Preliminary investigations into the causes for alternative routes to the evolution of competitive ability in populations of *Drosophila* selected for adaptation to larval crowding

A Thesis

Submitted for the Degree of

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By

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Dedicated to

the MCUs who made this thesis possible

DECLARATION

I hereby declare that the work embodied in this thesis entitled "*Preliminary investigations into the causes for alternative routes to the evolution of competitive ability in populations of Drosophila selected for adaptation to larval crowding*" has been carried out by me under the supervision of Prof. Amitabh Joshi, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore and that it has not been submitted for any degree or diploma to any other institution.

Following prevalent scientific practice, acknowledgement has been accorded wherever due. Any omission, which might have occurred by oversight or error of judgment, is deeply regretted.

Place: Bangalore Manaswini Sarangi

March 31, 2013

CERTIFICATE

This is to certify that the work embodied in this thesis entitled "*Preliminary investigations into the causes for alternative routes to the evolution of competitive ability in populations of Drosophila selected for adaptation to larval crowding*" has been carried out by Manaswini Sarangi under my the supervision at Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Prof. Amitabh Joshi

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I thank my supervisor Prof. Amitabh Joshi for his scholarly guidance, suggestions and motivation for undertaking this project. His consistent encouragement and valuable inputs during the execution of all the experiments further enhanced my interest with a much better understanding in the issue that I tried to address in this thesis.

I convey my heartiest gratitude to Dr. Snigdhadip Dey for his endeavour and support in materializing the project. Regular discussions with him have cleared and strengthened my concepts in the subject. I would also relish all the fun we had during the last two years or so.

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CONTENTS

Chapter 1

Introduction

Natural selection, as first independently conceptualized by Darwin (1859) and Wallace (Darwin and Wallace 1858), aimed to explain the evolutionary changes in biological populations by differential survival and reproduction among individual organisms. The principle of natural selection has also provided insights into how life-histories themselves evolve, in addition to focusing attention on the life-history as the interface between organismal phenotypes and fitness (Roff 1992; Stearns 1992, 2000). The life-history is determined by the process of development, age of attaining reproductive maturity, lifespan, and the number and probability of survival of offspring (Reznick and Travis 1996). Life-history theory attempts to predict the evolution of optimization of the schedule of survival and reproduction in a given ecological scenario (Stearns 1992, 2000; Roff 1992).

In an evolutionary utopia, life-histories should evolve to maximize overall fitness i.e. organisms would start reproducing soon after birth, producing very large numbers of offspring and survive infinitely. Such a scenario, however, is not possible due to resource limitation, manifested as trade-offs among fitness-related traits. The understanding of the evolution of life-history, thus, requires identification of main fitness-related traits and the genetic correlations among them in the ecological scenario of interest. In this regard, laboratory selection experiments on life-history related traits have been extremely useful in investigating the genetic architecture for fitness-related traits in a well defined and controlled ecology (reviewed in Prasad and Joshi 2003). In particular, such studies on *Drosophila melanogaster* have provided important insights into the manner in which ecology and genetics interact to shape trajectories of adaptive evolution (reviewed in Prasad and Joshi 2003). Patterns of correlated response to selection have varied among

studies selecting for postponed senescence (Rose 1984; Nusbaum et al 1996; Patridge and Fowler 1992), for starvation resistance (Chippindale et al 1996, 1998; Harshman et al 1999), for dessication resistance (Gibbs et al 1997). Selection for rapid egg-to-adult development (Zwaan et al 1995; Nunney 1996; Chippindale et al 1997; Prasad et al 2000, 2001) and for adaptation to crowding (Mueller and Ayala 1981; Mueller and Sweet 1986; Joshi and Mueller 1988, 1993, 1996; Santos et al 1997; Borash et al 1998) on the other hand yielded relatively similar patterns of correlated responses across different studies. Selection for adaptation to crowding is of particular interest because density–dependent selection is an important interface between evolution and population ecology. The basic premise of density–dependent selection is that genotypic fitnesses are functions of population density and that often no one genotype will be the most fit at both low and high densities. Following the early development of the notions of density–independent (*r*-selection) and density–dependent selection (*K*-selection) by MacArthur (1962) and MacArthur and Wilson (1967), formal population genetics model of density–dependent evolution were developed (Gadgil and Bossert 1970; Roughgarden 1971; Clarke 1972; Anderson and Arnold 1983; Asmussen 1983), along with a verbal theory attempting to explain life-history variants via the *r* and *K*- selection dichotomy (Pianka 1970). Central to much of this theory was the notion of an *r*-*K* trade off that essentially suggested that different sets of traits increasing population growth rates at either low or high densities, respectively, would be favored by selection under uncrowded versus crowded conditions.

The first systematic attempt to study *r*– and *K*– selection empirically were through laboratory selection of *Drosophila melanogaster* populations maintained either at low density by culling (*r*–populations) or at high density by allowing populations to reach

their carrying capacity (*K*– populations). This work revealed a trade-off between *r* and *K* for the first time (Mueller and Ayala 1981): the *K*–populations had a higher per capita rate of population growth than the *r*– populations when assayed at high densities, and vice-versa.

Subsequent studies on the same set of populations revealed that, relative to the *r*–populations, the *K*–populations had evolved greater competitive ability (Mueller 1988), larval feeding rate (Joshi and Mueller 1988), pupation height (Mueller and Sweet 1986; Joshi and Mueller 1993) and a reduced efficiency of food to biomass conversion, requiring more food than their *r* populations counterparts to successfully complete development to become adults of similar size at eclosion (Mueller 1990). Essentially the same pattern of trait evolution was seen in a later study using populations of *D. melanogaster* from a different geographical origin and imposing selection only for adaptation to larval crowding, unlike in the *r*– and *K*– populations where the *K*–populations experienced higher densities than the *r* populations in both larval and adult stages (Joshi and Mueller 1996). The overall similarity in the results of these two sets of studies led to the notion that chronic crowding in organisms with primarily scramble competition would likely lead to the evolution of greater efficiency of food acquisition, possibly even at the cost of efficiency of food utilization (Joshi and Mueller 1996).

In addition to food depletion, a crowded *Drosophila* culture also becomes more inhospitable over time due to accumulation of nitrogenous waste and, not surprisingly, crowding-adapted populations were seen to have evolved greater tolerance to toxic levels of urea and ammonia (Shiotsugu et al 1997; Borash et al 1998). Populations adapted to

larval crowding showed an interesting pattern of different strategies for coping with crowding at different times in a crowded culture. Larval progeny of early eclosing flies from a crowded culture in these populations tended to be less urea tolerant and faster feeding than those of late eclosing flies (Borash et al 1998), a polymorphism that is probably maintained in part due to inadvertent imposition of positive assortative mating for development time in the larval crowding-adapted populations (Archana 2010).

More recent studies suggest that there are alternative routes to the evolution of adaptation to larval crowding in *Drosophila* populations. *D. ananassae* and *D. nasuta* were subjected to selection for adaptation to larval crowding (Sharmila Bharathi 2007; Archana 2010) and it was found that feeding rates and tolerance to nitrogenous wastes did not evolve to be greater than their respective controls. Moreover, the selected populations had shorter egg–to–adult development time when assayed at both high and low densities. When assayed at high larval density, the selected populations had higher pre-adult survivorship and greater competitive ability as compared to their controls. The crowding-adapted *D. ananassae* populations were examined for a temporal polymorphism of the traits seen in the CU populations (Borash et al 1998) but no evidence for such a polymorphism was seen (Archana 2010).

This deviation of results of *D. ananassae* and *D. nasuta* crowding–adapted populations from that of CU populations was initially thought to be a species–specific difference, or perhaps due to the fact that the populations of these two species were relatively recently derived from the wild. Consequently, *D. melanogaster* populations (MCU populations: from the same ancestral population from which CU populations were derived; B-

populations, Rose 1984) were subjected to evolution under larval crowding and assayed for the above mentioned traits. The results were similar to that obtained from the other two species and different from those seen earlier in the CU populations. Feeding rates of the selected populations were not different from their respective controls, unlike the enhanced feeding rates of *K*-selected (Joshi and Mueller 1988) and CU populations (Joshi and Mueller 1996; Santos et al 1997). However, the difference in pre-adult development time between selected and control populations was not expressed at high density although the trend was for the crowding-adapted populations to be faster developing. Furthermore, when assayed for urea and ammonia tolerance the larval crowding selected populations did not differ significantly from their controls, unlike the CU populations, that were more tolerant to nitrogenous wastes (Shiotsugu et al 1997; Borash et al 1998).

Though slightly digressing, one can emphasize on the point that high larval density in limited amount of food can somewhat be seen as nutrition deficiency, both from the low availability of food and high levels of waste point of view. One can think of a parallel situation where there is larval nutritional stress provided by diluting the nutritional value of the food provided in the culture. In this case, running out of food over time is not the problem, rather individuals have to complete their development and eventually reproduce despite malnutrition. The mechanisms underlying this process of adaptation are thought to differ from the other form of nutritional stress that is imposed by extreme larval crowding but in better quality of food. Some of the recent experiments on populations of *D. melanogaster* adapted to chronic nutritional stress (Kolss et al 2009; Vijendraverma et al 2012) have shown interesting results. Egg to adult development time was found to be faster as compared to their control populations unlike no difference in between CU and

UU populations. Along with this, as the immediate consequence was an almost 50% decrement in dry body weight of freshly eclosed adults, whereas no body size difference was detected in between CU and their control populations. Moreover, average egg-to-adult growth rate was observed to be greater as compared to controls on poor quality food (Kolss et al 2009), as in the CU populations where till the pupal stage, growth rate was higher than their controls (Santos et al 1997).

Now, given all these differences in the traits that evolved in response to chronic larval crowding between the CU populations and our crowding-adapted MCU populations, I tried to investigate whether differences in the ecology of these two types of crowding–adapted *D. melanogaster* populations might be giving rise to different routes to evolution of greater competitive ability. In our laboratory, as compared to controls which are maintained in a density of approximately 70-80 eggs per vial in 6 ml of food, the selected MCU populations are reared in a density of approximately 600 eggs per vial in 1.5 ml of cornmeal food. The CU populations, on the other hand, were reared at approximately 1200 eggs in 6 ml of banana food. So, here in our crowding-adapted lines the '*intensity*' of crowding (egg density) is exactly double that of the CU population regime, and the total amount of food is much less. Due to the extremely low level of food in MCU cultures, nitrogenous waste may build up to toxic levels early on in the life of the culture, which might add a fitness cost to higher feeding rates due to increased rate of ingestion of toxic wastes (Mueller et al 2005). Thus, although both MCU and CU populations experience crowding, their laboratory ecology does actually differ in terms of how fast food is likely to run out and nitrogenous waste build up to toxic concentrations. Thus, in principle, the MCU and CU type of culture conditions could result in different levels of

selection acting on variation in these traits related to competitive ability: larval feeding rate, tolerance to nitrogenous waste and efficiency of converting food to biomass.

In this thesis, I report results from preliminary experiments aimed at testing the above hypothesis that the different routes to adapt to larval crowding seen in the MCU populations and the larval crowding-adapted populations of Joshi and Muller (1996) are due to the much lower amounts of total food available to larvae in the MCU culture regime. I also characterized the MCU populations in more detail, especially with regard to pre–adult traits.

All the experiments on the MCU populations reported earlier (Archana 2010) were done during the early generations of selection. The present experiments were carried out from generation 80 of MCU selection onwards.

Chapter 2 describes the details of the different stages of development of the crowding-adapted MCU populations with regard to development time and dry weight.

Chapter 3 discusses the degree of plasticity of the crowding-adapted MCU populations with respect to body size at eclosion, pre-adult survivorship and pre-adult development time when assayed at five different larval densities at a constant food level.

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Chapter 5 reports results from an experiment aimed at investigating how the differences in the distribution of pre-adult development time and body size at eclosion, and pre-adult viability are affected by different combinations of larval density and total food level.

All the assays reported here represent preliminary studies on how subtle changes in the ecology of laboratory populations of *Drosophila* under slightly different maintenance regimes can potentially affect the suite of traits that evolve in response to chronic larval crowding. In the final chapter, I discuss my findings in the perspective of how the balance of food available and build–up of nitrogenous waste can potentially affect the fitness of different traits underlying competitive ability. I also discuss avenues of future investigation along these lines in an attempt to provide a fuller explanation of the observed differences in the evolutionary trajectory of the MCU and CU populations.

Experimental Populations

Four laboratory populations of *D. melanogaster* were used for the experiment. The **M**elanogaster **B**aseline populations (MB_{1-4}) served as the controls which are maintained on a 21-day discrete generation cycle at 25˚C, constant light (LL) and on cornmeal medium. The egg density is kept at a moderate level by collecting about 70 eggs per vial in 6 ml of food. Each population consists of ~ 1500 breeding adults which are kept in Plexiglas cages (25 cm x 20 cm x 15 cm) with abundant food. On the 11th day after egg collection, the eclosed flies are transferred to the cages and provided with live yeast paste supplement from day 18 onwards. Three days after yeasting, on day 21, eggs are collected for the next generation. The selected populations, **M**elanogaster **C**rowded as larvae and **U**ncrowded as adults (MCU_{1-4}) were derived from their respective controls

(ie, MCU_1 was derived from MB_1, and so on). The selected populations are also maintained on a 21-day discrete generation cycle under similar conditions as controls except a much higher egg density which is about 600 eggs per vial in 1.5 ml food. Around 1500 breeding adults per population are maintained in the cages with abundant food. Once the flies start eclosing, they are transferred to the cages, followed by yeasting on day 18, 3 days after which egg collection is done for the next generation.

Standardization

Prior to any assay, both control and selected populations were subjected to common rearing conditions for one full generation to eliminate any non-genetic parental effects. About 70 eggs were collected per vial with 6 ml of food and 40 such vials were set up per population. On the 11th day after egg collection, the eclosed flies were transferred to cages. Subsequently, the populations were provided with live yeast paste for 3 days prior to the egg collection for assay. Progenies of these standardized flies were used for all assays.

Chapter 1

Introduction

Natural selection, as first independently conceptualized by Darwin (1859) and Wallace (Darwin and Wallace 1858), aimed to explain the evolutionary changes in biological populations by differential survival and reproduction among individual organisms. The principle of natural selection has also provided insights into how life-histories themselves evolve, in addition to focusing attention on the life-history as the interface between organismal phenotypes and fitness (Roff 1992; Stearns 1992, 2000). The life-history is determined by the process of development, age of attaining reproductive maturity, lifespan, and the number and probability of survival of offspring (Reznick and Travis 1996). Life-history theory attempts to predict the evolution of optimization of the schedule of survival and reproduction in a given ecological scenario (Stearns 1992, 2000; Roff 1992).

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All the assays reported here represent preliminary studies on how subtle changes in the ecology of laboratory populations of *Drosophila* under slightly different maintenance regimes can potentially affect the suite of traits that evolve in response to chronic larval crowding. In the final chapter, I discuss my findings in the perspective of how the balance of food available and build–up of nitrogenous waste can potentially affect the fitness of different traits underlying competitive ability. I also discuss avenues of future investigation along these lines in an attempt to provide a fuller explanation of the observed differences in the evolutionary trajectory of the MCU and CU populations.

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(ie, MCU_1 was derived from MB_1, and so on). The selected populations are also maintained on a 21-day discrete generation cycle under similar conditions as controls except a much higher egg density which is about 600 eggs per vial in 1.5 ml food. Around 1500 breeding adults per population are maintained in the cages with abundant food. Once the flies start eclosing, they are transferred to the cages, followed by yeasting on day 18, 3 days after which egg collection is done for the next generation.

