Evolution of Life-history Traits, Canalization and Reproductive Isolation in Laboratory Populations of *Drosophila melanogaster* Selected for Faster Pre-adult Development and Early Reproduction

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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Dedicated To

My Family, Friends

& Rahul

Contents

DECLARATION

I hereby declare that the work embodied in my thesis entitled "**EVOLUTION OF LIFE-HISTORY TRAITS, CANALIZATION AND REPRODUCTIVE ISOLATION IN LABORATORY POPULATIONS OF *DROSOPHILA MELANOGASTER* SELECTED FOR FASTER 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 that 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 findings of other investigators. Any omission, which might have occurred by oversight or error of misjudgment, is regretted.

Shampa Ghosh Modak

Place: Bangalore

Date: February 16, 2009

Evolutionary & Organismal Biology Unit

JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH

P. O. Box 6436, Jakkur, Bangalore, 560 064, India

16 February, 2009

CERTIFICATE

This is to certify that the work described in the thesis entitled "**EVOLUTION OF LIFE-HISTORY TRAITS, CANALIZATION AND REPRODUCTIVE ISOLATION IN LABORATORY POPULATIONS OF *DROSOPHILA MELANOGASTER* SELECTED FOR FASTER PRE-ADULT DEVELOPMENT AND EARLY REPRODUCTION**" is the result of investigations carried out by Ms. Shampa Ghosh Modak 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 life-history of an organism refers to the probability of survival and reproductive output at different life-stages and is, thus, intimately related to the realization of an individual's Darwinian fitness. Any phenotype must necessarily impinge upon the life-history if it is to have an adaptive significance. Hence, an understanding of the genetic architecture of fitness-related traits — how different phenotypes are genetically correlated with one another, and with components of the life-history — is fundamental to the understanding of adaptive evolution. Experimental evolution approaches using long-term studies of the evolutionary responses of *Drosophila melanogaster* populations to specific selection pressures in the laboratory have been an important means of studying the genetic architecture of fitness-related traits and life-history evolution over the past two decades or so.

In holometabolous insects like *Drosophila*, adult fitness is greatly dependent on the extent of resource acquisition and assimilation during the larval stage. Consequently, both pre-adult survival and the duration of larval development are important to overall fitness. In this thesis, I present results from an ongoing study of the evolutionary consequences of long-term selection in our laboratory for reduced pre-adult duration in *D. melanogaster*. Four populations (FEJs) were selected for faster pre-adult development and early reproduction (day 3 post-eclosion) for close to 400 generations, leading to a 25% reduction in pre-adult development time compared to the four ancestral control populations (JBs). A previous thesis from this laboratory detailed the evolution of the life-history of the FEJs over the first 200 generations of selection, and also the correlated evolution of various larval and adult traits related to resource acquisition and utilization. In an extension of this line of investigation, I studied various further changes in the life-history of the

FEJs between 200 and 300 generations of selection for faster development and also conducted crosses to examine the genetic control of development time and associated traits. In addition, I also obtained preliminary evidence of a tradeoff between developmental rate and pathogen resistance in these populations. In a major expansion of the scope of evolutionary questions addressed with these populations, I found evidence that long-term directional selection for rapid development has led to the evolution of (a) greater canalization of development time along two environmental axes, and (b) incipient reproductive isolation between selected and control populations. I also initiated a similar selection study with populations of *D. ananassae*, to ask whether the pattern of genetic correlations between development time and traits relevant to larval competitive ability in this species is similar to that seen in *D. melanogaster*. The results indicate that the genetic architecture of these traits is quite different in these two phylogenetically and ecologically similar species, thus recommending caution when extrapolating results from selection studies on *D. melanogaster* to other species with a similar ecology.

Studies of the FEJs had earlier revealed a peculiar pattern of evolution first toward but then later away from the optimal life-history of rapid development coupled with relatively high fecundity around day 3 post-eclosion, even if the latter meant reduced subsequent fecundity and lifespan. At generation 30, FEJs seemed to be evolving towards the predicted optimal life-history: they developed faster and had a shorter lifespan than JBs, but produced more eggs per unit dry weight in early life. However, at generation 70, FEJ and JB lifespan was the same, and FEJ fecundity per unit dry weight early in life was significantly lower than the JBs. It was hypothesized that this counter-intuitive evolution away from the optimal life-history was due to the FEJs having undergone a reduction in size and lipid content large enough to trigger a physiological switch

inducing greater resource allocation for somatic maintenance as opposed to reproduction. *D. melanogaster* is known to undergo nutritional-status-dependent allocation switching, thought to be adaptive under fluctuating food availability in the wild. The FEJs also showed a greater starvation resistance per unit lipid than the JBs, an observation consistent with the 'physiological switch' hypothesis since lipid mobilized to the ovaries for reproduction is unavailable for resisting starvation in *D. melanogaster*. The conclusion, thus, was that this past adaptation had become a constraint that the FEJs had not been able to overcome over the first 70 generations of selection. After 200 further generations, I found that ongoing selection had led to the FEJs circumventing the maladaptive constraint of the 'physiological switch'. FEJ lifespan at generation 270 was lower than the JBs and, despite undergoing further reduction in both total and fractional lipid levels, FEJs produced a significantly higher number of eggs per unit lipid than the JBs. In addition to producing more eggs per unit lipid, FEJ populations also showed a 70% higher starvation resistance per unit lipid compared to JBs. Thus, continued FEJ selection seems to have ultimately resulted in the evolution of greater efficiency of lipid usage in these populations. Alternatively, the FEJs may have evolved a reduced level of egg provisioning than the JBs as a means of reconciling the need for high early life fecundity and very low lipid levels at eclosion.

Another aspect of somatic maintenance, the pathogen resistance of adult flies was measured by assaying the time to death of FEJs and JBs in presence/absence of *E. coli* cultures on LB agar medium. *E. coli* induced a significant reduction in lifespan of both FEJs and JBs, but the percent reduction in lifespan due to presence of *E. coli* was significantly higher in FEJs, suggesting a possible tradeoff between developmental rate and pathogen resistance in *D. melanogaster*. It is very likely that this tradeoff is mediated by reduced energy reserves in the FEJs.

Canalization helps to maintain phenotypic constancy of a trait, buffering it against the effects of genetic and environmental noise. It is believed that this helps express phenotypes favoured in the most commonly experienced environment and suppresses deviations from the phenotypic optima. Thus, traits closely related to fitness have been predicted to get canalized in course of selection. In FEJs, the nature of the selection has imposed an extraordinary fitness premium on rapid development, which is not the case for the JBs. After 300 generations of selection, I studied the development time of the FEJs and JBs at nine different combinations of temperature and density (18°C, 25°C and 28°C crossed with 30, 70 and 300 eggs per vial). The objective of this experiment was to investigate whether or not development time has evolved to become more canalized across environments in FEJs compared to the JBs. The FEJs developed faster than JBs in all nine environments and had a smaller coefficient of variation (CV) of development time compared to the JBs. The changes in mean and CV of the trait across density were significantly less in FEJs compared to JBs. Along the temperature axis, mean trait value changed in a similar manner for both types of populations, but the FEJ CV remained more consistent across temperature than that of the JBs. This suggested a reduced sensitivity of FEJ development time to changes in both rearing temperature and density, reflecting environmental canalization and providing empirical support for the notion that long-term directional selection for a life-history trait can lead to its canalization.

The role of divergent ecological selection in speciation is well known, with the principal mode of action thought to be adaptation to different environments. The effect of divergent life-history evolution on reproductive isolation has not been explored thus far. I found that two complementary, asymmetric isolating mechanisms have mediated the evolution of incipient reproductive isolation

between the FEJs and JBs. The large size difference between the FEJ and JB flies evolved in response to about 400 generations of selection for faster development appears to play a crucial role in the isolation. Small FEJ males obtain few matings with large JB females, irrespective of the presence or absence of JB males, giving rise to unidirectional, pre-mating isolation mediated by sexual selection. Conversely, small FEJ females suffer increased mortality following mating with large JB males, resulting in viability-selection-driven, post-mating isolation in the opposite direction. The isolation is likely due to reduced size and activity levels in the selected populations, showing that early stages of ecological speciation can be a by-product of differential life-history evolution, even in the absence of major differences in habitat or resource use between populations. The results also show a complex interaction of sexual and natural selection, underscoring the need for detailed studies of the functional links between traits responding to divergent selection and the actual mechanisms of reproductive isolation.

Identifying genetic correlations among fitness-related traits by examining correlated responses to selection has been an important component in our understanding of life-history evolution in *Drosophila*. However, most such studies have been done on a single species, *D. melanogaster*, and there is little information about how conserved these correlations are across congeners. In view of this, I subjected four laboratory populations of *D. ananassae* to selection for faster development and early reproduction and studied the selection responses for 25 generations. Similar to *D. melanogaster*, developmental rate traded off with adult body size in *D. ananassae*. Faster development also resulted in a pre-adult survivorship cost in both the species, but the tradeoff seemed to be much stronger in case of *D. ananassae*. However, the tradeoffs between developmental rate and larval feeding rate, and between developmental rate and larval

competitive ability observed in *D. melanogaster* were not found in *D. ananassae*. These results indicate that the genetic architecture of fitness-related traits is not conserved in these two species despite their being quite similar ecologically and phylogenetically. Hence, one should be cautious when extrapolating results obtained from *D. melanogaster* even to fairly similar congeners.

Chapter I

Introduction

Experimental evolution provides a good framework for testing evolutionary theories and hypotheses (Rose *et al.* 1996; Prasad and Joshi 2003). Although field studies have been the traditional mode of investigating natural selection, experimental evolution through laboratory selection offers unique opportunities for understanding the details of the adaptive process and the interactions of micro-evolutionary factors that ultimately shape the evolutionary trajectory of populations. Experimental evolution offers the prospect of studying adaptive responses under well-defined selection pressures that are far less complex than those in the wild, giving the experimenter a relatively better chance of drawing clear inferences about cause-effect networks in adaptive evolution. Moreover, selection regimes can be replicated at the population level, increasing statistical power (Rose *et al.* 1996). In addition to these, since the population size to be chosen is at the discretion of the experimenter, working with a bigger population size can help effectively minimize confounding effects of genetic drift and inbreeding depression, which is often not possible in case of natural populations (Rose *et al.* 1996). In experimental evolution, the robustness of the responses to specific selection pressures can also be tested by repeating the selection experiments using sets of populations with different ancestries (Rose *et al.* 1996).

Experimental evolution has been extensively used to study adaptive evolution in bacteria and *Drosophila* (Rose *et al.* 1996). Laboratory selection studies involving *Drosophila* have enriched our knowledge of how complex life-histories evolve. These studies identified trade-offs among various components of fitness that seem to play a central role in the evolution of life-histories (Prasad and Joshi 2003). Complex life-cycles of holometabolous insects like *Drosophila* are characterised by trade-offs spanning different life-stages (Chippindale *et al.* 1997a) such that selection acting on pre-adult stages can potentially influence adult fitness. In *Drosophila*, acquisition of resources occurs mainly during the pre-adult stage, as a larva, and adult size is

largely controlled by the energy balance during the larval stage (Bakker 1961; Robertson 1963). Adult size in turn is a major determinant of adult fitness (Robertson 1957; Partridge and Farquhar 1983; Partridge and Fowler 1993). Thus, selection operating on pre-adult duration plays an important role in shaping the life-history of *Drosophila* (Chippindale *et al.* 1997a, Prasad 2004).

Studies involving selection for shorter pre-adult duration in *D. melanogaster* showed wide-ranging correlated changes in a host of larval and adult life-history related traits (Zwaan *et al.* 1995; Nunney 1996; Chippindale *et al.* 1997a, Prasad *et al.* 2000, 2001; Joshi *et al.* 2001; Shakarad *et al.* 2005). A previous study done in our laboratory involved simultaneous selection for faster development and early reproduction for about 100 generations (Prasad *et al.* 2000, 2001; Prasad 2004). Developmental rate was found to be negatively correlated with pre-adult survivorship, larval feeding rate and competitive ability. Additionally, rapid development traded off with components of adult fitness like fecundity and starvation resistance, most likely as a consequence of the evolution of smaller adult body-size (Prasad 2004). I continued the above-mentioned selection experiment for further 300 generations, while expanding the scope to address several fundamental evolutionary questions using this experimental system. The next two chapters present the results of assays of pre-adult and adult life-history traits on the faster developing populations beyond 200 generations of selection. The effects of selection on the durations of individual developmental stages and the viability cost in the larval and pupal stages are discussed in Chapter II, whereas a detailed discussion of the adult life-history of these populations is presented in chapter III. Apart from the characterisation of basic life-history traits like lifespan and fecundity, resistance to pathogens, a trait rarely considered in studies of life-history evolution, was also assayed in the faster developing populations and their controls. This

section of chapter III discusses how low resource levels in adults induced by faster development can also affect pathogen resistance. A final section at the end of chapter III discusses the pattern of lifetime fecundity commonly observed in *D. melanogaster*, with an emphasis on the timing of fecundity peaks in the life of flies and the underlying evolutionary causes.

Chapter IV explores the relationship of selection and canalization, an evolutionary issue which has not been addressed so far in experimental evolution. It has been hypothesized that the degree of canalization of a trait depends upon its impact on fitness and, hence, traits strongly acted upon by selection are likely to get canalized over time (Rendel 1967; Stearns and Kawecki 1994). Till date, only two studies have demonstrated that canalization of complex traits does seem to be proportionate to their relevance to fitness (Stearns and Kawecki 1994; Stearns *et al.* 1995). In the experimental system used for my study, development time is closely associated with fitness in the selected populations. Therefore, I investigated if selection has led to the canalization of development time in these populations.

Chapter V addresses the issue of possible ecological speciation as a result of prolonged divergent selection on life-history traits. The role of divergent ecological selection in speciation has been studied extensively in both natural populations and laboratory conditions (Rice and Hostert 1993; Rundle and Nosil 2005) but the role of divergent life-histories in speciation has rarely been addressed. I show that about 400 generations of selection for faster development and early reproduction have driven the evolution of incipient reproductive isolation between the faster developing populations and their ancestral controls, presumably mediated via body-size divergence. Since the reproductive isolation is still in its formative stages and not yet complete, F_1 progeny could be obtained through crosses between the selected populations and the controls. Characterisation of the F_1 traits revealed interesting details about the underlying genetics of

complex traits like development time, body-size and fecundity, and these results have been discussed in chapter VI.

Chapter VII addresses the broader issue of how conserved the pattern of genetic correlations among life-history related traits is in *Drosophila* species. Previous studies have established the pattern of genetic correlations among traits like development time, pre-adult survivorship, body size and larval competitive ability in *D. melanogaster* (Reviewed in Prasad and Joshi 2003). In this chapter, I investigate whether the same pattern of correlations among these traits is conserved in *D. ananassae*, a species related to *D. melanogaster*. Populations of *D. ananassae* were subjected to similar selection for faster development and early reproduction for 25 generations, and the correlated responses were studied. A comparative account of the pattern of genetic correlations among the studied traits in the two species is provided in chapter VII.

To summarize, this thesis uses the approach of experimental evolution to explore the life-historical consequences of prolonged selection for faster development and early reproduction in *Drosophila*, the transmission genetics of the relevant traits, the canalizing effect of selection, the generality of trade-offs seen in *D. melanogaster*, and also the evolution of reproductive isolation as a byproduct of long-term divergent selection.

The Experimental System

Populations of Drosophila melanogaster

The studies reported in chapters II to VI were conducted on eight laboratory populations of *D. melanogaster*; four of these are selected for faster development and early reproduction and the other four are ancestral controls. All eight populations are maintained at 25 (±1)^{o}C, ~90%

relative humidity, under constant light on a discrete generation cycle. The four control populations (JB_{1-4}) were first described by Sheeba *et al.* (1998). JBs are maintained on a 21 day discrete generation cycle on banana-jaggery food. The larval density is regulated at about 60-80 larvae per 8-dram vial (9 cm height × 2.4 cm diameter) with 6 mL food. The number of breeding adults is about 1500-1800 per replicate population and the adults are maintained in Plexiglas cages (25 cm × 20 cm × 15 cm) with abundant food. After 21 days from the previous egg collection, *i.e.* 10^{th}-11^{th} day of adult age, eggs are collected from these flies by placing Petri dishes with food into cages for 18 h. For each replicate population, the eggs collected off these food plates are then dispersed into 40 vials at a density of 60-80 eggs per vial. On the 12^{th}, 14^{th}, and 16^{th} day after egg collection, the eclosed flies are transferred into fresh food vials; on the 18^{th} day, all the flies are collected into Plexiglas cages containing a Petri dish of food on which a generous smear of yeast and acetic acid paste has been applied. Three days later, eggs are collected for the next generation. The four JB populations are descendants of a single wild-caught population of *D. melanogaster* — the IV population described by Ives (1970). They were first maintained in the laboratory for about 110 generations under constant light, 25 (±1)^{o}C and constant high humidity on a 14 day discrete generation cycle. Five populations (B_{1-5}) were then derived from IV populations and reared in the laboratory under similar conditions (Rose and Charlesworth 1981). After about 360 generations, a set of five populations were derived from B_{1-5} and christened UU_{1-5} (Uncrowded as larvae, Uncrowded as adults; described by Joshi and Mueller 1996). The UU populations were maintained under similar conditions, but on a 21 day discrete generation cycle rather than the 14 day cycle under which the IV and B populations were used to be maintained. After 170 generations of being maintained as UUs, JB_{1-4} populations were derived from UU populations (UU 1, 2, 3, 5).

The four populations selected for faster pre-adult development and early reproduction derived from the JBs are called FEJ_{1-4} (F: Faster developing; E: Early reproducing; J: JB derived populations) and were first described in detail by Prasad *et al.* (2000). FEJs are maintained under conditions similar to the JBs, except that 120 vials containing approximately 60-80 eggs are collected per population, and the vials are monitored for eclosion every 2 h after pupae have darkened. As soon as the earliest 25% or so flies in each vial (12-15 flies) have eclosed, they are transferred into fresh cages containing food plates supplied with a smear of yeast-acetic acid paste. These constitute the breeding adults for the next generation, comprising about 1200-1500 flies per replicate population. After three days, eggs are collected from FEJ cages by placing a Petri dish containing food inside the cage for 1 h. The eggs are then dispensed into vials at a density of 60-80 eggs per vial and the cycle is repeated. Thus, the major differences in the maintenance regime of JB and FEJ populations are that (i) for initiating the next generation, eggs are collected from FEJs three days post-eclosion, whereas in JBs egg collection is done on day 11 or 12 of adult age; (ii) egg-lay window for FEJ is 1 h, but JBs are allowed to lay eggs for 18 h; (iii) only the earliest ~25% of the eclosing flies constitute the breeding pool for the FEJ populations, whereas in JBs all the flies that eclose by the 12^{th} day after egg collection (this time period is sufficient for practically all the surviving individuals to have eclosed at moderate density *Drosophila* culture maintained at 25^{o}C) get the opportunity of contributing to the next generation. The various studies reported in this thesis were done between 230 and 370 generations of FEJ selection.

Each FEJ population has been derived from one JB population; thus selected and control populations bearing identical numerical subscripts are more closely related to each other ancestrally 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). Therefore, control and selected populations with identical subscripts were treated as blocks in the statistical analyses.

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

Table 1.1: The composition of 1 L of regular banana-jaggery food (used in the maintenance of FEJ and JB populations).

Populations of Drosophila ananassae

The study reported in chapter VII for testing the generality of genetic correlations across species was performed on eight populations of *D. ananassae.* Four of these populations that served as controls have been labeled AB_{1-4} (*Ananassae* Baseline). The AB populations, first described by Sharmila Bharathi *et al.* (2003) were derived from a single population established with ~300 wild inseminated females collected from Bangalore in 2001. This population was maintained for ~35 generations before being split into four replicate populations (AB_{1-4}). These populations are maintained on a 21- day discrete generation cycle at 25 (±1)^{o}C, about 90% relative humidity, constant light and on cornmeal medium. The larval density is regulated at about 60-80 larvae per 8-dram vial with 6 mL food. The number of breeding adults is about 1500-1800 per population and the adults are maintained in Plexiglas cages with abundant food and moist cotton. Eggs are collected from these flies by placing Petri dishes with food into cages for 18 h. The eggs

collected off these food plates are then dispersed into 40 vials at a density of 60-80 eggs per vial. On the 12th day after egg collection, the eclosed flies are transferred into Plexiglas cages containing a Petri dish of food and moist cotton. A generous smear of yeast and acetic acid paste is added to the food 4 days prior to egg collection.

At generation 64 of AB rearing, a set of populations were derived from the ABs and subjected to selection for faster pre-adult development and early reproduction (AF_{1-4}: *Ananassae* Faster development selection). AFs are maintained on a similar regime as ABs except that 120 vials containing approximately 60-80 eggs are collected per population, and the vials are checked for eclosion when pupae start darkening and as soon as 25% or so of the flies have eclosed, which is about 12-15 flies per vial, they are transferred into fresh cages containing food plates and moist cotton. These constitute the breeding adults for the next generation. The next day, the flies are supplied with a food plate covered with a generous smear of yeast and acetic acid paste. On the fourth day after yeasting, eggs are collected from AF cages by placing a food plate inside the cage for 2 h. The eggs are then dispensed into vials and the cycle is repeated. Thus the major differences in the maintenance regime of AB and AF populations are that (i) eggs are collected from AFs five days post-eclosion, whereas in ABs egg collection is done on day 12 or 13 of adult age; (ii) egg-lay window for AF is 2 h, but ABs are allowed to lay eggs for 18 h; (iii) only the earliest 25% or so of the eclosing flies constitute the breeding pool for the AF populations, whereas in ABs all the flies that eclose by the 12th day after egg collection get the opportunity of contributing to the next generation. The AF-AB system, thus, replicates the FEJ-JB system of *D. melanogaster*.

Since each AF population was derived from one AB population, selected and control populations bearing identical numerical subscripts are more closely related to each other ancestrally than to

other populations with which they share a selection regime (AB_i, and AF_i are more closely related than AB_i and AB_j or AF_i and AF_j ; i, j = 1-4). Therefore, control and selected populations with identical subscripts have been treated as blocks in the statistical analyses.

Ingredient	Amount
Cornflour (g)	150
Sugar (g)	40
Yeast (g)	40
Agar (g)	12
Charcoal* (g)	0.5
Ethanol (mL)	10
p-Hydroxymethyl benzoate (g)	1
Propionic acid (mL)	10

Table 1.2: The composition of 1 L of regular cornmeal food (used in the maintenance of AF and AF populations).

* Charcoal is added to darken the food, which is otherwise very light in colour, making it difficult to differentiate eggs from the food.

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. On the 12th day after egg collection, the flies 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 (or cornmeal food in case of *D. ananassae*) for 3 days prior to egg

collection for assays. The progeny of these flies, hereafter referred to as standardised flies, were then used for the various assays. For assays involving adult flies, eclosion of the assay flies from control and faster developing populations was synchronized by staggering the egg collection from the standardised flies according to the differences in the egg-to-adult development time of the control and selected populations.

Chapter II

Characterisation of Pre-adult Traits

Introduction

Pre-adult duration, the time spent before attaining sexual maturity, and survival during this stage are important components of an organism's fitness (Stearns 1992). Early attempts to study the evolutionary correlates of selection for fast development in laboratory populations of *Drosophila* failed to elicit successful responses (Sang and Clayton 1957; Clarke *et al.* 1961; Sang 1962). This observation led to the hypothesis that long-standing past selection for rapid development in the wild had probably led to the erosion of additive genetic variance for development time in the studied populations (Robertson 1963; Partridge and Fowler 1992). This explanation was based on the argument that, since *Drosophila* inhabit ephemeral habitats like rotting fruit in the wild, food scarcity and the concentration of nitrogenous waste would increase rapidly over the course of development in a habitat with high density of larvae, leading to selection for rapid development (Bakker and Nelissen 1963). However, more recent studies have been successful in selecting for faster development in *D. melanogaster*, suggesting that laboratory populations harbour sufficient additive genetic variance for development time. Two long-term selection experiments (Chippindale *et al.* 1997a; Prasad *et al.* 2000, 2001) and two studies of relatively shorter duration (Zwaan *et al.* 1995; Nunney 1996) yielded a fast and fairly large response to selection for faster development in *D. melanogaster*, leading to the speculation that the lack of response in the earlier studies was likely due to the small population size employed (Chippindale *et al.* 1997a). Given that selection for slower development is in the direction of lower fitness, it is difficult to interpret the results of such studies (Chippindale *et al.* 1997a). Hence I restrict my discussion to the evolutionary correlates of faster development in *D. melanogaster.*

In the first long-term study involving selection for rapid development reported by Chippindale *et al.* (1997a), 125 generations of selection led to an approximately 16% reduction in egg-to-adult

development time in the two sets of selected populations (ACO and ACB populations), and most of this reduction was accounted for by a reduction in the larval duration. Evolution of faster development was accompanied by correlated reductions in pre-adult survivorship and adult size. The other long-term study for faster development in *D. melanogaster*, using the FEJ populations, was conducted in our laboratory and yielded about 23% reduction in the overall development time in 110 generations (Prasad 2004). In this study, both larval and pupal durations underwent significant reduction (Prasad *et al.* 2001). An exhaustive account of the evolutionary responses of these populations over the first 100 generations or so is provided by Prasad (2004). In this chapter, I concentrate on the pre-adult traits like duration of different developmental stages and survivorship to adulthood in the FEJs and JBs beyond 200 generations of FEJ selection.

The contribution of different developmental stages to the evolved reduction in development time under selection for rapid development in *Drosophila* is not well understood. After 56 generations of selection, stage-specific durations were characterised in the FEJs and JBs populations (Prasad *et al.* 2001). At that time, egg duration (time from egg lay to egg hatch) did not differ significantly between FEJ and JB populations. The response to selection in the duration of the three larval instars was also not similar. Duration of the first and third larval instars showed significant reduction, but the second instar duration was more or less unchanged. Pupal duration was significantly shortened in FEJs as a result of selection. It was speculated that acceleration of the developmentally important stages such as the time between egg lay and egg hatch and second instar would lead to a high mortality cost. The observation that a mortality cost to rapid development was seen only after 50 generations of selection, is consistent with this speculation (Prasad *et al.* 2000). However, the pupal duration, a stage during which metamorphosis occurs, underwent significant reduction, suggesting that even stages involving major developmental

changes can get shortened as a result of selection. Thus, it was yet to be seen whether continued selection for a longer period can bring about reduction in the other developmentally critical stages like egg and second instar.

Evolution of faster development also led to correlated costs in pre-adult survivorship in FEJs, and this cost was almost entirely due to larval mortality. Reduce pupal duration did not induce significant pupal mortality in FEJ populations. This was in contrast to the finding of Chippindale *et al.* (1997a) who observed significant pupal mortality without substantial reduction in the pupal duration in their ACO and ACB populations. Chippindale *et al.* (1997a) speculated that reduced resource provisioning for pupal metabolism caused the mortality. An alternate explanation by Prasad *et al.* 2001 suggested that the simultaneous selection pressure for early completion of pre-adult development along with the selection for reproduction immediately after eclosion in the ACO and ACB populations would have constrained the reproductive maturation to be completed during the pupal stage, resulting in the observed pupal mortality. On the other hand, FEJ populations were thought to postpone some aspects of their reproductive maturation from the pre-adult pupal stage to the early adult stage during the three-day holding period before egg collection, thereby making a reduction of pupal duration evolutionary possible without a significant survivorship cost. This was further corroborated by the observation that the time to first mating after eclosion was significantly lengthened in FEJs compared to the JBs (Prasad 2004).

Prior to the present investigation, larval and pupal survivorship in the FEJs and JBs populations were last assayed at generation 50. Selection has been continued ever since during which overall development time and pre-adult survivorship in FEJ has continued to decrease (Prasad 2004).

The question that remained to be answered was how the increased pre-adult survivorship cost was distributed over larval and pupal stages.

I characterised the duration of different developmental stages and the pre-adult survivorship of the FEJs and JBs at around 250 generations of FEJ selection. The questions that I attempt to address using these data are:

(i) What are the relative contributions of different developmental stages to the overall reduction in development time in the FEJs after 250 generations of selection? Can prolonged selection for faster development lead to the reduction of duration of crucial developmental stages like the egg and the second larval instar?

(ii) Have 250 generations of selection affected the pupal survivorship? How is the current pre-adult survivorship cost distributed over the larval and pupal stages?

Materials and Methods

Development time

Total egg-to-adult development time, as well as the durations of the egg, the three larval instars and the pupal stage were assayed at generation 235. Standardised flies of each replicate FEJ 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 and new pupae formed were marked with colour pens on the outer walls of the vials and counted. The four-hourly 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 for new eclosions. The eclosed flies were removed after every check, frozen, and sexed under the microscope. Time of egg collection 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

Survivorship through the larval and pupal stages and overall egg-to-adult survivorship were assayed at generations 235 and 245. At generation 235, 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, and this measure thus included egg survival as well. Number of eclosed adults divided by the number of pupae formed in each vial yielded the mean pupal survivorship for that vial. At generation 245, 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.

Stage-specific development time

After 263 generations of selection, the contribution of different pre-adult life-stages to the overall egg-to-adult development time was assayed in the FEJs and JBs. After allowing the standardised flies to lay eggs for 1 h on a fresh food plate kept inside the cage, the plate was removed and a second plate containing food was introduced into the cage. Eggs were collected off the second food plate after 1 h with a moistened brush and placed into vials containing 6 mL of banana-jaggery food, collected at a density of exactly 30 eggs per vial.

Egg duration

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.

Larval instar duration assay

96 vials containing 30 eggs each were incubated for the assay. 32 h after the mid-point of the 1 h egg collection window, four vials from each population were removed from the incubator and the larvae killed by immersion in hot water. The dead larvae were collected and preserved in 70% ethanol at 4^{o}C. This procedure was repeated every 2 h, until 76 h had elapsed from the mid-point of the egg collection window. The numbers of first, second and third instar larvae in each vial was determined by counting the number of teeth in the mouth-hooks, characteristic of each instar. From these data, median times of each molt were obtained by extrapolation. The difference between the mean egg duration and the median time of first moult was taken as the duration of first instar, the difference between median of first and second moult yielded the duration of second instar and so on.

Pupation and egg-to-adult development time

From the remaining four vials out of 96 used for the larval instar duration assay, pupation and egg-to-adult development time were assayed, following the same procedure as described earlier.

Statistical analysis

Analysis of variance (ANOVA) was performed on the replicate population means for each trait studied and the data from different generations were analysed 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 egg and the individual instar durations. Since the survivorship 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 analysed separately.

Results

Development time at generation 235

Both larval and pupal duration were significantly reduced in the FEJs (Table 2.1), with larval duration accounting for 54.5% of the reduction in FEJ development time whereas the remaining 45.5% reduction was contributed by the reduction of the pupal duration (Figure 2.1a). Egg-to-adult development time of FEJ was ~23% shorter than JBs in both the sexes after 235 generations of selection (Figure 2.1b). Males took significantly longer to develop than females (Table 2.2).

