49	Selection	1	0.166	53.99	0.005
	Sex	1	0.569	1037.82	< 0.001
	Selection x Sex	1	0.002	0.78	0.442
Gen 54	Selection	1	0.181	76.71	0.003
	Sex	1	0.434	1037.85	< 0.001
	Selection x Sex	1	0.001	1.27	0.341

Table 3.1: Summary of the ANOVA results on the mean dry weight of RF and JB populations. In this three-way ANOVA selection regime and sex were treated as fixed factors crossed amongst themselves and also crossed with the random factor block. In this design, the effects of blocks and interactions involving the block cannot be tested for significance and have therefore been omitted.

Generation	Effect	*df*	MS	*F*	*P*
Gen 1	Selection	1	750.73	307.11	< 0.001
Gen 5	Selection	1	82.88	185.08	< 0.001
Gen 10	Selection	1	315.54	373.12	< 0.001
Gen 14	Selection	1	3490.66	14.43	0.0320
Gen 41	Selection	1	24.28	5.25	0.105
Gen 49	Selection	1	0.287	0.044	0.847
Gen 54	Selection	1	345.15	2.54	0.209

Table 3.2: Summary of the ANOVA results on the mean early-life fecundity of RF and JB populations. In this two-way ANOVA selection regime was treated as a fixed factor crossed with random blocks. In this design, the effects of blocks and interactions involving the block cannot be tested for significance and have therefore been omitted.

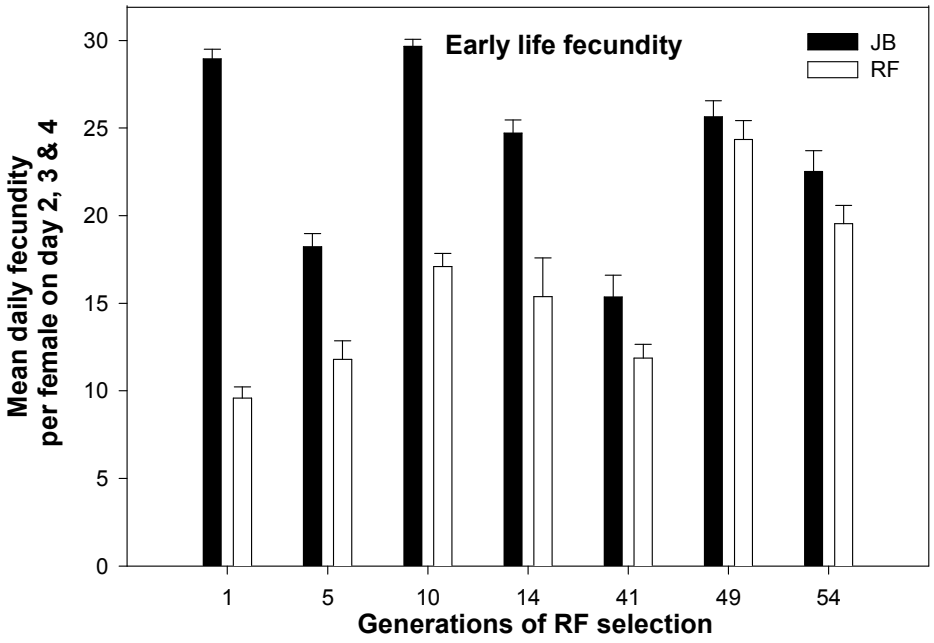


Figure 3.2: Mean (± s.e.) early life fecundity of single females averaged over day 2, 3 and 4 after eclosion over the generations of RF selection.

Generation	Effect	*df*	MS	*F*	*P*
Gen 5	Selection	1	241.38	342.73	< 0.001
Gen 10	Selection	1	104.47	125.07	0.0015
Gen 14	Selection	1	3297.12	61.50	0.0043
Gen 41	Selection	1	10.27	11.06	0.0448
Gen 49	Selection	1	64.79	16.85	0.0261
Gen 54	Selection	1	0.460	0.357	0.5921

Table 3.3: Summary of the ANOVA results on the average middle-life fecundity of RF and JB populations. In these two-way ANOVAs, selection regime was treated as a fixed factor crossed with random blocks. In this design, the effects of blocks and interactions involving the block cannot be tested for significance and have therefore been omitted.

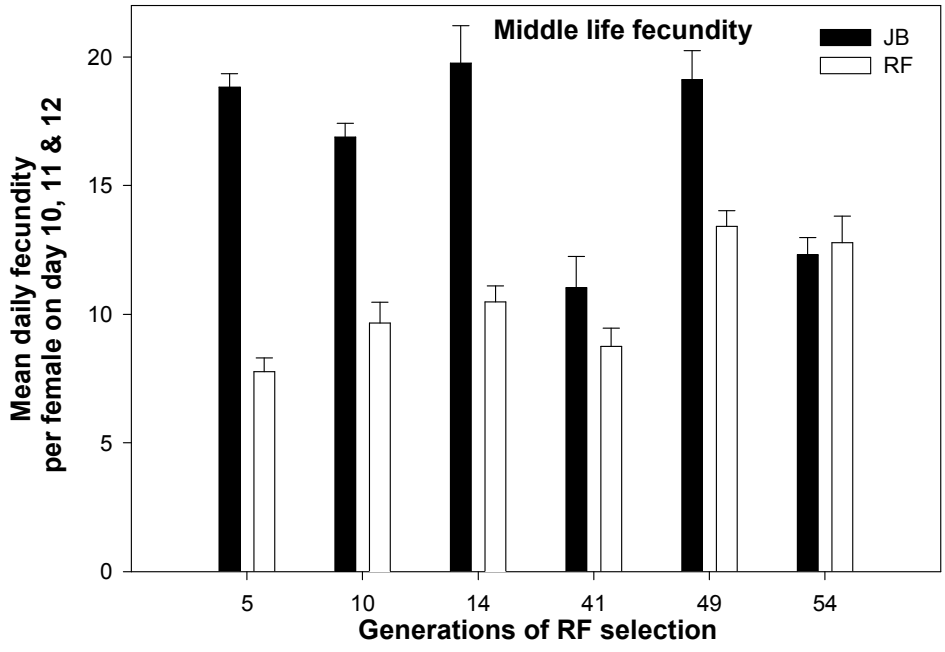


Figure 3.3: Mean (± s.e.) middle life fecundity of single females averaged over day 10, 11 and 12 after eclosion over the generations of RF selection.

DISCUSSION

As was the case for the pre-adult traits described in chapter II, the trait more closely correlated with adult fitness (fecundity) showed more complete convergence to ancestral values than dry weight at eclosion (figures 3.1 and 3.2). The FEJ populations were forward selected for early reproduction, relative to controls (day 3 versus day 12 post-eclosion), along with faster development. Over ~200 generations of selection, they evolved to develop around two and a half day faster than their JB counterparts. The evolution of early life fecundity in the FEJ populations had followed a strange trajectory. Over the first 30 generations of FEJ selection, early life fecundity stayed at par with the controls despite reducing dry weight (Prasad, 2004). At some point between generations 30 and 70 of FEJ selection, the FEJ populations appeared to evolve towards a non-optimal life history: fecundity per unit dry weight decreased, even as dry weight continued to reduce relative to controls. It was then hypothesized that this was due to a "physiological switch" that responded to lipid levels in the FEJ populations falling below some threshold and biasing the allocation pattern in the FEJ adults towards somatic maintenance rather than early life fecundity (discussed in detail in Prasad, 2004). Eventually, however, the FEJ populations evolved around this constraint and at generation 270 of FEJ selection had higher fecundity per unit lipid than controls, together with a concomitant lifespan reduction (Modak, 2009). However, at the start of RF selection, absolute fecundity both in early and middle life was considerably less in the RF populations than in controls (figures 3.2 and 3.3). Yet, both early life and middle life fecundity in the RF populations had converged to control levels by generations 49 and 54, respectively of RF selection. This observation differs from the finding of Teotonio and Rose (2000) that early life fecundity in the ACO

populations reverted back to only about 75% of the ancestral value over 50 generations of reverse selection.

The behaviour of dry weight at eclosion over 54 generations of RF selection (figure 3.1) was similar to that seen with the ACO populations by Rose and Teotonio (2000): the convergence was not complete, with the reverse selected populations attaining about 75% of the dry weight of the heavier controls. The incomplete reversion of dry weight at eclosion is to a degree similar to that seen for pre-adult development time (chapter II) and is not surprising given the consistently close relationship between faster development and reduced dry weight at eclosion (Zwaan, 1995; Nunney, 1996; Chippindale *et al*., 1997; Prasad *et al*., 2000).

Why early life fecundity in the RF populations converged fully whereas it did not in the ACO populations of Teotonio and Rose (2000) is not clear at this time. The ACO populations were selected for rapid development starting from a set of base populations (CO) with a different selective history in the context of age at reproduction than the JB controls of our FEJ populations. Moreover, the "early reproduction" part of selection on the FEJ and ACO populations was quite different. In the FEJ regime, eggs were collected to initiate the next generation three days after eclosion (Prasad *et al*., 2000), whereas in the ACO regime eggs for the next generation were collected as soon as enough eggs were laid, within 24 h (Chippindale *et al*., 1997). This small difference in selection regime led to some large differences in correlated responses, especially in terms of pre-adult life-stage specific development time and survivorship (Prasad *et al*., 2000, 2001). It is possible that the combination of different selective history with regard to age at reproduction in the respective controls and the different selection for early reproduction in the ACO and FEJ populations led to the evolution of different

genetic architecture for early life fecundity in these two sets of populations, eventually leading to differences in the degree of convergence seen in the two lineages upon reverse selection.

Chapter-IV

Antibody Staining, Cell Number and Cell Size in FEJ populations

INTRODUCTION

Development is one of the complex processes in any organism in which a fertilized egg cell after repeated division give rise to a complete organism. The genetic control of development has been particularly well studied in *D. melanogaster*, where the process of early embryo development after fertilization includes nuclear division without cytokinesis resulting in a multinucleate call called a syncytium. The common cytoplasm allows morphogen gradients to play a key role in pattern formation. At the tenth nuclear division, cells migrate to the periphery of the embryo. At thirteenth division, ~6000 or so nuclei are partitioned into separate cells. This stage is the cellular blastoderm (Turner and Mahowald, 1977; Foe and Alberts, 1983; Gilbert, 2006).

After the formation of cellular blastoderm, a cascade of gene activation sets up the *Drosophila* body plan. The maternal-effect genes, such as *bicoid* and *nanos*, are required during oogenesis. The transcripts or protein products of these genes are found in the egg at fertilization, and form morphogen gradients. The maternal-effect genes encode transcription factors that regulate the expression of the gap genes. The gap genes roughly subdivide the embryo along the anterior/posterior axis. These gap genes encode transcription factors that regulate the expression of the pair-rule genes. The pair-rule genes divide the embryo into pairs of segments. These pair-rule genes also encode transcription factors that regulate the expression of the segment polarity genes. The segment polarity genes set the anterior/posterior axis of each segment. The gap genes, pair-rule genes, and segment polarity genes are together called the segmentation genes, because they are involved in segment patterning (Gilbert, 2006).

Later in development, these segments acquire individual identity by the action of one more set of genes called homeotic genes. These genes encode transcription factors that control the expression of genes responsible for particular anatomical structures, such as wings, legs and antennae. As a whole, development in *Drosophila* involves a cascade of expression of a large number of genes, in a spatio-temporally controlled manner, ultimately resulting in the formation of a complete organism (Gilbert, 2006). This spatial and temporal regulation of gene expression is very important from the point of view of development of a normal fly. A change in the expression of these genes with respect to either space or time results in abnormalities.

In the FEJ populations which had been selected for faster development and early reproduction for more than 325 generations in our laboratory before this study was done, we observed a great variety of phenotypic changes compared to their control JB populations, such as significant reduction in duration of all the life-stages, reduced fecundity, longevity, lipid content *etc.*. More interestingly, the FEJ flies were consistently observed to be more lethargic than their controls and they were found to have some leg as well as wing malformations (K. M. Satish, personal observation). I have observed some of the wing malformations, such as notch formation in the wing blades, improperly opened wing blades and improperly folded wings. In the normal flies, the wings are folded properly and held on their thorax running towards the abdomen tip while resting whereas, in some of the FEJ flies I observed the wings to be held open even when they are resting. These observations led me to speculate that these abnormalities could be the result of change in the expression pattern of some of the developmentally important genes. Therefore, using antibody staining technique, I studied the expression pattern of some of the proteins coded by the developmentally

important genes *engrailed, wingless and Ubx* both in the embryos as well as in the wing discs of third instar larvae in JB and FEJ populations.

Most animals are constructed of segments, although it may not necessarily be externally visible, as in vertebrates. Much of the epidermis of *Drosophila* develops as a chain of alternating anterior (A) and posterior (P) compartments. Populations of cells in these compartments differ from each other because the selector gene *engrailed (en)* is active in the cells of P but not in cells of A. Early in development, the state of *en* expression is fixed in sets of cells *i.e,* 'on' in P compartment and 'off' in A. During growth, borderlines between A and P compartments act as engines to produce positional information. The *engrailed* gene is required for the maintenance of segmental pattern (Nüsslein-Volhard and Wieschaus, 1980; Kornberg, 1981). In the embryo, Engrailed protein is expressed in fourteen evenly spaced domains that become the anterior part of each parasegment. In the wing imaginal discs, it is expressed in posterior compartments (Brower, 1986).

The *Drosophila* segment polarity gene *wingless (wg)* encodes an intercellular signaling molecule that transmits positional information during development of the embryonic epidermis. The cell signaling molecule wingless (Wg) belongs to a family of secreted glycoproteins and is involved in a large variety of cell-fate decisions throughout the life of *Drosophila* (Wodarz and Nusse, 1998). In the embryonic ectoderm, the segmentally repeated expression pattern of *wg* is established by pair-rule segmentation genes (Nüsslein-Volhard and Wieschaus, 1980; Siegfried and Perrimon, 1994) and is required for specification of cell-fates along the anteroposterior axis of each segment (Peifer and Bejsovec, 1992). Wingless is expressed in a narrow stripe of

cells along the dorsal–ventral axis. Wingless acts to specify cell fates in the dorsal and ventral compartments (reviewed by Howes and Bray, 2000).

Ultrabithorax (Ubx), located in the left (proximal) end of the bithorax complex, is the most extensively studied of the *Drosophila* homeotic loci. In the wing disc, a derivative of T2, Ubx is expressed only in the peripodial membrane (White and Wilcox, 1985; Emerald and Shashidhara, 2000).

The final size reached by an adult organism is a consequence of changes in the size and number of cells during its development (Kawli, 2000). Growth is associated with an increase in biomass through the stimulation of the biosynthesis of cellular components. Growth can occur in the absence of cell division by cell enlargement and by the decomposition of extracellular matrix. However, the most common type of growth during development is coupled to cell division (Roush, 1996; Neufeld and Edgar, 1998; Polymenis and Schmidt, 1999).

When a cell divides, it will normally split into two daughter cells of equal size. These daughter cells then grow until they have reached the same size as the mother cell before they enter the next round of cell division. Therefore, growth which is increase in biomass, is typically tightly coupled with cell-cycle progression (Oldham *et al.,* 2000). During normal growth, the size of the cells remains constant. Therefore, a cell must be able to determine when it has reached a certain size to initiate the next round of cell division. In yeast, for example, this critical size is dependent on the availability of nutrients. When yeast cells were placed on poor media, they divided at a smaller critical size (Johnston *et al.,* 1977). Starved *Drosophila* larvae typically develop into small flies

that contained fewer and smaller cells than flies reared under non-starving conditions (Robertson, 1959, 1963; Simpson, 1979).