Standardization

Prior to any assay, both control and selected populations were subjected to common rearing conditions for one full generation to eliminate any non-genetic parental effects. About 70 eggs were collected per vial with 6 ml of food and 40 such vials were set up per population. On the 11th day after egg collection, the eclosed flies were transferred to cages. Subsequently, the populations were provided with live yeast paste for 3 days prior to the egg collection for assay. Progenies of these standardized flies were used for all assays.

Chapter 2

Stage-specific development time and body size in populations of *D. melanogaster* selected for adaptation to larval crowding

Introduction

Crowding in the larval stage of a *Drosophila* culture causes increased competition among individuals for acquiring sufficient resources to successfully complete all stages of development before the environment becomes inhospitable due to lack of food and build–up of nitrogenous wastes (Mueller 1997). As discussed earlier, the CU populations had adapted to larval crowding and evolved increased competitive ability largely through a higher larval feeding rate than their controls (Joshi and Mueller 1996; Santos et al 1997). A positive correlation between feeding rate and competitive ability had been seen in earlier studies, too (Bakker 1961, 1969). Moreover, the CU populations were also found to be more tolerant to toxic levels of urea and ammonia than their controls (Shiotsugu et al 1997; Borash et al 1998). In contrast to the evolutionary route to the increased competitive ability shown by the CU populations, the MCU populations had evolved higher competitive ability than the MB populations, but there was no correlated evolution of either a higher larval feeding rate or an enhanced level of urea or ammonia tolerance relative to the MB controls (Archana 2010). In principle, there could be two possible strategies for increasing competitive ability without a change in larval feeding rate. One possibility would be to develop faster than other individuals and thereby attain the critical size faster, before food potentially runs out in a crowded culture. The other possibility would be to evolve a lower critical size, thus allowing its attainment earlier. These two possible mechanisms for attaining increased competitive ability are not mutually exclusive, and the degree to which they are realized will depend on the details of the ecology of the culture and its interplay with patterns of genetic variance and covariance among the relevant traits. Earlier work showed that, unlike the

CU populations (Santos et al 1997), the MCU populations had evolved to complete pre-adult development about 8 h faster than the MB controls after 43 generations of MCU selection (Archana 2010). However, this difference was seen only when assayed at low (30 eggs per vial) but not at high larval density (800 eggs per vial) (Archana 2010).

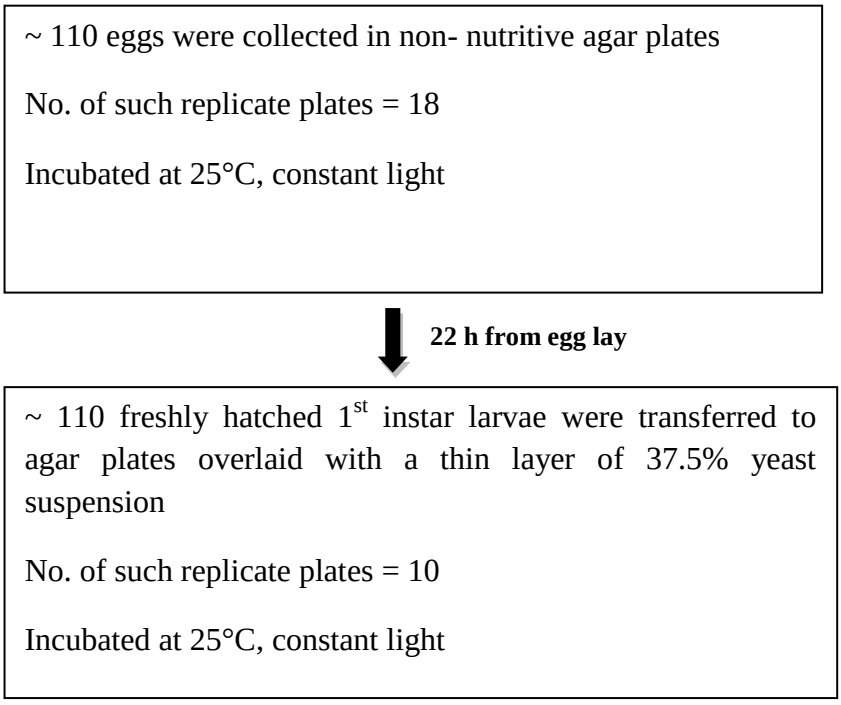
In other previous studies in which *D. melanogaster* populations were directly selected for reduced pre-adult development time, the largest reductions were observed in the first and third larval instar durations, and in the pupal phase, especially during the first 100 generations of selection (Prasad et al 2001). In the MCU cultures, food runs out at a point where most larvae would likely be in early to mid–third instar. As the minimum critical size is attained early in the third instar (Robertson 1963; de Moed et al 1999), reduction in the duration of the pre-critical growth phase would likely be favored by selection in the MCU populations because it would increase the probability of individuals attaining the critical size before food runs out or becomes inhospitable. On the other hand, reduction in the post-critical growth phase or pupal duration may not directly enhance survival but could evolve due to either pleiotropy driven correlations with pre-critical growth rate, or perhaps due to selection on reproductive fitness favoring individuals of slightly older age at the time of egg collection for initiating the next generation. Moreover, reduction of time till attainment of minimum critical size could be achieved via an increased pre-critical growth rate, or a reduction of minimum critical size, or both.

In this chapter, I report results from assays aimed at characterizing the duration of and growth rates at different pre-adult life stages in the MCU and MB populations as well as the mean time at which larvae from these populations attain the minimum critical size for successfully completing development.

Materials and Methods

Stage–Specific Development Time

This assay was conducted after 106 generations of MCU selection. Prior to the assay, MB and MCU populations were standardized by rearing both the control and selected lines under similar (MB-type) environmental condition for one generation so as to eliminate any non-genetic parental effects. The standardized flies were yeasted for three days prior to egg collection. To ensure approximately the same physiological age of eggs, a fresh food plate was kept in cage for one hour. After an hour this plate was discarded and another fresh food plate was placed in the cage for next one hour, after which eggs for the assay were collected. The procedure detailed below was followed thereafter.



24 h later

3 larvae were randomly collected from each plate, for a total of 30 larvae. The procedure was repeated for the next 8 h in an interval of 2h. The collected larvae were immediately freezed for subsequent identification of the instar stage based on the mouth hook structure (Ashburner 1989)

12 h later from the last larvae collection time point

Same procedure was repeated as in the previous step for next 10 h in an interval of 2 h

18 h later from the last larvae collection time point

Out of the 10 plates/ population, 5 randomly chosen plates were kept to allow pupation to occur and the other 5 plates were used for collection of wandering stage larvae

To allow larvae to wander off the food, plates were covered with transparent plastic sheets. The plates were then closely monitored for the first larva to wander off the food and onto the sheet at which point, it was collected and immediately freezed for subsequent dry weight measurement. The procedure was repeated at an interval of 2 h thereafter till no larvae were further observed to wander on the sheet for continuously 6 h

Remaining 5 plates were monitored for pupa formation and pupae were collected roughly between 172 to 196 h from egg lay after which they were transferred to vials containing non-nutritive agar

Vials were then closely monitored for eclosion and adults were collected at an interval of 2 h from the eclosion time of the first adult. Vials were monitored till no adults eclosed for a period of about 96 h

Characterization of Larval Stages

Larvae collected at different time points were dissected out for their mouth parts and imaged subsequently under a Nikon Eclipse E200 microscope. The larvae were scored being in the first, second or third instar depending upon the number of teeth present and the shape of their mouth hooks (Fig. 1). Larvae observed to possess four mouth hooks (one pair of the present instar and another pair belonging to the next instar) were scored to be in the transition zone. From these data, I calculated mean development time from egg to first instar, third instar, wandering larval and adult stage, respectively.

Dry Weight

Wandering stage larvae were collected at 2 h interval as described in the previous section to pre–empt their moulting into pre-pupae. These larvae were dried in hot air oven at 64˚C for 36 h and then weighed in batches of 20 each using a Sartorius (CP 225D) fine balance. For weighing, larvae were placed on a piece of aluminum foil whose weight was taken separately and later subtracted from the total weight of larvae and foil to give the dry weight of the larvae. Fifteen such batches of 15 larvae each were set up per population.

The freshly eclosed adults which were sexed during egg-to-adult development time checks were dried in hot air oven at 64℃ for 36 h and were then weighed in 10 to 15 batches of five flies each, males and females separately (number of replicates varied from one block to another and from male to female depending upon the maximum number of flies eclosing in a particular vial).

Critical Minimum Feeding Time

In this assay, the minimum duration of feeding on yeast required for larvae to successfully complete development was calculated. Eggs for the experiment were collected from the standardized flies which were yeasted for three days prior to egg collection. Eggs were collected in a narrow time window (as described earlier) to synchronize the age of eggs. Eggs from each population were separated into 20 batches of 100-110 eggs each. Each batch was spread on a non-nutritive agar plate for hatching. The plates were then monitored for hatching and freshly hatched first instar larvae were transferred to Petri-plates containing a thin layer of non-nutritive agar overlaid with 37.5% suspension of yeast. Twenty such plates were set up per population each containing approximately 80 larvae. Larvae were removed from the yeast plates at 2 h intervals between 62 to 80 h from egg collection. At each time point, 90 larvae were removed randomly from these plates and were moved to agar vials and their survivorship till eclosion was recorded.

Statistical Analyses

The mean time for transition from first to second instar, second to third instar, mean development time to wandering stage, mean pre-adult development time and mean dry weight at different larval stages were subjected to ANOVA treating selection regime as fixed factor and block as a random factor. For mean adult dry weight at eclosion ANOVA was done with selection and sex as fixed factors and block as random factor. ANOVA was also done on mean survivorship of larvae at different time points in the late second to mid-third instar larval stage with selection and time as fixed factors and block as a random factor. All statistical analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (Statsoft Inc., 1995).

Results and Discussion

Stage–Specific Development Time

Both MB and MCU populations reached second instar larval stage in approximately the same time, MCU populations in 47.32 h and MB populations in 47.4 h (Table 1). However, the MCU populations developed faster through the second instar phase and reached third instar stage earlier than MBs by 2.98 h (Table 1), taking 66.85 h to reach third instar stage, whereas MB populations did so after 69.8 h. Moreover, when scored for time till reaching wandering stage, MCU populations were faster than MB populations by 5.29 h (Table 1), taking 115.3 h to reach wandering phase from egg collection compared to 110.07 h for the MB populations. The mean pre-adult development time of MCU populations was found to be less than MBs (Table 1), with females developing faster than males across both selection regimes (Table 1). The mean pre-adult male development time in males was 226.67 h and 218.51 h in MB and MCU populations, respectively. In females, the mean pre-adult development time was 224.0 h and 213.45 h for MB and MCU populations, respectively (Fig. 2).

Stage–Specific Dry Weight

MCU larvae consistently weighed more than MB larvae during the time spanning 62 to 80 h from egg collection, which corresponds to late second instar through approximately middle third instar stage (Table 2.a, Fig. 3). However, at wandering phase, MB larvae weighed more than MCU larvae (Table 2.b, Fig. 3). Moreover, MCUs eclosed as lighter adults than MBs (Table 2.b, Fig. 3), with females weighing more than males across both selection regimes.

Critical Minimum Feeding Time

When larvae were assayed for survivorship after getting transferred to non-nutritive agar plates at 3 h intervals, at all time points between 62 to 80 h from egg collection, MCU larvae had consistently higher survivorship than MB larvae (Table 3, Fig. 4).

Results from this assay clearly suggest that the MCU populations attain their critical minimum size faster than the MB populations (Fig. 4). Moreover, at any given time from the late second to mid third instar, the MCU larvae are significantly heavier than their control larvae (Fig. 3a). At some point thereafter, the MB larvae become heavier and eventually eclose as heavier adults. At the beginning of the third instar, MCU populations have about a 3 h head start on the MB populations but the survivorship data from the assay in which larvae were removed from food at different time points suggest that the MCU populations are attaining minimum critical size more than 3 h before the MB populations (Fig. 4), possibly indicating that, in addition to a faster pre-critical growth phase growth rate, the MCU populations also have reduced minimum critical size. If the post-critical feeding duration is relatively constant (Robertson 1963), this may explain why essentially at the end of the post-critical feeding phase, MB larval weights catch up with those of the MCU populations. In the MCU cultures, post-critical size growth rates may also not be under very strong selection as there is typically very little food left in the crowded culture vials by the time most larvae are in mid-third instar. This may possibly explain why post-critical size growth rates in MB populations are higher than MCU populations, resulting in their being heavier as wandering larvae.

Given that larval feeding rates trade-off with efficiency of conversion of food to biomass (Joshi and Mueller 1996), the need to acquire critical size early, before food runs out, in the MCU cultures may also explain the lack of evolutionary response in increased feeding rates in the MCU populations. Moreover, due to the overall low food levels in the MCU cultures, nitrogenous waste likely builds up to toxic levels early on, further adding a fitness cost to higher feeding rates in the MCU populations due to increased rate of ingestion of toxic wastes (Mueller et al 2005).

Overall, these results suggests that larval density can interact with absolute food levels in a crowded culture to favor alternative routes to the evolution of greater competitive ability via either increased feeding rate and waste tolerance (as in the CU populations, Joshi and Mueller 1996; Borash et al 1998) or through rapid pre-critical size growth rate and possibly due to greater efficiency of food conversion, as seems to be the case in MCU populations.

Table 1 Results of ANOVA on mean time for transition from first to second instar, second to third instar larval stage, mean development time to wandering stage and mean pre-adult development time of MB and MCU populations, with selection as fixed factor and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect		df	MS	*F*	*P*
Selection	L1 to L2	1	0.054	0.747	0.451
	L2 to L3	1	17.834	43.273	0.007
	WL	1	56.093	63.287	0.004
	Adult	1	350.074	103.581	0.002
Sex	Adult	1	59.888	116.596	0.002
Selection × Sex	Adult	1	5.726	3.660	0.152

Table 2.a Results of ANOVA on mean dry weight of larvae at different stages of larval development, in a span of 62 to 80 h from egg collection in MB and MCU populations, with selection and time as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	95.298	8832.264	< 0.001
Time	6	238.881	58.033	< 0.001
Selection × Time	6	2.944	58.033	0.430

Table 2.b Results of ANOVA on mean dry weight of wandering larva and mean adult dry weight at eclosion of MB and MCU populations, with selection as fixed factor for wandering larva dry weight and, selection and sex as fixed factors for dry weight at eclosion with block as a random factor for both analyses. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect		df	MS	*F*	*P*
Selection	WL	1	24.733	832.245	< 0.001
	Adult	1	3.300	10.936	0.045
Sex	Adult	1	463.613	697.489	< 0.001
Selection × Sex	Adult	1	0.464	0.293	0.626

Table 3 Results of ANOVA on mean survivorship of larvae on non-nutritive agar plates when separated at different time points from egg collection in MB and MCU populations, with selection and time as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	0.315	297.848	< 0.001
Time	6	0.538	29.609	< 0.001
Selection × Time	6	0.024	2.365	0.073

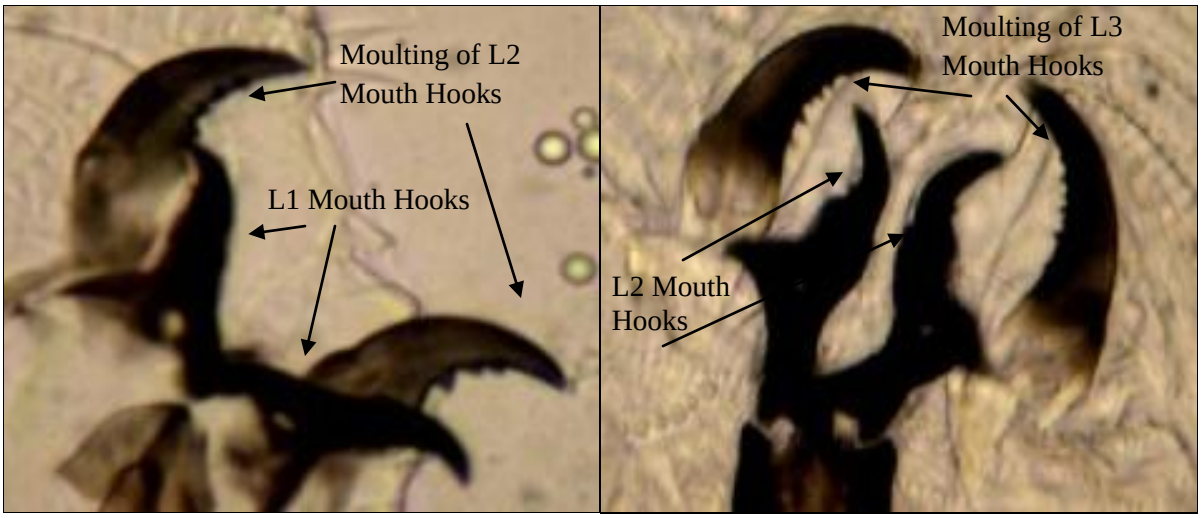


Fig. 1 Larval mouth hooks in transition state. Left panel: L1 to L2 transition, Right panel: L2 to L3 transition.