	Effect	*df*	MS	*F*	*P*
Egg to pupa	selection	1	1629.844	1353.857	< 0.0001
Pupa to adult	selection	1	1144.368	9529.078	< 0.0001

Table 2.1: Summary of results of separate ANOVAs done on mean larval and pupal duration in the FEJs and JBs after 235 generations of FEJ selection. Block effects could not be tested for significance.

Effect	*df*	MS	*F*	*P*
Selection	1	11011.231	3817.13	< 0.0001
Sex	1	47.6	2805.04	< 0.0001
Selection × sex	1	4.196	5.391	0.1029

Table 2.2: Summary of results of ANOVA done on mean egg-to-eclosion development time in FEJs and JBs after 235 generations of selection. Only fixed factor effects could be tested for significance.

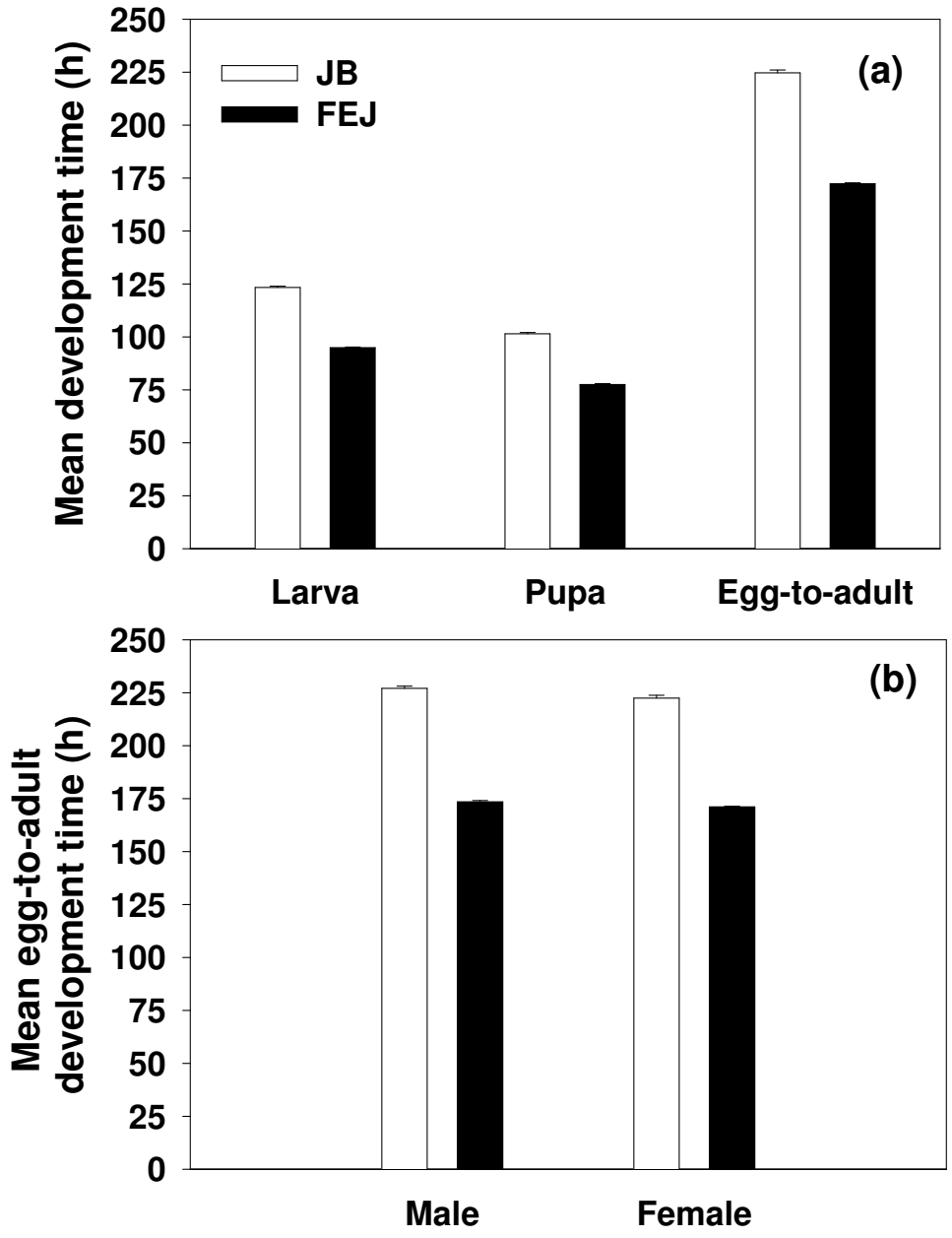

Figure 2.1: Mean (± s.e.) (a) duration of different pre-adult stages and (b) sex-specific egg-to-adult development time in FEJ and JB populations after 235 generations of FEJ selection.

Pre-adult survivorship at generations 235 and 245

Pre-adult survivorship of the JBs and FEJs varied considerably across the two assays, presumably due to unaccountable micro-environmental variation. However, pre-adult survivorship in the FEJs was significantly lower than the JBs at both the assay generations (Tables 2.3, 2.4). At generation 235, mean FEJ egg-to-adult survivorship was 13.7% lower than that of the JBs (Figure 2.2a), whereas at generation 245, the difference between the pre-adult survivorship of the FEJs and JBs was 24% (Figure 2.2b). Although the mean difference in survivorship between the two selection regimes was higher at generation 245, greater block variation led to a reduced *P*-level at this generation compared to generation 235. In both generations 235 and 245, the survivorship cost in the FEJs was largely due to larval mortality, while pupal survivorship did not differ significantly between the two selection regimes (Tables 2.3, 2.4).

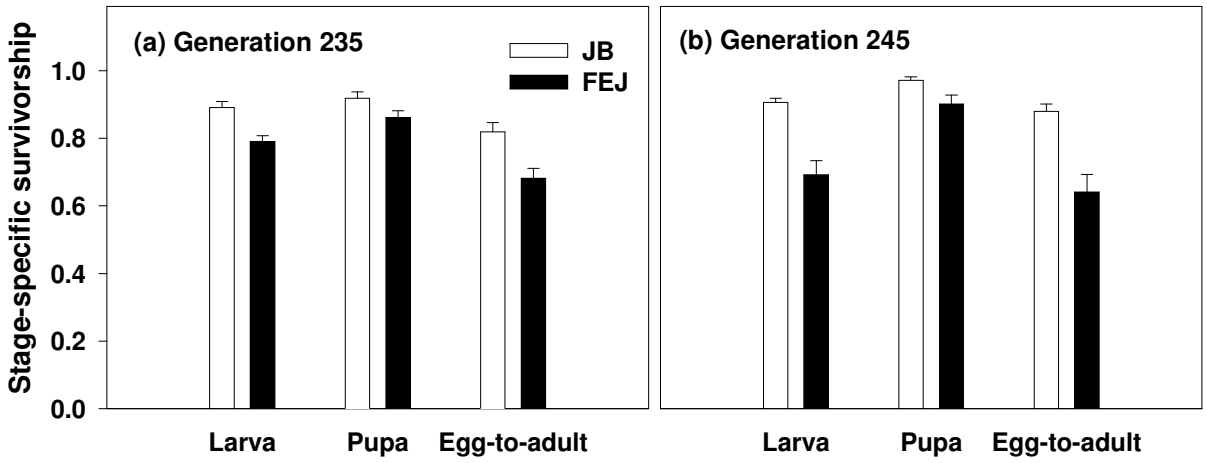

Figure 2.2: Mean (± s.e.) stage-specific survivorship in FEJ and JB populations after (a) 235 and (b) 245 generations of FEJ selection.

Stage	Effect	*df*	MS	*F*	*P*
Larval	selection	1	0.053	16.195	0.0276
Pupal	selection	1	0.03	9.619	0.0532
Total	selection	1	0.064	19.723	0.0212

Table 2.3: ANOVA results of stage-specific survivorship at generation 235. Block effects could not be tested for significance.

	Effect	*df*	MS	*F*	*P*
Larval	selection	1	0.1341	12.1447	0.0399
Pupal	selection	1	0.0498	7.1757	0.0751
Total	selection	1	0.1764	11.9949	0.0405

Table 2.4: ANOVA results of stage-specific survivorship at generation 245. Block effects could not be tested for significance.

Stage-specific development time

At generation 263, the duration of all developmental stages showed significant reduction in the FEJs, compared to controls (Table 2.5). Stages like egg and second larval instar in FEJ, which did not show reductions in the earlier assays, were also found to be significantly shortened after prolonged selection for over 260 generations (Figure 2.3).

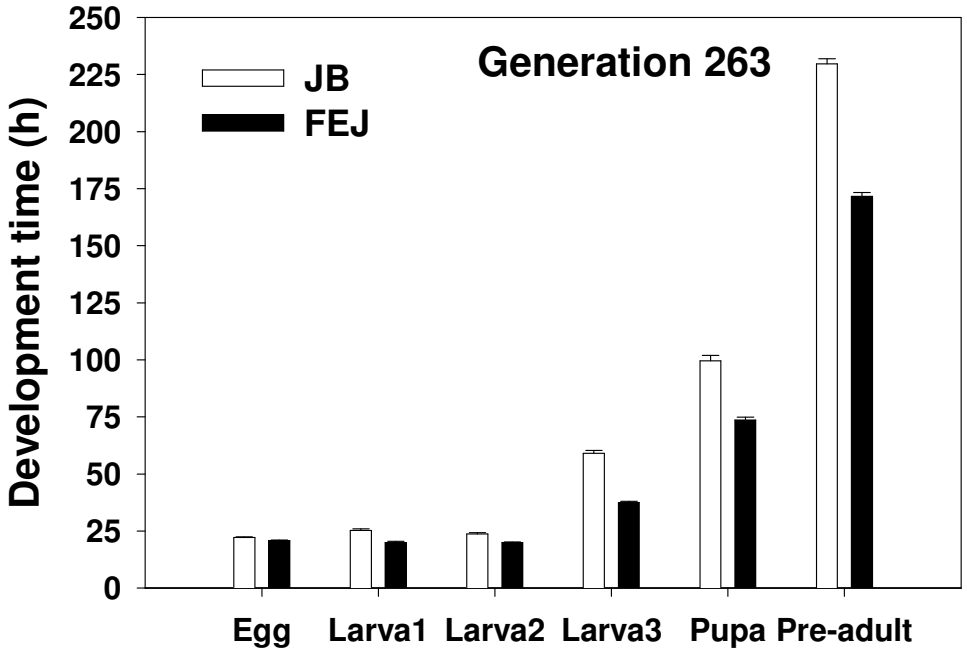

Figure 2.3: Mean (± s.e.) stage-specific duration of pre-adult developmental stages after 265 generations of FEJ selection.

Developmental stage	Effect	*df*	MS	*F*	*P*
Egg	selection	1	4.1508	28.3673	0.0129
Instar 1	selection	1	55.9639	22.9842	0.0173
Instar 2	selection	1	29.08442	48.1763	0.0061
Instar 3	selection	1	921.8295	181.3421	0.0009
Pupa	selection	1	1343.0142	150.9196	0.0012
Pre-adult	selection	1	921.8295	181.3421	0.0009

Table 2.5: Summary of results of separate ANOVAs done on stage-specific development time in the FEJs and JBs after 265 generations of selection. Block effects could not be tested for significance.

Discussion

At generation 263, the difference between mean egg-to-adult development time in the FEJs and JBs was 52 h. Unlike generation 56, the egg and second instar durations showed significant reductions at generation 263. There was a two-fold increase in the difference between the JBs and FEJs in the duration of first instar and pupal stages in past 200 generations. The difference between duration of the third instar in the JBs and FEJs increased by 30% during this period.

The survivorship cost at generation 50 was about 10% (Prasad *et al.* 2000), which kept on increasing further as selection proceeded. At generation 245, the mean egg-to-adult survivorship in the FEJs was 24% lower than the JBs. Surprisingly, pupal survivorship was still comparable between the FEJs and JBs, which suggested that the increasing difference between pre-adult survivorship of the FEJs and JBs was caused by rising larval mortality in FEJs. Reduction in the duration of vital developmental stages, along with further shortening of first and third larval instar, could possibly have exacted this additional survivorship cost.

Attainment of sexual maturity got further delayed in the FEJ populations in the last 100 generations (S. Ghosh Modak personal observation*).* A two-fold increase in the difference of pupal development time between the FEJs and JBs compared to generation 56 did not induce significantly increased pupal mortality in FEJ. This observation along with further postponement in the time to initiate mating supports the previous suggestion (Prasad *et al.* 2001) that perhaps the FEJs have been able to push some important aspects of adult maturity from pupal to adult stage. This study thus suggests that the pupal stage in *D. melanogaster* can readily evolve in response to directional selection for shorter development without much survivorship cost, provided there is no strong selection for reproduction soon after eclosion. However, the same

cannot be said about the embryonic and larval stages, which seem to be much more sensitive to large reductions in their duration, reflecting in substantial survivorship costs. A better understanding of the molecular underpinnings of the details of developmental changes, along with temporal profiling of development in larvae and pupae, and tracing the gonadal maturity of freshly eclosed adults in the FEJs and their controls could shed more light on this issue.

Chapter III

Characterisation of Adult Traits

SECTION A: Characterisation of adult life-history of FEJ after 270 generations of selection

Introduction

In organisms with complex life-histories, trade-offs can span across different life-stages such that fitness at a particular stage can be conditional upon traits expressed at a different life-stage (Chippindale *et al*. 1997a; Prasad and Joshi 2003). Exploring the evolution of complex life-histories under controlled laboratory conditions thus provides an opportunity to detect genetic correlations among traits expressed at different stages of the life-cycle. In *Drosophila*, adult body size and energy reserves are largely dependent on pre-adult food acquisition and assimilation, linking the pre-adult and adult components of fitness. Evolution of rapid pre-adult development constrains larval resource acquisition, thereby impinging upon adult traits such as body size and fecundity (Nunney 1996; Chippindale *et al.* 2003). Likewise, selection for faster development shaped the evolution of adult life-history in FEJs, showing an interesting zig-zag pattern in the evolutionary trajectory of these populations during first 100 generations of selection (Prasad 2004).

Given that the FEJs are selected for faster development and early reproduction, lifespan and fecundity beyond day 3 post-eclosion do not contribute to their fitness. Hence, the optimal life-history for FEJs would be to develop fast, mature sexually early, and produce a large number of eggs on day 3 post-eclosion, even if it reduces future lifespan and reproductive output (Prasad 2004). Studies during the early phase of selection suggested that the FEJs were indeed evolving towards the predicted optimal life-history over the first 30 generations of selection (Prasad 2004). After 10 generations of selection, the lifespan of mated FEJ females had declined by 20%

relative to the JBs, whereas male lifespan did not differ between the two sets of populations. By generation 30, both mated males and females lived significantly shorter than their JB counterparts, but FEJ females produced more eggs per unit body weight compared to the JBs in early life, indicating a well adapted life-history under the given selection regime. The longevity of virgin FEJs and JBs assayed after 20 generations of selection did not show any significant difference suggesting that the longevity reduction in FEJs relative to JBs in mated condition was indeed mediated through reproduction. However as selection proceeded, FEJs subsequently moved towards a life-history that did not conform to an optimal allocation pattern. At generation 70, FEJ longevity was found to have reverted back to the JB level, while FEJs produced significantly less eggs per unit body weight than the JBs in early life, suggesting the evolution of a very maladaptive strategy under the FEJ regime (Prasad 2004).

The following hypothesis was proposed to explain this surprising turn from an optimal life-history toward a maladaptive one during the course of FEJ selection (Prasad 2004). The altered adult life-history of the FEJs was thought to be due to the presence of a physiological mechanism, perhaps via a 'lipid switch', which determines the relative allocation of lipids to reproduction versus somatic maintenance based on the amount of lipid present in the body. Above a certain threshold level of lipid, allocation is expected to be biased towards reproduction whereas below the threshold allocation is biased towards somatic maintenance. Such switching in relative allocation to reproduction versus somatic maintenance in *Drosophila* in response to nutritional status has been documented previously in phenotypic manipulation studies (Chippindale *et al.* 1997b) and in flies grown under crowded conditions (Zwaan *et al*. 1991). In the course of FEJ selection, the FEJs evolved a substantially reduced body size and in lipid content. It was postulated that in the initial phase of selection lipid levels of FEJs declined but

remained above the 'switch' threshold, favouring allocation towards reproduction, as evident at generation 30; between generations 30 and 70, however, lipid levels fell below the 'switch' threshold, altering the allocation pattern, such that resources were preferentially allocated for somatic maintenance as opposed to reproduction. This was thought to be the reason for the reversal of the FEJ lifespan to JB levels despite experiencing an initial reduction during selection. The preferential allocation for somatic maintenance in FEJs was further corroborated by the observation that FEJs had a significantly higher starvation resistance per unit lipid compared to the JBs at generation 50 and also at generation 125. The 'lipid switch' might have evolved in the ancestors of these flies under natural selection in wild conditions, where nutritional levels presumably fluctuate over time (Prasad 2004). Thus, a physiological mechanism, presumably evolved due to past selection, became a constraint under the changed context of selection in the laboratory.

Within the framework of the 'lipid switch' hypothesis, genotypes that would have been able to allocate a little more towards early fecundity rather than longevity were likely to have been favoured by ongoing selection in the subsequent generations. The expectation, therefore, was that, given enough time, the FEJs might evolve to circumvent the maladaptive effects of the lipid switch. In view of this, I studied various adult traits of FEJ and JB populations after 270 generations of selection. The questions that I attempt to address in this section are:

(1) Did selection bring about further changes in the adult body weight and lipid content of FEJs over a further 200 generations of selection?

(2) How were the longevity, fecundity and starvation resistance of the FEJ populations relative to JBs after 270 generations of FEJ selection? Was the ongoing selection able to move FEJs away from their previously evolved maladaptive life-history?

Materials and methods

Dry weight and lipid at eclosion

Eggs were collected from standardised flies of at a density of 30 eggs per vial. Freshly eclosed adults were collected and freeze-killed. Males and females were separated in batches of 20 flies per vial and eight such vials were set up per population for each sex. The flies were then dried at 70^{o}C for 36 h and weighed, and the weights divided by 20 to obtain the average weight per fly. Lipid content of the flies used in the dry weight assay was also estimated. Lipid estimation was done following Zwaan *et al.* (1991). After taking the dry weight measures, the flies were placed in excess of di-ethyl-ether and lipid was extracted over a period of 48 h. Ether was changed every 12 h. The flies were then removed from the ether, dried at 70^{o}C for about 12 h and weighed to obtain lipid-free dry weights. The difference between dry weight before and after the extraction was taken as the total lipid content. Fractional lipid content was estimated by dividing the mean lipid content by dry body weight.

Lifespan

Lifespan of flies was assayed under mated conditions. Freshly eclosed flies obtained from the eggs of standardised flies were introduced into vials containing about 4 mL of banana jaggery food, at a density of four males and four females per vial. Each population contained ten such vials containing eight flies. Mortality of these flies was checked daily, and they were transferred to fresh food vials every alternate day.

Starvation resistance

Starvation resistance of freshly eclosed flies was assayed in unmated condition. Freshly eclosed flies were placed into vials containing 6 mL of non-nutritive agar to prevent desiccation. Six such vials per population containing five flies each were set up for each sex. The set up was done within 6 h after eclosion to ensure virginity. Mortality was scored at 4 h intervals until all five flies in the vial died. Mean time to death under starvation was calculated for each vial and the vial means were averaged to obtain the replicate population means.

Fecundity

At generation 250, daily fecundity of the populations was assayed till day 27 of adult age and the total number of eggs laid during this period was compared between the two selection regimes. Since females laid few eggs beyond this age in both the populations and many individuals died by this age in FEJ, reducing the sample size, the assay was discontinued beyond this point and total number of eggs laid till this age were treated as a representation of the lifetime egg production by the two populations. Flies eclosed from eggs collected from standardised flies were used for the assay. One day post-eclosion, one male and one female were introduced into a vial containing 2 mL of banana-jaggery food. Twenty such vials were set up per population. The eggs laid in each vial by the female in a 24 h period were counted under a microscope and the flies were transferred to fresh vials containing food at about the same time every day. If a male died or escaped then it was replaced with a virgin male of the same age. If a female escaped during transfer to fresh food vials, the data from such vials were not used in the analysis. The total number of eggs laid by per female was averaged across 20 vials and used for the analysis.

After 270 generations of selection, fecundity at early age was measured for FEJ and JB. The number of eggs laid was counted for 2nd and 3rd day of their adult life. For each vial, the average of the two days was taken and treated as the mean early fecundity, and the replicate population means were subsequently calculated.

Statistical analysis

The data for lifespan, starvation resistance, dry weight and lipid were analysed 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 were analysed using a two-way ANOVA with selection regime as a fixed factor crossed with random blocks.

Results

Dry weight and lipid at eclosion

FEJ body weight underwent further reduction since generation 70, with the flies weighing 59% less than the JBs at eclosion in both the sexes at generation 270 (Figure 3.1a). ANOVA revealed significant effects of selection and sex on dry weight (Table 3.1). Females were heavier than the males in both the regimes, but selection led to a reduced degree of sexual dimorphism in FEJ reflected in the significant selection × sex interaction (Table 3.1).

Absolute lipid content of the FEJs at eclosion was about one-sixth of that in the JBs (Figure 3.1b). ANOVA results showed significant main effects of selection and sex. Females had higher absolute lipid than the males. The selection × sex interaction was also significant, owing to a reduced degree of sexual dimorphism in FEJs for lipid content compared to JBs. JB males had

13% less lipid than the JB females whereas body-lipid in FEJ males was only 4% less than their female counterparts (Table 3.1).

Contrary to previous observations (Sharmila Bharathi *et al.* 2003), fractional lipid content of males was significantly higher than the females in both the FEJs and JBs. 25% and 22% of the total dry body-mass of JBs comprised of lipid in males and females respectively (Figure 3.1c). FEJs had very low fractional lipid content at eclosion, with males and females showing 9.7% and 8% fractional lipid (Figure 3.1c, Table 3.1).

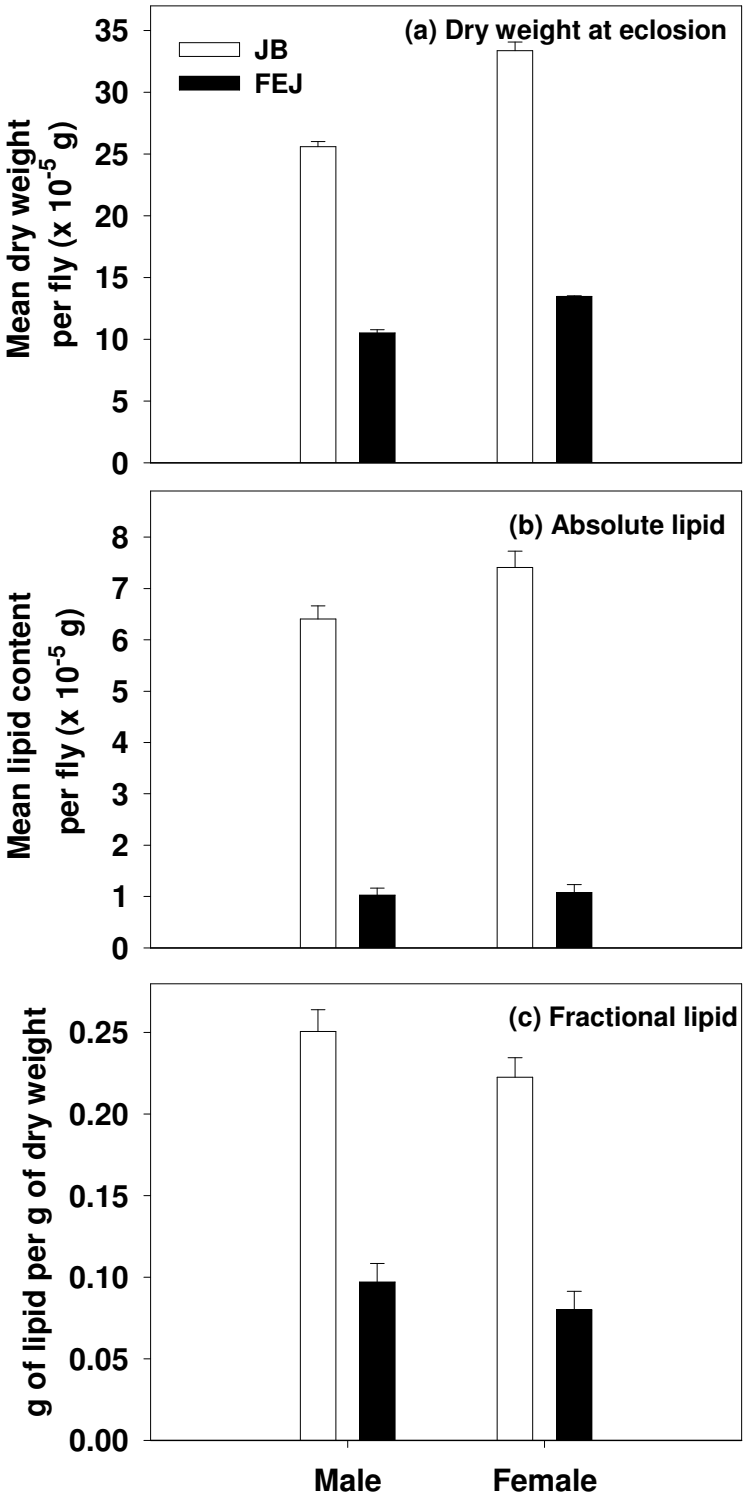

Figure 3.1: Mean (± s.e.) (a) dry weight, (b) absolute lipid, and (c) fractional lipid content per fly at eclosion in FEJs and JBs after 270 generations of FEJ selection.

Trait	Effect	*df*	MS	*F*	*P*
Dry weight at eclosion	Selection	1	1223.653	981.699	< 0.0001
	Sex	1	114.87	473.319	0.0002
	Selection × sex	1	23.235	287.187	0.0004
Absolute lipid	Selection	1	137.094	680.783	0.0001
	Sex	1	1.119	29.469	0.0123
	Selection × sex	1	0.919	23.023	0.0172
Fractional lipid	Selection	1	0.088	127.679	0.0015
	Sex	1	0.002	35.617	0.0094
	Selection × sex	1	0.0001	2.174	0.2368

Table 3.1: Summary of results of separate ANOVAs on mean dry weight, absolute lipid and fractional lipid at eclosion. Block effect and interactions involving block could not be tested for significance.

Longevity

After 270 generations of selection, lifespan of the FEJs was significantly less than that of the JBs (Table 3.4). The mean lifespan of JB males and females was 40.8 and 36.8 days, respectively, whereas average lifespan of FEJ males and females was 34.4 and 28.4 days (Figure 3.2).

Effect	*df*	MS	*F*	*P*
Selection	1	218.396	13.999	0.0333
Sex	1	100.987	7.258	0.0742
Selection × sex	1	3.509	0.391	0.5762

Table 3.2: ANOVA results for mean lifespan of FEJ and JB populations at generation 270. Only fixed factor effects could be tested for significance.

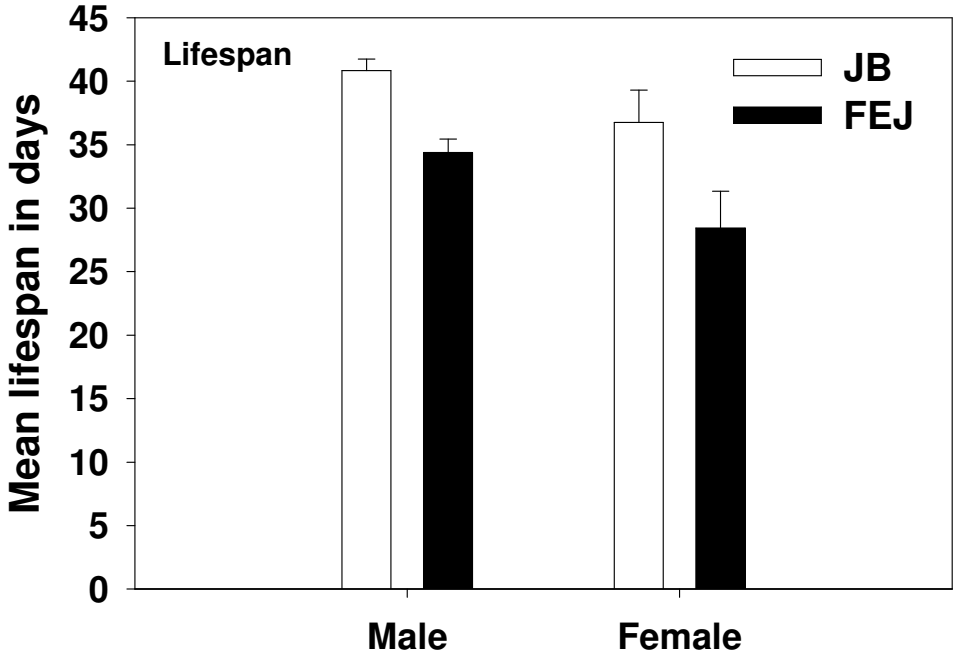

Figure 3.2: Mean (± s.e.) lifespan of FEJ and JB populations at generation 270.

Starvation resistance

Among virgins, females lived significantly longer than males under starvation (Table 3.3). JB males and females, on average lived for 121 and 141 h, respectively, under starvation (Figure 3.3a). The corresponding figures for FEJ males and females were 55 and 68 h, respectively (Figure 3.3a).

Starvation resistance per unit lipid was estimated by dividing the starvation resistance in hours of starvation by lipid content at eclosion. Although the absolute starvation resistance of FEJs was much lower compared to the JBs, when scaled by the lipid content present at eclosion, FEJs showed a much greater starvation resistance (Table 3.3). Starvation resistance per unit lipid in FEJs was 66% higher than the JBs for males, with females of FEJs showing a 71% higher starvation resistance per unit lipid than their JB counterparts (Figure 3.3b).

Trait	Effect	*df*	MS	*F*	*P*
Absolute starvation resistance	Selection	1	19488.781	300.502	0.0004
	Sex	1	1109.482	67.977	0.0037
	Selection × sex	1	58.404	2.409	0.2185
Starvation resistance per unit lipid	Selection	1	7109.245	26.425	0.0143
	Sex	1	99.307	9.814	0.0519
	Selection × sex	1	87.483	11.308	0.0436

Table 3.3: ANOVA results for mean absolute starvation resistance and starvation resistance per unit lipid of FEJs and JBs and relative starvation resistance at generation 270. Block effects could not be tested for significance.