FEJ flies are smaller in their body size and their larval feeding rate is also significantly lower than the controls. There could, therefore, be change in the rate of cell division in the FEJ larvae compared to control JB larvae. Therefore, I studied cell number and cell size in wing imaginal discs of FEJ and JB populations. Armadillo and alpha-Catenin are components of a multiprotein complex that both maintains and initiates formation of sheets of epithelial cells. These proteins are part of the adherens junctions, a biochemical crossroad where cells are attached to one another. Immonostaining with anti-Armadillo antibody can mark the cell boundaries and then cells can be counted. This technique was utilized to mark cell boundaries in FEJ and JB third instar larval wing imaginal discs in order to be able to count the number of cells and estimate their size.

MATERIAL AND METHODS

Embryo and larval collection for antibody staining

Standardized flies of JB and FEJ population in the cages were supplied with a Petri-plate containing agar medium containing sugar and yeast. The flies were allowed to lay eggs on this agar medium for ~14 h. These eggs, aged from 1 to 14 h, from each of the cages containing FEJ or JB flies were collected separately using a moistened paint brush and immediately transferred onto a sieve. These eggs were then washed thoroughly with distilled water to remove contaminants and then dechorionated by treated with 50% sodium hypochlorite solution. Immediately thereafter, the eggs were

fixed in solution containing 1:1 heptane and 5% para-formaldehyde. The fixed embryos were transferred to tube containing 100% methanol and stored at -20^{o}C until use.

For harvesting larvae, eggs from each of cages containing JB and FEJ standardized flies were transferred into vials containing approximately around 6 mL banana-jaggery food medium at a density of around 30 eggs per vial. Ten such vials were set up per population. The wandering third instar larvae from each FEJ and JB population were taken out of these vials using a moistened paint brush and put into a Petri dish containing 1X PBS (Sigma-Aldrich, cat# P3813-10PAK, St. Louis, Missouri, USA) solution. The larvae were washed thoroughly with 1X PBS to remove the food particles adhering to their body. Then the larvae from each of the population were dissected using a fine scissor and forceps in presence of 1X PBS in a watch glass under a binocular microscope. Using the forceps the mouth parts of the larva are held firmly and the larva was given a cut at 3/4th of the abdomen. Then using a fine needle and with the help of a fine forceps, the whole larval body was turned inside out exposing all the internal parts outside. The dissected larvae were then fixed in 4% para-formaldehyde solution for 20-30 minutes and immediately processed further.

Staining of the embryos

The fixed embryos, stored inside a microfuge tube containing methanol at -20^{0}C were taken out and thawed to room temperature. Methanol from the tube was removed and sterile distilled water was added to the embryos in order to rehydrate. Then the embryos of different populations were separately blocked using 0.3% PBTx solution (Appendix) for 20 minutes at room temperature with shaking. Embryos were then transferred to tubes containing the primary antibody (anti-Engrailed and anti-Wingless)

at appropriate dilution (1:300 for anti-Engrailed and 1:200 for anti-Wingless) prepared in 0.1% PBTx with bovine serum albumin (BSA) (Appendix) and incubated at 4^{o}C overnight without shaking. After the incubation, the embryos were washed thrice using 0.1% PBTx solution to remove excess antibody. After the primary antibody wash, the embryos were transferred to tubes containing secondary antibody (M-Alexa-594) at 1:500 dilution in 0.1% PBTx, tubes were wrapped with aluminum foil and were incubated at room temperature for 1 hour with shaking. After the incubation with secondary antibody the embryos were washed four times with 0.1% PBTx. After the secondary antibody washing, the embryos were transferred into 1X PBS and stored at 4^{0}C until use. The antibody stained embryos were taken out of PBS solution and were mounted on clean glass slides using 50% glycerol made in 1X PBS. The mounted embryos were then examined under the fluorescent microscope using appropriate filter. Axio Vision 4.3 software (Carl Zeiss Microimaging GmbH, Oberkochen, Germany) was used for capturing images of the antibody stained embryos.

Staining of the wing imaginal discs

The larvae fixed in 4% para-formaldehyde solution were rinsed with 1X PBS solution. These larvae of different populations were then transferred to different microfuge tubes containing 1 ml. of 0.1% PBTx solution containing BSA for permeablization and blocking. These tubes were incubated for 3 h at room temperature. Larvae were then transferred to tubes containing 100 μL of primary antibody solution at appropriate dilution (1:300 for anti-Engrailed, 1:200 for anti-Wingless, and 1:10 for anti-Ubx) prepared in 0.1% PBTx with BSA (Appendix) and incubated at 4^{o}C overnight without shaking. After the incubation, the larvae were washed thrice using 0.1% PBTx solution with duration of 20 minutes per each wash. After the primary

antibody wash, the larvae were transferred to tubes containing 250 μL of secondary antibody (M-Alexa-594) solution at 1:500 dilution prepared in 0.1% PBTx, tubes were wrapped with aluminum foil and were incubated at room temperature for 1.5 h under shaking. After the incubation with secondary antibody, the larvae were washed four times with 0.1% PBTx. After the secondary antibody washing the larvae were transferred into 1X PBS and stored at 4^0C until use. The antibody stained larvae were taken out of PBS solution and were placed on clean slide. Using fine needles the wing imaginal discs were dissected out from the larvae and were placed on the slide in presence of 50% glycerol made in 1X PBS. Then a cleaned cover slip is slowly placed on the dissected discs and sealed using nail polish. These mounted imaginal discs were then observed under a fluorescent microscope using appropriate filter. Axio Vision 4.3 software (Carl Zeiss Microimaging GmbH, Oberkochen, Germany) was used for capturing images of the antibody stained wing imaginal discs.

Anti-Armadillo staining of wing imaginal discs

Larvae from FEJ and JB populations of block 1 and 2 for anti-Armadillo staining were collected as mentioned above. The larvae were fixed in 4% para-formaldehyde solution were rinsed with 1X PBS solution. These larvae of different populations were then transferred to different microfuge tubes containing 1 mL of 0.1% PBTx solution containing BSA for permeablization and blocking. These tubes were incubated for 3 h at room temperature. Larvae were then transferred to tubes containing 100 μL of primary anti-Armadillo antibody solution at 1:100 dilution prepared in 0.1% PBTx with bovine serum albumin (Appendix) and incubated at 4^oC overnight without shaking. After the incubation, the larvae were washed thrice using 0.1% PBTx solution with duration of 20 minutes per wash. After the primary antibody wash, the larvae were

transferred to tubes containing 250 μL of secondary antibody (M-Alexa-594) solution at 1:500 dilution prepared in 0.1% PBTx, tubes were wrapped with aluminum foil and were incubated at room temperature for 1.5 h under shaking. After the incubation with secondary antibody the larvae were washed four times with 0.1% PBTx. After the secondary antibody washing the larvae were transferred into 1X PBS and stored at 4^0C until use. The antibody stained larvae were taken out of PBS solution and were placed on clean slide. Using fine needles the wing imaginal discs were dissected out from the larvae and were placed on the slide in presence of 50% glycerol made in 1X PBS. Then a cleaned cover slip was slowly placed on the dissected discs and sealed using nail polish. These mounted imaginal discs were then observed under a fluorescent microscope using appropriate filter with 100X magnification. Axio Vision 4.3 software (Carl Zeiss Microimaging GmbH, Oberkochen, Germany) was used for capturing images of the antibody stained wing imaginal discs. In the captured images cells could be visualized as anti-Armadillo had stained the boundaries of the cell. In both FEJ and JB larval imaginal discs, equal area was marked at the pouch region and the cells were counted manually and their size was estimated.

RESULTS

Engrailed protein expression pattern

Antibody staining of embryos with anti-Engrailed revealed no significant difference in the pattern of Engrailed expression between FEJ and JB populations. In embryos of both FEJ and JB populations Engrailed protein expression was observed in the fourteen evenly spaced domains that later become the anterior part of each parasegment (figure 5.1). In late third instar larval wing imaginal discs too, similar result was observed. There was no significant difference in Engrailed protein expression pattern between the FEJ and JB populations. In wing imaginal discs of both JB and FEJ populations it was found to be expressed in the posterior compartment (figure 5.2).

Wingless protein expression pattern

Antibody staining of embryos with anti-Wingless revealed no significant difference in the pattern of Wingless protein expression between the FEJ and JB populations. In embryos of both FEJ and JB populations Wingless protein expression was observed in the fourteen evenly spaced domains that later become the anterior part of each parasegment, similar to pattern seen with anti-engrailed expression pattern (figure 5.3). Late third instar larval wing imaginal discs of FEJ and JB populations stained with anti-wingless antibody showed no significant difference in their expression pattern. Wingless protein was found to be expressed in a narrow strip of cells along the dorsal–ventral axis in the imaginal discs of both the FEJ and JB populations (Figure 5.4).

Ubx protein expression pattern

Anti-Ubx immuno-stained late third instar larval imaginal discs of JB and FEJ populations showed no significant difference in the expression pattern of Ubx protein. In both FEJ and JB populations, Ubx expression was seen in the peripodial membrane cells (Figure 5.5).

Cell number and cell size

Immunostaining of wing imaginal disc with anti-Armadillo enabled marking of each cell present in the disc when observed under fluorescent microscope at 100X magnification. Counting of these cells manually in equal area taken on pouch region of different imaginal discs showed a significant difference in the cell number per unit area between JB and FEJ wing discs. Wing imaginal discs of FEJ populations had a smaller number of larger cells, whereas wing discs of JB populations had a larger number of smaller cells for the same area (figure 5.6).

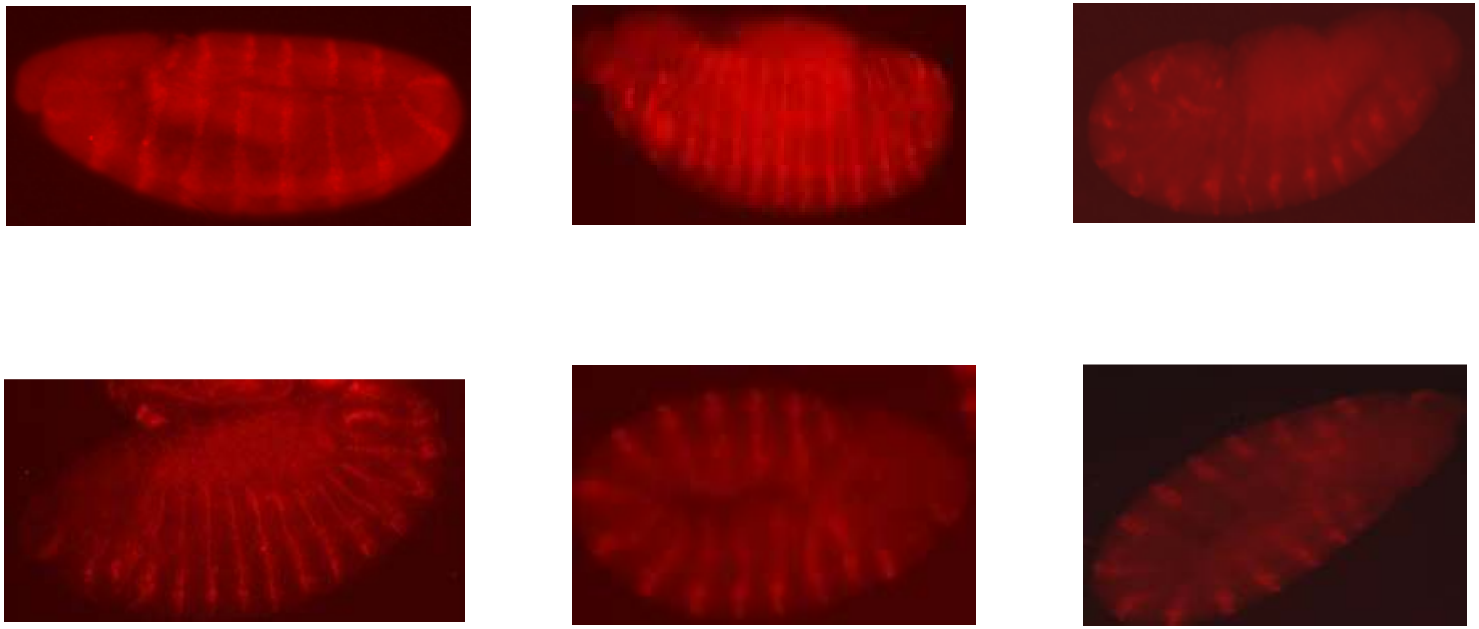


Figure 5.1: Representative images of anti-engrailed stained embryos showing localization of Engrailed protein in 1 to 14 h old embryos. Upper panel – Embryos from FEJ populations. Lower panel- Embryos from JB populations.

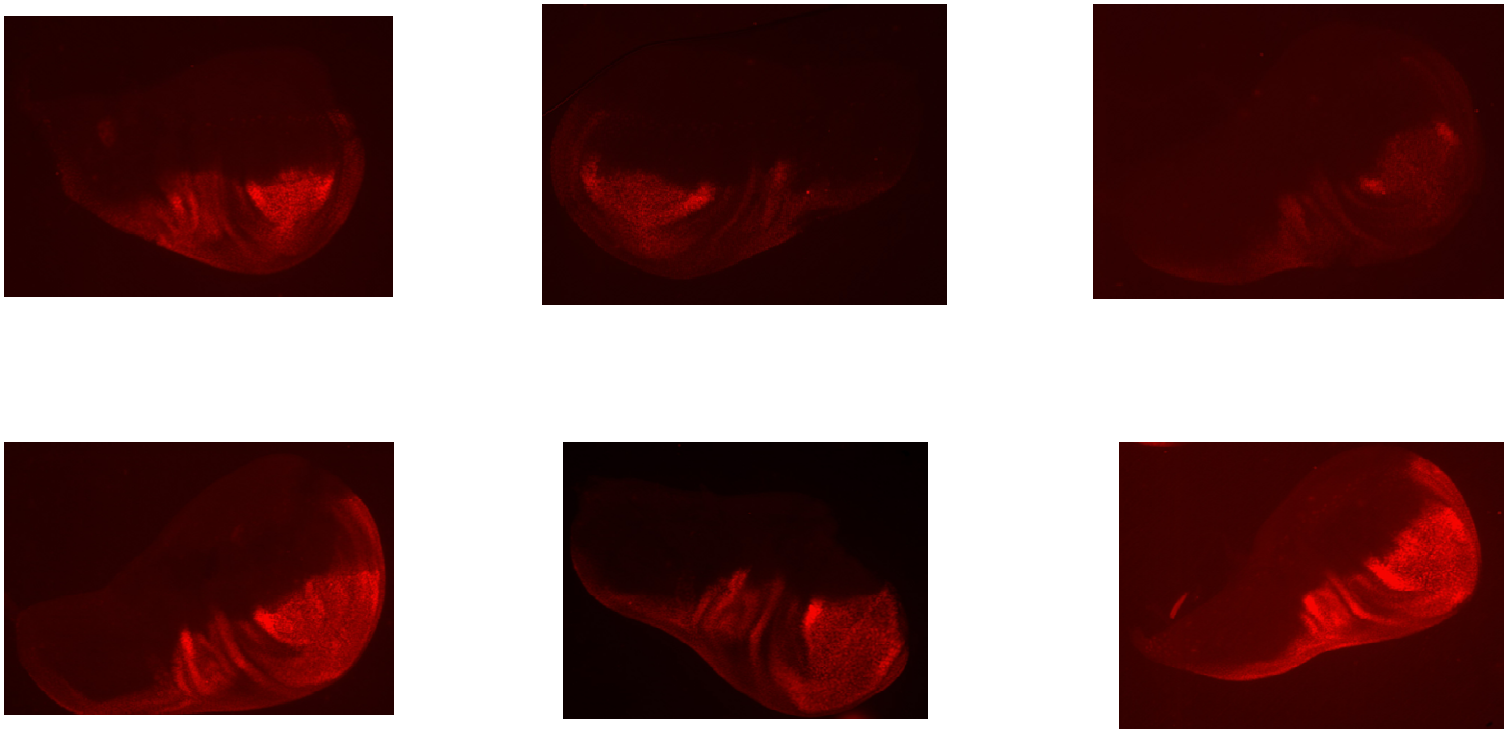


Figure 5.2: Representative images of anti-engrailed stained late third instar stage wing imaginal discs showing localization of Engrailed protein. Upper panel – Wing discs from FEJ populations. Lower panel- Wing discs from JB populations.

