Investigations on Density-Dependent Selection and its Effects on Population Dynamics and Stability in Fruitflies

A thesis submitted for the degree of

Doctor of Philosophy

by

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CERTIFICATE

This is to certify that the work presented in this thesis titled "**Investigations on density-dependent selection and its effects on population dynamics and stability in fruitflies**" has been carried out by Ms. Neha Pandey under my supervision at the Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, and that the results in this thesis have not previously formed the basis for the award of any other degree, diploma, or fellowship.

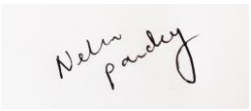
Joshi

Date: 6th June 2022 Prof. Amitabh Joshi

DECLARATION

I declare that the matter presented in my thesis titled "**Investigations on density-dependent selection and its effects on population dynamics and stability in fruitflies**" is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the 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, is regretted.



Neha Pandey

Place: Bengaluru

Date: 6th June 2022

For Juno and Chhutki

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CONTENTS

THESIS SYNOPSIS

Investigations on density-dependent selection and its effects on population dynamics and stability in fruitflies

Density-dependent selection and the evolution of population stability are two important areas where evolutionary biology and population ecology interface. In the mid-1970s, it was widely realized that even simple discrete-time population dynamics models could yield complex and unstable dynamics if the maximal (intrinsic) per capita population growth rate was high. This, in turn, led to increasing interest in understanding ecological factors that might promote the evolution of population stability. One of the most plausible proposed mechanisms for the evolution of relatively stable dynamics in populations was through density-dependent selection, especially in the presence of tradeoffs between realized per capita population growth rates at high and low densities, respectively. The demonstration of such a tradeoff in *Drosophila melanogaster* populations, and the development of *D. melanogaster* populations adapted to many generations of larval crowding in the laboratory, paved the way for experimental tests of whether adaptation to chronic crowding could lead to the evolution of population stability.

The first experimental test of this prediction, however, did not support the notion that density-dependent selection led to the evolution of greater stability: populations of *D. melanogaster* that had evolved greater competitive ability than ancestral controls, as a result of having been subjected to high larval density for many generations, nevertheless showed no evidence of greater constancy stability than controls. However, a subsequent study, using populations of *D. ananassae* adapted to larval crowding experienced under a different combination of egg number and food amount per rearing vial than in the earlier used *D. melanogaster* populations, exhibited significantly greater constancy and persistence than their ancestral control populations, but whether or not this was due to an underlying *r-K* tradeoff was unclear. Since the crowding adapted populations of *D. melanogaster* and *D. ananassae* also differed in the traits through which they evolved greater competitive ability, it was speculated that perhaps the precise suite of traits that evolves in response to chronic

larval crowding experienced at different combinations of egg number and food amount may affect whether or not population stability evolves as a correlated response to density-dependent selection. In this thesis, I describe results from three interlinked experiments aimed at addressing the above speculation, and also a stand-alone study on the phenomenon of 'larval stop'. All the data chapters (Chapters 2-5) are formatted as manuscripts, some already submitted; consequently, materials and methods are described in detail in the individual data chapters.

In the introductory Chapter 1, I lay out the backdrop to my experiments, outlining the development of thinking about density-dependent selection and its possible role in mediating the evolution of population stability. I also discuss how these lines of thought came to empirical fruition in studies of laboratory populations of *Drosophila* subjected to chronic larval crowding. In Chapter 2, I describe a population dynamics experiment aimed at asking whether *D. melanogaster* populations that adapted to chronic larval crowding experienced at a combination of 600 eggs in 1.5 mL of food per vial had evolved greater constancy and persistence stability than ancestral controls maintained at low larval density. Compared to controls, the selected populations exhibited significantly greater constancy, persistence, equilibrium population size, average population size, and reduced sensitivity of realized per capita population growth rate to density. Selected and control populations did not differ significantly in maximal per capita population growth rate. The results were similar to those seen earlier with populations of *D. ananassae* that had experienced chronic larval crowding under very similar conditions. In Chapter 3, I describe results from a similar population dynamics experiment using another set of *D. melanogaster* populations that also adapted to chronic larval crowding, but experienced at a combination of 1200 eggs in 6 mL of food per vial. These populations shared the same ancestral controls as those used in the study described in Chapter 2. This set of crowding adapted populations exhibited only significantly greater persistence, but not constancy, compared to controls. The only other significant difference between the selected and control populations was in reduced sensitivity of realized per capita population growth rate to density in the selected populations. Selected and control populations did not differ significantly in equilibrium population size, average population size, and maximal per capita population growth rate, although equilibrium population size and average population size were consistently higher in the selected populations. These two studies, thus, clearly revealed that differences in the

precise combination of egg number and food volume at which chronic larval crowding was experienced could not only affect the traits that evolved in response to the crowding, but also the demographic and stability attributes of the crowding adapted populations.

In Chapter 4, I report results of assays looking at the sensitivity to density of pre-adult survivorship, female fecundity at day 21 post-egg lay, dry weight at eclosion in males and females, and adult mortality from eclosion till day 21 post-egg lay, in the two sets of crowding adapted populations discussed in Chapters 2 and 3. These assays were aimed at asking whether the demographic and stability differences between these two sets of crowding adapted populations could be linked to differences between them in how sensitive important life-history traits were to larval or adult density. The only significant difference I found was that, at high but not low larval densities, pre-adult survivorship of populations adapted to larval crowding at 600 eggs in 1.5 mL of food was greater than that of populations adapted to larval crowding at 1200 eggs in 6 mL of food. This difference could, in part, explain why the former sustain a higher realized per capita growth rate than the latter at a range of moderately high densities in the population dynamics experiments described in Chapters 2 and 3, and therefore the difference in their stability attributes.

In Chapter 5, I revisit the phenomenon of 'larval stop' (physiological suspension of larval feeding activity and development in the absence of food), first described in the 1980s and not studied much thereafter. I examined the presence and magnitude of larval stop in both one set of crowding adapted populations and their controls, and also in a set of populations selected for rapid egg to adult development and early reproduction and their controls. In both sets of selected populations, I found evidence for fairly low levels of larval stop, which may be due to a combination of evolution of rapid pre-adult development and a discrete generation maintenance cycle.