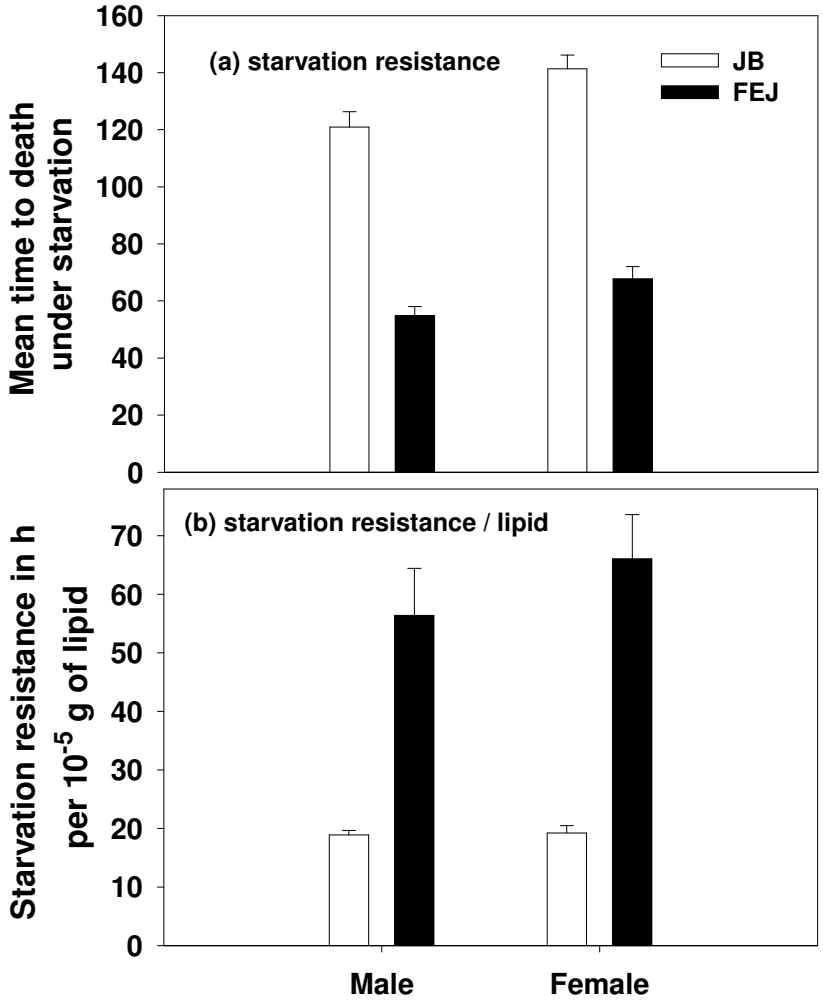

Figure 3.3: Mean (± s.e.) (a) absolute starvation resistance and (b) starvation resistance per unit lipid of FEJs and JBs after 270 generation of selection.

Fecundity

The mean total number of eggs laid by JB females over the first 27 days of adult life was 297, whereas FEJs laid 113 eggs on average during this period, after 250 generations of FEJ selection (Figure 3.4a).

Mean early fecundity of FEJ females after 270 generations was about one-third of that of the JBs (Figure 3.4b). Similar to starvation resistance, when early fecundity was scaled by the amount of lipid present in the body, eggs produced per unit lipid in the FEJs were three-fold higher relative to the JBs (Figure 3.4c). ANOVA results showed significant effects of selection in all the three cases (Table 3.4).

Trait	Effect	*df*	MS	*F*	*P*
Total fecundity	selection	1	67521.641	51.953	0.0055
Early fecundity	selection	1	750.73	307.112	0.0004
Eggs per unit lipid	selection	1	59.921	20.649	0.0199

Table 3.4: Summary of separate ANOVAs done on total fecundity at generation 250, early fecundity and early fecundity per unit lipid at generation 270. Block effects could not be tested for significance.

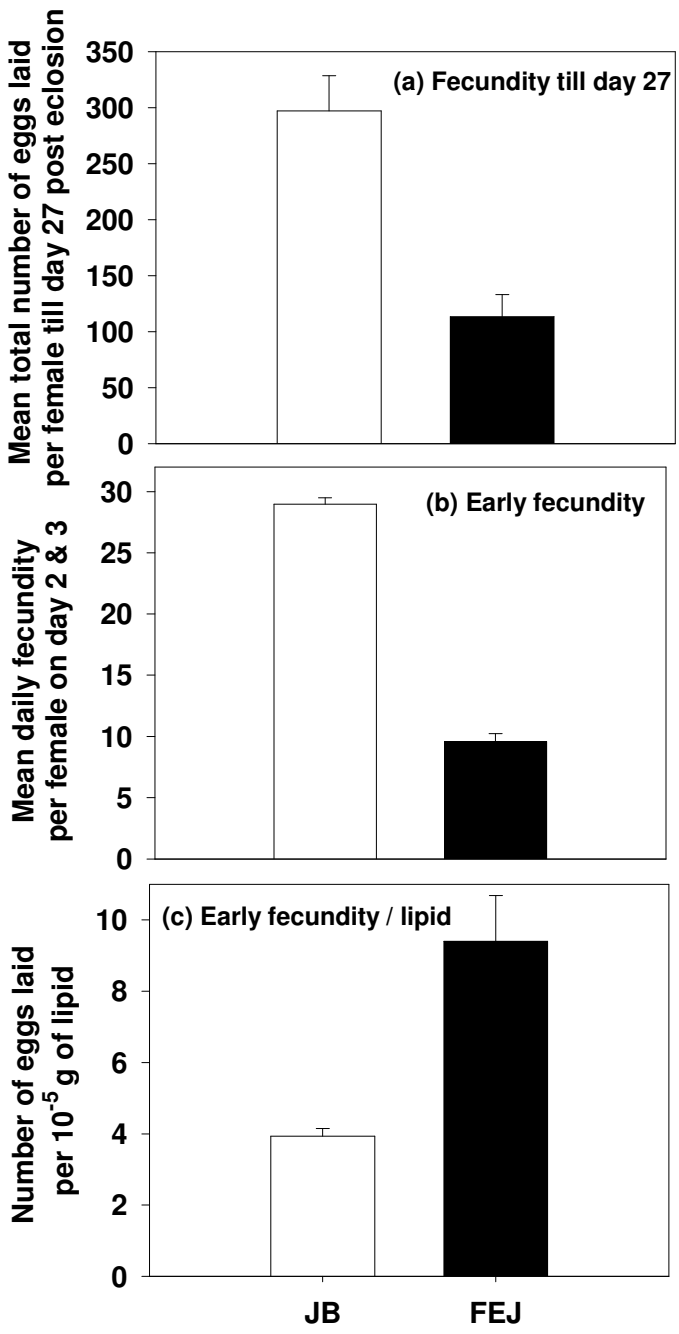

Figure 3.4: Mean (± s.e.) (a) total number of eggs laid per female till 27th day of adult age at generation 250, (b) eggs laid in 24 h averaged over day 2 and 3 post-eclosion, and (c) number of eggs laid in 24 h, averaged over day 2 and 3 post-eclosion, per unit lipid present at eclosion per female in the FEJs and JBs after 270 generations of FEJ selection.

Discussion

Adult size in *Drosophila* is thought to have evolved as a compromise between the need to develop fast under competitive conditions in nature and the fitness cost associated with small body size resulting from fast development (Robertson 1957; Partridge *et al.* 1987a; Santos *et al.* 1997). However, in the FEJ regime, there is an extraordinary fitness premium on developing fast, such that body size was greatly reduced over generations. This, in turn, affected various adult traits. Along with the evolution of small body size, absolute lipid content of freshly eclosed FEJs also underwent a correlated reduction. The lipid content underwent a greater degree of reduction than the body size, as reflected in the significant reduction of fractional lipid content in the FEJs at generation 125 (Prasad 2004). With continuous reduction of development time, absolute and relative body-lipid at eclosion decreased further in FEJs after 270 generations of selection.

In such a condition, according to the 'lipid switch' hypothesis, one would expect the FEJs to have continued to show a preferential allocation for somatic maintenance rather than reproduction. On the contrary, the results from generation 270 assays showed that FEJ lifespan was significantly lower than that of the JBs, whereas eggs produced per unit lipid in early life was three times higher in FEJs compared to JBs. Though the FEJs experienced an additional 30% reduction in their lifetime fecundity in last 200 generations, early fecundity remained relatively unchanged during this period. At generation 70, early fecundity in FEJ was about one-third of that of the JBs, which seemed to be the case at generation 270 as well. Thus, early fecundity did not get affected by further reductions in body size and lipid content in last 200 generations. It is possible in principle, that the fecundity at the time of egg collection in FEJ *i.e.* day 3 post-eclosion would have already reached a minimum by generation 70. Any further reduction in fecundity at this age would have risked extinction of these populations. Hence,

selection would have favoured individuals who could invest to produce enough eggs at this age, curtailing fecundity at later ages and lifespan. This is corroborated by the fact that, after 270 generations of selection, mean number of eggs produced per unit lipid in early life was three-fold higher in the FEJs compared to the JBs.

Thus, the FEJs seemed to have reverted back to a more optimal life-history between generations 70 to 270 of selection, suggesting that the 'lipid switch' constraint eventually got circumvented by ongoing selection. Studies on reverse evolution had suggested earlier that the genetic imprint of history tends to be transient in the face of selection, especially for traits closely related to fitness (Teótonio and Rose 2000; Teótonio *et al.* 2002; Joshi *et al.* 2003). Supporting this view, this study suggests that constraints evolved due to past adaptation can, in time, be ameliorated by selection even though in short term they can cause evolution of maladaptive strategies.

The other surprising revelation from this study is that both egg production per unit lipid (indicating allocation to reproduction) and starvation resistance per unit lipid (indicating allocation to somatic maintenance) were much higher in the FEJs compared to the JBs after prolonged selection. There are multiple possible explanations for this observation. First, starvation resistance of these populations at generation 270 was assayed in virgin flies and, hence, this assay might have failed to reflect any existing trade-off between the lipid allocation for starvation resistance and reproduction that would otherwise be found in reproducing flies. The second possibility is that severe resource crisis in FEJs would have led to the evolution of a greater efficiency of lipid usage in these populations over the course of their laboratory evolution beyond generation 70, favouring both the traits at the same time. Alternatively, even though FEJs produced more eggs per unit lipid, the provisioning for each egg might have become lower than that of the JBs in response to reduced lipid levels, leading to a greater productivity per unit lipid

present in these populations. Further studies estimating the lipid content of eggs in FEJs and JBs can possibly provide clearer explanations for this observation.

This study shows how selection operating on stages affecting resource acquisition can lead to changes in resource allocation patterns bringing significant changes in the life-history. It also shows that adaptive changes of the past can become constraints with a changing selective scenario, impeding adaptive evolution, but that such constraints can eventually get ameliorated by ongoing selection. Selection experiments, if continued for long, can thus reveal interesting changes in the evolutionary trajectory of populations, increasing our knowledge about the subtlety of the microevolutionary process.

SECTION B: A possible trade-off between developmental rate and pathogen resistance in *D. melanogaster*

Introduction

In recent years, possible costs of investment in immune responses have been receiving attention in the context of life-history evolution, with immunocompetence now believed to trade off with major life-history related traits (Sheldon and Verhulst 1996; McKean and Nunney 2001; Møller *et al.* 2001; Zuk and Stoehr 2002; Šimková *et al.* 2008). Since pathogens and parasites are ubiquitous in nature, the ability to resist them is likely to be an important component of fitness, along with other life-history related traits. In *Drosophila,* many species of which breed on or around decaying fruits and domestic garbage dumps, exposure to microbial pathogens and, hence, selection for pathogen resistance, is expected to be high. Laboratory selection experiments over the past few decades have greatly enhanced our understanding of life-history evolution and patterns of trade-offs among life-history-related traits in *Drosophila* (reviewed in Prasad and Joshi 2003). There is also evidence that reproductive activity reduces immunocompetence in *D. melanogaster* (McKean and Nunney 2001). There are, however, no studies on the genetic correlations between pathogen resistance and major life-history traits in *Drosophila*.

The view that pathogen resistance may trade off with other traits important to fitness is strengthened by the observation that although directional selection plays an important role in immune system evolution (Schlenke and Begun 2003), organisms generally do not exhibit maximal immune responses (reviewed in Zuk and Stoehr 2002). The evolutionary cost of maintaining and mounting immune responses is thought to be due to conflicting demands on

resource allocation to immune function versus other life-history traits, leading to suggestions that immune function may trade off with traits like competitive ability and developmental time (Roff 1992). Empirical evidence for such trade-offs between immune function and life-history traits is, as yet, meagre. *D. melanogaster* larvae selected for increased resistance against parasitoid wasps have been shown to be less competitive under crowding (Kraaijeveld and Godfray 1997). Immunocompetence has also been shown to trade off with reproductive investment in yellow dung flies, *Scathopaga sterocoraria* (Hosken 2001), and selection for higher resistance in the Indian meal moth, *Plodia interpunctella*, resulted in correlated evolution of longer development time (Boots and Begon 1993).

Selection for faster development in *Drosophila* typically results in the evolution of smaller adult body size (Chippindale *et al.* 1997a; Prasad *et al.* 2000). Reduced energy reserves due to faster pre-adult development are likely to provide the basis for a trade-off between developmental rate and adult traits requiring allocation of reserves accumulated during the larval stage (Prasad and Joshi 2003). It has recently been shown that recording the time to death of *Drosophila* reared in the presence and absence of *E. coli* constitutes a reliable mass-scale assay of immune competence: rearing in the presence of live *E. coli*, but not streptomycin attenuated or heat-killed *E. coli*, reduces the time to death of adult flies under starving conditions (Sharmila Bharathi *et al.* 2004, 2007). In this study, I examine the genetic correlation between developmental rate and pathogen resistance in *D. melanogaster*, by assaying pathogen resistance in FEJ and JB populations.

Materials and methods

I assayed the time to death in the presence and absence of growing *E. coli* culture on virgin male and female flies from the FEJs and JBs. Eggs collected from standardised flies were distributed

into vials at a density of 60-80 eggs per vial, and freshly eclosed (within 6 h post-eclosion) adults from these vials were used to set up the pathogen resistance assay, following the methods of Sharmila Bharathi *et al.* (2004). For each combination of block × selection regime × sex, eight vials were set up with 3 mL of Luria Bertani (LB) agar medium containing ampicillin on which *E. coli* strain DH5α carrying an amp^R gene was streaked. These vials were then incubated at 37°C for 24 h to allow bacterial growth. At this point, a lawn of bacteria was visible on the surface of the medium in the vials. Eight control vials containing LB agar with ampicillin, but not inoculated with *E. coli*, were also set up in a similar manner for each block × selection regime × sex combination. In each treatment or control vial, either five males or five females were placed, and the vials were then monitored every 2 h, and the death of any fly during the previous 2 h period recorded. This process was continued until all the flies had died. Thus, time to death was recorded for a total of 1280 flies (4 blocks × 2 selection regimes × 2 sexes × 2 assay conditions × 8 vials, with 5 flies each).

Statistical analysis

To compare pathogen resistance across selection regimes and sexes, I transformed the primary data on time to death of individuals in the vials with *E. coli* by dividing it by the mean time to death averaged across the control vials for that particular block × selection regime × sex combination, as in Sharmila Bharathi *et al.* (2004). The transformed data on individual flies were subjected to ANOVA, treating vials as a random factor nested within the three-way interaction between the fixed factors selection regime and sex, and the random factor, block.

Results

FEJs had a lower mean time to death (14 h) compared to the JBs (48 h) in the presence of *E. coli*, as well as in the absence of the pathogen (FEJ mean = 33 h; JB mean = 74 h). Scaling by time to death in the absence of the pathogen revealed that the JBs lived 65% and FEJs only 43% as long as their respective counterparts in the control vials (Figure 3.5). The ANOVA on this measure of pathogen resistance revealed a significant main effect of selection regime, but no significant effect of sex or the selection × sex interaction. Pathogen resistance of JB males and females was 66% and 65% respectively, whereas that of the FEJ males and females was 45% and 41%, respectively.

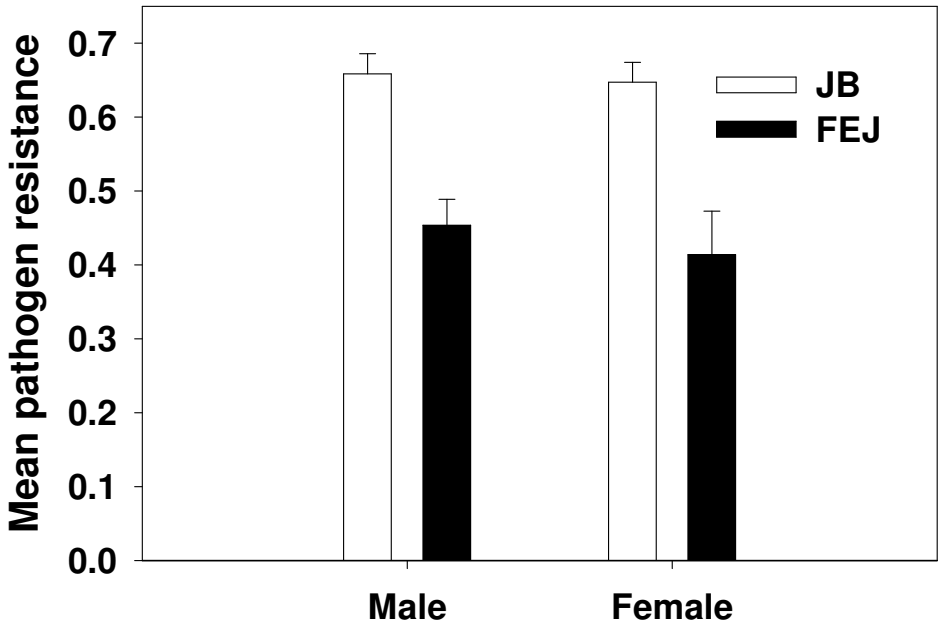

Figure 3.5: Mean (± s.e.) pathogen resistance measured as time to death in presence of *E. coli* expressed as a fraction of the time to death in absence of *E. coli*.

Effects	*df*	MS	*F*	*P*
Selection	1	7.682	16.858	0.0261
Sex	1	0.106	0.655	0.4776
Block	3	0.347	5.949	0.0008
Selection × sex	1	0.033	1.702	0.283
Sex × block	3	0.162	2.775	0.0447
Selection × block	3	0.456	7.818	< 0.0001
Selection × sex × block	3	0.019	0.328	0.8051

Table 3.5: Summary of results of ANOVA on mean pathogen resistance in the FEJs and JBs. Vial effect and interactions involving vial could not be tested for significance.

Discussion

It is clear from the results that the detrimental effect of *E. coli* was significantly more severe in FEJs, compared to JBs, reflecting a lower pathogen resistance in the FEJs. This result indicates a negative genetic correlation between developmental rate and pathogen resistance, complementing observations that selection for increased pathogen resistance leads to longer development time (Boots and Begon 1993). The physiological underpinnings of this trade-off between developmental rate and pathogen resistance, however, remain obscure. Given the substantially smaller size and lipid content of the FEJs, the trade-off is probably mediated through reduced resource availability for investment in immune function. It is also possible, however, that the FEJs, due to their much shorter duration of development (78% that of the JBs at the time of the assay), may have poor immunocompetence due to developmental anomalies. An alternative possibility that cannot be ruled out at this juncture is that the FEJs actually mount an equal or greater immune response than JBs to *E. coli*, and then succumb faster to the starving

conditions of the assay vials as they have less lipid reserves to start with. This possible explanation could be tested by directly assaying components of the immune response in JBs and FEJs. However, the present results clearly show that FEJs ultimately have reduced fitness in the presence of *E. coli*, compared to the JBs, at least under the starving conditions of this assay. Functionally, therefore, there is a possible trade-off between developmental rate and pathogen resistance in these populations, although whether it is underpinned by a trade-off between immunocompetence and developmental rate is not as yet clear.

SECTION C: Evolution of early the fecundity peak in *D. melanogaster*: a case study

Introduction

The distribution of fecundity along the age axis in wild-caught *Drosophila*, as well as those maintained in the laboratory tends to be positively skewed (David *et al.* 1974). Mated females of *D. melanogaster* start laying eggs within one or two days post-eclosion. Oviposition soon peaks sharply within a few days, followed by a decline as the female ages. The distribution of fecundity over age thus takes the shape of a triangle. Generally, such a triangular shape of the lifetime reproductive schedule characterized by an early peak, is a typical feature of iteroparous insects (Roff 1992; Kindlemann *et al*. 2001).

Survival in wild populations is highly stochastic owing to risks of predation and food scarcity, such that the cumulative probability of survival to an advanced age tends to be low (Rosewell and Shorrocks 1987). Hence, selection in nature is expected to favour individuals who invest more in early reproduction even if it inflicts costs on later reproduction and potential longevity (Williams 1974). This might have led to the evolution of an early fecundity peak in wild insect populations including *Drosophila.* However, under the benign environment of laboratory cultures, apart from aging, potential risks like predation etc. that can curtail the late-life survival or reproductive output do not exist. Hence, in laboratory populations with overlapping generations, eggs laid at any time in life have a good chance of making it to the next generation. Populations maintained under such conditions also generally show a fecundity peak in early life. On the other hand, in laboratory populations reared with discrete generations, only eggs laid on a particular day contribute to the next generation. Optimality arguments would suggest that, in

such cases, fecundity on that particular day should evolve at the cost of fecundity on other days and longevity beyond that day. The trends observed from life-history studies do not conform to this simplistic expectation. For example., selection for late-fertility in O populations of Rose (1984) led to reduced early-life fecundity compared to the controls, but the early-life fecundity of the Os remained higher than their own late-fecundity even after several hundred generations of selection. Thus, irrespective of the relevance to fitness, an early peak in fecundity seems to be a fairly fixed trait in *Drosophila* populations.

The FEJs populations show a fecundity peak on day 3 post-eclosion. Given that these populations are selected for reproduction at this age, this is not surprising. On the other hand, the JBs maintained on a 21-day discrete generation cycle have evolved a second peak around day 11 post-eclosion, presumably as a response to selection for reproduction at this age (Sheeba *et al.* 2000; Prasad and Joshi 2003). However, even after being selected for reproduction at a later age for 300 generations, the magnitude this second peak has remained much smaller compared to the peak found in their early life (Sheeba *et al*. 2000; Prasad and Joshi 2003). In principle, individuals in JB populations that could have saved resources till about day 11 post-eclosion, and thus increased the second peak of daily fecundity at the expense of the first, would have been favoured under the given 21-day discrete generation regime. Clearly, that has not happened, similar to the finding made by Rose (1984) while selecting for late reproduction. Hence, I investigated what factors constrain the evolution of an ideal lifetime fecundity schedule for populations selected for reproduction at ages other than very early adult life? What controls this early fecundity peak in *D. melanogaster*? Are there mechanisms intrinsic to the fly's development or physiology that constrain the lability of the peak? I seek to find answers to these questions in this section.

In order to address these questions, I experimentally manipulated the age at which the flies experience their first mating. Flies were kept as virgins after eclosion and different batches of flies were supplied with mates at different ages. The daily fecundity of individual females was scored till the 27th day of their adult life. The fecundity for the whole tenure was plotted and observed to find out if the flies show a shift in the peak owing to the delay in mating onset, or they lose the peak from their fecundity profile altogether once the normal timing of the peak is over. The experiment was carried out on both the FEJs and JBs to check if populations selected for different reproductive schedules show any difference in their responses to such manipulations.

Materials and methods

Eggs were collected from standardised flies by placing a fresh food plate in the cages for 4 h. The eggs were then dispensed into vials containing 6 mL banana-jaggery food at a density of 60-80 eggs per vial. Forty such vials were set up per population. Egg collection for the FEJs and JBs was staggered according to the existing differences in their egg-to-adult development time, and flies eclosing at the middle of the emergence distribution were used for the assay. Males and females were separated using light carbon dioxide anesthesia soon after eclosion and kept in single sex-vials containing about 30 flies each. After the collection of the required number of flies was completed, three sets of treatment vials containing ~3 mL banana-jaggery food were set up with flies. In the first set, one male and female pair was introduced; hereafter this will be referred to as the normal mating regime. The second set called the delayed mating on day 5, comprised of a single female fly in a vial that was provided with a mate (previously unmated male) from the 5th day from the set up of the assay onwards. In the third set, each vial contained a female kept as a virgin throughout the tenure of the experiment. The above mentioned

treatments were employed for all the four replicate populations of the JBs and FEJs, and 20 vials were set up for each treatment. In a fourth treatment set, females were provided with a male from day 9 onwards, but this was done using only two replicate populations of JBs *i.e.* $JB_{3,4.}$ The daily fecundity of each female was scored by counting the number of eggs laid over a period of 24 h and the flies were transferred to fresh food vials daily at about the same time. The experiment was continued for 27 days. When a male died or escaped, it was replaced with a virgin male of the same age. If a female escaped during transfer to fresh food vials, the data from such vials were not used in the analysis.

Statistical analysis

Fecundity on specific days was compared among the first three different treatments, separately for each selection regime. Hence, separate ANOVAs were performed for the FEJs and JBs with fecundity of a specific age viz. day 2 and 6 for the JBs and day 3 and 6 for FEJs. Treatment was considered a fixed factor crossed with random blocks. For post-hoc comparisons among the three treatments for a given selection regime, Tukey's HSD test was used. No statistical analysis was performed for the set having onset of mating from day 9, but the data has been presented for visual representation.

Results

Delayed onset of mating pushed the fecundity peaks to a later age in both sets of populations. A distinct fecundity peak on day 2 was shown by JBs only under the normal mating treatment *i.e.* when they were allowed to mate right after eclosion (Figure 3.6). JB females housed with mates from eclosion onwards showed highest fecundity on day 2, with females laying 32 eggs on average on this day. Eggs laid by females in other treatment groups were negligible at this age,

with the mean number of eggs laid per female by virgins and those in the delayed mating group being 4 and 2 eggs, respectively. Flies allowed to mate from day 5 onwards showed a fecundity peak on day 6. Fecundity on day 6 was significantly higher for this treatment group (Table 3.6), with average fecundity being 41, whereas the mean number of eggs laid per female were 14 and 12 in virgins and flies under normal regime of mating onset, respectively (Figure 3.6). Eggs laid by the virgins showed a gradual increase at early age, but the daily egg output became steady within a few days and no distinct peak was observed in the egg output of the virgin females throughout life (Figure 3.6).

The fecundity pattern of FEJs under the manipulation experiment showed a similar trend, although the magnitudes of fecundity peaks were much smaller compared to the peaks observed in the JBs. Instead of day 2, FEJ fecundity peaked on day 3 of adult life (Figure 3.7); the smaller magnitude of the peak notwithstanding, FEJ females under normal mating regime laid significantly more eggs on day 3 (Table 3.7) than those laid by females experiencing delayed mating onset or virgins of the same age. In the treatment group, FEJ females introduced to mates 5 days post-eclosion laid 13 eggs on average (Figure 3.7), which was significantly higher (Table 3.7) than those laid by the other groups at the same age. As in the case of the JBs, virgin fecundity seemed to lack any distinct peak throughout life in the FEJs (Figure 3.7).

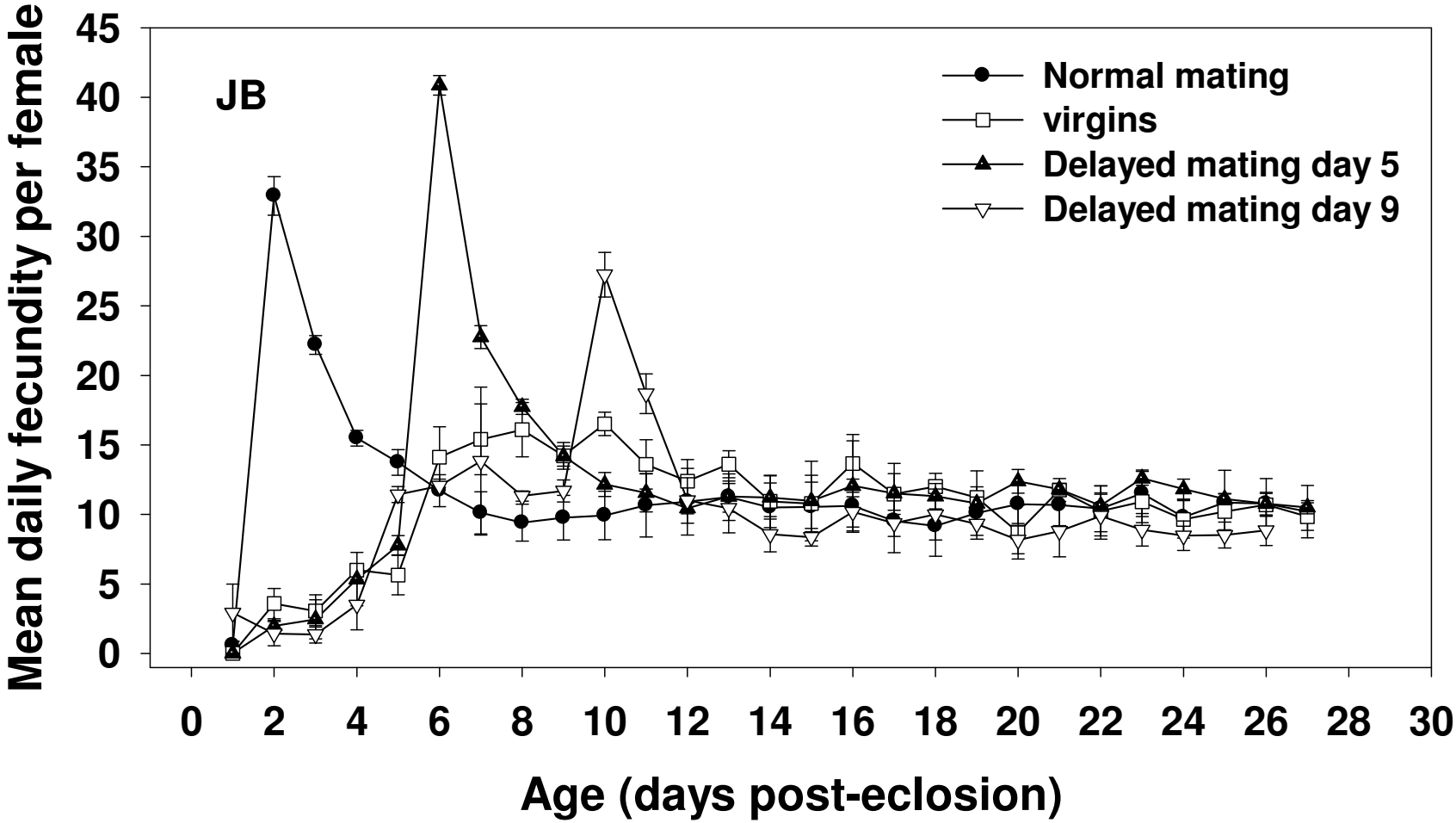

Figure 3.6: Mean (± s.e.) daily egg output per female in the JBs. The error bars in first three groups (normal mating, virgin & delayed mating on day 5) show standard errors for the four replicate population means. For the fourth group *i.e.* delayed mating on day 9, the means and standard error were calculated using two replicate population means.