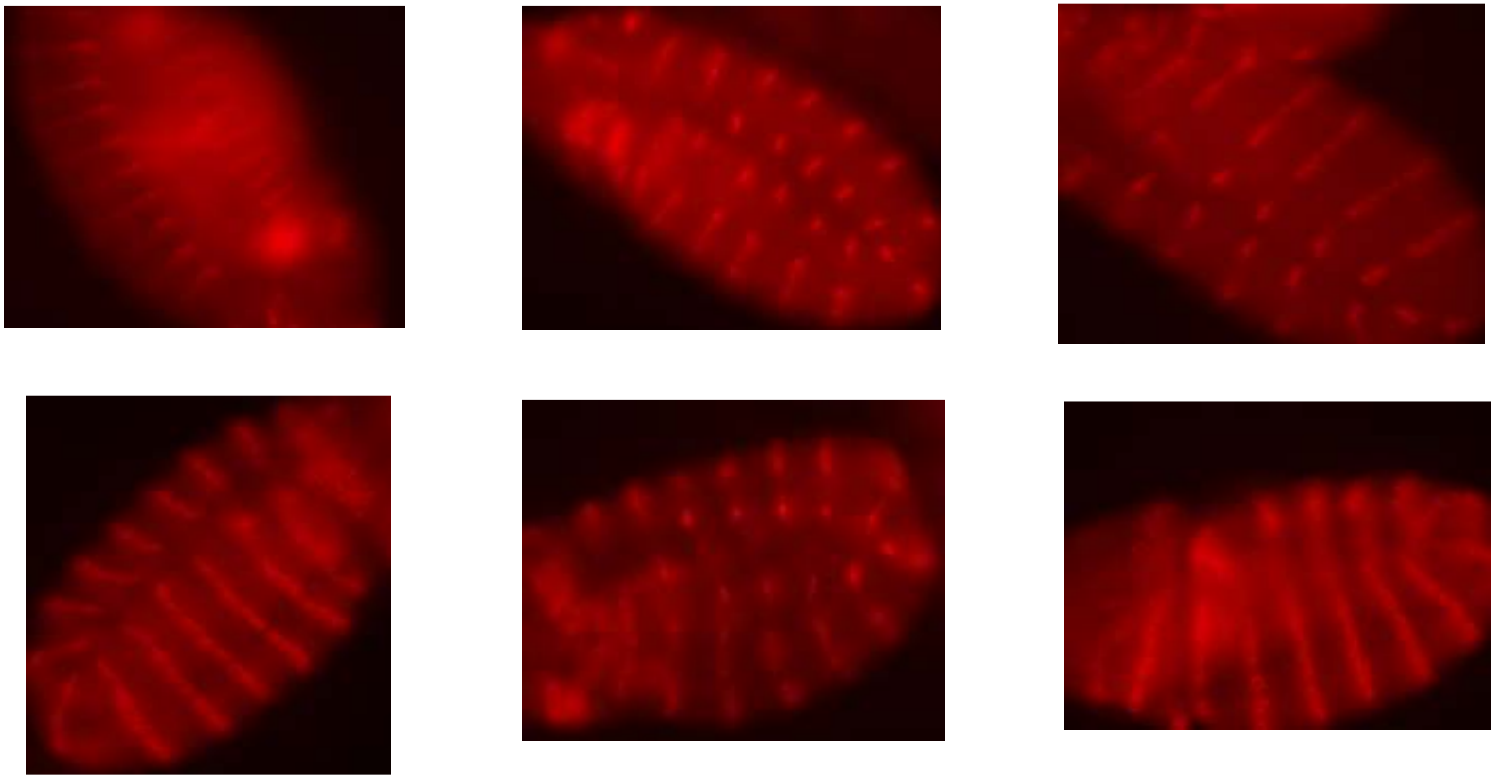


Figure 5.3: Representative images of anti-wingless stained embryos showing localization of Wingless protein in 1 to 14 h old embryos. Upper panel – Embryos from FEJ populations. Lower panel- Embryos from JB populations.

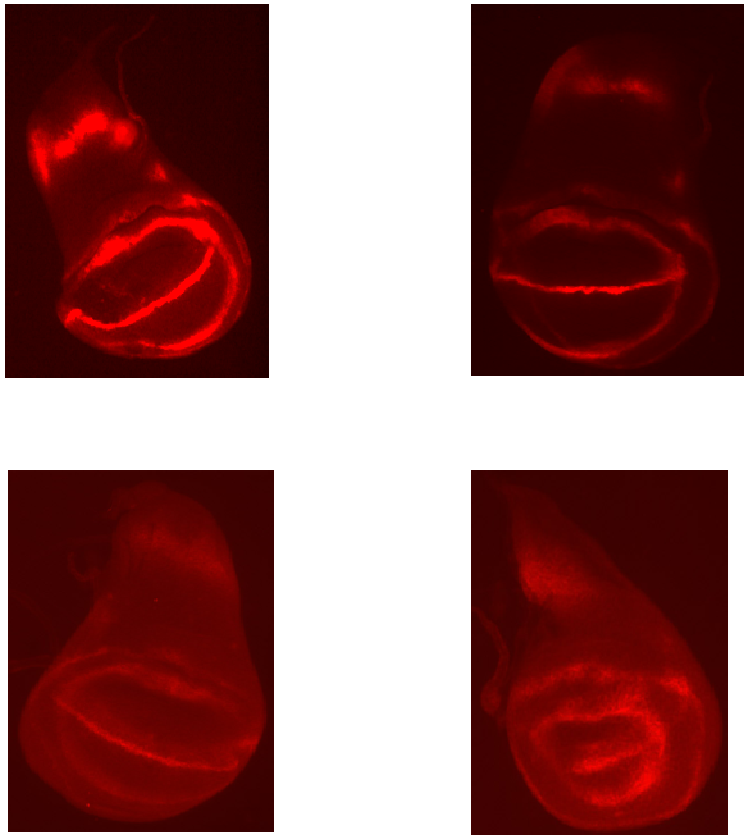


Figure 5.4: Representative images of anti-wingless stained late third instar stage wing imaginal discs showing localization of Wingless protein.

Upper panel – Wing discs from FEJ populations. Lower panel- Wing discs from JB populations.

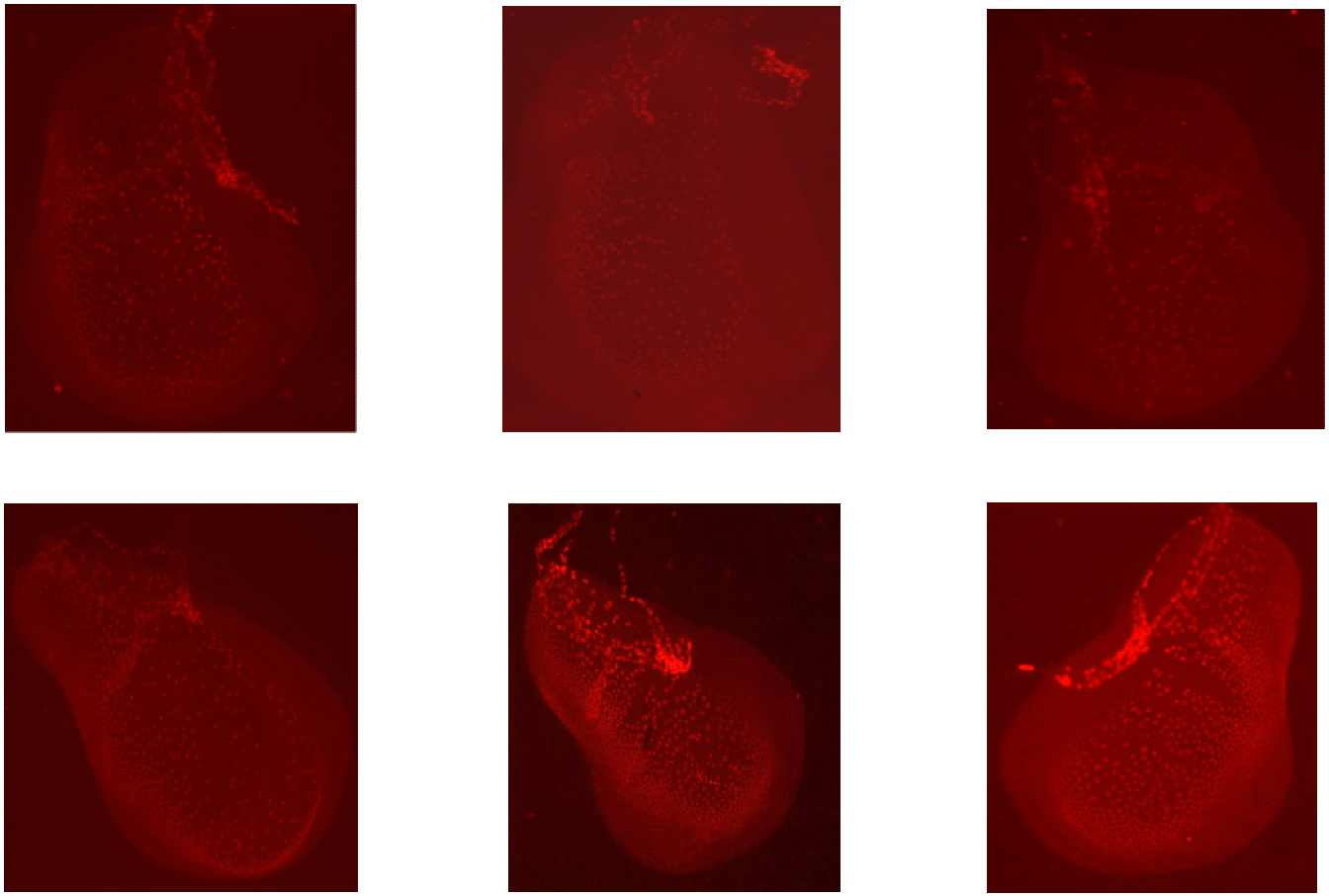
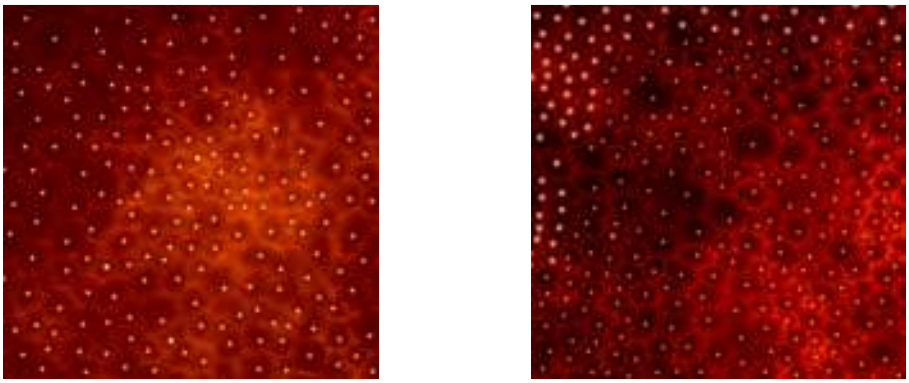


Figure 5.5: Representative images of anti-Ubx stained late third instar stage wing imaginal discs showing localization of Ubx protein. Upper panel – Wing discs from FEJ populations. Lower panel- Wing discs from JB populations.



A **B**

Figure 5.6: Representative images of pouch region of third instar larval wing imaginal disc showing the cell size and number in (A) FEJ, and (B) JB populations. White dots are manually marked to count individual cells.

DISCUSSION

Immunostaining of embryos and imaginal discs

There was no significant difference in the expression pattern of the proteins studied in JB and FEJ populations both in embryos and wing imaginal discs. This could be because the proteins which were selected in this study, namely Engrailed, Wingless and Ubx, were all developmentally very important ones and proper expression of these genes are very important from the point of view of proper development of the flies. FEJ populations might not have diverged so much that they show altered expression patterns of these developmentally important proteins. I did, however, notice that there were some differences in their expression levels between FEJ and JB populations (K. M. Satish, personal observation) but I did not do any follow up studies aimed at quantifying these

differences. This experiment was a preliminary step to study the expression pattern of some developmentally important proteins. More studies may have to be conducted in this line in future to study the expression levels of these and other proteins in these populations.

Cell number and cell size

For a given area, the number of cells in FEJ wing imaginal discs was less and the size was larger compared to that of JB wing imaginal discs. A particular size of an organ could be achieved either by faster cell multiplication or by greater enlargement of the cell, and it appears that the FEJ populations are following the latter mechanism. Cell division is costly in energy terms (O'Connor, 1954) and energy is typically a constrain in FEJ populations as they are under strong selection pressure for faster development which leading to a concomitant decrease in size and energy reserves, compared to controls (Prasad, 2004). Over the course of selection, FEJ populations might have evolved the mechanisms by which they are conserving the energy acquired during development by avoiding excess cell division. Instead, they are probably achieving the final size of the organ by relying more on cell growth and enlargement.

Chapter-V

dnpf

Gene Expression

in FEJ Populations

INTRODUCTION

In any organism, the information present in a gene is used for the synthesis of a functional gene product through a process known as gene expression. These products are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is a functional RNA. The process of gene expression is used by all known life to generate the macromolecular machinery for life. Gene expression mainly involves transcription of the gene in which the transcript of the gene is formed and translation in which a protein is synthesized using the transcript other cellular molecules. Several steps in the gene expression process may be modulated, including the transcription, RNA splicing, translation, and post-translational modification of a protein. Modulating gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Gene regulation may also serve as a substrate for evolutionary change, since control of the timing, location, and extent of gene expression can have a profound effect on the functions of the gene in a cell or in a multicellular organism.

Studying the abundance of mRNA is the most commonly used method for studying the level of gene expression. Several methods are available for studying expression of a gene at transcript level *e.g.* northern blotting, RT-PCR (Reverse transcription – polymerase chain reaction), real time PCR (qRT-PCR) and DNA microarray (Roth, 2000).

The *Drosophila neuropeptide F* gene (*dnpf*), a homolog of mammalian NPY, has been found to be involved in modulating larval feeding behavior. It is known that the young third instar larvae feed voraciously, but their feeding rate decreases as they mature

and eventually, late in the third instar, they start wandering in search of a pupation site. Wu *et al.* (2003) have shown that in wild type third instar larvae, *dnpf* gene expression is high at early third instar stage when larvae feed voraciously and its expression is low at late third instar stage at which time the larvae stop feeding and start preparing for pupation. They have also shown that over-expression of *dnpf* gene at late third instar stage makes the larvae continue to feed well beyond the age at which they would normally initiate pupariation. Moreover, repression of the same gene at early third instar stage makes the larvae cease feeding and pupariate well before the age at which they would normally initiate pupariation. It has been shown earlier that FEJ populations have significantly reduced mean feeding rate in the early third instar, and also a much reduced third instar duration compared to the JB controls (Prasad *et al*., 2001). This led me to speculate that perhaps FEJ larvae have lower expression of *dnpf* gene in the early third instar compared to that of JB larvae. Therefore, I studied *dnpf* gene expression levels in third instar larvae of both selected FEJ and the control JB populations. This study was performed after 280 generations of FEJ selection for faster pre-adult development and early reproduction.