Finally, in Chapter 6, I draw together the various results and insights obtained from my studies reported here and briefly discuss some potentially promising avenues for future research aimed at furthering our understanding of the effects of density-dependent selection on population dynamics and stability.

Chapter 2, 3 and 4 are available as preprints on bioRxiv and Chapter 4 has been submitted to the Journal of Biosciences and is currently under review. The doi for these manuscripts are provided on the title pages of each chapter.

CHAPTER 1

General Introduction

INTRODUCTION

Fluctuations in population size have long been of central interest to population biologists who have debated whether populations are regulated by density-dependent effects on demographic factors or only by external density-independent factors (review in Kingsland 1995, Mueller and Joshi 2000). Since stability was observed to be common in natural populations (Hassell *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 a, Mueller *et al* 2000), it generated interest in ecological and evolutionary explanations of population stability. The early models of population dynamics had considered populations to be genotypically homogenous groups (review in Joshi *et al* 2001) and did not consider the possibility of population dynamics evolving in response to selection. The theory of density-dependent selection incorporated within-population variation and differential fitness responses of genotypes to population density (MacArthur 1962, MacArthur and Wilson 1967), which paved the way for the hypotheses that population stability could evolve in response to density-dependent selection through the evolution of life-history traits that influence demographic parameters such as *r* and *K* (see below). The aim of my thesis is to experimentally examine this explanation of population stability and test whether life-history evolution in response to density-dependent selection can lead to the evolution of greater population stability. In this chapter, I provide a background on the present understanding of population dynamics, evolution of stability, and the role of density-dependent selection in shaping life history and population dynamics. I then introduce my research questions and the experimental system I used, sets of *Drosophila melanogaster* populations selected for adaptation to larval crowding, which were studied to address whether life-history evolution under crowding influences population stability.

Population dynamics, regulation, and density-dependent selection

Under natural selection, individuals in a population implicitly compete and those with higher fitness are favored, elevating mean fitness, something often equated with maximizing the population's growth rate in its particular environment (Fisher 1930, Wright 1931, Kingman 1961, but see Lewontin 2004). Although it may be expected that populations will grow exponentially over generations, continuous growth for a long time is usually not observed, and population size seems to be regulated, showing fluctuations within largely stable limits (Spencer 1864, Turchin 1995). Early explanations of such stability included the role of density-dependence in population regulation (Verhulst 1838: in Bacaer 2011, Pearl

and Reed 1920, Nicholson 1957) or invoked biotic and abiotic factors (Howard and Fiske 1911, Uvarov 1931: review in Kingsland 1995, Mueller and Joshi 2000). The early models of population dynamics (Verhulst 1838: in Bacaer 2011, Pearl and Reed 1920, Hairston *et al* 1970, Gilpin *et al* 1976, Hallam and Clark 1981: review in Joshi *et al* 2001) had considered populations to be genotypically homogenous groups and did not consider the possibility of population dynamics evolving. The first attempt to link population heterogeneity to growth rate was made by Fisher (1930) in a dynamic natural selection model derived from the theory of population growth, and subsequent experiments by Lewontin (1955) showed that fitness of genotypes was influenced by population density and composition (reviewed in Lewontin 2004, Christiansen 2004). Despite these developments, evolutionary theory did not integrate the effect of population density on genotypic fitness and evolution till the late 1960s (Mueller 1997).

MacArthur and Wilson (1967) developed a formal evolutionary framework of genotypic fitness and population growth rate based on population density, known as density-dependent selection theory, thus formally integrating the fields of population ecology and evolutionary genetics as part of their theory of island biogeography (MacArthur 1962, MacArthur and Wilson 1967). Density-dependent selection theory (henceforth DDST) proposed that population density was a critical component of the ecological context that mediates the relative fitness advantage of different genotypes in a heterogeneous population (MacArthur 1962, MacArthur and Wilson 1967, see also Elton 1927). DDST predicted that population growth would be high at low densities since per-capita food availability is high and, hence, genotypes with higher rates of growth would be favored at low density (*r*-selection). In contrast, since intra-specific competition would be high at high density, genotypes with higher competitive ability are expected to be favored (*K*-selection). The terms *r* and *K* here referred to parameters of the logistic model of density-dependent population growth, denoting the maximal per capita population growth rate and the equilibrium population size, respectively. Such density-dependent selection was also proposed to be mediated by possible trade-offs between *r* and *K*, such that the same genotype could not excel at both low and high density (MacArthur and Wilson 1967), and this *r*-*K* trade-off was later demonstrated experimentally in *Paramecium* (Luckinbill 1979) and *Drosophila* (Mueller and Ayala 1981 b, Mueller *et al* 1991).

Initial formulations of DDST were based on the logistic equation of population growth (Verhulst 1838: in Bacaër 2011, Pearl and Reed 1920, Anderson 1971, Charlesworth 1971, Roughgarden 1971, reviewed in Joshi *et al* 2001, see also Mallet 2012) and Fisher's (1930) idea that equated fitness of a genotype to the per-capita rate of population growth (Malthusian parameter) which was dependent on population density, such that the average fitness of a genotype would approximate 0 in the long run, implying possible asymptotic growth at carrying capacity (Christiansen 2004). While the earlier models of density-dependent selection did not incorporate within-population variation in demographic parameters, and presumed that intra- and inter-genotypic competition will have similar effects on an individual (Hairston *et al* 1970, Gilpin *et al* 1976, Hallam and Clark 1981: discussed in Joshi *et al* 2001), subsequent models based on Lotka-Volterra equations incorporated the genotypic identity (Clarke 1972, Matisse and Jayakar 1976, Asmussen 1983, Anderson and Arnold 1983), calling it density-frequency-dependent selection (reviewed in Joshi *et al* 2001, Christiansen 2004). With this formal theoretical framework, which linked density-dependent selection to density-dependent population regulation (see Turchin 1995, Mueller and Joshi 2000), it became possible to explore evolutionary causes for the stability observed in many populations.

