Ecological details mediate different paths to the evolution of larval competitive ability in *Drosophila*

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Dedicated to
all those
Drosophila larvae and adults
who made this thesis possible

Contents

DECLARATION

I declare that the matter presented in my thesis entitled "Ecological details mediate different paths to the evolution of larval competitive ability in *Drosophila*" is the result of studies carried out by me at the Evolutionary Biology Laboratory, Evolutionary and Integrative Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Amitabh Joshi and that this work has not been submitted elsewhere for any other degree.

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

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Place: Bangalore
Date: 26th Oct 2018

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CERTIFICATE

This is to certify that the work described in the thesis entitled "**Ecological details mediate different paths to the evolution of larval competitive ability in *Drosophila***" is the result of investigations carried out by Ms. Manaswini Sarangi in the Evolutionary Biology Laboratory, Evolutionary and Integrative Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Amitabh Joshi
(Professor)

Place: Bangalore
Date: 26th Oct 2018

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Synopsis

The broad aim of my doctoral research is to investigate further the manner in which seemingly trivial ecological details of how larval crowding is experienced can alter the evolutionary route by which *Drosophila* populations evolve greater competitive ability when subjected to chronic larval crowding in the laboratory. I build up on earlier work I did for my MS thesis in which I was able to identify ecological differences in how crowding was imposed as the major cause of earlier observed differences, across multiple experimental evolution studies, in the traits contributing to the evolution of enhanced competitive ability in *Drosophila* populations adapted to larval crowding. Among other things, that work indicated that evolutionary responses to being maintained in crowded larval cultures in *Drosophila* are likely to depend on the precise combination of egg number and food amount used to impose the crowding. Specifically, it was suggested that evolution at high larval density, but in relatively large amounts of food, would facilitate the evolution of increased larval feeding rates and metabolic waster tolerance, whereas evolution at high larval density, but in relatively small amounts of food, would proceed via the evolution of greater efficiency of food to biomass conversion and rapid development, involving no evolutionary change in larval feeding rate or waste tolerance. The reason for this difference was hypothesized to be related to the time dynamics of the build up of metabolic waste in the feeding band (upper 1 cm of food in the culture vials), and how it might differ in vials with greater or smaller food levels. The idea was that, in a crowded culture with a large absolute amount of food, there is quite a lot of food below the feeding band into which metabolic waste could diffuse, thereby resulting in a relatively slow build up of metabolic waste within the feeding band itself. By

contrast, crowded cultures with small amounts of food essentially consist largely of just a 1 cm deep feeding band, which consequently can accumulate waste at high concentrations even in the earlier stages of a culture. One of the outcomes of these speculations arising from my Masters' research was the setting up of two new selection regimes in our laboratory (CCU and LCU populations), which were subjected to larval crowding (as in the already existing MCU selection regime) but at various combinations of egg number and food amount. The MCUs were reared in vials at 600 eggs/1.5 mL of food, and had earlier been seen to evolve very different traits than the CU populations of L. D. Mueller, that were reared at over 1500 eggs in 6-7 mL of food per vial. The new CCU populations were subjected to crowding at exactly the same density as the MCUs, but with more food, at 1200 eggs/3 mL food per vial, whereas the new LCU populations approximated the CUs, being subjected to larval crowding with 1200 eggs/6 mL of food per vial. The backdrop to this work, and an introduction to the populations used in the study, is provided in Chapter 1.

In this thesis, I report results from studies on larval competitive ability, fitness-related traits (pre-adult viability and development time, and dry weight at eclosion) and how they are affected by different combinations of egg number and food amount, larval feeding rates measured under various conditions, tolerance to metabolic wastes like urea and ammonia, and the ability to survive on poor nutritional quality food, in the MCU, CCU and LCU populations, along with the ancestral MB controls. I also test earlier hypotheses regarding the diffusion or build up of metabolic waste in different zones (feeding band or below it) of the food column in a crowded culture vial. I first show the evolution of enhanced competitive ability, relative to MB controls, in all three sets of crowding adapted populations (Chapter 2). I then present

results from a study examining pre-adult survival and the distribution of pre-adult development time and dry weights of flies eclosing at different times in three different kinds of crowded cultures, corresponding to the MCU-, CCU- and LCU-type maintenance regimes. In part, these results show that crowding at different combinations of egg number and food amount can have very different consequences for fitness-related traits and can, therefore, possibly mediate evolution along very different trajectories (Chapter 3). I next discuss results from multiple assays of feeding rate and waste tolerance from which I conclude that although there is no clear evidence for the evolution of enhanced larval urea or ammonia tolerance in the MCU, CCU or LCU populations, there is clear evidence that feeding rate differences between selected populations and controls are phenotypically plastic, and to a much greater degree in MCUs, depending critically on the conditions under which the feeding rate measurements are made. This is important because feeding rate has long been used as a surrogate for competitive ability in *Drosophila* studies, and has always been measured in one way – one single larvae feeding on yeast suspension in a Petri-dish. My results clearly show that this classical method for measuring feeding rates can be very misleading as it does not necessarily reflect what feeding rates are like in the actual culture vials (Chapter 4). Finally, I show that the MCU populations do not perform better than controls on nutritionally poor food, suggesting that adaptation to limiting rich food and abundant poor food are quite different, even though an earlier study had shown that *Drosophila* populations adapted to abundant poor food also evolved a greater ability to compete for limiting rich food (Chapter 5). I then conclude with a brief discussion of further avenues of work suggested by these studies (Chapter 6).

Chapter 1: Introduction

For most of their historical development as distinct fields of biology, population ecology and population genetics have remained largely separate (Kingsland 1995, Mueller 1997), even though they have had obvious overlaps as reflected in Fisher's (Fisher 1930) equation of the Malthusian parameter of population growth with Darwinian fitness (henceforth, fitness). The two major areas where theory in population genetics and population ecology has meaningfully intersected are the dynamics of age-structured populations (Fisher 1930, Hamilton 1966, Charlesworth 1970, 1994, Charlesworth & Williamson 1975, Charlesworth and Hughes 1996) and density-dependent selection (MacArthur 1962, MacArthur & Wilson 1967, Gadgil & Bossert 1970, Roughgarden 1971, Clarke 1972, Asmussen 1983, Anderson & Arnold 1983). The latter is especially significant, as selection at high densities is essentially selection for increased competitive ability (Joshi *et al* 2001), and competition is an important factor shaping not just evolution but also population dynamics and stability, and community structure, as well as affecting the ecological and evolutionary outcomes of other species interactions (Arthur 1982, Case 1999, Dey *et al* 2012). Indeed, competition between organisms was an important component of Darwin's conception of the 'struggle for existence' (Darwin 1859).

Competitive ability, like fitness, is a composite phenotype and can be altered by changes in a number of traits, many of which are also linked by tradeoffs of varying strengths (Prasad & Joshi 2003). Moreover, increased competitive ability can result from increased ability to inhibit the population growth of the competitor (effectiveness: Joshi & Thompson 1995), or by an increased ability to resist the inhibitory effects of the competitor on one's own population growth (tolerance: Joshi & Thompson 1995), or a combination of both. Earlier work has shown that these two

constituents of competitive ability can in part be independent in both interspecific (Peart 1989; Goldberg & Landa 1991) and intraspecific (Mather & Caligari 1983; Eggleston 1985; Hemmat & Eggleston 1988, 1990) competition, that they are heritable (Eggleston 1985), and that they can evolve separately (Joshi & Thompson 1995), at least in *Drosophila* species. Various traits, including some closely related to the life history, can affect effectiveness and tolerance and, thereby, competitive ability (Joshi *et al* 2001). Moreover, the effects of increased competitive ability on population dynamics and stability appear to depend critically on the set of traits through which the increased competitive ability evolved (Dey *et al* 2012). As such, it is important to understand in some detail how various ecological factors, especially the details of how crowding is experienced, can influence the traits through which enhanced competitive ability evolves. In this thesis, I build upon work I did earlier for my Master's thesis (Sarangi 2013) in which I showed, *inter alia*, that the total amounts of food present in a crowded *Drosophila* culture interacts with larval density in mediating the evolution of increased competitive ability through very different sets of traits.

Competition as an agent of natural selection

Competition for resources refers to mutually negative interactions between two or more organisms, populations or species when they have similar requirements for survival, growth and/or reproduction. The definition for competition however has been a matter of discussion by many in the past (Grime 1979, Thompson 1987, Tilman 1987, Thompson & Grime 1988) and how competition as best defined is often highly context specific (Joshi & Thompson 1995, 1996, 1997). Typically, population growth rates are affected under competition, and at an individual level too, the

absolute fitness of individual organisms gets compromised. In scenarios where some resource is limiting, individuals may undergo an increased energy expenditure in merely acquiring resources, which in turn affects their chances of survival, development or reproduction. Therefore competition for resources, whether it is within or between species, plays a key role in mediating the process of natural selection, as also noted by Darwin (Darwin 1859). Again, resources under demand can be of several kinds, like food, water, mate and/or space for occupying a territory. My work focuses on the intra–specific mode of competition for food among larvae of *Drosophila* populations facing larval crowding. The effects of intra–specific competition on any individual or population are, by definition, an example of negative density–dependence.

Density–dependent selection theory

Given the above perspective, the theory of density–dependent selection has been of particular significance since it serves as an interface between population genetics and population ecology. The basic proposition of density–dependent selection theory is that genotypic fitnesses are functions of population density, and that the same genotype is unlikely to be the fittest at both low and high densities (Mueller 2009). Density-dependent selection was first developed as a verbal theory by MacArthur (1962), MacArthur & Wilson (1967), wherein the notions of density–independent (*r*–selection) and density–dependent selection (*K*–selection) were put forward, with selection at low densities assumed to favour traits contributing to a high population per capita growth rate (*r*), whereas selection under chronic crowding was believed to favour traits yielding higher carrying capacity (*K*). The theory of density-dependent selection was then, unfortunately, elaborated to explain different life-

history patterns (Pianka 1970). This abuse of the theory has been criticized in detail in the past (Stearns 1977; Parry 1981; Boyce 1984; Mueller 1995, 1997) and we will not concern ourselves with it further. A more consequential sequel to the verbal theory was the development of population genetics models for studying evolution of traits under density–dependent and density–independent scenarios (Gadgil & Bossert 1970, Roughgarden 1971, Clarke 1972, Anderson & Arnold 1983, Asmussen 1983). All of these models centered around the idea of an *r–K* trade-off, implying that populations chronically experiencing low or high density will evolve to perform better at their usual density, at a cost of performance at other densities. To test predictions from these models, a few experiments were carried out, that are discussed in the section below.

Empirical studies on adaptation to larval crowding in Drosophila: the canonical story

The first empirical evidence for an *r-K* tradeoff came from a study of laboratory populations of *Drosophila melanogaster* (Mueller & Ayala 1981). In this study, one set of three populations was maintained at low density, i.e., the *r*–selected populations, while another set of three populations was maintained at carrying capacities, i.e., the *K*–selected populations. The *K*–selected populations showed greater per capita population growth rate than the *r*–selected populations when assayed at high densities, but showed a reduced growth rate when assayed at low densities. The *K*–selected populations were also found to have evolved greater larval competitive ability (Mueller 1988a), increased larval feeding rates (Joshi & Mueller 1988), and increased pupation height (Mueller & Sweet 1986, Joshi & Mueller 1993). Interestingly, and contrary to the predictions of classical density-dependent theory

(MacArthur & Wilson 1967), the *K*–selected populations were seen to have evolved reduced food-to-biomass conversion efficiency (Mueller 1990). One problem with this earliest study of *r*– and *K*–selection in *Drosophila* was that the *K*–selected populations experienced crowding both as larvae and as adults, and the *r*– and *K*–population maintenance differed in having discrete versus overlapping generations, respectively.

A subsequent study using *D. melanogaster* also addressed the question of long-term adaptation to larval crowding while correcting for the above-mentioned confounding factors. This new selection experiment used *D. melanogaster* populations originating from a geographical region different from that of the *r*– and *K*– selected populations. Three sets of populations were derived (first described in Mueller *et al* 1993), one set selected for adaptation to larval crowding (the CU populations), another set selected for adaptation exclusively to crowding at adult stage (the UC populations) and the third set was maintained at moderate density of larvae and adults which served as controls (the UU populations) (Figure 1). This selection study was carried out over a long period of time and provided enormous insight into the pathways by which populations maintained at high larval densities undergo adaptive evolution. Adaptation to larval crowding in the CU populations was shown to occur via the evolution of a suite of traits broadly similar to that of the *K*–selected populations. Relative to the uncrowded control UU populations, CU populations were shown to have greater pre-adult survivorship and faster pre-adult development when assayed at high larval density (Mueller *et al* 1993, Santos *et al* 1997, Borash & Ho 2001). However, they did not differ from controls in either of these traits when assayed at low density (Santos *et al* 1997, Borash & Ho 2001). The CU populations

also showed increased larval feeding rates (Joshi & Mueller 1996), longer foraging path lengths (Sokolowski *et al* 1997) and increased tolerance of larvae to nitrogenous wastes like urea (Shiotsugu *et al* 1997, Borash *et al* 1998) and ammonia (Borash *et al* 1998). Moreover, like the *K*–selected populations, the CU populations also required more food to successfully pupate as compare to UU populations, indicating reduced efficiency of food utilization (Joshi & Mueller 1996). Thus, a canonical view developed that the evolution of greater larval competitive ability in *Drosophila* occurred largely through an increase in the efficiency of food acquisition and greater tolerance to metabolic waste, at the cost of efficiency of utilizing the ingested food (Joshi & Mueller 1996; Mueller 1997, 2009, Joshi *et al* 2001, Mueller & Cabral 2012). In particular, feeding rate was seen to be a strong correlate of larval competitive ability and was, consequently, treated almost as a surrogate for competitive ability in *Drosophila* (Borash *et al* 1998, Joshi *et al* 2001, Shakarad *et al* 2005; Rajamani *et al* 2006; Fellowes *et al* 1998, 1999; Kraaijeveld *et al* 2001).

A further nuance to this view came from a study showing that larvae from the CU populations exhibited a heritable polymorphism with respect to tolerance to metabolic waste and feeding rate, respectively. Early eclosing larvae in crowded CU cultures were a fast feeding, fast developing, waste sensitive type, whereas late eclosing larvae were a slower feeding, slower developing, waste tolerant type (Borash *et al* 1998). Further observations that selection for tolerance to ammonia (and urea) was accompanied by slower larval feeding rates (Borash *et al* 2000a) and shorter foraging path lengths (Mueller *et al* 2005) were also supportive of this view that faster feeding and waste tolerance were antagonistic traits, though both contributed to fitness in different phases of a crowded culture. Epistatic selection, however, on its own is unlikely to maintain such polymorphisms in the face of decay of linkage

disequilibrium due to recombination unless the selection is extremely intense. A subsequent study strongly suggested that the polymorphism observed by Borash *et al* (1998) was likely to be an artifact of the maintenance regime of the CU populations, specifically showing that the manner in which eclosing flies from CU culture vials were collected resulted inadvertently in promoting positive assortative mating for development time, a phenomenon that could help maintain the early-late polymorphism (Archana 2010).

Adaptation to larval crowding in Drosophila: a twist to the canonical story

By about 2000, the above-described canonical view on adaptation to larval crowding in *Drosophila* was well established, having been seen in three different sets of experiments (Mueller & Ayala 1981, Mueller 1988a,b, Joshi & Mueller 1988, Mueller 1990, Bierbaum *et al* 1989, Mueller et al 1991, Mueller *et al* 1993, Joshi & Mueller 1996, Santos *et al* 1997, Shiotsugu *et al* 1997, Borash *et al* 1998, Borash & Ho 2001, Mueller 1997, 2009). However, a subsequent study on adaptation to larval crowding in two different species of *Drosophila*, i.e., *D. ananassae* and *D. nasuta nasuta,* had an entirely different story to tell (Nagarajan *et al* 2016). These two studies involved relatively recently wild caught populations that were subjected to long-term selection for adaptation to larval crowding, implemented in a manner (ACU: *D. ananassae*; NCU: *D. n. nasuta*) slightly different than the earlier used *K*–selected or CU populations. In particular, the ACU and NCU populations, though subjected to high larval density, were maintained at total food levels and egg numbers per vial that were considerably lower than those in the case of the *K*–selected and CU populations used in the earlier studies. In crowding adapted populations of both species, the evolution of greater competitive ability was achieved through a combination of faster pre-adult development (when assayed at both low and high larval densities) with a

reduced minimum critical feeding time (indicating a greater time efficiency of food conversion to biomass), relative to their respective controls (Nagarajan *et al* 2016) In complete contrast to the canonical story, these crowding adapted populations did not evolve either increased levels of larval foraging behavior, i.e., feeding rates and foraging path lengths, or increased tolerance to nitrogenous waste like urea or ammonia (Nagarajan *et al* 2016).

The evolution of greater competitive ability through a very different set of traits than seen earlier in *D. melanogaster*, that was actually closer to the canonical predictions of *K*-selection theory, in *D. ananassae* and *D. n. nasuta* populations adapted to larval crowding was surprising, and a subsequent study with *D. melanogaster* (Sarangi *et al* 2016) attempted to discriminate between various possible explanations for the discrepancy in the results from these various selection studies. Species-specific differences, or differences between recently wild caught populations and the long-term laboratory–adapted *K*–selected or CU populations, in the genetic architecture of traits relevant to fitness under larval crowding were two possible reasons for the contrasting results seen in the ACU and NCU studies, as compared to the earlier studies on *D. melanogaster*. Moreover, the total food level and the absolute number of eggs in the ACU and NCU maintenance regimes were much lower than those of the *K*–selected and CU populations, and this ecological difference in how larval crowding was imposed could also be one of the potential reasons for driving the differences in the traits observed across studies (Sarangi *et al* 2016). The new long-term selection study used *D. melanogaster* populations (MCU populations) from the same ancestry as the CU populations, but subjected to larval crowding at low absolute food levels, as in the case of the ACU and NCU populations (Sarangi *et al* 2016).

Results from the MCU selection experiment showed evolution of greater competitive ability through traits similar to that observed in the ACU and NCU populations (Sarangi *et al* 2016). The MCU populations showed evolution of greater competitive ability, higher pre-adult survivorship (at high larval densities), faster pre-adult development (at both low and high densities), reduced duration of larval stage, reduced time to attain minimum critical size and increased time efficiency of food utilization (Sarangi *et al* 2016). However, they did not show any increase in larval feeding rates, larval foraging path length, pupation height or tolerance to nitrogenous wastes (Sarangi *et al* 2016). The fact that MCU and CU populations shared a common ancestry, but the MCU populations, nevertheless, showed a similar pattern of trait evolution to ACU and NCU populations, clearly implicated the ecological differences in how larval crowding was imposed in the maintenance regimes of CU versus ACU, NCU and MCU populations as the most likely factor for the different results in the ACU/NCU versus the CU studies (Sarangi *et al* 2016).

Context-specificity of genetic correlations

As we can see, laboratory selection experiments on life-history related traits in *Drosophila* have been important in understanding the genetic architecture of populations undergoing adaptive evolution under well-defined and relatively controlled ecological conditions (reviewed in Prasad & Joshi 2003). However, the correlated responses of traits connected to the trait(s) under selection have been observed to vary depending upon either the intensity of selection, maintenance regimes and/or the nature of the trait(s) under selection. One such example of a trait is larval feeding rate. As mentioned earlier, populations selected for adaptation to larval crowding evolved greater competitive ability mostly via increase in feeding rates

(Joshi & Mueller 1988, 1996) Similarly the converse was also true; i.e., when populations subjected to direct selection for greater larval feeding rates, they evolved to be better competitors as larvae (Burnet *et al* 1977). Similarly, when *D. melanogaster* populations were selected for increased parasitoid resistance, they evolved slower rates of larval feeding and reduced larval competitive ability (Fellowes *et al* 1998, 1999). The same pattern of reduced larval feeding rate accompanying reduced larval competitive ability was also observed to be true in two different sets of *D. melanogaster* populations selected for faster development (Prasad *et al* 2001, Shakarad *et al* 2005, Rajamani *et al* 2006). Thus, the strong positive correlation between larval feeding rate and competitive ability has been validated across many studies. Contrary to results from all of the above studies, the correlation was found to be broken, as seen from the results in the ACU, NCU and MCU populations. Similarly, patterns of correlated responses to selection have also been found to be quite diverse among other studies, like in selection for postponed senescence (Rose 1984, Nusbaum *et al* 1996, Patridge & Fowler 1992), starvation resistance (Chippindale *et al* 1996, Harshman *et al* 1999), desiccation resistance (Gibbs *et al* 1997), etc. Moreover, in *Drosophila,* it is known that trade-offs can potentially stretch across life-stages as well (Chippindale *et al* 1996, 1998). In the context of adaptations to crowding, the *K*–selected populations showed an increased longevity under high adult densities, greater body size at eclosion under high larval densities (Bierbaum *et al* 1989) and greater starvation resistance (Mueller *et al* 1993) relative to control *r*–selected populations. Yet, the CU populations had no difference in body weight at eclosion but had greater lipid content under high larval densities (Santos *et al* 1997, Borash & Ho 2001), and also showed higher starvation resistance (Borash & Ho 2001) and a tendency of higher fecundity compared to control UU

populations. In the ACU and NCU populations, by contrast, there was no difference in body weight at eclosion, lipid content or fecundity, relative to controls. Yet, the ACU populations showed greater starvation resistance and NCU populations had a longer lifespan in comparison to their respective controls (Archana 2010). Similarly, the MCU populations also showed evolution of increased longevity (Shenoi *et al* 2016a) when assayed adults were reared at various densities as larvae, relative to control MBs. MCU males also showed increased lipid content; there was also an increase in overall courtship levels in the MCU populations (although there was no difference in mating frequency or mating success, Shenoi *et al* 2016b, Shenoi & Prasad 2016), but the MCU adults were observed to be lighter than controls (Shenoi *et al* 2016a, Sarangi 2013). A trade-off within the larval stage was earlier seen between feeding rate and waste tolerance in the CU populations (Borash *et al* 1998), along with a reduction in efficiency of food utilization (in *K*–selected and CU populations, Mueller 1990, Joshi & Mueller 1996). Conversely, in the ACU, NCU and MCU populations, within the larval stage no such trade-offs were detected, but MCU adult body weights were found to be lower than controls, especially when larvae were reared at high densities.

Related to the issue of competition for limiting but rich food is the mechanism of adaptation to unlimited but poor quality food. In one set of experiments wherein *D. melanogaster* populations were selected for adaptation to chronic malnutrition, evolved populations showed faster pre-adult development (when assayed at low density in both standard and poor food), higher larval growth rate (when assayed at low density in poor food), higher pre-adult survivorship (when assayed at low density, poor food) and greater larval competitive ability than controls (when assayed at low

density in agar-yeast bottle cultures), but had shorter foraging path lengths without any change in feeding rates (raised at low density, standard food, assayed on Petri-dishes with yeast solution) (Kolss *et al* 2009, Vijendravarma *et al* 2012 a, b). In the adult stage, the body size of these selected populations was much lower than controls (when assayed at low density in both standard and poor food), resulting in lower fecundity (early life, when raised and assayed at low density in standard food), and lower starvation resistance (when raised at low density in standard food) without any change in mean longevity, relative to controls (when assayed at low density in poor food) (Kolss *et al* 2009).

Essentially, what is seen across experimental evolution studies, is that genetic correlations among fitness-related traits are typically labile, often tending to change depending upon the ecology of the trait under selection (Service & Rose 1985, Service *et al* 1988, Lenski *et al* 1991, Chippindale *et al* 1993, Leroi *et al* 1994 a,b, Lenski & Travisano 1994, Teotonio & Rose 2000, Matos *et al* 2002, Archer *et al* 2003, Phelan *et al* 2003, Rose *et al* 2005). One lesson from many past studies of this kind has been that working out the details of why and how correlations between fitness-related traits change across environments or time typically leads to considerable insights into the subtlety with which genetics and ecology interact to generate evolutionary trajectories.

Background and objectives of the present study

Given the differences between the traits through which competitive ability evolved in the ACU/NCU/MCU versus the CU populations, and the apparent breakdown of the larval feeding rate correlation with competitive ability in the former, I had earlier taken up a study of the MCU and MB populations for my

Masters' thesis (Sarangi 2013). I first undertook a more detailed phenotypic characterization of the MCU populations and also verified their greater competitive ability. In addition, I showed that altering food level and egg number, while keeping density constant, does affect the means and distributions of various fitness-related traits in these populations, thereby further confirming the view that small ecological details of how crowding is experienced could, in principle, mediate the differences in evolved responses to larval crowding seen across earlier studies with the ACU/NCU/MCU populations, as compared to the CU populations.

Based on the work reported in my Masters' thesis, I decided to set up two new selection experiments, in addition to the ongoing MCU populations. One selection regime (CCU) maintained the same density as the MCU, but with twice the amount of food, whereas the other (LCU) approximated the mode of crowding imposed on the CU populations in earlier studies in the Laurence Mueller laboratory. The experiments described in the present thesis examine the evolution of competitive ability in all three sets of populations, MCU, CCU and LCU and, moreover, aim at teasing apart the effects of different combinations of egg number and food level on pre-adult survivorship and the distributions of pre-adult development time and dry weight at eclosion in these three sets of populations, relative to the ancestral MB controls. I also examined larval feeding rates in far greater detail than previous studies, including measuring feeding rates in larvae while in the crowded culture vials. I also looked for evidence of the evolution of waste tolerance in these three sets of crowding adapted populations and, finally, I asked whether the MCU populations were superior to MB controls when raised on abundant but poor quality food.

Experimental Populations

All studies reported in this thesis used two or more sets of populations of *D. melanogaster,* the ancestral controls (MB) and one or more of the three sets of populations subjected to selection for adaptation to larval crowding under varying maintenance regimes (MCU, LCU or CCU). Each of these four sets of selected or control populations consisted of four replicate populations each, subjected to identical maintenance regimes (Fig. 1). The details of their regular maintenance regimes are described next.

MB: **M**elanogaster **B**aseline, served as ancestral control populations

These populations are reared at a moderate larval density starting with about 70 eggs per vial (9.5 cm h × 2.4 cm d) in 6 mL of cornmeal medium (Table 1). Forty such replicate vials are set up per population. On the 11^{th} day from egg collection, the eclosed adults are transferred from culture vials to Plexiglas cages (25 × 20 × 15 cm^3). Thus, with ~75–80 % pre-adult survivorship, each population comprises of ~2000 breeding adults every generation. On the 18^{th} day from egg collection, each population is provided with a Petri-dish containing excess supplement of live yeast-acetic acid paste on cornmeal food medium (henceforth, 'food plates). Prior to this, food plates are changed at the 12^{th}, 14^{th} and 17^{th} day from egg collection that initiated the generation. Fresh food plates are provided on day 21 (from egg collection) on which the flies are allowed to lay eggs for duration of ~18 h after which eggs are counted in numbers of 70 ± 10 and dispensed into 40 such replicate vials in order to initiate the next generation. Both the vial cultures and cages (larval and adult stages, respectively) are maintained at 25 ± 1°C, ~90 % relative humidity and constant light

on a 21-day generation cycle. The origin and derivation of these control MB populations, is outlined Figure 1 and described in Sarangi *et al* (2016).

MCU: **M**elanogaster **C**rowded as larvae and **U**ncrowded as adults is one of the sets of populations in our laboratory selected for adaptation to larval crowding

The rearing density in these populations is about 600 eggs in 1.5 mL of cornmeal media per vial (9.5 cm h × 2.4 cm d). Twelve such replicate vials are set up per population. Once eclosions begin, which is around 8^{th} day from egg collection, the adults are transferred from vials into Plexiglas cages, everyday until day 18 from egg collection (a period of about 8-10 days). The rearing density was initially about 800 eggs in 1.5 mL of food, which was subsequently reduced over a few generations to about 600 eggs in 1.5 mL of food to avoid population size crashes. Additionally, the total number of replicate vials set up per population was reduced from 24 to 15 and then to 12, due to increase in overall pre-adult survivorship over the course of selection, thereby avoiding adult crowding in the cages. All the other details of the maintenance regime are identical to those of the MBs. Some of the key properties of MB, MCU and CU maintenance regimes are detailed in Sarangi *et al* (2016).

CCU: Equal-density **C**ontrols to **M**CU - **C**rowded as larvae and **U**ncrowded as adults, is one more set of populations selected for adaptation to larval crowding at the same density but twice the amount of food and number of eggs, compared to the MCUs.

The maintenance regime is identical to the MCU populations except that the rearing density is about 1200 eggs in 3 mL of cornmeal food per vial (9.5 cm h × 2.4 cm d). Twelve such replicate vials are set up per population. Once eclosions begin, which is

around 8^{th}–9^{th} day from egg collection, adults are transferred from culture vials to Plexiglas cages, daily till the 20^{th} day from egg collection.

LCU: **L**arry Mueller CU-type, **C**rowded as larvae and **U**ncrowded as adults is the third set of populations selected for adaptation to larval crowding

The rearing density is about 1200 eggs in 6 mL of cornmeal food (9.5 cm h × 2.2 cm d). Twelve such replicate vials are set up per population. Similar to the maintenance of the MCU and CCU populations, once eclosions begin, which is around 8^{th}–9^{th} day from egg collection, adults are transferred from culture vials to Plexiglas cages, daily till the 20^{th} day from egg collection.

The MB, MCU, CCU and LCU regimes are comprised of four replicate populations each one population each of MCU, CCU and LCU was derived from one MB population. Hence, MB–*i*, MCU–*i*, CCU–*i* and LCU–*i* (*i* = 1..4) are related to each other and are treated as independent blocks, representing ancestry, in the statistical analysis.

I also used one marked mutant population of *D. melanogaster* as a common competitor for the MB, MCU, CCU and LCU populations, i.e., the **OE** (**O**range **E**ye) population, described briefly in Chapter 2. The flies used for the nitrogenous waste build-up assay, described in Chapter 4, were the. **VBC** ('**V**aga**B**ond' **C**ontrol) populations described in Tung *et al* 2017.

Standardization

All the populations were subjected to one generation of common, control type, rearing conditions, prior to any assay. This was done to eliminate any non-genetic parental effects. The common rearing condition comprised of 70 ± 10 eggs in ~ 6 mL cornmeal food per vial, with 40 such replicate vials set up per population. On the 11th day post egg-collection, the eclosed adults were transferred from culture vials to Plexiglas cages. Each of the standardized populations was given food supplemented with live yeast-acetic acid paste on a Petri-dish for 3 days prior to egg collection for any assay. The method of standardization slightly varied for experiments discussed in Chapter 5 (refer to materials and methods in Chapter 5).

Table 1 Composition of cornmeal medium (1 L)

Ingredients	Amount
Cornmeal	100 g
Yeast	40 g
Sugar	40 g
Agar	12 g
Activated charcoal	0.5 g
Water	1 L

For preparation of 1 L cornmeal medium, all the ingredients are weighed and thoroughly mixed together in 1 L of water. With continuous stirring, the mixture is then allowed to homogenize on heat. Once the froth dissipates, 120 mL of extra water is added to this mixture to maintain consistency of the food media. The mixture is then pressure cooked for 20 minutes, after which the prepared medium is cooled down to 60°C. Preservatives, 10 mL of propionic acid and 1 g of methyl-p-

hydroxybenzoate dissolved in 10 mL of ethanol, are then added to this cooled media and mixed thoroughly before dispensing into culture vials or Petri-dishes.

Table 2 Composition of assay egg-laying medium (1 L)

Ingredients	Amount
Yeast	36 g
Sugar	35 g
Agar	24.8 g
Water	1 L

For 1 L of assay egg-laying medium cook, all the ingredients are weighed at first. Agar is then allowed to boil. In the meanwhile, yeast and sugar are mixed together with water using a mixer grinder. This smooth mixture of yeast and sugar is then added to the boiling agar, following which the entire medium is again allowed to cook and boil. The prepared medium is then cooled to 60 °C. Preservative, 2.4 g of methyl-p-hydroxybenzoate dissolved in 23 mL of ethanol, is then added to this cooled medium.

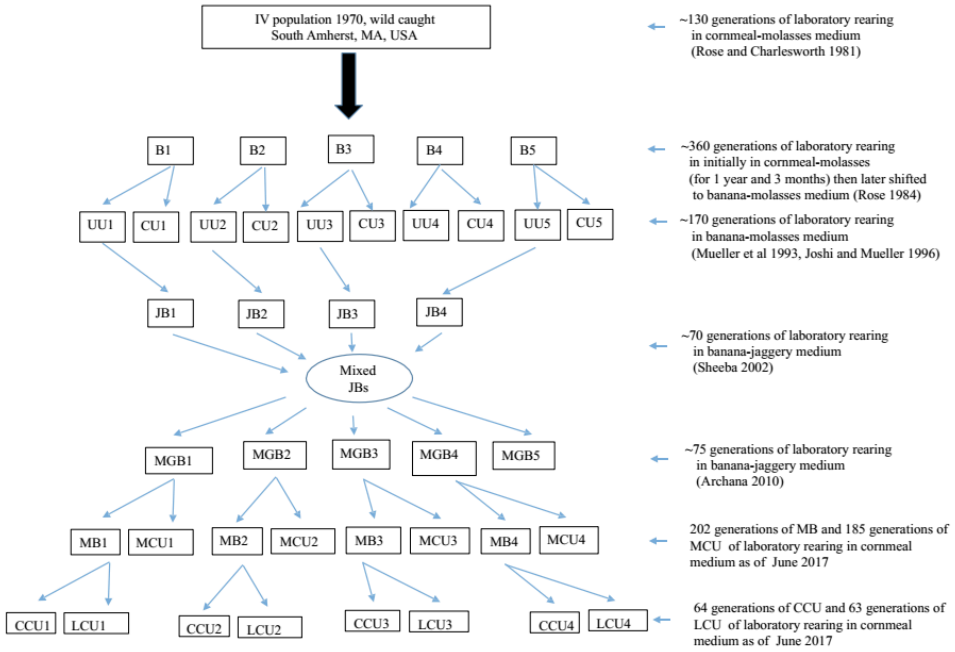

Figure 1. A schematic depiction of the ancestry and derivation of the four sets of populations (MB, MCU, CCU and LCU) used in this study. All the types of population depicted here were maintained on a 21-day discrete generation cycle, except for the IV and B populations that were on a 14-day discrete generation cycle (adapted and modified from Sarangi *et al* 2016).

Chapter 2: Evolution of larval competitive ability

Introduction

Individuals living in crowded *Drosophila* cultures face primary competition with respect to scarcity of food (Mueller 1988), in addition to also having to deal with other stresses like buildup of metabolic waste to potentially toxic levels (Borash *et al* 1998). Hence, the ability to compete successfully for limited resources becomes a fundamental aspect of fitness under such conditions. Competitive ability is as a composite trait and is essentially a reflection of fitness under high density conditions; as such, it can be viewed as the primary target of selection under crowding (Mueller 1997), though it can also be conceptualized in terms of its 'effectiveness' and 'tolerance' aspects (*sensu* Joshi & Thompson 1995), or further analyzed in terms of specific phenotypic traits that can contribute differentially to these two aspects (Joshi *et al* 2001, Dey *et al* 2012). In *Drosophila*, intra-specific competition is widely believed to be primarily of the scramble type and, therefore, individuals can compete with though the expression of traits like enhanced feeding rates, tolerance to metabolic waste, faster developmental rate, and/or enhanced food to biomass conversion efficiency (Prasad & Joshi 2003). The process of larval competition for food has been thoroughly studied via experiments in *Drosophila*, both in terms of intra– and inter–specific competition (Bakker 1961, Ayala 1969, Burnet *et al* 1977, Mather & Caligari 1983, Nunney 1983, Hemmat & Eggleston 1988, 1990, Mueller 1988, Peart 1989, Goldgberg & Landa 1991, Krijger *et al* 2001, Vijendravarma *et al* 2012b, Nagarajan *et al* 2016, Sarangi *et al* 2016). Results from these studies point to the fact that natural selection favors greater competitive ability when food resources are limiting, although the traits through which competitive ability evolves can vary in different ecological settings.

In the current study, since two new sets of crowding adapted populations (CCU and LCU) were initiated, it is important to first ascertain whether they exhibit a direct response to selection through the evolution of increased competitive ability, before proceeding to use them to examine finer aspects of how different combinations of egg number and food level might be mediating the evolution of competitive ability via different phenotypic routes. Consequently, in this chapter, I report results from pre-adult competition assays wherein the competitive ability of MCU, CCU and LCU populations, relative to the ancestral MB controls, was assessed against a common competitor, which was a population of *D. melanogaster* mutant for eye color, Orange Eye. These assays were performed at larval densities and food levels mimicking the maintenance regimes of each of four sets of populations under study.

Materials and Methods

The relative pre-adult competitive ability of the MB, MCU, CCU and LCU populations was determined by performing assays examining their pre-adult survivorship relative to a common marked mutant strain at both low and high larval densities. The assays were carried out after 150 and 170 generations of MCU selection, which corresponded to 29 and 50 generations, respectively, of CCU and LCU selection. The MB populations were at 168 and 188 generations at the time these two assays were conducted. The common marked mutant strain was from a population of Orange Eye (OE) mutants. OEs were first obtained from a spontaneous mutation that occurred in a white eye mutant of population of *Drosophila melanogaster* which itself was established following spontaneous mutation to white eye in one of the JB populations that were ancestral to the MB populations used in the present study as controls. The OE population was at 91 and 113 generations at the

time of the assays, and had been maintained on a 21-day generation cycle on cornmeal medium under constant light conditions with 25 °C and close to 90% humidity, conditions identical to the MB controls. The different assay environments are described below.

Assay Environments

These two pre-adult competition assays were carried out at four different environments per replicate population per selection regime:

1) *MB culture-type*, Moderate larval density in 8-dram vial: each of these vials (9.5 cm height, 2.4 cm diameter; used for MB, MCU, CCU selection regime cultures) had 70 eggs in 6 mL cornmeal medium. Each vial contained 35 eggs from the test population (MB, MCU, CCU or LCU) and 35 eggs from the common competitor population (OE). Four such vials were set up per population.
2) *MCU culture-type*, High larval density in 8-dram vial: 600 eggs were placed into each of these vials with 1.5 mL cornmeal medium. Each vial contained 300 eggs from a test population and 300 eggs from the OE population. Four such replicate vials were set up per population.
3) *CCU culture-type*, High larval density in 8-dram vial: 1200 eggs were placed into each of these vials with 3 mL cornmeal medium. Each such vial contained 600 eggs from the test population and 600 eggs from the OE population. Four such replicate vials were set up per population.
4) *LCU culture-type*, High larval density in 6-dram vial: 1200 eggs were placed into 6-dram vials (9.5 cm height, 2.2 cm diameter; similar to vials used for CU selection regime cultures, Joshi & Mueller 1996) in 6 mL cornmeal medium.