MATERIALS AND METHODS

Collection of larvae for total RNA isolation

Eggs were collected from cages containing JB and FEJ standardized flies and were transferred into vials containing approximately 6 mL banana-jaggery food medium at a density of around 30 eggs per vial. Ten such vials were set up per population. Larvae were collected at three time points namely early, middle and late third instar from JB and FEJ populations. The determination of which age constituted early, middle and late was done

by scaling by third instar duration for both JB and FEJ larvae, so as to match physiological ages to the best of my ability. For RNA isolation, larvae were taken out of these vials using a moistened paint brush and were put into a Petri dish containing 1X PBS solution (Sigma-Aldrich, cat# P3813-10PAK, St. Louis, Missouri, USA). The larvae were washed thoroughly with 1X PBS to remove the food particles adhering to their body. Larvae were then rolled on a tissue paper towel to remove excess water and were transferred to a microfuge tube. Around 15 of these larvae per time point were immediately frozen using liquid nitrogen and used for RNA isolation immediately.

Total RNA isolation from larvae

Frozen larval samples were crushed finely using dispensable plastic sterile pestles. Crushed samples were then subjected to total RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The quality of the isolated RNA was assessed by agarose gel electrophoresis and quantitated using a spectrophotometer.

Reverse transcription and PCR

2 μg of total RNA from each sample was used to perform reverse transcriptase (MBI Fermentas, Lithuania) reaction (RT) using oligo-dT as primer. cDNA was synthesized following the manufacturer's instructions. Reverse transcription product was diluted to 1:5 and 1 μL of this was used as the template and multiplexing PCR was performed using gene specific primers (Appendix) for *dnpf* and *elf-*α in which the latter housekeeping gene was used as internal control. Three replicate samples per population

were used in each PCR reaction performed. The PCR products obtained were electrophoresed on a 1.5% agarose gel. The gel image was taken after electrophoresing for about 2 h in gel documentation unit (Bio-Rad, Hercules, CA, USA). The relative *dnpf* gene expression was calculated using software Quantity One (Bio-Rad, Hercules, CA, USA) as the ratio of intensity of *dnpf* and *elf-α* bands on the agarose gel. The data were further analyzed statistically.

Statistical Analysis

Ratio of band intensity data were subjected to three-way analysis of variance (ANOVA) with selection and physiological age being treated as fixed factors crossed with random blocks. Since the data obtained were fractional, they were subjected to arcsine square-root transformation to meet the normality assumption of ANOVA. Replicate population means of the data were used for analysis. All analyses were implemented using Statistica for Windows release 5.0 B (StatSoft, 1995).

RESULTS

It was noticed that in all the PCR reactions performed with different samples of both JB and FEJ populations, the intensity of *elf-α* gene band remained almost same, although there was difference in the intensity of *dnpf* gene band between JB and FEJ samples (Figure 5.1), indicating minimal manual error, such as in pipetting. The ANOVA revealed no significant difference in the expression levels of *dnpf* gene between JB and FEJ populations (figure 5.2; table 5.1). However, the expression in JB populations was as expected i.e., in the early third instar larvae the expression was high compared to that of

middle instar larva and the expression level went down as the larva developed further. On the other hand, no such gradual decreasing trend was observed in FEJ populations, which showed a relatively constant low level of *dnpf* expression relative to the JB controls (Figure 5.1).

DISCUSSION

Wu *et al* (2003) have reported that the *dnpf* gene expression is high at early third instar stage at which the larva feeds voraciously and it is low at late third instar larva at which point they wander in search of place for pupation. A qualitatively similar expression profile was seen in our control populations whereas, in FEJ populations there was no such trend of decreased expression from early third instar to late third instar stage. FEJ populations had undergone a strong directional selection for more than 280 generation by the time this experiment was carried out and as response to this, they have undergone drastic reduction in many characters such as starvation resistance, desiccation resistance, pathogen resistance, urea tolerance, larval feeding rate *etc*. At the cost of these trade-offs in different characters, FEJ populations are developing faster than their control JB populations. In the process of becoming faster and faster, these flies might have taken short routes in developmental pathways or might have truncated some of the steps in those developmental pathways. This low expression profile of *dnpf* might be because of such a short cut or truncation in the developmental pathway. It was also observed in FEJ populations that they pupate very early compared to JB larvae. Low expression levels of *dnpf* gene in FEJ third instar larvae right at the beginning of the third instar stage could be one of the reasons for their early pupation.

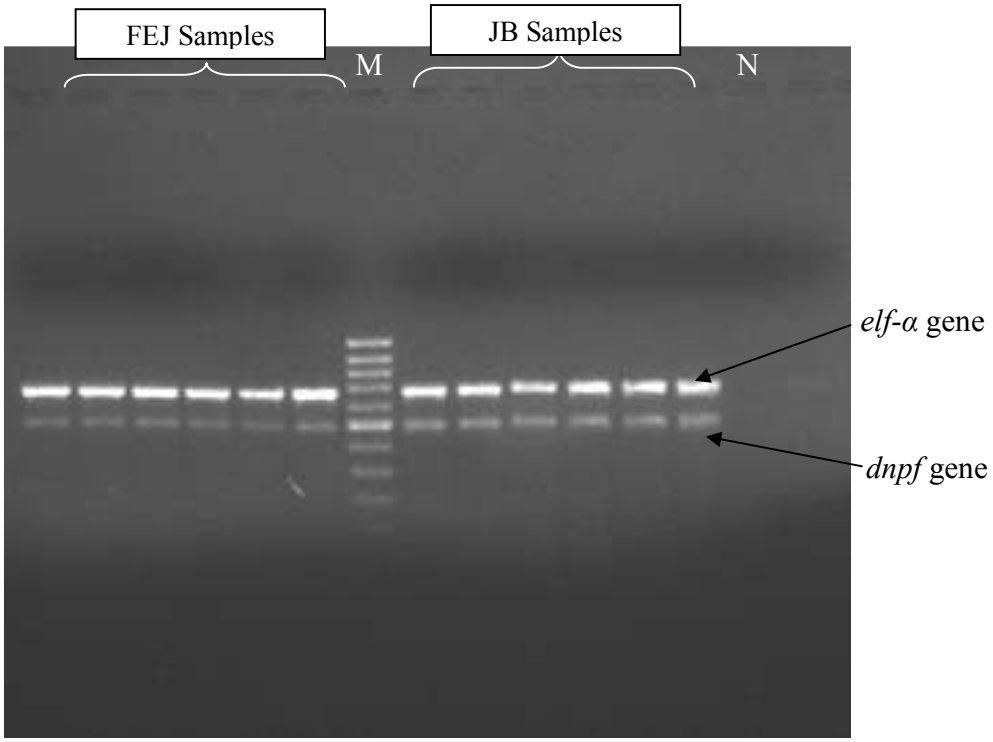


Figure 5.1: Representative gel picture of multiplexing PCR product of amplification of *elf-α* and *dnpf* genes from cDNA prepared from early third instar larvae of JB and FEJ populations. Bands which are on the left hand side of the marker lane (M) are of FEJ samples and that are on the right hand side of the marker lane are of JBs. Lane N is negative control for PCR reaction.

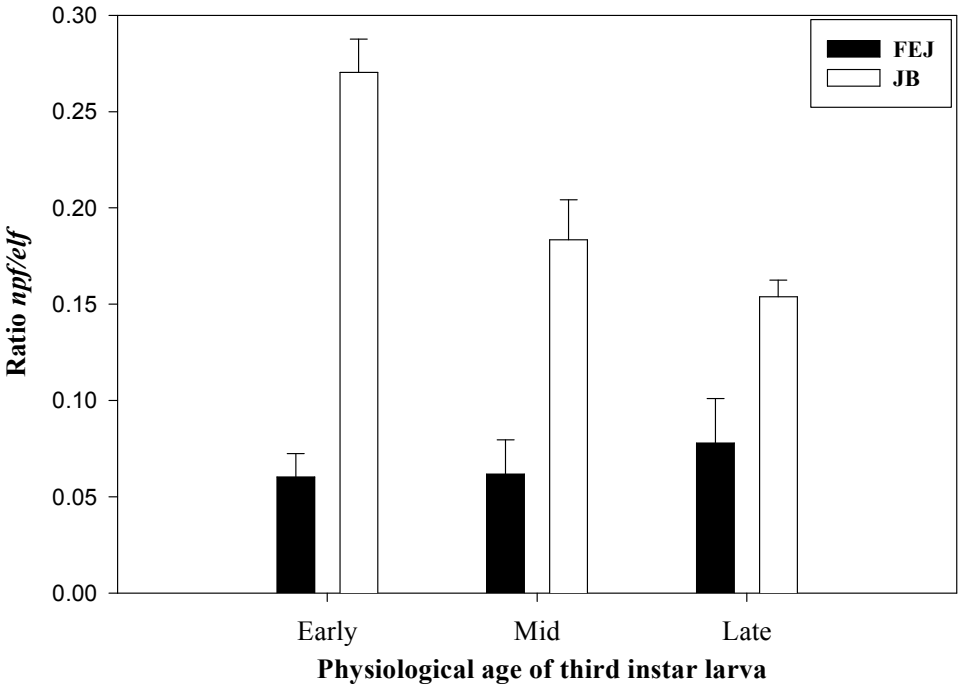


Figure 5.2: Mean (± s.e.) *dnpf* expression at different physiological ages of third instar larvae of FEJ and JB populations.

Effect	***df***	**MS**	***F***	***P***
Selection	1	0.1310	6.933	0.231
Physiological age	2	0.0006	0.178	0.848
Selection × Phy. Age	2	0.0023	2.067	0.326

Table 5.1: Summary of results of ANOVAs done on mean ratios of *dnpf* and *elf-α* band intensities in early, middle and late third instar larval stages of FEJ and JB populations. The effect of block cannot be tested for significance and has, therefore, been omitted from the table.

Chapter-VI

Microarray Analysis of FEJ and JB populations

INTRODUCTION

The generation of vast amounts of DNA sequence information coupled with advances in technologies developed for the experimental use of such information, allows the description of biological processes from a global genetic perspective. One such technology is DNA microarray, that permits the simultaneous monitoring of the relative expression levels of thousands of genes at a time (Schena *et al.,* 1995). Microarrays came onto the scene of molecular biology research in the mid-1990s, and have quickly been established as an essential tool for gene expression profiling in relation to physiology and development. When used in conjunction with classical genetic approaches and the emerging power of bioinformatics, they can be much more than a tool because they can induce us to change our perspective on the process under study. Moreover, microarray tehnology provides a new tool with which molecular ecologists and evolutionary biologists can survey genome-wide patterns of gene expression within and among species or populations (Gibson, 2002).

Microarray technology has been used in the past for the analysis of expression changes in single-celled organisms (Shalon *et al.,* 1996; DeRisi *et al.,* 1997; Lashkari *et al.,* 1997; Chu *et al.,* 1998), mammalian cell cultures (DeRisi *et al.,* 1996; Schena *et al.,* 1996; Iyer *et al.,* 1999*)* and human and mouse tissues (Perou *et al.,* 1999) with success in identifying groups of correlated genes.

Most of the microarray studies, till date, have focused on fold-change in transcript abundance as the measure of gene expression. Often, these studies employed a common reference sample as the standard against which experimental treatments were compared.

That is, one experimental sample is competitively hybridized with a reference sample that consists of pooled RNAs from multiple treatments and the fold difference between two experimental samples is inferred by comparing the two ratio measurements.

There are several different types of DNA microarrays. The two most commonly used technologies are cDNA microarrays and oligonucleotide expression arrays. cDNA microarray requires only a large library of cDNAs as a source of clones to be arrayed on a glass slide. From several thousands of the clones, a unique set of expressed sequence tags is selected for amplification. These products are robotically deposited at a density of around 20-30 clones per square millimeter on the end of a special glass microscopic slide or filter. The cDNA microarray probe is then hybridized to radioactively or fluorescently labeled cDNA prepared by reverse transcription of mRNA isolated from the test cells or tissues of interest. Competitive hybridization of two samples labeled with different dyes, commonly Cy3 or Cy5, allows an estimate of the ratio of transcript abundance in the two RNA samples being compared for each spot on the microarray independently.

Second method, oligonucleotide technology which is pioneered by Affymetrix GeneChip® differs from cDNA microarray in two important respects (Lockhart *et al.,* 1996). First, the probes are a set of up to 20 short around 25mer oligonucleotides that are specific for each gene or exon, along with the related set with single base mismatch incorporated at the middle position of each oligonucleotide. These probes are synthesized *in situ* on silicon chip by photolithographic deposition. Second, the arrays are hybridized to a single biotinylated amplified RNA sample and the intensity measure for each gene is

computed by an algorithm that massages the difference between the match and mismatch measurements and averages over each oligonucleotide.

Regulating gene expression spatially and temporally is a key mechanism by which an organism activates the information encoded in its genome to effect developmental changes, and differences in this regulation can cascade through development, resulting in different morphological or physiological character states (Johnson and Porter, 2001). In our laboratory populations of *D. melanogaster* selected for faster pre-adult development and early reproduction, many significant phenotypic changes have been observed compared to the control JB populations. At least some subset of these changes in the phenotypic values of different traits could be the result of changes in the expression of the genes that govern them. Since quantitative traits are controlled by many genes, changes in any of the genes controlling the trait can contribute to the change in the phenotypic value of that trait. Studying the expression of these genes individually in the faster developing FEJ populations in comparison to control JB populations is both time consuming and cumbersome. Moreover, studying the expression of all the genes together leads to the better understanding of the underlying phenomena due to the possibility of identifying broad trends in expression pattern change at different life-stages in selected and control populations. Therefore, one pair of FEJ and JB populations was subjected to microarray analysis using Affymetrix GeneChip Drosophila_2 which can be used to study the expression levels of about 18,500 genes at a time.

MATERIALS AND METHODS

Microarray analysis was performed for block-3, namely the JB-3 and FEJ-3 populations. The different life stages chosen for the microarray analysis were late third instar larva, pupa twelve hours after pupariation (pupa 12 HAP), pupa 24 HAP and freshly eclosed (< 4 h after eclosion) male and female adult flies separately. Late third instar larva, pupa 12 HAP and pupa 24 HAP life stages were selected for the analysis because the contribution of reduction in the third instar duration to the total reduction in the development time was maximum followed by pupal duration compared to the other life-stages. Moreover, the pupal stage is very active stage with respect to gene expression because, during this stage metamorphosis occurs during which there will be lot of changes in both expression patterns as well as expression level of the genes. It had been noticed in FEJ populations that they take more time till first mating after eclosion compared to the JB populations. At that time it was speculated that FEJs have been able to push some important aspects of adult sexual maturation from pupal to adult stage. Therefore, to study the gene expression profiles in these populations soon after eclosion, freshly eclosed adults were chosen for microarray analysis. These experiments were repeated with three biological replicates for each life-stage chosen except adult males and females (only one sample was analyzed) using RNA isolated from 3 batches of larvae or pupae from either FEJ-3 or JB-3 population.