Evolutionary explanations of stable population dynamics

A theoretical study on population dynamics using a simple discrete-generation model demonstrated that increase in intrinsic growth rate has a destabilizing effect on the equilibrium population size, resulting in complex dynamic behaviours like oscillations and chaos (May 1974). This was an interesting finding since adaptive evolution is expected to increase the fitness and thus growth rate of a population, and it is therefore hard to imagine selection for reduced intrinsic growth rate. In other words, all else being equal, selection would be expected to favour higher intrinsic growth rate and, consequently, relatively unstable dynamics. Since many studies on natural and experimental populations reported that population stability was more common than what would be expected from May's results (Hassell *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 a, Ellner and Turchin 1995), these contradictions between theoretical expectations and observed data generated interest in the mechanisms which bring stability in population dynamics. To reconcile May's findings with commonly observed stable populations, several types of hypotheses about how population stability could evolve were proposed. Hypothesis invoking group selection suggested that if there are some patches occupied with unstable

populations while other patches have populations with stable dynamics, then the patches with unstable populations will go extinct over time and will be recolonised by populations that have stable dynamics (Thomas *et al* 1980). This hypothesis may work in very narrow conditions (Mueller and Joshi 2000). Another kind of hypothesis suggested that stability could evolve by direct selection for lower rates of growth (Hansen 1992, Ebenman *et al* 1996), but this would be in contradiction to the Darwinian theory (i.e. all else being equal, selection will favour higher fecundity and therefore higher growth rates) and, accordingly, such evolution of stability in populations kept for many tens of generations on a destabilizing food-regime was not observed (Mueller *et al* 2000). It also is possible that inbreeding in a highly fecund outbred population could render it stable by reducing fecundity, but such an effect would not last long if there is mixing with other high-fitness populations (Mueller and Joshi 2000). The third set of hypotheses suggested that stability can evolve as a by-product of selection for certain life-history traits (Mueller and Ayala 1981 b, Mueller *et al* 2000, Prasad *et al* 2003) that enhance survival and reduce fecundity which enhances population stability (Turelli and Petry 1980, Stokes *et al* 1988, Gatto 1993, Ebenman *et al* 1996, Prasad *et al* 2003). These hypotheses were built around life-history trade-offs that could mediate the evolution of greater population stability as a by-product of life-history evolution. A very plausible hypothesis within this third category was that of density-dependent selection leading to the evolution of greater stability through the evolution of higher *K*, especially in the presence of *r*-*K* tradeoffs, which had been earlier observed (Luckinbill 1979, Mueller and Ayala 1981 b, Mueller *et al* 1991). This set of hypotheses about stability evolving via trade-offs is the only one for which there is experimental support. Rapidly developing populations of *Drosophila* were shown to have evolved greater constancy, but not persistence (*sensu* Grimm and Wissel 1997), relative to ancestral controls, most likely because of the correlated evolution of reduced body size and fecundity (Prasad *et al* 2003, Dey *et al* 2008) Only two studies attempted to see whether adaptations to larval crowding resulted in the evolution of population stability, and yielded contradictory results (Mueller *et al* 2000, Dey *et al* 2012). My studies reported in this thesis are an attempt to empirically examine the issue of population stability evolving in response to density-dependent selection in greater detail.

Life-history evolution under r- and K-selection, and consequences for population stability

The evolution of life histories in different ecological contexts is thought to be a key contributor to the diversity seen in the living world (Roff 1992, Stearns 1976, 1992,

Charlesworth 1994). The life history of an organism constitutes characteristics relating to the timing of growth (e.g. stage-specific development time, age at sexual maturity), somatic maintenance (e.g. survival, lifespan), and reproduction (e.g. number, size, and temporal distribution of offspring). Since an organism has limited energy and resources at its disposal, it needs to differentially allocate these resources to maintenance, growth, and reproduction over its lifetime, depending on its ecology and other constraints (Williams 1966, Gadgil and Bossert 1970, Schaffer 1974, Houle 2001). Natural selection on such differential allocation of resources can lead to evolutionary diversification of optimal life-histories in different ecological contexts (Gadgil and Solbrig 1972, Hirshfield and Tinkle 1975), which can potentially affect fitness components that affect population growth rates (Mueller 1997). Such consequences for population growth have made the understanding of ecology-specific life-history evolution of keen interest in population ecology (Cole 1954, Stokes *et al* 1988, Mueller *et al* 2000, Mueller and Joshi 2000, Reznick *et al* 2002, Prasad *et al* 2003, Dey *et al* 2008, Dey *et al* 2012). As mentioned above, life-history evolution in response to density-dependent selection has been proposed to explain the evolution of stability (Mueller and Ayala 1981 b, Mueller *et al* 2000), which I will further explore in my thesis.

The formalization of density-dependent selection theory (Anderson 1971, Charlesworth 1971, Roughgarden 1971: reviewed in Joshi *et al* 2001, see also Mallet 2012) was followed by many studies investigating the life-history traits that would evolve in *r*- versus *K*-type environments and if those traits traded-off at low versus high density. These studies provided good support to the predictions of DDST (Luckinbill 1978, 1979, Mueller and Ayala 1981 b, Mueller 1988, Joshi and Mueller 1988, Mueller 1990, Mueller *et al* 1991). However, empirical support for the role of density-dependent selection in mediating the evolution of population stability has been limited. An experimental study on sets of *Drosophila* populations reared at low and high density, respectively, (Mueller and Ayala 1981 b, Mueller *et al* 1991) reported a trade-off between *r*- and *K*-selected traits, such as between population growth rates at high versus low density. Consequently, stability reported in other studies (Hassell *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 a, Ellner and Turchin 1995) was hypothesized to emerge not just from proximal effects of population density (as proposed earlier by Nicholson in 1933, 1954) but also through long term effects of density dependence and evolutionary changes in *r* and *K* due to persistent selection in high density-environments (Mueller and Ayala 1981 b). The "*r*" and "*K*" populations of