Each such vial contained 600 eggs from the test population and 600 eggs from the OE population. Four such replicate vials were set up per population.

All vials were monitored closely for the first eclosion of adults. Checks were conducted at an interval of 24 h from the day of first eclosion till no flies eclosed for 4 days at a stretch. Freshly eclosed adults were collected, scanned under a dissecting microscope and scored for eye color.

Competitive ability

The proportion of the test population (MB, MCU, CCU or LCU) and common competitor proportion OE that survived till adulthood was calculated. The relative pre-adult competitive ability of the MB, MCU, CCU and LCU populations was then examined by comparing their mean survivorship in the three different crowded assay environments, and also by examining the mean survivorship of OEs, in the respective different environments. The survivorship of OEs when in competition with a test population can be taken to reflect the inverse of the 'effectiveness' aspect of the competitive ability of the test population, whereas the test population's own survivorship when in competition with the OEs can be taken to reflect the 'tolerance' aspect of competitive ability of the test population.

Statistical analyses

The mean survivorship data of all the populations were arcsin squareroot transformed and then subjected to mixed-model ANOVA. Selection and assay environment were treated as fixed factors and block as a random factor, crossed with the other two. Separate ANOVAs were performed on mean pre-adult survivorship of

MB, MCU, CCU, LCU populations and that of OE populations. Post-hoc comparisons were done using Tukey's HSD at $\alpha = 0.05$ level of significance. All statistical analyses were carried out using STATISTICA^{TM} using Windows Release 5.0B (Statsoft Inc. 1995).

Results

Gen. 150 (MCU) Gen. 29 (CCU, LCU)

Overall, mean survivorship declined in all three crowded assay environments, but the reduction in survivorship under crowding was maximum for the MBs and the minimum for the MCUs, especially in the MCU-type assay environment of 600 eggs in 1.5 mL food (Fig. 1(a)). Survivorship of all three crowding-adapted sets of populations was the lowest in the CCU-type assay environment (1200 eggs in 3 mL food), underscoring the fact that survivorship could differ even in assay treatments with exactly the same egg per unit volume food density (Fig. 1 (a)). The ANOVA revealed significant main effects of selection and assay environment, as well as a significant interaction between the two (Table 1(a)). Post-hoc analysis on selection regime means revealed that only the difference in mean survivorship between MB and MCU was significant (Tukey's HSD; $\alpha = 0.05$ level of significance), with MCUs having a greater survivorship than MBs. Although there was a tendency of CCU and LCU populations having a greater survivorship than the MB controls, these differences were not statistically significant. Examining the different levels of assay environment showed that mean survivorship was significantly greater in the low density assay environment than in any of the three high larval density environments, which did not differ significantly among themselves (Fig. 1(a)). Post-hoc comaprisons across different combinations of selection and assay environment revealed that at the

moderate larval density of 70 eggs/6 mL food, there was no difference in mean survivorship of MB, MCU, CCU and LCU populations in competition with OEs. On the other hand, in the 600 eggs/1.5 mL food assay environment, the MCU populations had a significantly greater pre-adult survivorship than MB, CCU and LCUs, with the MCUs showing ~36.21 % greater survivorship than the control MBs at this density, which coincided with their rearing density. In assay environment of 1200 eggs/3 mL and 1200 eggs/6 mL, MCUs had significantly greater survivorship than only the MBs. There was, moreover, no significant difference in mean survivorship between MB and CCU or LCU populations, although both CCU and LCU populations showed about 9% greater survivorship than MBs in the assay environments corresponding to their respective rearing densities (Fig. 1(a)).

OE survivorship was very low in all three crowded assay environments (Fig. 1(b)), indicating that the 'tolerance' of the OE population was quite poor compared to even the MBs. The only significant ANOVA effect was that of assay environment (Table 1(b)), with the only significant difference in mean OE survivorship being between the low density assay environment and each of the three crowded assay environments (Fig. 1(b)). These results, taken together, suggest that the MB, MCU, CCU, LCU differed far more in their 'tolerance' than in their effectiveness, and that the effectiveness of all the test populations was lower in the 1200 eggs/6 mL food assay environment, compared to the other two crowded environments (Fig. 1(b)).

Gen. 170 (MCU) Gen. 50 (CCU, LCU)

The overall pattern of results in the second competitive ability assay (Fig. 2(a)) was fairly similar to the first assay (Fig. 1(a)), although absolute survivorship

values were marginally lower. Overall, mean survivorship declined in all three crowded assay environments, but the reduction in survivorship under crowding was maximum for the MBs and the minimum for the MCUs, especially in the MCU-type assay environment of 600 eggs in 1.5 mL food and in the LCU-type assay environment of 1200 eggs/6 mL food (Fig. 2(a)). Survivorship of all three crowding-adapted sets of populations was the lowest in the CCU-type assay environment (1200 eggs in 3 mL food), although for CCU and LCU populations, the difference between survivorship in 1200 eggs/ 3 mL food and 600 eggs/3 mL was much smaller than in the first competition assay (Fig. 2 (a)), presumably due to the further evolution of competitive ability in these populations during the intervening 20 generations. The ANOVA revealed highly significant effects of selection and assay environment, as well as the interaction between the two (Table 2(a)).

The MCU populations had significantly greater mean pre-adult survivorship than MB, CCU and LCU populations, as in the first assay. Examining the different levels of assay environment showed that mean survivorship was significantly greater in the low density assay environment than in any of the three high larval density environments, which did not differ significantly among themselves, although, overall, the 1200 eggs/3 mL assay environment again had the lowest mean survivorship among all the three crowding assay environments (Fig. 2(a)). Unlike in the first assay, the CCU and LCU populations also showed significantly greater mean pre-adult survivorship than MBs at certain assay environments. As earlier, there was no difference in mean survivorship between any of the test populations examined at 70 eggs/6 mL food. At 600 eggs/1.5 mL food, MCU, CCU and LCU populations had a significantly greater survivorship than MBs (~32.88 % survival difference between

MB and MCU populations). MCU survivorship was also significantly greater than that of CCU and LCU populations. At 1200 eggs/3 mL food, MCU, CCU and LCU populations again had a significantly greater survivorship than control MBs (~17.21 % survival difference between MB and CCU populations). There was a significant difference in survivorship between MCU and LCU populations, with MCUs having a greater mean survivorship, however, MCU and CCU populations did not differ significantly. At 1200 eggs/6 mL food assay environment, MCU and LCU populations had a significantly greater survivorship than MB populations (~12.76 % survival difference between MB and LCU populations).

OE survivorship was again very low in all three crowded assay environments (Fig. 2(b)), indicating that the 'tolerance' of the OE population was quite poor compared to even the MBs. The only significant ANOVA effect was again that of assay environment (Table 2(b)), with the only significant difference in mean OE survivorship being between the low density assay environment and each of the three crowded assay environments (Fig. 2(b)). These results, taken together, further confirmed that the MB, MCU, CCU, LCU differed far more in their 'tolerance' than in their effectiveness, even after 20 more generations of selection, although the differences in effectiveness and tolerance among the test populations had slightly reduced compared to what was seen in the first assay.

Discussion

Competitive ability, as discussed earlier, is a composite trait that can potentially evolve through different combinations of specific phenotypes. The combination of traits underlying greater competitive ability can be different

depending upon the ecology that the individual or the population is subjected to. Previous studies showed correlated increase in larval feeding rates (Joshi & Mueller 1988, Joshi & Mueller 1996) and tolerance to nitrogenous waste (Shiotsugu *et al* 1997, Borash *et al* 1998) accompanying increased competitive ability in crowding adapted *D. melanogaster* populations. However, recent studies on *D. ananassae* and *D. n. nasuta*, under selection for larval crowding, showed that competitive ability could increase without any correlated change in feeding rates or nitrogenous waste tolerance, but rather via faster attainment of critical size and thereby reduced developmental time (Nagarajan *et al* 2016). This was shown to be true for the MCU populations of *D. melnaogaster* as well (Sarangi 2013, Sarangi e*t al* 2016). In the present experiment, we once again examined competitive ability of the MCU and MB populations, but along with the new crowding-adapted *D. melanogaster* populations (CCU and LCU), and over three different kinds of crowded assay environments, corresponding to the maintenance regimes of the MCUs and the, the CCUs and LCUs. It is clear from the results that both the CCU and LCU populations evolved to become more competitive than the ancestral MB controls by about 50 generations of selection (Fig. 2(a,b)). The MCU populations, having been subjected to selection for much longer, were of course more competitive than their CCU and LCU counterparts in both the assays, and this competitive superiority was more prominent in terms of tolerance as compared to effectiveness with regard to the OE population, and expressed in all three crowded assay conditions, though it was more marked in the 600 eggs/1.5 mL food environment which corresponded to the MCU maintenance regime (Figs. 1(a,b),2(a,b)). This observation could perhaps be explained by the greater reduction in development time in the MCU populations, compared to the CCU and LCU populations (see Chapter 3). Thus, the results of these assays clearly

indicate that selection in both the CCU and LCU maintenance regimes, at 1200 eggs/3 mL food and 1200 eggs/6 mL food, respectively, does lead to ongoing adaptation to larval crowding as evidenced by increased larval competitive ability. In subsequent chapters I examine some of the traits that could be underlying differences in the routes by which competitive ability evolves to become greater in these new selected populations compared to what had been seen earlier in the MCU populations.

These results also confirm earlier findings using only the MB and MCU populations, that assay environments with equal larval density but different absolute amounts of food and egg numbers can exert very different effects on competitive survival (Sarangi 2013). In the present experiments, overall survivorship of all test populations (MB, MCU, CCU and LCU) was lower in the 1200 eggs/3 mL food environment than in the 600 eggs/1.5 mL environment, even though the larval density was the same in both assay environments.

Tables

Table 1(a) Results of ANOVA on mean pre-adult survivorship of MB, MCU (Gen. 150), CCU (Gen. 29) and LCU (Gen. 29) populations when competed against the OE population across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.086	4.578	0.032
Assay environment	3	0.781	110.780	<0.001
Selection × Assay environment	9	0.021	5.075	<0.001

Table 1(b) Results of ANOVA on mean pre-adult survivorship of OE populations (Gen. 91) from the same assay as in Table 1(a), when competed against the test populations across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.025	3.809	0.051
Assay environment	3	3.174	302.168	<0.001
Selection × Assay environment	9	0.005	2.032	<0.074

Table 2(a) Results of ANOVA on mean pre-adult survivorship of MB, MCU (Gen. 170), CCU (Gen. 50) and LCU (Gen. 50) populations when competed against the OE population across different assay environments (4 levels), (b) Results of ANOVA on mean pre-adult survivorship of OE populations (Gen. 113) from the same assay as in Table 2(a), when competed against the test populations across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.100	85.159	<0.001
Assay environment	3	0.789	345.154	<0.001
Selection × Assay environment	9	0.016	7.556	<0.001

Table 2(b) Results of ANOVA on mean pre-adult survivorship of OE populations (Gen. 113) from the same assay as in Table 2(a), when competed against the test populations across different assay environments (4 levels). In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.015	3.631	0.057
Assay environment	3	2.932	709.500	<0.001
Selection × Assay environment	9	0.005	2.098	<0.066

Figures

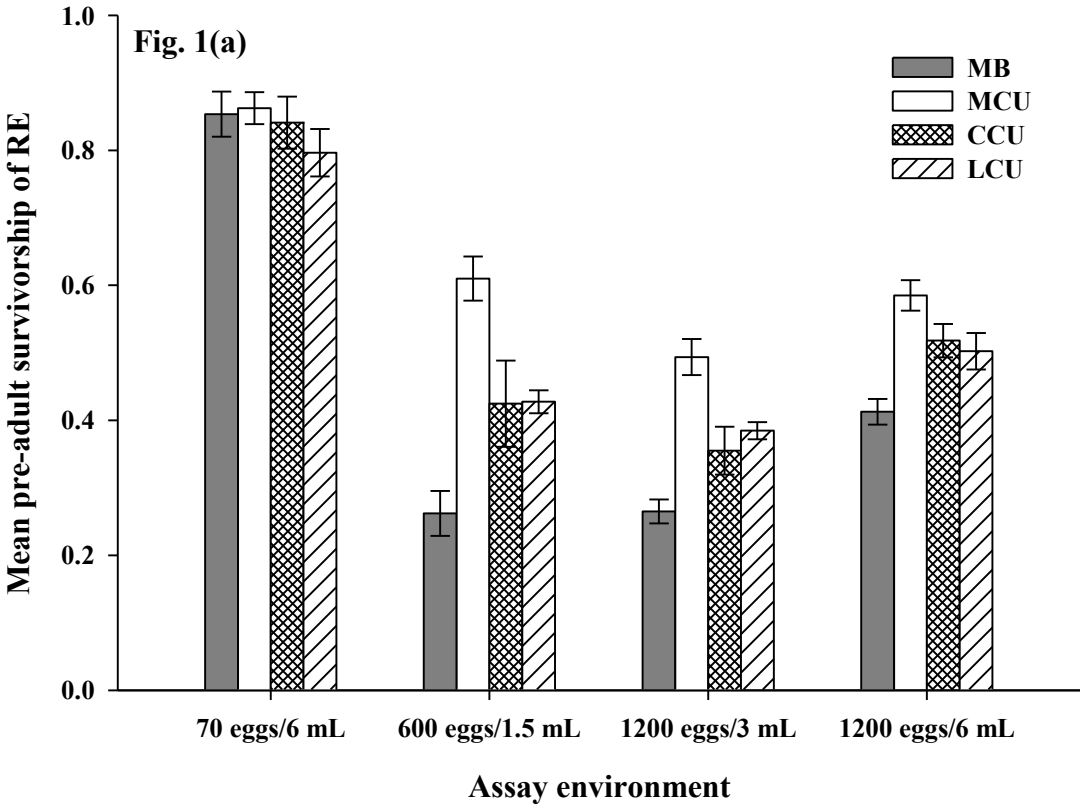
Fig. 1(a)
MB
MCU
CCU
LCU
Mean pre-adult survivorship of RE
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

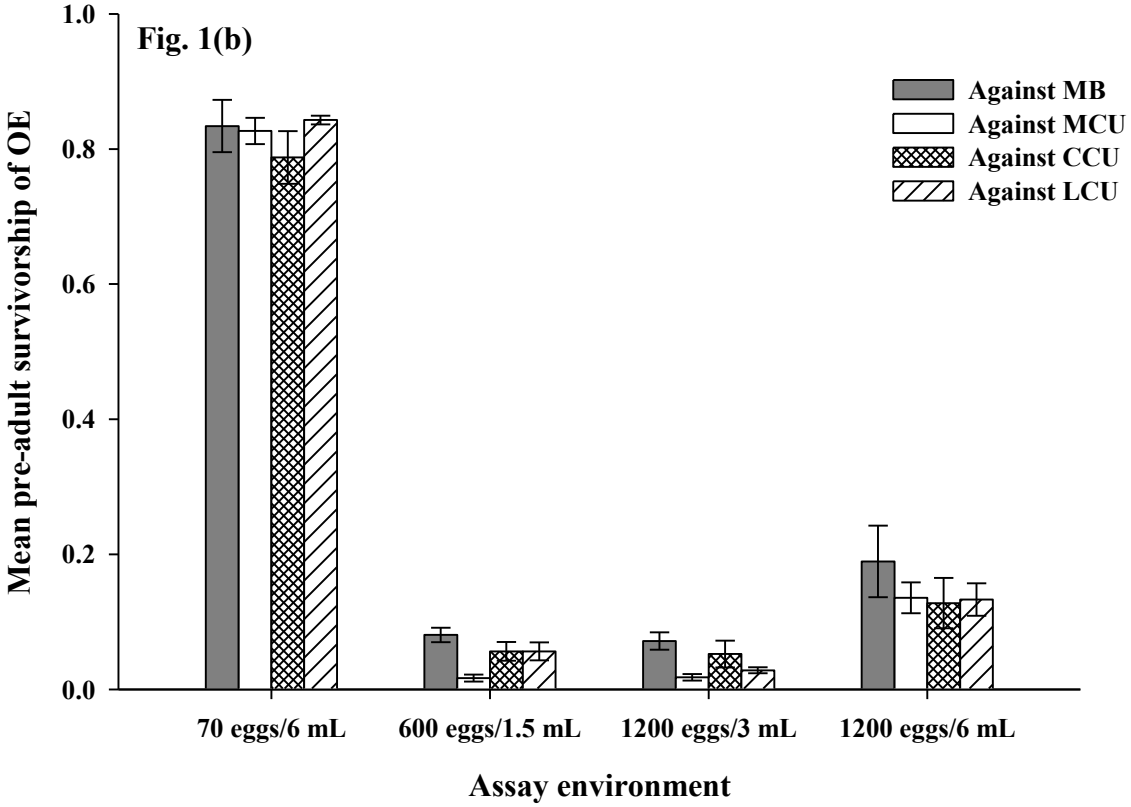
Fig. 1(b)
Against MB
Against MCU
Against CCU
Against LCU
Mean pre-adult survivorship of OE
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 1(a) Mean pre-adult survivorship of MB, MCU (Gen. 150), CCU (Gen. 29) and LCU (Gen. 29) populations in the competition assay at different assay environments. Error bars are the standard errors around the means of the four replicate populations in each selection regime, (b) Mean pre-adult survivorship of OE population (Gen. 91) in the same assay as in Fig. 1(a), when competed against MB MCU, CCU and LCU populations across different assay environments. Error bars are the standard error around the means of the four replicate populations in each selection regime.

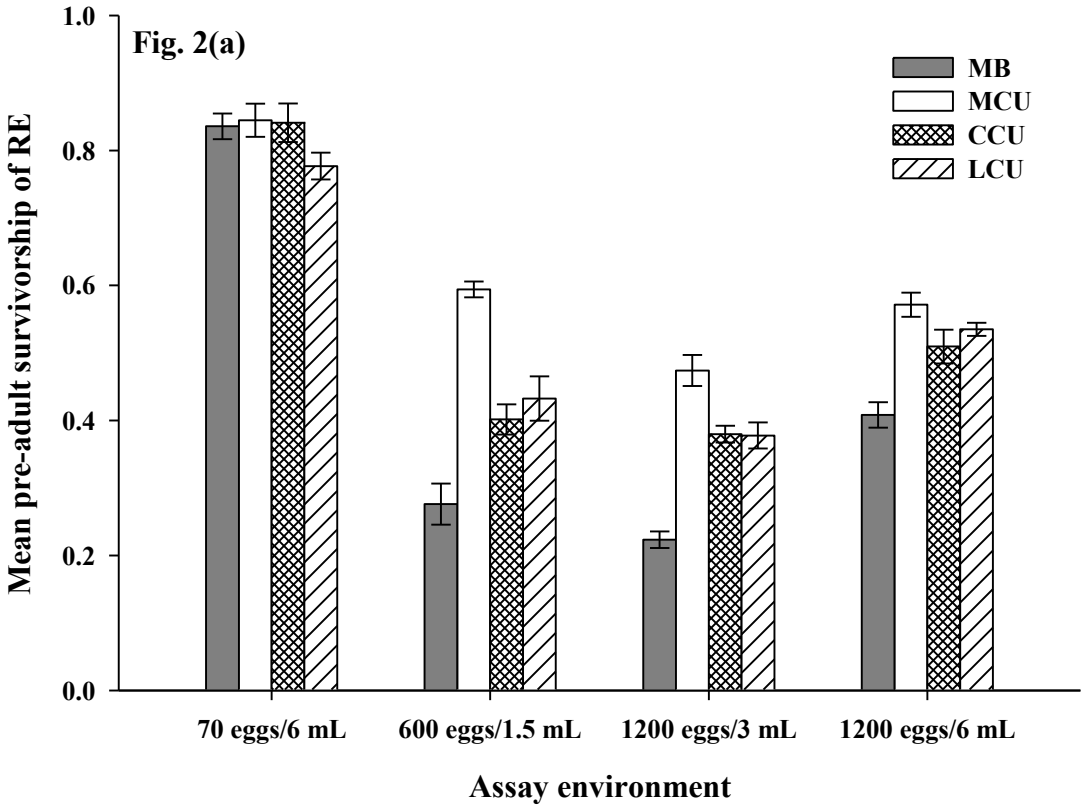
Fig. 2(a)
MB
MCU
CCU
LCU
Mean pre-adult survivorship of RE
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

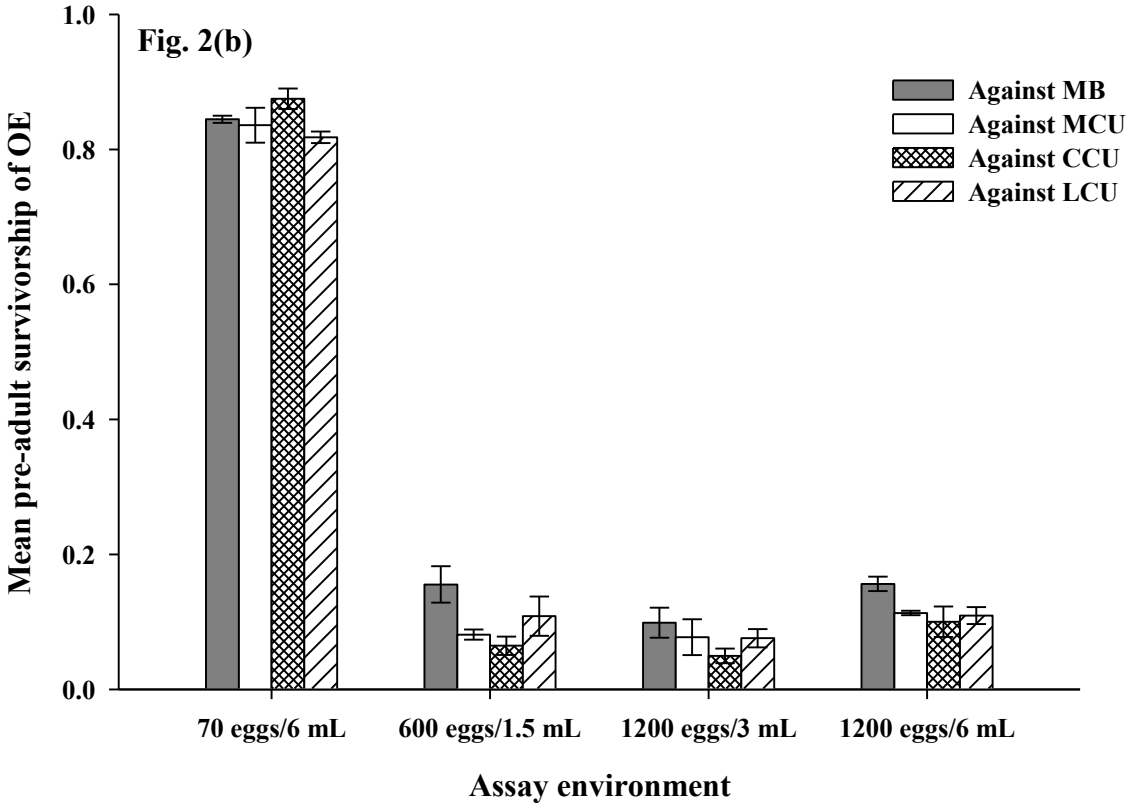
Fig. 2(b)
Against MB
Against MCU
Against CCU
Against LCU
Mean pre-adult survivorship of OE
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 2(a) Mean pre-adult survivorship of MB, MCU (Gen. 170), CCU (Gen. 50) and LCU (Gen. 50) populations in the competition assay at different assay environments. Error bars are the standard errors around the means of the four replicate populations in each selection regime, (b) Mean pre-adult survivorship of OE population (Gen. 113) in the same assay as in Fig. 2 (a), when competed against MB MCU, CCU and LCU populations across different assay environments. Error bars are the standard error around the means of the four replicate populations in each selection regime.

Chapter 3: Effect of different egg numbers and food amounts on fitness-related traits

Introduction

The aim of the experiments reported in this chapter was to examine pre-adult survivorship, pre-adult development time and dry weight at eclosion in the three sets of crowding adapted populations and their controls, and also to investigate if there were any differences among the various selection regimes in how these important fitness-related traits were affected by larval crowding imposed in different ways, using various combinations of egg number and food amount.

Due to the centrality of life-histories in mediating between various phenotypes and their fitness effects, understanding how the distributions of life-history related traits are affected by different ecological factors is an important step towards understanding how ecology alters fitness functions and, potentially, modulates evolutionary responses (Stearns 2000). Hence, understanding ecological effects on life-history traits is an important part of attempting to explain phenotypic evolution. From this perspective, understanding the nature of selective forces acting on a certain set of populations demands a detailed examination of its ecology. Competition is one such phenomenon in which importance of ecology stands out, and, as has been discussed in the previous chapter, increasing the population density or decreasing the amount of food are some of the ways of imposing competition among individuals in a population.

Traditionally, population density – the number of individuals per unit resource – has been used as a standard surrogate for the strength of competition (Case 1999). However, many studies across a variety of taxa indicate that the situation may not always be that straightforward and that density and food amount can interact in subtle

ways to influence fitness-related traits, especially in species with complex life-histories. For example, a study in mosquitoes, *Anopheles gambiae s.s*., showed that although food was given at optimal rates, density still played a role in driving mortality of larvae reared at high densities (Jannat & Roitberg 2013), with low density rearing producing larger adults and high density rearing giving rise to smaller sized adults (Lyimo *et al* 1992, Koella & Lyimo 1996, Gimmig *et al* 2002). In contrast, a study using monarch butterflies, *Danaus plexippus* L., showed that when the larvae were crowded but had constant access to food, adults that emerged were significantly larger than controls or those under food shortage without crowding (Atterholt & Solensky 2010). Another study in the lepidopteran *Sesamia nonagrioides* showed that larval crowding did not increase mortality rates but resulted in prolonged larval developmental period, reduced pupal weight, fecundity and longevity (Fantinou *et al* 2008). In contrast, a study in the European grapevine moth *Lobesia botrana* found that although larval crowding led to increased larval mortality, it did not affect other life-history related traits like probability of emergence from pupa, sex ratio, pupal mass, fecundity or longevity (Thiery *et al* 2014). Turning to vertebrates with metamorphosis, studies on the wood frog, *Rana sylvatica*, showed that populations reared at low densities, with increased levels of food, led to reduction in growth whereas high density reared populations showed enhanced growth with increased food levels (Wilbur 1977). There were significant interaction effects of food level and population density on growth rate and body weight at metamorphosis. This experiment gave suggestive evidence that food and density do not act in isolation and that density effects, apart from food limitation, can contribute to varied outcomes in traits examined. It was also shown in a different study that larvae from smaller eggs reared at low density had an increased larval duration but larger body size at

metamorphosis, in contrast to increased larval duration but smaller body size at metamorphosis for larvae from smaller eggs but reared at high density (Berven & Chadra 1988). Thus, it is clear that the effects of increase or decrease in food amounts versus increase or decrease in population density are not always additive.

Apart from single generation experiments, recent results from long-term studies on adaptation to larval crowding in *Drosophila* also suggested that the interaction of absolute food amounts and larval density may be mediating the evolution of greater competitive ability through different sets of traits across different selection experiments involving adaptation to larval crowding at relatively high versus low food levels. Specifically, the differences in traits that evolved to enhance competitive ability between studies on the *K*-selected (Mueller & Ayala 1981, Joshi & Mueller 1988, 1993) and CU populations (Joshi & Mueller 1996, Borash *et al* 1998) of *D. melanogaster* and the NCU, ACU and MCU populations of *D. n. nasuta*, *D. ananassae* and *D. melanogaster*, respectively (Nagarajan *et al* 2016, Sarangi *et al* 2016), were suggested to be driven by the fact that in the earlier set of studies larval crowding was imposed at relatively high absolute food levels, as compared to the later set of studies (Sarangi *et al* 2016). The crowding adapted populations in the earlier set of studies evolved greater larval feeding rates and waste tolerance, with reduced food to biomass conversion efficiency and no change in pre-adult development time, when assayed at low density. In contrast, the ACU, NCU and MCU populations evolved a faster attainment of minimum critical size, increased efficiency of food to biomass conversion and a significant reduction in pre-adult development time, when assayed at low density, with no change in larval feeding rate or waste tolerance.

A subsequent one-generation study of the MCU and MB populations showed that two assay environments, 1200 eggs/3 mL and 600 eggs/1.5 mL food, that have the same larval density, nevertheless had fairly different effects on pre-adult survivorship, development time and dry weight at eclosion, clearly indicating that fitness-related traits in these populations were affected by an interaction between egg number and food amount (Sarangi 2013). Pre-adult survivorship was lower in 1200 eggs/3 mL than in 600 eggs/1.5 mL environments, while pre-adult development time and dry weight at eclosion were higher. It was also seen that the spread in development time was approximately 200 h in the cultures at 600 eggs/1.5 mL but was closer to 500 h in the 1200 eggs/6 mL environment. Basically, eclosions started around the same time in both assay environments, but in the 1200 eggs/3 mL environment, there was a small but non-trivial number of adults eclosing very late, thereby increasing the mean pre-adult development time. The mean dry weight at eclosion was higher in early eclosing flies from the 1200 eggs/3 mL environment, compared to the early eclosing flies in the 600 eggs/1.5 mL environment. Moreover, the very late eclosing flies in the 1200 eggs/3 mL environment were heavier than those with intermediate development times in the same cultures.

Sarangi (2013) speculated that perhaps crowded cultures with only 1.5 mL food accumulate metabolic waste in the feeding band near the surface of the food faster than cultures with the same larval density but more food, which provides a larger volume for waste to diffuse into while feeding activity remains concentrated closer to the food surface. Given that greater metabolic waste concentrations are likely to reduce the optimal feeding rate of *Drosophila* larvae (Mueller *et al* 2005), this could potentially explain the evolution of enhanced feeding rate and waste

tolerance in populations experiencing chronic crowding, but at relatively high food levels (Sarangi 2013). The present experiments, conducted at two time points in the relatively early stages of CCU and LCU evolution, attempted to follow up on these speculations by examining how pre-adult survivorship, development time and dry weight at eclosion were affected by crowding experienced at various egg number and food amount combinations in the MB, MCU, CCU and LCU populations.

Materials and Methods

These experiments were conducted on all four replicate populations each of the MB, MCU, CCU and LCU selection regimes. One experiment was carried out when the MCUs had undergone 134 generations and CCU and LCU populations had undergone 11 generations of selection. The second, identical, experiment was carried out after 153 generations of MCU and 32 generations of CCU, LCU selection (occasionally, due to population crashes and subsequent restoration from backup flies from previous generations in the MCU populations, a greater number of generations of selection elapsed for the CUU/LCU populations than in the MCU populations in the same span of time). Each of the MB, MCU, CCU and LCU populations were subjected to multiple assay environments utilizing different combinations of egg number and food amount, as described below. After 3 days of yeast supplement with food to the adults, a cornmeal (details of the media given in Table 1, Chapter 1) plate was kept inside each of the cages of standardized populations. The flies were then allowed to lay eggs for 1 h, after which the plate was discarded. A second egg-laying food plate (details of the media given in Table 2, Chapter 2) was then placed in each of the cages for 12 hours. Eggs were then collected off these plates and the exact number of eggs for each assay environment were counted and then transferred to thin

strips of agar, which were then dispensed into the vials. The details of the larval densities and food levels used are described as below:

1) ***Moderate larval density, MB-type environment*:**

   70 eggs were collected in 6 mL cornmeal medium, and placed in 8-dram vials (used for MB-type maintenance cultures). Four such vials were set up per population. This served as the uncrowded control assay environment for the three crowded assay environments.

2) ***High larval density, MCU-type environment*:**

   600 eggs were collected in 1.5 mL cornmeal medium and placed in 8-dram vials (used for MCU-type maintenance cultures). Four such vials were set up per population.

3) ***High larval density, CCU-type environment*:**

   1200 eggs were collected in 3 mL cornmeal medium and placed in 8-dram vials (used for CCU-type maintenance cultures). Four such vials were set up per population.

4) ***High larval density, LCU-type environment*:**

   1200 eggs were collected in 6 mL cornmeal medium and placed in 6-dram vials (used for CU (Joshi and Mueller 1996) and LCU-type maintenance cultures). Four such vials were set up per population.

Pre-adult survivorship

The total number of flies successfully eclosing in each vial was recorded. Mean egg-to-adult survival was calculated for each combination of selection, assay environment and replicate popualtion.

Pre-adult development time

All experimental vials were monitored closely for the first eclosion of flies. Pre-adult development time was recorded at intervals of 24 h from the time of first eclosion till no flies eclosed for 4 days at a stretch. Numbers of male and female eclosing flies were scored separately at each of the checks. From these data, the mean egg-to-adult development time for each selection, sex, assay environment and their combinations were calculated. For examining the distributions of development time, data from all the replicate vials in each of the assay environments were pooled, and the frequency distributions of the number of flies eclosed in each 24 h window was plotted.

Dry weight at eclosion

Freshly eclosed adult males and females were collected separately for each day of eclosion. These adults were dried in hot air oven at 70 °C for 36 h and were then weighed in batches of 5 flies each, males and females separately (number of replicates varied from one population to another, from males to females, across days of eclosion and also depending upon the maximum number of flies eclosing in any particular vial). The dry weights of these adults were measured using a Sartorius (CP225D) fine balance. Means of dry weights were calculated for each selection, sex, assay environment, development time window and their combinations.

Statistical analysis

For all the traits described here, mixed-model analyses of variance (ANOVA) was carried out. Selection, assay environment and sex (for pre-adult development time and dry weight at eclosion) were treated as fixed factors, crossed with one

another and random blocks, representing ancestry (MB, MCU, CCU and LCU populations with same numerical subscripts were treated as random blocks). For pre-adult survivorship, arcsine square root transformation was performed on the data prior to ANOVA. Means of all the traits were considered for all the fixed factors and their interactions. All of ANOVA statistics were performed on STATISTICA^{TM} for Windows Release 5.0B (StatSoft Inc. 1995). Multiple comparisons were carried out using Tukey's honest significant difference test (HSD) at 0.05 level of significance.

Results

Gen. 134 (MCU) Gen. 11 (CCU, LCU)

Pre-adult survivorship

Overall, survivorship was significantly reduced in all three crowded assay environments, relative to the uncrowded assay environment, although mean survivorship did not differ significantly between the crowded environments (Fig. 1(a)). The ANOVA revealed significant main effects of assay environment and selection, but no significant interaction between the two factors (Table 1). Overall, averaged across all assay environments, MCU pre-adult survival was ~9.29 % greater than control MB populations, and this difference was statistically significant. MCUs also showed significantly greater pre-adult survivorship than LCU populations across all assay environments (Fig. 1(a), Tukey's HSD at $\alpha = 0.05$ level of significance). Although not statistically significantly different, CCU and LCU mean pre-adult survivorships were ~4.35% and ~2.94% greater than those of MB populations when averaged across all assay environments. More details on differences in pre-adult survivorship between populations at all assay environments are given in Table 11.

Pre-adult development time

The main points to note about the development time data from this experiment are that (a) overall, mean pre-adult development time increases with food amount in the three crowded assay environments (Figs. 2(a,b), 3(b)), and that (b) eclosions begin at about the same time from egg-lay in all assay environments, but go on for a longer time in crowded environments, with the range of development time increasing quite dramatically with food amount (Figs. 4,6(a,b)). These observations are potentially consequential because previous work suggests that extended duration of pre-adult life in crowded *Drosophila* cultures alters the joint buildup of waste and decline of available food in a manner that can significantly shape the evolution of larval traits in response to crowding (Borash *et al* 1998). Thus, it is entirely possible that crowded treatments that differ markedly in the distribution of pre-adult development time could give rise to the evolution of adaptations to larval crowding through very different patterns of the evolution of various larval traits related food acquisition and utilization, and waste production and tolerance. It is also clear from a comparison of the development time spread in the assay environments with 600 eggs/1.5 mL food and 1200 eggs/3 mL food that crowded treatments with the same larval density can differ in how long food remains available for 'late' individuals to eclose, potentially setting the stage for slightly different evolutionary trajectories of adaptation to larval crowding even at the exact same larval density.

Turning, next, to the details, the ANOVA revealed significant main effects of selection, assay environment and sex (Table 2). The selection main effect was driven largely by the fact that the MCUs, on average, were significantly faster developing than the MBs and LCUs by ~16.46 h and ~10.25 h, respectively. The other two

crowding adapted populations (CCU, LCU) also showed faster development than the MBs by ~9 h and ~6.21 h, respectively, but those differences were not statistically significant. The main effect of assay environment was clearly reflected in a monotonic increase of development time from the uncrowded assay environment to the crowded assay environments with greater food amounts (Figs. 2(a,b),4), and all pairwise comparisons across the four assay environments were statistically significant, including between the two environments that had the same larval density but differed in egg number and food amount. The main effect of sex was as expected, with females developing a few hours faster than males, on an average (Fig. 2(a,b)).

All two- and three-way interactions among the fixed factors were also significant, except for the selection by assay environment interaction (Table 2). Male female differences in mean development time were reversed between MBs and crowding adapted populations (Fig. 3(a)), and between the 1200 eggs/6 mL food assay environment and the other three assay environments, with males developing significantly faster (by ~2.8 h) than females, on an average, averaged in 1200 eggs/6 mL cultures (Fig. 3(b)). The three-way interaction was mostly driven by small differences in how the sexes varied in development time across selection regimes and assay environments (Fig. 2(a,b)).

ANOVA on the variance in pre-adult development time revealed a significant main effect of assay environment (Table 3(a), Fig. 4, 6(a)), with the 1200 eggs/6 mL food assay environment showing significantly greater variance in pre-adult development time, on average, as compared to other assay environments which, however, did not differ significantly among themselves in variance for development

time. The total duration of eclosion in 1200 eggs/6 mL cultures was also found to be the longest as shown by ANOVA (Table 3(b). Fig. 4(g, h), Fig. 6(b)). Overall, the range in the duration of eclosion was shortest in 70 eggs/6 mL, ~ 51.75 h, then increased to ~ 132 h in 600 eggs/1.5 mL, which further increased to ~245.25 h in 1200 eggs/ 3 mL and ~433.46 h in 1200 eggs/ 6 mL cultures, when averaged across all populations, and all these pairwise differences were statistically significant (Tukey's HSD at $\alpha = 0.05$ level of significance).