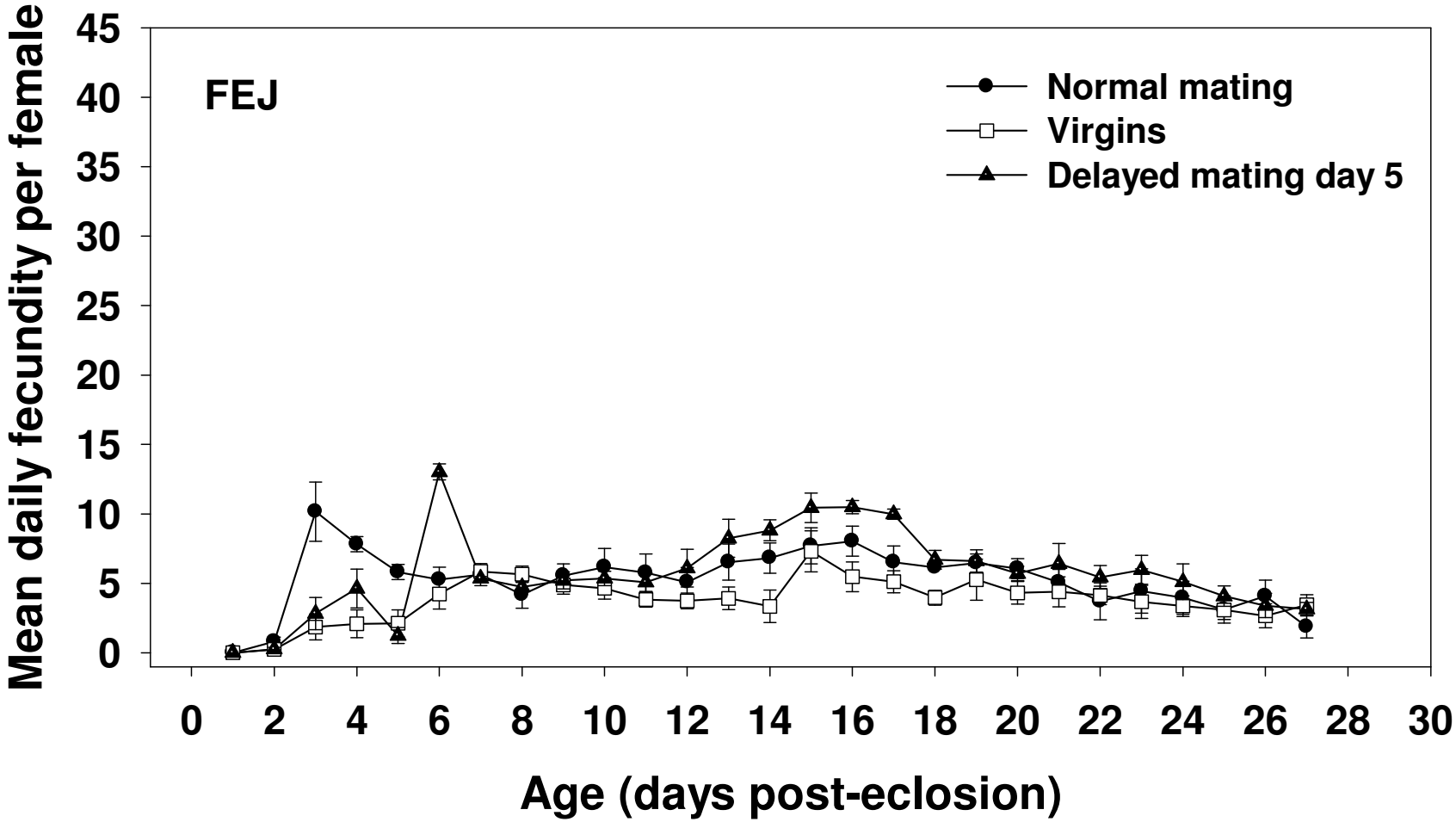

Figure 3.7: Mean (± s.e.) daily egg output per female in the FEJs, averaged across the four replicate populations (blocks). The error bars represent the standard errors for the four replicate population means.

	Effect	*df*	MS	*F*	*P*
Day 2 fecundity	Mating regime	2	1204.273	249.753	<0.00001
Day 6 fecundity	Mating regime	2	1046.776	143.99	<0.00001

Table 3.6: Results of separate ANOVAs for mean fecundity per female on day 2 and 6 in the JBs under three different mating regimes. Only the fixed factor effect could be tested for significance.

	Effect	*df*	MS	*F*	*P*
Day 3 fecundity	Mating regime	2	82.483	37.526	0.0004
Day 6 fecundity	Mating regime	2	92.259	76.382	<0.0001

Table 3.7: Results of separate ANOVAs for mean fecundity per female on day 3 and 6 in the FEJ under three different mating regimes. Only the fixed factor effect could be tested for significance.

Discussion

Peak fecundity in female *D. melanogaster* appears to be tightly synchronized with the onset of mating. Egg output data of virgins observed in this study support the suggestion that the reproductive machinery is probably designed to produce eggs at a constant rate (Novoseltsev *et al.* 2003). It is the introduction of the mates that results in a burst of egg output by the females, irrespective of their age. Flies allowed to mate right after eclosion in the experiment showed a fecundity peak within 2-3 days of adult life as normally observed in the populations of this lineage. Virgins failed to show any distinct peak, whereas flies experiencing a delay in their first mating by 5 days showed the peak on day 6. Further pushing the onset of mating to a later age *i.e.* at day 9, too, resulted in a peak in fecundity on day 10. Although the peaks in the JBs on day

2 and day 6 were very high, the magnitude had come down in the treatment in which the peak appeared on day 10. This is presumably an effect of aging. Further delaying the age of mating onset beyond a certain point might cause the peak to disappear altogether.

The elevated egg-laying rates of inseminated females have been reported by earlier studies (David 1963; Manning 1967; Partridge *et al.* 1986) and a specific male accessory gland protein present in the ejaculate has been shown to increase the egg-laying rate in flies mating for the first time (Wigby and Chapman 2005). In nature, apart from potential risks of predation and environmental adversities, the probability of finding a mate is not always high. Therefore, synchronizing the peak reproductive output with the onset of mating probably ensures that individuals do not waste too many unfertilized eggs until the time they mate and thus increase their fitness by laying a large number of fertilized eggs quickly after the first mating. Hence, the peak does not seem to be genetically programmed to occur in early life. Rather, selection seems to have shaped the responsiveness of the female reproductive machinery in such a way that it results in the maximum egg output soon after the commencement of mating, irrespective of the female's age, at least over the first 10 days or so of adult life, which may be the only relevant part of adult life given low life-expectancy in the wild (Rosewell and Shorrocks 1987). Thus the timing of fecundity peak in female *Drosophila* is likely to have evolved in nature as a result of selection driven by the stochasticity of mate-availability. Although individual variation is expected to exist for the age of finding a partner and mating onset, a majority of flies probably get chances to mate early in life in natural populations with an approximate 1:1 sex ratio. Thus, in studies involving wild-caught *Drosophila*, most often the fecundity schedule remains positively skewed, reflecting the average pattern. In laboratory populations, mating for most individuals almost invariably takes place soon after eclosion which triggers a burst of egg output.

Such coupling of the maximal reproductive output with the first instance of mating presumably driven by long history of selection in nature constrains the evolution of a reproductive pattern, optimal under current selection conditions, as observed in JB and O populations.

To summarize, past selection for coupling of the fecundity peak with the mating onset seems to hinder the lability of the reproductive peak in *D. melanogaster* and thus constrain the evolution of optimal lifetime fecundity patterns in laboratory populations subjected to selection for reproduction at relatively later ages. Long history of selection in the wild, where chances of finding a mate might not always be high seems to have led to this coupling, possibly ensuring the maximization of fertilized egg-output thereby increasing the fitness of the flies in natural populations.

Chapter IV

Effects of Temperature and Density on Development Time: Canalizing Influence of Selection

Introduction

The stability of phenotypic expression in the face of genetic and environmental perturbation is maintained by canalization (Waddington 1942, 1961). Canalization describes the processes by which phenotypic variation is reduced through developmental mechanisms (Stearns and Kawecki 1994). According to Waddington (1953), there are a number of probable pathways along which the development of a trait can proceed, depending on the epigenetic landscape, but there are mechanisms that canalize the trait expression to produce a certain optimal phenotype. Natural selection is thought to favour a system of canalization that helps to repress deviations from a phenotype optimal in the most common selecting environment (Eshel and Matessi 1998).

For a trait undergoing selection, deviations from an optimal phenotype by definition reduce its fitness (Stearns and Kawecki 1994). Any developmental mechanism that restricts the expression of the trait to be closer to the optimum is therefore expected to be favoured by selection (Rendel 1967), and the degree of canalization of a trait is predicted to be positively correlated with its impact on fitness (Waddington 1941; Schmalhausen 1949). Although considerable theory has been developed examining the relationship between selection and canalization, there still is a paucity of empirical data pertaining to the issue. One of the few empirical studies in this area examined age and size at eclosion, early and late fecundity, and lifespan in *D. melanogaster*, finding that, as predicted, canalization is greater for traits to which fitness is more sensitive (Stearns and Kawecki 1994; Stearns *et al.* 1995).

In the context of FEJ selection, an extraordinary fitness premium has been imposed on rapid development. The sharp cut-off for development time selection in FEJ ensures that flies not among the first 25% to eclose have zero fitness. In the JBs, by contrast, flies eclosing till 12 days

post egg collection, which practically includes the entire distribution of eclosing flies for *D. melanogaster* reared at 25^{o}C in uncrowded cultures, get to be part of the breeding population. Such a contrast in the fitness impact of pre-adult development time between the two sets of populations provides an interesting basis for studying the relationship of selection and canalization for development time. The question that I address here is whether prolonged selection for faster development has led to the canalization of development time in the FEJs. I therefore studied the development time of the FEJs and JBs after 300 generations of FEJ selection under nine combinations of temperature and density, namely 18^{o}C, 25^{o}C and 28^{o}C crossed with 30, 70 and 300 eggs per vial, and compared the relative degree of environmental canalization in the two sets of populations.

There is still much debate on how to define and infer canalization from empirical data (*e.g.* see Debat and David 2001; Dworkin 2005). In this study, rather than the absolute level of canalization for the trait, I am interested in the relative degree of canalization in development time shown by the FEJs and JBs across environments, a question which can be addressed in a relatively straightforward manner. One common way of empirically assessing canalization is via reaction norms, the array of phenotypes that a genotype produces when exposed to a gradient of an environmental factor (Woltereck 1909). Although reaction norms were originally used to compare the environmental sensitivity of different genotypes, they have subsequently been used to depict the plasticity of population means as well (Gebhardt and Stearns 1988; Stearns and Kawecki 1994; Stearns *et al.* 1995; Davidowitz and Nijhout 2004), and also to compare the plasticity of multiple species (David *et al*. 1997; Gibert *et al*. 2004). To infer canalization, I broadly followed the approach of Dworkin (2005), but without his emphasis on genotypes. According to Dworkin (2005), canalization can be addressed in two ways, namely the 'reaction

norm of the mean' (RxNM) and the 'variation' approaches. In the RxNM approach, the mean trait value of a line or population is plotted along an environmental axis. The more canalized the population or line is, the less the mean trait value changes across environments, thus showing a less curved reaction norm. For linear reaction norms, the smaller the slope, the greater canalization it reflects. In the 'variation' approach, the focus is on within line/population variation instead of mean trait value. The more uniform the variation a population shows against environmental changes, the more canalized it is.

Under the standard rearing environment condition, *i.e.* 25^{o}C and ~70 eggs per vial, not only do the FEJs show a significantly lower mean development time than JBs, there is no overlap in the distribution of the trait in the two populations. Difference in the variances of the two populations may, in principle, be attributable to the differences in trait values in FEJ and JB, rather than reflecting an actual contrast in the variability of the trait. Therefore, for comparing trait variation across selection lines and environments, I used the coefficient of variation (CV) rather than variance or standard deviation, as suggested by Dworkin (2005). The CV, being dimensionless, is often used while comparing the extent of variation in two populations, or the variability of two traits independent of the differences in their means (Sokal and Rohlf 1998). Stearns and Kawecki (1994) and Stearns *et al*. (1995) used the inverse of CV as a measure of canalization in various life-history traits. In this study, CV within populations was plotted across temperature and density gradients. The more the CV varied across environments, the less canalization it reflected.

Materials and methods

Development time

Eggs were collected from standardised flies following the method described in chapter II and dispensed into vials containing 6 mL of banana-jaggery food. Eggs were collected at a density of 30, 70 or 300 eggs per vial and incubated at one of three different temperatures — 18°C, 25°C and 28°C — for each density. Seven such vials were set up per replicate population for each of the nine combinations of temperature and density, for a total of 504 vials. The vials were monitored for the first eclosion and thereafter checked regularly at 4 h intervals and the number of eclosing males and females were recorded. The observation was continued until no fly eclosed for two consecutive days. Development time in hours was calculated by subtracting the mid-point of egg-lay from the time of eclosion.

Reaction norms for mean development time

Mean egg-to-adult development time for males and females was calculated separately for each vial, and vial means were averaged to obtain population means. Reaction norms for mean development time were plotted separately for temperature and density.

Linear regressions were fitted to reaction norms and the slopes of the regression lines were calculated. The greater the slope, the larger the deviation from a horizontal reaction norm, thus reflecting reduced canalization. Given that the aim of this comparison was to check how much the reaction norms deviated from horizontal, the signs of the slopes were ignored and only their absolute magnitudes considered.

Within-vial CV (coefficient of variation) for development time

In this study, each vial represented a sample distribution of development time for a given population at a particular combination of temperature and density. Hence, I calculated the standard deviation of development time for each vial and divided it by the respective vial mean to obtain the within-vial CV for the trait. Separate CVs were calculated for male and female development time and averaged across vials to derive the population mean for 'within-population' CV for each sex. Mean CV was expressed as a percentage by multiplying by 100 and plotted separately along temperature and density axes.

Statistical analysis

Both mean development time and CV for the trait were subjected to a five-way mixed model ANOVA with selection, temperature, density and sex treated as fixed factors crossed with random blocks. The units of analysis were replicate population means.

The slopes of the temperature reaction norms were subjected to ANOVA, treating selection and density as fixed factors crossed with random factor block. Similarly, for analyzing the slopes of the density reaction norms, selection and temperature were treated as fixed factors crossed with random blocks. Post-hoc comparisons were performed using Tukey's HSD test.

Results

Mean development time

FEJs developed significantly faster than JBs under all nine environmental conditions (Figure 4.1, Table 4.1). Across all nine assay environments, males took significantly longer than females to develop (Table 4.1). Mean development time decreased significantly with increasing temperature

(Figure 4.1, Table 4.1). Relative changes in mean development time across temperature were similar in the two selection regimes. From 25°C to 28°C, both FEJ and JB mean development time underwent ~19% reduction. From 25°C to 18°C, FEJ and JB means increased by 67.6% and 68.7%, respectively. Mean development time was less affected by increasing density than by changes in temperature. Development time did not undergo much change from 30 to 70 eggs per vial. However, the effect of higher density was much greater on the JBs (Figure 4.2). As the egg density rose from 70 to 300 eggs per vial, mean development time in the FEJs increased by only 1.6%, whereas the JB mean development was significantly lengthened by 9%. Although significant two-way and three-way interactions involving sex were seen (Table 4.1), trends for the two sexes were not qualitatively different in any of the comparisons.

Slopes of reaction norms

The FEJs consistently showed slopes closer to zero (horizontal reaction norm) than JBs, along both the temperature and density axes (Tables 4.2 to 4.5; Figures 4.1, 4.2), reflecting reduced deviation of mean development time across environmental changes. Males showed significantly higher slopes than females across temperature (Table 4.4). Multiple comparisons revealed that the slope of temperature reaction norms for the JB differed significantly across density, while the slopes of the FEJ temperature reaction norm remained similar at all three egg densities (Table 4.2). JB females showed significantly higher density slopes for development time than males, whereas in the FEJs, density slopes were similar across sexes resulting in a significant selection × sex interaction for slope (Table 4.5). The density reaction norms of the FEJ means remained similar at all three temperatures. In contrast, the JB reaction norms got significantly steeper at

18°C compared to 25°C and 28°C, indicating that the effect of density on mean development time in JB was most pronounced at 18°C (Figure 4.2).

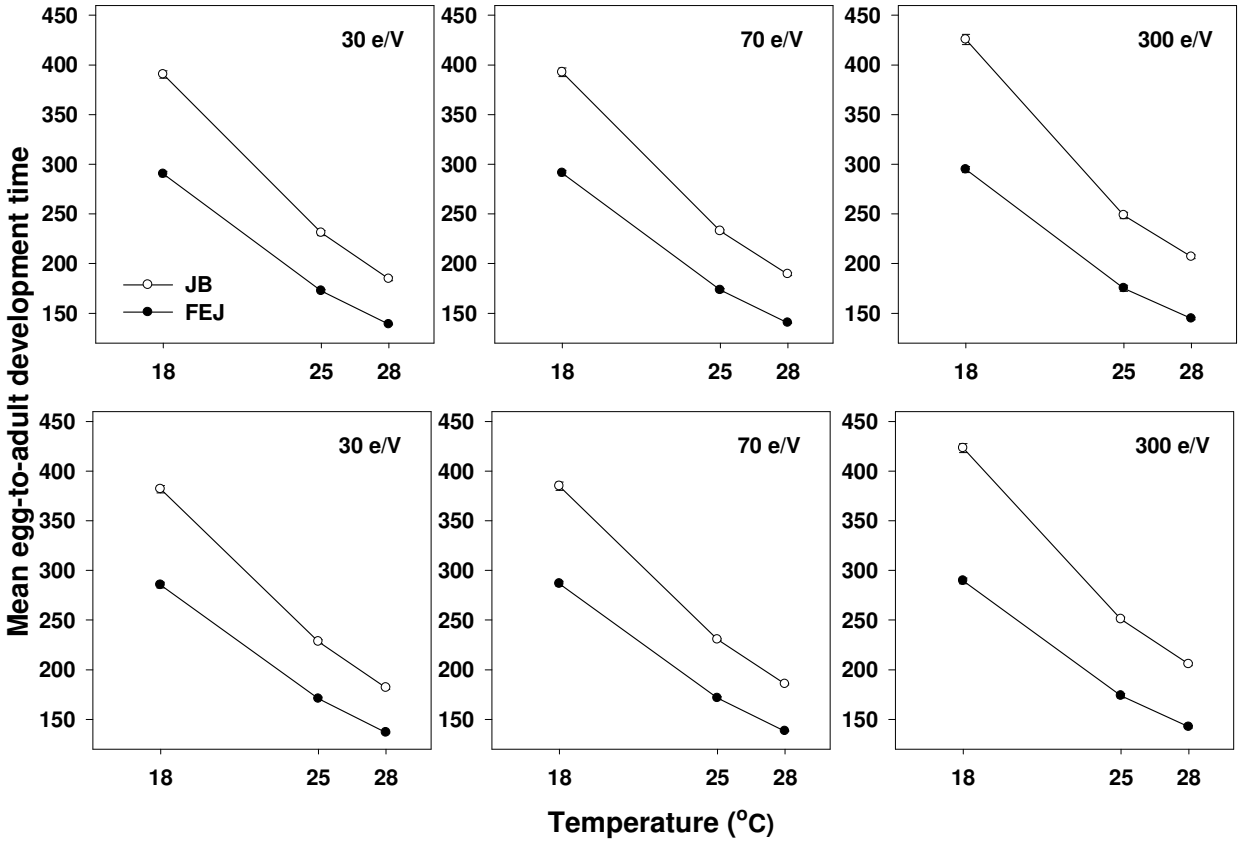

Figure 4.1: Mean (± s.e.) egg-to-adult development time of FEJs and JBs across temperature, for the three egg densities. The upper row shows the plot for male development time; the lower row shows development time of females. Error bars depict standard errors across the means of the four replicate populations; standard errors being very small, most of the error bars are not visible in the given scale. (e/V: eggs per vial)

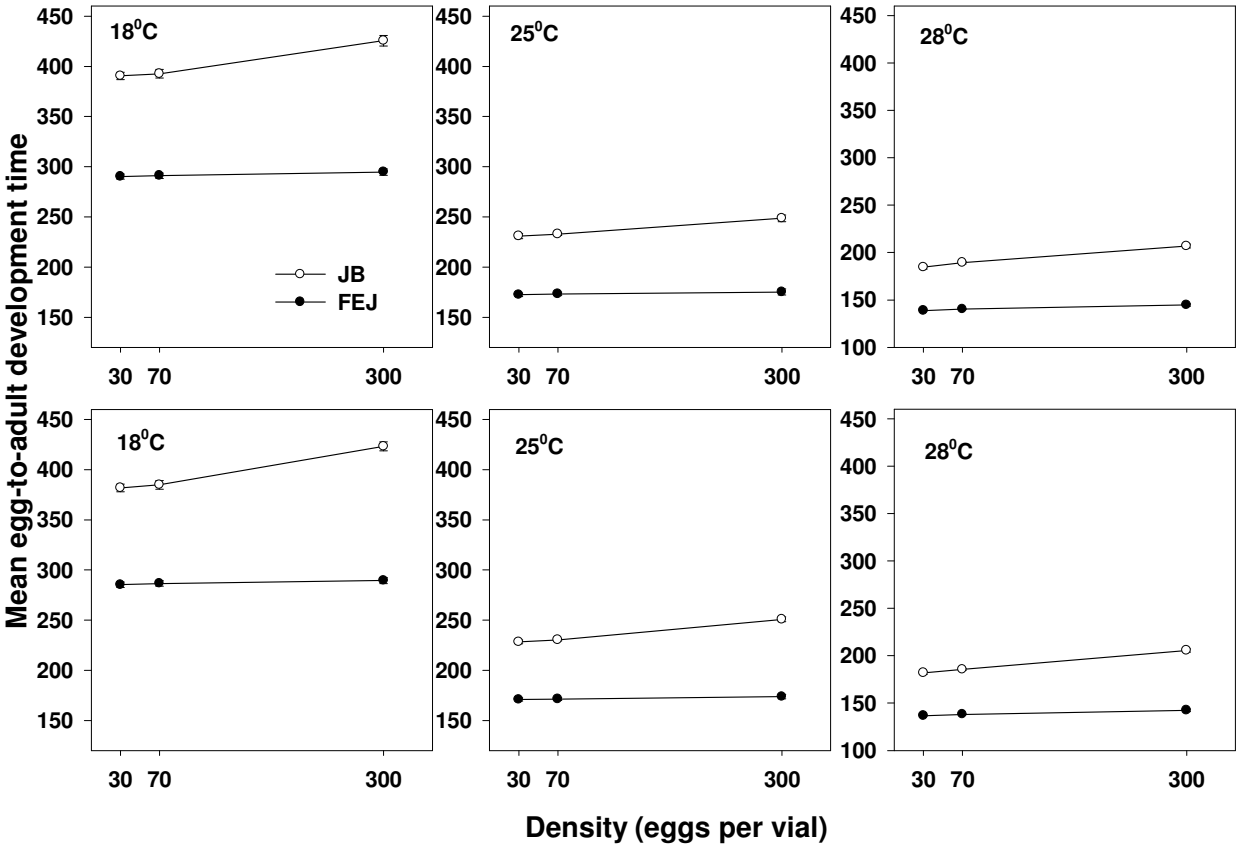

Figure 4.2: Mean (± s.e.) egg-to-adult development time of FEJ and JB populations across density at each treatment temperature. The upper panel shows the plot for male development time; the lower panel shows development time of females. Error bars depict standard errors across the means of the four replicate populations; standard errors being very small, most of the error bars are not visible in the given scale. (e/V: eggs per vial)

Effect	*df*	MS	*F*	*P*
Selection	1	205094.281	5115.049	<0.001
Temperature	2	422861.594	2184.462	<0.001
Density	2	3523.17	149.966	<0.001
Sex	1	337.231	109.109	0.002
Selection × temperature	2	11255.293	1995.867	<0.001
Selection × density	2	1925.725	71.384	<0.001
Temperature × density	4	136.151	11.526	<0.001
Selection × sex	1	1.164	0.727	0.456
Temperature × sex	2	61.56	67.178	<0.001
Density × sex	2	17.256	9.585	0.014
Selection × temperature × density	4	128.987	14.783	<0.001
Selection × temperature × sex	2	3.418	30.959	0.001
Selection × density × sex	2	18.933	85.599	<0.001
Temperature × density × sex	4	1.673	1.012	0.439
Selection × temperature × density × sex	4	1.519	5.297	0.011

Table 4.1: Results of ANOVA for mean development time. Only fixed factor effects could be tested for significance.

JB	30	20.655	(± 0.366)	FEJ	30	15.279	(± 0.289)
JB	70	20.535	(± 0.429)	FEJ	70	15.252	(± 0.321)
JB	300	22.371	(± 0.329)	FEJ	300	15.213	(± 0.319)

Table 4.2: Mean slopes of temperature reaction norms of development time at each egg density, averaged across sex. Figures in the parentheses depict standard error across the means of the four replicate populations.

JB	18	0.146	(± 0.018)	FEJ	18	0.019	(± 0.003)
JB	25	0.076	(± 0.007)	FEJ	25	0.012	(± 0.003)
JB	28	0.084	(± 0.003)	FEJ	28	0.021	(± 0.005)

Table 4.3: Mean slopes of density reaction norms of development time at each rearing temperature, averaged across sex. Figures in the parentheses depict standard error across the means of the four replicate populations.

Effect	*df*	MS	*F*	*P*
Selection	1	423.2131	2879.609	<0.0001
Sex	1	1.7108	165.9474	0.001
Density	2	3.9785	12.1248	0.0078
Selection × sex	1	0.0582	50.3503	0.0057
Selection × density	2	4.4634	16.9701	0.0034
Sex × density	2	0.0394	2.2889	0.1825
Selection × sex × density	2	0.0609	2.5516	0.1578

Table 4.4: Results of ANOVA for temperature slopes of mean development time. Only fixed factor effects could be tested for significance.

Effect	*df*	MS	*F*	*P*
Selection	1	0.0861	70.555	0.0035
Sex	1	0.0005	11.1879	0.0442
Density	2	0.0066	15.9557	0.0039
Selection × sex	1	0.0012	85.2878	0.0027
Selection × density	2	0.0054	15.5406	0.0042
Sex × density	2	0.0001	1.6654	0.2659
Selection × sex × density	2	0.0001	2.4434	0.1674

Table 4.5: Results of ANOVA for density slopes of mean development time. Only fixed factor effects could be tested for significance.

CV of development time

The CV of development time was significantly lower in FEJs than JBs overall (Table 4.6; Figures 4.3 and 4.4), which is not surprising given 300 generations of directional selection for

rapid development in the FEJs. The ANOVA revealed significant main effects of selection, temperature and density on the CV, and a significant selection × density interaction (Table 4.7). Pooled over the selection lines, the CV was significantly higher at 28^{o}C compared to18^{o}C. Increase in the CV at 28^{o}C compared to 25^{o}C and 18^{o}C, was much greater in JB relative to the FEJs (Figure 4.3).

At egg densities of 70 and 300, the CV of the JBs was significantly higher than that of the FEJs (Figure 4.4), but at 30 eggs per vial, the difference between JBs and FEJs was not significant. Within each selection regime, no considerable change in CV was observed as egg density rose from 30 to 70 eggs per vial (Figure 4.4). However, from 70 to 300 eggs per vial, a significant increase in the CV was observed in the both JBs and FEJs (Figure 4.4). The FEJs underwent a 45% increase in the CV of development time from 70 to 300 eggs per vial, whereas JBs showed 132% rise in CV across the same density range.

Differences in CV across temperature were most pronounced at 300 eggs per vial (Figure 4.3). Multiple comparisons of the three-way interaction among selection × temperature × density showed a significant increase of the JB CV at 28^{o}C, compared to the CV at 25^{o}C, at 300 eggs per vial.