Sample collection for total RNA isolation

Late third instar larval collection

Eggs were collected from JB-3 and FEJ-3 standardized flies of at a density of ~30 eggs per vial containing ~6 mL of standard banana-jaggery food medium. 20 such vials were set up for each population. These vials were incubated at 25 ± 1^{o}C, ~90% relative humidity and under continuous light condition. The ages of FEJ and JB flies were chosen to match the age of the flies when eggs are collected from them during the regular maintenance protocol. Late third instar larvae (wandering stage) from JB-3 and FEJ-3 populations were collected from these vials separately and transferred to a Petri dish with 1X PBS to wash off the food material adhering to them. Then, the excess PBS solution was removed from their body by rolling them gently on a tissue paper towel. Around 15 larvae per population were picked up randomly using a paint brush and transferred into a 1.5 mL microfuge tube containing 0.5 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA). These vials were immediately frozen using liquid nitrogen and immediately transferred to -80^{0}C deep- freezer and stored until use.

Pupal collection

Eggs were collected from JB-3 and FEJ-3 standardized flies of at a density of ~30 eggs per vial containing ~6 mL of standard banana-jaggery food medium. 24 such vials were set up for each population. The ages of FEJ and JB flies were chosen to match the age of the flies when eggs are collected from them during the regular maintenance protocol. These vials were incubated at 25 ± 1^{o}C, ~90% relative humidity and under continuous light

condition. Once larvae started crawling inside the glass vials, they were closely monitored every hour and freshly formed pupae were marked on the vials from outside. After 12 h or 24 h of marking, these marked pupae were gently transferred to a Petri dish with 1X PBS using a moistened paint brush. Then, excess PBS was removed by transferring pupae on to a tissue paper towel. Around 15 randomly chosen pupae from each population were transferred into 1.5 mL microfuge tubes containing 0.5 mL of Trizol reagent separately. These tubes were frozen using liquid nitrogen and transferred immediately to -80^{0}C deep freezer till use.

Adult collection

Eggs were collected from JB-3 and FEJ-3 standardized flies of at a density of ~30 eggs per vial containing ~6 mL of standard banana-jaggery food medium. 24 such vials were set up for each population. The ages of FEJ and JB flies were chosen to match the age of the flies when eggs are collected from them during the regular maintenance protocol. These vials were incubated at 25 ± 1^{o}C, ~90% relative humidity and under continuous light condition. Once pupae darkened, the vials were closely monitored and freshly eclosed flies (less than four hours after eclosion) were collected from all the vials from entire development time distribution. Then they were immediately frozen by keeping on dry ice and males and females were separated on the dry ice block. Around 15 males and 15 females were transferred into 1.5 mL microfuge tubes and stored in -80 ^{0}C deep freezer until use.

RNA isolation

Samples were taken out from -80 ^{0}C freezer and were immediately processed. Samples were crushed finely using dispensable plastic sterile pestles. Crushed samples were subjected to total RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. The quality of the isolated RNA was assessed by agarose gel electrophoresis and quantitated using a spectrophotometer.

Microarray analysis

8 μg of total RNA isolated from JB-3 and FEJ-3 samples were separately converted to double stranded cDNA using an Affymetrix (Santa Clara, CA, USA) one-cycle labeling kit following the manufacturer's instructions. The synthesized double stranded cDNA was column purified and was further subjected to RNA amplification using Affymetrix IVT labeling kit. The amplified RNA (cRNA) generated in this manner was quantitated by using a NanoDrop® ND-100 UV-Vis spectrophotometer (NanoDrop technologies, Washington DC, USA). The normalized cRNA (15 μg) was fragmented into 50-200 base pair (bp) fragments and hybridized to *Drosophila* gene chips, and washed, stained and scanned as per Affymetrix protocols. The experiment was repeated with three different biological replicates for each life-stage chosen, except adults *i.e.* using RNA isolated from 3 batches of larvae or pupae from either FEJ-3 or JB-3 populations.

Data analysis

Data were initially analyzed by using the Gene Chip Operating Software (GCOS) of Affymetrix. The present calls (the number of genes picked up on the gene chip) ranged

between 40 and 46% in different samples analyzed. All the CEL (cell intensity) files generated by GCOS for different life stages chosen were uploaded to Avadis™ software version 4.3 (an Affymetrix approved software manufactured by Strand Life Sciences, Bangalore, India) separately for further analysis. The CEL files of JB-3 were grouped as control whereas those of FEJ-3 were grouped as treatment for each life-stage chosen for the study. Data were normalized using RMA (Irizarry *et al.,* 2003 a, b) and PLIER (Affymetrix, 2005) algorithms available in Avadis™ and subject to differential expression analysis as per the manufacturer's instructions. Genes identified by both the algorithms as being more than 2.0 fold differentially expressed with $P \leq 0.05$ were selected for further analysis. Genes which were 2.0 fold differentially expressed in all the life-stages studied were taken and cluster analysis was performed by using web available softwares Cluster and TreeView (Eisen *et al.,* 1998). Further, gene ontology (GO) enrichment analysis was performed for up-regulated and down-regulated genes separately using web available software Database for Annotation, Visualization and Integrated Discovery (David) analysis (Dennis *et al.,* 2003; Huang *et al.,* 2009). Further, data obtained after analysis of adult male and female data separately using Avadis were used to find out the function of individual genes manually by using information from flybase which is a data base of *Drosophila* genes and genomes.

RESULTS

Differential gene expression

Preliminary analysis done by Avadis™ revealed many differentially expressed genes in all the life-stages chosen for the study in FEJ-3 population compared to the control JB-3 population. Many genes were found to be ≥ 2.0 fold differentially expressed in both the directions (up and down). In late third instar larva, pupa 12 HAP and pupa 24 HAP the number of down-regulated genes were more than the up-regulated genes in FEJ-3 compared JB-3 population (table 6.1). However, in adult males and adult females, the number of down-regulated genes in FEJ-3 was slightly less than the number of up-regulated genes.

Life Stages	**No. of genes showing ≥ 2 fold differential expression**	
	Down regulated	**Up regulated**
Late 3rd instar larva	627	198
Pupa 12 HAP	967	219
Pupa 24 HAP	251	77
Adult males	251	300
Adult females	413	428

Table 6.1: List of number of genes with ≥ 2.0 fold differential expression in different life-stages of FEJ-3 population in comparison to the control JB-3 population.

Hierarchical clustering

Cluster and TreeView analyses performed for hierarchical clustering arranged the genes which were ≥2 fold differentially expressed in all the life-stages studied, according to similarity in the pattern of gene expression across life-stages. In these analyses, the pre-adult life-stages clustered together, whereas adult males and adult females clustered together in a separate group. The analyses also revealed that, if the ≥2 fold cut off was not apllied, 318 genes were consistently differentially expressed in all the life-stages studied in the FEJ-3 population (figure 6.1). Among these 318 genes, a substantially larger number of genes were up-regulated (red colored data points in figure 6.1) rather than down-regulated (green colored data points). When a differential expression fold change cut off of ≥2 was imposed on the 318 genes with altered expression across all the life-stages studied, only 109 genes clustered. It was observed that after imposing cut off in expression level of ≥2 fold, the number of up-regulated genes (red data points) reduced, whereas the number of down-regulated genes remained more or less unchanged from that found when no cut-off in fold change was imposed.

Gene ontology (GO) term enrichment

GO term enrichment analyses using DAVID software for the up-regulated genes obtained after the hierarchical clustering resulted in genes enrichment in different GO terms which clustered together. Nine genes which were enriched in terms related to epigenetic control formed a cluster. Those enrichment terms include transcription cofactor activity, chromatin regulation, chromatin modification *etc* (figure 6.2). Another cluster was formed with 36 genes many of which were enriched in GO terms related to growth and

development such as, cell differentiation, cell development, gamete generation, sexual reproduction, oogenesis *etc* (figure 6.3). Other GO terms enriched with up-regulated genes include, translation, developmental process, phagocytosis, golgi associated vesicles and regulation of progression through cell cycle (table 6.2). The down-regulated genes enriched in GO terms, include genes involved in metabolism of xenobiotics by cytochrome P-450 and Glutathione metabolism.

GO term enrichment analyses of ≥ 2 fold up-regulated genes in larval stage by using DAVID resulted in the enrichment of the GO Glycolysis and tricarboxylic acid cycle (TCA cycle), translation and oxidative phosphorylation, with varying number of genes in each term (table 6.3).

GO term analysis of ≥ 2 fold up-regulated genes of pupal stage resulted in the enrichment of GO terms such as nervous system development, cellular localization, cell-cell adhesion, imaginal disc development, cytoskeleton organization, cell cycle process, cell migration, cell communication, pattern specification and notch signaling.

Results of GO term enrichment analysis of ≥ 2 fold differentially expressed genes in only larval stage revealed that many genes of insulin signaling pathway were down regulated (table 6.4) and at the same time many genes of ecdysone pathway were up-regulated (table 6.5).

Gene expression in adults

Gene ontology enrichment analysis of the adult microarray data revealed that in FEJ-3 freshly eclosed adult females, many genes were down-regulated with ≥ 2 fold change and they clustered into many clusters with many genes in each cluster. One such cluster obtained consisted of 38 genes which were involved in cell cycle regulation (figure 6.4), while another cluster obtained consisted of 12 genes involved in oocyte development and maturation (figure 6.5). When ≥ 2 fold up-regulated genes were analyzed using DAVID, 10 genes clustered and they were all involved in immune defense (figure 6.6). In FEJ-3 freshly eclosed males it was observed that many genes were down-regulated with ≥ 2 fold change and also many genes were up-regulated ≥ 2 fold change. DAVID analysis of ≥ 2 fold down regulated genes resulted in many clusters. In one of those clusters, 8 genes were clustered together which were involved in sexual reproduction and gamete generation (figure 6.7). Similarly, when up-regulated genes were analyzed many clusters resulted, in one among them 8 genes clustered together which were involved in immune defense (figure 6.8).

Gene Ontology Terms Enriched	**Number of Genes clustered**
Translation	18
Developmental Process	41
Phagocytosis	9
Golgi Associated vesicle	5
Regulation of progression through cell cycle	9

Table 6.2: List of enriched Gene Ontology terms when in genes which were ≥ 2 fold up-regulated and commonly differentially expressed in all the life stages analyzed through microarray.

Gene Ontology Terms Enriched	Number of Genes clustered
Glycolysis and TCA cycle	12
Translation	61
Oxidative Phosphorylation	19

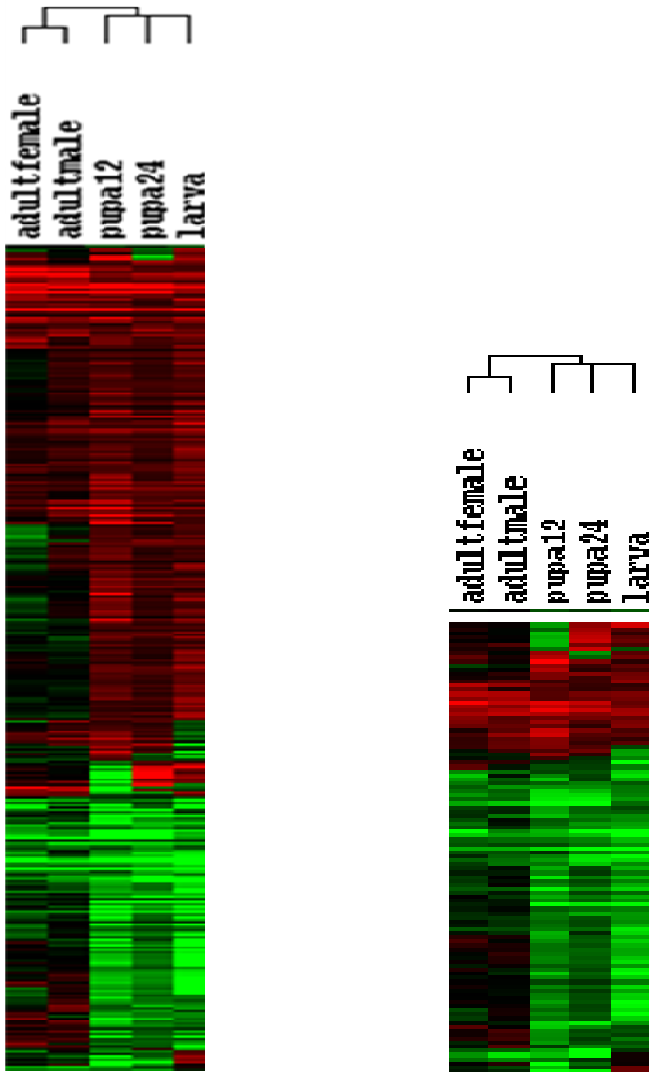
Table 6.3: List of Gene Ontology terms enriched when ≥ 2 fold up-regulated genes from only larval stage were analyzed using DAVID software.

Gene Name	Fold Down-Regulated
Insulin peptide 6 (DILP6)	1.94
Insulin receptor (Chico)	1.26
IMPL2	1.50
Akt	5.95
Thor	1.55
EIF 4E7	2.30
EIF 4E	2.30
EIF 4E5	3.96
EIF 4E3	1.75

Table 6.4: List of differentially expressed insulin signaling pathway genes and their expression level in FEJ-3 late third instar larval stage in comparison to JB-3.

Gene Name	Number of Folds Up-Regulated
Shade	2.13
Shadow	1.69
Phantom	2.03

Table 6.5: List of ecdysone pathway genes and their expression level in FEJ-3 late third instar larval stage in comparison to JB-3.



A **B**

Figure 6.1: Hierarchical clustering of differentially expressed genes common in all the life stages chosen for microarray analysis. (A) All the genes differentially expressed and common to all stages used for microarray study without cut off of expression level and (B) with a cut off of ≥ 2 fold change in expression and common to all the stages. Red data points indicate the up-regulated genes and green indicates down regulated genes.

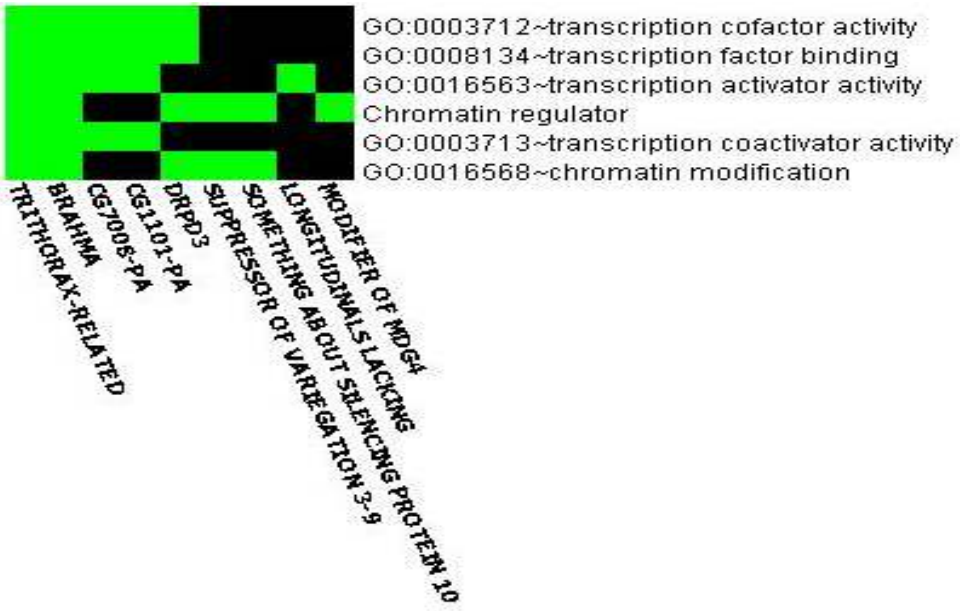


Figure 6.2: Heat map of 9 up-regulated genes in FEJ-3 population with ≥ 2 fold change and common to all stages used for the microarray analysis obtained after GO term enrichment analysis. Green data point represents corresponding gene-term association positively reported, black data point represents corresponding gene-term association not reported yet.