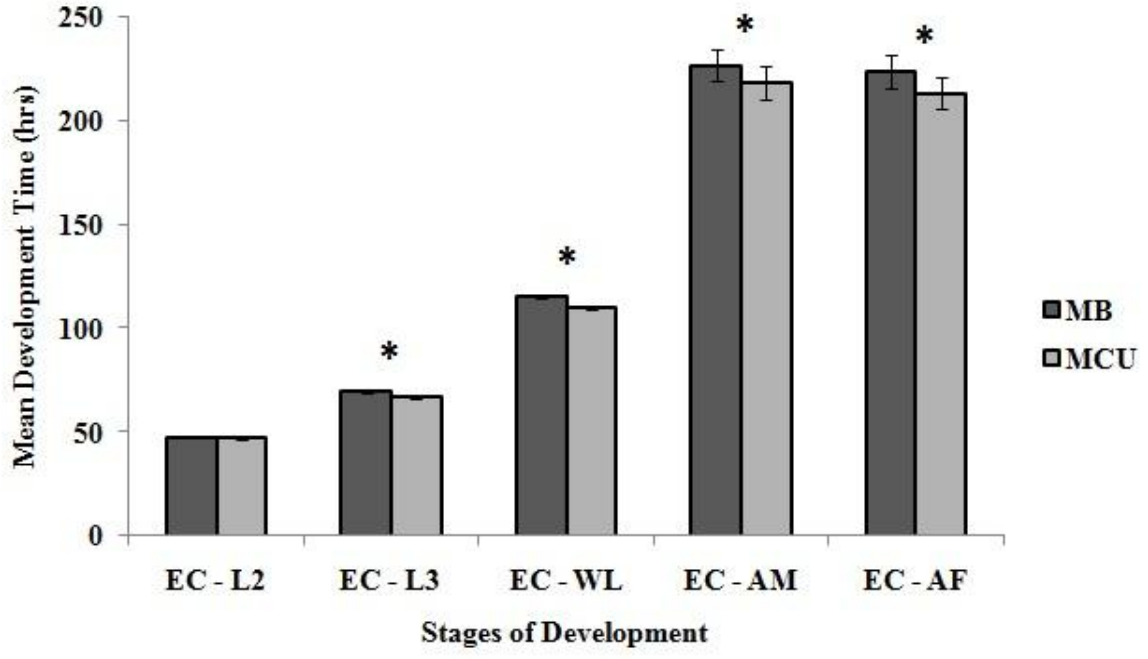


Fig. 2 Mean development time from egg lay to the beginning of the respective developmental stages. Error bars are the standard error around the means of four replicate populations. EC- egg collection, L2 - second larval instar, L3 - third larval instar, WL - wandering larva, AM – adult male at eclosion, AF – adult female at eclosion.

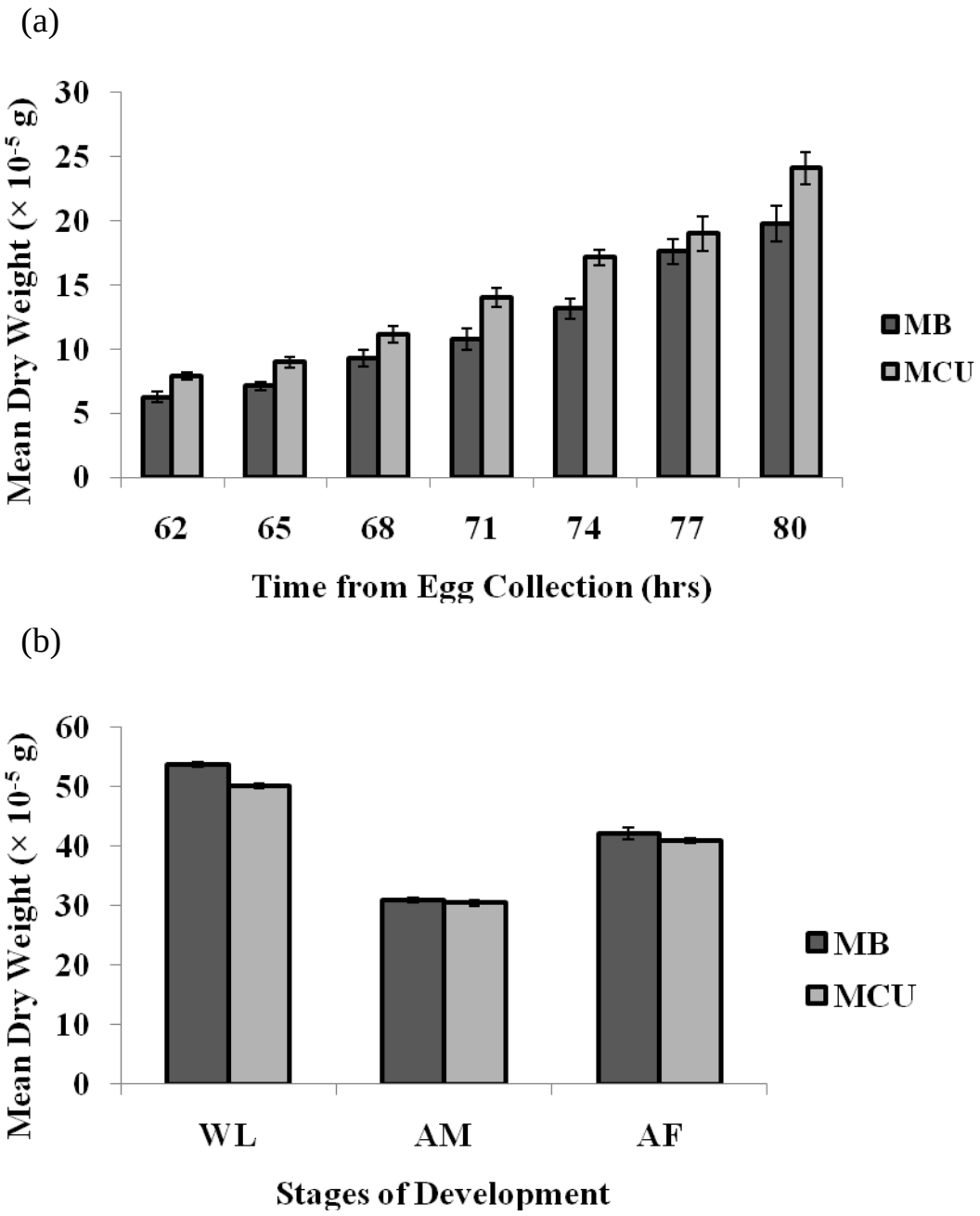


Fig. 3 (a) Mean dry weight of individual larva when measured at different time points from egg collection (b) Mean dry weight of individual wandering larva and adult male and female at eclosion. WL - wandering larva, AM – adult male at eclosion, AF – adult female at eclosion. Error bars represent the standard error around the means of four replicate populations, for both selection regimes.

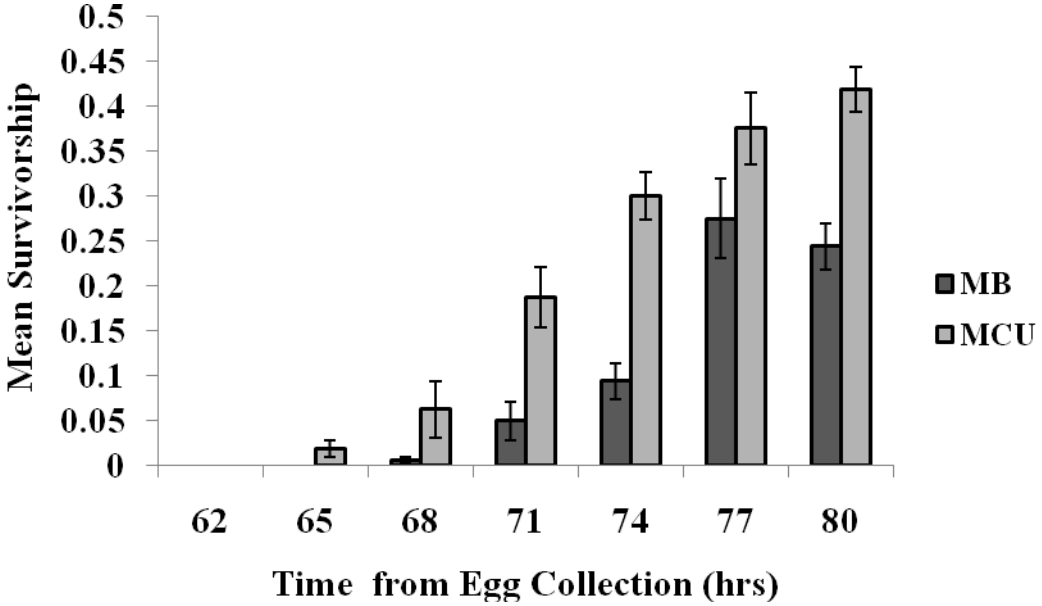


Fig. 4 Mean survivorship to eclosion of larvae when removed from food at different time points from egg lay and transferred to non-nutritive agar plates. Error bars are the standard error around the means of four replicate populations.

Chapter 3

Adaptation to larval crowding does not alter phenotypic plasticity of key fitness-related traits when assayed under different larval densities

Introduction

Phenotypes of organisms are subject to vary depending upon the environment, and this ability is referred to as phenotypic plasticity (Bradshaw 1965; Miner et al 2005). Being phenotypically plastic can be adaptively advantageous to organisms when ecological conditions are subject to fluctuations over time. Plasticity can be for variations in morphological traits or even different life-history traits. For example, frog tadpoles exhibit larger tails and smaller bodies upon exposure to predators (Buskirk and McCollum 2000), which ultimately enables them to react to and swim faster, thereby reducing the chances of being preyed upon (Buskirk and Relyea 1998; Buskirk et al 2003).

In *Drosophila*, the issue of phenotypic plasticity across larval densities, and the degree to which such plasticity can evolve, remains controversial. Bergland et al (2008) used quantitative genetic approaches, and reported extensive genetic variation for plasticity of adult body size (by measuring thorax length) with respect to varying yeast levels in inbred lines of *Drosophila*. Essentially, their study showed that across various yeast levels, there was a significant genotype × environment interaction for both ovariole number and thorax length. If this were generally true in *Drosophila*, one would expect evolution at different food levels or larval densities to shape phenotypic plasticity in different ways. However, in another study using relatively large outbred *Drosophila* populations, Mueller and Cabral (2012) reanalyzed data from Mueller et al (1991) and found no evidence for different patterns of phenotypic plasticity of body size across varying food levels in the *r*-selected and *K*-selected populations. Mueller and Cabral

(2012) suggest that the discrepancy between their results and that of Bergland et al (2008) could be due to the use of inbred lines by Bergland et al (2008). Moreover, under larval crowding, as in the *K*-selected populations, larvae are not just subjected to food limitation but also to other kinds of stresses like increasing level of nitrogenous waste in the medium (Borash et al 1998), and competition for pupation sites. Hence, along with the interaction of food level and larval density these factors could also potentially contribute to the observed discrepancy between the studies of Bergland et al (2008) and Mueller and Cabral (2012).

Due to discrepancy across studies discussed above, I decided to compare the pattern of phenotypic plasticity across larval density in some fitness-related traits in the MCU and MB populations. I assayed pre-adult survivorship and development time, and dry weight at eclosion of the MCU and MB populations across five different larval densities. At the time of this assay, the MCU populations had undergone 82 generations of selection.

The results showed that MCUs develop faster than the MBs in all the density regimes studied. Moreover, the developmental time distribution of these two sets of populations does not show any detectable qualitative difference. The MCUs also showed significantly higher pre-adult survivorship than the control MBs at both low and high densities. Additionally, MCUs are smaller adults at eclosion than their MB counterparts at all larval densities examined.

Materials and Methods

Assay Environments

For both MB and MCU populations, the following larval density assay environments were set up by collecting an exact number of eggs in vials (9.6 cm height, 2.2 cm inner diameter) with 1.5 ml of cornmeal medium: 20, 60, 160, 300 or 600 eggs per vial, with ten replicate vials each of the two lowest densities and six replicate vials each of the three highest densities.

Egg-to-Adult Development Time

Egg-to-adult development time was recorded by checking for the eclosion of adult flies and after the first fly eclosed, the vials were checked regularly at 6 h intervals and the number of eclosing males and females was recorded. From these data, the mean egg-to-adult development time for each sex × selection regime × assay environment combination was calculated. For examining the distributions of developmental times, data from all the replicate vials in each of the larval densities were pooled, and the frequency distribution of the number of flies eclosed in each 24 h window was plotted.

Egg-to-Adult Survivorship

The number of flies successfully eclosing from each vial was recorded and used to mean calculate egg-to-adult survival for each selection regime × assay environment combination.

Dry Weight at Eclosion

Dry weight of the freshly eclosed males and females was recorded by dividing freshly eclosed flies into batches of 5 males or females separately, and then freezing them. Later, the flies were dried at 70˚C for 36 h and weighed immediately using the Sartorius (CP 225D) fine balance in batches of five flies. Barring a few exceptions where the number of eclosing individuals was not adequate, three replicate batches of 5 males and 5 females for each combination of selection regime × sex × assay environment were weighed.

Statistical Analyses

The mean survivorship, development time and dry weights were subjected to mixed-model ANOVA treating selection regime, assay environment, and sex (only for development time and dry weight) as fixed factors crossed with one another and with random blocks. All statistical analyses were implemented on STATISTICA™ for Windows Release 5.0 B (Statsoft Inc., 1995).

Results and Discussion

Egg-to-Adult Development Time

The ANOVA revealed a significant main effect of selection regime, with MCUs developing faster than the MBs to about the same extent at all larval densities (Fig. 1, Table 1). Overall, pre-adult development time increased with increase in larval density (Fig. 1) and the main effect of assay environment was also significant (Table 1). However, there was no significant interaction between selection regime and assay

environment (Table 1). Sex did not show a significant main effect, but there were significant interactions between selection and sex, and density and sex, respectively (Table 1). Females developed significantly faster than males in MCU populations, whereas in MB populations males and females did not differ significantly in development times (Tukey's HSD test at 0.05 level of significance). Females developed significantly faster than males at larval densities of 20, 60 and 150 eggs per vial, whereas the male-female difference were not significant at the high densities of 300 and 600 eggs per vial (Tukey's HSD test at 0.05 level of significance).