Dry weight at eclosion

The only significant ANOVA effects for dry weight at eclosion were those of sex, assay environment and the assay environment by sex and assay environment by selection interactions (Table 4). Clearly, eclosing flies were heaviest in the uncrowded assay environment (significantly heavier than all 3 crowded environments), followed by the 1200 eggs/6 mL food environment (significantly different from uncrowded and the other two crowded environments), with the lightest flies being seen in the two equal density environments of 1200 eggs/3 mL and 600 eggs/1.5 mL food (Fig. 8(a,b)), with no significant difference in dry weight between them. As expected, on an average, females were significantly heavier than males at eclosion, but these differences were significant only in the two assay environments with 6 mL of food and lower overall density (Fig. 9(a)). Similarly, the differences in dry weight at eclosion, averaged across sexes, were greater among selection regimes in the two assay environments with 6 mL of food and lower overall density (Fig. 9(b)).

I also examined the temporal distribution and variance of the mean dry weight of freshly eclosing adults based on the day of eclosion. Males and females eclosing on

any given day, were pooled from all the vials in each selection × block × assay environment combination. Five adults were then randomly chosen to make one replicate. Upto 3 such replicates were weighed for any given day, and, if very few adults eclosed in a particular day, then they were pooled across subsequent days to make one replicate. There was no significant main effect of assay environment on the variance in dry weight at eclosion (Table 5(a), Fig. 10), neither was there any significant interaction effect between selection, assay environment or sex (Table 5(a)). In other words, regardless of how fast or slowly flies developed, the variance of their dry weights at eclosion was not much affected (Fig. 10). There was, however, a strong tendency of early eclosing adults (from the first and second days of eclosion) in the three crowded assay environments to be slightly heavier than their later eclosing counterparts (Fig. 10). ANOVA revealed a significant main effect of time window (Table 5(b)) and significant interaction effect of time window and assay environment (Table 5(b), Fig. 12), with adults eclosing in the early time window being significantly heavier than their late counterparts, although this could be misleading since the assay environments 70 eggs/6 mL and 600 eggs/1.5 mL had no adults emerging in the defined late window. Also, 1200 eggs/3 mL had very few adults emerging in the late window in some blocks, in some selection regimes. In other words, adults from 1200 eggs/6 mL assay environment were the only kind to fall in both the early and late windows in large numbers, wherein the early and late adults had little difference in their dry weight at eclosion (Fig. 10, Fig. 12). There was also a significant main effect of assay environment ($p < 0.001$, Table 5(b)) where averaged across both time windows and all populations, 70 eggs/6 mL cultures had significantly heavier adults than those from 600 eggs/1.5 mL and 1200 eggs/3 mL cultures, but did not significantly differ from those from 1200 eggs/6 mL cultures.

Gen. 153 (MCU) Gen. 32 (CCU, LCU)

Pre-adult survivorship

The broad pattern of results was very similar to that seen in the pre-adult survivorship assay done at generation 11 of CCU and LCU selection. Overall, survivorship was significantly reduced in all three crowded assay environments, relative to the uncrowded assay environment, although mean survivorship did not differ significantly between the crowded environments (Fig. 1(b)). The ANOVA revealed significant main effects of assay environment and selection, and a significant interaction between the two factors (Table 6). Overall, averaged across all assay environments, MCU pre-adult survival was ~11.36 % greater than control MB populations, and this difference was statistically significant; the MCU, CCU and LCU populations did not differ amongst themselves in mean survivorship, unlike in the generation 11 assay. Unlike in the generation 11 assay, even the CCU and LCU populations showed significantly greater survivorship than the MBs after a further 21 generations of selection, by ~5.76 % and ~4.91 %, respectively.

Interestingly, there was also a significant selection and assay environment interaction effect (Fig. 1(b), Table 6). MCU populations had significantly greater pre-adult survivorship than MBs at all the crowding environments but not at the 70 eggs/6 mL assay environment (MCU > MB by ~17.67 % at 600 eggs/1.5 mL). CCU populations had significantly greater pre-adult survivorship than control MBs only at 1200 eggs/3 mL assay environments (CCU> MB by ~13.76 %). LCU populations had significantly greater survivorship than MBs only at 1200 eggs/6 mL assay environments (LCU> MB by ~13.44 %) whereas they had ~7.86 % lower survivorship (not significant) than MBs at 70 eggs/6 mL (Fig. 1(b)). More details on

differences in pre-adult survivorship between populations at all assay environments are given in Table 11.

Pre-adult development time

Barring some minor difference, the gross pattern of results for pre-adult development time after 32 generations of CCU and LCU selection (Fig. 2(c,d)) was substantially similar to that seen in the assay after 11 generations of CCU and LCU selection (Fig. 2(a,b)). In this assay, too, mean pre-adult development time increased with food amount in the three crowded assay environments (Figs. 2(c,d)), and eclosions began at about the same time from egg-lay in all assay environments, but went on for a longer time in crowded environments, with the range of development time increasing quite dramatically with food amount (Figs. 5,7(a,b)). The differences in development time spread in the assay environments with 600 eggs/1.5 mL food and 1200 eggs/3 mL food seen in the earlier assay were still clearly seen (Figs. 5,7(b)), reconfirming that crowded treatments with the same larval density can differ in how long food remains available for 'late' individuals to eclose, potentially setting the stage for slightly different evolutionary trajectories of adaptation to larval crowding even at the exact same larval density.

Coming now to the details, unlike in the previous assay, the ANOVA revealed significant effects on mean development time only of selection and assay environment (Table 7). Averaged across sex and assay environment, both MCU and CCU adults developed significantly faster than MBs by ~16.79 h and ~14.06 h, respectively. LCU populations also showed ~13.05 h faster development than MBs, but the difference was not statistically significant (Fig. 2(c, d)). The main effect of assay environment

was clearly reflected in a monotonic increase of development time from the uncrowded assay environment to the crowded assay environments with greater food amounts (Figs. 2(c,d),5), but in this assay the differences in mean development time between the two equal density crowded environments (600 eggs/1.5 mL and 1200 eggs/3 mL food) were no longer significant, unlike in the assay after 11 generations of CCU and LCU selection. Somewhat surprisingly, there was also no significant difference, an average, between pre-adult development time in males and females. The results on variance and spread of mean pre-adult development time across selection regimes and assay environments were also substantially similar across this assay and the previous one (compare Figs. 7(a,b) and 6(a,b); Figs. 5 and 4; Tables 8(a,b) and 3(a,b).

Dry weight at eclosion

As was true in the case of pre-adult development time, the gross pattern of results for dry weight at eclosion after 32 generations of CCU and LCU selection (Figs. 8(c,d),11) was also substantially similar to that seen in the assay after 11 generations of CCU and LCU selection (Figs. 8(a,b),10), although in terms of absolute values, the dry weights of freshly eclosed flies in the two 6 mL food treatments tended to be higher in the generation 32 assay. In addition to the four ANOVA effects on dry weight at eclosion that were significant at generation 11 of CCU and LCU selection (Table 4), the main effect of selection regime was also significant in the assay after 32 generations of CCU and LCU selection (Table 9). In this assay, averaged across sex and assay environment, MCU and CCU adults were significantly lighter than control MBs whereas CCU and LCU adults were heavier than that of the MCUs (Fig. 8(c,d)). Overall, eclosing flies were still the heaviest in

the uncrowded assay environment (significantly heavier than all 3 crowded environments), followed by the 1200 eggs/6 mL food environment (significantly different from uncrowded and the other two crowded environments), with the lightest flies being seen in the two equal density environments of 1200 eggs/3 mL and 600 eggs/1.5 mL food (Fig. 8(c,d)).

The only significant ANOVA effects for dry weight at eclosion were those of sex, assay environment and the assay environment by sex and assay environment by selection interactions (Table 4). Clearly, eclosing flies were heaviest in the uncrowded assay environment (significantly heavier than all 3 crowded environments), followed by the 1200 eggs/6 mL food environment (significantly different from uncrowded and the other two crowded environments), with the lightest flies being seen in the two equal density environments of 1200 eggs/3 mL and 600 eggs/1.5 mL food (Fig. 8(a,b)), with no significant difference in dry weight between them. As expected, and as seen at generation 11 of CCU and LCU selection, females, on an average, were significantly heavier than males at eclosion, but these differences were significant only in the two assay environments with 6 mL of food and lower overall density (Fig. 9(c)). Similarly, the differences in dry weight at eclosion, averaged across sexes, were greater among selection regimes in the two assay environments with 6 mL of food and lower overall density (Fig. 9(d)).

The temporal distribution and variance of the mean dry weight of freshly eclosing adults based on the day of eclosion was also very similar across the two assays (compare Figs. 10,11 and Figs. 12,13(b)). However, unlike in the generation 11 assay, there were significant ANOVA effects of sex and the assay environment by

selection interaction on the variance in dry weight at eclosion (Table 10(a), Figs. 11,13(a)), but it is hard to ascribe clear biological significance to these findings. By and large, regardless of how fast or slowly flies developed, the variance of their dry weights at eclosion was not much affected (Fig. 11). There was, however, a strong tendency of early eclosing adults (from the first and second days of eclosion) in the three crowded assay environments to be slightly heavier than their later eclosing counterparts (Fig. 11). ANOVA revealed significant main effects of time window and assay environment (Table 10(b)) and significant interaction effect of time window and assay environment (Table 10(b), Fig. 13(b)), with adults eclosing in the early time window being significantly heavier than their late counterparts. Similar to the generation 11 assay, when, averaged across both time windows and all populations, 70 eggs/6 mL and 1200 eggs/6 mL cultures had significantly heavier adults than those from 600 eggs/1.5 mL and 1200 eggs/3 mL cultures, but did not significantly differ amongst themselves.

Discussion

The results reported in this chapter can be examined along two different axes to draw inferences that may help understand how differences in egg number and food amount while experiencing crowding might facilitate the evolution of enhanced competitive ability in different ways. First, one can look at differences among the crowding adapted MCU, CCU and LCU populations in the uncrowded assay environment to get a feel for how pre-adult survivorship, development time and dry weight at eclosion differ among these three sets of populations. The three traits examined are, respectively, the main component of pre-adult fitness (survivorship), a trait that may alter the way in which the deteriorating environment of a crowded

culture is experienced (development time) and a bridge between larval food acquisition and adult reproductive fitness, especially in females (dry weight at eclosion). These comparisons are, of course, rendered a bit tricky by the discrepancy between the numbers of generations of selection undergone by the MCUs compared to the CCUs and LCUs. Second, one can look at the one-generation effects of the three crowded assay environments (MCU-, CCU- and LCU-type), especially on the MBs, and gain some insight into how crowding experienced at different egg number and food amount combinations can differentially affect fitness functions.

If we recollect the backdrop to this work, the CU populations did not differ from their controls (UU) in pre-adult development time and survivorship when assayed at low density but developed faster and survived better than the UUs at high density (Mueller *et al* 1993, Shiotsugu *et al* 1997, Borash & Ho 2001). In contrast, the MCU populations reached their critical minimum size faster than the MB controls, while maintaining a higher body size till the pre–critical–size phase (Sarangi 2013), and showed faster development than controls even at low density (Sarangi *et al* 2016). The MCU also had higher pre-adult survivorship than the MBs at high but not at low density (Sarangi *et al* 2016). In the present study, the MCUs and LCUs, and even the CCUs, evolve to be significantly better survivors that MB, at least in the type of crowded culture most closely approximating their maintenance regime, while they are not better survivors than MBs at low density (Fig. 1(b)). There is, however, a slight tendency for the LCUs to show slightly poorer survivorship than MBs at low density, both in the assays discussed in this chapter and in Chapter 2. In the case of development and dry weight time assayed at low density, the MCUs develop much faster and are considerably lighter than MBs but, by 32 generations of selection, LCU

and CCU are also developing somewhat faster than MBs, although LCUs, especially males, are quite heavy (Figs. 10,11). As such, it is difficult to say clearly at this early point in LCU and CCU selection whether they are going to evolve a slightly different set of traits than the MCUs in response to crowding and, especially, whether the LCUs are going to evolve a set of traits more similar to the CU populations used in earlier studies. These are questions that need to be followed up as CCU and LCU selection progresses.

Focusing next on the three different crowded assay environments, the results from the two assay environments with equal larval density (600 eggs/1.5 mL and 1200 eggs/3 mL) clearly suggest that the two are not the same in their effects on fitness-related traits. The 1200 eggs/3 mL food environment yielded significantly lower pre-adult survivorship than 600 eggs/1.5 mL (Fig. 1(a, b)), and also resulted in increased mean pre-adult development time with eclosions occurring over a longer time duration (Fig. 2(a, b, c, d), Fig. 4, Fig. 5, Fig. 6(b), Fig. 7(b)). However, despite the difference in spread, there was no statistical difference in the variance of pre-adult development time between these two types of assay environments (Fig. 4, Fig. 5, Fig. 6(a), Fig. 7(a)). The increased mean development time in the 1200 eggs/3 mL cultures was the result of few adults eclosing very late (Fig. 4(e, f), Fig. 5(e, f)). Specifically, in addition to larvae developing significantly faster than control MBs over generations, the total duration of eclosion was also observed to shorten in the CCU populations in the 1200 eggs/3 mL assay environment. This is likely to be a direct response to the selection for adaptation to this assay environment. Moreover, adults from 1200 eggs/3 mL cultures showed a trend of being heavier than those eclosing from 600 eggs/1.5 mL cultures, but there was no statistical difference in the means of

their dry weights at eclosion (Fig. 8(a, b, c, d). Particularly, the adults eclosing in the early window were observed to be slightly heavier in the 1200 eggs/3 mL as compared to those from 600 eggs/1.5 mL, however the difference was again not statistically significant (Fig. 10, Fig. 11). The mean dry weights of the MCU and CCU populations were however lower than control MBs across all assay environments. Additionally, the adults from CCU populations were also significantly heavier than those from the MCUs.

Both these responses were further enhanced in the 1200 eggs/6 mL cultures, where the larval density was lower and the food level higher than the other two crowded assay environments. Overall, mean pre-adult survivorship was higher in these cultures compared to 600 eggs/1.5 mL and 1200 eggs/3 mL (Fig. 1(a, b)). The means and the variances in pre-adult development time and the means of dry weight at eclosion were also significantly greater than the other two assay environments, with the addition of the fact that the total duration of eclosion was also found to be much more prolonged (Fig. 2(a, b, c, d), Fig. 4, Fig. 5, Fig. 6(a, b), Fig. 7(a, b)). There was a strong trend of heavier adults eclosing in the early and late windows of 1200 eggs/6 mL cultures as compared to the rest of the distribution but the difference in variance was not statistically significant relative to other assay environments. But, on an average females were found to be heavier than their male counterparts in 1200 eggs/6 mL as well as in 70 eggs/6 mL assay environments, suggesting that indeed its not just the larval density which determines the extent of crowding but also the absolute amount of food, that facilitates if larvae can attain greater body weights or not.

The LCU populations are of specific interest because their maintenance regime approximates that of the CU populations used in earlier studies. Like in the CCUs in 1200 eggs/3 mL cultures, the total time duration over which eclosion occurs appear to shorten over generations in the LCU populations relative to control MBs in the 1200 eggs/6 mL assay environment. In fact, the fixed time window analysis showed that only the 1200 eggs/6 mL assay environment had a major late component to its distribution unlike 600 eggs/1.5 mL cultures where there were absolutely no eclosing adults in that window or the 1200 eggs/3 mL cultures with very few eclosing adults.

These observations are consistent with a speculation that there is likely higher effective competition for food in the 600 eggs/1.5 mL and 1200 eggs/3 mL cultures than in the 1200 eggs/6 mL cultures, where the larvae develop faster before the food runs out or becomes toxic. The nitrogenous build-up in the feeding band of these low food cultures is probably quicker than in the 1200 eggs/6 mL cultures. Unlike the 1200 eggs/6 mL, these cultures have lower absolute food levels where the diffused waste from the top-feeding layer probably gets accumulated faster in their entire food column (this issue will be discussed further in the next chapter). On the other hand, in the 1200 eggs/6 mL assay environments, since the waste probably accumulates in the feeding band at a relatively slower rate, it might permit larvae to survive and feed for a longer duration, thereby prolonging the pre-adult development time and eventually facilitating the larvae to emerge as heavier adults. Such late eclosing adults were however not observed in the 600 eggs/1.5 mL cultures, where the food runs out by the time the feeding larvae attain the minimum critical size for pupation. In case of 1200 eggs/3 mL cultures, however we did observe a small number of adults eclosing in the

late window, because although the food doesn't run out, but indeed could be accumulating a lot more nitrogenous waste in the food column rendering the environment inhospitable. This may also explain the overall reduction in pre-adult survivorship in 1200 eggs/3 mL cultures as compared to the other two crowding environments. This study, thus, underscores the importance of the interaction between egg number and the absolute amount of food, in particular, the height of the food column, in the culture vials where the larvae are subjected to crowding.

Tables

Table 1 Results of ANOVA on mean pre-adult survivorship of MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations measured across four different assay environments. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.029	7.726	0.007
Assay environment	3	1.143	123.290	<0.001
Selection × Assay environment	9	0.002	0.834	0.591

Table 2 Results of ANOVA on mean pre-adult development time of MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	1492.231	10.502	0.002
Assay environment	3	125005.700	356.118	<0.001
Sex	1	51.525	28.149	0.013
Selection × Assay environment	9	136.292	1.411	0.232
Selection × Sex	3	100.378	22.332	<0.001
Assay environment × Sex	3	67.000	36.191	<0.001
Selection × Assay environment × Sex	9	24.785	6.724	<0.001

Table 3(a) Results of ANOVA on the variance in pre-adult development time of MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	2452.094	0.333	0.801
Assay environment	3	112617.100	21.853	<0.001
Sex	1	1146.265	0.237	0.659
Selection × Assay environment	9	5543.088	1.237	0.314
Selection × Sex	3	1075.520	0.350	0.789
Assay environment × Sex	3	50.071	0.012	0.997
Selection × Assay environment × Sex	9	1678.311	0.591	0.79

Table 3(b) Results of ANOVA on total duration of eclosion of adults from MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	26702.132	7.722	0.007
Assay environment	3	894757.625	133.056	<0.001
Sex	1	1018.132	1.953	0.256
Selection × Assay environment	9	8498.965	2.519	0.030
Selection × Sex	3	582.132	0.831	0.509
Assay environment × Sex	3	16.632	0.015	0.997
Selection × Assay environment × Sex	9	804.632	1.201	0.334

Table 4 Results of ANOVA on mean dry weight of males and females at eclosion of MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	18.239	3.269	0.073
Assay environment	3	4195.35	621.659	<0.001
Sex	1	378.294	80.082	0.002
Selection × Assay environment	9	7.918	2.342	0.042
Selection × Sex	3	4.255	3.094	0.082
Assay environment × Sex	3	118.773	31.844	<0.001
Selection × Assay environment × Sex	9	1.291	1.164	0.355

Table 5(a) Results of ANOVA on the variance in dry weight of males and females at eclosion of MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	157.553	1.267	0.342
Assay environment	3	190.187	3.269	0.073
Sex	1	35.260	0.216	0.673
Selection × Assay environment	9	33.821	0.555	0.820
Selection × Sex	3	102.146	0.678	0.586
Assay environment × Sex	3	109.178	1.847	0.208
Selection × Assay environment × Sex	9	74.974	1.787	0.117

Table 5(b) Results of ANOVA on dry weight of adults (averaged across males and females) eclosing in fixed time windows from MB, MCU (Gen. 134), CCU (Gen. 11) and LCU (Gen. 11) populations across four assay environments, with selection regime, assay environment and time window as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	3.034	0.248	0.860
Assay environment	3	1239.376	87.806	<0.001
Time window	1	6213.711	430.732	<0.001
Selection × Assay environment	9	3.776	0.443	0.898
Selection × Time window	3	12.210	0.985	0.442
Assay environment × Time window	3	1206.662	112.038	<0.001
Selection × Assay environment × Time window	9	3.969	0.268	0.978

Table 6 Results of ANOVA on mean pre-adult survivorship of MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations measured across four different assay environments. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	0.034	20.628	<0.001
Assay environment	3	0.880	109.067	<0.001
Selection × Assay environment	9	0.013	5.756	<0.001

Table 7 Results of ANOVA on mean pre-adult development time of MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	1550.211	4.808	0.028
Assay environment	3	159069.2	277.202	<0.001
Sex	1	0.961	0.037	0.859
Selection × Assay environment	9	200.826	1.244	0.310
Selection × Sex	3	41.999	1.609	0.254
Assay environment × Sex	3	34.208	2.079	0.173
Selection × Assay environment × Sex	9	8.773	0.437	0.902

Table 8(a) Results of ANOVA on the variance in pre-adult development time of MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	34055.5	1.298	0.333
Assay environment	3	462372.7	26.921	<0.001
Sex	1	7400.894	0.327	0.607
Selection × Assay environment	9	12533.58	0.315	0.962
Selection × Sex	3	594.786	0.044	0.986
Assay environment × Sex	3	6740.727	0.409	0.749
Selection × Assay environment × Sex	9	2253.321	0.117	0.998

Table 8(b) Results of ANOVA on total duration of eclosion of adults from MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	4708.125	2.998	0.087
Assay environment	3	1191487.13	89.470	<0.001
Sex	1	1012.5	0.857	0.422
Selection × Assay environment	9	3370.125	1.320	0.272
Selection × Sex	3	556.5	0.652	0.601
Assay environment × Sex	3	148.5	0.216	0.882
Selection × Assay environment × Sex	9	444.5	0.994	0.467

Table 9 Results of ANOVA on mean dry weight of males and females at eclosion of MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	31.770	26.310	<0.001
Assay environment	3	2376.712	532.002	<0.001
Sex	1	236.639	161.235	0.001
Selection × Assay environment	9	5.044	2.709	0.021
Selection × Sex	3	0.915	1.142	0.383
Assay environment × Sex	3	84.356	67.775	<0.001
Selection × Assay environment × Sex	9	1.428	1.808	0.112

Table 10(a) Results of ANOVA on the variance in dry weight of males and females at eclosion of MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four 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 cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	68.589	1.035	0.422
Assay environment	3	385.147	3.074	0.083
Sex	1	334.033	37.406	0.008
Selection × Assay environment	9	115.230	2.487	0.032
Selection × Sex	3	84.526	1.509	0.277
Assay environment × Sex	3	122.206	1.526	0.273
Selection × Assay environment × Sex	9	89.447	1.445	0.218

Table 10(b) Results of ANOVA on dry weight of adults (averaged across males and females) eclosing in fixed time windows from MB, MCU (Gen. 153), CCU (Gen. 32) and LCU (Gen. 32) populations across four assay environments, with selection regime, assay environment and time window 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	3	14.408	3.022	0.086
Assay environment	3	648.357	134.700	<0.001
Time window	1	3945.130	1024.885	<0.001
Selection × Assay environment	9	1.740	0.493	0.865
Selection × Time window	3	18.490	3.539	0.061
Assay environment × Time window	3	647.841	85.577	<0.001
Selection × Assay environment × Time window	9	5.859	1.205	0.332

Table 11 Differences in mean pre-adult survivorship (in percentage) between all the populations and across all assay environments, detailed for assays done after Gen. 134, 153 of MCU and after Gen. 11, 32 of CCU, LCU selection.

Assay Environment	Population	Gen. 134 of MCU & Gen. 11 of CCU, LCU	Gen. 153 of MCU & Gen. 32 of CCU, LCU
70 eggs/6 mL	MB-MCU	-2.85	4.76
	MB-CCU	-2.23	1.99
	MB-LCU	-3.30	7.86
	MCU-CCU	0.62	-2.77
	MCU-LCU	6.16	3.10
	CCU-LCU	5.53	5.87
600 eggs/1.5 mL	MB-MCU	-13.32	-17.67
	MB-CCU	-6.56	-8.78
	MB-LCU	-3.42	-8.86
	MCU-CCU	6.76	8.88
	MCU-LCU	9.89	8.81
	CCU-LCU	3.13	-0.07
1200 eggs/3 mL	MB-MCU	-10.26	-19.26
	MB-CCU	-1.79	-13.76
	MB-LCU	-4.70	-5.20
	MCU-CCU	8.46	5.49
	MCU-LCU	5.55	14.06
	CCU-LCU	-2.91	8.56
1200 eggs/6 mL	MB-MCU	-10.71	-13.29
	MB-CCU	-6.81	-2.48
	MB-LCU	-6.92	-13.44
	MCU-CCU	3.89	10.81
	MCU-LCU	3.78	-0.14
	CCU-LCU	-0.10	-10.95

Figures

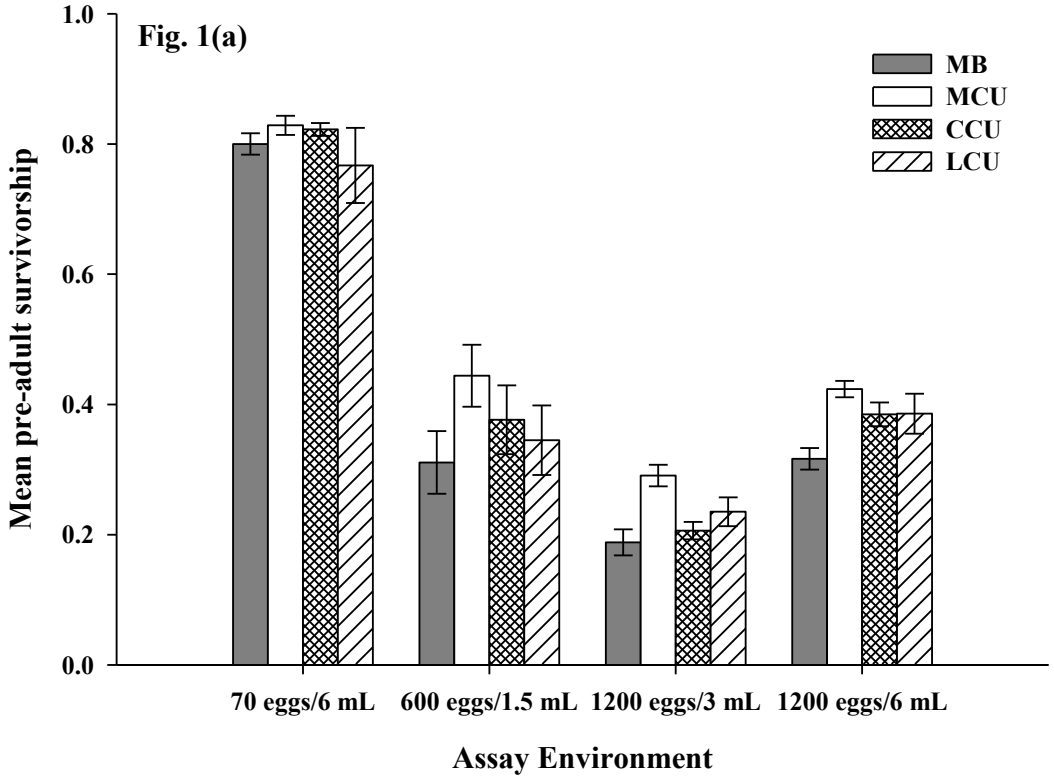
Fig. 1(a)
MB
MCU
CCU
LCU
Mean pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

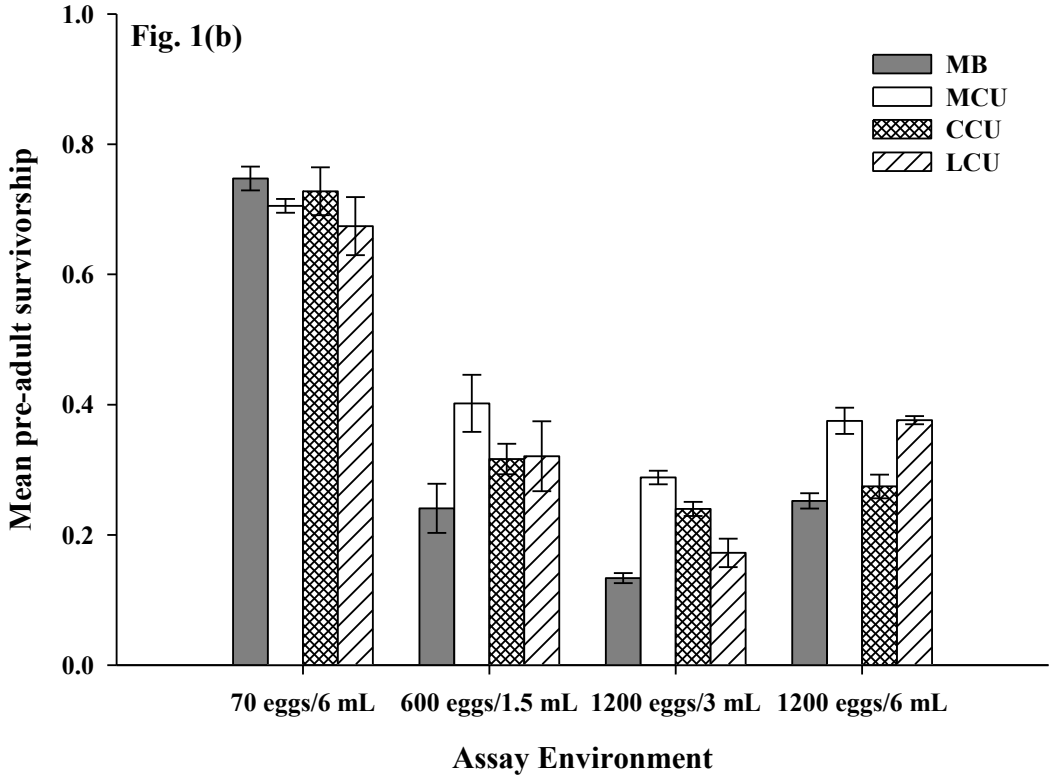
Fig. 1(b)
MB
MCU
CCU
LCU
Mean pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

Fig. 1 Mean pre-adult survivorship of the MB, MCU, CCU and LCU populations in four different assay environments after (a) Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection (b) Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

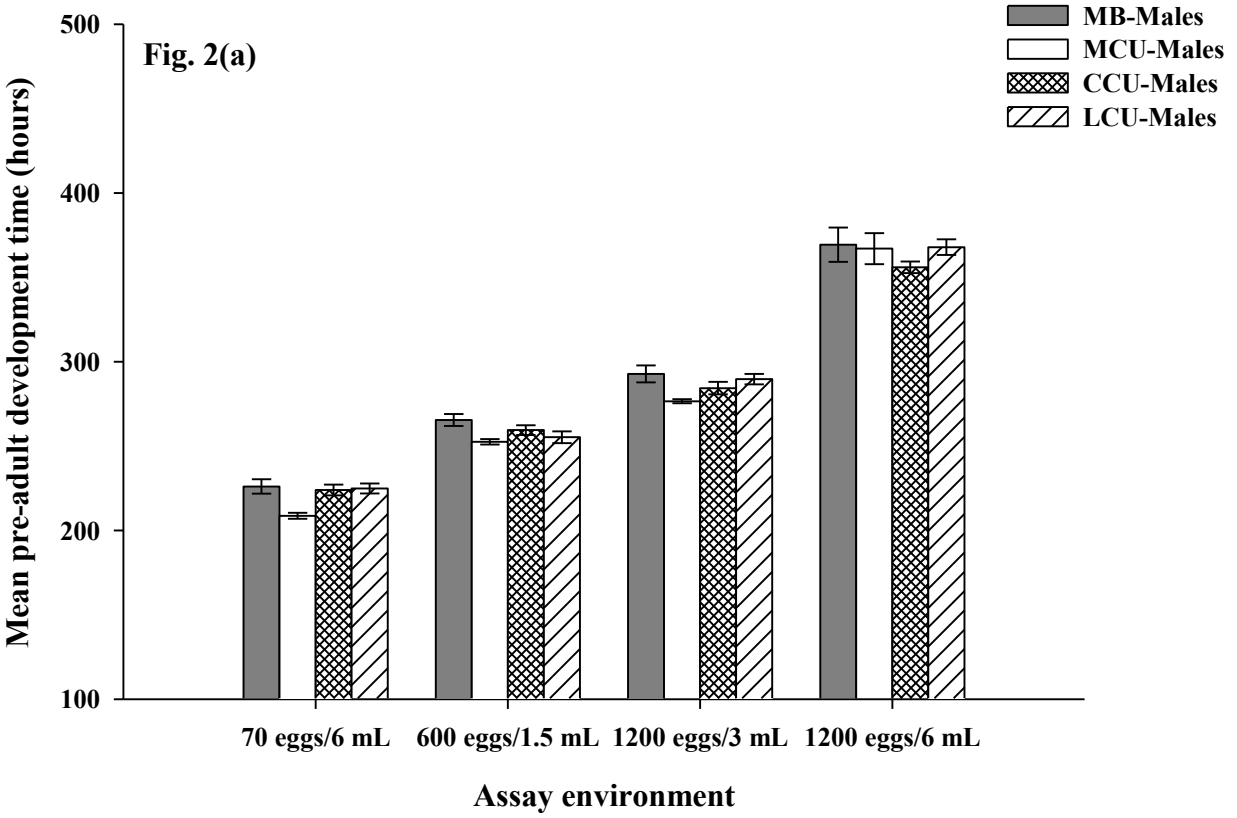
Fig. 2(a)
MB-Males
MCU-Males
CCU-Males
LCU-Males
Mean pre-adult development time (hours)
500
400
300
200
100
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

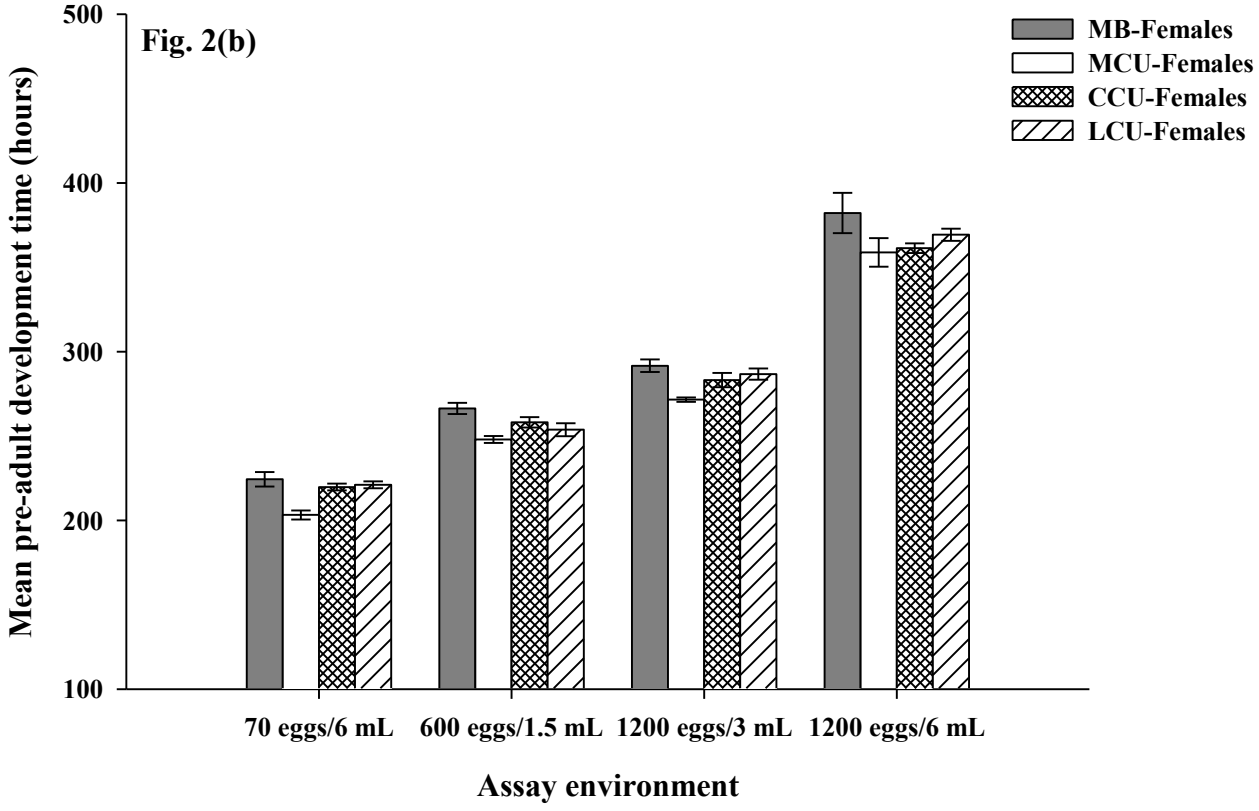
Fig. 2(b)
MB-Females
MCU-Females
CCU-Females
LCU-Females
Mean pre-adult development time (hours)
500
400
300
200
100
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

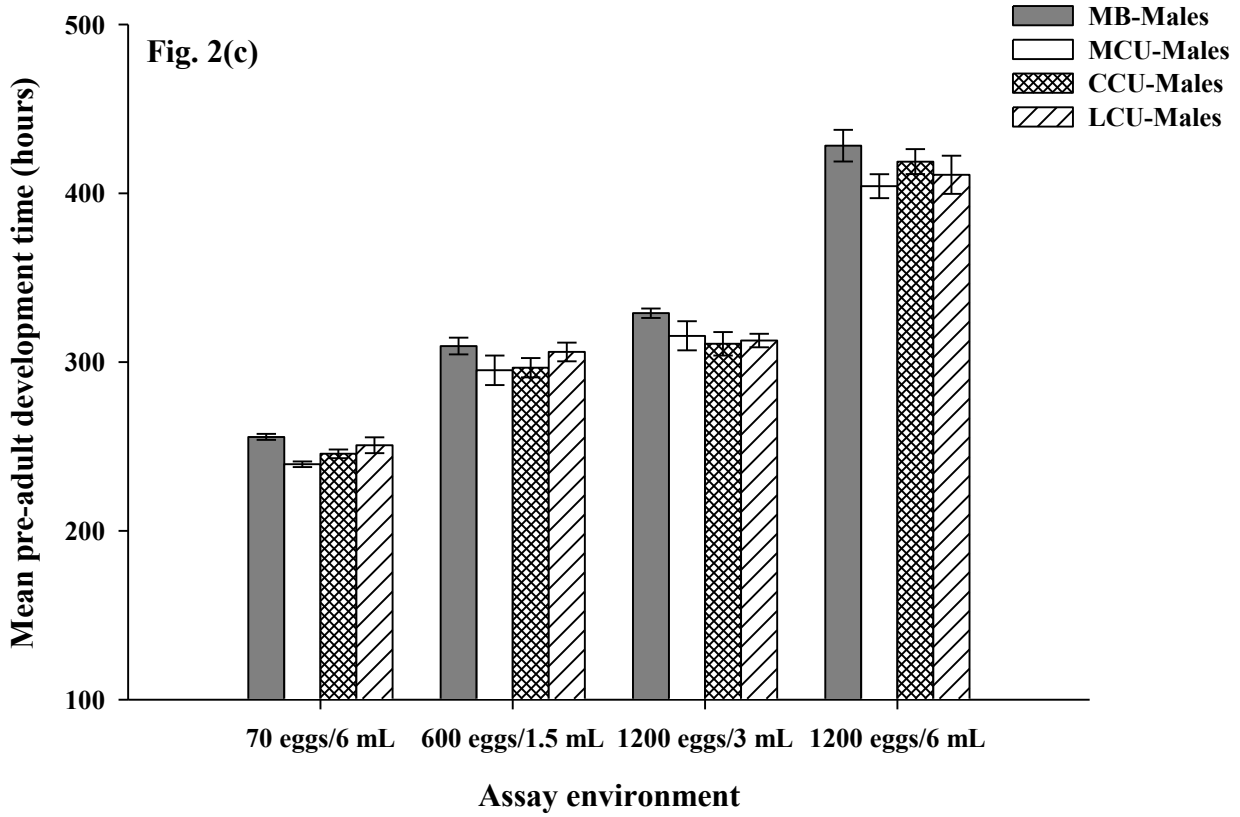
Fig. 2(c)
MB-Males
MCU-Males
CCU-Males
LCU-Males
Mean pre-adult development time (hours)
500
400
300
200
100
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