Drosophila were examined for stability, and were also not controlled separately for crowding at the larval or adult stage (Mueller 1987, see Mueller and Joshi 2000). Subsequently, Mueller *et al* (1993) selected new sets of populations of *D. melanogaster* in low and high larval crowding environments (UU and CU populations) partly mimicking *r* and *K* environments, and found that the selected populations (CU) evolved life-history traits similar to the earlier '*r*' and '*K*' populations (Mueller *et al* 1993, Joshi and Mueller 1996, Santos *et al* 1997, Borash and Ho 2001). Mueller *et al* (2000) also studied CUs for the evolution of population stability but did not find any signs of increased stability, relative to their controls. In both the *r*- and *K*-populations (Mueller 1990) and the CU and UU populations (Joshi and Mueller 1996), a key prediction of *K*-selection – the evolution of enhanced efficiency of food conversion to biomass – was not met. Theoretical analysis of a *Drosophila* specific model of larval competition also indicated that competitive ability could evolve without a concomitant increase in *K* (Mueller 1988).

To explain the limitations of *K*-selection in explaining adaptations to larval crowding in *Drosophila*, Joshi *et al* (2001) proposed that density-dependent selection can often occur through α-selection, rather than *K*-selection per se (Gill 1972, 1974, Case and Gilpin 1974), wherein the competitive ability of genotypes increases in high density without a change in the *K*-related traits (theoretically shown by Mueller 1988), such as greater conversion efficiency of resources into biomass, smaller size, or offspring production at high densities. They also suggested that α-selection may be more common than *K*-selection in high-density environments. Subsequently, Mallet (2012) also discussed the limitations of *K*-selection as an explanatory concept, and pointed out that the *r*-*K* parameterization of the logistic equation (as opposed to the *r*-α parameterization), and the equation of *K* with carrying capacity rather than equilibrium population size, has led to considerable confusion in the literature on density-dependent selection. Mallet (2012) suggests the usage of the *r*-α equation instead, where *r* is the intrinsic rate of growth (growth rate at optimal low density) and α is the intra-specific competition coefficient, reflecting the sensitivity of realized population growth rate to density. At equilibrium, population density is equal to r/α which is equivalent to *K*. It should be noted that *K*-selection is closely related to the notion of tolerance (the ability to withstand the inhibition from the other group) aspect of competitive ability, whereas α-selection is synonymous with the effectiveness component (the ability to inhibit the other group) of competitive ability (*sensu* Joshi and Thompson 1995, Joshi *et al* 2001). It has been speculated that tolerance can increase population stability by increasing

equilibrium population size, while effectiveness might not affect population stability (Joshi *et al* 2001, Dey *et al* 2012). Recently, experimental studies on *Drosophila ananassae, D. nasuta nasuta,* and *D. melanogaster* have shown that populations in crowding environments evolve traits that often contribute to greater *K* (Nagarajan *et al* 2016, Sarangi *et al* 2016) than α, and it seems that in addition to just density, other aspects of the ecology of density-dependent selection, such as the precise combination of egg number and food amount per rearing container, may also be affecting the nature of selection (i.e. α-selection or *K*-selection).

The first experimental evidence for population stability evolving as a by-product of life-history evolution, although not from density-dependent selection, came from a set of *Drosophila* populations (FEJs, first described by Prasad *et al* 2000) which were selected for rapid development and early reproduction (Prasad *et al* 2003), that were shown to have evolved constancy but not persistence stability (Dey *et al* 2008). A subsequent experimental study on the populations of *D. ananassae* (ACUs) that were selected for adaptation to larval crowding also found the evolution of population stability as a response to density-dependent selection, and such evolution of stability was suggested to have come through *r*-*K* trade-off (Dey *et al* 2012). Thus, two studies on *Drosophila* populations selected in larval crowding environments (see Mueller *et al* 2000 above, Dey *et al* 2012) yielded mixed results about the effects of density-dependent selection on population stability, which did not evolve in CUs (Mueller *et al* 2000) while both greater constancy and persistence evolved in ACUs (Dey *et al* 2012). Both these populations had evolved correlates of higher competitive ability, but CUs evolved traits that possibly contributed more to the effectiveness component (Joshi *et al* 2001) while ACUs evolved traits that contributed to higher tolerance component (Nagarajan *et al* 2016). It is worth noting here that CUs and ACUs were selected at different larval densities, corresponding to very different combinations of egg number and food amount per vial, which perhaps affected their ecology differently (more discussion in Nagarajan *et al* 2016) leading to the evolution of different sets of traits in CUs and ACUs. These findings suggest that if populations evolve traits that contribute to higher *K*, then such density-dependent selection could possibly lead to the evolution of population stability.

Drosophila as a study system to understand the evolution of population stability

Studying the evolution of population dynamics in natural settings is difficult due to logistical constraints and because many unknown environmental variables could affect the

response of traits and population size. In contrast, lab settings give more control over experimental design and allow biologists to specify population size and the nature of selection to implement, as well as to have many replicates that give suitable statistical power to analyses and inferences. Lab-maintained model organisms, such as the *Drosophila* populations used in this thesis, allow implementation of selection pressures of interest, easy tracking of life-history traits over generations, and total population census, to study life-history evolution and quantify population dynamics (reviewed in Mueller and Joshi 2000). *Drosophila* has been widely used as a study system in biology as it is easy to maintain in the laboratory and has short generation time, small size, high fertility, and short lifespan, which allows easy manipulation of genetics and selection regime. Its physiology, developmental biology, and genetics are well understood (reviewed in Flatt 2020). All these features make *Drosophila* an excellent study system to investigate the evolution of life-history traits in response to density-dependent selection and its consequences on population dynamics.