Development Time Distribution

At the lower densities of 20, 60 and 150 eggs per vial, the distribution of development time was narrower, whereas at higher densities (300 and 600 eggs per vial), the distribution showed a greater spread which was clearly seen at density regime of 600 eggs per vial in both MB and MCU populations (Fig. 2, 3; due to similar qualitative pattern in all the four blocks, data is shown only for one representative block of MCU and MB populations). The MCU flies at 600 eggs per vial showed earlier eclosion as compared to the MB flies both in case of males and females. Qualitatively, the development time distribution of MB and MCU populations did not differ at any of larval densities. Also, the development time distribution of the two sexes did not show any clear qualitative difference in either MCUs or MBs.

Egg-to-Adult Survivorship

There was a significant main effect of selection regime (Table 2) with MCUs having higher survivorship overall, compared to the MBs. There was also a significant effect of assay environment (Table 2), with overall survivorship showing a decrease with increasing larval density, especially at 300 and 600 eggs per vial (Fig. 4, Table 2). However, there was no significant selection × density interaction (Table 2), indicating that the selected MCU populations had significantly higher survivorship, irrespective of the density.

Dry Weight at Eclosion

Results from ANOVA showed a significant main effect of selection regime (Table 3) on the dry weight at eclosion (a surrogate of body size), with MCU adults being relatively smaller than their MB counterparts (Fig. 5). As one would expect, there was also a significant main effect of density (Table 3), with flies reared at lower densities weighing more than the flies reared at higher larval densities in both the MB and MCU populations (Fig. 5). Unlike for development time, there was a significant main effect of sex (Table 3), with females weighing more than males (Fig. 5). Selection and sex also showed a significant interaction effect (Table 3) showing that the difference in dry weight between MB and MCU flies was due to male rather than female body size reduction in the MCU populations (Tukey's HSD test at 0.05 level of significance; Fig. 6). Larval density and sex also showed a significant interaction (Table 3) on dry weight as females were significantly heavier than males only at lower larval densities but not at higher densities

(Fig. 6), indicating the disappearance of sexual dimorphism with increasing larval density (Tukey's HSD test at 0.05 level of significance).

Overall, the lack of strong selection × density interactions for survivorship, development time and dry weight at eclosion seen in this study tends to support the notion that in large outbred *Drosophila* populations there is little evolution of different patterns of phenotypic plasticity across larval density in populations routinely reared under crowded conditions (Mueller and Cabral 2012). The response of these traits to increase in larval density in the MCU populations does not appear to be very different from that of the MB populations: populations under both selection regimes are, thus, showing similar patterns of plasticity for these traits across different larval densities.

The difference in pre-adult development time between MCU and MB populations was approximately 12 h, and is consistent with earlier results on populations of *D. nasuta* and *D. ananassae* subjected to selection for adaptation to larval crowding (Archana 2010). This is in contrast to the results obtained from the larval crowding-adapted CU populations, where there was no reduction in development time of the CU populations (Santos et al 1997). As discussed in Chapter 2, in the MCU cultures food levels are much lower than in CU cultures, and MCU larvae need to reach minimum critical size fast, before food runs out. This necessity to attain the minimum critical size fast in the MCU populations may be the reason for evolving shorter pre-adult development time. That the CU populations did not evolve a faster egg-to-adult development time might be a consequence of the higher food level in the culture regime (~6 ml compared to 1.5 ml in MCU cultures). Even at high density of 600 eggs in 1.5 ml of food, both MB and MCU

populations showed eclosion spread out over about 6 days (Fig. 3), whereas in high density in ~ 6 ml of food, the CU populations and their controls' eclose over a period of more than 15 days (Borash et al 1998). Dry weight at eclosion was the only trait for which there was a significant interaction involving selection regime and larval density, indicating possible evolution of genotype X environment interaction for this trait. The significant selection × sex × larval density interaction (Table 3) is driven by the fact that in females the difference between MCU and MB dry weights reduces with increase in larval density, whereas in males the difference between MCU and MB dry weight is more or less maintained across all densities (Fig. 5).

Table 1 Results of ANOVA on mean pre-adult development time across five larval densities with selection, assay environment and sex as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	2740.068	91.344	0.002
Larval Density	4	6946.973	86.422	< 0.001
Sex	1	26.445	3.679	0.151
Selection × Larval Density	4	7.264	0.329	0.853
Selection × Sex	1	141.770	20.384	0.020
Larval Density × Sex	4	8.523	27.219	< 0.001
Selection × Larval Density × Sex	4	1.666	1.279	0.332

Table 2 Results of ANOVA on mean egg-to-adult survivorship across five larval densities, with selection and assay environment as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	0.026	20.906	0.020
Larval Density	4	0.547	146	< 0.001
Selection × Larval Density	4	0.001	0.432	0.783

Table 3 Results of ANOVA on mean dry weight of males and females at eclosion across five larval density regimes, with selection, density and sex as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	341.09	40.989	0.008
Larval Density	4	40498.99	246.570	< 0.001
Sex	1	4109.21	130.626	0.001
Selection × Larval Density	4	8.57	0.719	0.595
Selection × Sex	1	47.95	17.374	0.025
Larval Density × Sex	4	938.69	105.508	< 0.001
Selection × Larval Density × Sex	4	6.81	5.474	0.010

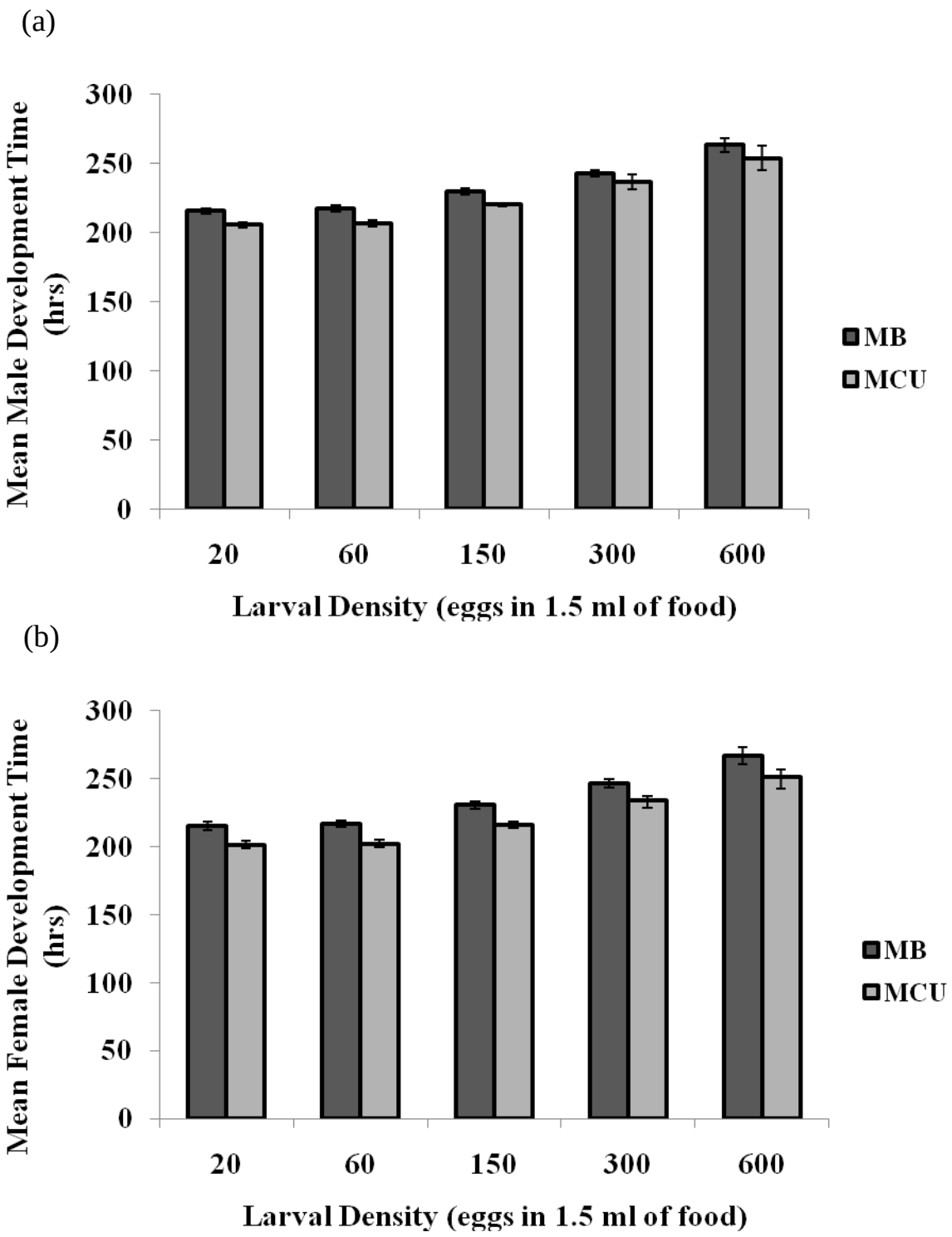


Fig. 1 Mean pre-adult development time of (a) males, and (b) females of the MB and MCU populations across five larval densities. Error bars are the standard errors around the means of four replicate populations.

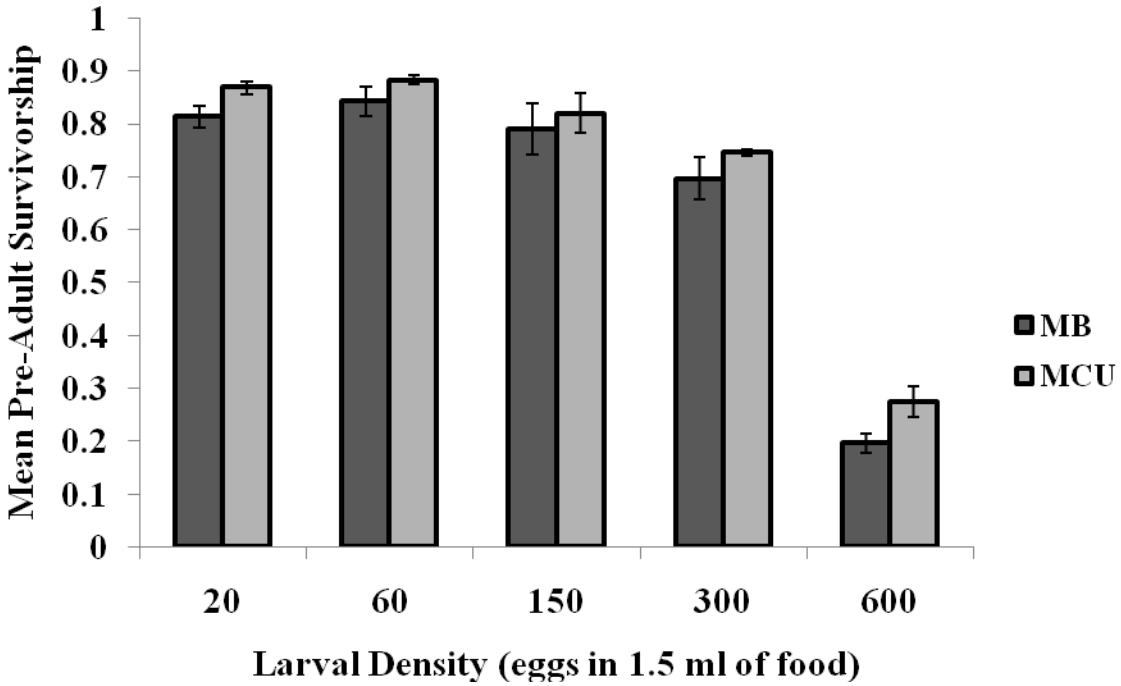
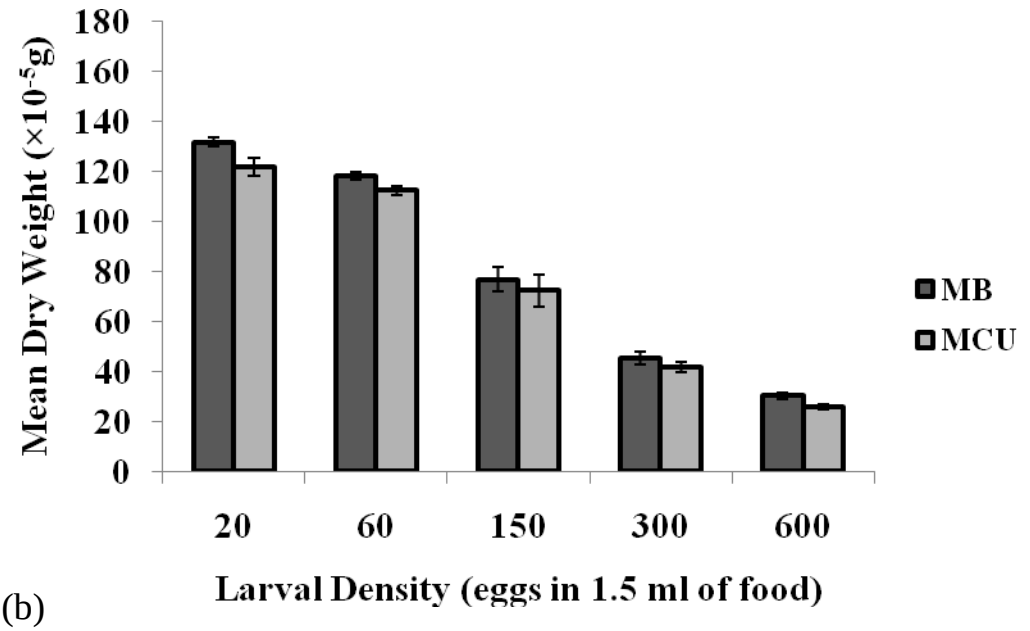


Fig. 4 Mean pre-adult survivorship of the MB and MCU populations in all the five larval densities. Error bars are the standard errors around the means of four replicate populations.