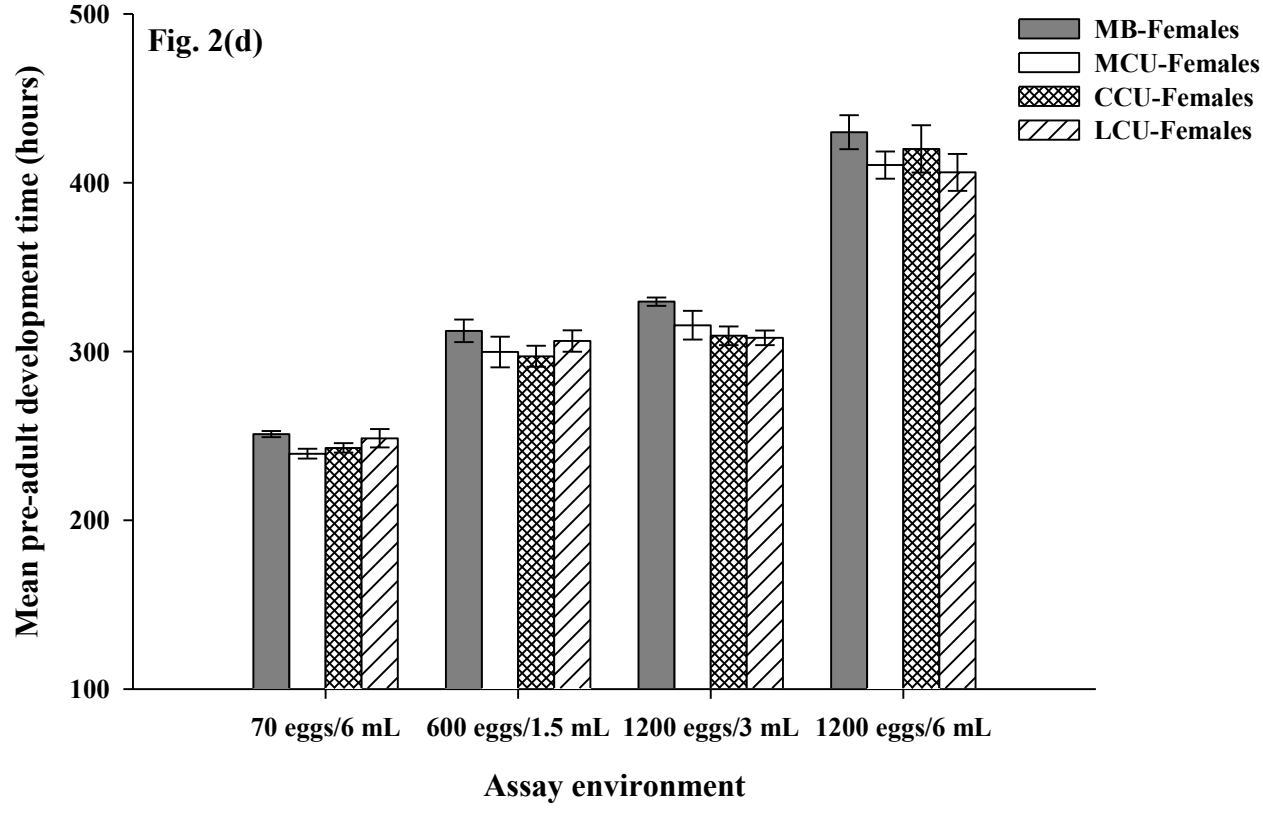
Fig. 2(d)
MB-Females
MCU-Females
CCU-Females
LCU-Females
Mean pre-adult development time (hours)
500
400
300
200
100
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 2 Mean pre-adult development time of the MB, MCU, CCU and LCU populations in four different assay environments after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection in (a) males (b) females and after Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection in (c) males (d) females. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

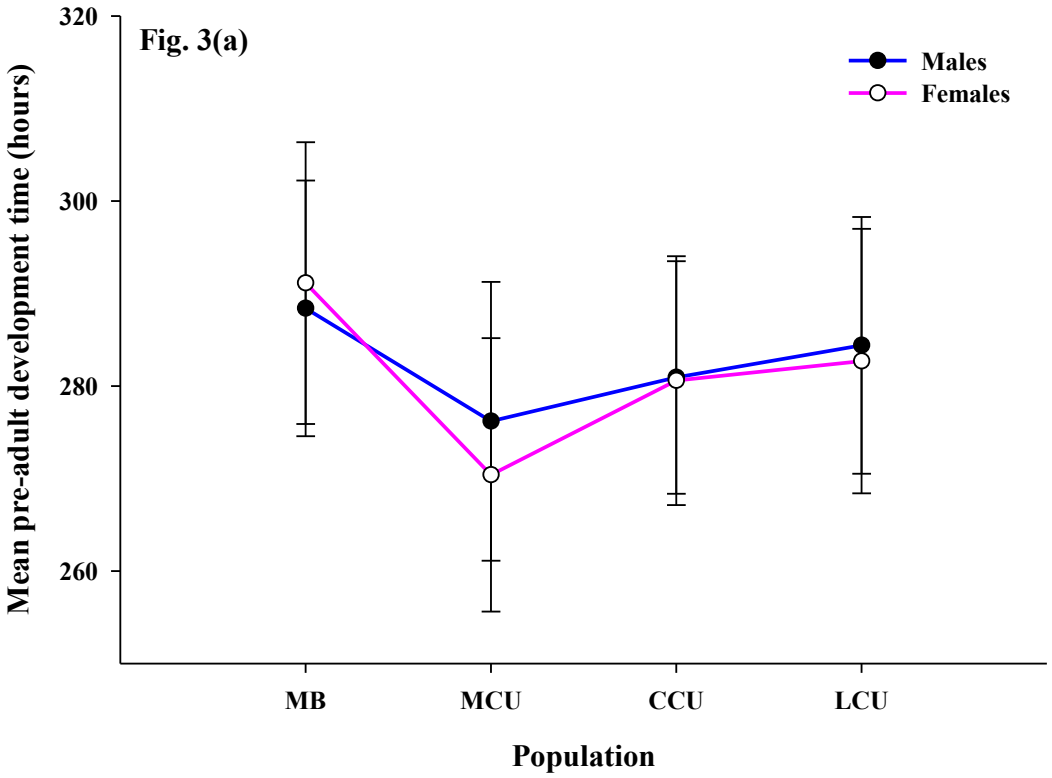
Fig. 3(a)
Males
Females
Mean pre-adult development time (hours)
320
300
280
260
MB
MCU
CCU
LCU
Population

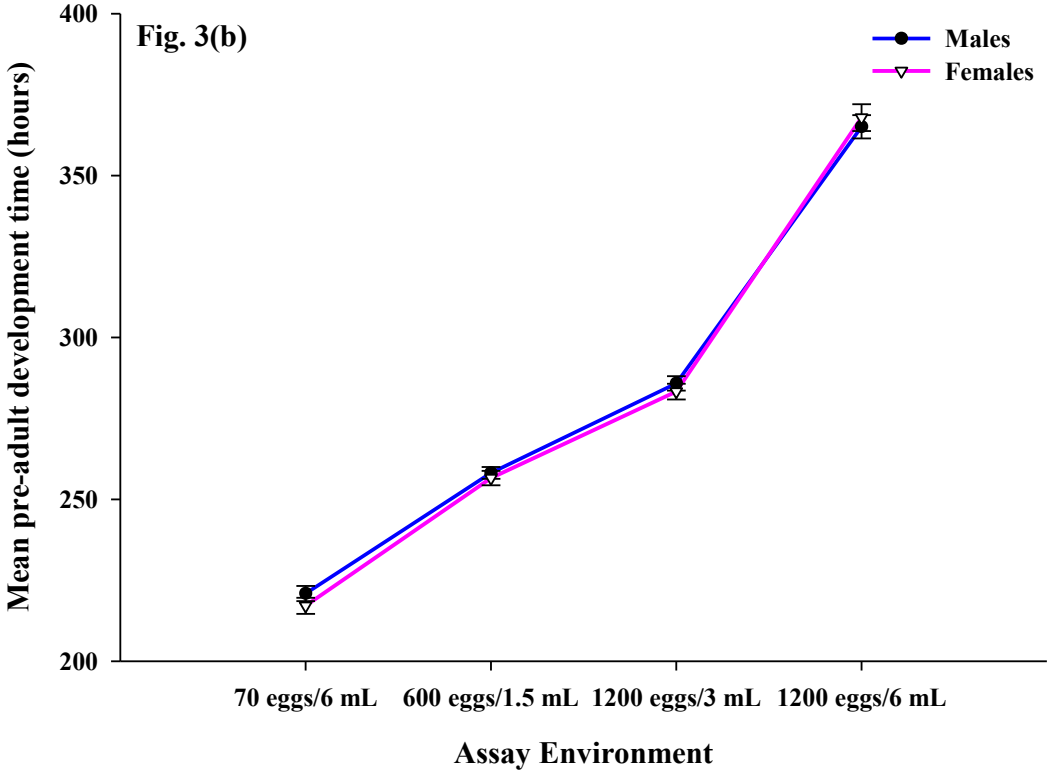
Fig. 3(b)
Males
Females
Mean pre-adult development time (hours)
400
350
300
250
200
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

Fig. 3 (a) Selection by sex interaction for the mean pre-adult development time of all four sets of populations averaged across four different assay environments, and (b) assay environment by sex interaction for the mean pre-adult development time averaged across all four sets of populations, after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

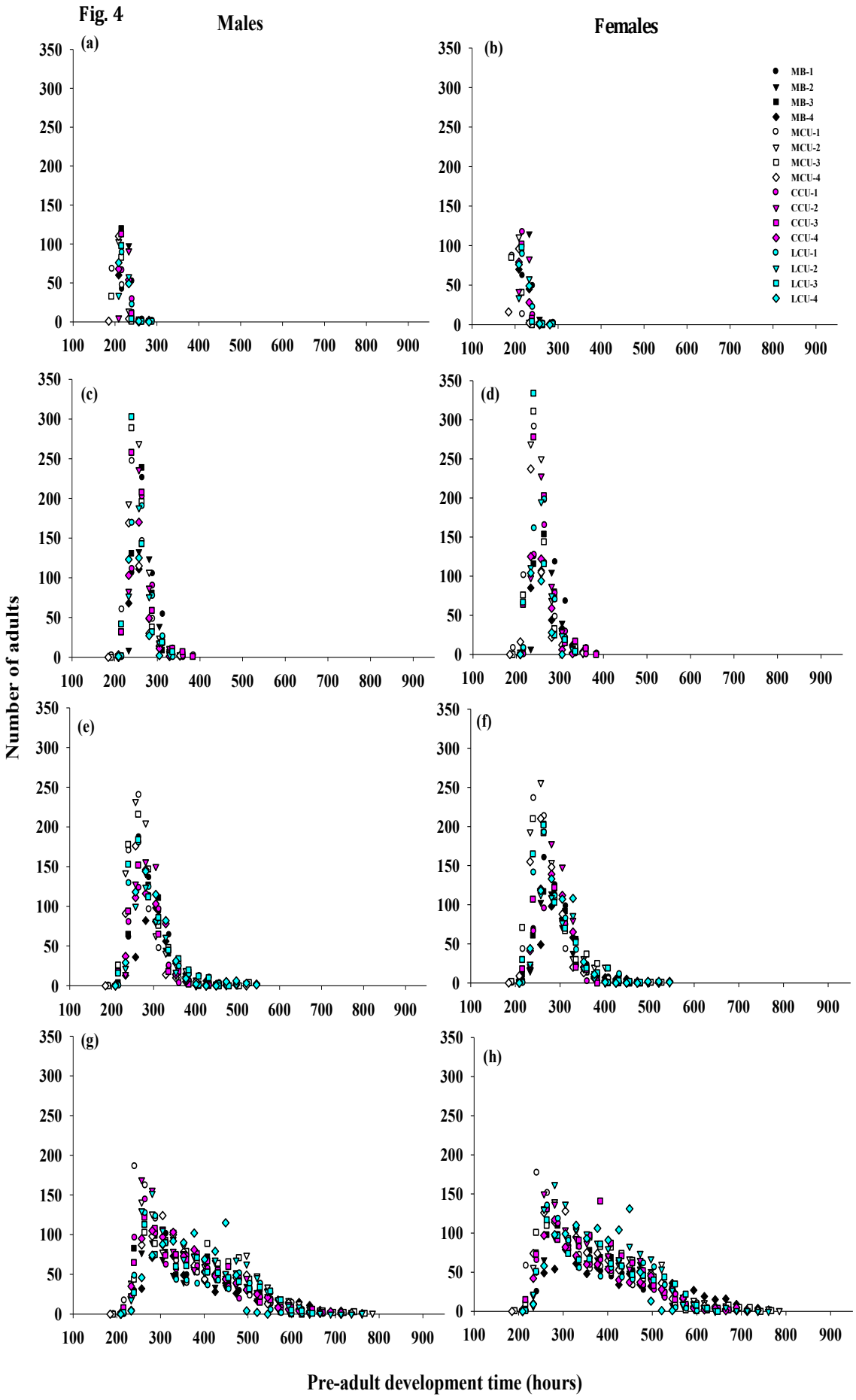

Fig. 4 Frequency distribution of pre-adult development time of males and females in MB, MCU, CCU and LCU populations in (a, b) 70 eggs/6 mL, (c, d) 600 eggs/1.5 mL, (e, f) 1200 eggs/3 mL and (g, h) 1200 eggs/6 mL assay environments. after Gen. 134 of MCU and Gen. 11 of CCU. LCU selection.

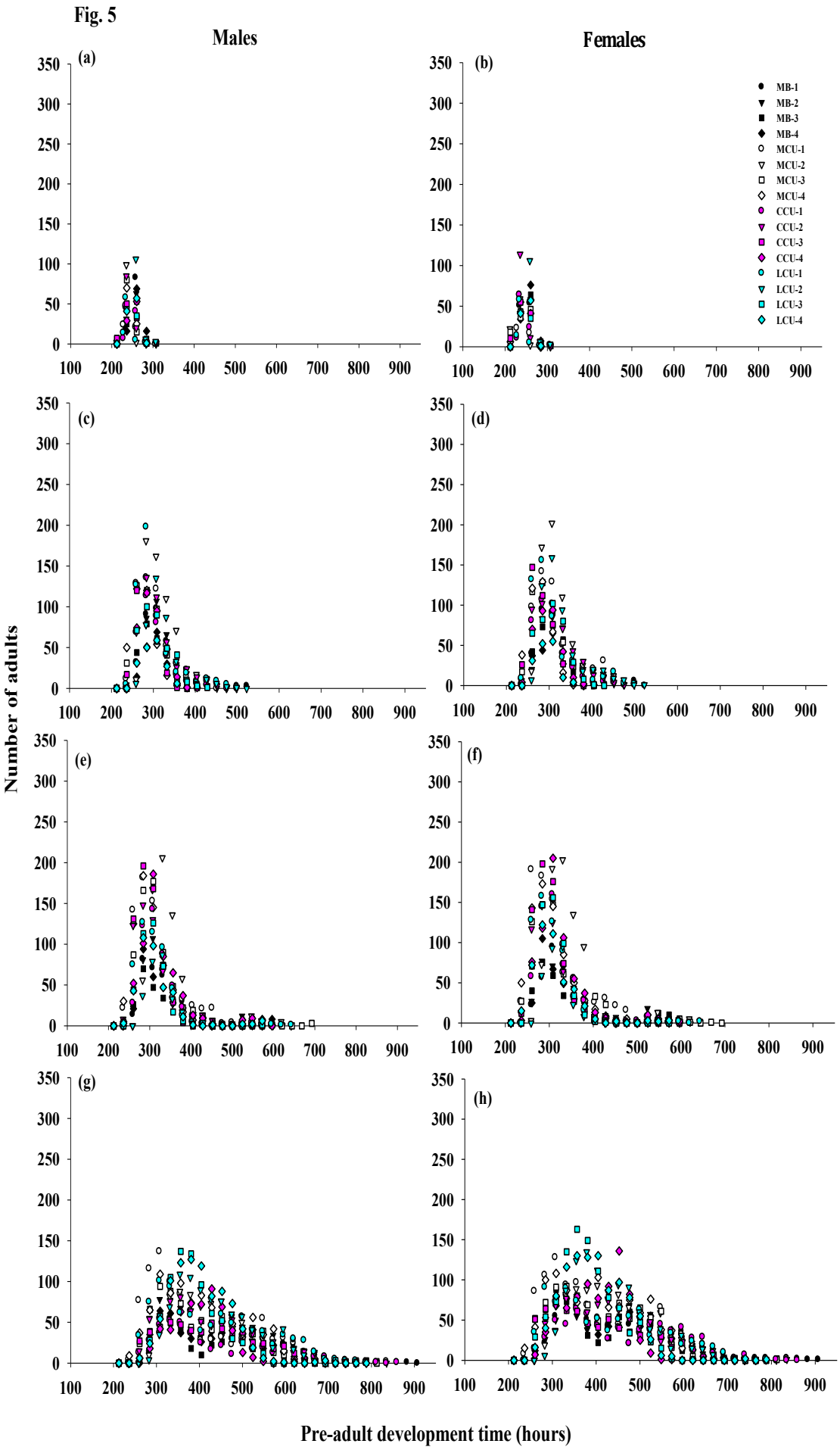

Fig. 5 Frequency distribution of pre-adult development time of males and females in MB, MCU, CCU and LCU populations in (a, b) 70 eggs/6 mL, (c, d) 600 eggs/1.5 mL, (e, f) 1200 eggs/3 mL and (g, h) 1200 eggs/6 mL assay environments. after Gen. 154 of MCU and Gen. 32 of CCU. LCU selection.

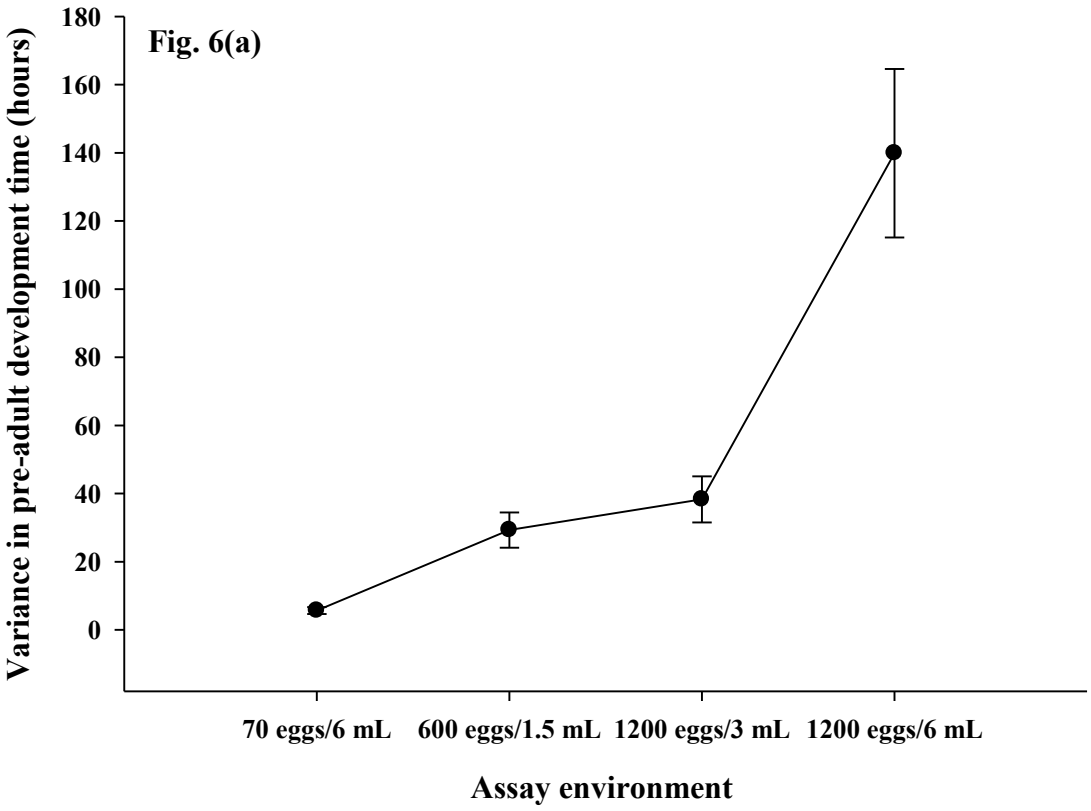
Fig. 6(a)
Variance in pre-adult development time (hours)
180
160
140
120
100
80
60
40
20
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

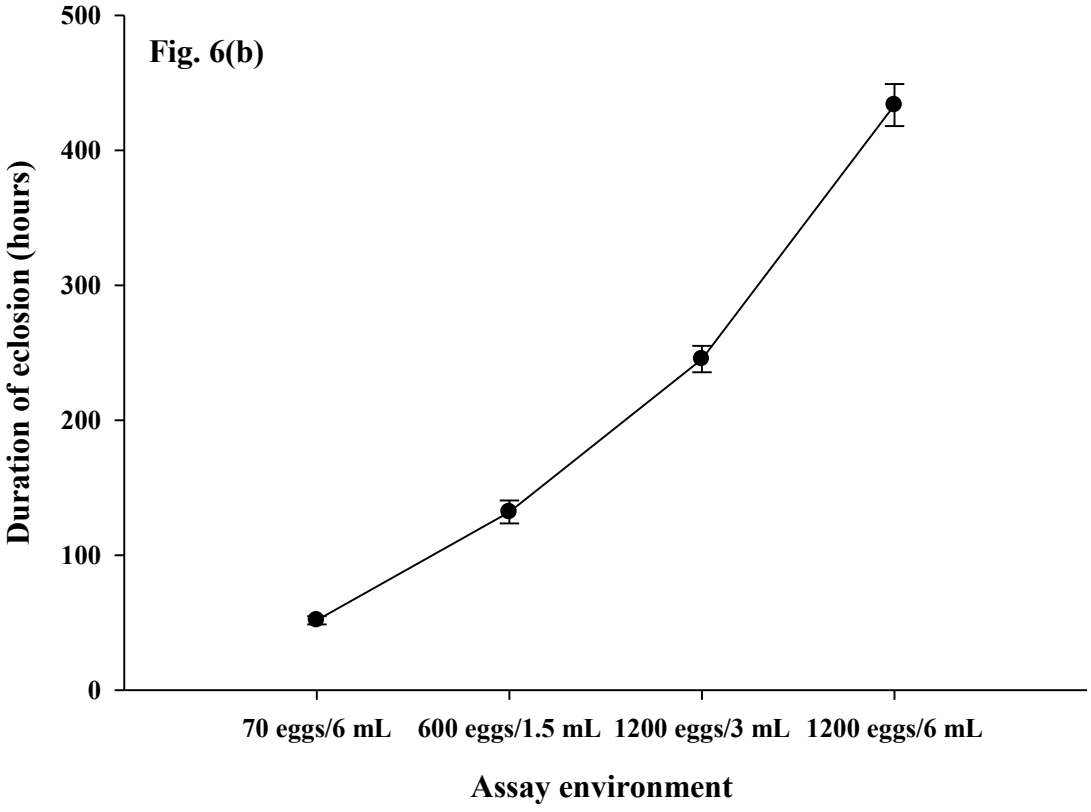
Fig. 6(b)
Duration of eclosion (hours)
500
400
300
200
100
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 6 (a) Variance in pre-adult development time, and (b) total duration over which adults eclosed, at different assay environments, when averaged across all four sets of populations after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

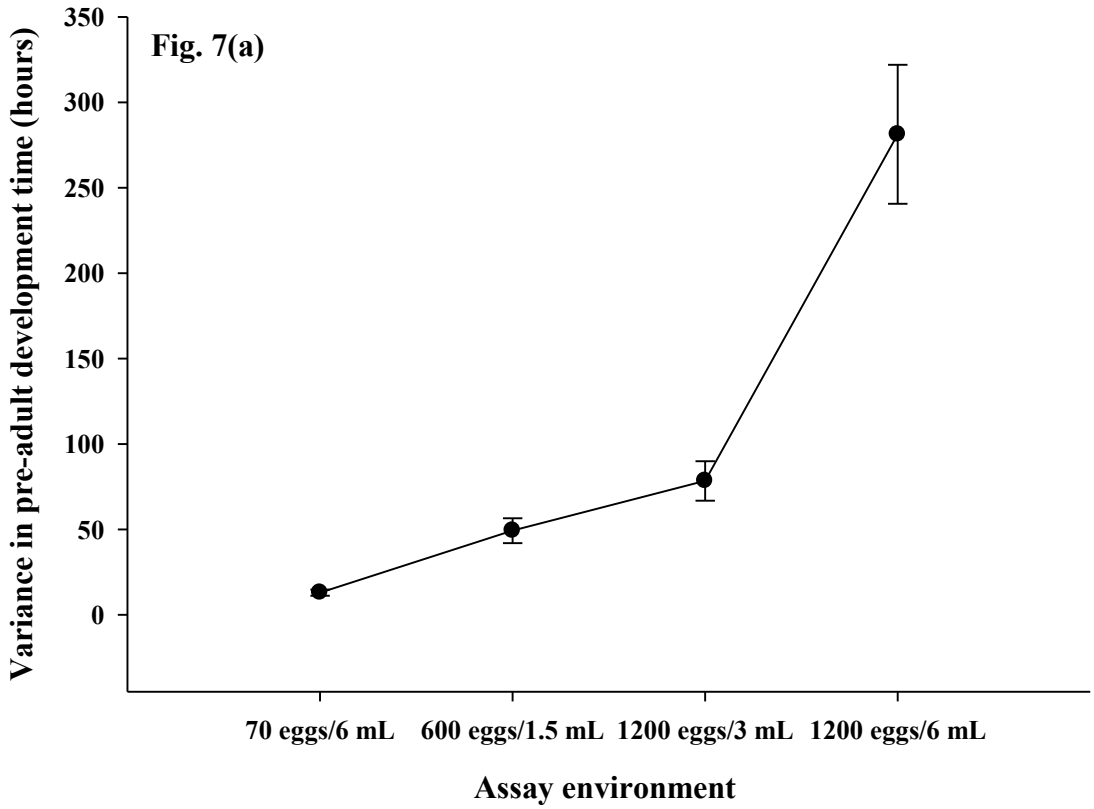
Fig. 7(a)
Variance in pre-adult development time (hours)
350
300
250
200
150
100
50
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

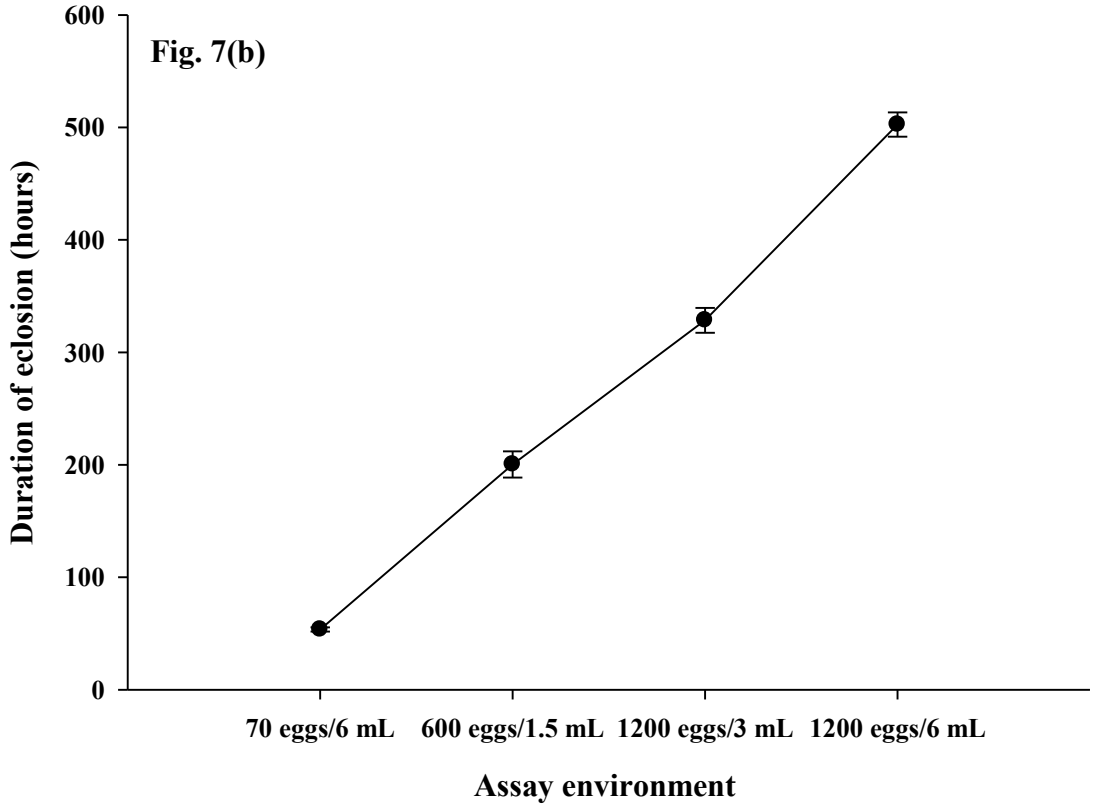
Fig. 7(b)
Duration of eclosion (hours)
600
500
400
300
200
100
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 7 (a) Variance in pre-adult development time, and (b) total duration over which adults emerged, at different assay environments when averaged across all four sets of populations after Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

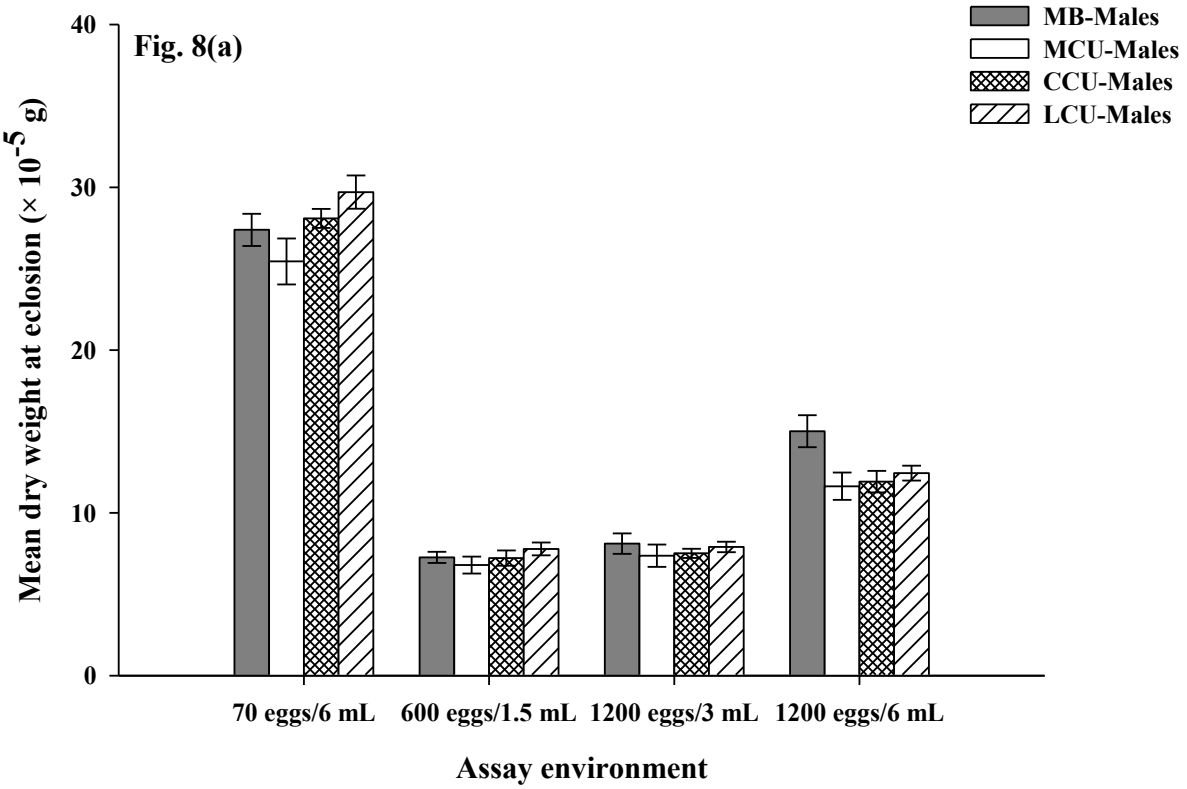
Fig. 8(a)
MB-Males
MCU-Males
CCU-Males
LCU-Males
Mean dry weight at eclosion (× 10-5 g)
40
30
20
10
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

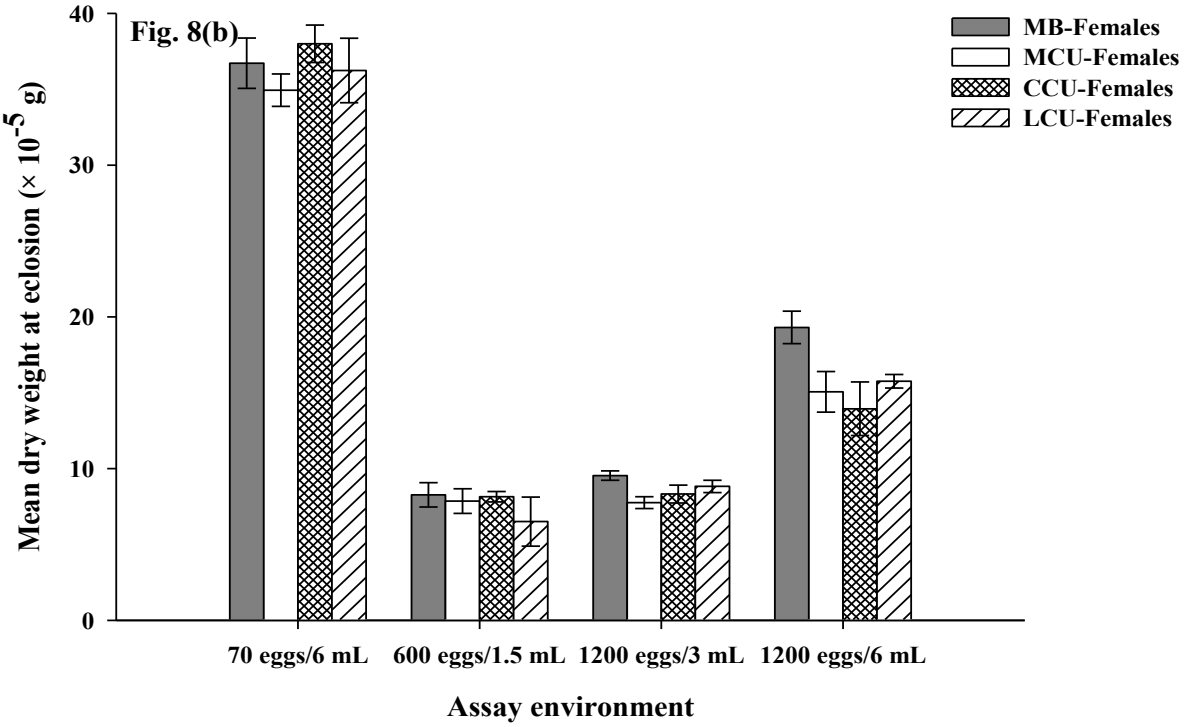
Fig. 8(b)
MB-Females
MCU-Females
CCU-Females
LCU-Females
Mean dry weight at eclosion (× 10-5 g)
40
30
20
10
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

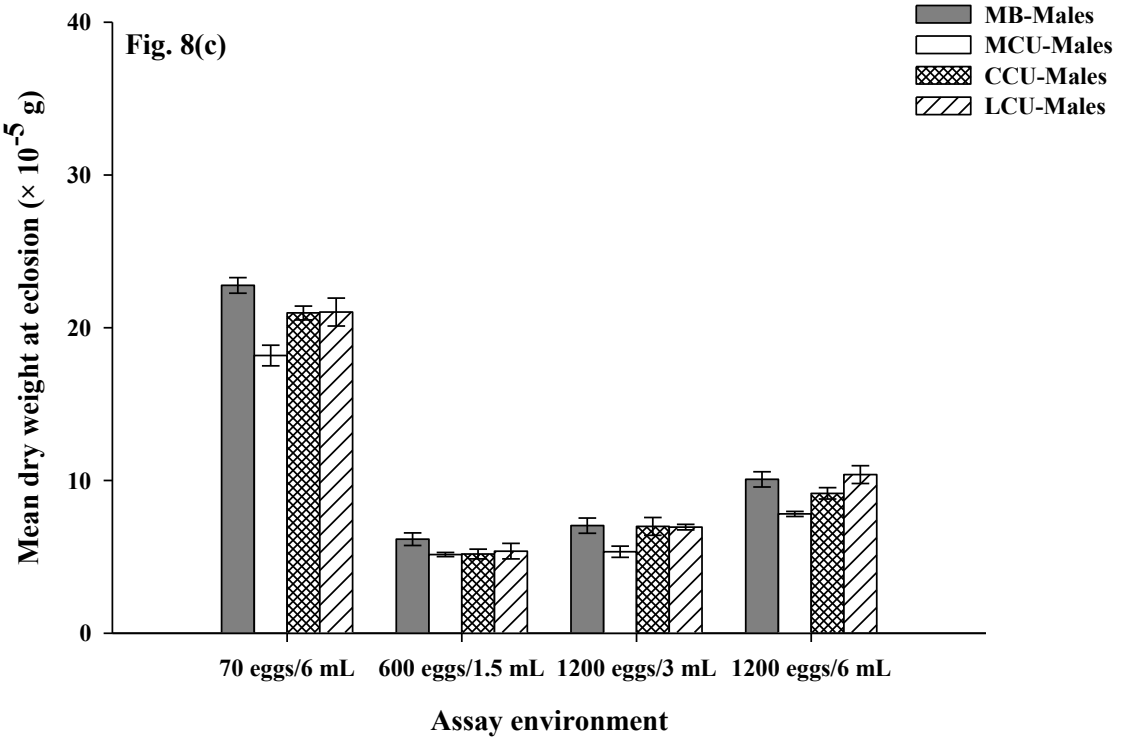
Fig. 8(c)
MB-Males
MCU-Males
CCU-Males
LCU-Males
Mean dry weight at eclosion (× 10-5 g)
0
10
20
30
40
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