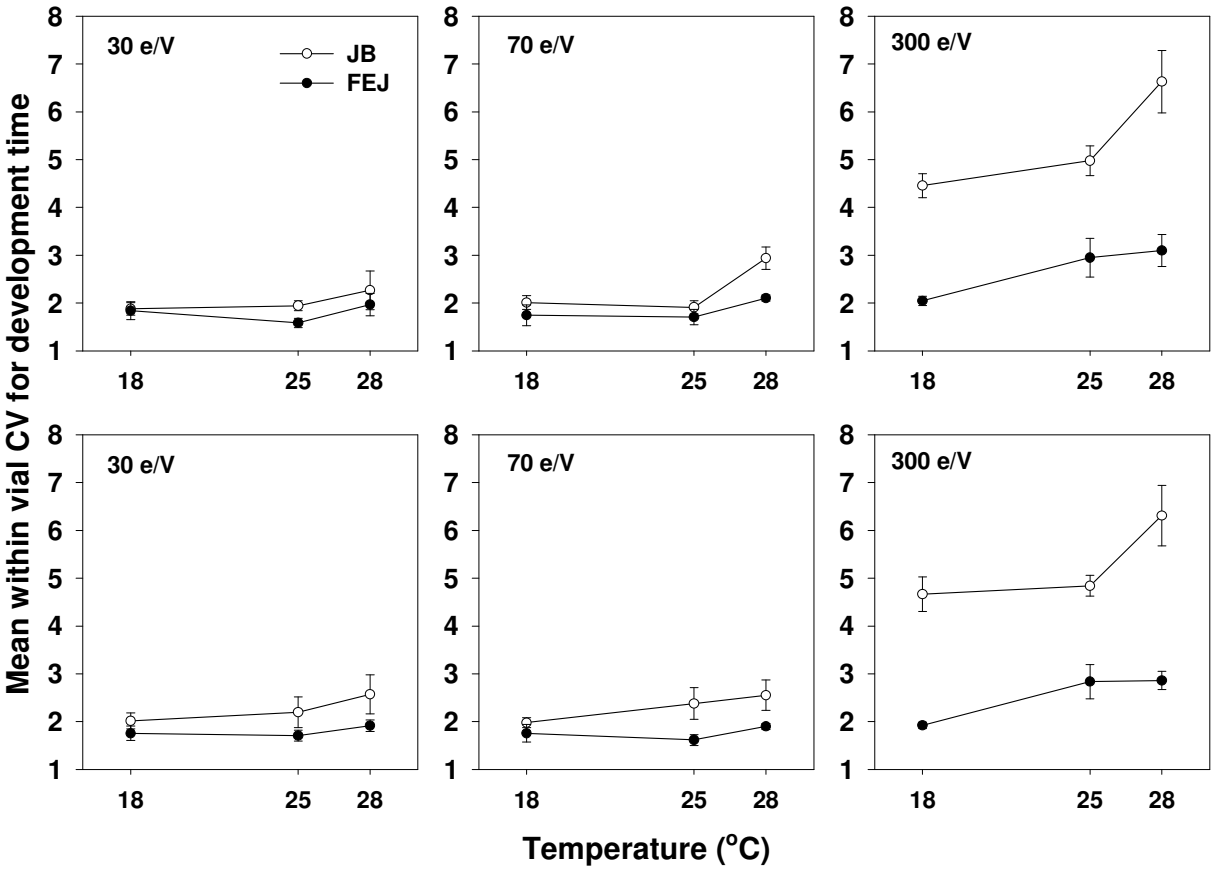

Figure 4.3: Mean within-vial CV (± s.e.) of development time along the temperature axis. The CV for males is shown in the upper row; the lower row shows the CV of females. Error bars depict standard errors across the means of the four replicate populations. (e/V: eggs per vial)

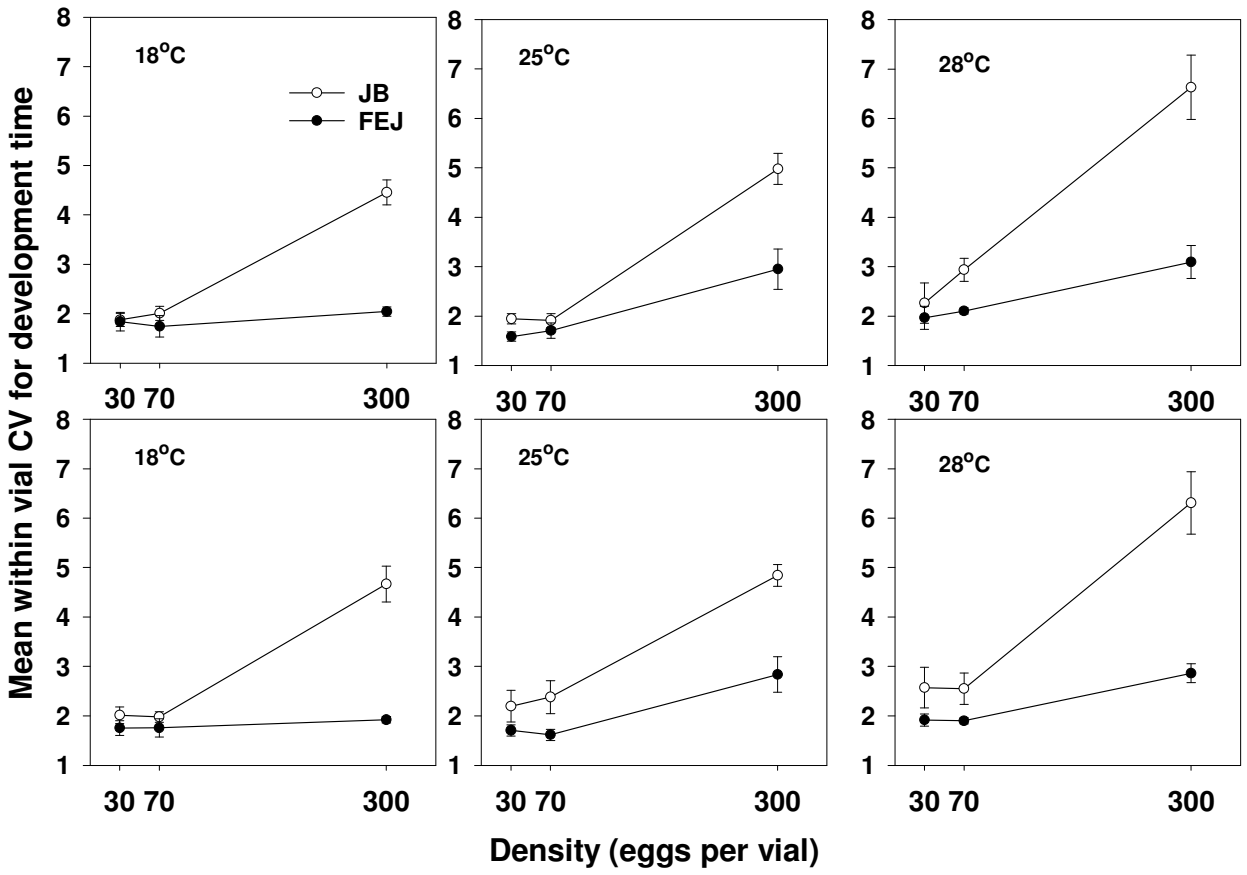

Figure 4.4: Mean within-vial CV (± s.e.) of development time across egg densities. The CV for males is shown in the upper row; the lower row shows the CV of females. Error bars depict standard errors across the means of the four replicate populations. (e/V: eggs per vial)

Effect	*df*	MS	*F*	*P*
Selection	1	49.987	36.537	0.009
Temperature	2	7.255	19.692	0.002
Density	2	61.321	283.762	<0.001
Sex	1	0.008	0.549	0.512
Selection × temperature	2	1.384	3.132	0.117
Selection × density	2	20.765	164.516	<0.001
Temperature × density	4	1.519	1.939	0.168
Selection × sex	1	0.179	1.024	0.386
Temperature × sex	2	0.175	3.102	0.118
Density × sex	2	0.167	1.396	0.318
Selection × temperature × density	4	0.605	1.913	0.173
Selection × temperature × sex	2	0.029	0.338	0.726
Selection × density × sex	2	0.022	0.227	0.804
Temperature × density × sex	4	0.098	1.561	0.247
Selection × temperature × density × sex	4	0.095	0.87	0.509

Table 4.4: Results of ANOVA for the CV of development time. Only fixed factor effects could be tested for significance.

Discussion

Canalizing effect of selection on development time

As evident from the trait means and the slopes of their reaction norms, mean development time of the FEJs remained more uniform than that of the JBs across densities, especially in the higher density range. As the egg density rose from 70 to 300 eggs per vial, relative increase in mean development time was eight times higher in the JBs than in the FEJs. Similarly, the relative increase in the among-individual trait variation across density was three times less in the FEJs compared to the JBs, as apparent from the changes in CV. Thus the FEJs not only have a significantly reduced phenotypic variability for development time than that of the JBs, but the

FEJ variability also remains more uniform across density. Bigger flies are more likely to be susceptible to crowding conditions; a crowded habitat consisting of bigger individuals like the JBs is expected to result in greater food scarcity and toxicity due to accumulation of nitrogenous waste than one which harbours smaller flies like the FEJs. However, the FEJs are also known to be less competitive with reduced larval feeding rates and urea tolerance (Prasad *et al.* 2001; Joshi *et al.* 2001; Shakarad *et al.* 2005). Despite this, a significantly reduced degree of deviation in development time variation indicates an increased buffering of development time against density changes in the FEJs.

Increasing temperature is well-known to accelerate *Drosophila* development and, expectedly, development time became shorter with increasing temperature in this study. The slopes of reaction norms of mean development time were consistently lower in the FEJs, suggesting reduced sensitivity of the trait mean to temperature and density changes in these populations. Although mean development time decreased with increasing temperature, variability as reflected in the CV increased along the temperature axis. From 25^{o}C to 18^{o}C, the CV underwent reduction, but it was not significant. Even though 25^{o}C is the optimal temperature for development of *D. melanogaster*, in terms of pre-adult survivorship, the species shows a higher tolerance zone on the colder side than on warmer side. Stress effects are generally observed on viabilities at developmental temperatures below 14^{o}C (David *et al.* 1983; Pétavy *et al.* 2001). In this experiment, too, significant viability differences were not found between 25^{o}C and 18^{o}C (S. Ghosh Modak and A. Joshi, unpublished data). Hence, it is not surprising to find a non-significant change in the development time CV in this range. Changing the rearing temperature from 25^{o}C to 28^{o}C, on the other hand, led to a significant increase in the CV in both FEJs and JBs. 28^{o}C is known to stress *D. melanogaster* development and above 28^{o}C viability goes down

and sexual development in males is adversely affected (David *et al.* 1983). Although significant reduction in viability was not observed at 28°C in this study (S. Ghosh Modak and A. Joshi, unpublished data), significant increase in trait variability indicated stress at this temperature. The increase in the CV of the JBs was much higher compared to that of the FEJs at 28°C, especially at 300 egg density (Figures 4.3, 4.4). A stressful combination of high temperature and density *i.e.* 28°C and 300 eggs per vial, probably led to the breakdown of canalization for JB development time, but the FEJ variability was affected to a much lesser degree, relative to that of the JBs (Figure 4.3). This clearly shows that the FEJs have evolved a better buffering of their development time against macroenvironmental changes.

Effect of macroenvironmental factors on mean vs. CV of development time

The other interesting point to note from the results of this experiment is the difference in the sensitivities of mean trait value and trait variability to changing density. Mean development time in the FEJ underwent a change of only 1.6% as egg density rose from 70 to 300 eggs per vial, whereas CV got inflated by 45%. Similarly, JB development time showed a 9.9% increase in its mean, while the CV increased by 132% in the same density range. However, the effect of density on changes in mean and CV were qualitatively similar as both showed increase with higher densities.

Changes in temperature on the other hand affected the mean development time more than its CV, except in the JBs at 28°C. Reducing the temperature from 25°C to 18°C lengthened the mean development time by 67.6% in the FEJs and 68.7% in the JBs, whereas the CV got reduced by 10.7% and 6.8% respectively. Increasing the rearing temperature from 25°C to 28°C decreased the mean development time by ~19% in both selection lines, while the corresponding increase in

the CV was 12% and 28% for the FEJs and JBs, respectively. Across temperatures, the mean and CV of development time showed opposite trends as mean trait value decreased but the CVs showed an increase with increasing temperature.

To summarize, this study presents an empirical validation of the prediction that the extent of canalization of a trait is proportionate to its relevance to fitness and that, therefore, strong directional selection for a trait can lead to its canalization. Stearns *et al.* (1995) had shown earlier that traits more closely related to fitness showed greater canalization in *D. melanogaster*. This study, similarly, shows that in different populations the same trait can show different extent of canalization depending on the fitness impact of the trait, determined by the nature of selection experienced by the population. Another point to note here is that the mean value and variability of a trait can respond in markedly different ways when subjected to a perturbing factor. This study thus underscores the necessity of measuring both population means and CVs to get a clearer picture of canalization.

Chapter V

Incipient Reproductive Isolation as a Complex By-product of Divergent Life-history Evolution

Introduction

In recent years, the focus of studies on speciation has been shifting from broad geography-based models of sympatry versus allopatry towards understanding the mechanisms that give rise to reproductive isolation (Rice and Hostert 1993; Schluter 2001; Rundle and Nosil 2005). In this new approach, one major model of species formation is ecological speciation, in which reproductive isolation arises as a by-product of divergent natural selection on traits not directly related to reproduction (reviewed by Rundle and Nosil 2005). Ecological speciation, of course, also includes the classical concept of allopatric speciation (Dobzhansky 1951; Mayr 1942, 1963). Both theoretical and empirical studies indicate that ecological speciation is likely to be reasonably common (Coyne and Orr 2004).

As a process, ecological speciation has three main components: some ecological basis for divergent selection, a mechanism of reproductive isolation, and some underlying genetic link between the traits under divergent selection and those responsible for reproductive isolation, whether through pleiotropy or linkage disequilibrium (Rundle and Nosil 2005). Of these three, the link between the selected and isolating traits is the least commonly addressed component of the process (McKinnon *et al*. 2004; Rundle and Nosil 2005). Most laboratory and field studies of ecological speciation have focused on adaptation to very different habitats in terms of resources and/or antagonists, and often even the traits involved in the reproductive isolation are not clearly identified (Rundle and Nosil 2005).

Another issue that is rarely directly addressed is whether sexual selection and viability selection can interact to produce reproductive isolation. Typically, sexual and viability selection are implicitly treated as being coincident with pre-zygotic and post-zygotic isolation, respectively

(Howard and Berlocher 1998). Thus, sexual selection is believed to act through differences in mate preference or mating success, whereas viability selection is thought to play a role in isolation through poorer survival of hybrids.

I looked for evidence of pre- and post-zygotic isolation between the FEJs and JBs. The FEJs have evolved to become substantially different from the JBs in many traits, showing reduced egg-to-adult development time and viability, larval growth rate, feeding rate, foraging path length and competitive ability, urea tolerance, dry weight and body size at eclosion, starvation resistance and fecundity (Prasad *et al*. 2000; 2001; Prasad and Joshi 2003; Joshi *et al*. 2001; Prasad 2004; Shakarad *et al*. 2005; Chapters II, III of this thesis). Hence, I was interested in examining if the evolutionary divergence between FEJ and JB populations, including the large difference in their body size (Figures 5.1, 5.2) has led to reproductive barriers between the two sets of populations. As this is a well-characterized laboratory system, whose evolutionary history over several hundred generations is known, the likelihood of being able to specifically link divergent and isolating traits seemed higher than is typically the case with field studies.

Figures 5.1: FEJ (top) and JB (bottom) males after 370 generations of FEJ selection.

Figures 5.2: FEJ (right) and JB (left) females after 370 generations of FEJ selection.

Materials and methods

Offspring of standardised flies were used for the experiments. As soon as the adults started eclosing, virgin males and females were separated and kept in single-sex conditioning vials with food, at a density of 20 flies per vial. For all the four experiments described below, 3 day old virgin flies were used.

Crosses in vials with varying male density

Reciprocal crosses at three different male densities were set up in vials containing food using virgin JB and FEJ flies from the same block. In the first set, one female was housed with one male. In the second set, one female was exposed to five males, and in the third set, a single female was housed with ten males. The four types of cross set up were (a) $JB_i♂ \times FEJ_i♀$, (b) $FEJ_i♂ \times JB_i♀$, (c) $JB_i♂ \times JB_i♀$, and (d) $FEJ_i♂ \times FEJ_i♀$. For each combination of cross, block and male density, 10 replicate vials were set up, yielding a total of 480 vials for the assay. Each vial was observed at 8 h intervals from the time of set up till 329 h, and the time of death of the female noted. The cumulative mortality of females at 80.5 h and 328.5 h was calculated.

Individual mate choice

Separate male and female choice assays were performed in vials with food. In the male choice assay, one male was exposed to two females, one from each selection regime. Ten such vials were set up for each combination of block and selection regime. Similarly, in the female choice assay, one female was provided with two males, one from each selection regime. Ten such vials were set up for each combination of block and selection regime. In all vials, the identity (selection regime) of the chosen mating partner in the first copulation observed was noted, and

the duration between the introduction of the flies into the vial till the first mating observed (mating latency) was also recorded. Only copulation events lasting for more than 3 minutes were recorded. From these data, the number of homogamic and heterogamic matings were obtained for each choice test. Additionally, from all the matings that took place in these assays, the frequencies of all four possible mating combinations were calculated.

Multiple mate choice

Forty flies — 10 males and 10 females each from the JB and FEJ populations belonging to the same block — were introduced together in a a glass Petri dish of 17 cm diameter containing a thin layer of banana-jaggery food. The number of copulating pairs of each of the four possible mating combinations (JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, FEJ♂ × FEJ♀) in an observation period of 1 h was recorded. The size difference between JB and FEJ flies was large enough to determine the mating combination without having to remove the copulating pairs from the Petri dish. The assay was replicated three times for each block, with a different set of 40 flies for each replicate. The numbers of each of the four classes of matings were calculated for each Petri dish and averaged across the three replicates for each block.

Hybrid survivorship and development time

To check for post-zygotic isolation, we assayed the egg-to-adult viability of F_1 and F_2 progeny from all four types of cross: JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, and FEJ♂ × FEJ♀. We also assayed the egg-to-adult development time of the F_1 progeny. These crosses were set up by putting 400 males and 400 females in a Plexiglas cage containing a Petri dish with banana food topped with a smear of yeast-acetic acid paste. After three days, eggs were collected from the

cages by placing a fresh Petri dish with food into the cage for 1 h. Since many of the females in the JB♂ × FEJ♀ cages were dead (see Results), eggs from this cross were collected over a time window of ~10 h. Eggs were dispensed into vials with 6 mL of food at a density of 30 eggs per vial and incubated. 10 vials were set up for each combination of cross × block. The vials were monitored closely and, once eclosion began, the vials were checked regularly at 2 h intervals and freshly eclosed flies were removed from vials. The number of eclosing adults was recorded. These observations were continued until no new flies eclosed for two consecutive days in any of the vials. From these data, the mean development time of F_1 flies was calculated. The number of flies eclosed in each vial was divided by 30 to obtain the F_1 egg-to-adult survivorship.

After collecting eggs from the cages for the F_1 survivorship assay, a second Petri dish with food was introduced into the same cage and flies were allowed to lay eggs for 18 h. Eggs were collected from this plate and dispensed into vials at a density of 60-80 eggs per vial containing 6 mL of food. A total of 40 such vials were set up per block and cross combination for rearing the F_1 generation. The F_1 flies eclosing in these vials were transferred into fresh cages and maintained as mixed-sex groups. They were provided with supplementary yeast acetic acid paste along with banana food for three days, and then eggs were collected from these cages by placing a fresh Petri dish with food into the cage for 1 h. These eggs were dispensed into vials with 6 mL of food at a density of 30 eggs per vial and incubated. For each combination of cross and block, 10 such vials were set up. Once all the flies in each vial eclosed, they were counted, and the number was divided by 30 to obtain the F_2 egg-to-adult survivorship.

Hybrid fecundity

Fecundity of unyeasted F_1 flies was assayed at two different ages, corresponding to the ages of egg collection under the FEJ and JB maintenance regimes. Thus, daily fecundity per female was assayed for day 2, 3 and 4 (corresponding to the FEJ egg collection) and also day 10, 11 and 12 (corresponding to the JB egg collection). Flies were collected from the F_1 cages and sorted under light carbon dioxide anesthesia and one male and one female were placed into a vial containing 3 mL of banana-jaggery food. Twenty such vials were set up for each cross × age × block combination. The assay was set up on the 2^{nd} day after eclosion and after 24 h, the pair was transferred into a fresh vial and the eggs laid over the 24 h period were scored and this was repeated for three consecutive days. Number of eggs laid daily per female was averaged over the three consecutive days to obtain the mean fecundity of the female around day 3. On day 10, a different batch of flies were removed from the F_1 cages and set up in the similar manner as described. The mean 11^{th} day fecundity was similarly calculated by averaging the number of eggs laid on day 10, 11 and 12 post-eclosion. Fecundity was averaged across the two ages (day 3 and day 11 post-eclosion) and analysed.

Statistical analysis

The cumulative mortality of females at 80.5 and 328.5 h for each combination of cross × male density was arcsine-square-root transformed and subjected to separate mixed-model ANOVAs, with cross and male density being treated as fixed factors crossed with random blocks.

The number of homogamic and heterogamic matings for each combination of selection regime, replicate population and sex were obtained from the individual mate-choice assay and subjected to replicated G-tests for goodness of fit (Sokal and Rohlf 1998; McDonald 2008), permitting

both block-wise and overall testing of the null hypothesis of the random-mating expectation of a 1:1 ratio of homogamic to heterogamic matings.

Similarly, the data for mate identity obtained from the multiple mate-choice assay for each combination of replicate population, selection regime and sex were subjected to replicated G-tests for goodness of fit, for both block-wise and overall testing of the null hypothesis of the random-mating expectation of a 1:1 ratio of homogamic to heterogamic matings.

For F_1 development time and fecundity, trait values were averaged across vials to obtain population means. For F_1 and F_2 survivorship assays, vial means were arcsine-square-root transformed and averaged across vials to obtain population means. For each trait, the replicate population means were subjected to a two-way ANOVA treating type of cross (4 levels) as a fixed factor crossed with random blocks.

Results

Cross-specific female mortality at different male density

In all four types of cross — JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, and FEJ♂ × FEJ♀ — the rate of increase of cumulative female mortality with time tended to increase with increasing number of male partners, although the effect was marginal in the JB♀ × FEJ♂ cross (Figure 5.3). In general, cumulative female mortality increased the fastest in the JB♂ × FEJ♀ cross, and this cross also showed the greatest sensitivity of female mortality to increasing male density: all FEJ females housed with JB males in 1:10 female:male ratio died within 80.5 h (Figure 5.3). ANOVA on cumulative mortality at both 80.5 h and 328.5 h revealed significant effects of cross, male density and the cross × male density interaction (Table 5.1). Post-hoc comparisons showed that cumulative female mortality at 80.5 h was significantly higher ($P < 0.05$) for the JB♂ ×

FEJ♀ cross than that for the remaining three crosses, and that that cumulative female mortality of the JB♂ × FEJ♀ cross was significantly less at 1:1 sex ratio than at either 1:5 or 1:10. At 328.5 h, the general pattern of cumulative female mortality was similar across all three male densities. Cumulative female mortality for JB♂ × FEJ♀ cross was significantly higher than the other crosses, and the cumulative mortality at 1:10 and 1:5 sex ratios for this cross was significantly higher than that at 1:1 sex-ratio. Moreover, at 328.5 h, the FEJ♂ × JB♀ cross showed significantly lower cumulative mortality than that observed in FEJ♂ × FEJ♀ cross, a difference not apparent at 80.5 h (Figure 5.3).

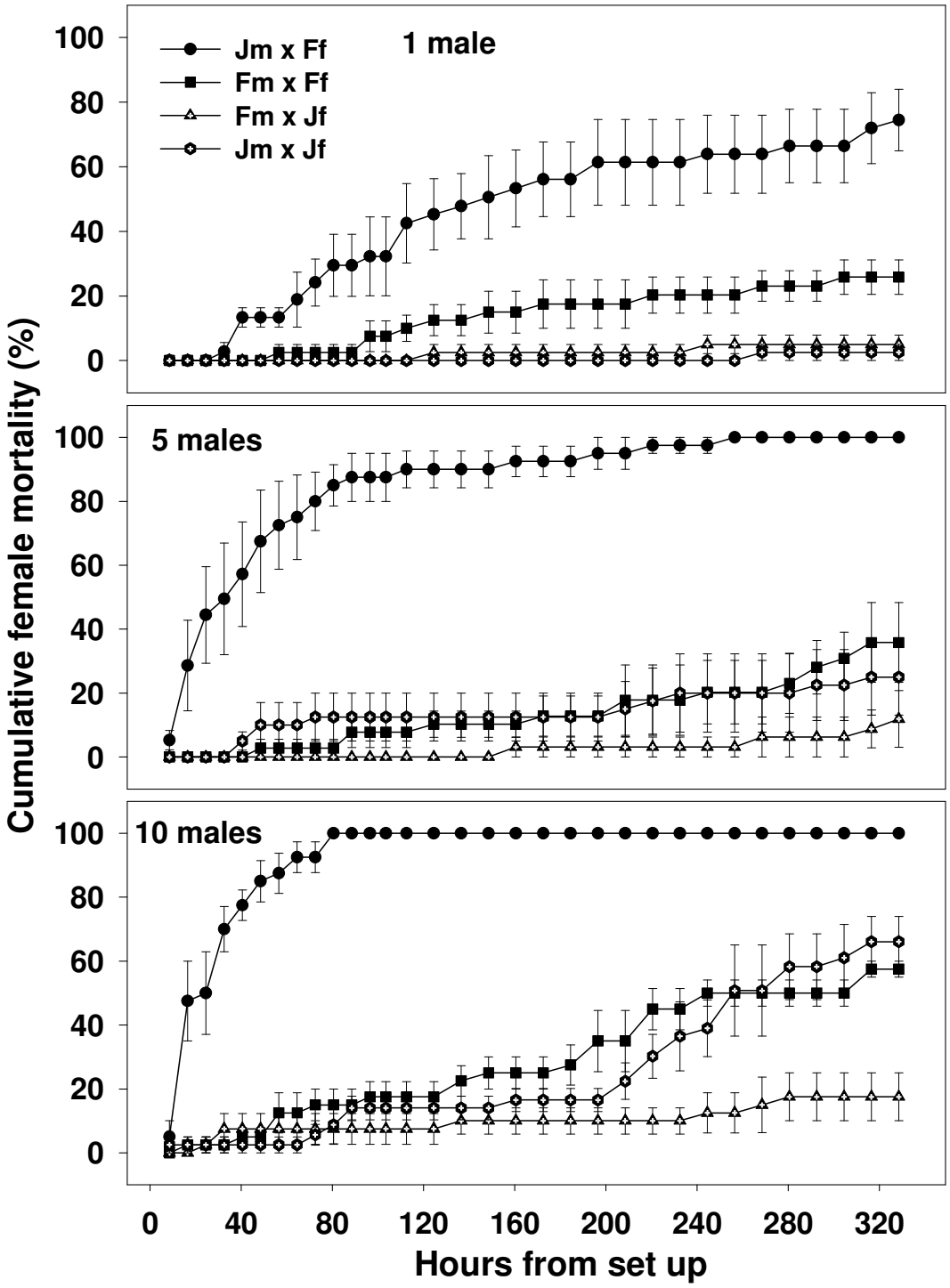

Figure 5.3: Mean (± s.e.) cumulative female mortality plotted across time from set up in the crosses with varying male density.

		80.5 h			328.5 h		
Source	*df*	MS	*F*	*P*	MS	*F*	*P*
Cross	3	2.9463	66.6103	<0.001	2.9318	130.2593	<0.001
Male density	2	0.9342	10.2518	0.012	0.7483	35.1552	<0.001
Cross × male density	6	0.1059	2.6822	0.049	0.1896	4.8722	0.004

Table 5.1: Summary of results of ANOVA done on mean cumulative female mortality at 80.5 h and 328.5 h in the crosses with varying male density. Only fixed factor effects could be tested for significance.

Mate choice assays

In both the individual and multiple mate choice assays, there was a similar, marked asymmetry in the pattern of homogamic and heterogamic matings (Figure 5.4). In Fig 5.4a, the data on mate identity were pooled across the male choice and female choice assays to permit comparison with the results of the multiple mate choice assay (Figure 5.4b). In both assays, there is a clear deviation from a 1:1:1:1 ratio of the four kinds of mating, and the frequency of mating between FEJ males and JB females is less than ten percent (Figure 5.4). Thus, regardless of whether it is the female or the male that has a choice, or a mixed situation of both sexes having a choice in the multiple mate choice assay, FEJ males are rarely able to mate with the much larger JB females. Indeed, in the more realistic conditions of the multiple mate choice assay, the frequency of matings between FEJ males and JB females was even less (3.7%) that that seen in the individual mate choice assay (Figure 5.4). However, the other heterogamic mating, that of JB males with FEJ females, does occur at a considerable frequency of ~30 percent in both assays (Figure 5.4).

In the individual mate choice assay, when the ratio of homogamic to heterogamic matings was tested for deviations from the 1:1 null expectation separately for each combination of selection regime, replicate population and sex, three of the four combinations showed a significant deviation from the null hypothesis of random mating (Tables 5.2, 5.3). FEJ males mated significantly more often with FEJ females, when given a choice. Similarly, JB females mated significantly more often with JB males, when given a choice. In both these cases, the deviation from a 1:1 ratio was significant overall (Tables 5.2, 5.3), and there was no heterogeneity among blocks (Table 5.3). In the case of FEJ females, the overall trend was of significantly greater matings with JB males, but there was also significant heterogeneity among blocks (Table 5.3), and only blocks 1, 2 and 3 showed a significant deviation from the 1:1 expectation (analysis not shown). JB males, when given a choice, mated more often with JB females (Table 5.2), but the difference was consistently not significant across blocks (Table 5.3). The results of the G-test on data from the multiple mate choice assay (Table 5.4) were similar to those from the individual mate choice assay (Table 5.3), except that the FEJ females in the former did not significantly deviate from a 1:1 ratio of homogamic to heterogamic matings (Table 5.4).

Since the mating latency was measured from the individual choice assay, the sample size for FEJ♂ × JB♀ was very small overall, and zero for some blocks. Hence, ANOVA was not done on these data. Overall, the mating latency results followed the pattern of the mate choice results, with the latencies for FEJ♂ × JB♀ being the highest across the male and female choice assays (Figure 5.5).

Male choice:

	JB♀	FEJ♀
JB♂	0.64 (± 0.09)	0.36 (± 0.09)
FEJ♂	0.25 (± 0.05)	0.75 (± 0.05)

Female choice:

	JB♂	FEJ♂
JB♀	0.97 (± 0.03)	0.03 (± 0.03)
FEJ♀	0.84 (± 0.11)	0.16 (± 0.11)

Table 5.2: Fraction of homogamic and heterogamic matings in the individual mate-choice assay. Figures in parentheses represent standard errors across four replicate population means.

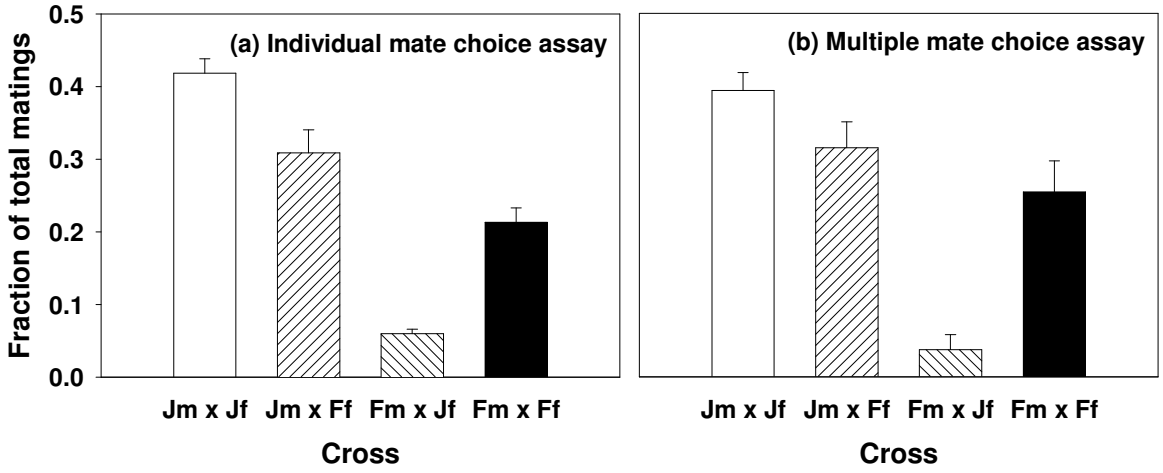

Figure 5.4: Mean proportion of each of the four types of matings (JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, FEJ♂ × FEJ♀), averaged over four replicate populations, expressed as a fraction of the total matings (a) observed over the four individual mate choice experiments (two male choices and two female choices) and (b) observed in the multiple choice assay (averaged over the three runs of the assay). The error bars represent standard errors across four replicate population means.