(a)



(b)

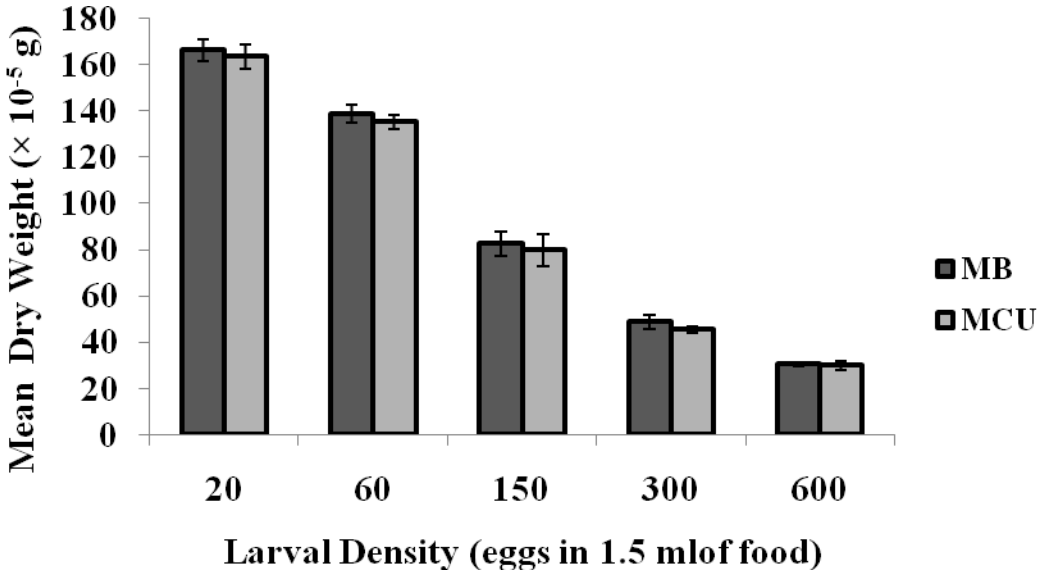
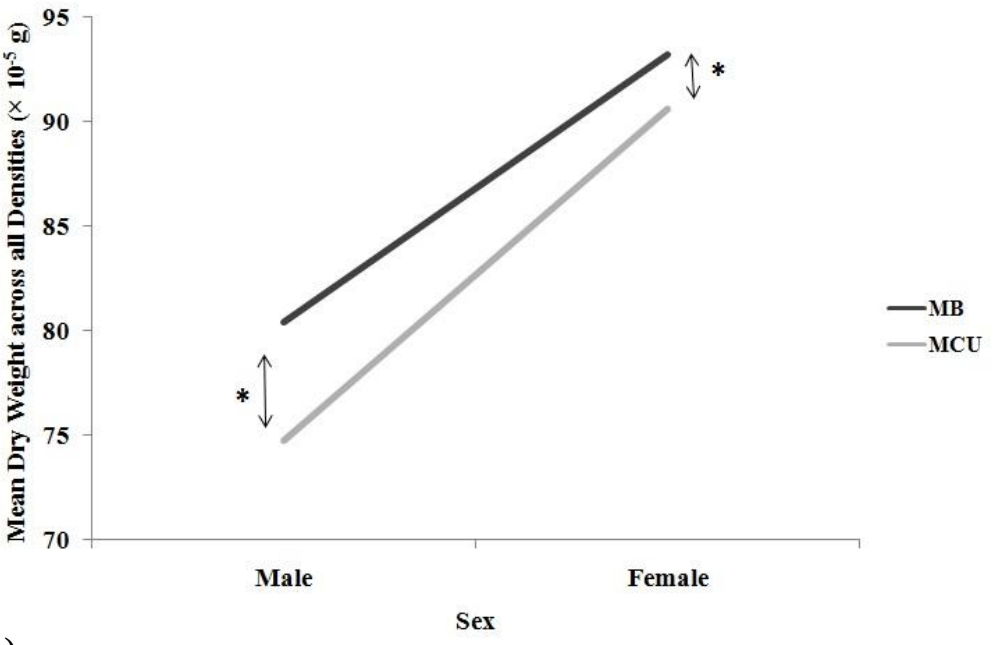


Fig. 5 Mean dry weight of freshly eclosed (a) male, and (b) female flies of the MB and MCU populations at five larval densities. Error bars are the standard errors around the means of four replicate populations.

(a)



(b)

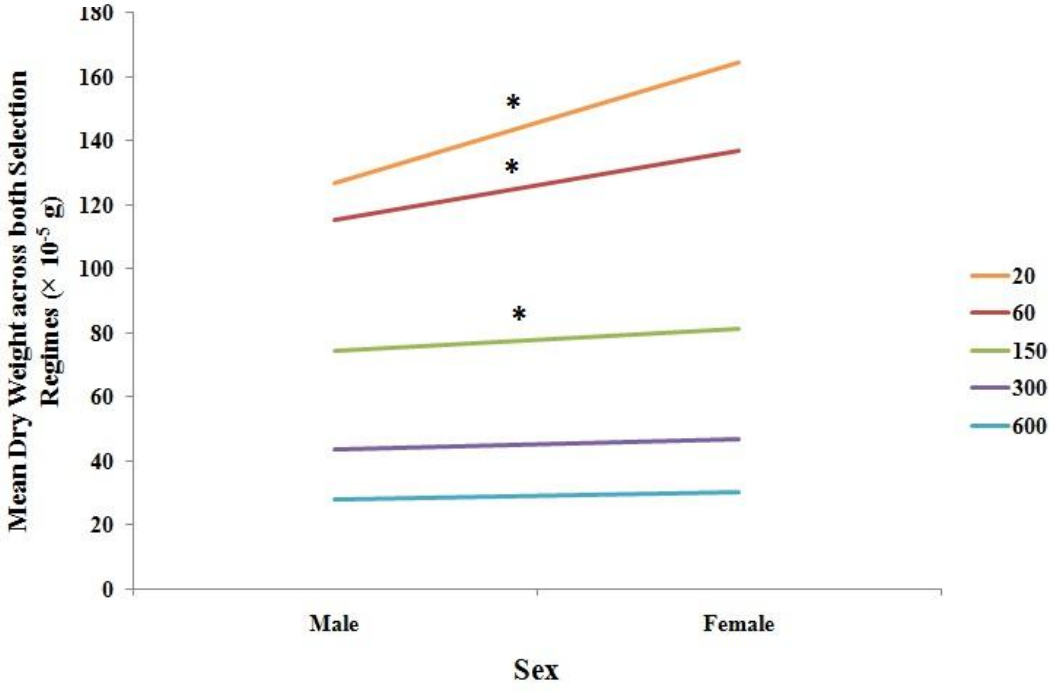


Fig. 6 Mean dry weight of freshly eclosed flies with (a) the selection × sex interaction on dry weight, and (b) the assay environment × sex interaction on dry weight.

Chapter 4

Examination of pre-adult competitive ability across different combinations of food level and larval density

Introduction

Under conditions of food limitation, as happens during larval crowding in *Drosophila*, competition for resources increases, placing a fitness premium on pre-adult competitive ability (Mueller 1997). Thus, pre-adult competitive ability is the primary trait under selection in a regime involving elevated levels of larval crowding. In principle, in crowded cultures, where food tends to run out and waste accumulates over time, individuals can out-compete others by either acquiring and/or utilizing resources more efficiently, or being more tolerant to high concentrations of nitrogenous waste or by evolving a reduced minimum critical size.

Competitive ability itself is, thus, a composite trait and can evolve via differing underlying mechanisms (Joshi and Thompson 1995; Dey et al 2012). Another way of conceptualizing these phenotypic routes to competitive ability is through notions of 'effectiveness' and 'tolerance', referring to the ability of an organism to suppress the population growth rate of its competitors or resist the suppression of its own growth rate by the competitors, respectively (Joshi and Thompson 1995; Joshi et al 2001). Thus, the different phenotypic traits contributing to increased competitive ability can be thought of as affecting primarily either effectiveness (e.g. increase larval feeding rate) or tolerance (e.g. increased waste tolerance) although some traits may affect both components of competitive ability simultaneously (Joshi et al 2001).

In an earlier assay, after 30 generations of selection, the MCU populations were observed to have greater pre-adult survival than the MB populations under competitive conditions at both low and high densities (Archana 2010). In the previous chapters, I have discussed how populations of *Drosophila* can adapt to crowded larval conditions by evolving enhanced competitive ability via different suites of life-history related traits. In the selected populations of Mueller and Ayala (1981) and the CU populations (Joshi and Mueller 1996, Borash et al 1998), the principle route to evolving greater competitive ability was through the evolution of increased larval feeding rate and nitrogenous waste tolerance, at the cost of a reduced efficiency of converting food to biomass (Mueller 1990; Joshi and Mueller 1996). In both these sets of crowding-adapted populations, pre-adult development time did not show any consistent pattern of evolution, unlike our MCU populations that develop to adulthood faster than their controls (Chapter 2). Across *Drosophila* species, faster development has been observed to be positively correlated with greater competitive ability (Krijger et al 2001). However, faster development in itself, especially beyond a point, can lead to reduced competitive ability (Shakarad et al 2005). Thus, the relationship between rapid pre-adult development and competitive ability is likely to be context- dependent in a complex way.

In this chapter, I report results from a pre-adult competition assay in which the competitive ability of MCU and MB populations was assessed against a common marked competitor population, at densities and food levels mimicking both MCU-type and CU-type (Joshi and Mueller 1996) rearing conditions.

Materials and Methods

An assay to determine the pre-adult competitive ability of the MB and MCU populations, relative to a common marked mutant strain, was carried out after 112 generations of MCU selection. A population of Orange eye mutants (OE) was chosen as the common competitor. The OE population was obtained from a spontaneous mutation that occurred in a white eye mutant population of *D. melanogaster* which itself was established following spontaneous mutation in one of the JB populations that were ancestral to the MB controls used in the present study. The OE population was maintained for over 48 generations on a 21 day discrete generation cycle on corn medium under constant light conditions with 25°C and close to 90% relative humidity.

Assay Environments

The competition assay was carried out using four assay environments per replicate population per selection regime:

Moderate larval density in 8 dram vial: 70 eggs each were placed in 8 dram vials (9.6 cm height, 2.2 cm inner diameter; used for MB and MCU selection regime cultures) in 1.5 ml cornmeal medium. Each vial contained 35 eggs from the test population (MB or MCU population) and 35 eggs from the common competitor population (OE). Six such vials were set up per population.

High larval density in 8 dram vial: 600 eggs each were placed into 8 dram vials in 1.5 ml cornmeal medium. Each vial contained 300 eggs from the test population (MB or MCU population) and 300 eggs from the common competitor population (OE). Five such vials were set up per population.

Moderate larval density in 6 dram vial: 70 eggs each were placed into 6 dram vials (9.6 cm height, 2.1 cm inner diameter; used for CU selection regime: Joshi and Mueller (1996) in 6 ml cornmeal medium. Each vial contained 35 eggs from the test population (MB or MCU population) and 35 eggs from the common competitor population (OE). Six such vials were set up per population.

High larval density in 6 dram vial: 1200 eggs each were placed into 6 dram vials in 6 ml cornmeal medium. Each vial contained 600 eggs from the test population (MB or MCU population) and 600 eggs from the common competitor population (OE). Five such vials were set up per population.

All vials were monitored closely for eclosion of flies. Freshly eclosed flies were collected, scanned under a dissecting microscope and scored for eye color. These collections were done twice a day and were continued till no flies eclosed for 4 days.

The low and high egg density treatment in the 6 dram vials were added to the competition assay to examine whether the results were affected by this difference in culture vial and total amount of food. The earlier studies on the CU populations in L.D. Mueller's

laboratory (Joshi and Mueller 1996, Borash et al 1998) used 6 dram vials with relatively large amounts of food and very high egg numbers for rearing CU larvae, as compared to our MCU populations that were reared in 8 dram vials with a density of 600 eggs in 1.5 ml of food.

Competitive Ability

For each vial, the fraction of OE eggs and either MB or MCU eggs that survived till adulthood was calculated. The relative pre-adult competitive ability of the MB and MCU populations was then examined by comparing their mean survivorship in the different assay environments, and also by examining the mean survivorship of the OE eggs in the various assay environments. These two ways of looking at competitive ability crudely reflect the dichotomy between the 'tolerance' and 'effectiveness' components of competitive ability, respectively (Joshi and Thompson 1995; Joshi et al 2001).

Statistical analyses

Mean egg-to-adult survivorship data for the MB, MCU and OE populations were arcsin squareroot transformed and subjected to ANOVA, wherein, selection and assay environment were treated as fixed factors and block as a random factor. Separate ANOVAs were done on mean pre-adult survivorship of MB and MCU populations and of the OE populations. All statistical analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (Statsoft Inc., 1995).

Results and Discussion

Overall, the MCU populations have greater egg-to-adult survivorship than MB populations, when competed against the OE larvae (significant main effect of selection regime: MCU > MB, Table 1). Assay environment also showed a significant main effect (Table 1), with mean survivorship declining at the two higher larval densities. The egg-to-adult survivorship in vials with 70 eggs in 1.5 ml food and 70 eggs in 6 ml food did not differ significantly, but was significantly greater than survivorship at higher densities (Tukey's HSD; $p < 0.05$). Similarly, survivorship did not differ significantly between vials with 600 eggs in 1.5 ml food and 1200 eggs in 6 ml food (Fig. 1). The survivorship of OE larvae was particularly poor at high densities when they were competed against MCU populations (Table 2, Fig. 2). OE survival in vials with MCU larvae at high densities was significantly lower than in vials with MB larvae at high density, whereas at low densities OE survival was similar, regardless of competitor identity (Table 2, Fig. 2, significant selection regime × assay environment interaction). These results clearly indicate that MCU larvae are more competitive than MB larvae.

In the CU-type of assay environment (1200 eggs in 6 ml of food), mean survivorship was significantly lower than in the MCU-type assay environment (600 eggs in 1.5 ml food) for MCU populations (Tukey's HSD test; $p < 0.05$) , even though the egg density per ml of food is half that of the MCU-type of environment. However, the difference was not statistically significant for MB populations. This observation raises the possibility that there is more to larval crowding than just the density of eggs per unit volume of food.

Moreover, even in the CU-type assay environment, the competitive ability of MCU populations was greater than the MB controls.

As discussed earlier, competitive ability can increase either through increased effectiveness or tolerance (Joshi and Thompson 1995; Joshi et al 2001). In case of the MCU populations, a greater competitive ability has evolved without the concomitant evolution of higher larval feeding rate or an increased tolerance to nitrogenous waste (Archana 2010). Earlier studies on crowding adapted populations of *D. melanogaster* showed correlated increase in feeding rates (Joshi and Mueller 1988, Joshi and Mueller 1996) and, tolerance to nitrogenous waste (Shiotsugu et al 1997; Borash et al 1998). Perhaps the significant reduction in pre-adult development time and maintenance of a higher growth rate till middle third instar (Chapter 2) enables the MCU larvae to achieve better competitive ability via increased tolerance to the suppression of population growth rate by competitors, as reflected in smaller reduction in MCU survivorship under crowding, compared to the MB populations (Fig. 1). However, the much greater reduction in OE survivorship when competing with MCU larvae, as compared to MB larvae, also suggests greater effectiveness of the MCU populations, although it is difficult to unambiguously identify traits mediating increased effectiveness.

Overall, these results show that competitive ability continues to be higher than controls for MCU populations after 112 generations of selection. Moreover, the increased competitive ability of MCU populations is exhibited both in an MCU-type and in a CU-type assay environment, showing its robustness across different levels of crowding and food levels. Compared to earlier results from generation 30 of MCU selection (Archana

2010), the MCU larvae no longer have higher survivorship than MB larvae when placed at low densities with a common competitor. This may indicate a trade-off between some aspect(s) of fitness at low versus high density that is becoming apparent as selection has proceeded. Most interestingly, the results suggest that pre-adult survivorship is not simply determined by overall egg density, as survivorship is lower in the CU-type assay environment than in the MCU-type environment even though the egg density is twice as high in the latter. This result indicates that, in principle, selection on different traits such as survivorship, development time and body size at eclosion can differ in selection regimes that vary in food levels, even if egg densities are similar. Given that the CU and MCU maintenance regimes differ in both egg density and the total food levels, it is possible that there is differential selection on various competitive fitness-related traits in the two regimes, possibly resulting in the observed differences in traits that evolve in response to these two types of selection regimes.