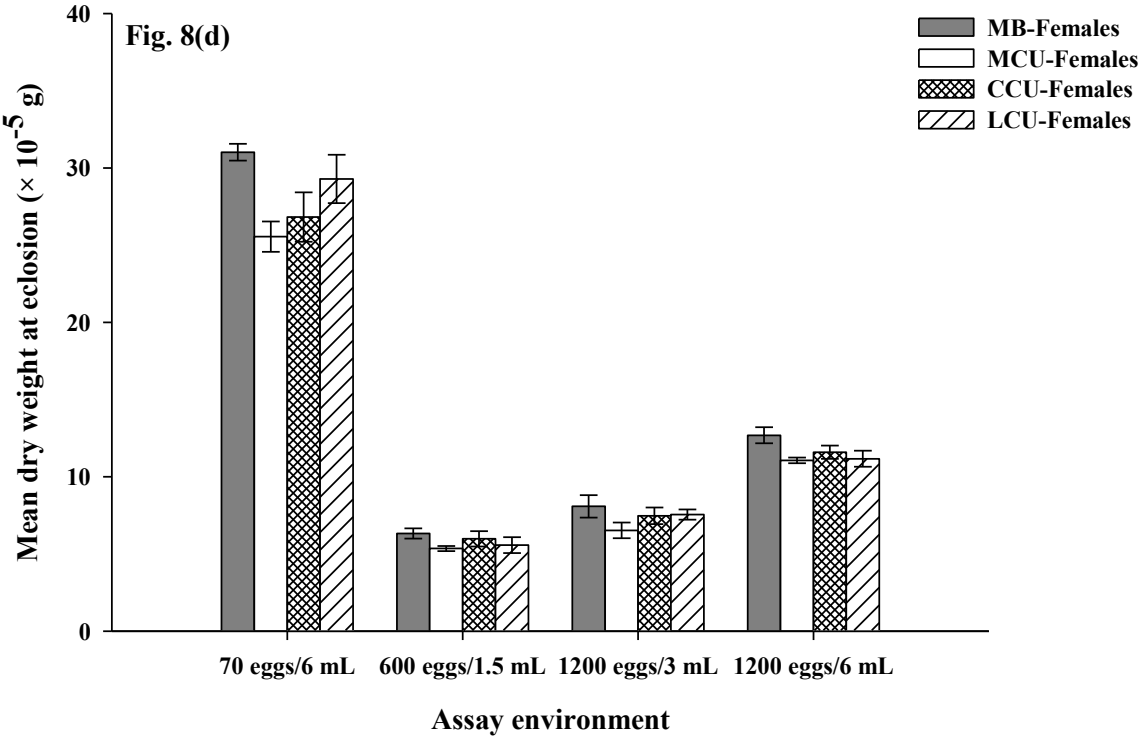
Fig. 8(d)
MB-Females
MCU-Females
CCU-Females
LCU-Females
Mean dry weight at eclosion (× 10-5 g)
0
10
20
30
40
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

Fig. 8 Mean dry weight at eclosion of the MB, MCU, CCU and LCU populations in four different assay environments after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection in (a) males (b) females and after Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection in (c) males (d) females. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

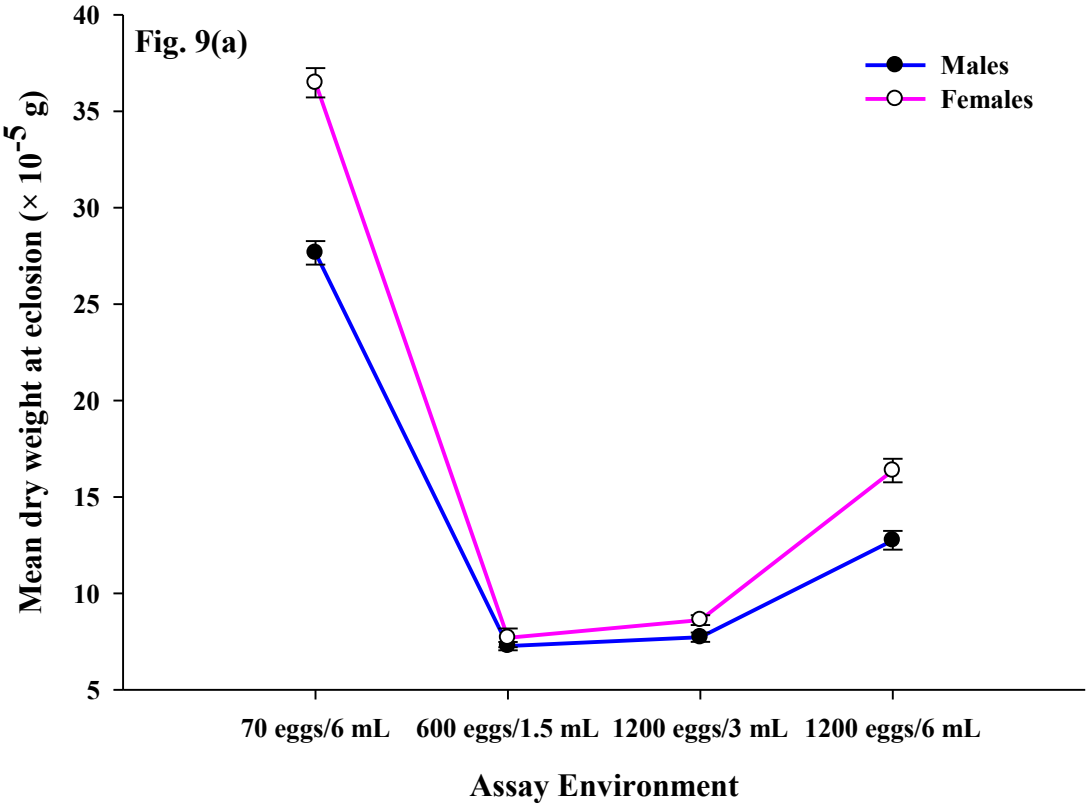
Fig. 9(a)
Males
Females
Mean dry weight at eclosion (× 10-5 g)
40
35
30
25
20
15
10
5
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

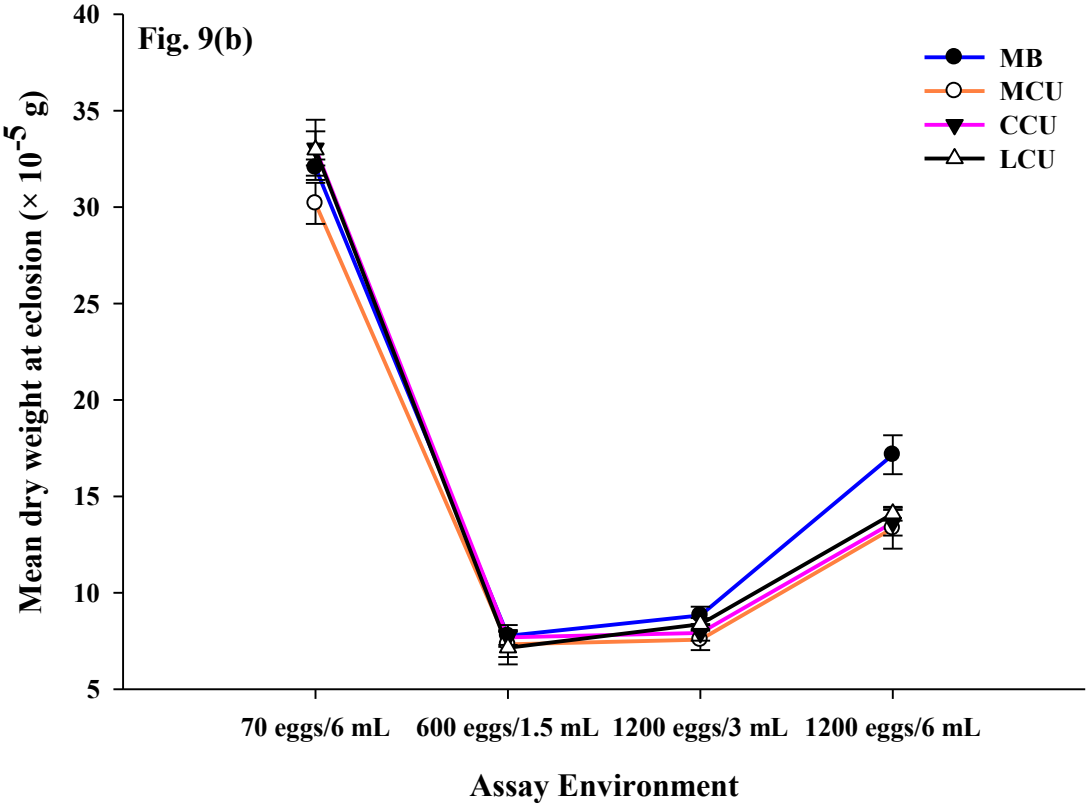
Fig. 9(b)
MB
MCU
CCU
LCU
Mean dry weight at eclosion (× 10-5 g)
40
35
30
25
20
15
10
5
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

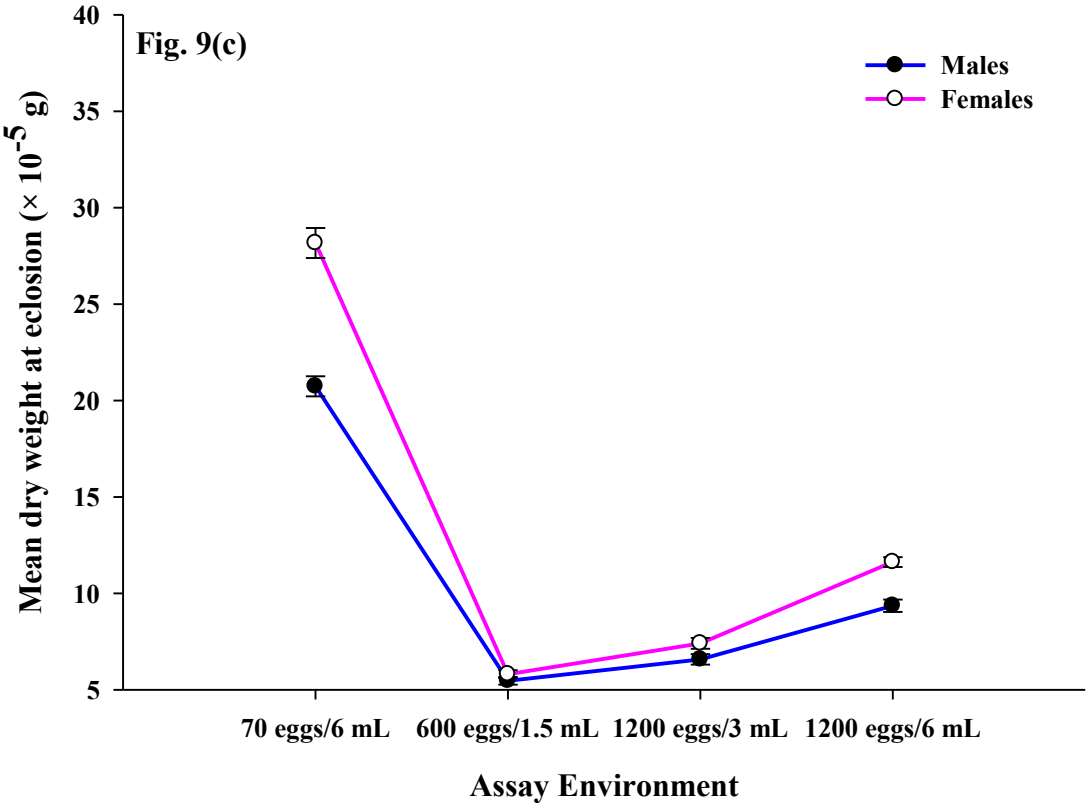
Fig. 9(c)
Males
Females
Mean dry weight at eclosion (× 10-5 g)
40
35
30
25
20
15
10
5
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

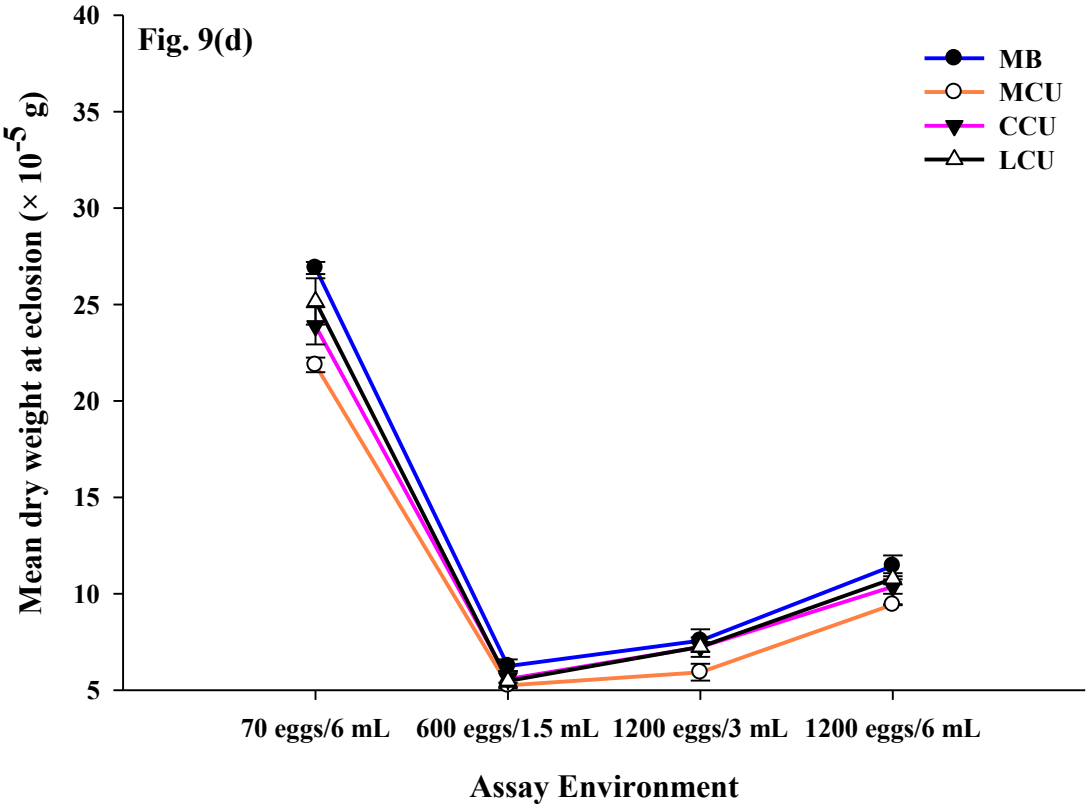
Fig. 9(d)
MB
MCU
CCU
LCU
Mean dry weight at eclosion (× 10-5 g)
40
35
30
25
20
15
10
5
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay Environment

Fig. 9 (a) Assay environment and sex interaction for the mean pre-adult development time of all four sets of populations averaged across four sets of populations and (b) Assay environment and selection interaction for the mean pre-adult development time averaged across both sexes, after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection. (c) Assay environment and sex interaction for the mean pre-adult development time of all four sets of populations averaged across four sets of populations and (d) Assay environment and selection interaction for the mean pre-adult development time averaged across both sexes, after Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

Fig. 10

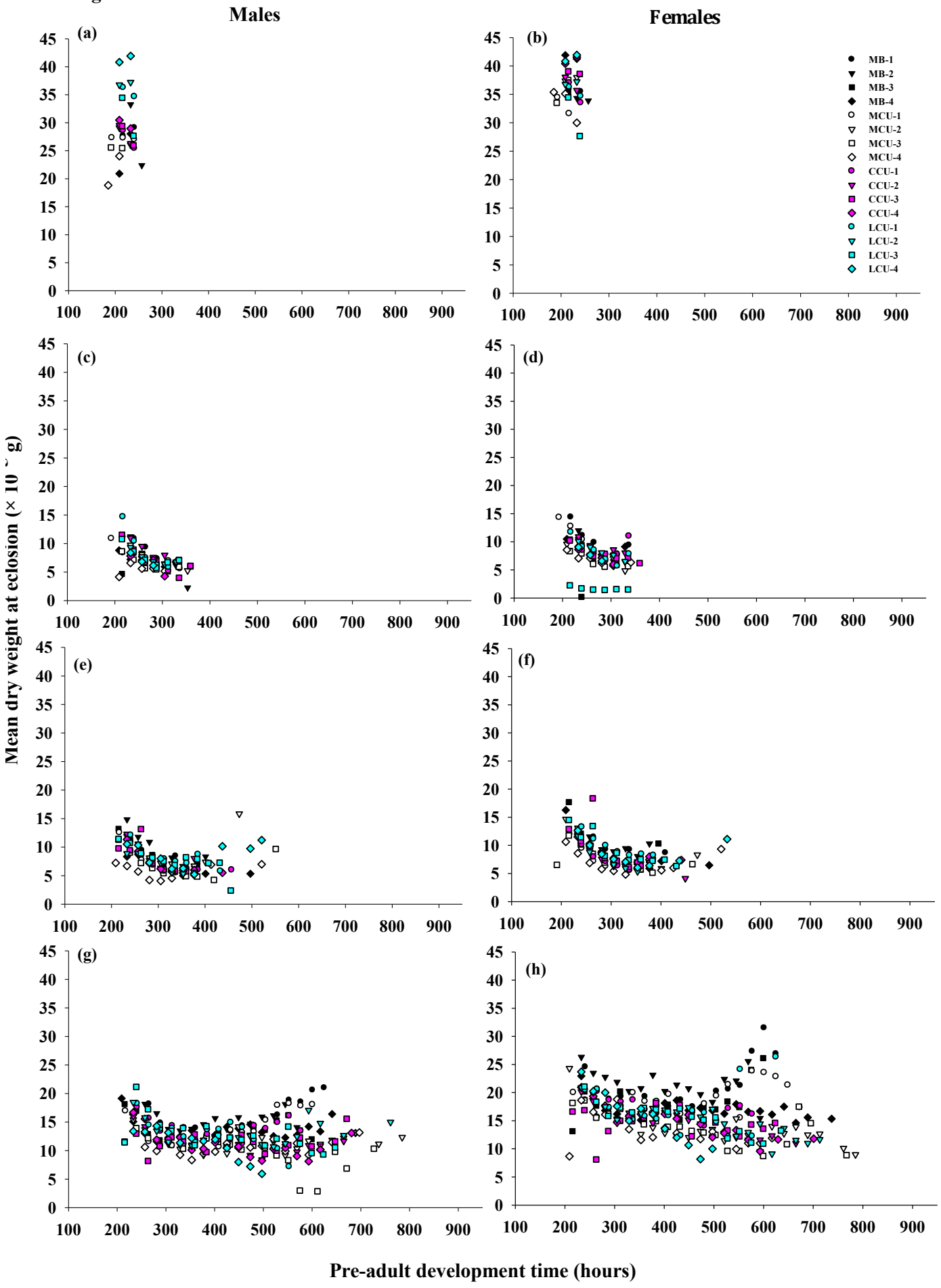

Fig. 10 Mean dry weight distribution of males and females from MB, MCU, CCU and LCU populations in (a, b) 70 eggs/6 mL, (c, d) 600 eggs/1.5 mL, (e, f) 1200 eggs/3 mL and (g, h) 1200 eggs/6 mL assay environments, after Gen. 134 of MCU and Gen. 11 of CCU, LCU selection.

Fig. 11

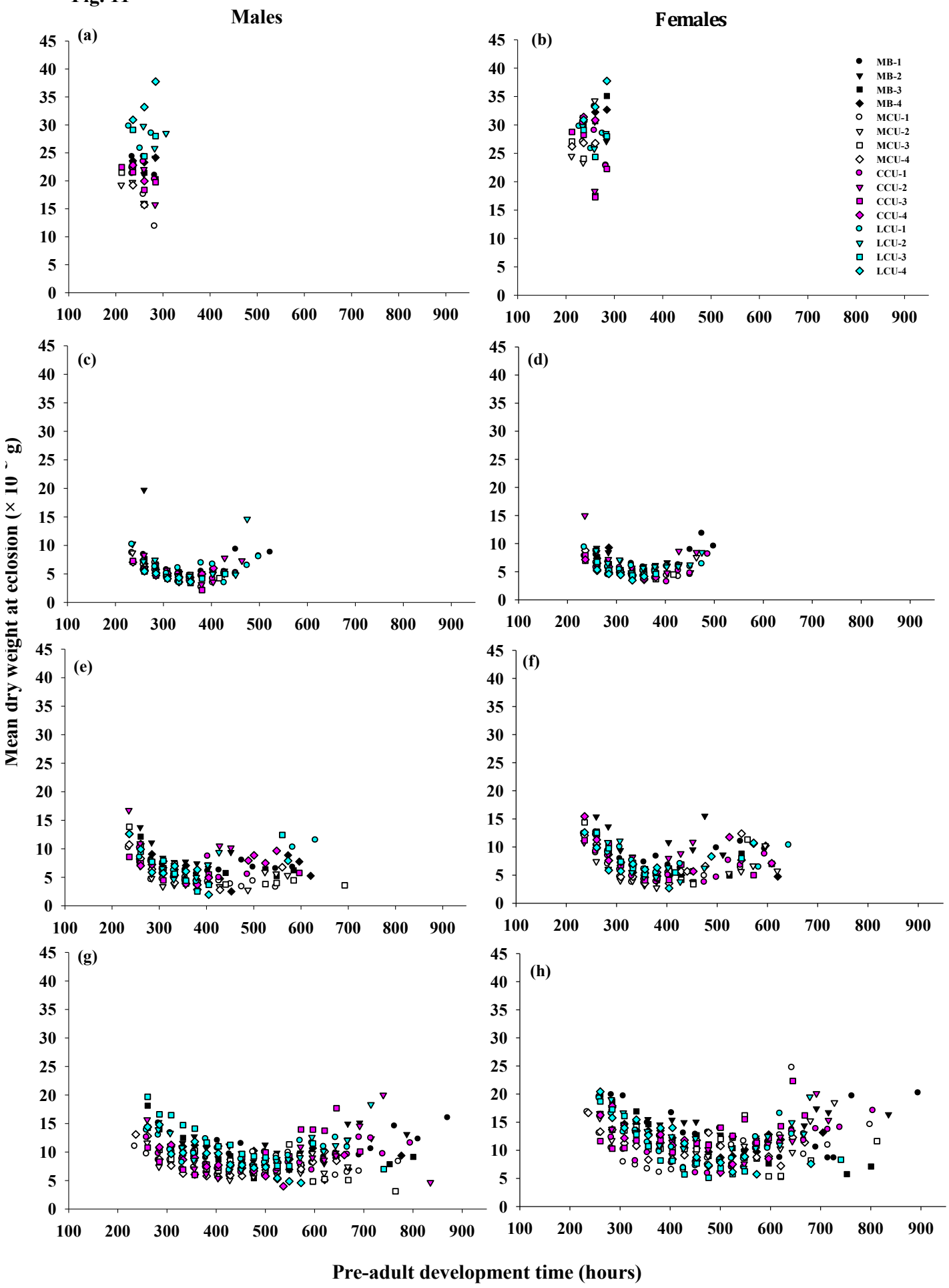

Fig. 11 Mean dry weight distribution of males and females from MB, MCU, CCU and LCU populations in (a, b) 70 eggs/6 mL, (c, d) 600 eggs/1.5 mL, (e, f) 1200 eggs/3 mL and (g, h) 1200 eggs/6 mL assay environments, after Gen. 154 of MCU and Gen. 32 of CCU. LCU selection.

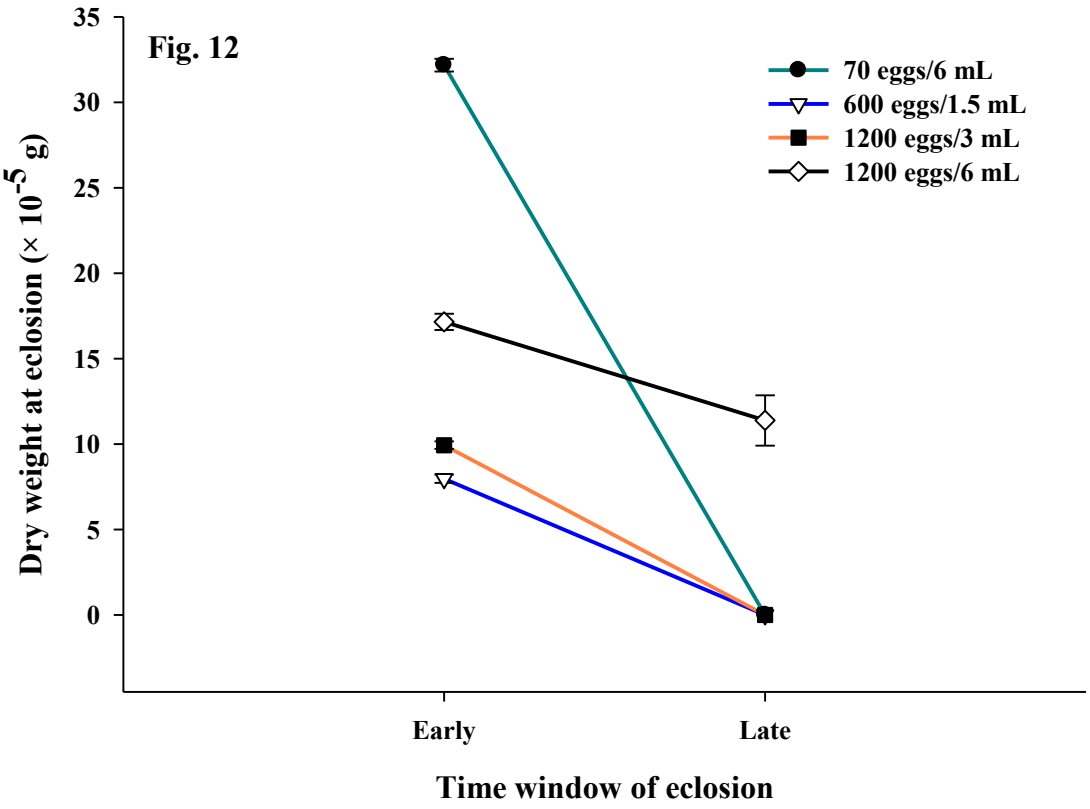

Fig. 12 Mean dry weight number of adults (averaged across males and females) eclosing in early (200-300 h) and late (600-700 h) windows, at different assay environments when averaged across all four sets of populations after Gen. 134 of MCU selection and Gen. 11 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at α = 0.05 level of significance.

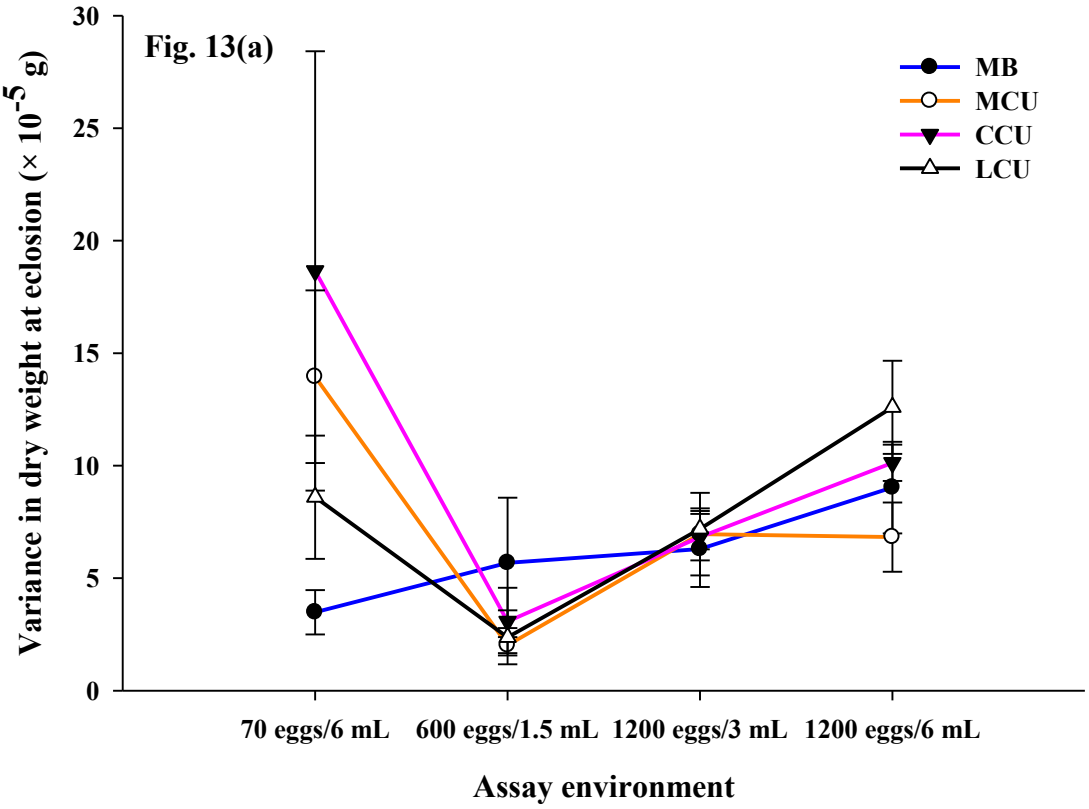
Fig. 13(a)
MB
MCU
CCU
LCU
Variance in dry weight at eclosion (× 10-5 g)
30
25
20
15
10
5
0
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Assay environment

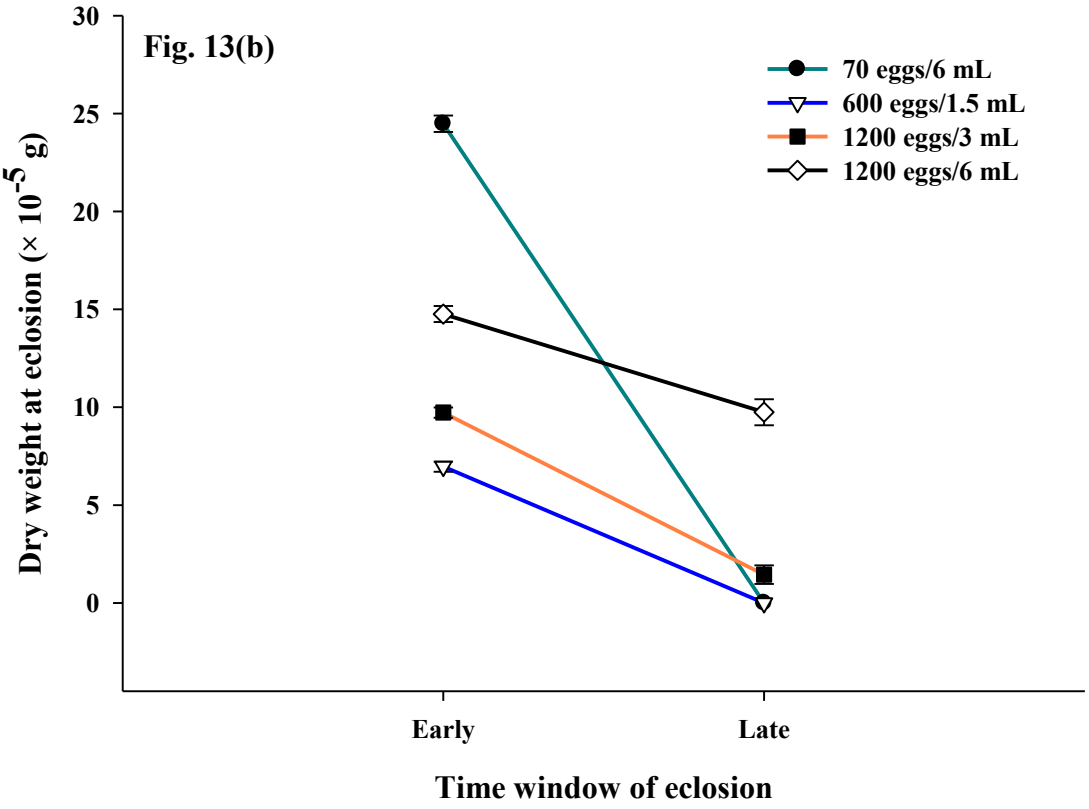
Fig. 13(b)
70 eggs/6 mL
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Dry weight at eclosion (× 10-5 g)
30
25
20
15
10
5
0
Early
Late
Time window of eclosion

Fig. 13 (a) Variance in dry weight at eclosion of all four sets of populations at different assay environments and (b) Mean dry weight of adults (averaged across males and females) eclosing in early (200-300 h) and late (600-700 h) windows, at different assay environments when averaged across all four sets of populations after Gen. 153 of MCU selection and Gen. 32 of CCU and LCU selection. Error bars are the standard errors around the means of the four replicate populations in each selection regime. Tukey's post-hoc test was done at $\alpha = 0.05$ level of significance.

Chapter 4: Larval feeding rate and waste tolerance

Introduction

Two major aspects in which the environment in a crowded *Drosophila* culture deteriorates over time within a generation is the disappearance of food and the increase in concentration of metabolic waste in the food (Borash *et al* 1998). Early studies of adaptation to crowding in *D. melanogaster* showed repeated evidence of the evolution of adaptations to these specific stresses of crowding through the evolution of enhanced larval feeding rates (Joshi & Mueller 1988, 1996, Santos *et al* 1997) and urea/ammonia tolerance (Shiotsugu *et al* 1997, Borash *et al* 1998). Feeding rate in *Drosophila* larvae has typically been measured as the number of cephalopharyngeal sclerite retractions performed by a larva per minute while feeding on a yeast suspension, usually on a small Petri-dish.

By the early 2005, larval feeding rates were repeatedly shown to be a strong correlate of larval competitive ability across multiple studies in different contexts and, indeed, were often used as surrogate for competitive ability. Bakker's (1961, 1969) work first drew attention to the importance to competitive ability of being a faster larval feeder in a food-limited crowded *Drosophila* culture, and pointed out that larvae consuming food rapidly would most likely attain the minimum critical size for pupation before the food ran out, thus being able to successfully eclose as viable adults. Subsequently, selection for faster feeding in *Drosophila* was shown to result in the correlated evolution of increased larval competitive ability and the ability to eclose as heavier adults than their slower feeding counterparts (Sewell *et al* 1975, Burnet *et al* 1977). Thereafter, the evolution of greater competitive ability in crowding adapted populations of *Drosophila* was shown to involve the evolution of increased larval feeding rates in both the *K*-selected (Joshi & Mueller 1988) and CU

populations (Joshi & Mueller 1996, Santos *et al* 1997). Further experiments also showed that feeding rates in larvae of the CUs reverted back to similar levels shown by control UU populations when the selected CU populations were maintained at moderate densities, suggesting a cost associated with increment of feeding rate at high densities (Joshi *et al* 2003). Reduced larval feeding rate has also been shown to be a strong evolutionary correlate of reduced competitive ability in the context of the evolution of both rapid pre-adult development (Prasad *et al* 2001 Shakarad *et al* 2005, Rajamani *et al* 2006) and increased parasitoid resistance (Fellowes *et al* 1998, 1999).

Along with larval feeding rates, larval foraging path lengths have also been shown to be correlated with competitive ability. Under high-density rearing conditions, the longer path length, i.e., the 'rover' phenotype was found to be favored, whereas the shorter path length, i.e., the 'sitter' phenotype was favored under low-density rearing conditions (Sokolowski *et al* 1997). Rover phenotypes were also shown to survive better under poor food conditions (Kaun *et al* 2007). Faster developing populations of *D. melanogaster* that had evolved slower feeding rates also exhibited reduced foraging path lengths and digging behaviour, relative to ancestral controls (Prasad *et al* 2001). However, in a different study, populations of *D. melanogaster* selected for adaptation to poor nutritional quality food in larval stages, evolved greater frequencies 'sitter' phenotype of the larvae (Vijendraverma *et al* 2012a). Larval tolerance to nitrogenous metabolic wastes like urea and ammonia has also been seen to evolve in the crowding adapted CU populations of *D. melanogaster* (Shitosugu *et al* 1997, Borash *et al* 1998). In fact, the reason I focused on examining larval feeding rate and tolerance to metabolic waste in some detail in the MCU, CCU and LCU populations was because, unlike the CU populations, the MCU populations

were earlier seen to have adapted to larval crowding without evolving increased larval feeding rate, foraging path length or waste tolerance, and it was speculated that this may be due to their experiencing crowding at low rather than relatively high food levels (Sarangi 2013, Sarangi *et al* 2016).

The relationship between larval feeding rate and metabolic waste tolerance is not completely straightforward. On the one hand, simple optimality modeling suggests that optimal larval feeding rates will be lower in the presence of increasing concentrations of metabolic waste in the medium (Mueller *et al* 2005), and this is also borne out by the observation that populations of *D. melanogaster* selected for tolerance to metabolic wastes like urea or ammonia do evolve reduced larval feeding rates as a correlated response (Borash *et al* 2000a). Similarly, Borash *et al* (1998) also demonstrated an interesting pattern of a genetic polymorphism with regard to larval feeding rate and waste tolerance. Offspring of early eclosing CU larvae from crowded cultures were faster feeders but not very tolerant of metabolic waste, as compared to controls, whereas offspring of late eclosing CU larvae from crowded cultures were more tolerant of toxic levels of metabolic waste but slower feeders, compared to controls (Borash *et al* 1998). On the other hand, *D. melanogaster* populations selected for rapid pre-adult development evolved both reduced larval feeding rates (Prasad *et al* 2001) and reduced metabolic waste tolerance (Joshi *et al* 2001), although this result could be due to an overall reduction of energy expenditure in these populations. In this chapter, I discuss results from experiments in which larval feeding rates under a variety of assay conditions and tolerance to urea and ammonia were examined in the MB, MCU, CCU and LCU populations.