Drosophila is a non-social, holometabolous insect, and has three larval instar stages (L1, L2, and L3), a sedentary pupal stage, and a mobile adult stage. The L1 and L2 larval stages span nearly 24 hours each while the L3 stage lasts for nearly 48 hours (Bakker 1959). After feeding for nearly four days, larvae stop feeding and leave the food for pupariation/pupation which spans four to four and half days, after which they eclose as adults. Food acquisition at the larval stage is crucial in *Drosophila* and is positively correlated with adult weight which can affect viability and reproductive success (Than *et al* 2021 and references therein). This importance of feeding and competition at the larval stage for survival and reproduction makes it an interesting and ideal stage in *Drosophila* where selection pressure can be experimentally induced to study the consequences of density-dependent selection to life-history evolution and population dynamics. The work in my thesis is based on experiments and assays conducted on populations that have been selected for adaptation to chronic crowding at the larval stage, at very different combinations of egg number and food amount per vial.

In our laboratory, we have three sets of selected populations of *D. melanogaster* which are selected for adaptation to larval crowding and have been studied for competitive ability (Archana 2010, Sarangi 2018), although these selected populations vary with each other in the way selection for adaptation to larval crowding has been implemented (details of selection in chapter 2, 3, 4) and in the number of generations for which they have undergone selection. All these selected populations and their controls share the same recent ancestry.

These selected populations were established to understand how differences in the nature and intensity of density-dependent selection can affect the evolution of different life-history traits that contribute to competitive ability (Archana 2010, Sarangi 2013, Sarangi 2018).

Backdrop and the objectives of the thesis

Although the proximal role of density-dependence in population regulation is widely recognized (Turchin 1995, Mueller and Joshi 2000), a consensus on the role of the density-dependent natural selection for population stability is yet to emerge due to contradictory results of the two studies carried out thus far (Mueller *et al* 2000, Dey *et al* 2012). The goal of my thesis was to conduct further empirical examination of whether density-dependent selection leads to the evolution of life-history traits such that population stability emerges as a correlated evolutionary response to such selection.

I have conducted separate population dynamics studies on two different sets of populations that share the same ancestry with CUs (population used in Mueller *et al* 2000) but differ in the larval ecology at which density-dependent selection was implemented. The MCU (*D.* ***m****elanogaster* **c**rowded as larvae and **u**ncrowded as adults, ~600 eggs/ 1.5 mL larval food) populations are selected at a larval density similar to Dey *et al* 2012 (ACUs: *D.* ***a****nanassae* **c**rowded as larvae and **u**ncrowded as adults, ~600 eggs/ 1.5 mL larval food) while the LCU (**L**arry Mueller CU-type, **c**rowded as larvae and **u**ncrowded as adults, ~1200 eggs/ 6 mL larval food) populations are selected at a larval density similar to CUs. Previous assays of life-history traits found that these differences in the ecological context of selection have led to differences in the life-history and other traits that have evolved in these selected populations (Sarangi 2018). My thesis addresses whether such differences in the ecology of selection lead to differences in the evolved population stability and whether such selection differences have also led to differences in demographic traits known to govern the population dynamics. Specifically, my thesis addresses whether density-dependent selection operating at the larval stage leads to the evolution of population stability by affecting life-history traits that govern population dynamics in *Drosophila* populations selected for adaptation to larval crowding, and how these effects might be mediated by the precise combination of egg number and food amount at which the larval crowding was experienced.

My thesis comprises of six chapters, including the present **Introduction** chapter; two independent studies on the evolution of population stability as a response to density-

dependent selection; a study of two different sets of crowding selected populations to investigate if adaptation to larval crowding leads to change in demographic traits that can explain the differences in population stability; a study on larval stop (a larval state representing physiological arrest of feeding activity) in two sets of populations, and the Conclusion chapter summarising and synthesizing the findings from these experiments.

In **Chapter 2**, I examine if the selection for adaptation to larval crowding leads to the evolution of higher constancy and persistence stability and if such evolution of stability is detected differently in contrasting food regimes. I conducted this study on MCUs which have evolved life-history traits similar to the ACU populations (Nagarajan *et al* 2016, Sarangi *et al* 2016) which showed the evolution of higher constancy and persistence stability, possibly through an *r-K* trade-off (Dey *et al* 2012). The rationale behind this study was to understand if the population stability evolves through correlated life-history trait evolution, then MCUs too would be expected to evolve a higher constancy and persistence stability through an *r-K* trade-off as they had evolved life-history traits similar to ACUs in response to adaptation to larval crowding. Interestingly, a previous study on population stability in MCUs had not found any evidence for increased stability although it involved census data from only ten generations (Vaidya 2013). I conducted the study of population dynamics on MCUs after 75 additional generations of selection after Vaidya (2013), and carried out a population census for 31 generations. It is possible that an additional 75 generations of density-dependent selection would have made MCUs more stable. In addition, I chose two more destabilizing food regimes (1 and 1.5 mL larval food) as compared to Vaidya (2 mL larval food, 2013) to detect the evolved constancy and persistence, as the more destabilizing regime can help detect the evolved stability because in destabilizing food regimes with slightly higher food levels the population may not show higher fluctuations and extinctions. Further, data from more generations in population dynamics study may help pick up the differences in the constancy and persistence stability than the smaller datasets in Vaidya (2013).

In **Chapter 3**, I study if the ecological difference in selection for adaptation to larval crowding can affect the evolution of population stability, as suggested previously (Dey *et al* 2012). I conducted a population dynamics study on the LCU populations which share the same recent ancestry with CUs and are maintained at a similar larval density. LCUs have also evolved similar life-history traits to CUs, although they do differ in some traits (Sarangi

2018). As persistence stability could not be studied in CUs previously because they were maintained at a very high population size in the population dynamics study of Muller *et al* (2000), the rationale behind my study was to see if the LCUs have evolved higher persistence stability to gather more empirical support for the hypothesis that stability can evolve as a correlated response to density-dependent selection. Moreover, it is possible that evolved constancy was not detected in CUs because the food regime in which they were studied was not destabilizing enough, as suggested in chapter 2 above and by Vaidya (2013) previously. I carried out the population dynamics experiment in LCUs for 26 generations to compare the constancy and persistence stability of populations.