Table 1 Results of ANOVA on mean egg-to-adult survivorship of MB and MCU populations when competed against the OE population across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	0.140	36.836	0.009
Assay Environment	3	0.504	132.432	< 0.001
Selection × Assay Environment	3	0.026	14.009	0.001

Table 2 Results of ANOVA on mean egg to adult survivorship of the OE population when competed against MB or MCU populations across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	0.173	17.581	0.009
Assay Environment	3	1.094	214.789	< 0.001
Selection × Assay Environment	3	0.073	11.780	0.001

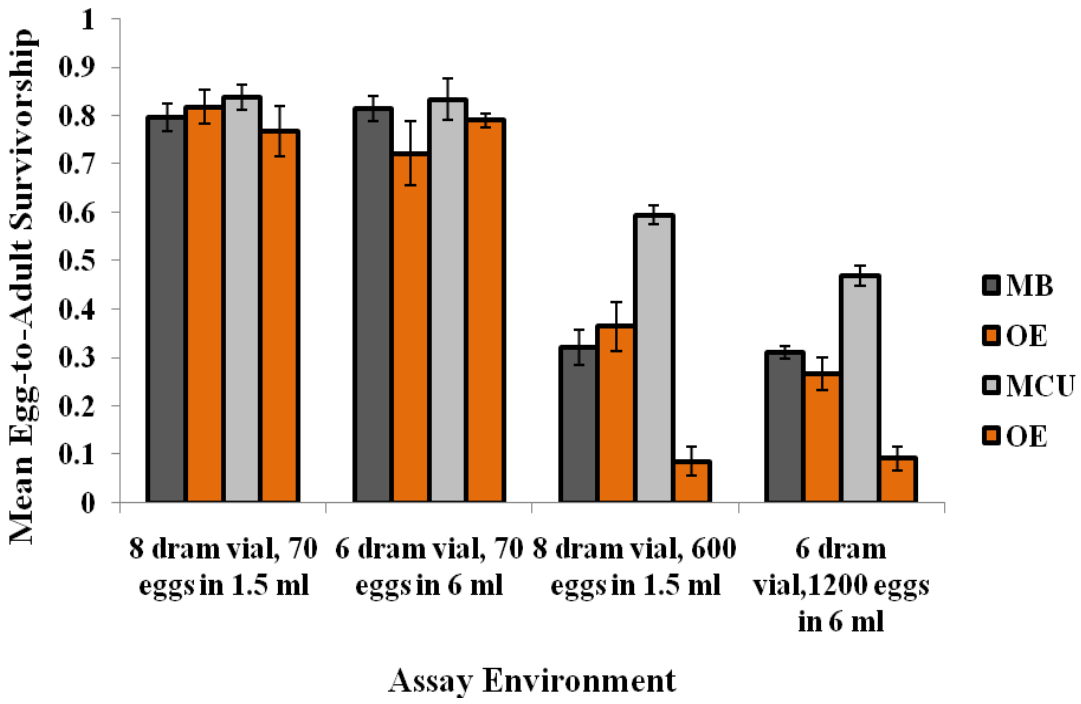


Fig. 1 Mean egg-to-adult survivorship of MB, MCU and OE populations in the competition assay at different larval density and food levels. Error bars are the standard errors around the means of the four replicate populations.

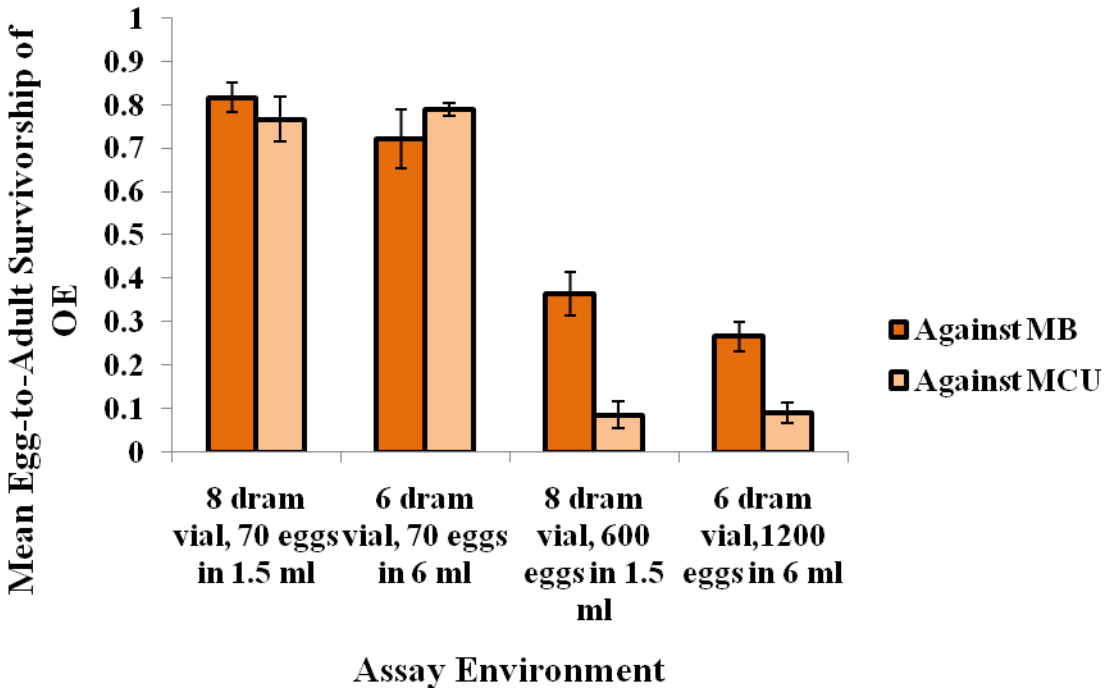


Fig. 2 Mean egg-to-adult survivorship of OE population when competed against MB and MCU populations across different larval density and food levels. Error bars are the standard error around the means of the four replicate populations.

Chapter 5

Total amount of food in addition to the egg density most likely determines the nature of selection acting on traits affecting competitive ability

Introduction

The results discussed in previous chapters show that the MCU populations differ from the CU populations used by Joshi and Mueller (1996) and Borash et al (1998) in having a much narrower distribution of development time (Chapter 3). The competition assay results also indicated that food levels, in addition to egg densities, may be affecting traits related to competitive ability (Chapter 4). Moreover, the MCU populations have adapted to their maintenance regime by having a greater viability and reduced body size than their MB counterparts (Chapter 3). I therefore wanted to compare the effects of MCU and CU-type rearing conditions on the distributions of traits related to competitive ability. The assay environmental conditions used in this experiment are similar to the competition assay (Chapter 4), except that monotypic cultures are used such that individuals of each populations are competing only against other individuals of the same populations, and that one treatment (1200 eggs in 3 ml food) has been added to the assay to provide an egg density control to the MCU-type treatment (600 eggs in 1.5 ml food), but with a greater amount of food. This treatment also acts as an egg number control for the CU-type treatment (1200 eggs in 6 ml food) but with half the egg density.

Materials and Methods

This assay was done after 113 generations of MCU selection. Each MCU and MB population was subjected to five different assay environments (see below). After three days of yeasting, a cornmeal food plate was kept inside the cages with the standardized

populations and the flies were allowed to lay eggs for duration of one hour, after which the plate was discarded. A second cornmeal food plate was then placed in the cage for 12 hours. Eggs were then collected off these plates and the exact number of eggs for each assay environment were counted, and transferred to thin strips of agar which were then dispensed into respective vials. The details of the larval densities and food level are described as below (four of the five assay environments are the same as that in the competition experiment described in Chapter 4). At the time of writing this thesis, only 3 of the 4 blocks (Blocks 1,3 and 4) could be assayed and results from those 3 blocks are reported in this chapter.

CU-type Environment:

High larval density in 6 dram vial: 1200 eggs per 6 dram vial (9.6 cm height, 2.1 cm inner diameter; used for CU selection regime: Joshi and Mueller (1996) were collected in 6 ml cornmeal medium. Four such vials were set up per population.

Moderate larval density in 6 dram vial: This served as the control environment for the high density in the CU-type vials. 70 eggs per 6 dram vial were collected in 6 ml cornmeal medium. Six such vials were set up per population (except for block 4 which had five replicates per population).

MCU- type Environment:

High larval density in 8 dram vial: 600 eggs were collected per 8 dram vial (9.6 cm height, 2.2 cm inner diameter; used for MCU cultures) in 1.5 ml cornmeal medium. Five such vials were set up per population.

Moderate larval density in 8 dram vial: In this case, 70 eggs were collected per 8 dram vial in 1.5 ml cornmeal medium. Six such vials were set up per population (except for block 4 which had five replicates per population).

Egg-to-food ratio control to MCU-type Environment:

1200 eggs per 6 dram vial were collected in 3 ml cornmeal medium. Four such vials were set up per population.

Statistical Analyses

The mean survivorship, development time and dry weights were subjected to mixed-model ANOVA treating selection regime, assay environment, and sex (only for development time and dry weight) as fixed factors crossed with one another and with random blocks. All statistical analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (Statsoft Inc., 1995).

Results and Discussion

Egg-to-Adult Development Time

The ANOVA revealed a significant main effect of selection regime (Table 1), with MCU populations being faster developing overall than the controls (Fig. 1). Assay environment also showed a significant main effect (Table 1), with development time generally increasing from lower to higher densities (Fig. 1). Interestingly, development time was highest for the vials with 1200 eggs in 6 ml food, an egg density lower than either 600

eggs in 1.5 ml or 1200 eggs in 3 ml (Fig. 1). Thus, development time is not necessarily the highest for vials with the highest egg density. The only other significant effect was that of the selection regime × sex × assay environment interaction effect (Table 1), because MCU males had significantly shorter development time than MB males in three assay environments, whereas MCU females were faster developing than MB females at all assay environments (Table 2). This result differs from that found when comparing development times at different densities (Chapter 3) in 1.5 ml of food and reinforces the notion that other than egg density, the total amount of food can affect life-history traits in crowded *Drosophila* cultures (Chapter 4).

In addition to the mean development time, I also examined the frequency distributions of developmental time across different assay environments. If total food levels can affect the means of life-history traits even at similar egg densities, then examining food level effects on the distribution of such traits could provide insights into the underlying mechanisms by which such factors affect the life-history traits being studied. Overall, at lower densities (70 eggs in 1.5 ml and 70 eggs in 6 ml of food), the distribution of development time was narrower, with eclosions occurring over 3 days (data not shown). At higher densities (600 eggs in 1.5 ml and 1200 eggs in 3 ml and 6 ml of food), however, the distribution of development time showed a much greater spread (Figs. 2, 3 and 4). The development time distributions however, did not differ qualitatively between sexes in either MCU or MB populations (Fig. 2, 3 and 4). Although the first eclosions occurred around the same time in all three high density assay environments, the spread was approximately 200 hours in the 600 eggs in 1.5 ml MCU-type vials, whereas in the CU-type vials (1200 eggs in 6 ml food), eclosions were spread out over 500 hours, as also

reported earlier for the CU populations (Borash et al 1998). Interestingly, in the 1200 eggs in 3 ml food vials, the spread was qualitatively more close to that of the 600 eggs in 1.5 ml vials, but there were small numbers of flies eclosing very late, after 500 hours from egg collection (Figs. 2, 3 and 4). Thus, even at the same egg density, with a greater total amount of food, there is increased likelihood of some flies surviving to eclose very late in the culture, whereas in the MCU-type vials, eclosions essentially end by about 400-420 hours from egg collection (Figs. 2, 3 and 4).

Pre-Adult Survivorship

The ANOVA revealed no significant main effect of selection regime on mean pre–adult survivorship (Table 3). However, there was a significant selection regime × assay environment interaction (Table 3), with mean survivorship of MCU populations being greater than controls at two out of the three high density assay environments (600 eggs in1.5 ml and 1200 eggs in 3 ml: Tukey's HSD test; $p < 0.05$, Fig. 5). In the 1200 eggs in 3 ml environment, too, the MCU populations had higher survivorship than the MB populations but the difference was not statistically significant (Tukey's HSD test; $p > 0.05$). The ANOVA also revealed a significant main effect of assay environment, with mean survivorship generally being higher at low densities and lower at high densities (Table 3, Fig. 5). At the two low densities, survivorship of MCU populations did not differ significantly from the MB populations (Tukey's HSD test; $p > 0.05$), but the trend was for MCU survivorship to be less than the MB populations. Earlier in MCU selection (Gen. 82), the MCU survivorship was greater than controls at both low and high densities

(Chapter 3), indicating that perhaps a trade-off between pre-adult survivorship at low versus high density is evolving as MCU selection proceeds.

Dry Weight at Eclosion

The ANOVA did not show a significant main effect of selection regime (Table 4) on the dry weight at eclosion (a measure of body size), although the trend was towards MCU adults being relatively smaller than their MB counterparts. As expected, there was a significant main effect of assay environment (Table 4), with flies reared at the lower egg densities being substantially heavier than those reared at higher egg densities (Fig. 6). Interestingly, mean dry weight tended to increase with total amount of food in the 3 high egg density assay environments, even as egg density remained the same, or decreased (Fig. 6), once again indicating that there is more to crowding than just the egg density. Selection regime and assay environment had a significant interaction effect (Table 4), with the general trend being for MCU adults to be lighter than their MB counterparts at the three high egg densities, but not at the two low egg densities (Fig. 6). However, post-hoc comparisons using Tukey's HSD test did not yield significant difference between the MB and MCU populations in any of the five assay environments. Moreover, sex and assay environment also showed a significant interaction (Table 4), as females were heavier than males only at lower densities but not at higher densities (Tukey's HSD test; at significance level of 0.05), indicating the disappearance of sexual dimorphism with increasing larval density except in the 1200 eggs in 6 ml food high egg density vials (Fig. 7).

Given that mean dry weight at eclosion tended to increase with total food amount across the 3 high egg density assay environments (Fig. 6), I also examined the temporal distribution of the mean dry weight of freshly eclosing flies based on the day of eclosion. Males and females eclosing on any given day, were pooled from all the vials in each selection regime × block × high density assay environment combination. Five adults were then randomly chosen to make one replicate. Upto five such replicates were weighed for any given day and, if very few flies eclosed in a particular day, then flies were pooled across subsequent days.

In the MCU-type vials (600 eggs in 1.5 ml of food), the early eclosing flies from the first and second days of eclosion were slightly heavier; thereafter the body size was fairly constant over the next five or so days of eclosion (Fig. 8, 9 and 10). In the 1200 eggs in 3 ml vials, the mean dry weight decreased with day of eclosion till 9 to 10 days from first eclosion, and the earliest eclosing flies were heavier than in the MCU-type vials (Figs. 8, 9 and 10). At around the 14^{th} to 17^{th} day from first eclosion (with no emergence for a duration of approximately 4 to 5 days), again the mean dry weight was somewhat high (Figs. 8, 9 and 10). In the CU-type vials (1200 eggs in 6 ml vials), the earliest eclosing flies were heavier than in the other two assay environments (Figs. 8, 9 and 10). Once again, it seems clear that both the means and indeed the distributions of the key life-history related traits in crowded cultures of *Drosophila* are affected not just by egg density per se, but by the total food available as well.

Considering the overall results from the three high egg density vials, we can put together a possible explanation of what is happening in crowded cultures and why total food level seems to interact with egg density in affecting life-history related traits. Comparing the two assay environments with equal egg density (600 eggs in 1.5 ml and 1200 eggs in 3 ml food), we can see that while survivorship is lower in the 1200 eggs in 3 ml vials, both development time and dry weight at eclosion are higher than in the 600 eggs in 1.5 ml vials (Figs. 1, 5 and 6). The increased development time in the 1200 eggs in 3 ml vials appears to be due to a small number of very late eclosions (Figs. 2, 3 and 4), whereas the increased dry weight at eclosion is primarily due to the flies eclosing during the first two days of eclosion in the 1200 eggs in 3 ml vials being heavier than their counterparts in the 600 eggs in 1.5 ml vials (Figs. 8, 9 and 10). Both these effects are further heightened in the 1200 eggs in 6 ml vials (Figs. 2, 3, 4, 8, 9 and 10) in which egg density is reduced and food level increased relative to the other two high egg density treatments.