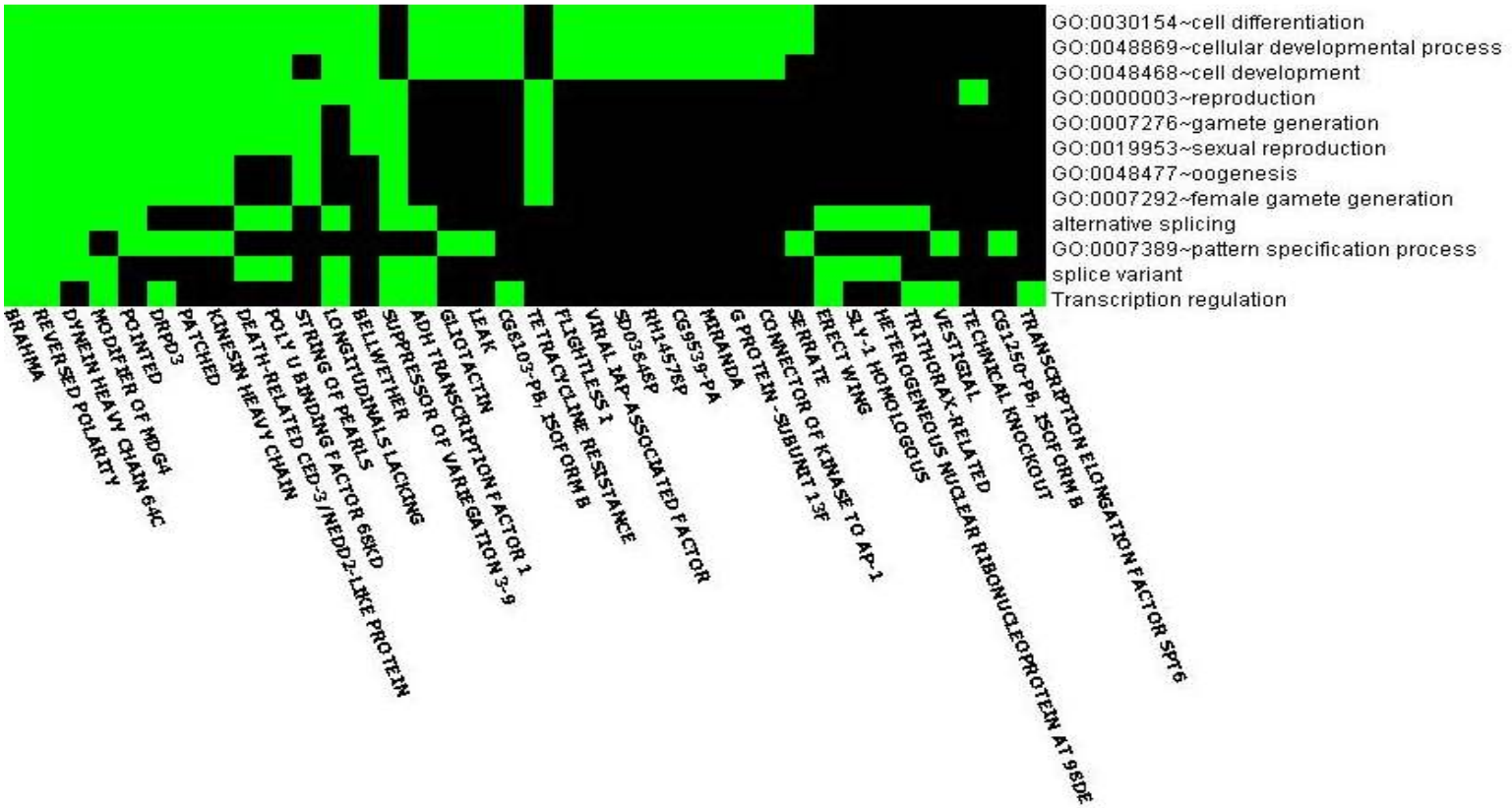


Figure 6.3: Heat map of 36 up-regulated genes in FEJ-3 population with ≥ 2 fold change and commonly differentially expressed in all the life stages analyzed through microarray, obtained after GO term enrichment analysis using DAVID. Green data point represents corresponding gene-term association positively reported, black data point represents corresponding gene-term association not reported yet.

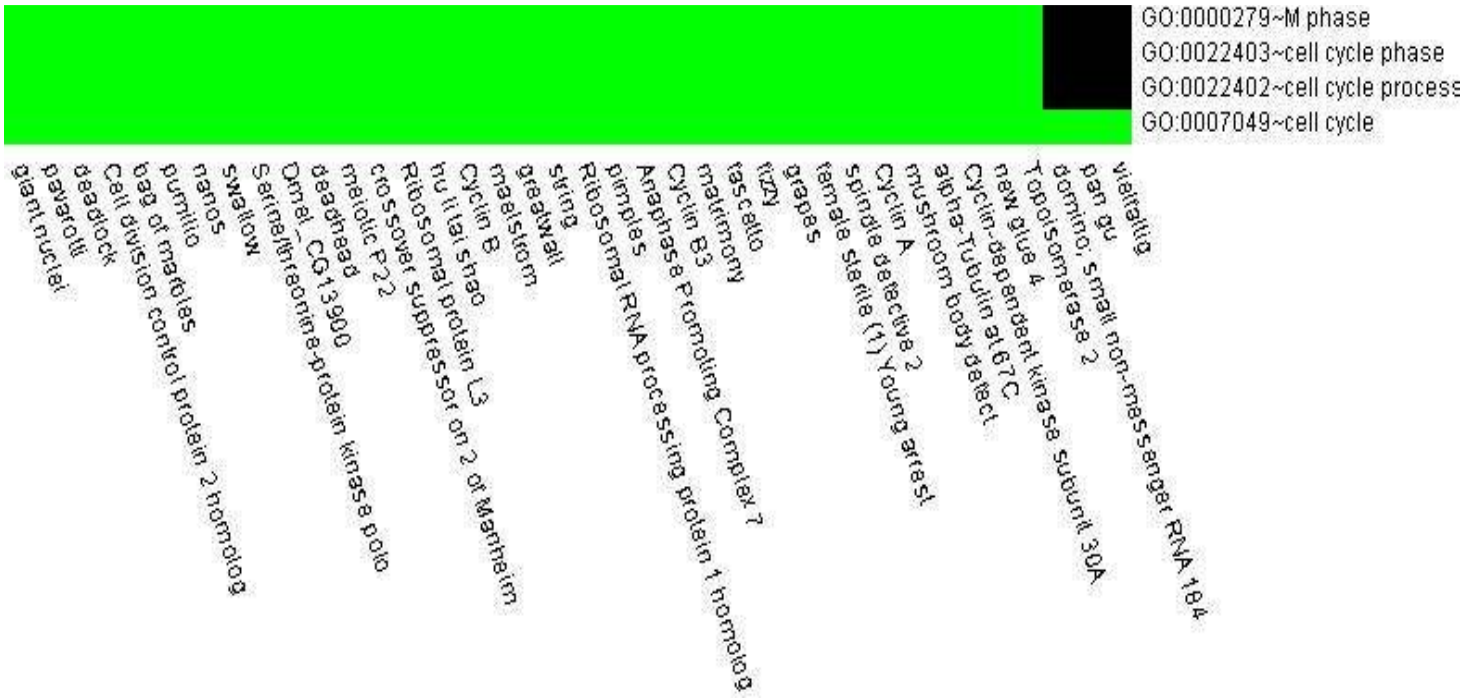


Figure 6.4: Heat map of 36 down -regulated genes in FEJ-3 population with ≥ 2 fold change obtained after GO term enrichment analysis of data obtained from microarray analysis of freshly eclosed adult females using DAVID. Clustered genes are involved in cell cycle. Green data point represents corresponding gene-term association positively reported black data point represents corresponding gene-term association not reported yet.

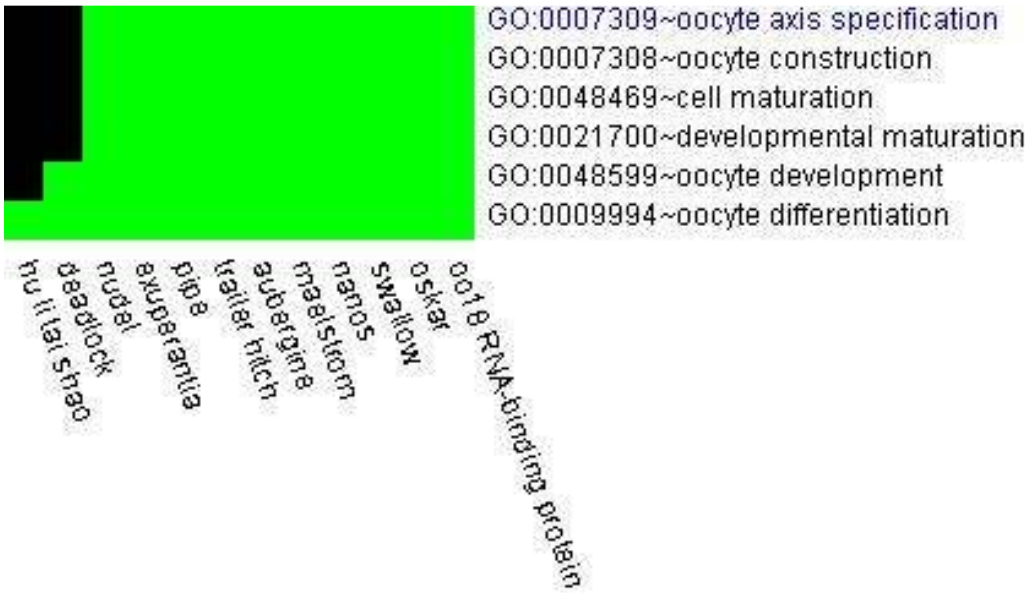


Figure 6.5: Heat map of 12 down -regulated genes in FEJ-3 population with ≥ 2 fold change obtained after GO term enrichment analysis of data obtained from microarray analysis of freshly eclosed adult females using DAVID. Clustered genes are involved in oocyte development. Green data point represents corresponding gene-term association positively reported black data point represents corresponding gene-term association not reported yet.

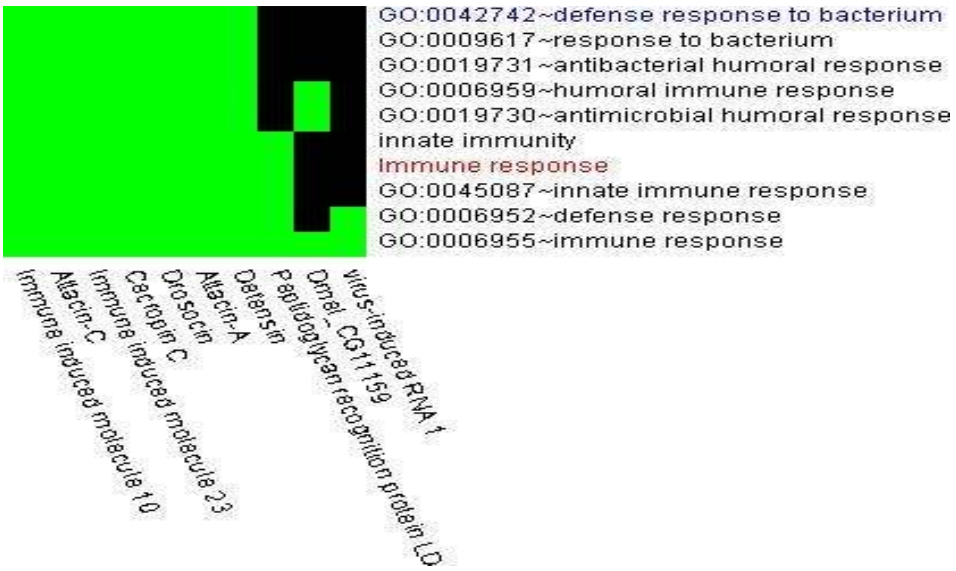


Figure 6.6: Heat map of 10 up regulated genes in FEJ-3 population with ≥ 2 fold change obtained after GO term enrichment analysis of data obtained from microarray analysis of freshly eclosed adult males using DAVID. Clustered genes are involved in immune defense.

Figure 6.7: Heat map of 8 down -regulated genes in FEJ-3 population with ≥ 2 fold change obtained after GO term enrichment analysis of data obtained from microarray analysis of freshly eclosed adult males using DAVID. Clustered genes are involved in sexual reproduction and gamete generation.

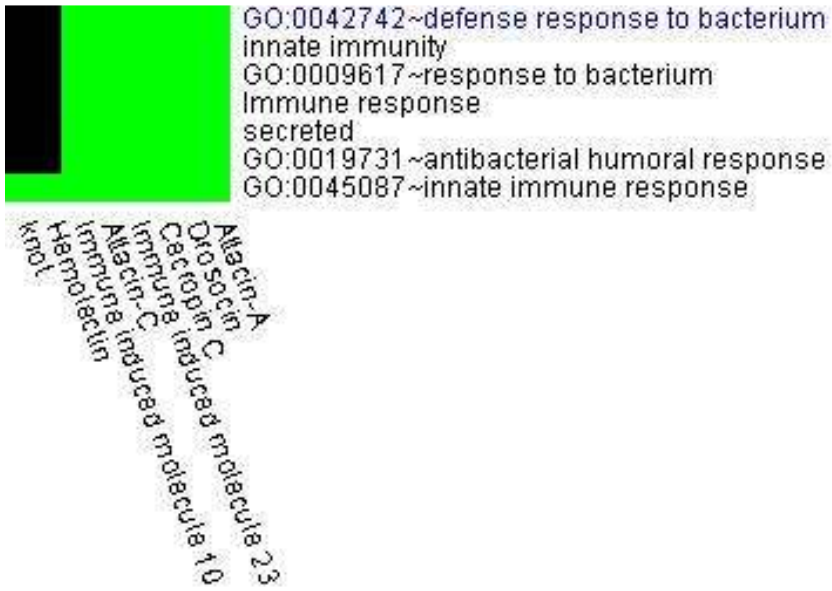


Figure 6.8: Heat map of 8 down -regulated genes in FEJ-3 population with ≥ 2 fold change obtained after GO term enrichment analysis of data obtained from microarray analysis of freshly eclosed adult males using DAVID. Clustered genes are involved in defense mechanism. Green data point represents corresponding gene-term association positively reported black data point represents corresponding gene-term association not reported yet.

DISCUSSION

Differential gene expression

Earlier studies performed on these populations in our laboratory have shown that the FEJ populations are differentiated from the control JB populations in a wide range of life historical, physiological and behavioural traits (Prasad, 2004; Modak, 2009). For the first time, I have looked for changes in the gene expression pattern at some selected life-stages in these populations through microarray analyses. It was noticed after the microarray analyses of different life stages of these populations that many hundreds of genes were differentially expressed in FEJ-3 population compared to JB-3 control population in all the life-stages studied. These changes in the gene expression levels in the FEJ populations, if consistent across other replicate populations, could provide the basis for further studies aimed at determining the proximate causal mechanisms of many of the observed differences between the FEJ and JB populations in gross phenotypes.