Materials and Methods

Feeding Rate Experiments

These experiments were conducted on all four replicate populations of either the MBs and MCUs only, or the MB, MCU, CCU and LCU populations. Each of the MB, MCU, CCU and LCU populations were subjected to multiple assay environments depending upon the type of experiment (described below in detail). Irrespective of experiment type, the feeding rates were measured as the number of cephalopharyngeal sclerite retractions in a 1-minute interval. The standardized populations were provided with 3 days of yeast supplement on cornmeal food. Following that, another cornmeal food plate was kept inside each of these standardized cage populations where the females were allowed to lay eggs for duration of 1 h, after which that plate was discarded. A second egg laying food plate (Table 2, Chapter 1) was then placed in each of the cages for duration of 12 h.

(1) ***For assays in petri-dish and slial environments***

Eggs were then collected off these plates and approximately 120 numbers were counted and then transferred to Petri-dishes containing a thin layer of non-nutritive agar. Fifteen such replicate plates were set up per population. Twenty-four hours later, approximately 100 freshly hatched larvae from these Petri-dishes were transferred into Petri-dishes containing a thin layer of non-nutritive agar overlaid with ~1.5 mL of 37.5% yeast suspension. Ten such culture Petri-dishes were set up per population. The larvae were then allowed to feed till they molted to mid 3rd instar stage, which was about 72 h from egg collection stage. The assays were conducted on 10 replicates per assay environment according to the experiment type. Details of each of these experiments are as follows:

1.1 ***On 35 mm Petri-dishes with yeast suspension***

One set of experiment was conducted with 1, 5, 10 and 20 as different larval group sizes at mid 3rd instar stage. In the group sizes with more than 1 larva, the feeding rates were measured by considering randomly one focal individual out of the group. These larvae were assayed from MB and MCU populations, after 144 generations of MCU selection. The other two sets of experiments were conducted on MB, MCU, CCU and LCU populations after 150 and 170 generations of MCU selection, and 29 and 49 generations of CCU, LCU selection, respectively. In each of these experiments, 1 larva per yeast plate was the unit of observation. After a period of 1 minute of acclimatization, feeding larvae were filmed for about 2-4 minutes. The previous assays from our laboratory had 90 mm Petri-dishes for feeding rate experiments unlike what has been used in earlier studies with the *K*-selected and CU populations. Those older experiments used 35 mm Petri-dishes, which is what we used here, thereby allowing for comparisons between previous and present assays.

1.2 ***In Slials***

Slials were prepared using 2 microscope glass slides (75 mm × 25 mm × 1 mm) joined together with a gap of about 1 mm between them (Fig. 11). Cornmeal food was then inserted to this cavity from one of the ends till about ¼ the height of the slide, which was then sealed from the rear end of the food. Larvae from the MB and MCU populations were studied using this assay environment with 5, 10 and 20 group sizes. These larvae were individually picked up from the culture Petri-dishes and gently placed inside the slials using a thin bristled paint- brush. The larval feeding was

filmed for about 5-8 minutes after all the larvae entered into the food after the acclimatization period. At the time of the assay, MCUs were post 150 generations of selection.

The slials were named as such since these were prepared using glass microscope slides with an intention to resemble a the low density vial ecology, where we aimed to get a glimpse of the nature of larval feeding in a restricted surface area with smaller group sizes and abundant availability of cornmeal food.

(2) ***In culture vials***

Eggs from MB, MCU, CCU and LCU populations were collected off the food plates on thin strips of non-nutritive agar dispensed into vials as follows:

2.1 MCU-type environment, where ~600 eggs were put in 8-dram vials with 1.5 mL cornmeal food. Four such replicate vials were set up per population

2.2 CCU-type environment, where ~1200 eggs were put in 8-dram vials with 3 mL cornmeal food. Four such replicate vials were set up per population

2.3 LCU-type environment, where ~1200 eggs were put in 6-dram vials with 6 mL cornmeal food. Four such replicate vials were set up per population

Feeding larvae were filmed at the following time points from egg collection stage, separately detailed for MB and other 3 crowding adapted populations. For all four types of populations, 2nd instar time points were the same, which were 52, 55, 58, 61 and 64 hours from egg stage. The 3rd instar time points in MB populations were 69, 76, 83, 90, 97, 104 and 111 hours from egg stage. For the 3 crowding adapted populations the 3rd instar time points were 66, 73, 80, 90, 101 and 108 hours from egg stage, in order to make allowance for their faster development. The low density, MB-

type environment i.e., 70 eggs/6 mL could not be used, since previous pilot experiments had shown that there were barely any observations of larvae feeding on the periphery of the food vials that could be successfully filmed by the camera. MCU populations were at 170 generations and CCU, LCU populations were at 49 generations of selection at the time of this assay.

The feeding rates of larvae from the control and selected populations were measured at physiologically matched ages, based on their development time differences. This was done by conducting each of these experiments at mid 3rd instar stage, 3 h later in control MB populations relative to the larvae from selected crowding populations. All of these experiments were filmed using Canon PowerShot S3 IS and Canon PowerShot S5 IS with appropriate illumination. The recorded videos were analyzed using VLC Media Player. Three readings were obtained for each focal larva and the mean counts for each video in a population were averaged to obtain the overall mean feeding rate for any particular assay environment in a given population.

Waste Tolerance and Waste Dynamics Experiments

Waste Tolerance Experiments

For these experiments, eggs were collected from each standardized population by placing a cornmeal food plate at first, which was removed after a window of 1 h, and then another egg laying plate was placed in each of the cages for a 12 h time window. These eggs were then reared in environments with different concentrations of nitrogenous wastes, viz. ammonia and urea. Details of each of these concentrations are given in Table 1.

(1) One set of experiment was conducted only with MB and MCU populations, after 143 generations of MCU selection. Moderate density, 70 eggs/6 mL was the rearing environment with different nitrogenous waste concentrations. Ten such replicate vials were set up per concentration per population.

(2) The other three sets of experiments were conducted on MB, MCU, CCU and LCU populations after 135, 157 and 177 generations of MCU selection, corresponding to generations 15, 36 and 56 of CCU, LCU selection, respectively. Details of the assay environments are given in Table 1. Experiment done at Gen. 135 of MCU, corresponding to Gen. 15 of CCU, LCU selection, had four replicate vials set up per concentration per population, whereas other experiments had ten replicate vials set up per concentration per population.

Vials were monitored for the eclosion of the first adult(s), following which checks were conducted every 24 h to score the number of adults successfully eclosing from each vial. The pre-adult survivorship was calculated as the proportion of adults eclosing per vial, then averaged across all replicate vials for a given concentration in any population. This served as a measure of how tolerant the population is, as a whole, to the presence of nitrogenous wastes in its culture environment.

Waste Dynamics Experiments

The purpose of these experiments was to examine whether urea build up in the upper zone of food where larvae feed in a culture vial is affected by the presence of food below the upper zone, and whether urea from the upper zone containing feeding larvae actually diffuses down the food column in a culture vial. It is not clear whether

the primary metabolic waste excreted by *D. melanogaster* is uric acid, urea or ammonia. In a moist environment, such as that of a crowded *Drosophila* culture, there is a possibility of ammonia being the nitrogenous waste excreted by larvae, and Borash *et al* (1998) did observe a significant increase in ammonia levels relative to that of urea in crowded *Drosophila* cultures, although urea levels did show a slight increase over time, as well. Moreover, Borash *et al* (1998) also mentioned that they could not rule out the possibility of ammonia being generated through microbial action in the food. On the other hand, urea levels have also been demonstrated to increase over time in crowded *Drosophila* cultures by Botella *et al* (1985) and in the current study reported in this thesis. Additionally, populations selected for tolerance to extreme levels of ammonia and urea were shown to have evolved cross-tolerance to urea and ammonia respectively, for egg-to-adult survivorship and developmental time, and there is evidence for pleiotropic control of tolerance to urea and ammonia (Borash *et al* 2000a, Borash & Shimada 2001). Thus, it would appear not to make too much difference whether one chooses ammonia or urea to measure tolerance. We chose to focus on urea partly for logistic reasons of very well standardized test-kits being available for urea measurements.

The following waste build-up and waste diffusion experiments were carried out in the Population Biology Laboratory, Indian Institute of Science Education and Research Pune, in collaboration with Prof. Sutirth Dey, and for both these assays, one of the four VBC ('Vagabond' Control) (Tung *et al* 2017) populations was used. The VBC population was derived from one of the JB populations from our laboratory and, hence, from the same lineage as the MB control populations of the present study. The VBC population maintenance is substantially the same as that of the MBs. The Urea

Assay Kit from Sigma Aldrich (Catalog Number MAK006) was used for both these experiments.

Waste Build Up in Feeding Band

In this experiment, we used two different food volume treatments at identical larval densities, 600 eggs/3 mL and 1200 eggs/6 mL, in order to assess how the build up of nitrogenous waste concentration might be affected by differences in the height of the food column in the culture vials. Essentially, the 1200 eggs/6 mL food treatment has food below the upper zone where larvae feed, whereas vials with 3 mL of food have very little food below the upper feeding zone. Larvae in both the treatments were cultured in vials (9.6 cm height, 2.2 cm inner diameter) open at both ends, with one end sealed with parafilm. Food from the feeding band was used as the sample for assessing urea concentration. The term feeding band refers to the zone from the surface of the food to a depth of 1 cm, which is where all larvae typically feed. At various time points (2, 3, 4, 5, 7 and 10 days after egg lay) after setting up the vials, the parafilm at the bottom was removed and the food carefully pushed out of the vial. Food from the feeding band was then gently removed using a scalpel, and a sample of 16 µL of food (without larvae) was used for the next step. One sample of food from each of six replicate vials per treatment was extracted at each time point and placed in an Eppendorf tube, and 35 µL of urea assay buffer (Sigma Aldrich: Urea Assay Kit, Catalog Number MAK006) was then added to each replicate sample. The samples were then homogenized by vortexing for 30-40 sec and then centrifuging at 4°C and 13,000g for 10 minutes, in order to thoroughly mix the food sample with the buffer. Next, after centrifuging, 1 µL of the supernatant was serially diluted to 1/100, and 5 µL of the final dilution was then used for the reaction. This amount was

added to the 5 μL make up buffer (from the kit) in one well of a 96 well plate, and to this 40 μL of the master mix from the kit was added to bring the final volume up to 50 μL. After addition of the master mix to each replicate sample, the clear bottom 96 well plate was immediately wrapped in aluminum foil in order to prevent light interference with the reaction. The samples were then further homogenized by shaking for about 3 minutes, following which they were incubated in dark at 37°C for 1 h. At the end of 1 h, the optical density (OD) in each well was measured at 570 nm using a plate reader (Synergy HT BioTek, Winooski, VT, USA). Standards were run with every experiment. The controls composed of urea assay make-up buffer, urea standard and master mix, same as the experimental sets, except it did not have the food in the final reaction mixture.

Waste Diffusion out of Feeding Band

For this experiment, I set up two kinds of vials, both with 8 mL of food in them. In one set of vials, 800 eggs per vial were added to the food, whereas the control set consisted of just food and no eggs. Six replicate vials each were set up for the treatment with eggs and the control. Food from just below the feeding band (1 cm from the surface) was extracted on day 7 from the start of the experiment (time of egg lay) and its urea concentration assessed, as described in the assay above. After carefully pushing out the food from the vial, the feeding band was removed and the remaining food was vertically cut into two equal halves using a scalpel. Food from the topmost layer of this food column below the feeding band was then carefully removed using a straw, and added to 35 μL of urea assay buffer in an Eppendorf tube. Each sample was then vortexed for 30-40 seconds along with 2 small ball bearings to thoroughly mix the food sample with the buffer, following which the samples were

centrifuged at 4°C and 13,000g for 10 minutes. 1 μL of the supernatant was then serially diluted to 1/100, and 10 μL of the final dilution of the sample was then used for the reaction. 40 μL of the master mix was added to make the final volume up to 50 μL. The urea concentration assay was as described for the nitrogenous waste build up experiment described in the preceding section.

Statistical Analyses

For feeding rate and waster tolerance data, mixed-model analysis of variance (ANOVA) was performed. For the feeding rate experiments, selection, assay environment, instar stage or time point were treated as fixed factors, depending upon the type of experiment. In case of the waste tolerance experiments, survivorship values were arcsine square root transformed prior to doing ANOVA. Selection and urea/ammonia concentration were treated as fixed factors. The fixed factors were crossed with one another and random blocks, representing ancestry (MB, MCU, CCU and LCU populations with same numerical subscripts were treated as random blocks). Means of all the traits were considered for all the fixed factors and their interactions. For the waste build up and waste diffusion assays, assay environment and day were used as fixed factors and only one population (VBC) was used, so there were no blocks in the experimental design. Replicates were represented by individual vials in each combination of assay environment and day. All of ANOVA statistics were performed on STATISTICA^{TM} for Windows Release 5.0B (Statsoft Inc. 1995). Multiple comparisons were carried out using either Tukey's honest significant difference test (HSD) for the mixed-model ANOVAs, or Holm-Sidak Step Down procedure for the waste diffusion assay data, in both cases at 0.05 level of significance.

Results

Feeding rate experiments

(1) *On 35 mm Petri-dishes with yeast suspension*

Gen. 144 (MCU)

MB and MCU feeding rates did not differ significantly from one another at any of the four group sizes (Fig. 1, Table 2). Selection did not have a significant main effect, nor there was any significant interaction between selection and assay environment (Table 2). Overall, there was a slight tendency for feeding rates to increase with increasing group size, however there was no main effect of assay environment (Fig. 1, Table 2).

Gen. 150 (MCU), Gen. 29 (CCU, LCU)

In this assay, ANOVA revealed a significant main effect of selection (Table 3), and post-hoc analysis revealed that the LCU populations had significantly greater feeding rates than both the MB and MCU populations (LCU > MB by ~12 and LCU > MCU by ~11 sclerite retractions per minute), whereas the MB, MCU and CCU populations did not differ significantly from one another. Fig. 2).

Gen. 170 (MCU), Gen. 49 (CCU, LCU)

In this assay there was a significant main effect of selection (Table 4), and post-hoc analysis revealed that larvae from both the CCU and LCU populations had significantly greater feeding rates than control MB populations (CCU > MB by ~ 15 and LCU > MB by ~20 sclerite retractions per minute), and that none of the other pairwise comparisons were significant (Figure 3, Table 4).

(2) ***In slials***

In this assay, MB and MCU feeding rates did not differ from one another across any of the three group sizes (Fig. 4), and the only significant ANOVA effect was that of assay environment (group size) (Table 5), with an overall increase in feeding rates from low to high group sizes. Post-hoc analysis revealed significantly higher mean feeding rates of larvae at group size 20 relative to group size 5.

(3) ***In culture vials***

Feeding rates were not separately averaged across 2nd and 3rd instar time points, rather all 12 time points spanning the two instars were treated as levels of one of the fixed factors, 'time point'. ANOVA revealed a significant main effect of selection (Table 6) with MCU, CCU and LCU larvae having significantly greater feeding rates than control MBs averaged across all three assay environments (Fig. 5(a,b,c)). Additionally, MCU larvae also showed significantly greater feeding rates than CCU and LCU larvae, averaged across all three assay environments. There was no significant main effect of assay environment but time point showed a significant main effect, with feeding rates, on the whole, tending to increase with time, perhaps to a slightly greater degree in the MCU-type environment of 600 eggs/1.5 mL food (Fig. 5, Table 6). Assay environment and time point interacted significantly in their effects on feeding rate (Table 6), with the lowest feeding rates (averaged across selection regimes) being in the 600 eggs/1.5 mL food environment between 52-69 h from egg lay, and the highest feeding rates being in the same food environment after 83 h (Fig. 5 d). Selection also interacted significantly with time point (Table 6), with MCU populations showing significantly greater

feeding rates than MBs at 58, 61, 64, 66, 80, 87 and 94 h, whereas LCU populations had significantly greater feeding rates than MBs at 87 and 94 h (Fig. 5(a, b, c)).

Waste Tolerance and Waste Dynamics Experiments

Waste Tolerance Experiments

(1) ***At low and high larval density environments***

Gen 136 (MCU), Gen. 15 (CCU, LCU)

Waste type: Ammonia, in 30 eggs/6 mL

There was no evidence for greater ammonia tolerance in the selected populations, as there was no significant interaction between selection and concentration of waste (Table 7(a)). The ANOVA revealed a significant main effect of selection (Table 7(a)), but this was driven by MCU populations showing significantly greater overall survivorship than LCUs (Fig. 6(a)). Concentration also had a significant main effect (Table 7(a)), where in general there was a trend of viability declining with increasing concentrations of ammonia added in the food (Fig. 6(a)).

Waste type: Ammonia, in 300 eggs/1.5 mL

At this higher density, too, there was no evidence for greater ammonia tolerance in the selected populations, as there was no significant interaction between selection and concentration of waste (Table 7(a)). There was a significant main effect of selection (Table 7(a)), with MCUs showing significantly greater viability, on average, compared to the MB, CCU and LCU populations (Fig. 6(b)). Concentration also had a significant main effect

(Table 7(a)), where in general there was a trend of viability declining with increasing concentrations of ammonia added in the food.

Waste type: Urea, in 30 eggs/6 mL

Here too, ANOVA revealed only significant main effects of selection and concentration of waste (Table 7(b)), but none of the crowding adapted population had greater viability than controls. Only the MCU populations showed significantly greater survivorship than LCUs, and there was a general trend of viability declining with increasing concentrations of urea added in the food (Fig. 6(a)).

Waste type: Urea, in 300 eggs/1.5 mL

Once again, ANOVA revealed only significant main effects of selection and concentration of waste (Table 7(b)), but none of the crowding adapted population had greater viability than controls. Only the MCU populations showed significantly greater survivorship, on an average, compared to MB, CCU and LCU populations, and there was a general trend of viability declining with increasing concentrations of urea added in the food (Fig. 6(b)).

(2) ***At moderate larval density environment***

Gen. 143 (MCU)

Waste type: Ammonia

The MB and MCU populations did not differ significantly in their mean pre-adult survivorship at different concentrations of ammonia in food in this assay, too (Fig. 7). The only significant ANOVA effect was that of

concentration (Table 8) with overall mean pre-adult survivorship declining from low to high concentrations of ammonia in food (Fig. 7).

Waste type: Urea

MCU populations had significantly greater mean pre-adult survivorship than MBs at all concentrations of urea, including the zero urea controls (Fig. 7, Table 8). Concentration also had a significant main effect (Table 8), reflecting a general decline in mean pre-adult survivorship from low to high concentrations of urea in food (Fig. 7).

Gen. 157 (MCU), Gen. 36 (CCU, LCU)

Waste type: Ammonia

The only significant ANOVA effect was that of concentration (Table 9), with overall mean pre-adult survivorship declining from low to high concentrations of ammonia in food (Fig. 8).

Waste type: Urea

The only significant ANOVA effect was that of concentration (Table 9), with overall mean pre-adult survivorship declining from low to high concentrations of urea in food (Fig. 8).

Gen. 177 (MCU), Gen. 56 (CCU, LCU)

Waste type: Ammonia

The only significant ANOVA effect was that of concentration (Table 10), with overall mean pre-adult survivorship declining from low to high concentrations of ammonia in food (Fig. 9).

Waste type: Urea

The ANOVA revealed a significant main effect of concentration (Table 10), with overall mean pre-adult survivorship declining from low to high concentrations of urea in food (Fig. 9). There was also a significant interaction between selection and assay environment, (Table 10) where CCU mean pre-adult survivorship was significantly higher than LCU populations at control treatment, there was however, no survival difference between any of crowding adapted populations and their control MBs (Fig. 9).

Waste Dynamics Experiments

Nitrogenous waste build-up

Among the two assay environments used for estimating the nitrogenous waste build up over time in crowded cultures, the overall level of urea in the feeding band was significantly higher in the 600 eggs/3 mL food vials than in the 1200 eggs/6 mL food vials Fig. 10, Table 11), despite the number of larvae being only half as many. Additionally, the ANOVA also revealed a significant assay environment and day interaction (Table 11). Post-hoc comparisons, however, revealed that differences were only significant at day 2 and 5, with significantly greater OD, corresponding to urea level, in the 600 eggs/3 mL food vials. These results clearly suggest that not having food below the feeding band zone, into which waste can potentially diffuse, leads to a substantially quicker build up of urea in the feeding band, even with only half the number of larvae excreting.

Nitrogenous waste diffusion

At day 7 from set up of the vials, culture vials with eggs in them had significantly higher OD values than control vials with just food and no eggs in the

zone below the feeding band (Table 11), showing that waste does indeed diffuse to the food below the feeding zone in culture vials with feeding larvae.

Discussion

As mentioned earlier, the experiments reported in this chapter were motivated by the observation that crowding adapted CU populations evolved greater larval feeding rate and waste tolerance (Borash *et al* 1998), whereas the crowding adapted MCUs did not (Sarangi *et al* 2016). Sarangi (2013) and Sarangi *et al* (2016) speculated that this difference may be driven by the much lower levels of food in the MCU culture vials, as compared to the CU vials because, in principle, metabolic waste levels in the 1.5 mL food vials in which MCUs are raised will build up much faster, making it counter-productive to feed faster. In the CU culture vials, on the other hand, at least in the initial stages of the crowded culture, there is considerable food below the feeding zone also, into which waste could potentially diffuse, leaving a lower concentration of metabolic waste in the food medium in the upper feeding band, thereby allowing faster feeders to benefit competitively without the detrimental effects of ingesting high levels of metabolic waste. In terms of providing support for this speculation, the results from the experiments reported here were a mixed bag.

Were the above speculation true, one prediction that could be made is that adaptation to crowding at high larval density, but with greater total food amounts per vial (compared to the 1.5 mL in the MCU cultures), would be more likely to involve the evolution of increased larval feeding rate and metabolic waste tolerance. This prediction is actually supported by some of the results reported in this chapter regarding the feeding rate assays carried out in Petri-dishes, as they were in all past

studies. Although the MCUs did not show greater larval feeding rates than the MB controls, either earlier (Sarangi *et al* 2016) or in these experiments (Figs. 1,2,3), the LCU populations did exhibit significantly greater feeding rate than control MBs after both 29 and 49 generations of selection (Figs. 2,3). Indeed, in the assay conducted after 49 generations of CCU and LCU selection, even the CCU populations exhibited significantly faster feeding rates than the MB controls (Figs. 2,3). The CCU maintenance regime involves exactly the same larval density as the MCUs, but with twice the amount of food (3 mL, rather than 1.5 mL). As shown in Chapter 3, this vial environment permits the successful eclosion of a small number of late eclosing individuals who may face increased levels of metabolic waste in the food, too. In this respect, the CCU maintenance environment is somewhat intermediate between that of the MCU (600 eggs/1.5 mL food) and LCU (1200 eggs/6 mL) food populations. On the other hand, if the speculation of Sarangi (2013) and Sarangi *et al* (2016) were correct, we would also expect the LCUs, and perhaps even the CCUs, to evolve greater tolerance to metabolic waste, but none of the waste tolerance assays reported in this chapter suggest even a possibility that this may have happened (Figs. 6,8,9). It is possible that, if our LCU populations evolve an early-late polymorphism similar to that seen in the CU populations by Borash *et al* (1998), the enhanced waste tolerance of the late eclosing types may be swamped by the lower tolerance of early types in a random sample. However, as mentioned earlier, it is quite likely that the LCU populations may not show such a polymorphism because their handling protocol does away with the vial-to-vial transfers during adult collection that most likely led to positive assortative mating for development time in the CU populations. Alternatively, it is possible that overall higher mean waste tolerance may not evolve in the LCU and CCU populations simply because of opposing selection during the

early and late phases of the culture, driven by the tradeoff between larval feeding rate and waste tolerance (Borash *et al* 2000a; Mueller *et al* 2005). In that case, it is possible that a greater variance of waste tolerance would be seen in the CCU and LCU populations, and that is something that can be examined in further studies. Interestingly, though, some component arguments of the Sarangi (2013) and Sarangi *et al* (2016) speculation are supported by the results in this chapter showing that metabolic waste does diffuse into the food column below the feeding band in the top 1 cm or so of the food (Table 11), and that metabolic waste buildup in the feeding band is significantly slower in cultures with greater total amount of food (6 mL, compared to 3 mL), even though the number of larvae in the 6 mL cultures was twice that of the 3 mL cultures (1200 eggs versus 600 eggs) (Fig. 10, Table 11).

The results of the feeding rate measurements done by recording feeding larvae in actual culture vials corresponding to the MCU-, CCU- and LCU-type cultures (Fig. 5) further suggest that the whole issue of what is the relevant measure of feeding rate in a crowded culture is far more context-specific than was hitherto believed. Since the 1970s, larval feeding rates have been typically measured by counting the number of cephalopharyngeal sclerite retractions of a single larva feeding horizontally in a agar plate with yeast suspension on the surface of the agar, while it moves around. This is in contrast to the situation in a crowded culture vial wherein there are numerous larvae in close proximity (indeed, touching) each other while they feed, often moving relatively little, in a more or less vertical position, with anterior ends digging down in the food. Moreover, the food itself does contain metabolic wastes and, possibly, other chemicals secreted by the larvae. As with all complex traits, the question, therefore, then arises as to what degree larval sclerite retraction rate measured under these two

very different kinds of ecological conditions, can be considered to constitute the 'same' trait. In the past, feeding rates measured in Petri-dishes have correlated well with competitive ability (reviewed in Prasad & Joshi 2001), but the present study suggests that, at least in the MCUs, the feeding rate difference they show vis a vis the MB controls is highly plastic and environment specific, being expressed only in culture vial conditions and not in Petri-dishes.

While a lot of further work will be needed to address this issue, a couple of points from the present results are worth noting. It appears that local larval density, perhaps modulated by touch, does affect feeding rate, with greater density perhaps inducing faster feeding. The results from the feeding rate assay in slials, which captures some aspects of the vial environment, including close proximity of larvae feeding vertically with anterior ends down, suggest that increasing the number of larvae in the slial may be increasing feeding rate (Fig. 4, Table 5). Similarly, especially at early time points (52-69 h in Fig. 5(d)), feeding rates in the two treatments with 1200 larvae seem to be greater than those in the 600 larvae treatment. With twice the number of larvae, the effective density of larvae in the feeding band will be twice as large in the 1200 eggs/6 mL and 1200 eggs/3 mL food treatments, compared to the 600 eggs/1.5 mL food treatment, presumably also resulting in more contacts between larvae. Other than touch and local density is the issue of waste concentration in the food. Nothing is known about whether urea/ammonia content in food affects feeding rates plastically; all we know is that selection for urea or ammonia tolerance also yields the evolution of reduced larval feeding rate (Borash *et al* 2000a), but it is also known that phenotypic correlations and genetic correlations can differ in both sign and magnitude (Falconer 1960). Waste concentration in the

food could, in principle, act as a signal to which larvae can evolve to respond, especially if it coincides with important events in the vial ecology like the imminent running out of food in, say, and MCU-type culture. In the study reported here, feeding rates in the 600 eggs/1.5 mL food cultures, become significantly greater than those in the 1200 egg cultures after about 83 h (Fig. 5(d)). By this time, the dynamics of waste buildup in the feeding band are likely to be very different in the three types of cultures, and this is an issue that needs to be studied further.

One final fascinating aspect of the feeding rate results reported in this chapter is that they suggest that, while faster feeding does still seem to be an important correlate of larval competitive ability in *Drosophila*, it is possible that adaptation to crowding imposed at different egg number and food amounts may involve the evolution of greater larval feeding rates as a phenotypically plastic response (e.g. the MCUs) or as more canalized response, exhibited in a variety of environments (e.g. the CCUs, LCUs). This is, at this point, very speculative but it opens up a potentially interesting area for further studies.

Tables

Table 1 Summary of protocol used for different waste tolerance experiments.

Assay Environment	Waste Type	Control	Conc. 1	Conc. 2	Conc. 3
After Gen. 135 of MCU and Gen. 15 of CCU, LCU selection					
30 eggs/6 mL	Ammonia	0g/L	15g/L	20g/L	25g/L
	Urea	0g/L	14g/L	16g/L	18g/L
300 eggs/1.5 mL	Ammonia	0g/L	10g/L	15g/L	20g/L
	Urea	0g/L	6g/L	8g/L	10g/L
After Gen. 143,157, 177 of MCU and Gen. 15, 36 and 56 of CCU, LCU selection					
70 eggs/6 mL	Ammonia	0g/L	15g/L	20g/L	25g/L
	Urea	0g/L	14g/L	16g/L	18g/L

Table 2 Summary of mixed-model ANOVA on feeding rate at four group sizes in MB and MCU populations (Gen. 144), assayed in a Petri-dish with yeast suspension on agar. Selection regime and assay environment (group size) were treated as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	87.548	1.898	0.262
Assay environment	3	226.350	2.039	0.178
Selection × Assay environment	3	81.499	1.191	0.366

Table 3 Summary of ANOVA on feeding rate in MB, MCU (Gen. 150), CCU (Gen. 29) and LCU (Gen. 29) populations, assayed in a Petri-dish with yeast suspension on agar (1 larva/ Petri-dish). Selection regime was treated as fixed factor and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	191.037	7.433	0.008

Table 4 Summary of ANOVA on feeding rate in MB, MCU (Gen. 170), CCU (Gen. 49) and LCU (Gen. 49) populations, assayed in a Petri-dish with yeast suspension on agar (1 larva/ Petri-dish). Selection regime was treated as fixed factor and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	344.054	18.087	<0.001

Table 5 Summary of mixed-model ANOVA on feeding rate at 3 group sizes in MB and MCU populations (Gen. 150), assayed in slials. Selection regime and assay environment (group size) were treated as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	1	256.748	1.077	0.375
Assay environment	2	352.124	6.082	0.036
Selection × Assay environment	2	148.577	1.429	0.310

Table 6 Summary of mixed-model ANOVA on feeding rate in MB, MCU (Gen. 170), CCU (Gen. 49) and LCU (Gen. 49) populations across 12 time points during 2nd and 3rd instar larval stages, when assayed in three different types of crowded cultures in vials. Selection regime, assay environment (combination of egg number and food amount) and time point were treated as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Selection	3	38614.011	37.643	<0.001
Assay environment	2	3292.310	4.742	0.058
Time point	11	28999.478	30.139	<0.001
Selection × Assay environment	6	59.891	0.119	0.992
Selection × Time point	33	677.328	1.585	0.042
Assay environment × Time point	22	4205.993	9.897	<0.001
Selection × Assay environment × Time Point	66	300.014	1.098	0.307

Table 7(a) Summary of mixed-model ANOVA on pre-adult survivorship of MB, MCU (Gen. 136), CCU (Gen. 15) and LCU (Gen. 15) populations at 30 eggs/6 mL food per vial and 300 eggs/1.5 mL food per vial, in food containing 3 different concentrations of ammonia at each assay environment. Selection and concentration were used as fixed factors and block was a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Waste type: Ammonia, Low Density				
Selection	3	0.050	4.703	0.030
Concentration	3	1.300	123.333	<0.001
Selection × Concentration	9	0.004	1.277	0.293
Waste type: Ammonia, High Density				
Selection	3	0.038	11.507	0.001
Concentration	3	0.537	171.481	<0.001
Selection × Concentration	9	0.001	1.666	0.146

Table 7(b) Summary of mixed-model ANOVA on pre-adult survivorship of MB, MCU (Gen. 136), CCU (Gen. 15) and LCU (Gen. 15) populations at 30 eggs/6 mL food per vial and 300 eggs/1.5 mL food per vial, in food containing 3 different concentrations of urea at each assay environment. Selection and concentration were used as fixed factors and block was a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Waste type: Urea, Low Density				
Selection	3	0.111	5.270	0.022
Concentration	3	1.526	81.370	<0.001
Selection × Concentration	9	0.012	0.905	0.534
Waste type: Urea, High Density				
Selection	3	0.044	24.659	<0.001
Concentration	3	0.456	7.214	0.009
Selection × Concentration	9	0.004	2.212	0.063

Table 8 Summary of mixed-model ANOVA on pre-adult survivorship of MB and MCU (Gen. 143) populations at 70 eggs/6 mL food per vial in food containing 3 different concentrations of either ammonia or urea. Selection and concentration were used as fixed factors and block was a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Waste type: Ammonia				
Selection	1	0.018	2.213	0.233
Concentration	3	0.474	140.110	<0.001
Selection × Concentration	3	0.006	2.159	0.163
Waste type: Urea				
Selection	1	0.132	177.737	<0.001
Concentration	3	0.418	154.277	<0.001
Selection × Concentration	3	0.001	0.586	0.638

Table 9 Summary of mixed-model ANOVA on pre-adult survivorship at 70 eggs/6 mL food per vial of MB, MCU (Gen. 157), CCU (Gen. 36) and LCU (Gen. 36) populations in food containing 3 different concentrations of either ammonia or urea. Selection and concentration were used as fixed factors and block was a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Waste type: Ammonia				
Selection	3	0.010	1.179	0.370
Concentration	3	1.104	308.079	<0.001
Selection × Concentration	9	0.001	0.826	0.597
Waste type: Urea				
Selection	3	0.009	1.292	0.335
Concentration	3	1.365	92.472	<0.001
Selection × Concentration	9	0.002	0.768	0.646

Table 10 Summary of mixed-model ANOVA on pre-adult survivorship at 70 eggs/6 mL food per vial of MB, MCU (Gen. 177), CCU (Gen. 56) and LCU (Gen. 56) populations in food containing 3 different concentrations of either ammonia or urea. Selection and concentration were used as fixed factors and block was a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	df	MS	*F*	*P*
Waste type: Ammonia				
Selection	3	0.008	0.610	0.625
Concentration	3	1.069	436.565	<0.001
Selection × Concentration	9	0.003	1.038	0.436
Waste type: Urea				
Selection	3	0.026	1.686	0.238
Concentration	3	1.298	161.494	<0.001
Selection × Concentration	9	0.010	2.324	0.043

Table 11 Results of ANOVA on nitrogenous waste build up in the feeding band over days 2, 3, 4, 5, 7 and 10 of culture from egg stage, and results of ANOVA on nitrogenous waste diffusion into food below the feeding band at day 7 of culture from egg stage.

Effect	df	MS	F	*P*
Nitrogenous Waste Build Up				
Assay Environment	1	27.892	5.978	0.017
Day	5	84.300	18.069	<0.001
Assay Environment × Day	5	16.462	3.528	0.007
Nitrogenous Waste Diffusion				
Assay Environment	1	19.857	2519.83	<0.001

Figures

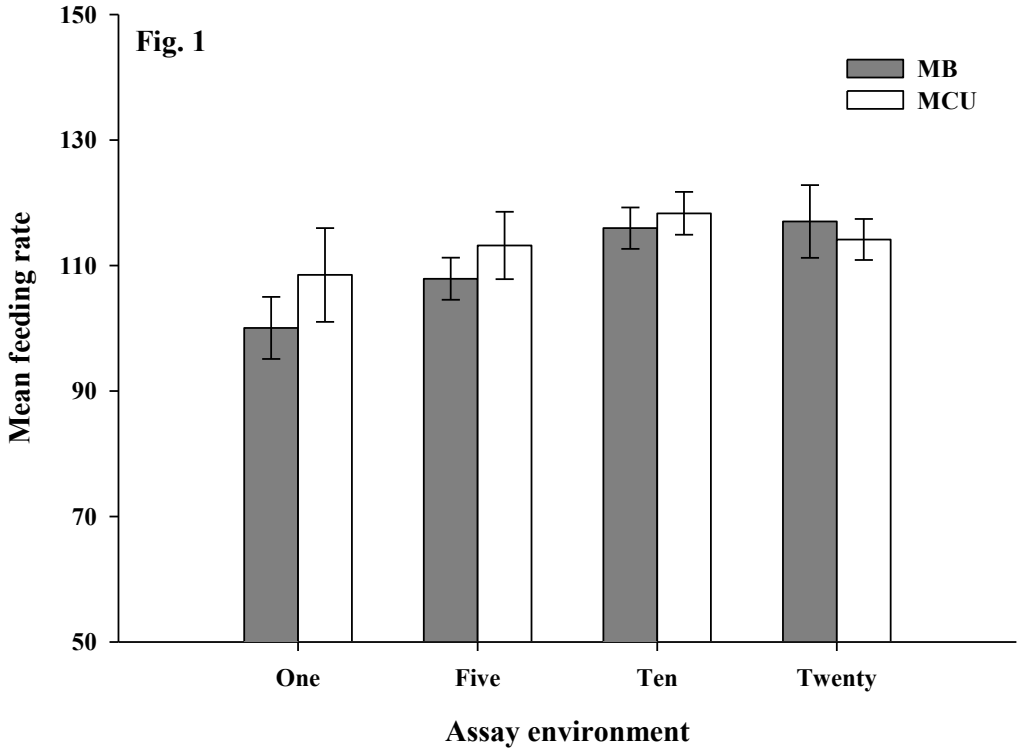

Fig. 1 Mean feeding rate of MB and MCU (Gen. 144) populations in four different assay environments (group sizes). These were assayed on Petri-dishes with yeast suspension on agar. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

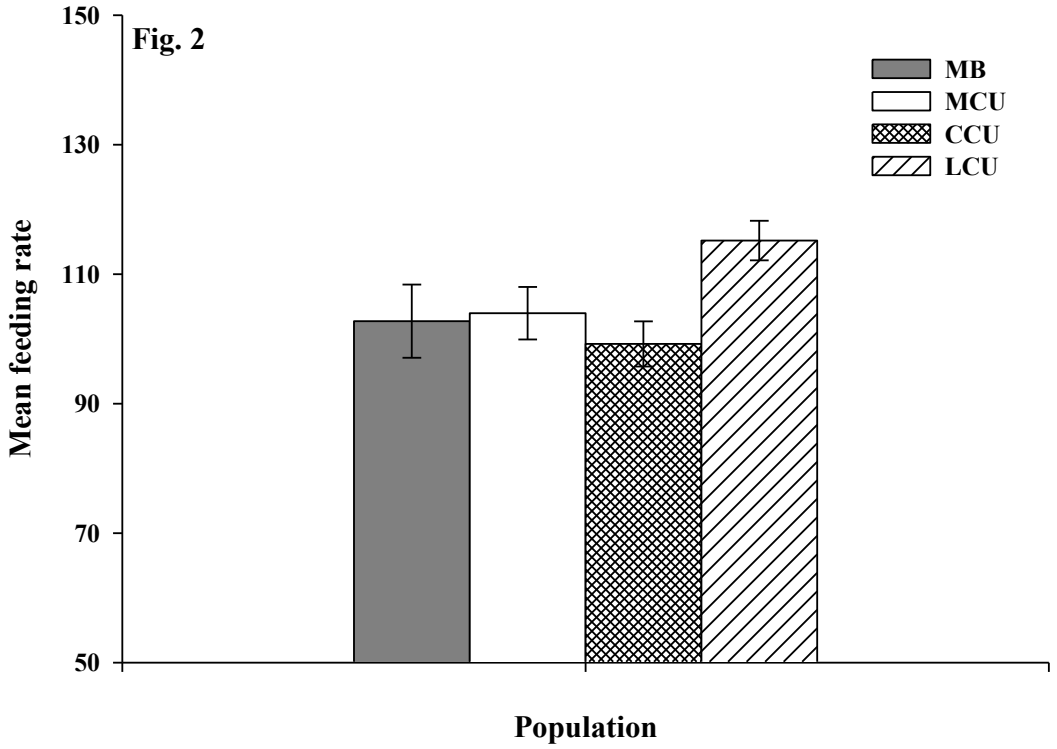

Fig. 2 Mean feeding rate of MB, MCU (Gen. 150), CCU (Gen. 29) and LCU (Gen. 29) populations, assayed on Petri-dishes with yeast suspension on agar (1 larva/petri-dish). Error bars are the standard errors around the means of the four replicate populations in each selection regime.