The most likely explanation for these results is that in vials with 600 eggs in 1.5 ml of food, the food is used up very fast and probably also accumulates toxic levels of nitrogenous waste quite quickly as the total amount of food available for waste to diffuse into is small even early on. *Drosophila* larvae typically feed within 1 cm depth from the surface. With 1.5 ml of food, the height of the food column is less than 1 cm. As food levels are increased to 3 ml or 6 ml, there is greater amount of food for waste to diffuse into and thus the effective waste concentration experienced by feeding larvae in the 1 cm depth feeding band is likely to be lower than that experienced by larvae in vials with only 1.5 ml of food. At the same time, since feeding is restricted to a 1 cm deep band, effective competition for food, especially in early stages of a crowded culture, will be higher in

vials with 1200 eggs in 3 ml, or 1200 eggs in 6 ml, compared to vials with 600 eggs in 1.5 ml food, because the volume of food in the 1 cm deep feeding band is constant, but the number of larvae feeding in that zone is increased in the two treatments with 1200 eggs. The higher effective competition for food in the two 1200 eggs cultures will tend to magnify the effect of small differences in feeding rate among larvae, resulting in a smaller proportion of larvae exceeding their critical minimum size but then going on to become bigger before the food runs out, explanining the increased dry weight of early eclosing adults in the two 1200 egg treatments (Figs. 8, 9 and 10). In addition, since the nitrogenous waste build up in the feeding band is probably slower in the 1200 egg treatments, it permits some individuals who probably may have slightly higher tolerance to nitrogenous waste to survive after a relatively long phase of larval feeding on the deteriorating food. Such late eclosing flies are not seen in the 600 eggs in 1.5 ml vials where the food runs out soon after the surviving larvae have crossed their minimum critical size.

This is also a likely explanation of why the CU populations evolved greater feeding rate (Joshi and Mueller 1996), urea tolerance (Shiostugu et al 1997) and a polymorphism involving different strategies for early and late eclosing flies (Borash et al 1998), and the MCU populations did not (Archana 2010). In MCU cultures with 600 eggs in 1.5 ml of food, there is no scope for urea tolerant late phenotypes to survive as food runs out and the eclosions are spread out only for about 5 days. Similarly, the early build up of nitrogenous waste probably renders higher feeding rates maladaptive due to the concomitant high rate of nitrogenous waste ingestion (Mueller et al 2005). Thus, the nature of selection acting on traits affecting competitive ability is likely to depend

critically on both egg density and total amount of food, specifically the height of the food column. This also underscores the fact that although measures like egg density are often of heuristic value when thinking about adaptation to crowding, ultimately the ecological details of different types of crowding cultures can affect the form of the fitness functions acting on different traits related to competitive ability, thereby mediating the evolution of greater competitive ability via different sets of trait changes.

Table 1 Results of ANOVA on mean egg-to-adult development time across five assay environments, with selection regime, sex and assay environment as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	150.841	68.708	0.014
Sex	1	66.517	2.933	0.229
Assay Environment	4	41172.301	170.358	< 0.001
Selection × Sex	1	28.349	2.017	0.291
Selection × Assay Environment	4	21.322	0.386	0.813
Sex × Assay Environment	4	5.719	1.341	0.334
Selection × Sex × Assay Environment	4	41.168	11.138	0.002

Table 2 Difference in mean pre-adult development time between MB and MCU populations when examined across the five different assay environments.

Sex	Assay Environment	Development Time Difference: MB – MCU (hrs)	
Males	70 eggs in 1.5 ml	8.00	*
	70 eggs in 6 ml	11.33	*
	600 eggs in 1.5 ml	13.09	*
	1200 eggs in 3 ml	7.20	
	1200 eggs in 6 ml	4.15	
Females	70 eggs in 1.5 ml	7.02	
	70 eggs in 6 ml	7.69	*
	600 eggs in 1.5 ml	14.49	*
	1200 eggs in 3 ml	8.71	*
	1200 eggs in 6 ml	19.61	*

** Significant at 0.05 level; Tukey's HSD test*

Table 3 Results of ANOVA on mean egg-to-adult survivorship across five assay environments, with selection regime and density as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	0.002	0.282	0.648
Assay Environment	4	0.557	46.41	< 0.001
Selection × Assay Environment	4	0.014	11.983	0.001

Table 4 Results of ANOVA on mean dry weight of males and females at eclosion across five assay environments, with selection regime, assay environment and sex as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	20.024	7.981	0.106
Sex	1	259.111	219.732	0.005
Assay Environment	4	1528	243.717	< 0.001
Selection × Sex	1	5.071	2.325	0.267
Selection × Assay Environment	4	6.904	4.943	0.026
Sex × Assay Environment	4	41.740	62.955	< 0.001
Selection × Sex × Assay Environment	4	1.702	0.443	0.775

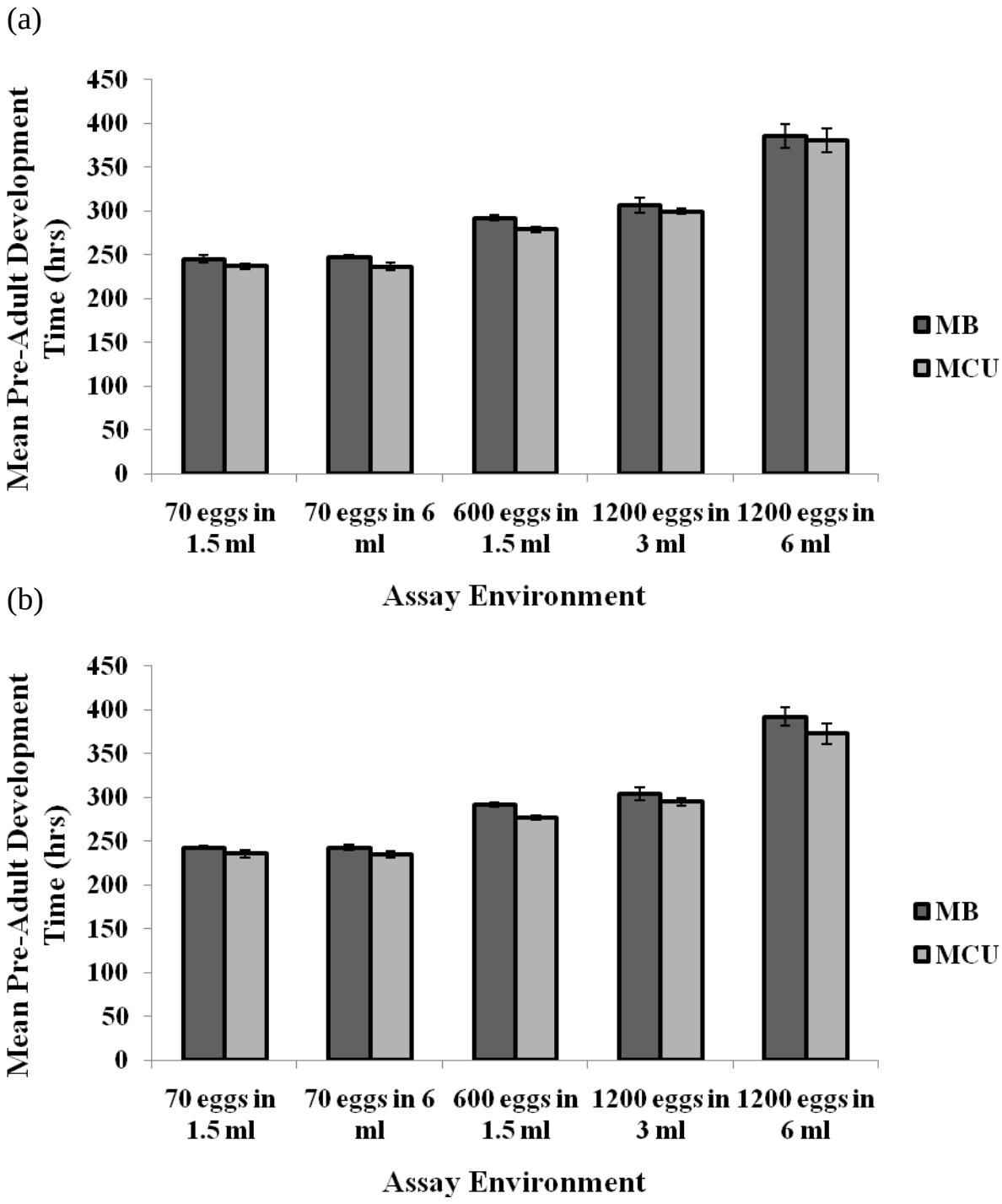


Fig. 1 Mean pre-adult development time in five different assay environments in MB and MCU populations for (a) males and (b) females. Error bars are the standard errors aound the means of the three replicate populations.

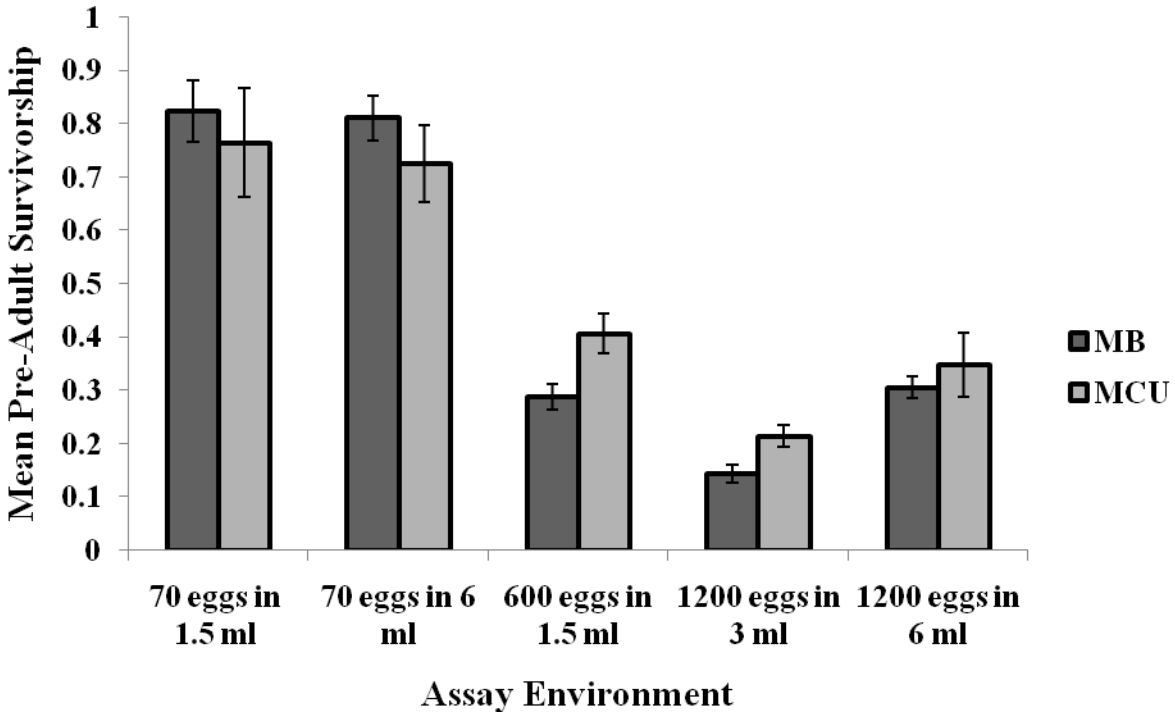
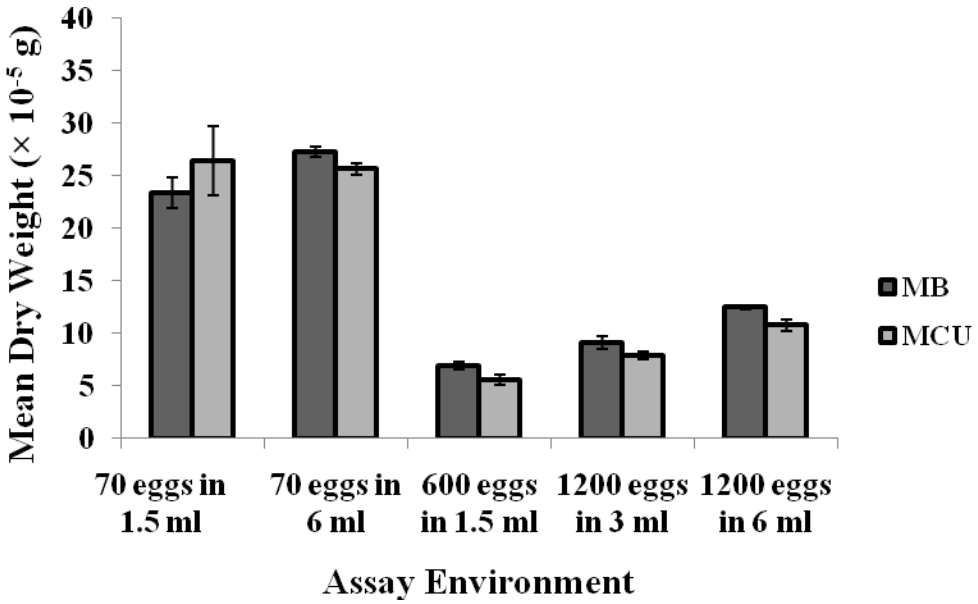


Fig. 5 Mean pre-adult survivorship of the MB and MCU populations in all the five density regimes. Error bars are the standard errors aound the means of the three replicate populations.

(a)



(b)

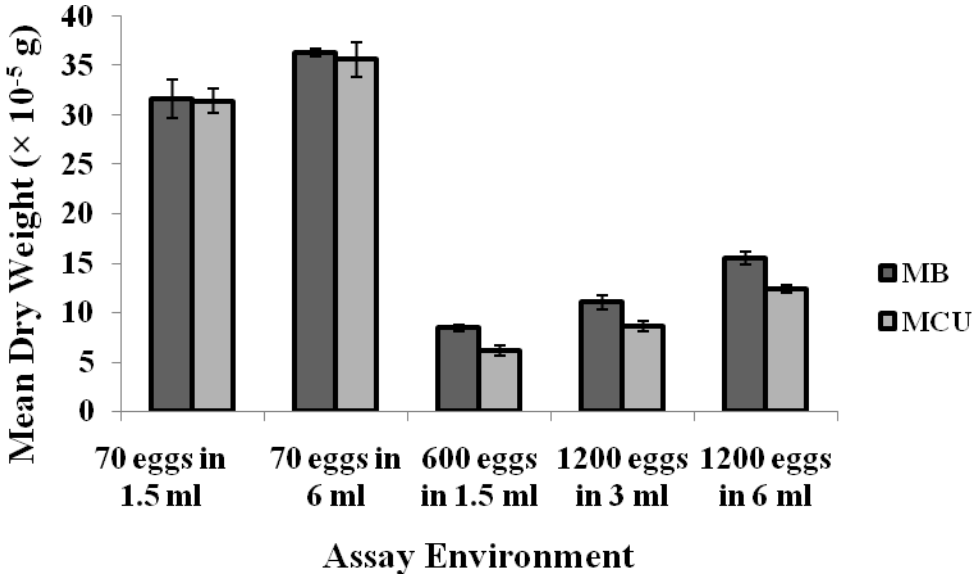


Fig. 6 Mean dry weight of freshly eclosed (a) male and (b) female flies of two populations (MB and MCU) in the five assay environments. Error bars are the standard errors aound the means of the three replicate populations.