Hierarchical clustering

When genes which were differentially expressed in all the life-stages studied in FEJ-3 population compared to JB-3 population were subjected to hierarchical clustering, they were arranged according to similarity in pattern of expression across the life stages studied (figure 6.1). It was noticed that in FEJ-3 population when all the genes which were differentially expressed in all the life stages studied were used for the analyses without imposing a cut-off of ≥ 2 fold change, there were more number of up regulated genes (red data points in figure 6.1) than the number of down regulated genes (green data points in figure 6.1). When these genes were analyzed by imposing a cut-

off of ≥ 2 fold change the number of down regulated genes remained almost same whereas, the number of up regulated genes drastically decreased. This observation indicates that the genes which were down-regulated had greater magnitude of change in expression compared to the up regulated genes which had lesser magnitude with respect to their expression level. These up regulated genes with lesser magnitude (less than 2 fold) of change in expression level could be having effects on other genes if they are located at upstream in the biological pathways, which have not been tested in the experiment that I have performed. This experiment is first of its kind on these populations, further analyses and studies are required to understand the effect of these small changes in the gene expression, and explore the possibility of up-regulated genes in FEJ populations being primarily genes of significant developmental importance. In the analyses that I have performed in this experiment, I have concentrated only on the genes which have larger changes in their expression *i e,* more than or equal to two fold change.

Gene Ontology (GO) term enrichment

When genes which were differentially expressed across all the life-stages studied were analyzed using DAVID software for gene ontology term enrichment, 9 up-regulated genes formed a cluster with GO terms related to epigenetic control, such as chromatin modification, chromatin regulator, transcription factor binding *etc.* (figure 6.2). These results suggest that there might be epigenetic control over some subset of genes which are differentially expressed in the FEJ populations which could be an indication of possible adaptive evolution mediated by changes in epigenetic control mechanisms.

Another cluster formed consisted of 36 genes many of which were enriched in GO terms such as cell differentiation, cell development, gamete generation *etc.,* (figure 6.3). Other genes clustered separately and enriched in GO terms involving translation, developmental processes, and the regulation of progression through cell cycle (table 6.2). All of these GO terms are related to growth and development. All the genes clustered in this cluster are up-regulated in FEJ-3 life stages compared to JB-3 population. This could be another possible mechanism by which FEJs are completing their developmental processes faster compared to JBs.

Down-regulated genes were enriched in GO terms such as metabolism by cytochrome P-450 and glutathione metabolism. A gene in this cluster namely glutathione-S-transferase is involved in response to oxidative stress. Earlier studies on FEJ populations in our laboratory (Prasad *et al.*, 2001; Prasad, 2004; Modak, 2009) have shown that these faster developing populations are less resistant to different stresses experienced as adults, namely starvation, desiccation and pathogen load. The down-regulation of the genes which are involved in response to oxidative stress could be one of the reasons for the reduced resistance to different stresses in FEJ populations.

In the larval stage, it was found that many genes involved in glycolysis, TCA cycle and oxidative phosphorylation were up-regulated in the FEJ population compared to the JB control. All of these biological cycles produce the energy molecule ATP. This molecule is utilized by the organisms to carry out other biological processes which are necessary for growth and development. In FEJ larvae, genes in energy generating cycles appear to be up-regulated, probably helping to cope with the energy requirements of completing development, and especially metamorphosis, faster than their JB counterparts.

The other interesting result observed in larval stage is that many genes involved in insulin signaling pathway namely *dilp2, chico, Akt1 etc.,* were down-regulated in the FEJ population (table 6.4), and at the same time genes involved in ecdysone pathway namely *Shade, shadow and phantom* were up-regulated (table 6.5). It is known that the antagonistic actions of ecdysone and insulin are a major determinant of the final size in *D. melanogaster* (Colombani *et al.*, 2005). In insects, insulin family molecules control growth and metabolism, whereas pulses of the steroid 20-hydroxyecdysone (20E) initiate major developmental transitions. In the present microarray study, I have found that genes involved in insulin signaling are down-regulated while ecdysone biosynthesis genes are up-regulated, and this could be a major reason for FEJ adults to be significantly smaller and lighter than the JB control flies.

In the pupal stage too, in the FEJ population many genes were up-regulated, including genes involved in different developmental processes such as nervous system development, imaginal disc development, cell communication, pattern specification *etc.* FEJ pupae are perhaps completing these developmental processes faster than the JB pupae, in part by up-regulating the genes involved in different pupal-stage-specific developmental processes.

In FEJ-3 adult females, it was noticed that 38 down regulated genes clustered together with GO terms related to cell cycle regulation (figure 6.4). Down regulation of all the genes in this cluster indicates that probably in the FEJ flies the cell division rate would have been decreased. This result is consistent with the results observed in cell number and cell size experiment in larvae (chapter IV). It was observed that FEJ larvae's imaginal discs were consisted of lesser number of larger cells compared to JB larvae's imaginal discs which were consisted of more number of smaller cells. Down-

regulation of the genes related to cell division regulation as evident from the microarray experiment could be one of the reasons for lower cell division in FEJ populations.

Another cluster in the data on adult females consisted of 12 genes which were down regulated in FEJ-3 population and were enriched in GO terms related to oocyte development and maturation (figure 6.5). It has been observed that FEJ flies produce hardly any eggs for about 24 h after eclosion, whereas JB flies start egg laying ~12 h after eclosion (Modak, 2009). Microarray results show that in freshly eclosed FEJ adult females genes related to oocyte development and maturity are down-regulated compared to JB females. Down-regulation of genes related to oocyte development and maturity could be a possible reason for the delayed egg production in FEJ flies compared to JB flies.

In freshly eclosed males of FEJ-3 population, genes involved in sexual reproduction and gamete generation were down-regulated compared to JB-3 population. It has been observed in earlier studies in our laboratory that adults of FEJ populations take increased time till first mating after eclosion (Prasad *et al.,* 2001). It was then speculated that perhaps the FEJs have been able to push some important aspects of adult maturity from pupal to adult stage (Prasad *et al.,* 2001), especially as sperm maturation is believed to increase the pupal duration in male *D. melanogster*, relative to females (Nunney, 1996). Down-regulation of genes related to sexual reproduction and gamete generation in FEJ population indicates that FEJ flies may not be as sexually mature as JB flies soon after eclosion. This further supports our earlier speculation that the FEJs might have pushed some important aspects of adult maturity from pupal to adult stage.

Genes related to immune defense were up-regulated both in males and females of the FEJ population (figures 6.6 and 6.8). It has been seen in earlier studies done on FEJ populations that they possess lower pathogen resistance as adults compared to JB populations (Modak *et al.,* 2009). In that study, it was not clear whether the FEJ flies died faster in the presence of pathogens due to poor immune defense, or because they mounted a strong immune defense and then died due to depletion of their meager lipid reserves under the starving conditions of the assay. This observation from the microarray data suggests that perhaps the latter explanation is correct.

Chapter-VII

Conclusions

In this thesis, I have presented results from two major sets of experiments in which I have used large outbred laboratory populations of *D. melanogaster* selected for rapid pre-adult development and early reproduction. First, I have addressed the question of reversal of evolution, an area in evolutionary biology that has rarely been addressed via the experimental evolution approach, by returning the populations of FEJ populations earlier selected for rapid pre-adult development and early reproduction back to their ancestral selective environment. In this section (chapters II and III), I have addressed the question of whether long-term selection constrains the reversion of the populations back to their ancestral phenotypic state, and if it does not, whether complete reversion of the populations occurs or not. I also compared my results with those from an earlier study of reverse evolution of populations selected for rapid pre-adult development (Teotonio and Rose, 2000). The FEJ populations I used had been subjected to forward selection for many more generations than the populations of Teotonio and Rose (2000) before being subjected to revere selection in the ancestral environment. My results, by and large, are consistent with the earlier observations and suggest that even intense, long-term directional selection for over 280 generations does not severely constrain the reverse evolution of the selected trait and other traits related to fitness. The results also show that different traits respond differently to reverse selection, with traits more closely related to fitness in the reverse selection environment tending to converge more completely and rapidly to ancestral values.

Second, I addressed the question whether long-term laboratory selection (over 350 generations) for rapid development and early reproduction on large out bred populations of *D. melanogaster* has led to changes in the pattern of gene expression at different life-stages. In this broad attempt to begin to study the developmental and

molecular underpinnings of the raid development phenotype (chapters IV, V and VI), I conducted different experiments on the faster developing populations (FEJ) to study changes in gene expression. I also conducted experiments to study rate of cell division in faster developing populations indirectly by studying the cell number and cell size in these populations. Further, I surveyed genome-wide patterns of gene expression in these populations. In the following paragraphs, I briefly discuss the major findings emerging from these studies.

The irreversibility of evolution has been an important debate in evolutionary biology (Teotonio and Rose, 2001), and has been viewed as an extreme type of evolutionary restriction (Bull and Charnov, 1985). One of the main questions to be addressed is the degree to which evolutionary history constrains reverse evolution. Experimental reverse evolution studies have rarely been conducted and one among them is the study by Teotonio and Rose (2000). This was the only major study done to study reverse evolution using laboratory selected large outbred laboratory *D. melanogaster* populations. Through this study, Teotonio and Rose (2000) showed that convergence of traits back to ancestral phenotypic values can occur within 50 generations of reverse selection but is not universal and depends on the previous history and trait studied. In my study, I have also shown that complete reversal of some of the traits studied occurs within 50 generations of reverse selection but is not universal. This also suggests that there still exists sufficient genetic variation in the long term selected FEJ populations which is necessary for their reverse evolution.

In chapter IV, I have shown that the expression pattern of proteins involved in early development and body pattern formation in *Drosophila* such as engrailed, wingless and Ubx do not change in FEJ populations even after long-term directional

selection for more than 350 generations. This could be because these proteins are developmentally very important and if their expression pattern is changed then the body plan itself might change. Although, FEJ populations have evolved to be smaller and lighter than the JB controls, and show various leg and wing abnormalities and deformations, the expression pattern of these developmentally important gene products does not appear to have changed. In the same chapter, I have also provided evidence suggesting that over the course of forward selection, FEJs appear to have evolved a slower rate of cell division, compensated in part by increased cell size. I speculate that this may be part of a larger syndrome to conserve energy that has evolved in the FEJ populations due to their extremely meager energy reserves (Modak, 2009). The final size reached by an organism is a consequence of changes in the size and number of cells during its development (Kawli, 2000). My finding supports the notion that growth can occur by more cell enlargement with lesser cell division (Neufeld and Edger, 1998).

In chapter V, I have shown that the gene involved in feeding behavior namely, *Drosophila neuropeptide F* (*dnpf*) has a different expression pattern in FEJ third instar larval stage compared to that of JBs. FEJ larvae have significantly slower feeding rate and they pupate significantly earlier compared to JB larvae (Prasad, 2004). This study suggests that the change in the expression pattern of *dnpf* gene in FEJ third instar larvae could be a reason for their early pupation compared to JB third instar larvae.

In chapter VI, I have presented the results of the microarray study that I carried out to study the changes in gene expression level in FEJ populations, compared to JB populations. In this study I have studied the gene expression levels of around 18,500 genes at four different life-stages in one FEJ and one JB population. I have shown that

as a result of long term directional selection hundreds of genes have changed in their expression levels in the FEJ population, compared to the control JB population, at both pre-adult and adult stage. I have also shown that the change in expression can occur in either direction, *i.e,* many genes were found to be down-regulated, while many other genes were found to be up-regulated in the FEJ population. Further, in this chapter I have shown that the genes involved in epigenetic control *i.e,* involved in biological functions such as cofactor activity, chromatin regulation, chromatin modification *etc* are up-regulated more than 2 fold in the FEJ population. This suggests that some of the evolved differences in FEJ and JB gene expression could be mediated via epigenetic control mechanisms. This could also be a probable reason for finding change in expression of such a huge number of genes in FEJ population. I have also shown that genes involved in growth and development are up-regulated in the FEJ population, which could be a probable reason for their faster development. The FEJ population also showed down-regulation of genes involved in metabolism of xenobiotics by cytochrome P-450 and glutathione metabolism. This may be a possible underlying reason for the reduced stress tolerance of the FEJ populations (Prasad, 2004). In the same chapter, I have further shown that the FEJ population exhibits up-regulation of genes involved in glycolysis and TCA cycle, translation and oxidative phosphorylation, a possible indicateor of an elevated metabolic rate for sustaining rapid developmental change, especially during metamorphosis.

It was earlier observed that FEJ flies do not lay eggs on day 1 after eclosion, whereas JB flies start laying eggs about 12 h after eclosion (Modak, 2009). Consistent with this, the microarray results show that in freshly eclosed females the genes involved in oocyte development and maturity are down-regulated in the FEJ population.

Similarly, I found that freshly eclosed FEJ males show down-regulation of genes involved in sexual reproduction and gametogenesis, consistent with an earlier speculation (Prasad *et al*., 2001) that the reduction of pupal duration in the FEJ populations might be partly due to their postponing some aspects of gonadal maturation from pupal to adult stage.

Overall, although the microarray and associated studies are of a somewhat preliminary nature, they represent the start of an attempt to dissect out the developmental and molecular underpinnings of the rapid development phenotype evolved under laboratory selection in the FEJ populations. The results also provide useful pointers to specific modules in development or ontogeny, like insulin signaling, that may be worth studying in more detail to understand how changes in these modules bring about the rapid development phenotype, and its associated phenotypic correlates.

References

Affymetrix, 2005. *Guide to probe logarithmic intensity error (PLIER) estimation*. Affymetrix Whitepaper, accessed 24 December 2008; available at http://www.affymetrix.com/support/technical/technotes/plier_technote.pdf.

Archana N. 2009. *The genetic architecture of fitness-related traits in populations of three species of Drosophila subjected to selection for adaptation to larval crowding.* Ph.D. thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, India (submitted).

Bossdorf, O., Richards, C. L. and Pigliucci, M. 2008. Epigenetics for ecologists. *Ecol. Lett.* **11**, 106–115

Brower, L. D. 1986, Engrailed gene expression in *Drosophila* imaginal discs. *The EMBO Journal* **5**, 2649-2656

Bull, J. J. and Charnov, E. L. 1985. On irreversible evolution. *Evolution* **39**, 1149-1155

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

Chippindale, A. K., Leroi, A. M., Kim, S. B. and Rose, M. R. 1993. Phenotypic plasticity and selection in *Drosophila* life-history evolution. 1. Nutrition and the cost of reproduction. *J. Evol. Biol*. **6,** 171-193.

Chippindale, A. K., Hoang, D. T., Service, P. M. and Rose, M. R. 1994. The evolution of development in *Drosophila melanogaster* selected for postponed senescence. *Evolution* **48,** 1880-1899.

Chippindale, A. K., Chu, T. J. F. and Rose, M. R., 1996 Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. *Evolution* **50,** 753-766

Chippindale, A. K., Alipaz, J. A., Chen, H. W. and Rose, M. R. 1997. Experimental evolution of accelerated development in *Drosophila*. 1. Developmental speed and larval survival. *Evolution* **51**, 1536-1551

Chippindale, A. K., Gibbs, A. G., Sheik, M., Yee, K. J., Djawdan, M., Bradley, T. J. and Rose, M. R. 1998. Resource acquisition and the evolution of stress resistance in *Drosophila melanogaster*. *Evolution* **52,** 1342-1352.

Chippindale, A. K., Alipaz, J. A. and Rose, M. R. 2004. Experimental evolution of accelerated development in *Drosophila*. 2. Adult fitness and the fast development syndrome. In *Methuselah flies: a case study in laboratory evolution* (eds. M. R. Rose, H. Passananti, M. Matos), pg. 413-435. World Scientific Publishing, New York, USA.