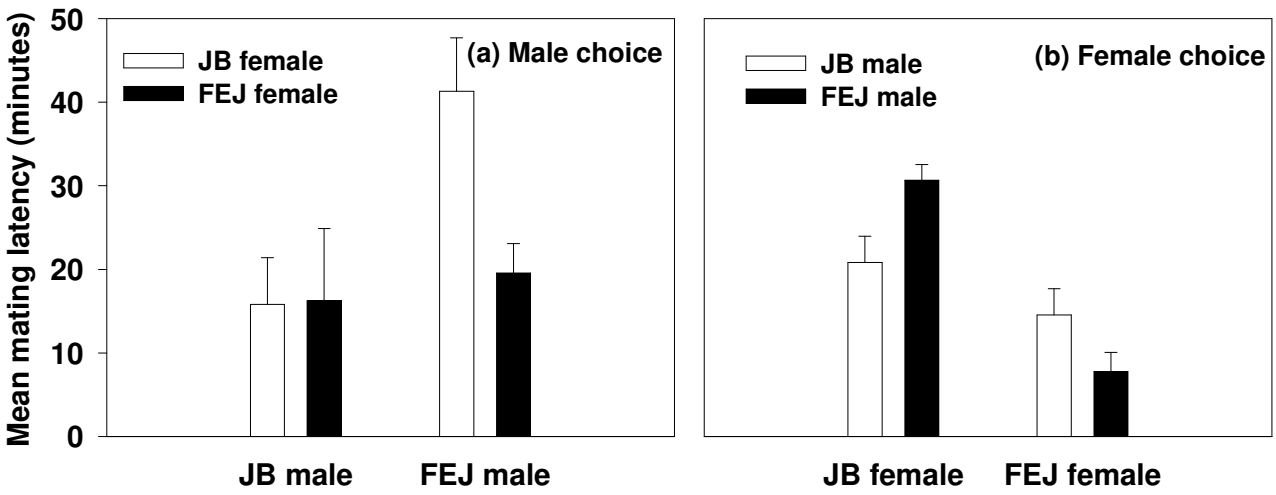

Figure 5.5: Mean (± s.e.) mating latency (time to first mating from the introduction of the flies in vials) in (a) male and, (b) female individual choice assays.

	Total G	*df*	*P*	Pooled G	*df*	*P*	Heterogeneity G	*df*	*P*
JB male	8.381	4	0.079	3.145	1	0.076	5.236	3	0.155
FEJ male	47.564	4	< 0.001	44.764	1	< 0.001	2.799	3	0.424
JB female	12.002	4	< 0.001	10.034	1	< 0.001	1.969	3	0.579
FEJ female	31.692	4	< 0.001	20.578	1	< 0.001	11.114	3	0.011

Table 5.3: Results of the replicated G-test for the individual mate choice assay.

	Total G	*df*	*P*	Pooled G	*df*	*P*	Heterogeneity G	*df*	*P*
JB male	3.247	4	0.517	1.97	1	0.16	1.277	3	0.735
FEJ male	42.609	4	< 0.001	36.057	1	< 0.001	6.553	3	0.088
JB female	78.245	4	< 0.001	70.774	1	< 0.001	7.471	3	0.058
FEJ female	7.714	4	0.103	1.447	1	0.229	6.266	3	0.099

Table 5.4: Results of the replicated G-test for the multiple mate choice assay.

Hybrid survivorship and development time

The pattern of egg-to-adult survivorship of F_1 and F_2 progeny from parental and hybrid crosses was very similar (Figure 5.6). In both generations, the ANOVA showed a significant effect of cross type on survivorship (Table 5.5), and multiple comparisons revealed that the only significant pair-wise differences were those between the progeny of the FEJ♂ × FEJ♀ cross and the progeny of the other three crosses.

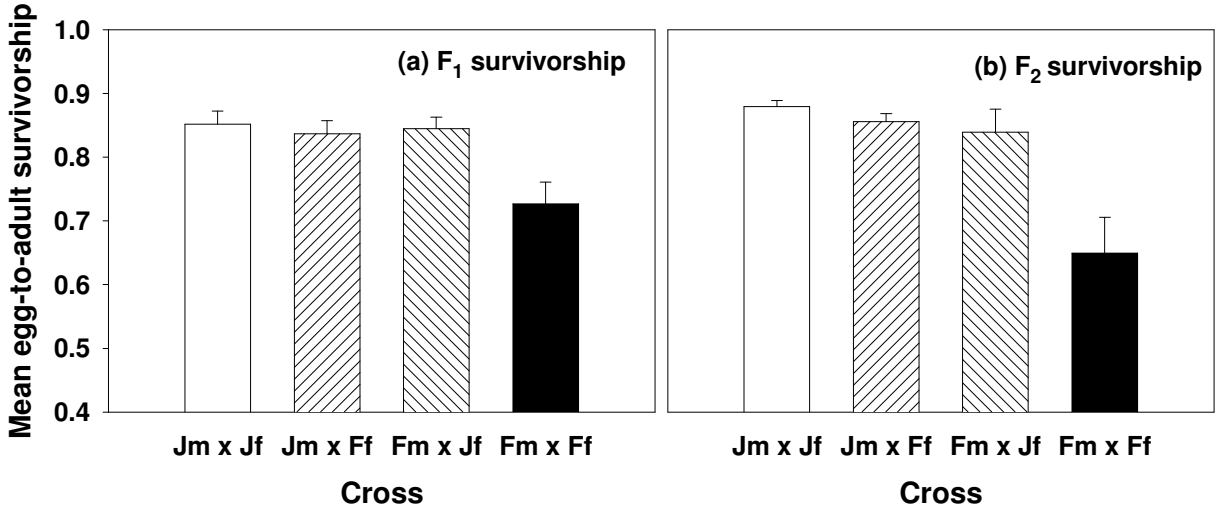

Figure 5.6: Mean (± s.e.) egg-to-adult survivorship of the (a) F_1, and (b) F_2 progeny raised from the four types of crosses (JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, FEJ♂ × FEJ♀).

Mean fecundity averaged over day 3 and day 11 post-eclosion was similar for the JBs and the hybrids (Figure 5.7a). The ANOVA revealed a significant effect of cross (Table 5.5), and multiple comparisons showed that the progeny of FEJ♂ × FEJ♀ cross had significantly lower fecundity ($P < 0.05$) compared to the other three crosses.

In contrast to survivorship and fecundity, the F_1 hybrids showed development time intermediate to that of the parental FEJs and JBs (Figure 5.7b). The ANOVA revealed a significant main effect of cross (Table 5.5), and multiple comparisons showed that the mean development time of

the F_1 hybrids was significantly different from both the parental types, but that the development time of the reciprocal hybrids did not differ significantly.

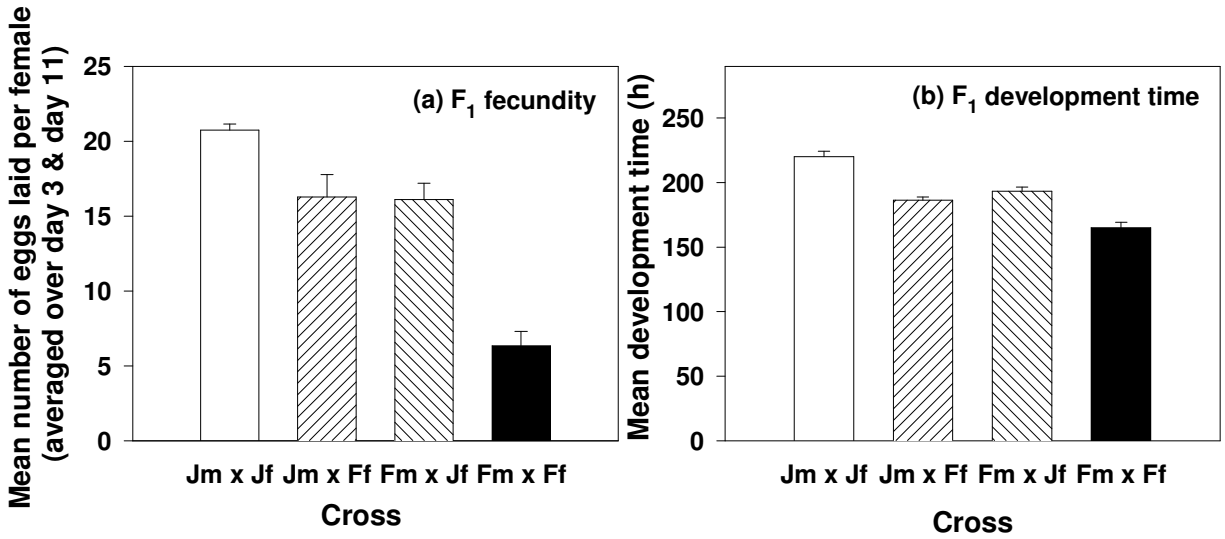

Figure 5.7: Mean (± s.e.) (a) fecundity per female averaged over day 3 and 11 post-eclosion and (b) egg-to-adult development time of the F_1 progeny raised from the four types of crosses (JB♂ × FEJ♀, FEJ♂ × JB♀, JB♂ × JB♀, FEJ♂ × FEJ♀).

Trait	Effect	*df*	MS	*F*	*P*
F_1 development time	Cross	3	2057.4141	202.592	<0.0001
F_1 survivorship	Cross	3	0.0218	11.263	0.0021
F_1 fecundity	Cross	3	147.5233	40.145	<0.0001
F_2 survivorship	Cross	3	0.0884	7.287	0.0088

Table 5.5: Results of separate ANOVA for various F_1 and F_2 traits. Only fixed factor effects could be tested for significance.

Discussion

The results of this study show the evolution of incipient reproductive isolation between the FEJs and JBs due to the complementary effects of two separate asymmetric isolating mechanisms, one pre-mating, involving sexual selection, and one post-mating, based on viability selection.

In both the individual mate choice and multiple mate choice assays, matings between the small FEJ males and the large JB females were rare (Table 5.2; Figure 5.4). FEJ males obtained very few matings with JB females in the presence of JB males, as is evident from the results of the JB female choice (JB♀ × JB♂, FEJ♂; Table 5.2) and multiple choice (JB♂, FEJ♂ × JB♀, FEJ♀; Figure 5.4b) assays. Given the well-known mating advantage conferred by large body size in situations of male-male competition in *Drosophila* (Partridge and Farquhar 1983; Partridge *et al*. 1987a,b; Markow 1988; Markow and Ricker 1992), this is not surprising given the almost two-fold size difference between JBs and FEJs. The fact that FEJ males were also rather unsuccessful at obtaining matings with FEJ females, when competing with JB males in the FEJ female mate choice assay (FEJ♀ × JB♂, FEJ♂; Table 5.2), is also consistent with this explanation.

More interestingly, even in the absence of male-male competition with the larger JBs in the FEJ male choice assay (FEJ♂ × JB♀, FEJ♀), FEJ males mated three times more often with FEJ females than JB females (Table 5.2). This result suggests that not only are FEJ males outcompeted by JB males, but JB females also seem to resist mating attempts by FEJ males quite effectively. Why this is so is not clear at this time. Female *Drosophila* are observed to preferentially mate with larger males (Ewing 1961; Markow 1988; Partridge *et al*. 1987a; Pitnick 1991), but the causal mechanism is not known (Partridge 1988). It is possible that JB females avoid mating with the small FEJ males because of some innate size preference. Alternatively,

FEJ males might be less attractive to JB females due to some other reason, such as differences in courtship song or pheromonal cues, or simply because they are not vigorous and active compared to JB males. It could also be that the small FEJ males are just not able to deal easily with mounting and copulating with the much larger JB females (*e.g.* see Maynard Smith 1956). With the present data, we cannot distinguish between these various possibilities. Nevertheless, it is clear that there is incipient asymmetric pre-mating isolation between FEJ males and JB females, mediated by sexual selection, that effectively renders one kind of heterogamic mating rare in the FEJ-JB system. The mating success results are mirrored by the finding that the longest mating latency in both male and female choice tests was observed for matings between FEJ males and JB females (Figure 5.5).

While there is no impediment to the other heterogamic mating between JB males and FEJ females, the results of the variable male density assay (Figure 5.3) reveal incipient asymmetric post-mating isolation between JB males and FEJ females, mediated by the greatly increased mortality of the FEJ females after such matings. While the present data do not permit us to assign a cause to this phenomenon, it is very likely that it is due to the much larger body size of the JB males, compared to the FEJ males and females (Figures 5.1, 5.2). *Drosophila* females are known to suffer reduced lifetime fitness as a consequence of mating, and this cost is mediated by harmful effects of both male courtship as well as seminal fluid proteins transferred to the female's body during mating (Partridge *et al.* 1987c; Partridge and Fowler 1990; Chapman *et al.* 1995). The cost of mating to the females is also known to rise with increased male body size (Pitnick and García-González 2002; Friberg and Arnqvist 2003). The high mortality of FEJ females after mating even with a single JB male (Figure 5.3) could be due to one, some or all of several reasons. JB males are larger than FEJ females, and the body size difference may be

causing mechanical injury during mating. It could also be that the amount of harmful seminal fluid proteins transferred by JB males is far greater than what the FEJ females have evolved to deal with over the course of several hundred generations of body size reduction in response to selection for faster development. In addition, the costs of being vigorously courted by large and energetic JB males might be excessive for the small and relatively inactive FEJ females that have coevolved for several hundred generations with small and inactive relatively FEJ males.

Thus, the two complementary asymmetric blocks to successful heterogamic matings effectively result in incipient pre-zygotic reproductive isolation between the faster developing FEJs and their ancestral controls, the JBs. We found no evidence for post-zygotic isolation, as hybrids between FEJs and JBs were as viable as the JBs (Figure 5.6), and nearly as fertile as that of the JBs (Figure 5.7a). The development time of the hybrids, however, was intermediate between the FEJs and JBs (Figure 5.7b). Thus, despite the considerable evolutionary restructuring of most aspects of the pre-adult and adult life-history, and many related traits, in the FEJs, a restructuring that has resulted in substantially reduced pre-adult survivorship (Figure 5.6), there does not seem to be any intrinsic genetic incompatibility between the FEJ and JB genomes that would reduce hybrid viability. It may be that the kinds of genetic difference needed to generate such genomic incompatibilities may be rather more extensive than often thought to be the case, as also suggested by Rice and Hostert (1993). These results, therefore, support the view that pre-zygotic isolation often precedes post-zygotic isolation, even in cases of allopatric speciation, in *Drosophila* (Kilias *et al.*, 1980; Coyne and Orr, 1989, 1997; Rice and Hostert, 1993) as is also seen in some other taxa (Coyne and Orr, 2004; Vines and Schluter, 2006).

The principal trait underlying the incipient pre-zygotic isolation between the FEJs and JBs appears to be body size which has undergone a large evolutionary reduction in the FEJs through

its being genetically correlated with development time, the primary trait under selection. Body-size divergence has been shown to be a potential driving force of reproductive isolation in both sympatric and allopatric populations of several families of fish (McKinnon *et al.* 2004; Vines and Schluter 2006, Bolnick *et al.* 2006), and the results from this study suggest that it can play a similar role in fruitflies too. Whether body size is playing a role directly, or indirectly through accessory gland proteins, or both, is not clear at this time, and suggests a fruitful line of further investigation.

Overall, this study provides a good example of divergent selection on a life-history trait resulting in the evolution of incipient pre-zygotic isolation, without any post-zygotic isolation, on a timescale of a few hundred generations. Being a laboratory study, it allowed me to narrow in on the link (in this case, via a genetic correlation) between the trait diverging under different selection pressures (development time as a result of the different FEJ and JB maintenance regimes) and the trait most likely mediating the reproductive isolation (body size). The results also reveal an interplay of sexual and viability selection in driving the incipient reproductive isolation between FEJs and JBs, thereby underscoring the necessity of appreciating the potential complexity of the mechanisms underlying the important evolutionary process of speciation.

Chapter VI

Study of F_1 Hybrids between The FEJ & JB Populations: Genetic Bases of Complex Life-history Traits

Introduction

The underlying genetics of many life-history traits is poorly understood. Although recent QTL studies have made some contributions in identifying genes regulating complex life-history traits (reviewed in Mackay 2004), the genetic dissection of most of these traits still remains largely incomplete. The major obstacle to study the hereditary basis of these complex traits is that they are affected by several genes and, hence many of the tools of classical genetics are not very helpful in this regard. For example, genetic bases of larval tolerance to urea and ammonia were studied through crosses between populations of *D. melanogaster* selected for larval ammonia and urea tolerance and their controls (Joshi *et al.* 1996; Borash and Shimada 2001).

Nunney (1996) investigated the genetic bases of larval development time and adult body weight in *D. melanogaster* by performing reciprocal crosses between faster developing lines and their controls. He selected two replicate populations for fast larval development. Larval development time along with adult body weight declined gradually in response to selection. After 15 generations of selection, reciprocal crosses were conducted between each selected line and its corresponding control to study the genetics of the observed selection responses. Analysis of F_1 dry weight did not indicate any maternal effect, X-chromosome effect or dominance of either of the body weight types. The development time results suggested a small X-chromosomal effect for the trait and there was also some indication of dominance acting in the direction of faster development. Both the effects, nonetheless, were only marginally significant and the bulk of the response for larval development time appeared to involve autosomal loci with primarily additive effects. However, having only two replicates provided low degrees of freedom (*df* 1) to the study and the authors also noted that the two selected lines responded in slightly different manners to selection. The detected X-linked effect for development time was very weak; one of the lines

showed a non-significant effect and the other showed a small but significant effect. The dominance contrast between the faster developing type and the control too showed a similar trend.

It is not clear whether the low significance of the X-chromosomal effect for development time and the observed dominance of the faster developing phenotype was due to lack of enough divergence between the parental populations owing to short duration of selection or the low statistical power of the analysis. Given the much larger divergence in development time and body weight between FEJ and JB populations, and the increased statistical power due to four replicates, I investigated the pattern of inheritance of egg-to-adult development time and dry weight at eclosion by conducting crosses between the FEJs and JBs after 310 generations of FEJ selection. In addition to these two traits, I also studied the fecundity of F_1 flies at two different ages. The difference in development time between the two sets of populations was about 55 h at the time of this study. FEJ dry weight and lifetime fecundity were close to one-third of the corresponding JB trait values. Thus, the two parental populations provided a good contrast to study the F_1 traits and interpret their genetic bases, based on the pattern of inheritance. This study, thus, allowed a much more rigorous and powerful testing of the genetic basis of development time, adult dry weight and fecundity in *D. melanogaster* compared to the earlier study of Nunney (1996).

In contrast to development time and body weight, fecundity of a female is expected to be influenced by the genetics of both the female and its male partner. However, an investigation of fecundity of the P_1 flies involving similar reciprocal crosses between FEJ and JB populations suggested that the contrast between the fecundity of the two populations could be explained largely by the female's identity (S. Ghosh Modak personal observation). JB females laid

significantly more eggs compared to FEJ females irrespective of whether they mated with JB or FEJ males. Similarly, FEJ females laid fewer eggs when mated by either JB or FEJ males. Therefore, F_1 fecundity data were analysed in a manner similar to the development time and dry weight data.

Materials and methods

The crosses

The crosses for the experiment were carried out in cages. Eggs were collected from the standardised flies and reared in vials at a density of 60-80 eggs per vial. The number of rearing vials for each replicate population was 40. The vials were incubated and monitored for eclosion. As eclosion started, males and females were separated with light carbon dioxide anesthesia at every 6 h interval to ensure virginity. After adequate numbers of flies of each sex had been separated, around 400 males and 400 females of the relevant combination belonging to the same block were introduced into an adult cage. Four such combinations including two reciprocal crosses and the two parental crosses were set up for each block: (a) $JB_i♂ \times FEJ_i♀$, (b) $FEJ_i♂ \times JB_i♀$, (c) $JB_i♂ \times JB_i♀$, and (d) $FEJ_i♂ \times FEJ_i♀$. From now onwards these cages will be referred to as cross cages.

F_1 development time

After setting up the crosses, the flies were provided with a fresh food plate with a generous smear of yeast-acetic acid paste. Three days later, eggs of approximately identical age were collected from the cross cages. A plate containing fresh food was placed into the cage for 1 h. This plate was then replaced by another food plate for 1 h from which eggs were collected and dispensed into vials with 6 mL of food at density of 30 eggs per vial and incubated: these eggs

constituted the F_1 generation. In one of the reciprocal crosses *i.e.* JB♂ × FEJ♀, high female mortality was observed (see chapter V). The number of eggs obtained was low, and hence, the egg collection window had to be increased to 4 h for collecting the required number of eggs. 10 assay vials per block were set up for each cross and monitored simultaneously for the first eclosion. Once eclosion started, the vials were checked regularly at 2 h intervals and freshly eclosed flies were removed from vials. The number of eclosing males and females were recorded. These observations were continued until no new flies eclosed for two consecutive days in any of the vials. From these data, the mean development time of F_1 generation was obtained.

Dry weight at eclosion for F_1 flies

Freshly eclosed F_1 adults obtained from the development time assay vials were stored at –20^{o} C within 2 h of eclosion. Thereafter, the flies were dried at 70ºC for 36 h and males and females were grouped in separate batches of 5 flies each. Eight such batches were set up for each combination of cross × sex × block and weighed.

F_1 fecundity

For rearing F_1 adults, eggs were collected from each of the cross cages and dispensed into 40 vials containing ~6 mL food at a density of 60-80 eggs per vial and incubated. 12 days after egg collection, the eclosed F_1 adult flies were transferred into cages containing food. Each F_1 cage contained about 1800 males and females.

The fecundity of F_1 flies was assayed at two different ages, under unyeasted condition. The assay ages were matched with the usual egg collection age in the FEJ and JB populations, respectively. Hence, daily fecundity per female was assayed for day 2, 3 and 4 and also day 10, 11 and 12 post-eclosion. Flies were collected from the F_1 cages and sorted under light carbon dioxide

anesthesia and one male and one female were placed into a vial containing 3 mL of banana-jaggery food. Twenty such vials were set up for each cross × age × block combination. The assay was set up on the 2nd day after eclosion and after 24 h, the pair was transferred into a fresh vial and eggs laid over the period of 24 h were scored. This process was repeated for three consecutive days. The number of eggs laid daily per female was averaged over the three consecutive days to obtain mean fecundity around day 3. On day 10, a different batch of flies were removed from the F_1 cages and set up in the similar manner as described. The mean 11th day fecundity was similarly calculated by averaging the number of eggs laid on day 10, 11 and 12 post-eclosion.

Statistical analysis

Development time and dry weight data were analysed in a similar fashion. The sex-specific vial average for the trait was calculated and the replicate population means were obtained by averaging the vial means. The population means were subjected to three-way mixed model ANOVA. Cross (4 levels) and sex (2 levels) were treated as fixed factors crossed with random blocks. The significant fixed factor effects and interactions involving them were subjected to Tukey's HSD test for multiple comparisons.

The hybrid females arising from the two reciprocal crosses inherited one X-chromosome each from either parent such that each carried one FEJ X-chromosome and another JB X-chromosome. Hence, a difference between the trait values of the two types of hybrid female indicated non-genetic maternal effects. In absence of any maternal effect detected in the females, significant difference between the F_1 males arising from the two hybrid crosses indicated an X-chromosome effect because the two F_1 males carried different X-chromosomes, inherited from their respective mothers (FEJ or JB). The third type of comparison revealed information about

the dominance pattern of JB or FEJ phenotype. The mid-parent value (MP) was calculated separately for each sex and trait. Separate paired *t*-tests were conducted between the MP and mean trait value of each type of hybrid to investigate the dominance pattern. If the trait mean for a particular hybrid was significantly different from MP, and closer to a parental value, then it would indicate significant dominance of that particular parental type.

Fecundity data were analysed separately for day 3 and day 11. For fecundity, X-chromosome effect could not be tested with this design as it is a female-specific trait. Maternal effects and dominance patterns were investigated in a manner similar to that outlined above for development time and dry weight.

Results and discussion

Development time

Mean development time of the F_1 hybrids was intermediate between FEJ and JB development time (Figure 6.1). ANOVA results showed significant main effects of cross and sex. The cross × sex interaction was also significant (Table 6.1). Mean development time of both the F_1 hybrids was significantly different from the parental values. Mean development time of the two types of hybrid female was similar. Mean development time of females arising from the FEJ♂ × JB♀ cross was 189 h whereas females derived from the other reciprocal cross, *i.e.* JB♂ × FEJ♀, took 188 h to develop. This ruled out any significant maternal effect on development time. Male progeny of FEJ♂ × JB♀ took 197 h time to develop while those arising from JB♂ × FEJ♀ cross showed a mean development time of 184 h and this difference was significant. Development times of the male hybrids are characteristic of their respective matrilineages. Since the F_1 male derived from the FEJ♂ × JB♀ cross inherited its only X-chromosome from its JB mother, and

vice versa, this indicates a significant X-linked effect on development time. This study thus supports Nunney's (1996) preliminary finding of X-chromosome involvement in development time of *D. melanogaster.* In *Drosophila*, males generally take longer to develop than the females, which has been repeatedly observed in the FEJs and JBs as well. However in the F_1 flies derived from JB♂ × FEJ♀ cross, female development time was significantly longer than that of the males (Figure 6.1). This unusual finding can be explained as follows. In this particular cross, the male progeny got its only X-chromosome from the FEJ mother whereas the female got one additional X-chromosome from the JB father. This additional X-linked component for slow development time would have contributed to the lengthening of development time in the females leading to longer development time of the female progeny. This observation once again reinforces the suggestion of X-linked influence on development time in *D. melanogaster*. However, only a subset of loci controlling development time is likely to be on the X-chromosome, since the difference between the parental development time and the hybrids also indicate a much larger autosomal component to the trait (Figure 6.3). Similar to Nunney's (1996) finding, a small amount of dominance for the faster developing phenotype was observed (Figure 6.3), but it was not statistically significant.

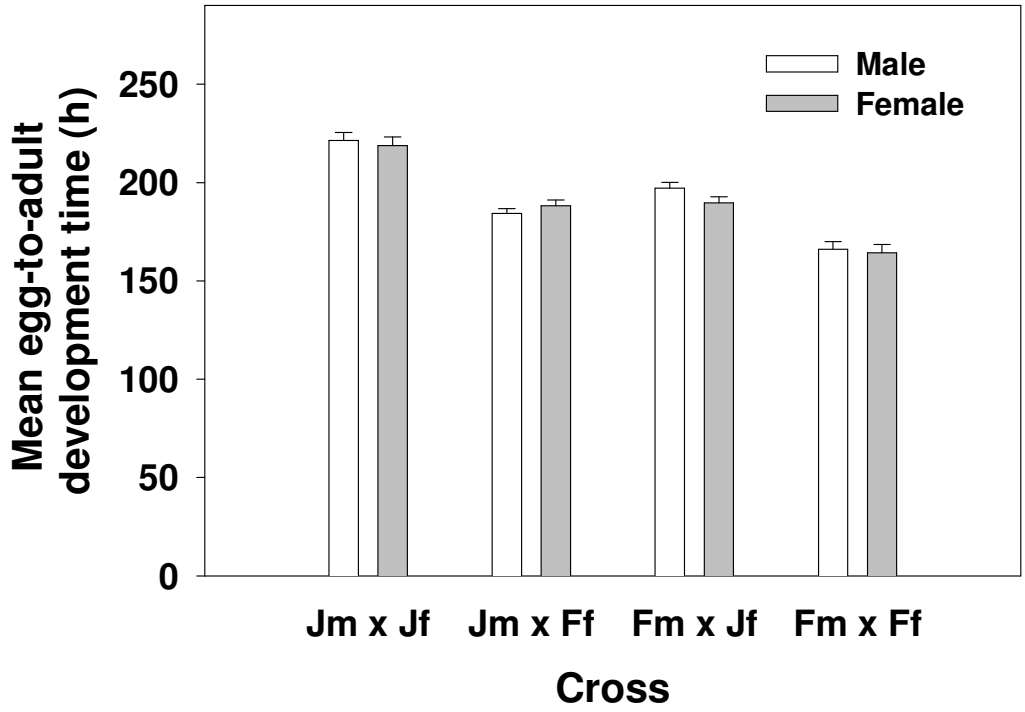

Figure 6.1: Mean (± s.e.) egg-to-adult development time of the F_1 progeny.

Effect	*df*	MS	*F*	*P*
Cross	3	4114.8281	202.5915	< 0.0001
Sex	1	30.7349	14.8116	0.0309
Cross × sex	3	42.5891	34.6589	< 0.0001

Table 6.1: Summary of ANOVA results done on mean development time of F_1 progeny. Only fixed factor effects could be tested for significance.

Dry weight at eclosion

Dry weight at eclosion was significantly influenced by cross, sex and the interaction of the two factors. Both males and females in FEJs were significantly lighter than their JB counterparts. No

other pairwise comparisons for selection × sex interaction were statistically significant. The degree of sexual dimorphism for dry weight was highest in JBs and least in FEJs; that in hybrids was intermediate (Figures 6.2, 6.3). The dry weights of F_1 females obtained from the two reciprocal crosses were similar (Figures 6.2, 6.3), but the F_1 males obtained from JB♂ × FEJ♀ cross were lighter than the male progeny derived from FEJ♂ × JB♀ cross. Although statistically not significant, this trend nonetheless suggested the possibility of a weak X-linked component for body weight. A small dominance of the JB phenotype was observed for body weight, but pairwise *t*-tests did not reveal statistical significance. Thus, body-weight in *D. melanogaster* appears to be largely governed by autosomal loci with additive effects, with perhaps a small X-linked component. The dry weight results of my study were partly in contrast with findings of Nunney (1996) since he did not observe any detectable X-linked effect for body weight.

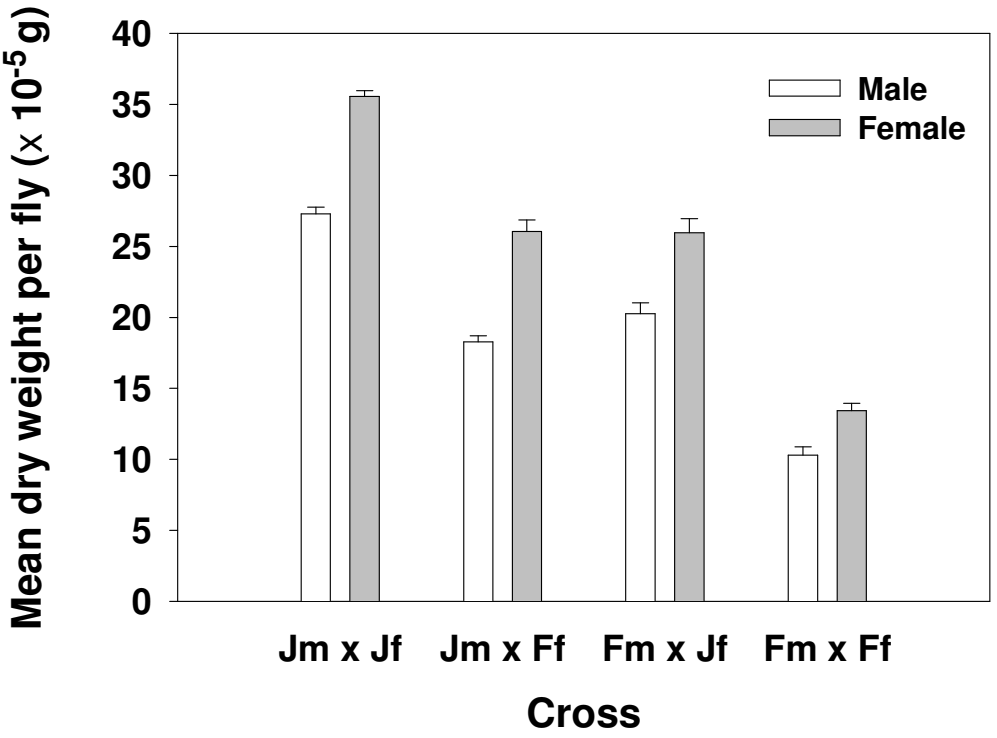

Figure 6.2: Mean (± s.e.) dry weight of F_1 flies at eclosion.