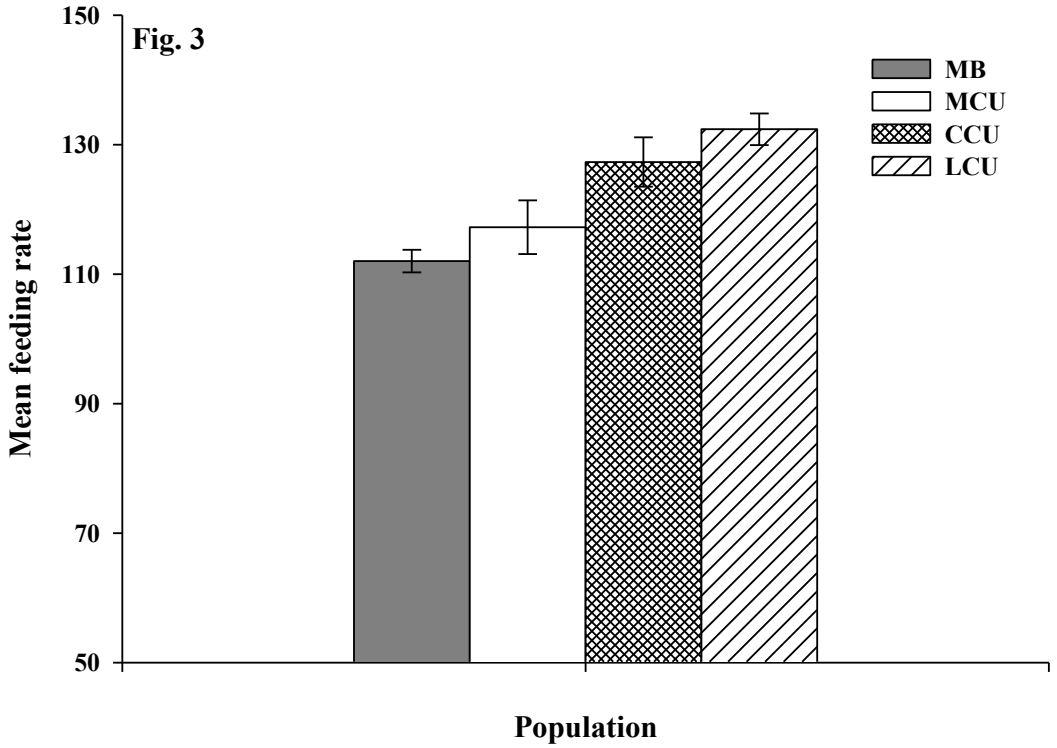

Fig. 3 Mean feeding rate of MB, MCU (Gen. 170), CCU (Gen. 49) and LCU (Gen. 49) populations, assayed on Petri-dishes with yeast suspension on agar (1 larva/petri-dish). Error bars are the standard errors around the means of the four replicate populations in each selection regime.

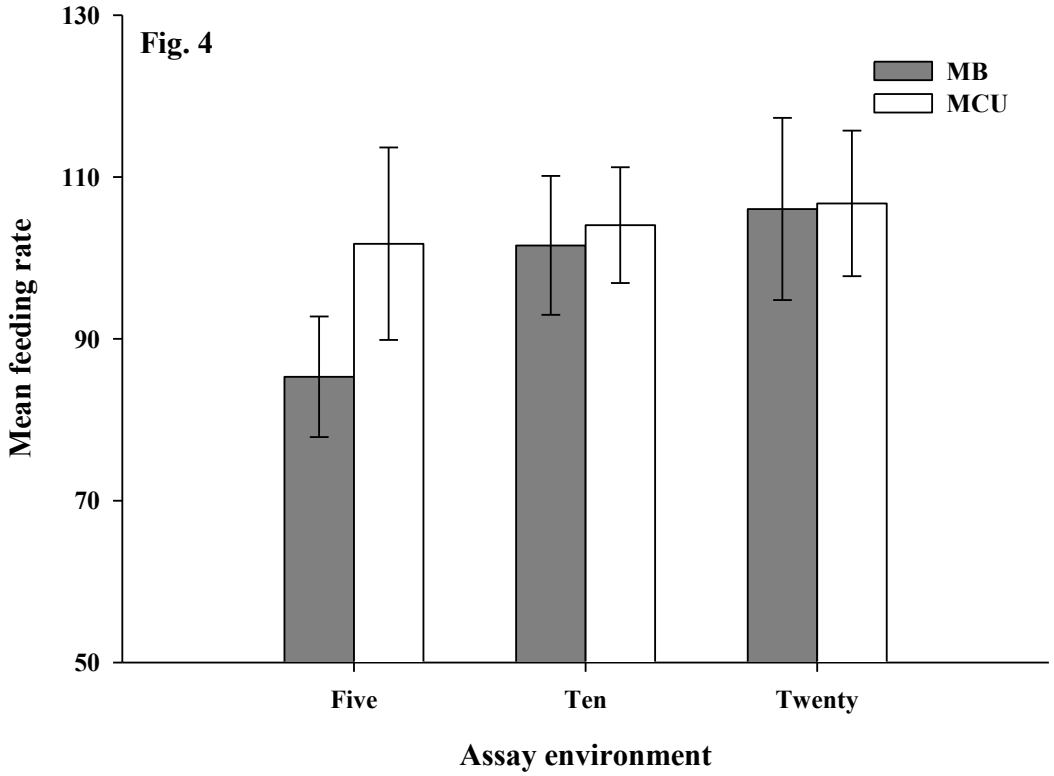

Fig. 4 Mean feeding rate of MB and MCU (Gen. 150) populations at 3 group sizes when assayed in slials. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

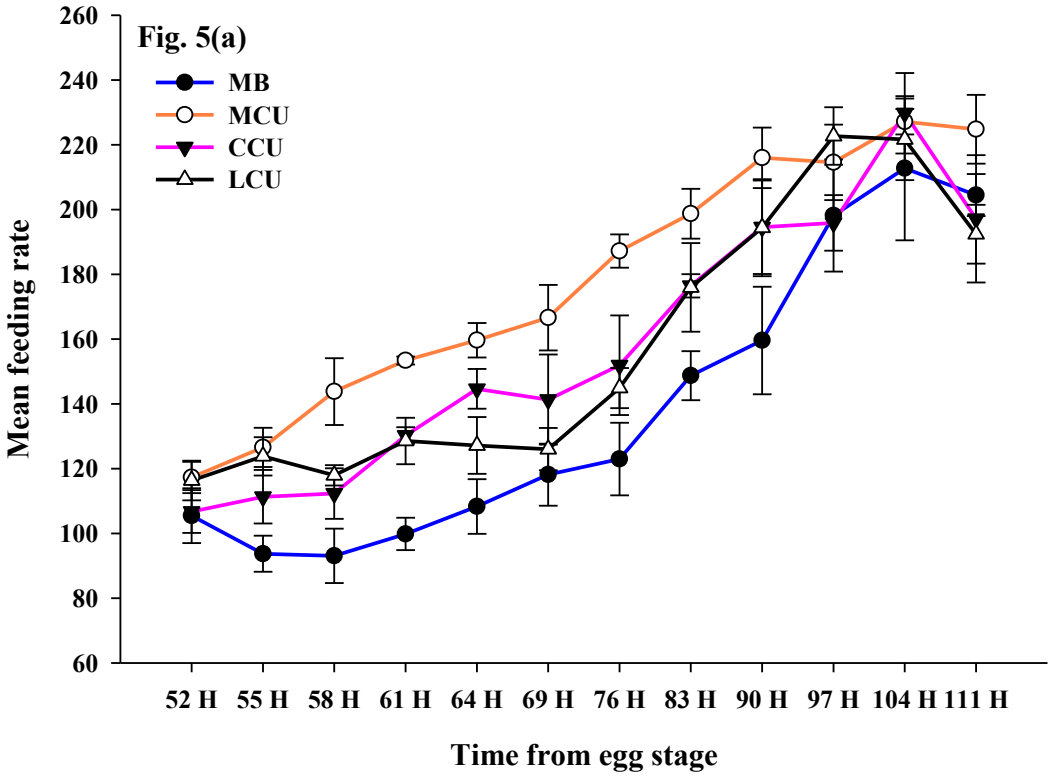
Fig. 5(a)
MB
MCU
CCU
LCU
Mean feeding rate
260
240
220
200
180
160
140
120
100
80
60
52 H
55 H
58 H
61 H
64 H
69 H
76 H
83 H
90 H
97 H
104 H
111 H
Time from egg stage

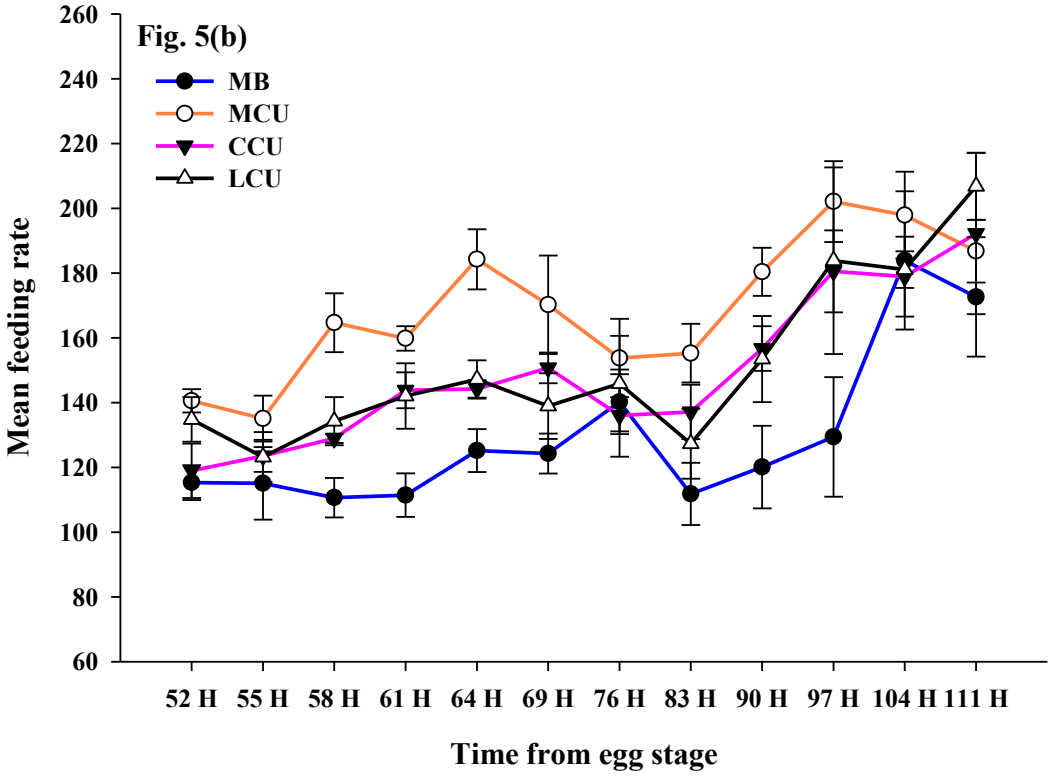
Fig. 5(b)
MB
MCU
CCU
LCU
Mean feeding rate
260
240
220
200
180
160
140
120
100
80
60
52 H
55 H
58 H
61 H
64 H
69 H
76 H
83 H
90 H
97 H
104 H
111 H
Time from egg stage

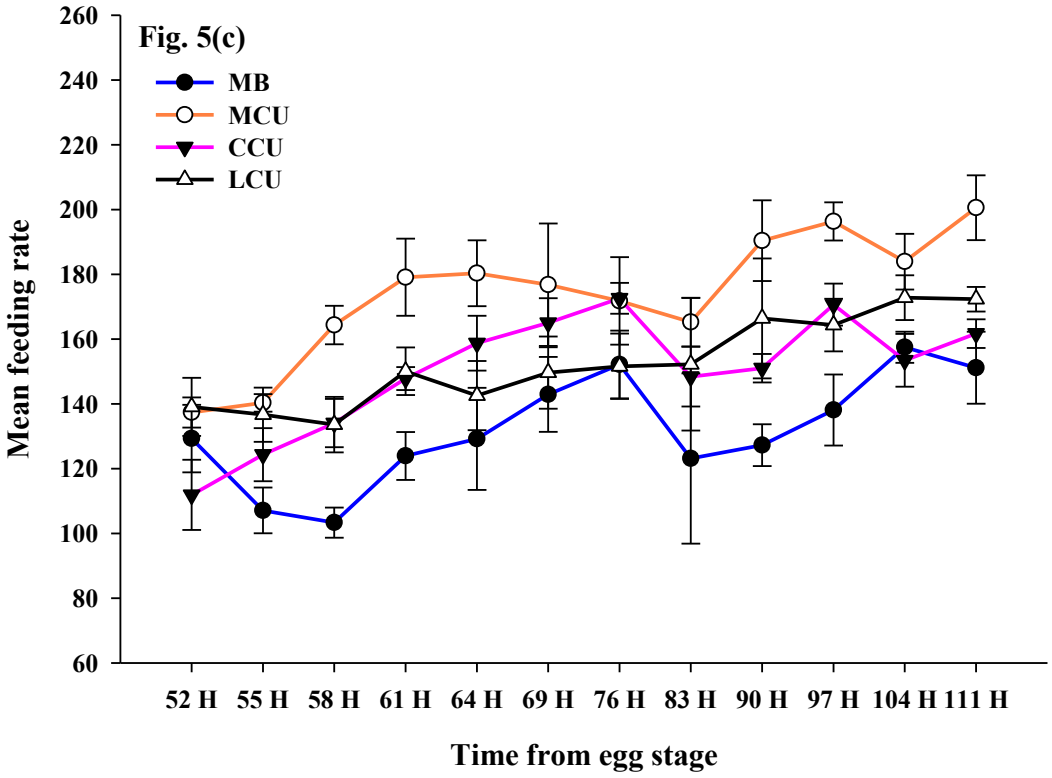
Fig. 5(c)
MB
MCU
CCU
LCU
Mean feeding rate
260
240
220
200
180
160
140
120
100
80
60
52 H
55 H
58 H
61 H
64 H
69 H
76 H
83 H
90 H
97 H
104 H
111 H
Time from egg stage

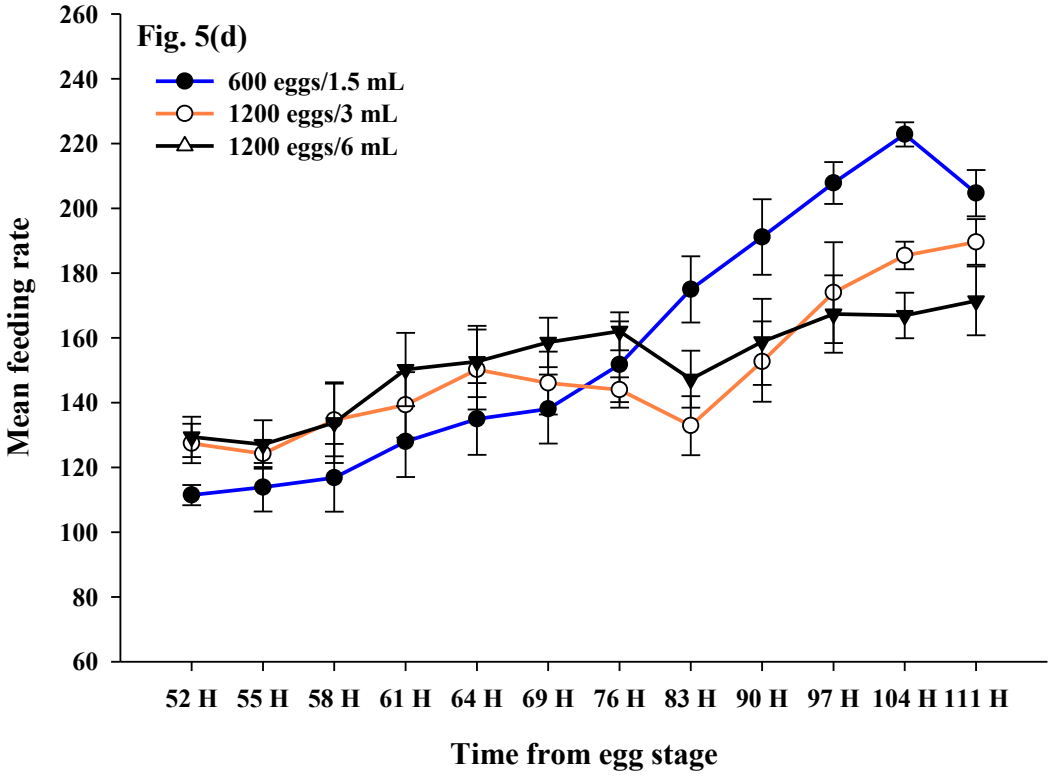
Fig. 5(d)
600 eggs/1.5 mL
1200 eggs/3 mL
1200 eggs/6 mL
Mean feeding rate
260
240
220
200
180
160
140
120
100
80
60
52 H
55 H
58 H
61 H
64 H
69 H
76 H
83 H
90 H
97 H
104 H
111 H
Time from egg stage

Fig. 5 Mean feeding rate of MB, MCU (Gen. 170), CCU (Gen. 49) and LCU (Gen. 49) populations, assayed in three different crowding cultures in vials across 12 time points are shown in each of these populations measured at assay environments (a) 600 eggs/1.5 mL (b) 1200 eggs/3 mL (c) 1200 eggs/6 mL and (d) shows the assay environment and time point interaction averaged across all populations. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

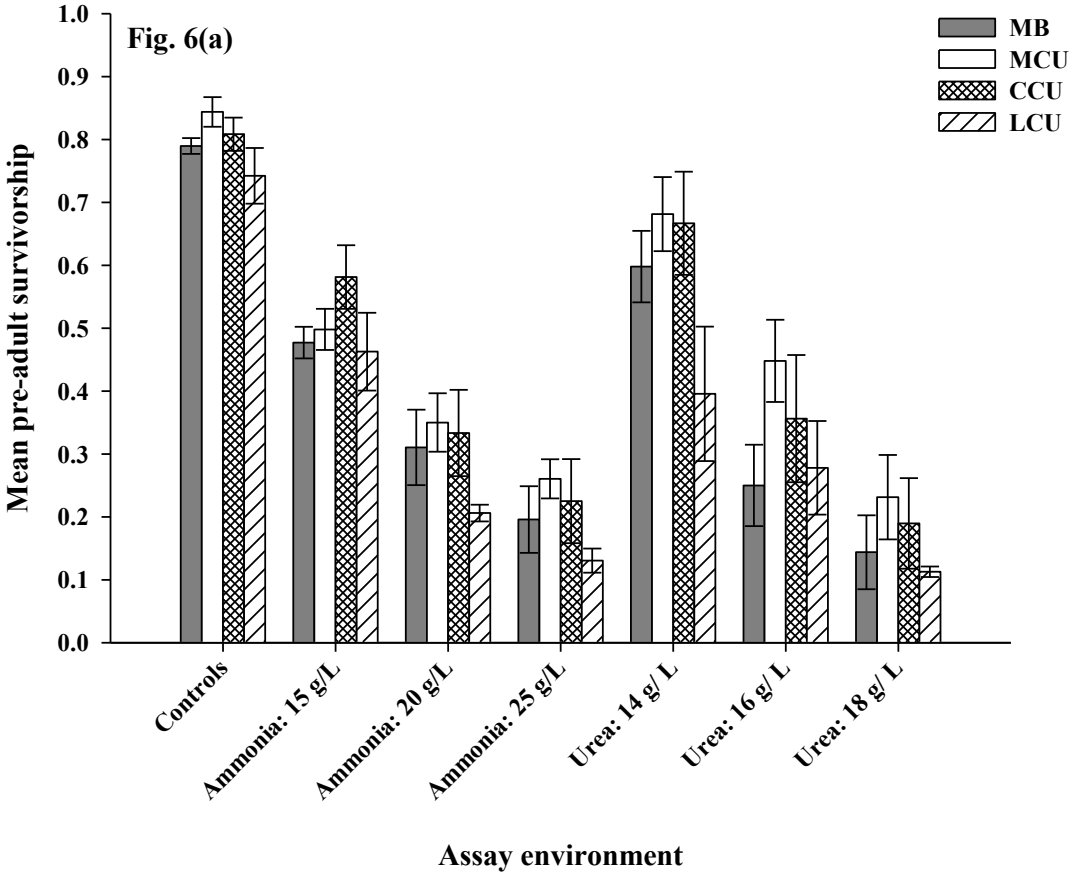
Fig. 6(a)
MB
MCU
CCU
LCU
Mean pre-adult survivorship
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Controls
Ammonia: 15 g/L
Ammonia: 20 g/L
Ammonia: 25 g/L
Urea: 14 g/ L
Urea: 16 g/ L
Urea: 18 g/ L
Assay environment

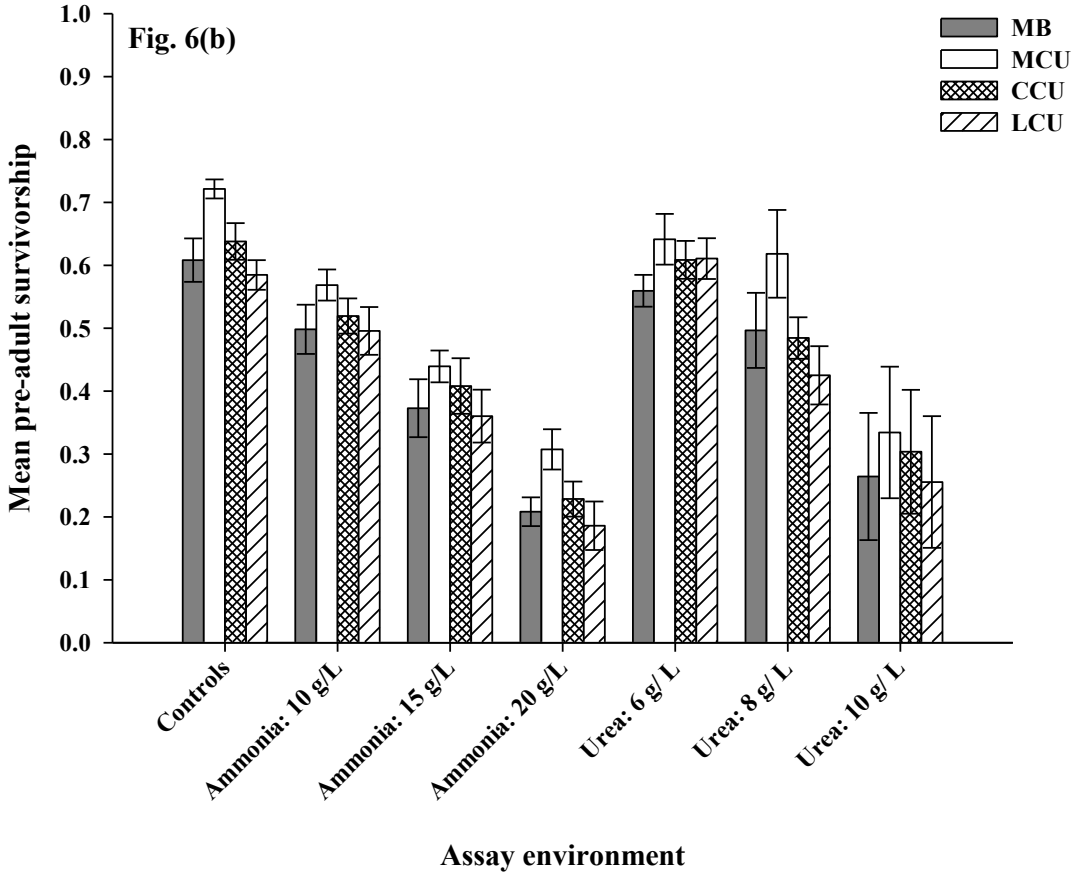

Fig. 6 Mean pre-adult survivorship of MB, MCU (Gen. 136), CCU (Gen. 15) and LCU (Gen. 15) populations at (a) 30 eggs/6 mL food per vial and (b) 300 eggs/1.5 mL food per vial in food, containing 3 different concentrations of ammonia and urea at each assay environment. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

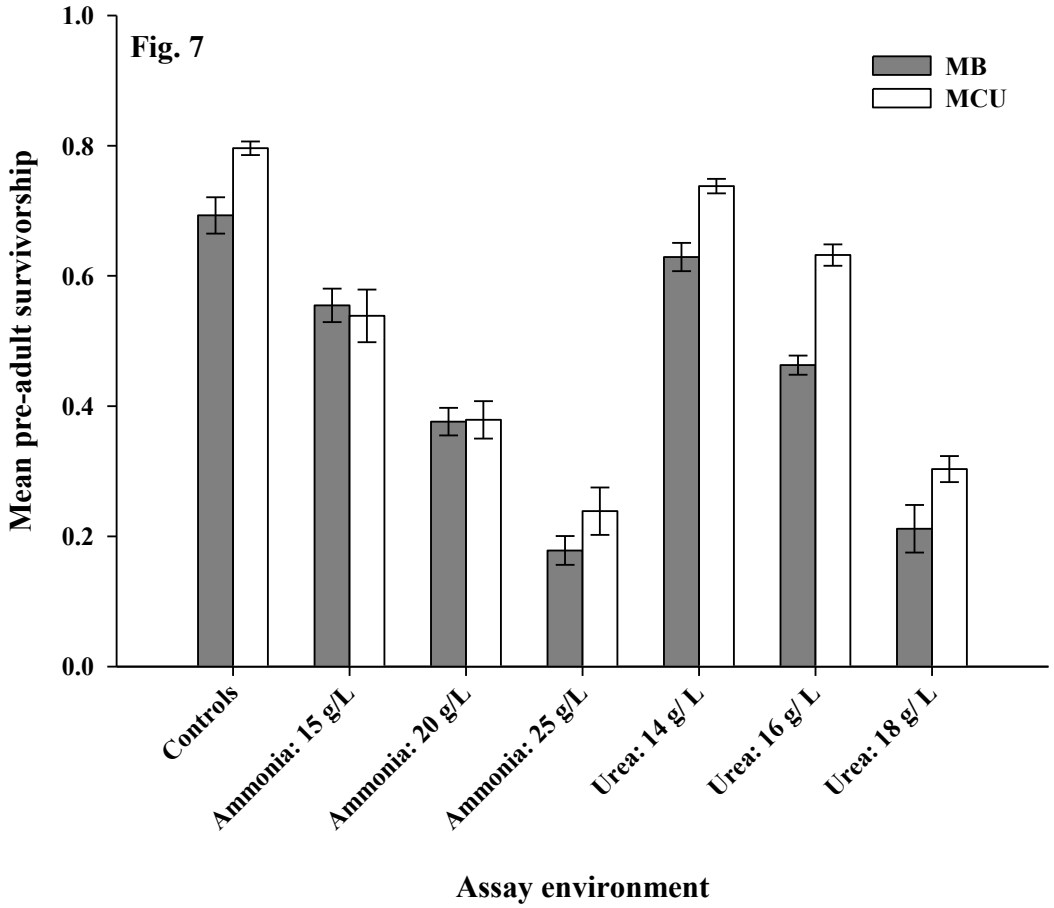

Fig. 7 Mean pre-adult survivorship of MB and MCU (Gen. 143) populations at 70 eggs/6 mL food per vial in food, containing 3 different concentrations of ammonia and urea. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

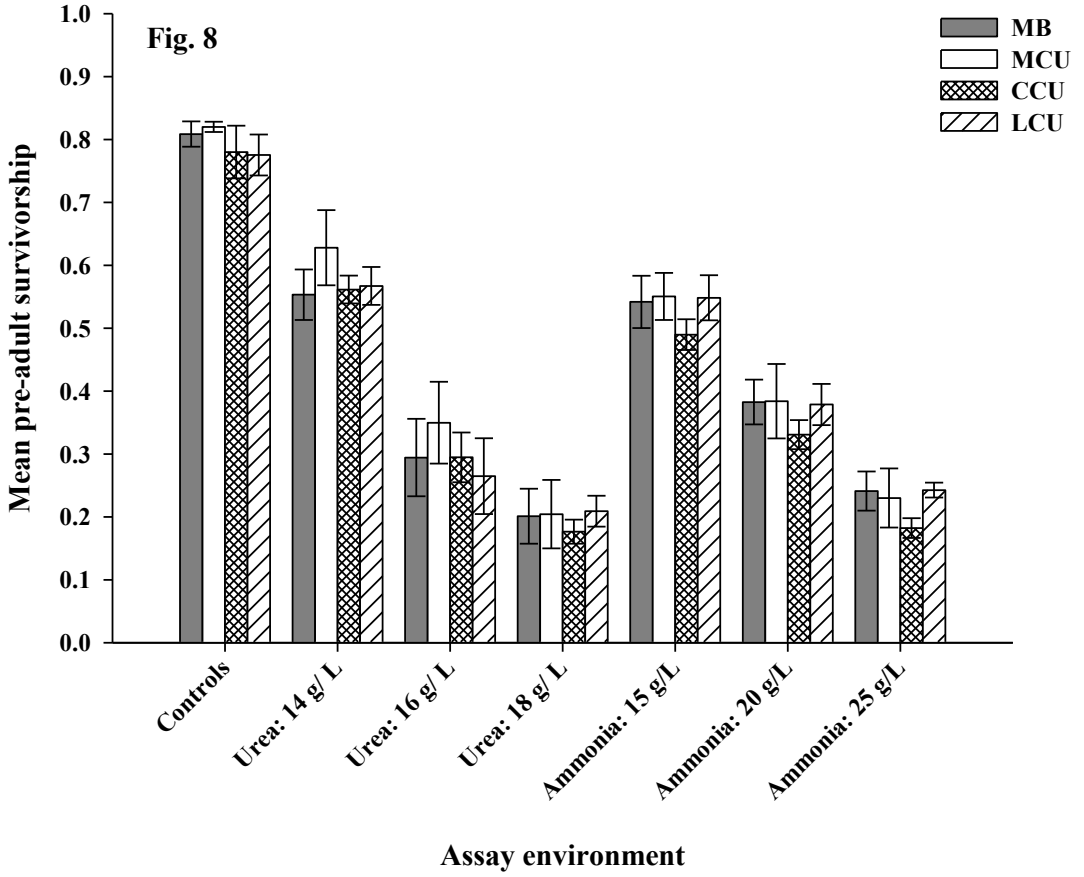

Fig. 8 Mean pre-adult survivorship of MB, MCU (Gen. 157), CCU (Gen. 36) and LCU (Gen. 36) populations at 70 eggs/6 mL food per vial food per vial in food, containing 3 different concentrations of ammonia and urea. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

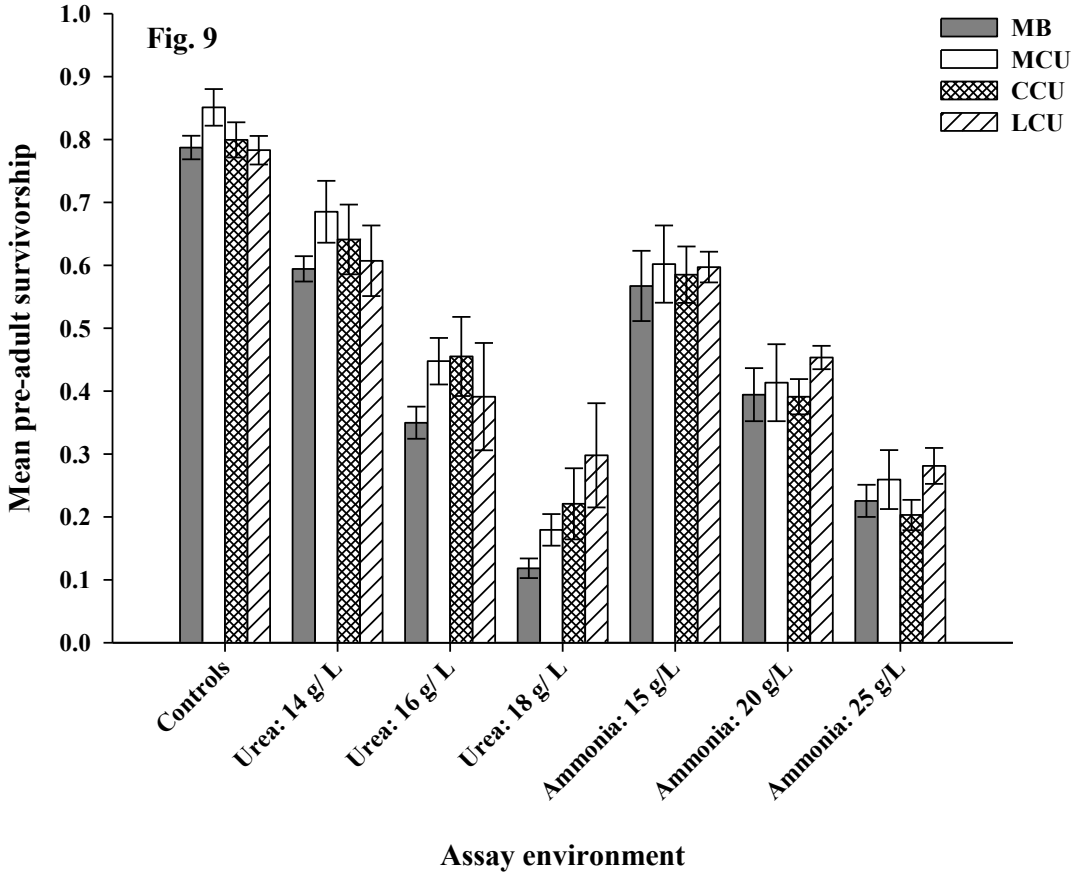

Fig. 9 Mean pre-adult survivorship of MB, MCU (Gen. 177), CCU (Gen. 56) and LCU (Gen. 56) populations at 70 eggs/6 mL food per vial food per vial in food, containing 3 different concentrations of ammonia and urea. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

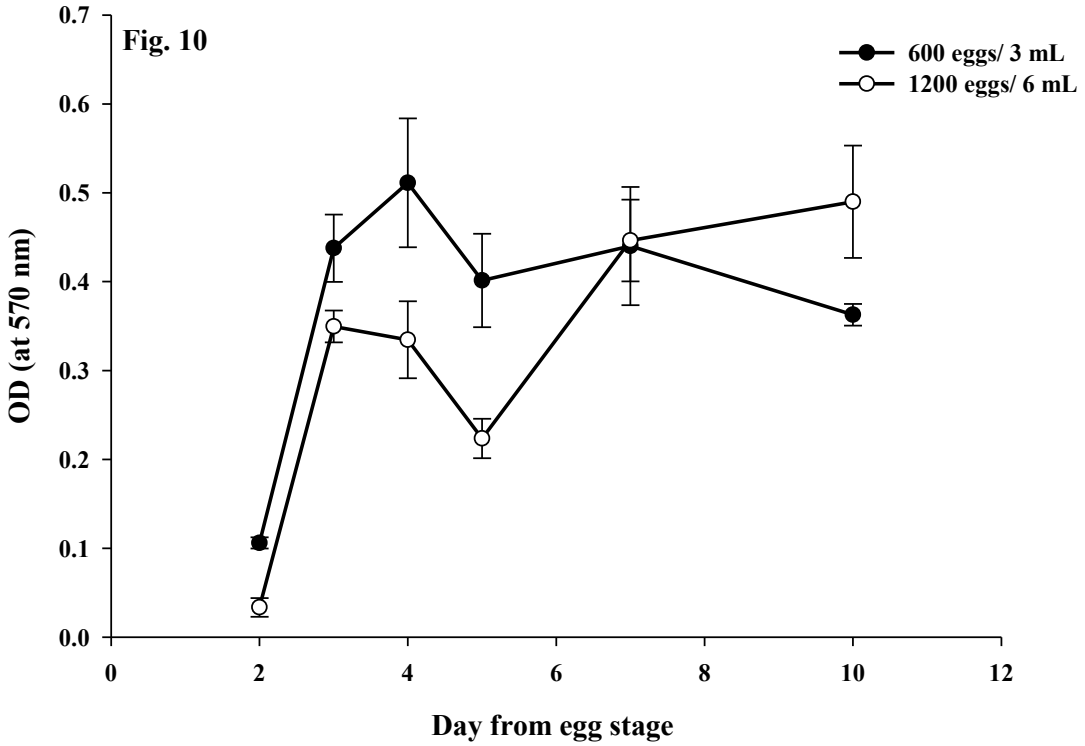

Fig. 10 Nitrogenous waste build up (measured in OD at 570 nm) across day 2, 3, 4, 5, 7 and 10 of culture from egg stage. Error bars are standard errors around the six replicate vial samples at each combination of assay environment and time point.