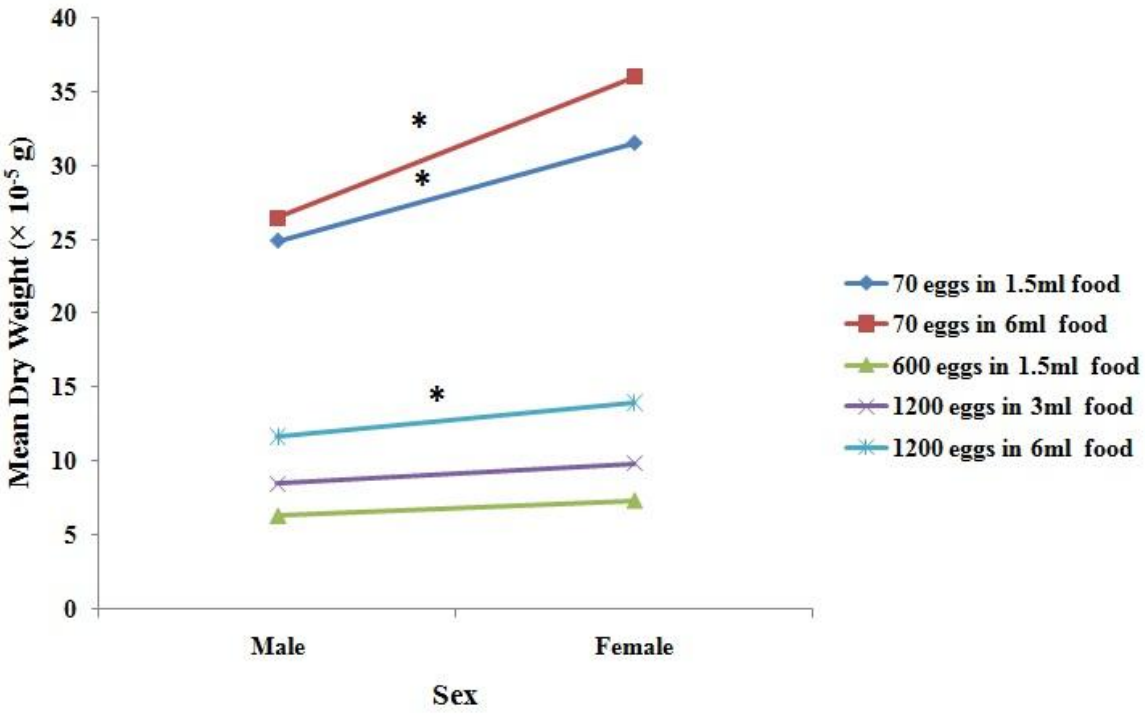


Fig. 7 Sex x assay environment interaction: the mean dry weight of females averaged across selection regime and block is significantly greater than males only at relatively low egg densities. Asterisks represent male and female differences that were significant at $p < 0.05$ using Tukey's HSD test.

Chapter 6

Conclusion

The aim of the studies reported in this thesis was to carry out preliminary investigations to identify aspects of the laboratory ecology of crowded cultures of *Drosophila* with an intent to ultimately try and explain different routes to the evolution of competitive ability in different populations subjected to selection for adaptation to larval crowding under slightly different regimes (MCU populations: Archana 2010; CU populations: Joshi and Mueller 1996; Borash et al 1998). The two sets of selected populations had the same ancestry, but were reared under slightly different maintenance regimes, especially with regard to the total amount of food available in each vial. In this chapter, I briefly summarize the important results from the experiments conducted and try to emphasize the complex interaction of food level and larval density in affecting the fitness consequences of various traits related to competitive ability in a fruitfly culture.

One major result from the stage specific development assay (Chapter 2) was that MCU populations reached their critical minimum size faster than their controls, while maintaining a higher body size till the pre–critical–size phase. However, in the post–critical–phase, MCU larvae weighed less and eventually eclosed as lighter adults as compared to their MB counterparts. This can be explained by the fact that, in the standard MCU cultures, post-critical–size growth rates may not be under very strong selection as there is typically very little food left in the crowded culture vials by the time most larvae reach critical size. Therefore, results from this assay indicate that MCU larvae have probably evolved to be more efficient at food to biomass conversion, at least during the pre-critical phase of the larval stage, compared to their ancestral controls. It also seems

likely that the MCU populations have evolved a lower minimum critical size as opposed to either evolving increased feeding rates (Joshi and Mueller 1988, 1996; Santos et al 1997), or urea and ammonia tolerance (Shiotsugu et al 1997; Borash et al 1998) as seen in the earlier studies on the CU populations.

When assayed for phenotypic plasticity across a gradient of larval densities at the same constant food level, neither pre-adult survivorship, development time, nor dry weight at eclosion, showed any evidence for different patterns of phenotypic plasticity for these traits between MCU and MB populations. This supports the notion that adaptation to larval crowding does not involve changes in the phenotypic plasticity of body size across food levels (Mueller and Cabral 2012). However, in the experiment discussed in Chapter 5, there is some evidence for genotype × environment interaction for some life-history related traits especially development time. But, in this study, different assay treatments differed in larval densities and/or total food amount and that could be driving the interaction, rather than any evolution of different patterns of phenotypic plasticity in the strict sense.

Results presented in Chapter 4 showed that MCU competitive ability continues to be higher than their controls after 112 generations of selection, as also seen in earlier phase of selection (Archana 2010). Moreover, the increased competitive ability of MCU populations was exhibited both in MCU-type and CU-type assay environments, suggesting that higher competitive ability of MCU populations is robust even in an

environment that was seen to result in a different trajectory to the evolution of higher competitive ability (in the CU populations).

The experiment mentioned in Chapter 5 was designed to explore the impact of different kinds of crowded environments on major fitness-related traits like pre-adult survivorship and, development time, and dry weight at eclosion. Difference in the level of crowding was imposed by varying the egg density (600 eggs in 1.5 ml food versus 1200 eggs in 6 ml food) as well as by varying the height of the food column in vials while keeping the egg density constant (600 eggs in 1.5 ml food versus 1200 eggs in 3 ml food). Results from this experiment revealed a lower pre-adult survivorship but a greater mean development time and dry weight at eclosion in vials with 1200 eggs in 3 ml or 6 ml food, as compared to the MCU-type environment (600 eggs in 1.5 ml food). Similarly, in vials with 1200 eggs in 3 ml or 6 ml food, both dry weight at eclosion and pre-adult development time were observed to have a much greater variance as compared to the MCU-type environment. Moreover, in the two treatments with 3 ml or 6 ml of food, flies eclosing during the first two days of eclosion were heavier than their counterparts in the MCU-type vials. This suggests that upon increasing the total amount of food available per larva and the overall height of the food column, the individuals with faster feeding rates get an advantage of reaching the critical size earlier, while there is still food left in the medium to gain body weight during the post–critical–size phase, thereby eclosing as heavier adults and also depleting the food, thus depressing overall survivorship. At the same time, those individuals with slightly higher waste tolerance also survive with a prolonged development time, since the waste build–up is relatively slow in the 1200 eggs

in 3 ml and 6 ml food compared to the MCU-type environment. In the MCU-type environment the absolute food level (i.e. 1.5 ml) itself is lower than the normal feeding band (approx. 1 cm deep in *Drosophila* cultures). Hence, the amount of nitrogenous waste in this case probably reaches somewhat high levels early on, enough to nullify a possible fitness benefit of higher feeding rates (Mueller et al 2005). Additionally, since food runs out very fast over time in MCU-type vials, it does not permit better survival of individuals with a high waste tolerance. These results, thus, potentially explain all the discrepancies between the MCU and CU populations in terms of selection responses.

These experiments basically indicate the egg density, along with the amount of the food in a crowded culture, to be the two most important factors in determining the fitness consequences of variation in traits like larval feeding rate, nitrogenous waste tolerance, and efficiency of food to biomass conversion in adaptation to larval crowding. In this context, it will be worthwhile to try and tease apart the evolutionary effects of these two factors by altering the height of the food column while keeping the egg density constant, or vice-versa. This, in principle would allow one to examine which among the two is the most relevant in deciding the evolutionary trajectories of different life-history traits during adaptation to larval crowding. Hence, it might be worth conducting a new selection experiment in which the amount of food and the egg number can be doubled, keeping the egg-to-food ratio same as that of MCU-type regime.

References

Anderson, W. W. and Arnold, J. 1983 Density-regulated selection with genotypic interactions. *Am. Nat*. **121:** 649–655

Archana, N. 2010 *The genetic architecture of fitness-related traits in populations of three species of Drosophila subjected to selection for adaptation to larval crowding*. Ph.D. thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

Asmussen, M. A. 1983 Density-dependent selection incorporating intraspecific competition. II. A diploid model. *Genetics* **103:** 335–350

Bakker, 1961 An analysis of factors which determine success in competition for food among larvae of *Drosophila melanogaster*. *Archs. Neerl. Zool.* **14**: 200–281

Bakker, 1969 Selection for the rate of growth and its influence on competitive ability of larvae in *Drosophila melanogaster*. *Neth. J. Zool.* **19**: 541–595

Bergland, A. O., Genissel, A., Nuzhdin, S. V. and Tatar, M. 2008 Quantitative trait loci affecting phenotypic plasticity and the allometric relationship of ovariole number and thorax length in *Drosophila melanogaster*. *Genetics* **180:** 567–582

Borash, D. J., Gibbs, A. G., Joshi, A. and Mueller, L. D. 1998 A genetic polymorphism maintained by natural selection in a temporally varying environment. *Am. Nat*. **151:** 148–156

Bradshaw, A. D. 1965 Evolutionary significance of phenotypic plasticity in plants. *Adv. Genet.* **13:** 115–155

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

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

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

Clarke, B. 1972 Density-dependent selection. *Am. Nat.* **106:** 1–13

de Moed, G. H., Kruitwagen, C. L. J. J., de Jong, G. and Scharloo, W. 1999 Critical weight for the induction of pupariation in *Drosophila melanogaster*: genetic and environmental variation. *J. Evol. Biol.* **12:** 852–858

Darwin, C. R. and Wallace, A. R. 1858 On the tendency of species to form varieties; and on the perpetuation of varieties and species by natural means of selection. *J. Proc. Linn. Soc. Lond. Zool.* **3:** 45–50

Darwin, C. 1859 *On the origin of new species by means of natural selection.* John Murray, London, UK.

Dey, S., Bose, J. and Joshi, A. 2012 Adaptation to larval crowding in *Drosophila ananassae* leads to the evolution of population stability. *Ecol. Evol.* **2:** 941–951

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

Gibbs, A. G., Chippindale, A. K., and Rose, M. R. 1997 Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster*. *J. Exp. Biol.* **200:** 1821–1832

Harshman, L. G., Hoffmann, A. A. and Clark, A. G. 1999 Selection for starvation resistance in *Drosophila melanogaster*: physiological correlates, enzyme activities and multiple stress responses. *J. Evol. Biol.* **12:** 370–379

Joshi, A. and Mueller, L. D. 1988 Evolution of higher feeding rate in *Drosophila* due to density-dependent natural selection. *Evolution* **42:** 1090–1092

Joshi, A. and Mueller, L. D. 1993 Directional and stabilizing density-dependent natural selection for pupation height in *Drosophila melanogaster*. *Evolution* **47**: 176–184

Joshi, A. and Thompson, J. N. 1995 Alternative routes to the evolution of competitive ability in two competing species of *Drosophila*. *Evolution* **49:** 616–625

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

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

Kolss, M., Vijendravarma, R. K., Schwaller, G., Kawecki, T. J. 2009 Life-history consequences of adaptation to larval nutritional stress in *Drosophila*. *Evolution* **63:** 2389–2401

Krijger, C. L., Peters, Y. C. and Sevenster, J. G. 2001 Competitive ability of neotropical *Drosophila* predicted from larval development times. *Oikos* **92:** 325–332

MacArthur, R. H. and Wilson, E. O. 1967 *The theory of island biogeography*. Princeton University Press, Princeton, NJ, USA

MacArthur, R.H. 1962 Some generalized theorems of natural selection. *Proc. Natl. Acad. Sci. USA* **48:** 1893–1897

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K. and Relyea, A. R. 2005 Ecological consequences of phenotypic plasticity. *Trends Ecol. Evol.* **20:** 685–692

Mueller, L. D. and Ayala, F. J. 1981 Trade-off between *r*-selection and *K*-selection in *Drosophila* populations. *Proc. Natl. Acad. Sci. USA* **78:** 1303–1305

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

Mueller, L. D. and Sweet, V. F. 1986 Density-dependent natural selection in *Drosophila*: evolution of pupation height. *Evolution* **40:** 1354–1356

Mueller, L. D. 1988 Evolution of competitive ability in *Drosophila* by density-dependent natural selection. *Proc. Natl. Acad. Sci. USA* **85:** 4383–4386

Mueller, L. D. 1990 Density-dependent natural selection does not increase efficiency. *Evol. Ecol.* **4:** 290–297

Mueller, L. D., González-Candelas, F. and Sweet, V. F. 1991 Components of density-dependent population dynamics: models and tests with *Drosophila*. *Am. Nat*. **137:** 457–475

Mueller, L.D. 1997 Theoretical and empirical examination of density-dependent selection. *Annu. Rev. Ecol. Syst.* **28**: 269–88

Mueller, L. D., Folk, D. G., Nguyen, N., Lam, P., Rose, M. R. and Bradley, T. 2005 Evolution of larval foraging behavior in *Drosophila* and its effects on growth and metabolic rate. *Physiol. Entomol.* **30:** 262–269

Mueller, L. D. and Cabral, L. G. 2012 Does phenotypic plasticity for adult size versus food level in *Drosophila melanogaster* evolve in response to adaptation to different rearing densities. *Evolution* **66:** 263–271

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

Nusbaum, T. J., Mueller, L. D. and Rose, M. R. 1996. Evolutionary patterns among measures of aging. *Exp. Gerontol.* **31:** 507–516

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

Pianka, E. R. 1970 On *r*- and *K*-selection. *Am. Nat.* **104:** 952–956

Prasad, N. G., Shakarad, M., Gohil, V. M., Sheeba, V., Rajamani, M. and Joshi, A. 2000 Evolution of reduced pre-adult viability and larval growth rate in laboratory populations of *Drosophila melanogaster* selected for shorter development time. *Genet. Res.* **76:** 249–259

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

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

Reznick, D. and Travis, J. 1996 The empirical study of adaptation in natural populations. In *Adaptation* (eds. M. R. Rose and G. V. Lauder), pp. 243–289. Academic Press, San Diego, CA, USA

Robertson, F. W. 1963 The ecological genetics of growth in *Drosophila*. 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. *Genet. Res*. **4:** 74–92

Roff, D. A. 1992 *The evolution of life histories*. Chapman & Hall, New York, NY, USA

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

Roughgarden, J. 1971 Density-dependent natural selection. *Ecology* **52:** 453–468

Santos, M., Borash, D. J., Joshi, A., Bounlutay, N. and Mueller, L. D. 1997 Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. *Evolution* **51:** 420–432

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

Sharmila Bharathi, N. 2007 *Variation and covariation in life-history related traits in some species of Drosophila*. Ph.D. thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

Shiotsugu, J., Leroi, A. M., Yashiro, H., Rose, M. R. and Mueller, L. D. 1997 The symmetry of correlated responses in adaptive evolution: an experimental study using *Drosophila*. *Evolution* **51:** 163–172

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

Stearns, S. C. 1992 *The evolution of life histories*. Oxford University Press, New York, NY, USA

Stearns, S. C. 2000 Life history evolution: successes, limitations, and prospects. *Naturwissenschaften* **87:** 476–486

Van Buskirk, J. and Relyea, R. A. 1998 Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. *Biol. J. Linn. Soc*. **65:** 301–328

Van Buskirk, J. and McCollum, S.A. 2000 Functional mechanisms of an inducible defence in tadpoles: morphology and behavior influence mortality risk from predation. *J. Evol. Biol*. **13:** 336–347

Van Buskirk, J., Anderwald, P., Lupold, S., Reihardt L. and Schuler, H. 2003 The lure effect, tadpole tail shape, and the target of dragonfly strikes. *J. Herpetol*. **37:** 420–424

Vijendravarma, R. K., Narasimha, S., Kawecki, T. J. 2012 Adaptation to abundant low quality food improves the ability to compete for limited lich food in *Drosophila melanogaster*. *PLoS ONE* **7:** e30650

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