Effect	*df*	MS	*F*	*P*
Cross	3	12844.8066	206.4921	<< 0.0001
Sex	1	7720.0019	642.3735	0.0001
Cross × sex	3	273.1778	9.9751	0.0032

Table 6.2: Summary of ANOVA results done on mean dry weight of F_1 flies at eclosion. Only fixed factor effects could be tested for significance.

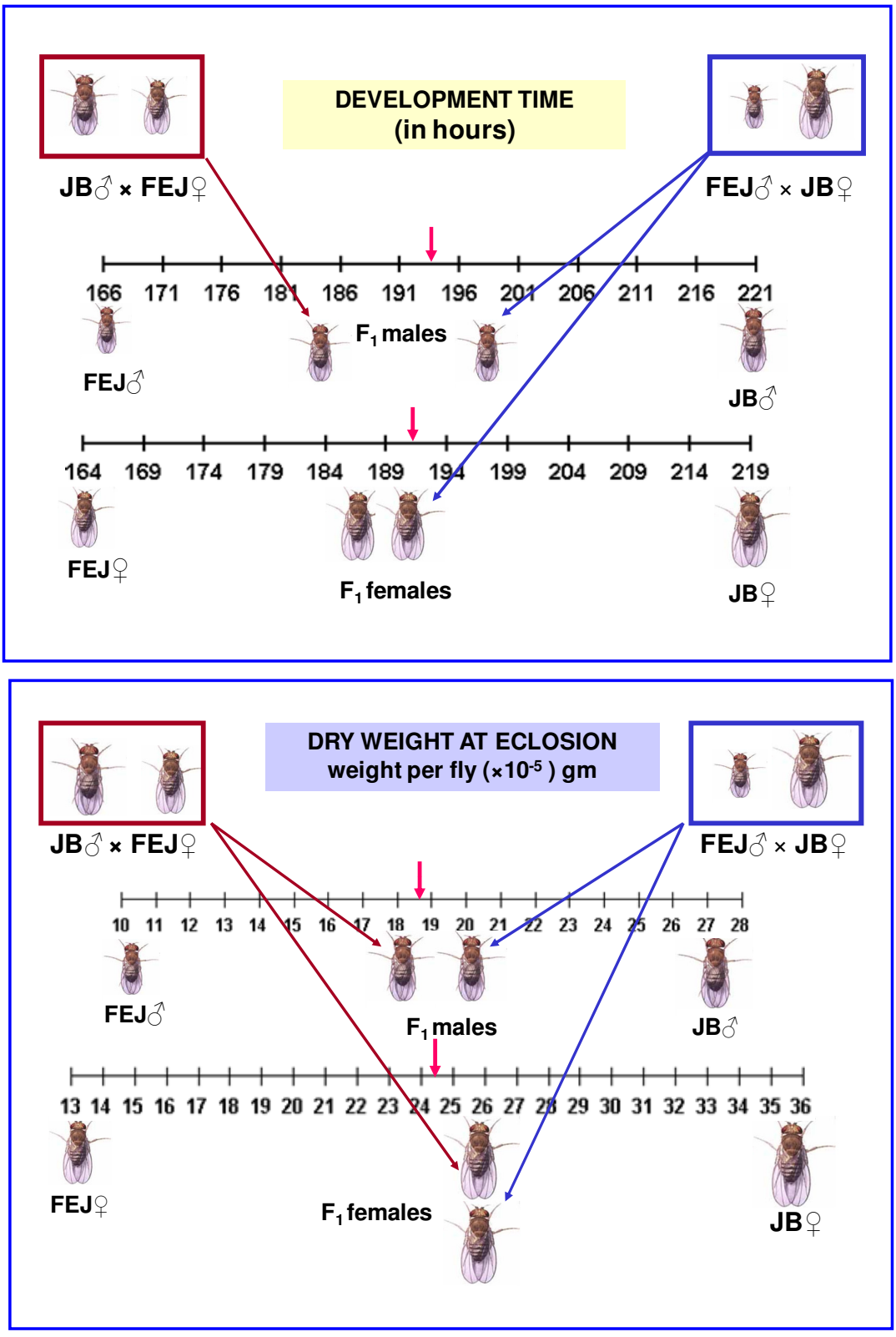

Figure 6.3: A pictorial representation of the results of crosses between FEJ and JB populations for development time and dry weight at eclosion.

Fecundity

The ANOVA results revealed significant effects of cross and the cross × age interaction on fecundity interaction (Table 6.3). Mean early fecundity was highest in JB females (26.13), followed by F_1 females arising from FEJ♂ × JB♀ cross (20.4) and JB♂ × FEJ♀ (15.89); the lowest early fecundity was recorded in the FEJ females (8.68) (Figure 6.4). The two types of hybrids derived from crosses involving a JB mother (JB♂ × JB♀ and FEJ♂ × JB♀) did not differ significantly difference in mean early fecundity. Early fecundity of the JB females was significantly higher than females derived from crosses with an FEJ mother (FEJ♂ × FEJ♀; JB♂ × FEJ♀). Early fecundity of females arising from FEJ♂ × JB♀ was higher than that of the other F_1 hybrid but this difference was not significant. Although statistical significance could not be detected, the overall data nonetheless suggested a small maternal effect in controlling early fecundity, and a partial dominance of the JB alleles.

The trend was different for fecundity around day 11 (Figure 6.4). Fecundity at this age was not statistically different for the JB females and the F_1 hybrids arising from either of the reciprocal crosses. Day 11 fecundity was significantly less in FEJ females compared to the three other groups. These results support the view held by Leips *et al.* (2006) in their QTL study that suggested that different loci contribute to the variation in fecundity at different ages in *D. melanogaster*.

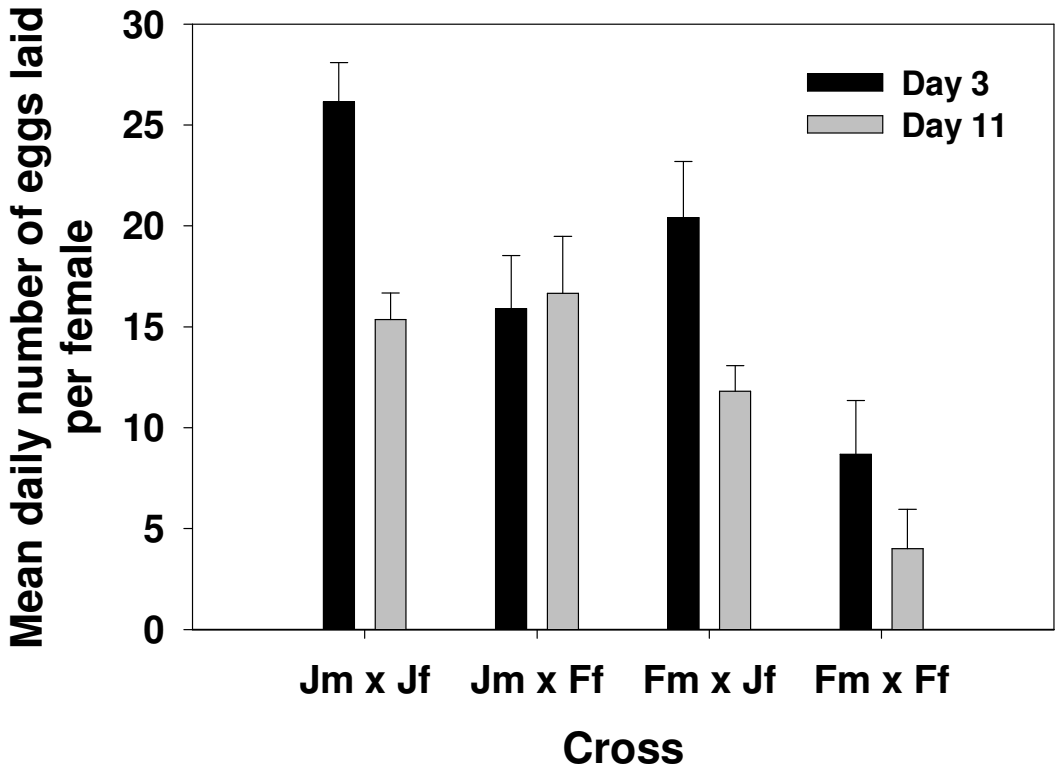

Figure 6.4: Mean (± s.e.) daily fecundity of F_1 females on day 3 and 11 post-eclosion.

Effect	*df*	MS	*F*	*P*
Cross	3	295.0466	40.145	< 0.0001
Age	1	271.1538	2.622	0.2039
Cross × age	3	51.346	6.793	0.0109

Table 6.3: Summary of ANOVA results done on mean fecundity of F_1 females on two different ages (day 3 and 11 post-eclosion). Only fixed factor effects could be tested for significance.

This study, thus, adds to our knowledge of the genetic bases of three important life-history traits in *D. melanogaster* namely pre-adult development time, body weight and age-specific fecundity. Body weight is known to be a good reflector of body size in *Drosophila.* Development time and

body size are highly correlated in *Drosophila* (Zwaan *et al.* 1995; Nunney 1996; Chippindale *et al.* 1997a; Prasad *et al.* 2001). My study shows that the underlying genetic control of the two traits also is fairly similar since both the traits show a large autosomal control with a small X-linked component (development time having a greater X-linked influence than body weight), without any detectable maternal effect. QTL studies in conjunction with this kind of segregation analysis in future could help us to understand the genetics of complex traits better.

Chapter VII

Selection for Faster Development in D. ananassae: Comparing The Genetic Architecture of Fitness across Species

Introduction

The interaction between the form and intensity of natural selection and the details of the genetic architecture of fitness is what largely determines the evolutionary trajectory of life-histories. The genetic architecture of fitness refers to the network of genetic correlations among traits related to fitness. Thus, knowledge of genetic correlations among traits associated with fitness is fundamental to the understanding of life-history evolution. Laboratory selection studies over the past few decades have yielded a lot of information about the sign and relative magnitude of genetic correlations among several life-history and other related traits in *Drosophila* (reviewed in Prasad and Joshi 2003). However, one major concern about our understanding of the genetic architecture of fitness in *Drosophila* is that it is primarily based on studies of genetic correlations in a single species, *D. melanogaster*. It is not clear whether or not these genetic correlations are largely conserved across congeneric species of *Drosophila*. Thus, some knowledge of the consistency of these genetic correlations across species is crucial to deciding upon the degree to which evolutionary predictions based on our knowledge of life-history evolution in *D. melanogaster* are generalizable.

One of the most well studied sets of genetic correlations in *D. melanogaster* involves those linking egg-to-eclosion development time and other related traits such as pre-adult survivorship, dry weight at eclosion and larval feeding rate (reviewed in Prasad and Joshi 2003). Direct selection for rapid development in *D. melanogaster* consistently showed a correlated reduction in adult body size (Zwaan *et al*. 1995; Nunney 1996; Chippindale *et al.* 1997a; Prasad *et al.* 2000), and the trade-off between developmental rate and adult body size is also supported by indirect lines of evidence (Partridge and Fowler 1993; Betran *et al.* 1998). Two studies involving long-term selection for faster development, including the FEJ selection study discussed extensively in

this thesis, also showed a trade-off between developmental rate and pre-adult survivorship, albeit after a considerable period of selection (Chippindale *et al.* 1997a; Prasad *et al.* 2000). Two shorter-term studies, however, did not show any detectable survivorship cost to fast development (Zwaan *et al*. 1995; Nunney 1996). These results suggest that reduction of pre-adult development time in *D. melanogaster* beyond a point imposes a survivorship cost. A slightly counter-intuitive result from the FEJ-JB study was that the larval feeding rate of the FEJs underwent a correlated decrease over about 50 generations of selection for rapid development (Prasad *et al*. 2001). A different selection line (FLJ), derived from the JBs and selected for rapid development and increased lifespan, showed that the larval feeding rate declined rapidly within 10 generations of selection (Rajamani *et al.* 2006), suggesting that the trade-off between feeding rate and developmental rate was already present in the JBs and did not arise as a result of long-term selection. Larval feeding rate in *D. melanogaster* is a well-known correlate of competitive ability (Joshi and Mueller 1988, 1996; Burnet *et al.* 1977) and, not surprisingly, the FEJs were also found to be significantly poorer larval competitors than the JBs (Shakarad *et al*. 2005).

In this chapter, I discuss results from a study on *D. ananassae* which addressed the question of whether the genetic correlations between developmental rate, pre-adult survivorship, dry weight at eclosion and larval feeding rate in this species are similar to those seen in *D. melanogaster*. *D. ananassae* is phylogenetically related to *D. melanogaster*, belonging to the Ananassae sub-group of the Melanogaster species group. Moreover, the two species occupy a similar ecological niche in nature, living in and around human habitation. Hence, the two species are expected to have evolved under similar selection pressures in the wild, and therefore one would intuitively expect the patterns of genetic correlations among fitness-related traits to be similar in these two species. If, however, the genetic architecture of fitness in the two species is substantially different, it

would imply that the results from selection studies on *D. melanogaster* should be extrapolated, if at all, with great caution.

To address this question of the generality of the genetic architecture of developmental rate and related traits, I subjected four laboratory populations of *D. ananassae* to selection for faster development and early reproduction, following the FEJ selection protocol. The four selected populations are referred to as AF_{1-4} (*Ananassae* Faster development) and the matched ancestral controls as AB_{1-4} (*Ananassae* Baseline). The populations have been described in detail in chapter I of the thesis. In this chapter, I compare the selection responses of the AF populations with those of the FEJs, and discuss the genetic architecture of fitness in the two species.

Materials and methods

Generation 10 Assays

Stage-specific development time

Standardised flies were allowed to lay eggs for 1 h on a plate containing fresh food. At the end of this period, this plate was replaced by another plate containing food and kept for 1 h. The eggs were removed from the second food plate with a moistened brush and placed on agar pieces. Eggs were counted under a microscope, and exactly 30 eggs were dispensed into individual vials containing 6 mL of cornmeal food. Ten such vials, each containing 30 eggs, were set up for each replicate population and incubated.

As the larvae started crawling out of the food and wandering for pupation sites on the glass wall of the vial, the vials were being monitored, and the number of pupae formed at every 2 h interval were marked using colour pens and counted. This process was continued till no new pupae formed for 2 consecutive days. The time of egg collection was subtracted from the time of pupa

formation to calculate the larval duration. Mean larval duration for each vial was calculated and averaged across vials to obtain population means.

After the pupae had darkened, the vials were monitored for the first eclosion. Thereafter, the vials were checked regularly at 2 h intervals and the number of eclosing males and females were recorded. These observations were continued until no new flies eclosed for 2 consecutive days in any of the vials. By subtracting time of egg collection from the time of eclosion of male and female flies, sex-specific egg-to-adult development time was obtained.

Mean pupation time of each vial was subtracted from the mean egg-to-adult development time (pooled over the sexes) scored for the respective vial to obtain the mean pupal duration. The population means were calculated by averaging vial means.

Egg-to-adult survivorship

Mean egg-to-adult survivorship was calculated by dividing the number of flies eclosed in each vial used in the development time assay with number of eggs collected *i.e.* 30.

Dry weight at eclosion

Flies eclosing in the vials used for development time assay were collected at every 2 h interval and freeze-killed. They were sexed later and grouped into batches of five flies each. Eight such batches were set up per population for each sex. The flies were then dried at 70^{o}C for 36 h and weighed.

Larval feeding rate

Feeding rates of AB and AF larvae were measured at physiologically matched ages. The egg collection for the two selection regimes were staggered by 5 h. Eggs were collected from both

ABs and AFs with a 4 h long window. At the end of this time period, strips of food were cut and placed into agar plates. 24 h later, 35 newly hatched larvae were transferred into a Petri dish containing a thin layer of non-nutritive agar overlaid with 1.5 mL of 37.5% yeast suspension. Four such Petri dishes were set up per population. At the time of the assay, AF larvae were 77 h old, whereas AB larvae were 82 h old, both being in their early third instar. Larvae were sampled from all the plates and the mouthparts were checked under the microscope to confirm their developmental stage; by this time larvae from both sets of populations had entered third instar. At this point, 20 larvae from each population were assayed for feeding rates, following the procedure of Joshi and Mueller (1996), by placing them individually in a small Petri dish (5 cm diameter) containing a thin layer of agar overlaid with a thin layer of 10% yeast suspension. After allowing for a 15 second acclimation period, feeding rates were measured as the number of cephalopharyngeal sclerite retractions in 1 min. Selected and control populations matched by subscripted indices were assayed together, with one larva from the selected population and one from the control population being assayed alternately.

Generation 20 assays

Egg-to-adult development time

After 20 generations of selection, development time assays were set up at two different densities. Eggs were collected from standardised flies over a window of 14 h and dispensed into vials. Two sets of vials were set up: one set containing 30 eggs per vial with 6 mL of food and another with 400 eggs per vial containing 1.8 mL of food. Eggs used in setting up both the assays were collected off food plates kept in the cages containing standardised flies for ~14 h. Eight such vials were set up per replicate population. Once eclosion started, the number of emerging flies

was scored every 6 h. Sex-specific development time was recorded only for the assay done at 30 eggs per vial.

Dry weight at eclosion

Flies eclosing in the vials used for development time assay done at the two densities, namely 30 and 400 eggs per vial, were collected at every 6 h interval and freeze-killed. They were sexed later and grouped into batches of five flies each. For both densities, eight such batches were set up per population for each sex. The flies were then dried at 70^{o}C for 36 h and weighed.

Larval feeding rate

Larval feeding rate was assayed following the same protocol as described for generation 10.

Generation 25 assays

Egg-to-adult survivorship

At generation 25, standardised flies were allowed to lay eggs for ~14 h on a food plate, and the eggs were collected at densities of 70 and 400 eggs per vial with 1.8 mL of food. For each density, eight such vials were set up per population. The number of eclosing flies was scored and divided by the respective number of eggs collected to obtain the mean egg-to-adult survivorship for each vial.

Larval competition assay

The pre-adult stages of the ABs and AFs were competed against a common competitor strain with a morphological marker for convenience of identification. The population used as the competitor was white eye mutant (WE) of *D. melanogaster* obtained from *de novo* mutations

arising in one of our JB populations, and maintained on a JB protocol. The assay was set up at two different densities: 70 and 400 eggs per vial containing 1.8 mL of cornmeal medium. The low density (70 eggs per vial) bitypic cultures contained 35 eggs of the test population and 35 WE eggs whereas the high density assay vials (400 eggs) consisted of 200 eggs from the test population and another 200 from the WE population. For each density, eight such vials were set up per population. The number of eclosing *D. ananassae* flies was recorded. Vials set up for the survivorship assay (30 and 400 eggs per vial) served as the monotypic controls and were run parallel to the bitypic cultures.

Statistical analysis

Data from all the assays were analysed by mixed-model ANOVAs. For post-hoc comparisons, Tukey's HSD test was used.

Larval duration, pupal duration and larval feeding rate data were subjected to separate two--way ANOVAs treating block as a random factor and selection regime as a fixed factor crossed with block. Analyses of egg-to-adult development time and dry weight data obtained from generation 10 included an additional fixed factor, sex, crossed with selection and block. For pre-adult survivorship data at generation 10, each vial mean for survivorship was subjected to arcsine square-root transformation for meeting the normality assumption of ANOVA. The transformed data were averaged across vials to obtain population means and ANOVA was performed on the population means, treating selection as a fixed factor crossed with block.

ANOVA for egg-to-adult development time and dry weight at generation 20, and survivorship at generation 25, included an additional fixed factor, density, crossed with selection regime and block.

For assessing larval competitive ability, the number of eclosed flies belonging to the test population under competitive condition in bitypic cultures was divided by half of the number of eclosed flies in the corresponding monotypic cultures. This scaling was performed on the population means and three-way ANOVA was performed with selection and density as fixed factors crossed with random blocks.

Results

Development time at generation 10

AF populations showed significant reduction in mean egg-to-adult development time compared to the AB populations, early in AF selection. At generation 10, the mean development time for the AF was 208.6 h whereas ABs took 217.4 h to develop (Figure 7.1a). The ANOVA revealed significant effects of selection, sex and the selection × sex interaction (Table 7.1). Females developed faster than the males and experienced greater reduction in development time than the males. Male development time in the AFs showed a reduction of 7.9 h whereas AF females were 9.5 h faster than their AB counterparts (Figure 7.1b).

Effect	*df*	MS	*F*	*P*
Selection	1	307.72	95.4086	0.002
Sex	1	119.86648	491.5942	< 0.001
Selection × sex	1	2.5554	42.8122	0.007

Table 7.1: Summary of ANOVA results for mean egg-to-adult development time of the AFs and ABs at generation 10 of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

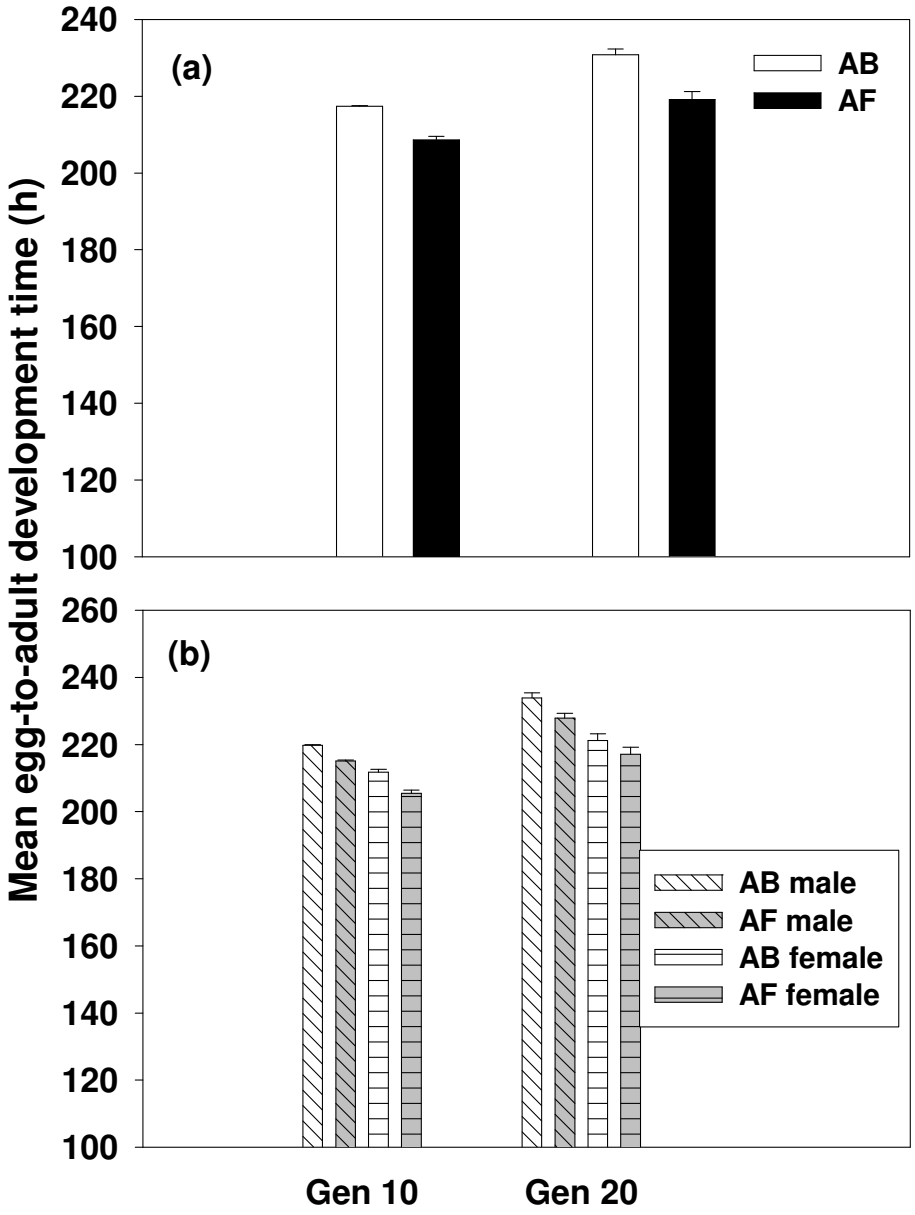

Figure 7.1: Mean (± s.e.) (a) egg-to-adult development time, and (b) sex-specific egg-to-adult development time of the AFs and ABs, after generations 10 and 20 of AF selection, respectively.

Although both larval and pupal durations got significantly reduced in AFs (Table 7.2), 77% of the reduction in development time was due to shorter larval duration whereas decrease in pupal duration accounted only for the remaining 23% of the reduction. From egg collection, the ABs

took 119.6 h and the AFs took 112.8 h to pupate (Figure 7.3). Pupal durations for the ABs and AFs were 97.8 and 95.8 h, respectively (Figure 7.3).

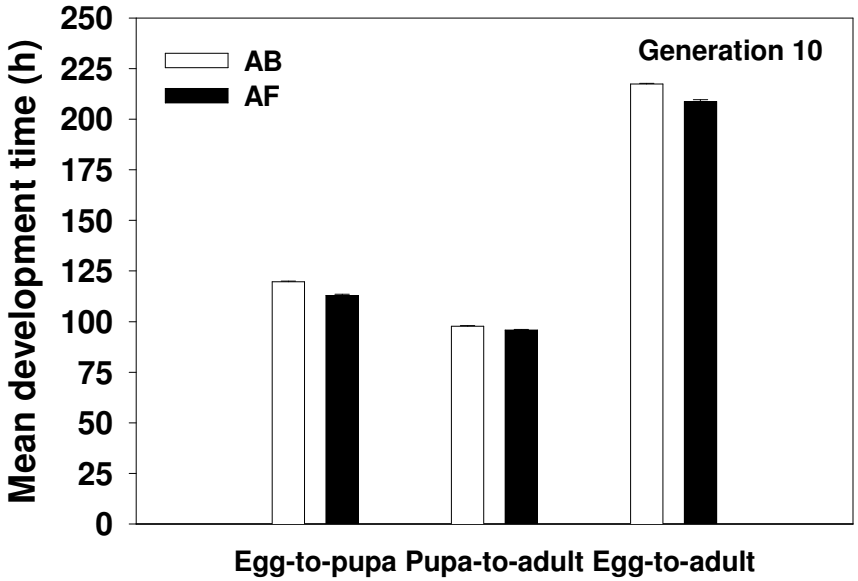

Figure 7.2: Mean (± s.e.) stage-specific development time of AF and AB populations reared at 30 eggs per vial, at generation 10 of AF selection.

Stage	Effect	*df*	MS	*F*	*P*
Larval	Selection	1	91.4554	151.5959	0.001
Pupal	Selection	1	8.0701	30.8758	0.011
Total	Selection	1	307.72	95.4086	0.002

Table 7.2: Results of separate ANOVAs done on mean duration of different pre-adult stages of the AFs and ABs at generation 10 of AF selection.

Egg-to-adult development time at generation 20

The difference in mean egg-to-adult development time between ABs and AFs increased by only a further 2 h between generations 10 and 20, such that the AFs developed 11 h faster than the

ABs at a rearing density of 30 eggs per vial. Mean development time for ABs and AFs was 230.5 and 219 h, respectively (Figure 7.1a). AF males developed 12.7 h faster than the AB males, whereas the female development time in the AFs showed a reduction of 10.8 h (Figure 7.1b).

At the higher density of 400 eggs per vial, the AFs developed 20 h faster than the ABs. The ABs took 257.8 h to develop, on average, whereas mean development time in the AFs was 237.6 h (Figure 7.3). Selection regime interacted significantly with density (Table 7.3), as AF development time was less affected by increasing density than that of the ABs. From 30 to 400 eggs per vial, AB development time got lengthened by 10% compared to a 7% increase in development time for the AFs.

Effect	*df*	MS	*F*	*P*
Selection	1	995.5834	95.0017	0.002
Density	1	2111.2319	74.9967	0.003
Selection × density	1	76.4934	12.6869	0.037

Table 7.3: Summary of ANOVA results for mean egg-to-adult development time of AF and AB populations at generation 20 of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

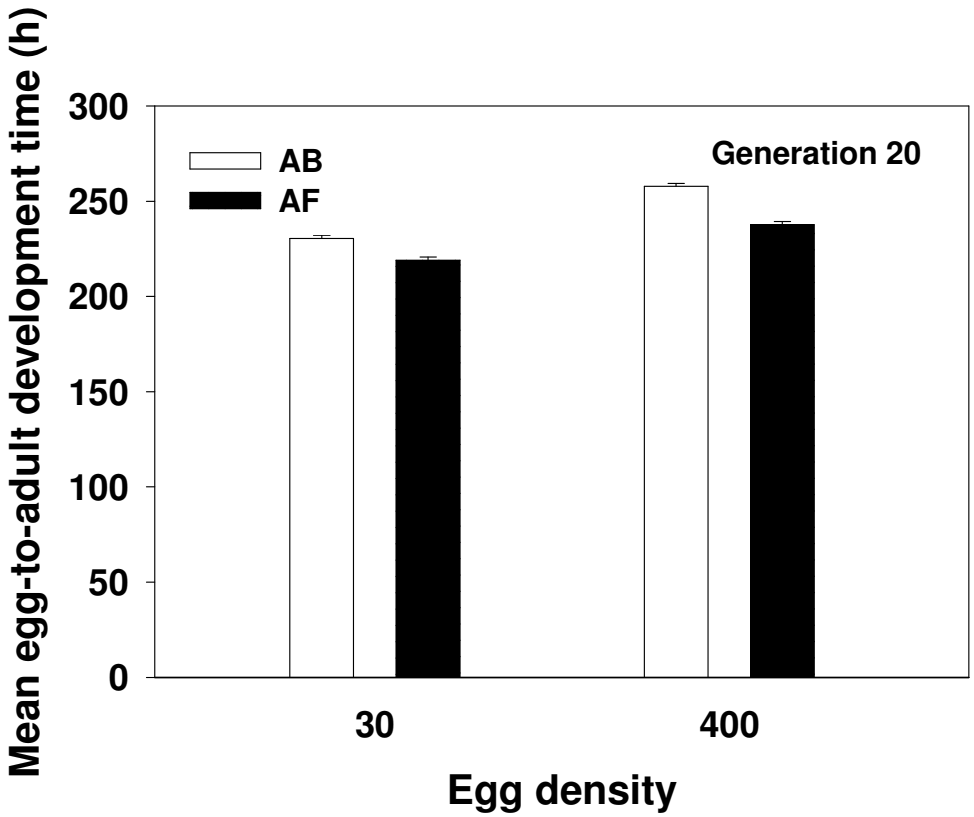

Figure 7.3: Mean (± s.e.) egg-to-adult development time of AF and AB populations, at generation 20 of AF selection.