Since in the above two chapters (Chapter 2 and 3), the MCU and LCU populations differed in their population stability properties, I conducted assays of life-history traits to explore the differences in the components of fitness of these populations (**Chapter 4**) which have been theoretically shown to influence population stability (Tung *et al* 2019). For this study, I assayed traits such as pre-adult survivorship, fecundity, and adult survivorship at contrasting larval/adult densities that are attainable in the population dynamics experiment. These assays were conducted in two different sets of populations selected for adaptation to larval crowding (MCUs and LCUs).

In **Chapter 5**, I have examined the consequences of larval crowding on larval stop – a trait or larval state representing physiological suspension of feeding activity and development. Such physiological suspension may last for up to ~340 hours under larval crowding. This trait was first studied by Ménsua and Moya (1983) in *Drosophila* populations, where the physiological arrest was noticed in the third instar when larvae were exposed to high crowding, and development was seen to resume after the food was provided. As larval stop could prolong the pre-adult development time, it could increase the viability of larvae under larval crowding if food becomes available in the future. Despite such possible fitness consequences of larval stop in a larval crowding environment, this trait has not been studied much, especially in populations that are maintained in discrete generation cycles. It is possible that the evolution of faster pre-adult development time may lead to the loss of the larval stop trait. Since faster development has been shown to trade-off with many life-history traits, in this chapter I examined if direct selection for faster development (FEJ: **F**aster developing and **e**arly reproducing **JB**, first described in Prasad *et al* 2000) and a correlated decrease in pre-adult development time due to adaptation to larval crowding

(Sarangi *et al* 2016) can lead to loss of the larval stop trait in the MCU and FEJ populations and if the responses of the MCUs and FEJs differ with each other as they have evolved faster development though different evolutionary routes. We expected that *Drosophila* populations that are maintained in discrete generation cycle may not show such a phenomenon as the individuals developing later than a fixed number of days (11 days: MBs and 12 days: JBs (Control for FEJs) are not taken in the breeding pool. We also expected that FEJs and MCUs may have lost this trait because both have evolved faster development, while MCUs have also adapted to larval crowding.

In the **Conclusion** chapter, I summarise the results obtained from the above experiments and then discuss how these experiments have furthered our understanding of population stability and how density-dependent selection shapes population dynamics by altering certain life-history traits. These studies widen the empirical support for density-dependent selection as an explanation of the evolution of population stability and advance our understanding of how natural selection shapes population dynamics. Further, I suggest some future directions that follow from these studies, and also point out the limitations and confounding factors to keep in consideration to further test the role of density-dependent selection in shaping population stability.

Details on Materials and Methods

In the following sections, I have summarised protocols for work that was done prior to the main assays described in Chapters 2, 3, 4, and 5 and the media preparation for the maintenance of the selected populations and media used for assays.

Standardization and egg collection prior to assays

Since environmental variation and non-genetic maternal effects could alter the trait differences between the selected and control populations, I reared all the selected and control populations in a common low-density environment for a generation to get rid of such effects. Prior to all the assays all the studied populations were standardized, eggs of both the control and selected populations were collected at a density of ~70 eggs in ~6 mL of food in 40 vials (9.5 cm × 2.4 cm) to get a good representation of the original population (~1800 adults). On the 11^{th} day from egg collection, the crowding populations and their

controls (MB, MCU and LCU) were transferred to Plexiglas cages (25 × 20 × 15 cm^3) in standardization process. The eclosing adults from JBs were transferred on the 12th day from egg collection in Plexiglas cages and eclosing FEJ adults were transferred on the 7th day into the Plexiglas cages. For standardization, FEJs eggs were collected 5 days after JBs egg collection because the adults had to be age-matched for assay egg collection. All these populations were provided with their maintenance food and live acetic acid yeast paste for three days before the egg collection for assays.

Media preparation for maintenance and egg-laying, and egg counting

The crowding-adapted population are maintained in cornmeal food medium (MCUs, LCUs, and MBs) and faster developing and early reproducing flies and their controls (FEJs and JBs) are maintained in banana-jaggery medium, and I have described the media preparation below.

For one litre cornmeal food medium preparation, the ingredients are given in Table 1. First, the weighed ingredients, i.e. agar, cornmeal, yeast, sugar, and activated charcoal are thoroughly mixed in one litre of water, then this mixture is brought to a boil. Once the froth forms in this boiling mixture an extra 120 mL of water is added and this mixture is then pressure cooked for the next twenty minutes. Following this, the mixture is cooled down to 60^{o}C, and the preservative, i.e. methyl-p-hydroxybenzoate dissolved in the ethanol is added, and propionic acid is added.

For one litre banana-jaggery food medium preparation, the ingredients are given below (Table 2). First in the 1 litre water, weighed agar and jaggery is dissolved on heat. Then 22 mL alcohol is added in the weighted yeast and some water from extra water is used to obtain a smooth mix with the mixer, this mix is further added in the boiling water, agar and jaggery. Later, a smooth mix with peeled banana and barley is obtained by putting in the remaining water from extra water and grinding it. This mix is then added to the boiling mixture. Following this the mixture is brought to boil and cooled down to 60^{o}C, and the preservatives i.e. methyl-p-hydroxybenzoate dissolved in the 23 mL of ethanol is added.

Since in most of the assays in the following chapters I required a lot of eggs for the assays and the eggs in the experiment were required to be undamaged and without food, I use slightly different media from the usual maintenance food medium for the assay egg-laying (double-agar medium), following Sarangi (2018). Further, to keep the egg-laying window

constant across assays egg-laying was allowed for 12 hours prior to assay egg collection. Prior to egg laying in the sterile double agar media, the populations were allowed to lay eggs on a dummy plate (regular food plate with cut sides) for an hour to get rid of the eggs that may have matured earlier within the female reproductive tract (Bakker 1959). After egg-laying on the double-agar medium eggs were scraped off with the help of water, scalpel, and brush, and counted on agar sheets for assays.