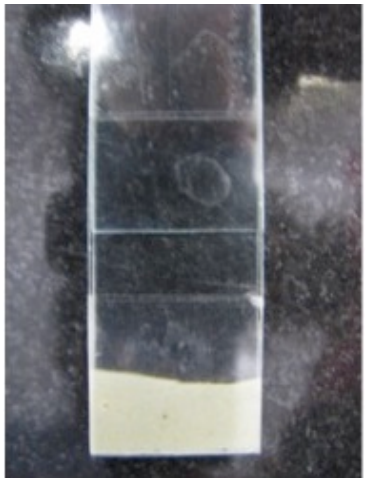

Fig. 11 The above image shows a slial prepared using microscopic glass slides, with cornmeal food inserted into it.

Chapter 5: Quality vs. quantity of food: differential responses in fitness-related traits of crowding adapted populations when assayed on poor quality food

Introduction

Extreme food limitations in quantity or sub-optimal quality of food are stresses often faced by organisms in nature (Hoffman & Parsons 1991). Adapting to such nutritionally stressful environments has been observed to affect various aspects of the life-history of organisms (Hoffman & Parsons 1991, Iason & Van Wieren 1999, Blanckenhorn 2000, Dearing *et al* 2005) The existing literature shows that two different approaches have been taken by various investigators to study this process. One is to focus on physiological or plastic responses to nutritional stress experienced within one's lifetime (Koteja 1996, Gluckman *et al* 2005, reviewed in Rion & Kawecki 2007), while the other is via experimental evolution studies (Chippindale *et al* 1996, Harshman & Schmid 1998, Harshman *et al* 1999, Baldal *et al* 2005, Harbison *et al* 2005, reviewed in Rion & Kawecki 2007, Kolss *et al* 2009, Vijendravarma *et al* 2012 a,b,c). I will focus on the latter.

Previous studies on populations subjected to direct selection for adaptation to chronic malnutrition have shown the evolution of 15% greater egg to adult survival and 17% faster development in poor food as compared to their control populations (Kolss *et al* 2009). Additionally, the selected flies were also significantly lighter than their controls when raised on poor food. Interestingly, the selected populations that had adapted to poor quality food also showed greater larval competitive ability than their controls when assayed in a novel medium under conditions of limited but nutritionally rich food (Vijendravarma *et al* 2012b). Interestingly, selected populations had almost 18% reduction in critical size compared to their controls, which was accompanied by a similar reduction in adult body weight as well (Kolss *et al* 2009, Vijendravarma *et al* 2012c). The selected populations showed shorter

foraging path lengths but no difference in feeding rate compared to their controls, (Vijendravarma *et al* 2012a), although this report used a method to measure feeding rates very different to what has been typically used in studies of adaptation to larval crowding in *Drosophila* (e.g. Joshi & Mueller 1988, 1996; Borash *et al* 2000a; Archana 2010; this thesis). The method of Vijendravarma *et al* (2012a), moreover, results in large among-replicate variation, potentially obscuring biologically relevant differences between selected populations and controls. In addition, the competitive index used by Vijendravarma *et al* (2012a) is not adequate to differentiate between pre-adult survivorship and competitive ability, rendering their findings about feeding rate and competitive ability in their malnutrition-adapted populations difficult to compare directly with other studies on adaptation to larval crowding in *Drosophila*. If the results of Vijendravarma et al (2012a) are in fact comparable to those from the other studies on adaptation to crowding, it would be another example of the correlation between feeding rate and competitive ability being context-specific.

On a similar note, extreme food limitation can also be viewed as a different form of nutritional stress where competition among individuals for limiting but otherwise nutritious food is the central focus. Experimental evolution studies on *Drosophila* involving selection for adaptation to larval crowding have provided some interesting results. Previous studies have shown that *Drosophila* cultures at high larval density evolved greater competitive ability (Mueller 1988), increased larval feeding rate (Joshi & Mueller 1988, 1996), increased pupation height (Mueller & Sweet 1986, Joshi & Mueller 1993), reduced food to biomass conversion efficiency (Mueller 1990, Joshi & Mueller 1996), and greater tolerance to toxic levels of urea and ammonia (Shiotsugu *et al* 1997, Borash *et al* 1998, Borash *et al* 2000b, Borash &

Ho 2001). More recent studies suggested that there are alternative routes to the evolution of adaptation to larval crowding in *Drosophila* populations. *D. ananassae, D. n. nasuta* and *D. melanogaster* when subjected to larval crowding but with very limited food (unlike the food level in cultures of the previous studies), did not evolve greater feeding rates and tolerance to nitrogenous wastes (Nagarajan *et al* 2016, Sarangi *et al* 2016). Rather, they evolved shorter egg to adult development time when assayed at both high and low densities. Egg to adult survivorship was higher than their respective controls for all the three species. So, here it can be emphasized that high larval density in limited amount of food can be seen as nutrition deficiency, both from the low availability of food and high levels of waste point of view. In contrast, a situation where stress is provided by diluting the nutritional value of the food given in the culture, running out of food over time is not the problem, rather individuals have to complete their development and eventually reproduce despite ongoing 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 in limited but normal food. Here, I ask whether the evolution of higher competitive ability in populations of *D. melanogaster* selected for adaptation to larval crowding also result in the correlated evolution of increased survival ability in poor nutrient medium. I show that the MCU populations have in fact reduced egg to adult survivorship when assayed on poor quality larval food. In addition, they show prolonged pre-adult development and significant reduction in their body weight at eclosion when reared on poor quality food.

Materials and Methods

Study populations

All the experiments discussed in this chapter were carried out using all four replicate populations each of the MBs and MCUs. The MCU populations had undergone over 127 generations of selection at the time of these assays.

Assay environment and Standardization

Experiments were carried out on cornmeal medium, which was different in its constitution from the cornmeal medium used for the regular maintenance of the MB and MCU populations. For the experiment, 1 L of standard cornmeal medium was prepared using 50 g cornmeal, 15 g agar, 60 g glucose, 30 g sucrose, 12.5 g dry yeast, 0.5 g $MgSO_4$, 0.5 g $CaCl_2$, 30 mL ethanol, 6 mL propionic acid and 1 g methyl-p-hydroxybenzoate in 1 L of water. The poor food medium had the same composition, but only one-fourth the quantities of cornmeal, sugar and yeast per 1 L of water, as compared to the standard medium.

Prior to the assays, both the control and selected populations were subjected to common rearing conditions (the novel standard cornmeal medium) for two full generations to eliminate any non-genetic parental effects. I collected 70 ± 10 eggs per 8-dram vial in 6 mL of food, and 40 such vials were set up per population. Eclosing adults were collected into cages on the 11^{th} day from egg collection, followed by 3 days of being provided with food supplemented with live yeast-acetic acid paste. Eggs laid by second generation standardized flies over a window of 3 h were collected in batches of 200 each and were dispensed into plastic bottles (10 cm length × 5 cm diameter) containing 30 mL of either standard or poor food. There were 4 replicate

bottles for each combination of population and food type. All assays were conducted at 25 ± 1°C, under constant light and ~90 % relative humidity.

Egg to pupa survivorship and development time

Culture bottles were monitored in a regular manner for the appearance of the first pupae. From that time point onwards, the number of new pupae on the walls of each bottle was recorded at 6 h intervals till no more pupae were formed for 24 h. From these primary data, egg to pupa survivorship and development time were calculated.

Egg to adult survivorship and development time

After the pupae darkened, the culture bottles were checked every 6 h and the number of eclosing adults of each sex noted till no adults eclosed for a 24 h period. From these primary data, egg to adult survivorship and male and female egg to adult development time were calculated.

Dry weight of freshly eclosed adults

The freshly eclosed adults that were sexed and collected during the course of the experiment were dried in a hot air oven at ~70 °C for 36 h. They were then weighed in 3 batches of either 5 males or 5 females per population per food type using a Sartorius (CP225D) fine balance.

Statistical Analysis

For all traits considered here, mixed-model analysis of variance (ANOVA) was performed. For survivorship data, the mean survivorship values were arcsine squareroot transformed prior to doing ANOVA. Selection, type of food and sex (in case of development time and dry weight at eclosion) were treated as fixed factors, and population means of all the traits were considered for the analyses. The fixed factors were crossed with one another and with random blocks, representing ancestry

(MB and MCU populations with same numerical subscripts were treated as random blocks). All of ANOVA statistics were performed on STATISTICA^{TM} for Windows Release 5.0B (Statsoft Inc. 1995). Multiple comparisons were carried out using Tukey's honest significant difference test (HSD) at 0.05 level of significance.

Results

Egg to pupa survivorship

The MB and MCU populations did not differ significantly from one another in mean egg to pupa survivorship in either standard or poor food. There was also no difference in mean egg to pupa survivorship between the two food types (Fig. 1(a)). The ANOVA also revealed no significant effects of selection, food type, or their interaction (Table 1). However, there did appear to be a tendency for the MCU survivorship to be lower at poor food as opposed to standard food (Fig 1(a)).

Egg to pupa development time

Mean egg to pupa development time, overall, was significantly affected by food type (Table 1), but the effect was similar on both the MB and MCU populations, which, moreover, did not differ much among themselves (Fig. 1(b)). The ANOVA, too, revealed no significant main effect of selection, nor a significant interaction of selection and food type (Table 1). There was, again, a slight tendency for mean MCU egg to pupa development time to be more affected by poor than standard food, as compared to the MB populations.

Egg to adult survivorship

Mean egg to adult survivorship of both the MB and MCU populations declined in poor food compared to standard food (Fig. 2(a)), with the decline being much larger and statistically significant (Tukey's HSD test) in the case of the MCU

populations. The ANOVA revealed both a significant main effect of food type and a significant interaction between food type and selection, but no significant main effect of selection (Table 2).

Egg to adult development time

Overall, mean egg to adult development time was considerably prolonged by poor food across both sexes and selection regime (Fig. 2(b)), though the main effect of food type was only marginally significant ($p = 0.051$; Table 2). The ANOVA also revealed significant effects of sex and the interaction of sex and food type (Table 2), driven by a greater development time in females than males under poor food conditions in both selection regimes (Fig. 2(b)). Compared to MB males and females, the MCU males and females both showed a significantly greater prolongation of development in poor food, compared to standard food, although the effect was more pronounced in the case of females (Fig. 2 (b)). This pattern of differences drove significant ANOVA effects of the interaction betweem selection and food type and the three-way interaction between selection, sex and food type (Table 2).

Dry weight at eclosion

Overall, the mean dry weight at eclosion in MB populations was significantly greater than the selected MCU populations. Mean dry weight at eclosion of females was significantly greater than males, and mean dry weight at eclosion in poor food was significantly less than that in standard food (Fig. 3). This pattern was reflected in significant main effects of selection, food type and sex in the ANOVA (Table 3). Additionally, a significant interaction of sex and food type in the ANOVA (Table 3) was driven by the reduction due to poor food in dry weight at eclosion being significantly greater in females than in males in both MB and MCU populations (Fig. 3).

Discussion

I undertook this experiment because of an earlier report that *D. melanogaster* populations adapted to abundant amounts of poor nutritional quality food also evolved greater ability to compete for limiting but high quality food as a correlated response to selection (Vijendravarma *et al* 2012b). Examining the symmetry of correlated responses to selection can often provide insights into the underlying genetics of fitness related traits and whether the responses to selection in different contexts are affected by gene × environment interactions (Bohren *et al* 1966, Shiotsugu *et al* 1997). The results of this experiment clearly indicate asymmetry in correlated responses to selection for adaptation to limiting but nutritionally rich food as compared to abundant but nutritionally poor food. The MCU populations, adapted to larval crowding in which they have a rich source of food but in a very limited quantity, were not able to perform as well as their ancestral control populations (MBs) in a poor nutritional quality larval food medium, even though it was available in abundance ample amount.

Specifically, the MCU populations overall showed a prolonged egg to adult development time when assayed in poor food, with females in particular taking longer time to develop than males (Fig. 2(b)). This stands in contrast to what was observed earlier in the MCU populations on nutritionally rich food, where they exhibit reduced pre-adult development time, relative to controls, including a reduction in the minimum larval feeding duration (Sarangi *et al* 2016). But, interestingly, this is concordant with earlier anecdotal observations that males tend to develop faster under conditions of nutritional stress, the mechanisms being unknown. On the other hand, populations selected for adapting to poor quality food showed faster development

compared to their control populations when assayed on poor food (Kolss *et al* 2009). Moreover, in the MCU populations, the prolongation of pre-adult development time on poor food was significant only in the pupal stage since the mean egg to pupa development time was similar between MB and MCU populations in either type of food medium (Fig. 1(a)). The process of metamorphosis occurs during this stage making it quite crucial for the overall development of the individual as such; therefore, rearing on poor food probably affects some aspects of pupal metabolism that eventually delays their development to adult.

Egg to adult survivorship in MCUs in poor food was lower as compared to standard food (Fig. 2(a)), once again contrasting with what was seen in populations adapted to poor quality food, which showed higher egg to adult viability than controls when raised on poor food (Kolss *et al* 2009). Indeed, the selected populations of Kolss *et al* (2009) showed evolution of faster pre-adult development and simultaneous evolution of enhanced egg to adult viability in poor food condition, circumventing a well documented trade-off between these two traits in high quality food (Prasad *et al* 2001, Chippindale *et al* 1997, Sarangi *et al* 2016). Moreover, I also observed a significant reduction in body weight at eclosion of MCU populations in poor food compared to standard food and the reduction in body weight was much more severe in females, as compared to males (Fig. 3). This was also true in case of the poor food adapted populations (Kolss *et al* 2009).

The food medium in which the experiments were carried out during this study was novel to both the control and selected populations. Although, prior to the assays, both control and selected populations had undergone two complete generations of common rearing conditions in the novel standard type of food medium, it is possible, even if unlikely, that some of my results are an artifact of different responses to a

novel medium in the MB and MCU populations. The present study cannot rule out this possibility. Overall, though, the results of this study suggest that the manner in which selection acts on populations adapting to extreme food limitation is very different from how it acts on populations adapting to poor quality but abundant food, although the precise mechanisms and traits mediating this asymmetry in correlated responses remain unknown at this time.

Tables

Table 1 Results of three-way ANOVA on egg to pupa survivorship and development time of MB and MCU populations in standard and poor food. Selection regime and food type were treated as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	*df*	MS	*F*	*P*
Survivorship				
Selection	3	0.016	3.376	0.163
Food type	3	0.020	1.001	0.390
Selection × Food type	3	0.003	4.941	0.112
Development time				
Selection	3	1516.128	0.008	0.930
Food type	3	1717.205	74.794	0.003
Selection × Food type	3	682.396	3.429	0.161

Table 2 Results of ANOVA on egg to adult survivorship and development time of MB and MCU populations in standard and poor food. Selection regime and food type were treated as fixed factors and block as a random factor for survivorship; selection regime, food type and sex were treated as fixed factors and block as random factor for development time. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	*df*	MS	*F*	*P*
Survivorship				
Selection	3	0.025	0.062	0.819
Food type	3	0.030	11.928	0.040
Selection × Food type	3	0.005	12.081	0.040
Development time				
Selection	3	3449.181	0.424	0.561
Food type	3	114.816	9.819	0.051
Sex	3	4634.518	64.330	0.004
Selection × Food type	3	23.032	23.737	0.016
Selection × Sex	3	3769.394	2.981	0.182
Food type × Sex	3	68.315	19.866	0.021
Selection × Food type × Sex	3	41.922	18.360	0.023

Table 3 Results of ANOVA on mean dry weight at eclosion in the MB and MCU populations in standard and poor food. Selection regime, food type and sex were treated as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions cannot be tested for significance and are therefore omitted from the table.

Effect	*df*	MS	*F*	*P*
Selection	3	4.884	31.683	0.011
Food type	3	0.909	1502.582	<0.001
Sex	3	1.785	136.005	0.001
Selection × Food type	3	2.496	0.121	0.750
Selection × Sex	3	0.815	1.878	0.264
Food type × Sex	3	1.592	48.770	0.006
Selection × Food type × Sex	3	0.706	0.724	0.457

Figures

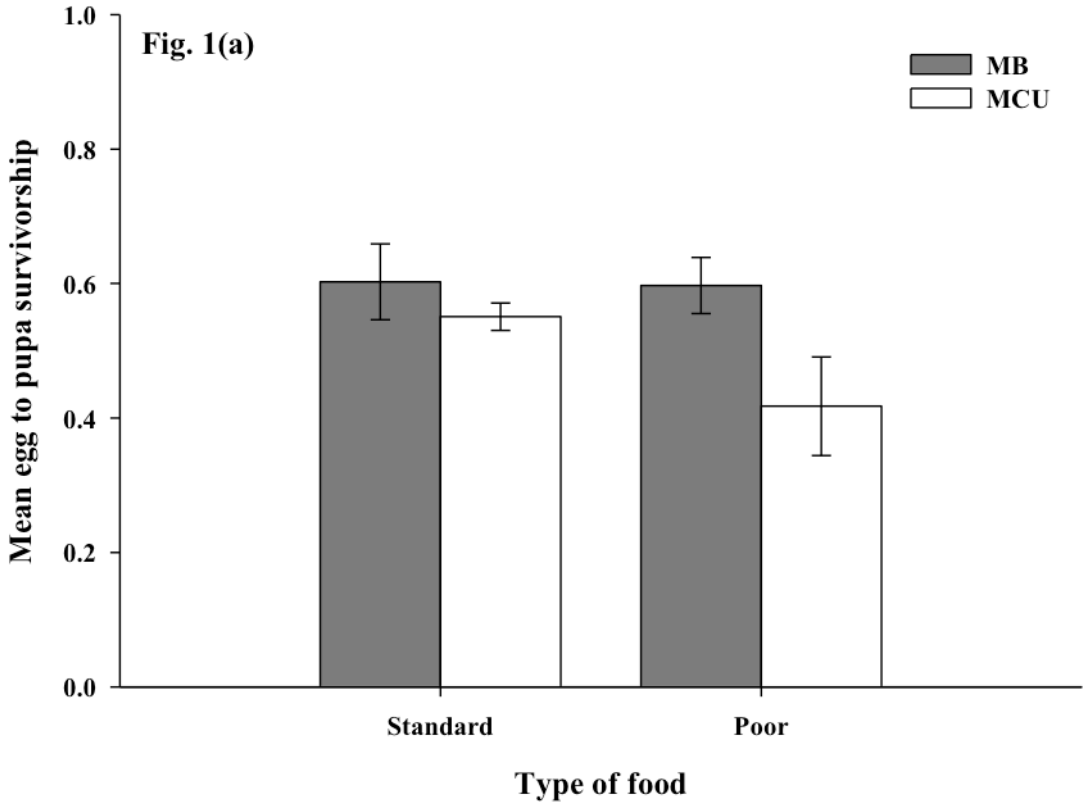
Fig. 1(a)
MB
MCU
Mean egg to pupa survivorship
1.0
0.8
0.6
0.4
0.2
0.0
Standard
Poor
Type of food

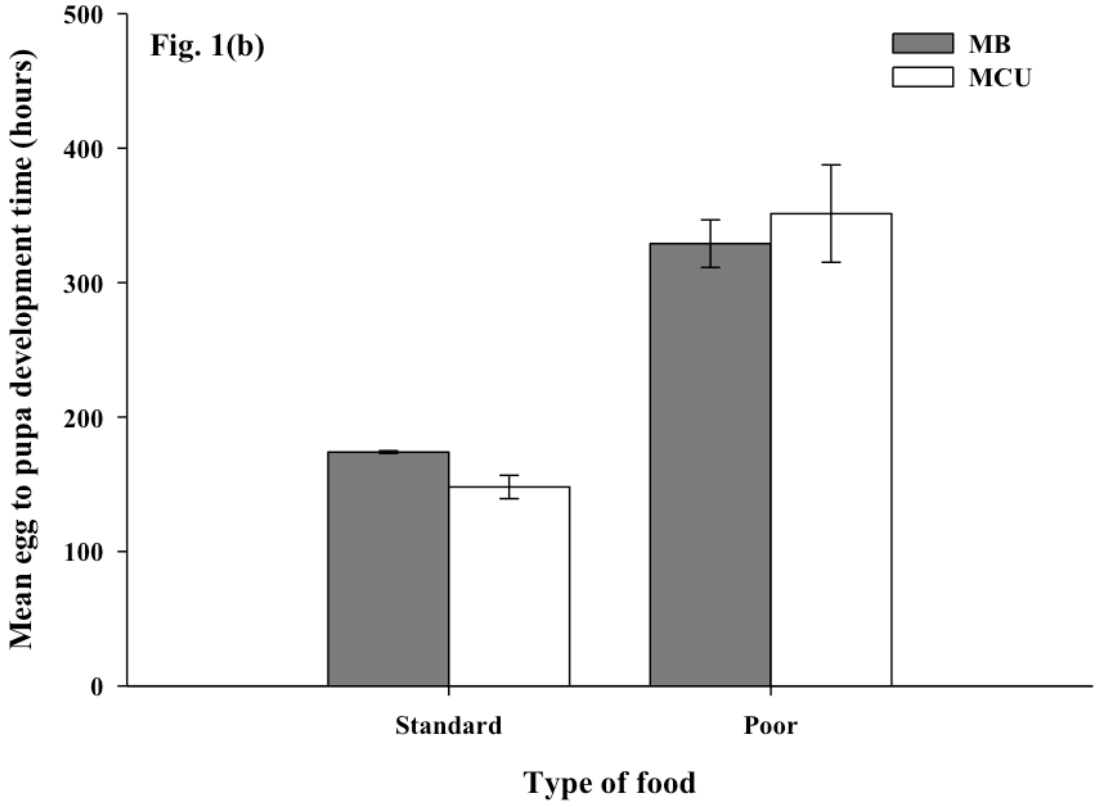
Fig. 1(b)
MB
MCU
Mean egg to pupa development time (hours)
500
400
300
200
100
0
Standard
Poor
Type of food

Figure 1. Mean egg to pupa (a) survivorship and (b) development time of MB and MCU populations in standard and poor food. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

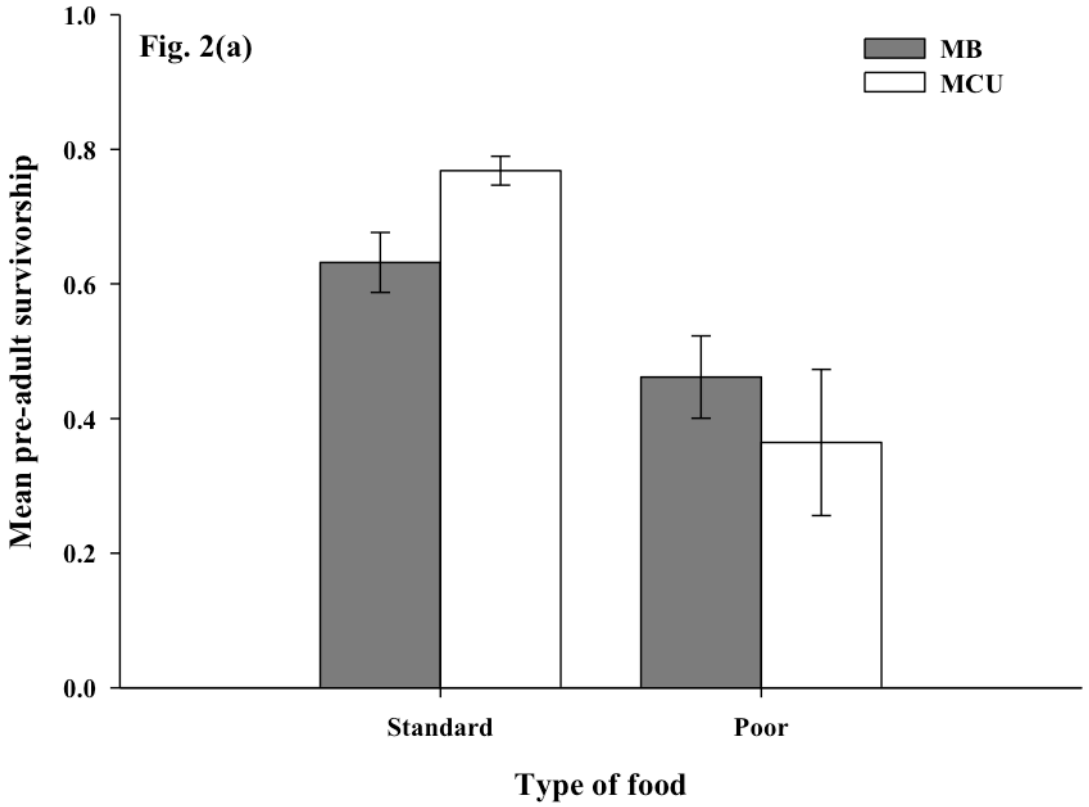
Fig. 2(a)
MB
MCU
Mean pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
Standard
Poor
Type of food

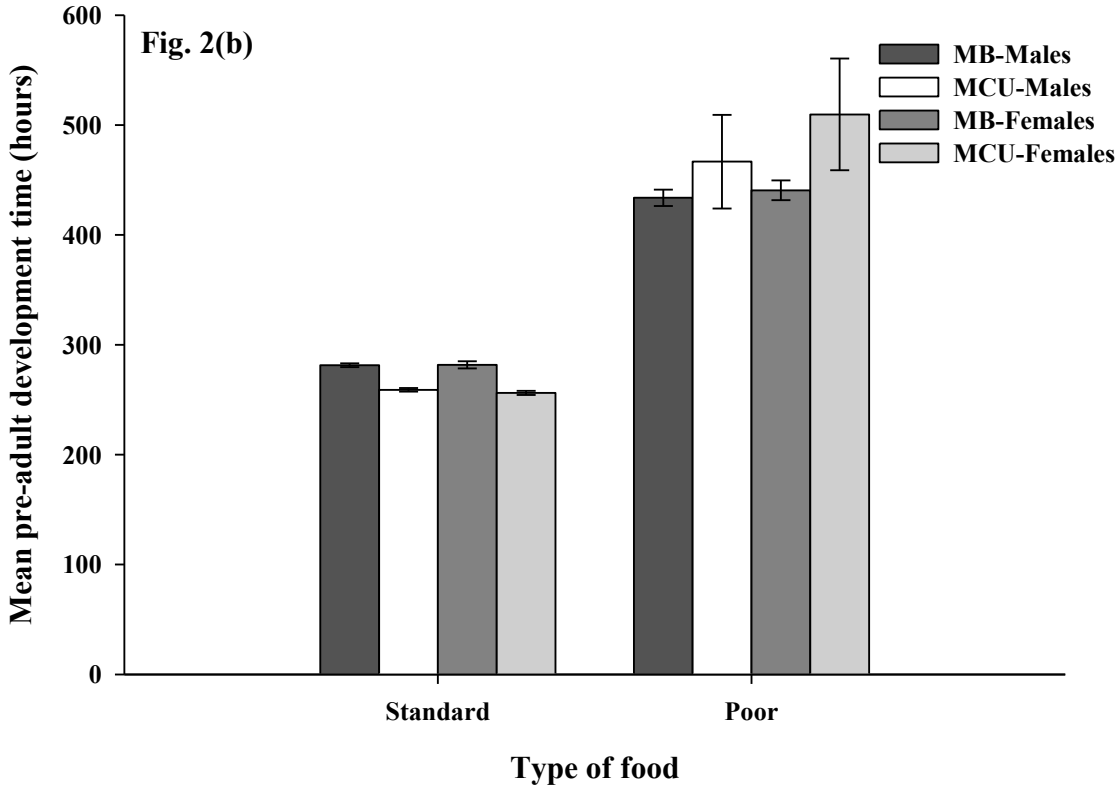
Fig. 2(b)
MB-Males
MCU-Males
MB-Females
MCU-Females
Mean pre-adult development time (hours)
600
500
400
300
200
100
0
Standard
Poor
Type of food

Figure 2. Mean egg to adult (a) survivorship and (b) development time of MB and MCU populations in standard and poor food. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

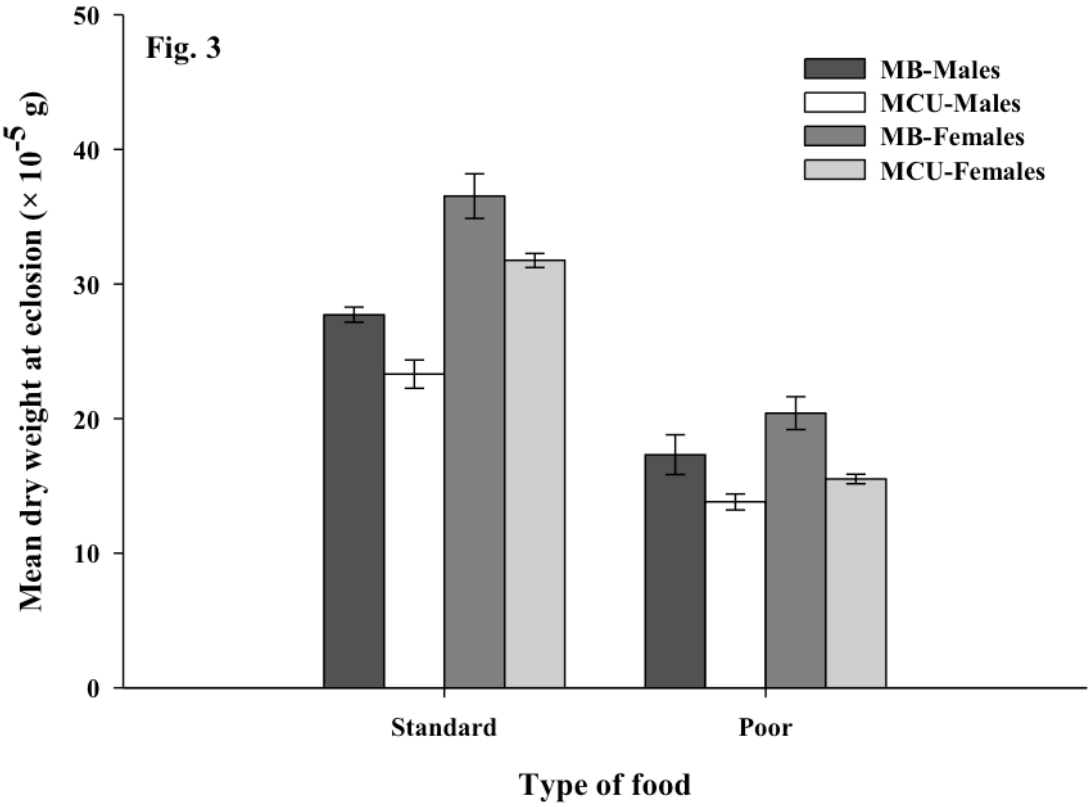

Figure 3. Mean dry weight of freshly eclosed adults of MB and MCU populations in standard and poor food. Error bars are the standard errors around the means of the four replicate populations in each selection regime.

Chapter 6: Conclusions and future directions

The broad aim of the studies reported in this thesis was to investigate some specific aspects of a tentative explanation built up in my Masters' research (Sarangi 2013, Sarangi *et al* 2016) about why the set of traits seen to evolve under larval crowding in *Drosophila* was different between L. D. Mueller's CU populations (Joshi & Mueller 1996, Borash *et al* 1998) and the NCU, ACU and MCU populations in our laboratory (Nagarajan *et al* 2016, Sarangi *et al* 2016). The tentative explanation was based on the notion that in a crowded culture with a large absolute amount of food, there is quite a lot of food below the feeding band into which metabolic waste could diffuse, thereby resulting in a relatively slow build up of metabolic waste within the feeding band itself. By contrast, crowded cultures with small amounts of food essentially consist largely of just a 1 cm deep feeding band, which consequently can accumulate waste at high concentrations even in the earlier stages of a culture. It was, therefore, suggested that evolution at high larval density, but in relatively large amounts of food (as in the CUs), would facilitate the evolution of increased larval feeding rates and metabolic waste tolerance, whereas evolution at high larval density, but in relatively small amounts of food (as in the MCUs), would proceed via the evolution of greater efficiency of food to biomass conversion and rapid development, involving no evolutionary change in larval feeding rate or waste tolerance. Consequently, for my PhD research, I established two new selection regimes, the CCU and LCU populations, which were also subjected to larval crowding at 1200 eggs/3 mL food per vial and 1200 eggs/6 mL of food per vial, respectively. Thus, the CCUs had the same larval density as the MCUs, but twice the amount of food, whereas the LCUs approximated the egg number and food amount combination experienced by the CUs. The results reported in Chapter 2 clearly show the evolution of greater larval competitive ability in these new sets of crowding adapted

populations, setting the stage for further investigations of correlated responses to selection in these populations, along with the MCUs and MBs. In this chapter, I briefly discuss the major implications of the findings that emerged from several experiments on these three sets of crowding adapted populations, and their ancestral MB controls.

One aspect of interest when linking adaptations to crowding to population dynamics is that of an *r-K* trade-off (Dey *et al* 2012). Of course, the CCU and LCU populations are still at relatively early stages of selection and fitness-related trade-offs are often seen only after selection has progressed substantially. Earlier, after 82 generations of selection, the MCUs actually showed greater pre-adult survivorship than the MB controls at both low and high densities (Sarangi *et al* 2016), whereas in the present studies they do not significantly differ from controls in survivorship at low density (Chapters 2,3). It now appears as though the MCU populations might be evolving greater population stability via an *r-K* trade-off, in experiments wherein derivatives of these populations were allowed to grow freely without egg or adult density being controlled (Neha Pandey & Amitabh Joshi, *unpubl*. *data*), something also earlier seen in the *D. ananassae* ACU populations, subjected to larval crowding in a manner very similar to the MCUs (Dey et al 2012). By contrast, the CU populations showed no evidence of an *r-K* trade-off (Borash & Ho 2001) or greater population stability (Mueller *et al* 2000). It would, therefore, be interesting to conduct population dynamics experiments to see whether the LCU populations evolve an *r-K* trade-off and greater population stability in the future.

The experiments discussed in Chapter 3 clearly confirm, in greater detail and with more populations, the earlier observation (Sarangi 2013) that crowded cultures of *Drosophila* with different combinations of egg number and total food can actually have very different effects on traits closely related to fitness, such as pre-adult survivorship and the distribution of development time and weights of flies eclosing at different time points. They also underscore the fact that considering larval density alone as a major surrogate for the strength of competition may not be advisable. For example, even though larval density was identical between MCU-type (600 eggs/1.5 mL food) and CCU-type (1200 eggs/3 mL food) cultures, there were large differences between these two assay environments in pre-adult survivorship, and the means and variances of pre-adult development time and body weights at eclosion. It is now clear, from the earlier work of Sarangi (2013) and the experiments reported in this thesis that larval density by itself is not a very good indicator of how a population will evolve to adapt to chronic larval crowding and that, in particular, it is a combination of effective larval density in the feeding areas of a food patch, and the total amount of substrate available for metabolic waste to diffuse into, that will have a major effect on which traits respond to selection under chronic crowding. This insight adds a new layer of subtlety to our understanding of the possible evolutionary consequences of predominantly scramble competition in species inhabiting discrete food patches and is, therefore, potentially relevant to many invertebrate species.

The results on feeding rate in the MCU, CCU, LCU and MB populations under different assay conditions similarly add a new dimension to how we study the role of this potentially very important trait in the evolution of competitive ability. In particular, observation that feeding rate differences between selected populations

(MCU, CCU and LCU) and controls (MB) are phenotypically plastic to varying degrees, such that the fact that MCUs are actually the fastest feeders of the lot, especially during the third instar, is only seen when feeding rates are measured in the culture vials and not when measured in Petri-dishes on single larvae, which has been the standard feeding rate assay technique since the 1970s. This is important because feeding rate is a very important correlate of competitive ability in *Drosophila*, and has long been used as a surrogate for competitive ability. Given the centrality of competition to evolutionary ecology and of feeding rates to competitive ability in organisms with scramble competition, it is important to now try and understand factors that affect the plastic feeding rate response and why the degree of plasticity in the feeding rates may be different in populations adapted to crowding at different levels of egg number and food amount. It will be particularly important to assess the role that urea/ammonia levels might be playing as a marker of the imminent running out of food running in certain types of crowded cultures but not others, for example. The roles of immediate crowding, and perhaps touch, in mediating a plastic feeding rate response may also be worth investigating.

The lack of evolution of enhanced urea or ammonia tolerance in the MCU, CCU and LCU populations, and in the earlier studied ACU and NCU populations (Nagarajan *et al* 2016) remains mysterious at this point, especially given the earlier observations of the evolution of greater metabolic waste tolerance than controls in the CU populations (Shiotsugu *et al* 1997, Borash *et al* 1998). Of course, in addition to differences in egg number and food amount between the CU and ACU, NCU and MCU populations, there was also a consequential difference in how eclosing adults from the crowded larval culture vials were collected. In the CU populations, the initial

collection of eclosing adults was into vials for the first few days whereas in all the crowding adapted populations in our laboratory (including the LCUs), the eclosing adults are directly collected into cages. It has been shown earlier that this kind of vial to vial collection of eclosing adults increases the frequency of assortative mating for development time compared to a maintenance regime wherein eclosing adults are directly transferred to cages, and it was argued that it was this assortative mating that facilitated the earlier seen polymorphism of early eclosing fast feeders and late eclosing waste tolerant flies (Borash *et al* 2000b) in the CU populations (Archana 2010). It is possible that the response to selection for metabolic waste tolerance in the CU populations was enabled in part by assortative mating and this is something that could be examined theoretically. It would also be good to have an empirical feel for how exactly urea/ammonia levels build up in the feeding band of different kinds of crowded cultures, and how toxic those levels are. Just looking at the level of pre-adult mortality induced by crowding and by the substantially higher mortality seen at the highest levels of urea/ammonia in the waste tolerance assays suggests that the waste levels in crowded cultures may not be extremely toxic, perhaps indicating relatively weak selection for waste tolerance under crowding, the evolutionary response to which that got amplified by assortative mating in the CU populations.

The observation that the MCU populations perform worse than the MBs when reared on abundant but poor nutritional quality food suggests that the physiological mechanisms for dealing with nutritional stress under crowding versus due to poor food may be quite different in *Drosophila*. Asymmetric correlated responses to selection are often indicators of complex underlying genetic architecture and genotype-by-environment interactions. The fact that selection for adaptation to

abundant poor quality food leads to the correlated evolution of greater ability to compete for limiting rich food (Vijendravarma *et al* 2012b), but not vice versa, suggests that there may be a lot more to understand in the ecology and genetics of how populations adapt to different kinds of nutritional stress.

Competition, whether within- or between-species, is an important ecological phenomenon that also has varied evolutionary consequences. The work reported in this thesis suggests that we need to go beyond considerations of just density when evaluating the evolutionary consequences of varied forms of competition. We also need to examine in some detail how seemingly trivial aspects of the ecological backdrop against which crowding is experienced can markedly affect the precise manner in which a population evolves to adapt to crowding and become more competitive.

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