Egg-to-adult survivorship

Pre-adult survivorship at a density of 30 eggs per vial in the AFs was not significantly different from the ABs at generation 10, but was significantly lower at generation 25 (Figure 7.4). At generation 25, the AFs showed significantly lower survivorship than the ABs at both egg densities *i.e.* 70 and 400 eggs per vial (Figure 7.5). The ANOVA results showed significant main effects of selection regime and density, but there was no significant selection regime × density interaction (Table 7.4), indicating that survivorship in both the ABs and AFs was equally affected by increased density.

Effect	*df*	MS	*F*	*P*
Selection	1	0.0409	72.5554	0.003
Density	1	0.0488	28.8313	0.013
Selection × density	1	0.0014	0.3876	0.578

Table 7.4: Summary of ANOVA results for mean egg-to-adult survivorship in the AFs and ABs at low and high density at generation 25 of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

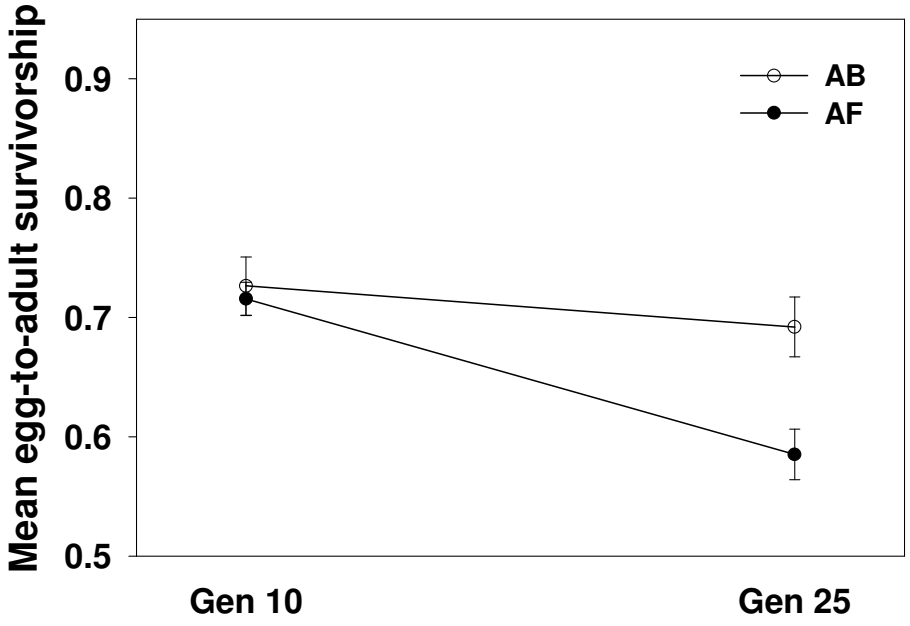

Figure 7.4: Mean (± s.e.) egg-to-adult survivorship of AF and AB populations reared at 30 eggs per vial, at generations 10 and 25 of AF selection.

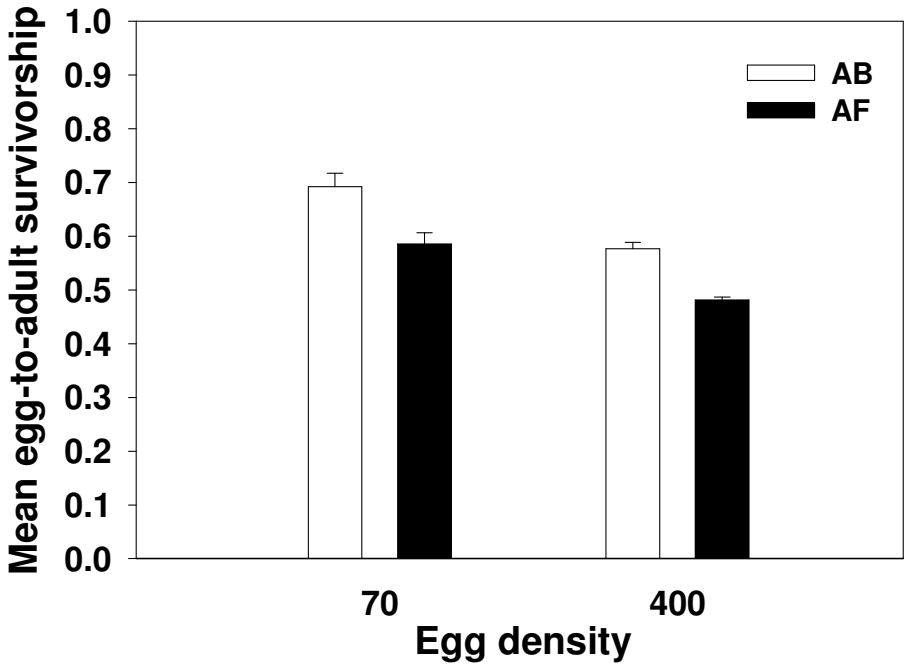

Figure 7.5: Mean (± s.e.) egg-to-adult survivorship of AFs and ABs at low and high densities, at generation 25 of AF selection.

Dry weight at eclosion

Freshly eclosed adult AF flies weighed significantly less than AB flies at generation 10 (Figure 7.6a). Females were significantly heavier than males in both the selection regimes (Figure 7.6b), and the selection × sex interaction was not significant (Table 7.5).

At generation 20, AF flies at eclosion were lighter than ABs when reared at 30 eggs per vial, but heavier than ABs when reared at 400 eggs per vial (Figure 7.7), as reflected in the significant selection regime × density interaction (Table 7.6). There was no significant main effect of selection, but significant effects of density and sex (Table 7.6), as flies from both the regimes were lighter at eclosion when reared at higher density, and females were consistently heavier than males in both ABs and AFs.

Effect	*df*	MS	*F*	*P*
Selection	1	1.96	125.44	0.001
Sex	1	43.2306	131.8342	0.001
Selection × sex	1	0.81	1.9199	0.26

Table 7.5: Summary of ANOVA results for mean dry weight at eclosion of the AFs and ABs at generation 10 of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

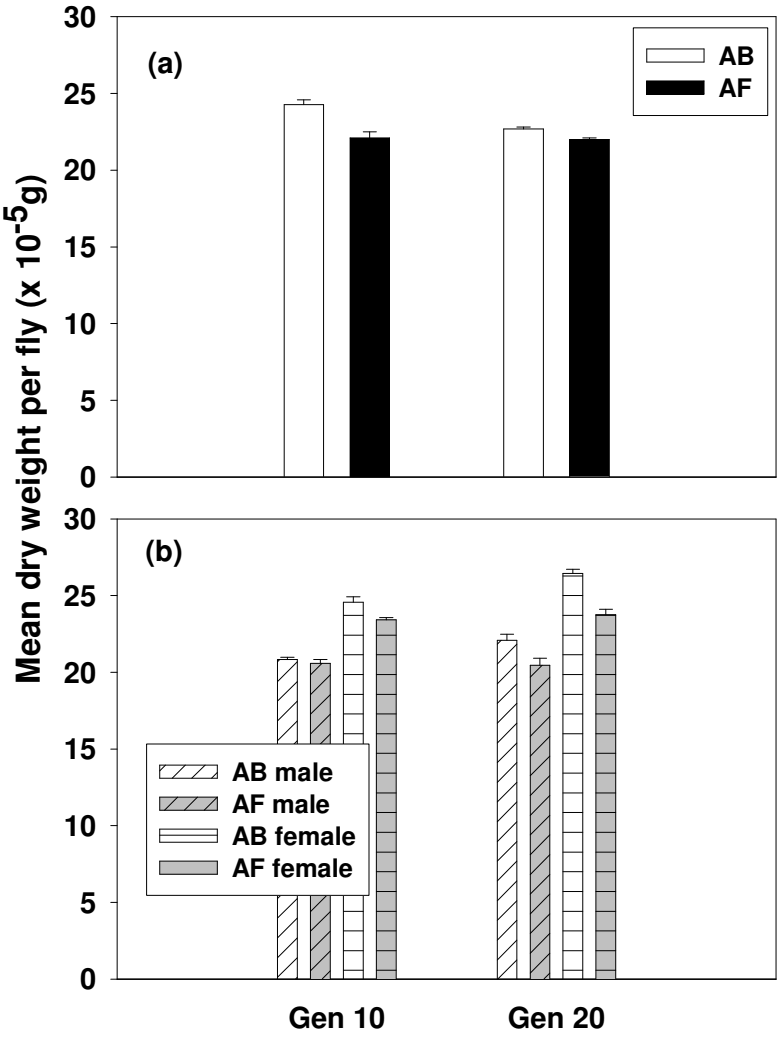

Figure 7.6: Mean (± s.e.) (a) dry weight, and (b) sex-specific dry weight, of freshly eclosed AF and AB adults reared at a density of 30 eggs per vial, at generations 10 and 20 of AF selection.

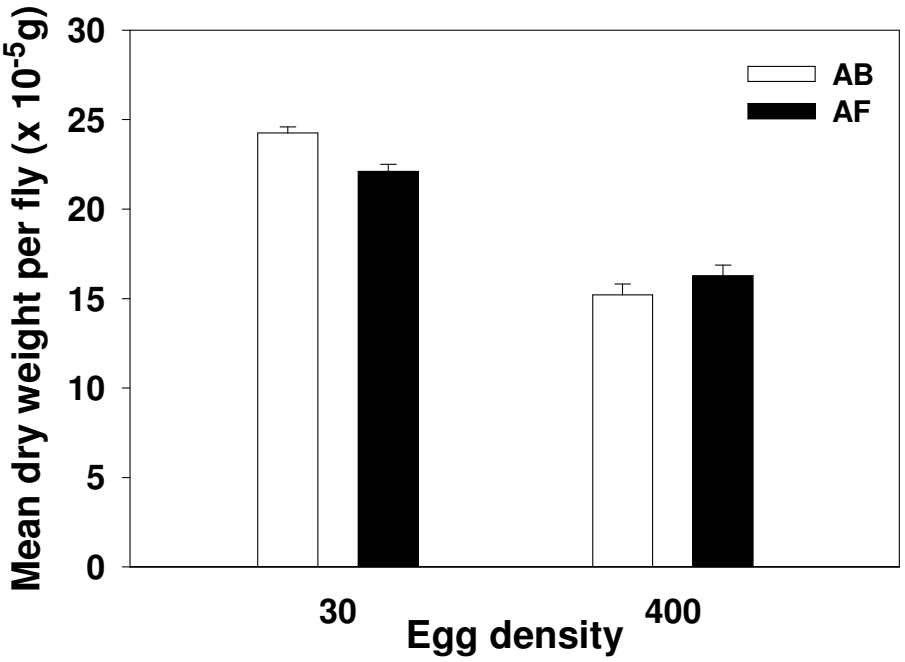

Figure 7.7: Mean (± s.e.) dry weight at eclosion of AF and AB populations at generation 20 of AF selection, at two different rearing densities.

Effect	*df*	MS	*F*	*P*
Selection	1	59.4731	5.1606	0.108
Density	1	11086.5352	118.4821	0.001
Sex	1	2028.7123	242.7107	< 0.001
Selection × density	1	519.024	12.4566	0.038
Selection × sex	1	0.8477	0.2589	0.65
Density × sex	1	81.4141	6.897	0.078
Selection × density × sex	1	45.0261	6.9638	0.077

Table 7.6: Summary of ANOVA results for mean dry weight at eclosion of AF and AB populations reared at two densities at generation 20 of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

Larval feeding rate

The feeding rate of larvae did not evolve over the first 20 generations of AF selection. The ANOVA results showed no significant difference between mean feeding rates of the AFs and ABs. Mean feeding rates for the ABs and AFs were 122.43 and 120.44, respectively, at generation 10. At generation 20, the mean feeding rates were 147.41 and 140.39, respectively (Figure 7.8).

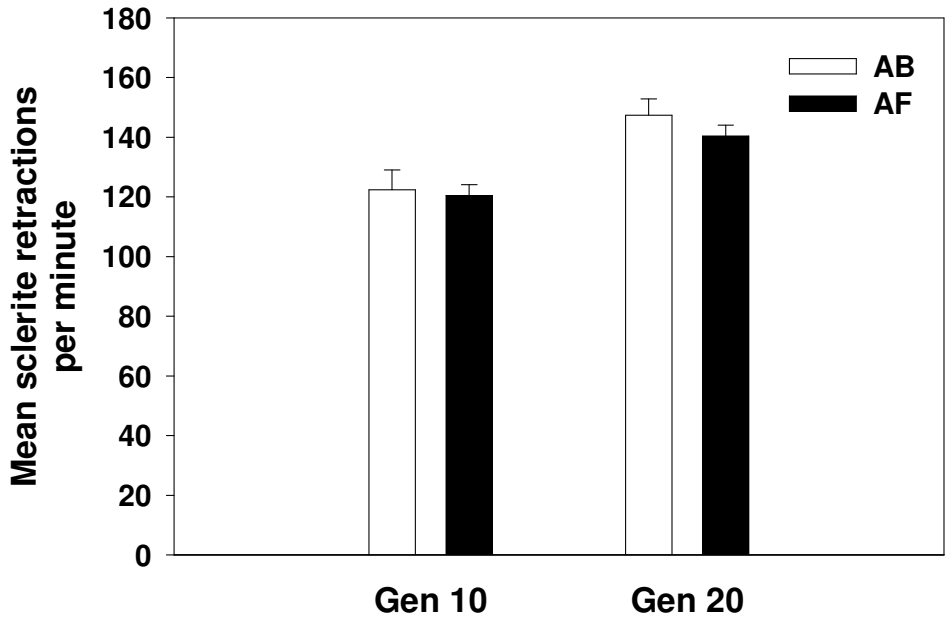

Figure 7.8: Mean (± s.e.) larval feeding rate of AF and AB populations at third instar.

Larval competitive ability

As mentioned earlier, in the '*statistical analysis*' section, survivorship of the test population *i.e. D. ananassae* AB or AF under competitive condition in bitypic culture with WE was scaled by the survivorship of the corresponding population in monotypic culture. The ANOVA done on this measure of competitive ability showed no difference between the AB and AF populations at

either low or high densities (Table 4.8; Figure 7.9). A significant main effect of density was found, as both ABs and AFs showed significantly lower competitive ability against WE at 400 eggs per vial compared to 70. Mean AB and AF survivorships were 71% and 64%, respectively, at the density of 70 eggs per vial; those for 400 eggs per vial were 52% and 45% respectively.

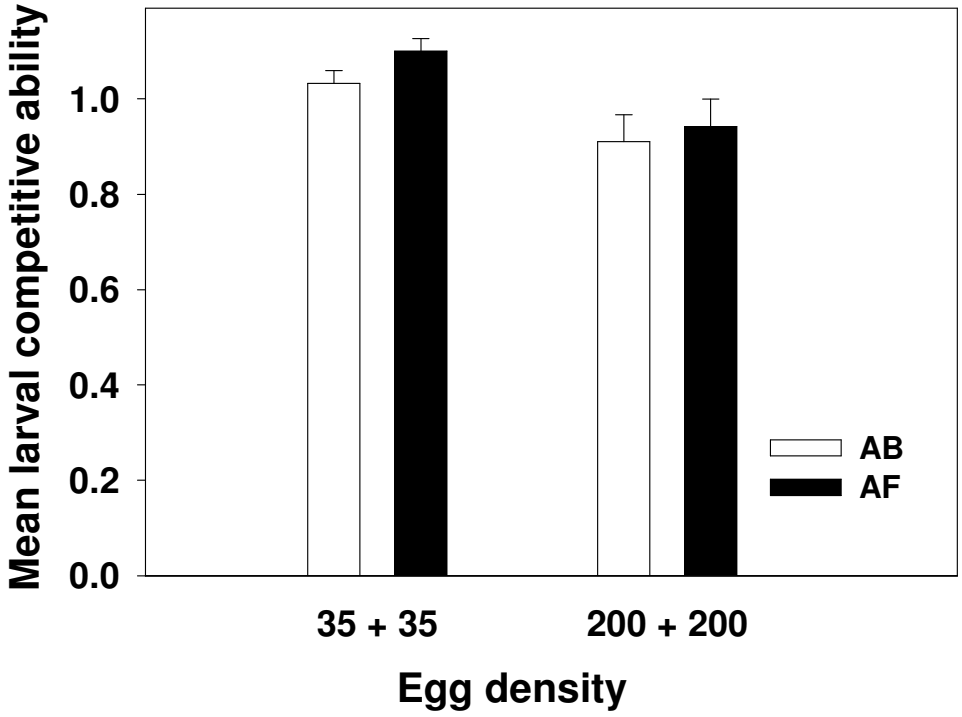

Figure 7.9: Mean (± s.e.) larval competitive ability of AB and AF populations at low and high densities at generation 25 of AF selection.

Effect	*df*	MS	*F*	*P*
Selection	1	0.0099	0.7879	0.44
Density	1	0.0785	15.4145	0.029
Selection × density	1	0.0014	3.6868	0.151

Table 7.6: Summary of ANOVA results for mean larval competitive ability of AF and AB populations after 25 generations of AF selection. Only fixed factor effects and interactions among them could be tested for significance.

Discussion

In *D. melanogaster*, selection for rapid development led to the correlated evolution of reduced pre-adult survivorship, dry weight at eclosion, larval feeding rate and larval competitive ability (Prasad *et al*. 2000, 2001; Shakarad *et al*. 2005; Rajamani *et al*. 2006). I now discuss the results obtained over the first 25 generations of *D. ananassae* AF selection against the backdrop of the previous studies on the *D. melanogaster* FEJs.

The initial response to selection for faster development was quite rapid in AFs as pre-adult development time underwent a 9 h reduction in the first 10 generations of selection. By contrast, the reduction in mean development time of FEJ populations after 10 generations of selection was only 6 h (Prasad *et al.* 2000). As in the FEJs, pre-adult survivorship in the AFs also traded off with developmental rate, but the survivorship cost became apparent after 20 generations of AF selection, as compared to the FEJs in which a significant reduction in survivorship was detected only after 50 generations of selection, by which time development time had reduced by about 26 h (Prasad *et al.* 2000). At generation 20, development time of AFs got further reduced by only 2 h, suggesting that perhaps the AFs were already close to a plateau in the direct response to selection. By contrast, the difference between FEJ and JB development time kept increasing almost linearly through the course of selection till about generation 90 (Prasad 2004). As in the FEJs (Prasad *et al.* 2000), the difference in male and female development times reduced as selection proceeded in the AFs, going from 6.3 h at generation 10 to 4.1 h at generation 20.

As in *D. melanogaster*, a trade-off between developmental rate and dry weight at eclosion was observed in *D. ananassae* populations, with dry weight at eclosion showing a significant decline after 10 generations of AF selection. However, at generation 20, AF dry weight was lower than

that of the ABs when reared at 30 eggs per vial, but higher than that of ABs when reared at 400 eggs per vial, suggesting a G×E interaction masking the trade-off at high density; such effects are known in *D. melanogaster* with regard to the early fecundity and late fecundity trade-off (Leroi *et al*. 1994). Why exactly the AFs are heavier than ABs at high density is not clear, although it is likely that the differences in AFs and ABs in the distribution of food intake per larva and their interaction with minimum critical size are playing a role (*e.g.* see Mueller 1988).

In contrast to the results from *D. melanogaster*, neither larval feeding rate nor larval competitive ability traded off with developmental rate in *D. ananassae*. Thus, it appears that in *D. ananassae* it is possible to evolve faster development without compromising on traits like feeding rate and larval competitive ability. It is also possible that part of the reason for the earlier appearance of a survivorship cost to rapid development in the AFs is that they underwent a reduction in development time without a concomitant, and presumably energy-saving, reduction in larval feeding rate. Perhaps the FEJs staved off an early appearance of a survivorship cost to rapid development by reducing energy expenditure on traits like feeding rate, which led to a concomitant decline in competitive ability.

Overall, the trade-off between developmental rate and dry weight seems to be conserved across the related species *D. melanogaster* and *D. ananassae*. This is not surprising, given that a large reduction in larval duration, especially if it is in the third instar as seen in the FEJs (Prasad *et al*. 2001; Chapter II of this thesis), will result in less time for accumulation of biomass. However, the earlier appearance of the trade-off between developmental rate and pre-adult survivorship, and the lack of a trade-off between developmental rate and larval feeding rate, suggest that the network of genetic correlations between these traits in *D. ananassae* is quite different from that earlier seen in *D. melanogaster*. While the details of why additive genetic variances and

covariances associated with these traits differ across the species are not addressed here, the implication of these results is that one should be cautious when extrapolating results obtained from *D. melanogaster* even to fairly similar congeners. This study also emphasizes the need to study adaptive responses across different species to understand the diversity of ways in which life-histories can evolve.

Chapter VIII

Conclusions

In this thesis, I have addressed diverse evolutionary issues using laboratory populations of *D. melanogaster* selected for rapid development and early reproduction. Although the study was initiated primarily with the objective of investigating the evolution of the life-history of these populations under continued directional selection, observations during the course of my study led me to address a new set of broader evolutionary questions that went beyond the evolution of an optimal life-history. In addition to documenting the further reshaping of the pre-adult life-history of the faster developing populations, with reductions in both egg and second instar duration, I also showed that ongoing selection had been able to circumvent the earlier documented, apparently maladaptive, evolution of the adult life-history toward increased allocation for somatic maintenance rather than early life reproduction. In another study, I experimentally explored the relationship of selection and canalization, an issue on which there was substantial speculation but little empirical evidence, and showed that long-term selection had indeed led to the evolution of greater canalization of development time in the faster developing populations. I also found evidence for a possible trade-off between developmental rate and pathogen resistance in *D. melanogaster.* My work also shows that divergent evolution of life-histories can result in incipient reproductive isolation, an aspect rarely explored in studies of speciation. The results of my selection experiment on *D. ananassae* demonstrates that even fairly close congeneric species can show strikingly different genetic architecture of fitness-related traits, underscoring the necessity to study various species to understand the diversity of ways in which life-histories can evolve in response to different selection pressures. In this chapter, I briefly discuss the major novel findings emerging from my studies in the light of broader conceptual issues in evolutionary genetics.

Past experimental evolution studies on *D. melanogaster* have shown that although responses to selection can be significantly affected by past selection history (Teótonio and Rose 2000; Teótonio *et al.* 2002), the genetic imprints of history can be fairly shallow and are often rapidly erased by ongoing selection (Joshi *et al*. 2003). The proposed 'lipid switch', which determines the relative allocation of resources to reproduction versus somatic maintenance presumably evolved in the wild as an adaptive response to fluctuating nutritional conditions. However, with the altered environmental scenario in the FEJ maintenance regime, the switch became maladaptive, leading to the evolution of a non-optimal life-history in these populations (Prasad 2004). Eventually, over a long period of continued selection, the FEJs overcame this historical constraint and once again began to evolve toward a more optimal resource allocation pattern (Chapter III, section A). The evolution of a maladaptive life-history and its subsequent disappearance in FEJs, thus, exemplifies that sometimes genetic imprints of history can constrain adaptive evolution in the short term, but can get ameliorated by ongoing selection.

However, past selection history can also give rise to constraints that exhibit much greater robustness in the face of ongoing selection. The JBs, and their ancestors the UU populations (described by Joshi and Mueller 1996), were selected for reproduction around day 11 post-eclosion for over 300 generations, beginning from ancestors (B populations of Rose 1984) that had been selected for reproduction at about 4 days post-eclosion ever since their derivation from a wild population in Massachussets (IV population: Ives 1970). Selection for reproduction at day 11 post-eclosion (*i.e.* on a 3 week discrete generation cycle) in the UU-JB lineage led to the evolution of a small subsidiary fecundity peak around this age, while the populations retained a large fecundity peak early in adult life, around day 3 post-eclosion, reflecting a non-optimal fecundity pattern (Prasad 2004). My study suggests that the synchronization of the peak egg

output with the first instance of mating has led to the constancy in the timing of this peak over several hundred generations of selection for reproduction around day 11 post-eclosion (Chapter III, section C). Perhaps such a coupling of the female reproductive output with the onset of mating evolved as an adaptation in the wild, where the probability of finding a mate might not always be high. If true, this is another example of a past adaptation becoming a constraint, because this early peak in fecundity does not add to fitness under the JB maintenance regime. In fact, it probably reduces fitness by using up resources that could be saved to produce more eggs around day 11 post-eclosion. The burst of egg output following the first mating in *D. melanogaster* has been shown to be caused by a component of the seminal fluid (Chen and Buhler 1970; Burnet *et al.* 1973). It is possible that past selection in *D. melanogaster* has led to the fixation of this coupling between peak female fecundity and the first mating, such that it is impossible to uncouple the two events and push the reproductive peak to a later life through selection. While the role of constraints in evolution is well appreciated in theory, there are relatively few experimental studies documenting such constraints (Roff 1992; Stearns 1992). My work on FEJ life-history evolution provides two contrasting examples of past adaptations constraining the attainment of an optimal life-history. In one case, selection for a few hundred generations was able to overcome the constraint, whereas in the other, the constraint proved more resilient. These results, thus, not only demonstrate that past selection history can influence responses to ongoing selection, but also indicate that the ability of selection to circumvent the constraints created by past adaptations can be very case-specific.

The possible involvement of immune function in trade-offs with life-history related traits is increasingly being recognized as an important aspect of life-history evolution (McKean and Nunney 2001; Zuk and Stoehr 2002; Šimková *et al.* 2008). Even in model organisms such as

Drosophila, however, there is little empirical information on the genetic correlations between immune function and life-history traits. One hypothesis about immune function related trade-offs is that they are mediated via the conflicting demands of resource allocation to immune defense and other life-history related traits (Zuk and Stoehr 2002; Šimková *et al.* 2008). In *Drosophila*, selection for faster development is known to consistently lead to the correlated evolution of smaller body-size, indicating reduced resources available to the flies. Thus, developmental rate can be expected to trade off with immune function. Testing this hypothesis by assaying the pathogen resistance of FEJ and JB populations showed significantly lower pathogen resistance of the FEJ populations, suggesting a negative genetic correlation between developmental rate and pathogen resistance (Chapter III, section B), and indicating that resistance to microbial pathogens can indeed decrease as a correlated response to selection on life-history traits in *Drosophila*.

The study reported in Chapter IV provided empirical validation of the prediction that traits strongly acted upon by selection evolve to become more canalized over time. Most studies investigating the environmental sensitivity of traits typically consider mean trait values (*e.g.* see discussion in David *et al.* 2006). For inferring canalization, however, information on trait variation is equally important. Assaying the FEJ and JB development time across various combinations of temperature and density revealed that the trait means as well as variability were markedly less in FEJs. More importantly, both mean and variability of development time in FEJs showed less variation across environments, compared to the JBs, indicating increased canalization of development time in the FEJs.

The concept of canalization was originally proposed to describe the buffering of morphological phenotypes (Waddington 1942). Although considerable theoretical work has been done subsequently, with canalization being viewed as a general phenomenon controlling trait variation

(Eshel and Matessi 1998), the canalization of life-history traits has rarely been addressed (Stearns ans Kawecki 1994; Stearns *et al.* 1995). My study is the first experimental investigation of the effect of prolonged selection on the degree of canalization of a life-history trait. This approach can also be used in the context of other experimental evolution studies to verify the canalizing effect of selection on different life-history traits. Such studies can potentially enhance our understanding of phenotypic buffering and how it evolves, thus broadening the context of canalization from morphological traits to life-histories in general.

The observation of incipient reproductive isolation between the FEJs and JBs (Chapter V) is interesting for several reasons. It not only provides the first evidence for incipient ecological speciation driven by divergence of life-histories rather than specialization on to different habitats or hosts, but also provides rare evidence for the involvement of both sexual selection and viability selection in generating incipient reproductive isolation through the complementary action of two asymmetric barriers to successful reproduction between FEJ and JB individuals. FEJ females suffer high mortality following mating with JB males suggesting a viability selection driven post-mating isolation, whereas JB females seem to exercise a strong preference for JB males leading to a pre-mating isolation between FEJ males and JB females caused by sexual selection.

Speciation is, of course, a very old issue in evolution. More recently, however, the focus of speciation biologists has shifted from classifying the modes of speciation into broad geography-based categories to trying to understand and categorize modes of speciation based on the underlying mechanisms involved. In this context, divergent ecological selection has been implicated as an important agent that can drive speciation, but there are hardly any studies that have been able to identify the ecological basis for divergent selection, the mechanism of

reproductive isolation, and the underlying genetic link between the traits under divergent selection and those responsible for reproductive isolation (reviewed by Rundle and Nosil 2005). My study fills this gap by exposing the link (in this case, via a genetic correlation) between the trait diverging under different selection pressures (development time as a result of the different FEJ and JB maintenance regimes) and the trait mediating the reproductive isolation (body size). My results also reveal an interplay of sexual and viability selection in driving the incipient reproductive isolation between FEJs and JBs, thereby underscoring the necessity of appreciating the potential complexity of the mechanisms underlying the important evolutionary process of speciation.

Most studies on *Drosophila* life-history evolution so far have employed a single species, namely *D. melanogaster*, and there is practically no information about the genetic correlations among fitness components in other related species (Sharmila Bharathi 2007). My study of correlated responses to selection for faster development in *D. ananassae*, a close taxonomic relative of *D. melanogaster*, demonstrated that the genetic correlations among traits associated with development time are quite different in the two species, despite their phylogenetic and ecological closeness (Chapter VII). This finding strikes a cautionary note for drawing broad conclusions from results of selection studies, even across fairly close congeners. It also underscores the importance of studying different species to understand the diversity of ways in which life-histories can evolve in response to same selection pressure.

Overall, the various studies described in this thesis have added considerable fine detail to our understanding of several important evolutionary phenomena such as life-history evolution, overcoming evolutionary constraints due to history, the evolution of canalization, the generality of the genetic architecture of fitness-related traits across species, and the evolution of

reproductive isolation. These studies, thus, also serve to underscore the resolution and power of experimental evolution as a framework for investigating evolutionary processes and phenomena at various levels, as compared to more traditional approaches, such as the comparative method.

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