For one litre of double-agar medium preparation, the ingredients are given in Table 3. First, agar is weighed and put in the boiling water (half litre). Then a smooth mix of weighed yeast and sugar with the other half litre is prepared with a mixer and added to the boiling water and agar. After this, the medium is brought to a boil and cooled till 60^{o}C, following which the weighed methyl-p-hydroxybenzoate preservative is dissolved in the ethanol and added to the cooled double-agar media, and poured into petri dishes.

For one litre of agar medium, the ingredients are given in table 4. First, weighed agar is added to one litre of water and this mixture is brought to a boil. Following this, the mixture is brought to a boil and cooled down to 60^{o}C, and the preservative i.e. methyl-p-hydroxybenzoate is mixed in the ethanol. This medium is then poured into petri dishes and used for counting eggs.

Table 1: Quantities of ingredients required for one litre of cornmeal food medium.

Ingredients	Weight/ Volume
Water	1 litre
Agar	12 gram
Cornmeal	100 gram
Yeast	40 gram
Sugar	40 gram
Activated charcoal	0.50 gram
Extra water	120 millilitre
methyl-p-hydroxybenzoate	1 gram
Ethanol	10 millilitre
Propionic acid	10 millilitre

Table 2: Quantities of ingredients required for one litre of banana-jaggery food medium.

Ingredients	Weight/ Volume
Water	1 litre
Agar	12.40 gram
Jaggery	35 gram
Yeast	36 gram
Barley	25 gram
Banana weighed with peel	205 gram
Extra water	180 millilitre
methyl-p-hydroxybenzoate	2.40 gram
Ethanol	45 millilitre

Table 3: Quantities of ingredients required for one litre of double-agar medium.

Ingredients	Weight/ Volume
Water	1 litre
Agar	24.80 gram
Yeast	36 gram
Sugar	35 gram
methyl-p-hydroxybenzoate	2.40 gram
Ethanol	23 millilitre

Table 4: Quantities of ingredients required for one litre of agar medium.

Ingredients	Weight/ Volume
Water	1 litre
Agar	12.40 gram
methyl-p-hydroxybenzoate	2.40 gram
Ethanol	23 millilitre

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CHAPTER 2

Density-Dependent Selection at Low Food Levels Leads to the Evolution of Population Stability in *Drosophila melanogaster* Even Without a Clear *r*-*K* Trade-Off

Title: Density-dependent selection at low food levels leads to the evolution of population stability in *Drosophila melanogaster* even without a clear *r-K* trade-off

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ABSTRACT

Density-dependent selection, especially together with *r-K* trade-offs, has been one of the most plausible suggested mechanisms for the evolution of population stability. However, experimental support for this explanation has been both meagre and mixed. One study with *Drosophila melanogaster* yielded no evidence for populations adapted to chronic larval crowding having also evolved greater population stability. Another study, on *D. ananassae*, suggested that populations adapted to larval crowding evolved both greater constancy and persistence stability, and the data also suggested an *r-K* trade-off in those populations, though the evidence for the latter was not conclusive. Moreover, theoretical work suggested that density-dependent selection could result in the evolution of greater population stability, even in the absence of an *r-K* trade-off. Here, we show that populations of *D. melanogaster*, selected for adaptation to larval crowding at very low food amounts per vial, evolve enhanced constancy and persistence stability. The enhanced population stability in the crowding-adapted populations seems to have evolved through the increased equlibrium size (*K*) and reduced sensitivity of realized population growth rates to density (α).There was no clear evidence for reduced intrinsic population growth rate (*r*) in the more stable crowding-adapted populations. Our study adds to the growing evidence in support of the hypothesis that population stability can evolve in response to density-dependent selection through the evolution of certain life-history traits that are associated with higher *K* and less negative α. We discuss our results in the light of previous work, and suggest that a model-free framework might be of great heuristic value in understanding the evolution of population stability through changes in the density-sensitivity of life-history traits, whether or not these changes result from density-dependent selection.

Keywords: Population dynamics, constancy, persistence, sensitivity to density, life-history evolution, larval crowding, fruitflies.

INTRODUCTION

While the early understanding of population dynamics suggested that population size is regulated at a point equilibrium through the effect of density on demographic factors and through density-independent factors (reviewed in Kingsland 1995), a very interesting theoretical finding in the 1970s was that increase intrinsic growth rate in simple discrete-generation models of population dynamics destabilizes the equilibrium in population size, resulting in complex and unstable oscillatory dynamics, including chaos (May 1974). Since natural selection, all else being equal, could be expected to favour increased intrinsic growth rate, this finding implied that unstable dynamics might be fairly common. Subsequently, however, a synthesis of population dynamics data from several studies, including populations with high growth rates, suggested that population stability was more common than what would be expected from May's finding (Hassell *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 a, Ellner and Turchin 1995), which generated interest in the mechanisms through which population stability could evolve. Early evolutionary explanations of population stability invoked direct selection for reduced maximal or intrinsic growth rate (r in the logistic or Ricker models) (Hansen 1992, Ebenman *et al* 1996), or group-selection for stable populations (Thomas *et al* 1980). A more plausible explanation was that stability can evolve as a by-product of selection on life-history traits (Mueller and Ayala 1981 b), especially if the selected traits happened to trade off with fecundity, thereby increasing population stability (Turelli and Petry 1980, Stokes *et al* 1988, Gatto 1993, Ebenman *et al* 1996, Prasad *et al* 2003).

In particular, Mueller and Ayala (1981 b) suggested that population stability could evolve via density-dependent selection through evolutionary reductions in intrinsic growth rate (r) under chronic crowding, through a trade-off with K (equilibrium population size). This hypothesis was based on the theory of density-dependent natural selection suggesting population density as a critical component of an organism's ecology that mediates the relative fitness advantage of genotypes in a heterogeneous population (MacArthur 1962, MacArthur and Wilson 1967), first articulated in the context of selection in populations with cyclic population size dynamics by Elton (1927). Population genetic models of density-dependent selection, which were formalized in the 1970s-1980s (reviewed in Joshi *et al* 2001) proposed that the population growth rate will be high at low densities and will favor

genotypes with higher intrinsic rates of growth (*r*-selection), whereas increased intra-specific competition under crowding would favor genotypes with higher competitive ability (*K*-selection). Moreover, a trade-off between *r* and *K* was expected such that no one genotype would have high fitness at both low and high density (MacArthur and Wilson 1967), as demonstrated later in *Paramecium* (Luckinbill 1979) and *Drosophila* (Mueller and Ayala 1981 b, Mueller *et al* 1991). Thus, density-dependent selection theory linked the evolution of life-history traits to population density such that genotypes that rapidly acquire resources and convert them into offspring are favored in *r*-selection, while those with better efficiency of resource utilization are favored in *K*-selection (reviewed by Reznick *et al* 2002).