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. and Herskowitz, I. 1998. The transcriptional program of sporulation in budding yeast. *Science*, **282,** 699-705.

Colombani, J., Bianchini, L., Layelle, S., Pondeville, E., Dauphin-Villemant, C., AntoniewskiC., Carre, C., Noselli, S. and Leopold, P., 2005. Antogonistic actions of ecdysone and insulins determine final body size in *Drosophila*. *Science*, **310**, 667-670

Dennis, G J., Sherman, B. T, Hosack, D. A, Yang, J, Gao, W., Lane, H.C., Lempicki, R. A. 2003. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* **4,** P3.

DeRisi, J. L, Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A. and Trent, J. M. 1996. Use of cDNA microarray to analyze gene expression patterns in human cancer. *Nat Genet.* **14,** 457-460.

DeRisi J. L., Iyer V. R., Brown P. O. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale *Science* **278,** 680-686.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. 1998. Cluster analysis and display of genome-wide expression patterns**.** *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868

Emerald, B. S. and Shashidhara, L. S., 2000. Negative regulation of ultrabithorax expression by engrailed is required for the proper specification of wing development in *Drosophila melanogaster*. *J. Genet*. **79,** 61-70

Falconer, D. S. and Mackay, T. F. C. 1996. *Introduction to quantitative genetics*, 4th ed. Longman, Essex, UK.

Foe, V. E. and Alberts, B. M. 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* **61**, 31-70

Gadgil, M. and Bossert, W. H. 1970. Life historical consequences of natural selection. *Am. Nat.* **104,** 1-24

Gibson G. 2002. Microarrays in ecology and evolution: a preview. *Mol. Ecol.* **11**, 17-24.

Gilbert, S. F. 2006. *Developmental biology.* 8th edn. *Sinauer Associates Inc.* Sunderland, USA.

Hillesheim E. and Stearns S. C. 1992. Correlated responses in life-history traits to artificial selection for body weight in *Drosophila melanogaster*. *Evolution* **46,** 745-752.

Howes, R. and Bray, S. 2000. Pattern formation: wingless on the move. *Curr. Biol*. **10,** R222-R226.

Huang, D. W., Sherman, B. T. and Lempicki, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc.* **4,** 44-57

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B. and Speed T. P. 2003a. Summaries of Affymetrix GeneChip probe level data. *Nucl. Acids Res*. **31,** e15.

Irizarry, R. A., Hobbs, B., Collin, F., Barclay, B.Y.D., Antonellis, K. J., Scherf, U. and Speed, T. P. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4,** 249-64

Ives, P. T. 1970. Further studies of the South Amherst population of *Drosophila melanogaster*. *Evolution* **38,** 507-518.

Iyer, V. R, Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C., Trent, J. M., Staudt, L. M., Hudson, J. Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D. and Brown, P. O., 1999. The transcriptional program in the response of human fibrosis to serum. *Science* **283,** 83-87.

Johnson, N. A. and Porter, A. H. 2001. Toward a new synthesis: population genetics and evolutionary developmental biology**.** *Genetica* **112–113,:** 45–58.

Johnston, G. C., Pringle, J. R. and Hartwell, L. H. 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* **105,** 79-98.

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

Joshi, A., Shiotsugu, J. and Mueller, L. D. 1996 Phenotypic enhancement of longevity by environmental urea in *Drosophila melanogaster*. *Exp. Gerontol*. **31,** 533-544.

Joshi, A. 1997. Laboratory studies of density-dependent selection: adaptations to crowding in *Drosophila melanogaster*. *Current Science* 72: 555-561.

Joshi, A., 2000. Life-history evolution in the laboratory. *J. Indian Inst. Sci.,* **80,** 25-37

Joshi, A., Prasad, N. G. and Shakarad, M. 2001. *K*-selection, α-selection, effectiveness and tolerance in competition: density-dependent selection revisited. *J. Genet*. **80,** 63-75.

Joshi, A., Castillo, R. B. and Mueller, L. D. 2003. The contribution of ancestry, chance, and past and ongoing selection to adaptive evolution. *J. Genet*. **82,** 147-162.

Kawli, T. S. 2000. Size control in development: lessons from *Drosophila*. *J. Biosci.* **25**, 2-3.

Kohler, R. 1994. *Lords of the fly*. University of Chicago Press, Chicago, USA.

Kornberg, T. 1981. *engrailed*: a gene controlling compartment and segment formation in *Drosophila. Proc. Natl. Acad. Sci. USA* **78,** 1095-1099.

Lashkari, D. A., DeRisi, J. L., McCusker, J. H., Namath, A. F., Gentile, C., Hwang, S. Y., Brown, P. O. and Davis, R. W. 1997. Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proc. Natl. Acad. Sci. USA* **94,** 13057-13062.

Lebo, M. S., Sanders, L. E., Sun, F. and Arbeitman, M. N. 2009. Somatic, germline and sex hierarchy regulated gene expression during *Drosophila* metamorphosis. *BMC Genomics* **10,** 80.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Norton, H. and Brown, E. L. 1996. Expression monitoring by hybridization to high-density oligonucleotide. *Nature Biotechnol.* **14,** 1675–1680.

Luckinbill, L. S. and Clare, M. J. 1985. Selection for lifespan in *Drosophila melanogaster*. *Heredity* **55,** 9-18.

Modak, S. G. 2009. *Evolution of life-history traits, canalization and reproductive isolation in laboratory populations of Drosophila melanogaster selected for faster pre-adult development and early reproduction.* Ph.D. thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, India.

Modak, S. G., Satish, K. M., Mohan, J., Dey, S., Raghavendra, N., Shakarad, M. and Joshi, A. 2009. A possible tradeoff between developmental rate and pathogen resistance in *Drosophila melanogaster*. *Journal of Genetics*, **88,** 253-256

Mueller L. D. 1985. The evolutionary ecology of *Drosophila*. *Evol. Biol*. **19,** 37-98.

Mueller L. D. 1997. Theoretical and empirical examination of density-dependent selection. *Annu. Rev. Ecol. Syst*. **28,** 269-288.

Neufeld, T. P. and Edgar, B. A. 1998. Connections between growth and the cell cycle. *Curr. Opin. Cell Biol.* **10,** 784-790.

Nunney, L. 1996. The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: an example of a fitness trade-off. *Evolution* **50,** 1193-1204.

Nüsslein-Volhard, C. and Wieschaus, E. 1980. Mutations affecting segment number and polarity in *Drosophila. Nature* **287,** 795-801.

O'Connor, R. J. 1954. Observations on the nature of the relationship between cell division and carbohydrate metabolism. *J. Embryol. Exp. Morph*. **2,** 26-37

Oldham, S., Bihni, R., Stocker, H., Brogiolo, W. and Hafen, E. 2000. Genetic control of size in *Drosophila. Phil. Trans. R. Soc. Lond. B* **355,** 945-952.

Partridge, L., Hoffmann, A. and Jones, J. S. 1987. Male size and mating success in *Drosophila melanogaster* and *Drosophila pseudoobscura* under field conditions. *Anim. Behav.* **35,** 468-476

Partridge, L. and Fowler, K. 1992 Direct and correlated responses to selection on age at reproduction in *Drosophila melanogaster*. *Evolution* **46,** 76-91.

Partridge, L., Langelan, R., Fowler, K., Zwaan, B. J. and French, V. 1999. Correlated responses to selection on body size in *Drosophila melanogaster*. *Genet. Res*. **74,** 43-54.

Peifer, M. and Bejsovec, A. 1992. Knowing your neighbors: cell interactions determine intrasegmental patterning in *Drosophila*. *Trends Genet*. **8,** 243- 249.

Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., Lashkari, D., Shalon, D., Brown, P. O. and Botstein, D. 1999. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc. Natl. Acad. Sci. USA* **96,** 9212–9217.

Polymenis, M. and Schmidt, E. V. 1999. Coordination of cell growth with cell division. *Curr. Opin. Genet. Dev.* **9,** 76-80.

Prasad, N. G., Shakarad, M., Gohil, V. M., Sheeba, V., Rajamani, M. and Joshi A. 2000. Evolution of reduced pre-adult viability and larval growth rate in laboratory populations of *Drosophila melanogaster* selected for shorter developmeent time. *Genet. Res. Camb.* **76,** 2249-259.

Prasad, N. G., Shakarad, M., Anitha, D., Rajamani, M. and Joshi A., 2001. Correlated responses to selection for faster development and early reproduction in *Drosophila*: the evolution of larval traits. *Evolution* **55,** 1363-1372.

Prasad, N. G. and Joshi, A. 2003. What have two decades of laboratory life-history evolution studies on *Drosophila melanogaster* taught us? *J. Genet*. **82,** 45–76.

Prasad, N. G. 2004. *Life-history evolution in laboratory populations of Drosophila melanogaster subjected to selection for faster development and early reproduction.* Ph.D. thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India.

Purves, W. K., Orians, G. H., Heller, H. C. and Sadava, D. 1998. *Life: the science of biology*, 5th edn. Sinauer Associates Inc., Sunderland, USA.

Reinke, V. and White, K. P. 2002. Developmental genomic approaches in model organisms. *Annu. Rev. Genomics Hum. Genet*. **3,** 153-178.

Robertson, F. W. 1959. Studies in quantitative inheritance. XII. Cell size and number in relation to genetic and environmental variation of body size in *Drosophila*. *Genetics* **44,** 869-896.

Robertson, F. W. 1963. The ecological genetics of growth in *Drosophila*. VI. The genetic correlation between the duration of the larval period and body size in relation to larval diet. *Genet. Res*. **4,** 74-92.

Roff, D. A. 1992. *The evolution of life histories: theory and analysis.* Chapman and Hall, New York, USA.

Rose M. R. 1984 Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* **38,** 1004-1010.

Rose, M. R., Nusbaum, T. J. and Chippindale, A. K. 1996. Laboratory evolution: the experimental wonderland and the Cheshire Cat syndrome. In *Adaptation* (eds. M. R. Rose, G. V. Lauder), pp. 221-241. Academic Press, San Diego, USA.

Rose, M. R., Passananti, H. B. and Matos, M. (eds) 2004. *Methuselah flies: a case study in the evolution of aging*. World Scientific, New York, USA.

Rose, M. R., Passananti, H. B., Chippindale, A. K., Phelan, J. P., Matos, M., Teotonio, H. and Mueller, L. D. 2005. The effects of evolution are local: evidence from experimental evolution in *Drosophila*. *Integr. Comp. Biol*. **45,** 486-491.

Roth, C. M. 2002. Quantifying gene expression. *Curr. Issues Mol. Biol.* **4,** 93-100

Roush, W. 1996. Putting the brakes on bone growth. *Science* **273**, 579.

Santos, M., Ruiz, A., Barbadilla, A., Quezada-Diaz, J. E., Hasson, E. and Fontdevila, A. 1988. The evolutionary history of *Drosophila buzzatii*. XIV. Larger flies mate more often in nature. *Heredity* **61,** 255-262.

Schena, M., Shalon, D., Davis, R. W. and Brown, P. O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270,** 467-470.

Schena, M., Shalon, D., Heller, R., Chai, A. and Brown, P. O. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc. Natl. Acad. Sci. USA* **93,** 10614-9

Service P. M. and Rose M. R. 1985. Genetic covariation among life-history components: the effects of novel environment. *Evolution* **39,** 943-945.

Shalon, D., Smith, S. J. and Brown, P. O. 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res.* **6,** 639-645

Shakarad, M., Prasad, N. G., Gokhale, K., Gadagkar, V., Rajamani, M. and Joshi. A. 2005. Faster development does not lead to correlated evolution of greater pre–adult competitive ability in *Drosophila melanogaster*. *Biol. Lett.* **1,** 91–94

Sheeba, V., Madhyastha, N. A. A. and Joshi, A. 1998. Oviposition preference for novel *versus* normal food resources in laboratory populations of *Drosophila melanogaster*. *J. Biosci*. **23,** 93-100.

Siegfried, E. and Perrimon, N. 1994. *Drosophila* wingless: a paradigm for the function and mechanism of *Wnt* signaling. *BioEssays* **16,** 395-404.

Simpson, P. 1979. Parameters of cell competition in the compartments of the wing disc of *Drosophila*. *Dev. Biol.* **69,** 182-193.

StatSoft Inc. 1995. *Statistica Vol. I: General conventions and statistics I.* Statsoft Inc., Tulsa, USA.

Stearns S. C. 1992. *The evolution of life histories*, Oxford University Press, Oxford, UK.

Sucena, E. and Stern, D. L. 2000. Divergence of larval morphology between *Drosophila sechellia* and its sibling species caused by cis-regulatory evolution of *ovo/shaven-baby*. *Proc. Natl. Acad. Sci. USA* **97,** 4530-4534.

Teotonio H. and Rose M. R. 2000. Variation in the reversibility of evolution. *Nature* **408,** 463-466.

Teotonio, H. and Rose, M. R. 2001. Perspective: reverse evolution. *Evolution* **55,** 653-660.

Teotonio, H., Matos, M. and Rose, M. R. 2002. Reverse evolution of fitness in *Drosophila melanogaster*. *J. Evol. Biol.* **15,** 608-617.

Turner, F. R. and Mahowald, A. P. 1977. Scanning electron microscopy of *Drosophila melanogaster* embryogenesis. *Dev. Bio.* **57**, 403-416.

White, R. A. H. and Wilcox, M. 1985. Distribution of ultrabithorax proteins in *Drosophila*. *EMBO J.* **4,** 2035-2043.

White, K. P., Rifkin, S. A., Hurban, P. and Hogness, D. S. 1999. Microarray analysis of *Drosophila* development during metamorphosis. *Science* **286,** 2179-2184.

Wodarz, A. and Nusse, R. 1998. Mechanism of Wnt signaling in development. *Annu. Rev. Cell Biol.* **14,** 59-88.

Wu, Q., Wen, T., Lee, G., Park, J. H., Cai, H. N. and Shen, P. 2003. Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron* **39,** 147-161.

Zamudio, K. R., Huey, R. B. and Crill, W. D. 1995. Bigger isn't always better: body size, developmental and parental temperature and male territorial success in *Drosophila melanogaster*. *Anim. Behav*. **49,** 671-677.

Zwaan B. J., Bijlsma R. and Hoekstra R. F. 1995. Artificial selection for development time in *Drosophila melanogaster* in relation to the evolution of aging: direct and correlated responses. *Evolution* **49,** 635-648.

Zwaan, B. J. 1999. The evolutionary genetics of ageing and longevity. *Heredity* **82,** 589-597.

Appendix

Reagents used in the study

1. **1X PBS** (Sigma-Aldrich, cat# P3813-10PAK, St. Louis, Missouri, USA)

   Reconstitution contents of one pouch, when dissolved in one liter of distilled or deionized water, will yield 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M); pH 7.4, at 25 °C.

2. **0.1% PBTx** (For 100 ml solution)

   100 µl Triton-X (Sigma-Aldrich, cat# T8787, St. Louis, Missouri, USA) and 500 mg Bovine Serum Albumin (BSA) dissolved in 100 ml of 1X PBS.

3. **0.3% PBTx** (for 100 ml solution)

   300 µl (Sigma-Aldrich, cat# T8787, St. Louis, Missouri, USA) dissolved in 100 ml of 1X PBS.

4. **Primers used for the study of *dnpf* gene expression (chapter IV)**

   ***dnpf*:** FW- 5'-TACAGTCCGACGAACAATTG-3'

   RW 5'-CTTTCCCAGCACGTTAAACTATT-3'

   ***Elf-1α*:** FW 5'-ACATTGCCTGCAAGTTTTCC-3'

   RW 5'-AGGACTTGCGGTGACGATAC-3'