Such differential evolution of life-history traits in response to density was supported by subsequent experimental investigations of traits evolving in low versus high-density environments (Luckinbill 1978, 1979, Mueller and Ayala 1981 b, Mueller 1988, Joshi and Mueller 1988, Mueller 1990, Mueller *et al* 1991), although empirical tests for population stability evolving in response to density-dependent selection have been very few (Mueller *et al* 2000, Dey *et al* 2012).

Empirical tests of density-dependent selection leading to the evolution of enhanced population stability involve experimental manipulation of population densities to implement contrasting selection pressures. Mueller and Ayala (1981 b; see also Mueller *et al* 1991) implemented such selection in *Drosophila* and developed '*r*-populations' and '*K*-populations' corresponding to low and high density rearing, and found that population growth rates at high density traded off with population growth rates at low density; population stability was not examined in these sets of populations. Subsequently, Mueller *et al* (1993) selected populations of *D. melanogaster* in low and high larval crowding conditions (UU and CU populations, respectively) and found that the crowding-adapted populations (CU) evolved life-history and other traits similar to the earlier-studied '*r*' and '*K*' populations (Mueller *et al* 1993, Joshi and Mueller 1996, Santos *et al* 1997, Borash and Ho 2001). Interestingly, however, Mueller *et al*'s (2000) investigation of population stability in the CUs and UUs when placed in a destabilizing food environment for many generations did not find evidence of enhanced constancy stability (*sensu* Grimm and Wissel 1997); persistence stability was not assessed as very large populations were used in the study and no extinctions were observed. The CUs did evolve traits that indicated increased

competitive ability, but as in the case of the *K*-populations (Mueller 1990), these traits did not include the classic *K*-selected trait (MacArthur and Wilson 1967) of increased efficiency of food conversion to biomass (Joshi and Mueller 1996).

A subsequent experimental study on *D. ananassae* populations selected for adaptation to larval crowding (ACUs) presented the first evidence of both constancy and persistence stability evolving in response to density-dependent selection and it was suggested to have come about through an *r-K* trade-off, although the evidence for the latter was suggestive rather than conclusive (Dey *et al* 2012). Thus, these two studies (Mueller *et al* 2000, Dey *et al* 2012) yielded mixed results about the effects of density-dependent selection on population stability: stability did not evolve in the CUs (Mueller *et al* 2000) while it did in the ACUs (Dey *et al* 2012). The CU and ACU populations had experienced chronic larval crowding at very different combinations of egg number and food amount per rearing vial, with the ACUs being selected at very low food amounts (Nagarajan *et al* 2016). These differences in the ecology of experienced crowding had also led to the evolution of different sets of traits in the CUs and ACUs, with the CUs evolving higher larval feeding rates than controls (Joshi and Mueller 1996) whereas the ACUs were faster developing but did not differ from controls in larval feeding rate (Nagarajan *et al* 2016). In terms of effectiveness and tolerance (*sensu* Joshi *et al* 2001), the CUs had evolved traits indicative of greater effectiveness component (Joshi *et al* 2001) whereas ACUs had evolved traits likely to contribute to a higher tolerance component (Nagarajan *et al* 2016). Given these differences, Dey *et al* (2012) speculated that which specific traits evolve in response to larval crowding, and whether they primarily affect the effectiveness or tolerance components of competitive ability, might determine whether or not population stability evolves as a correlated response to density-dependent selection. However, this explanation for the difference in results between the studies of Mueller *et al* (2000) and Dey *et al* (2012) could only be suggestive, since the two studies differed not just in the food level and egg number combination at which they experienced larval crowding, but also in the species and food medium used. Consequently, in this study we investigated whether population stability has evolved in *D. melanogaster* populations adapted to larval crowding at the same combination of egg number and food amount, and food medium, as the ACU populations. The *D. melanogaster* populations we used share ancestry with the CU populations of Mueller *et al* (2000), rendering the comparison even more rigorous. We also investigated whether small differences in food amounts available to larvae in the population dynamics experiment

interacted with selection because higher resource levels during the larval stage can increase pre-adult survivorship, fecundity, and decrease the sensitivity of growth rate to population density (Mueller and Huynh 1994, reviewed in Dey and Joshi 2018), all of which can in turn influence population dynamics (Mueller and Huynh 1994, Vaidya 2013).

MATERIALS AND METHODS

We conducted population dynamics experiments, using *D. melanogaster* populations which were selected for adaptation to larval crowding, and their ancestral controls. We conducted these experiments at two different food amounts i.e. 1 mL and 1.5 mL per vial.

Experimental populations

We used eight large outbred lab-maintained populations (four selected and four controls) of *D. melanogaster* which are maintained on a 21-day discrete generation cycle in all light (LL) environment at 25^{o}C±1^{o}C and at around 80 percent humidity. The control populations, **MB**$_{1\text{-}4}$ (*Melanogaster* Baselines), are maintained at low larval density i.e. ~ 70 eggs per ~6 mL of cornmeal medium in 40 glass vials (per replicate population) of 2.2-2.4 cm inner diameter and 9.5 cm height. After eclosion (11th day from egg lay when all flies emerge) the adult flies are transferred at once in respective Plexiglas cages (25 cm ×20 cm × 15 cm^3) containing a food plate with a wet cotton ball to keep up humidity. The selected populations, **MCU**$_{1\text{-}4}$ (*Melanogaster* crowded as larvae and uncrowded as adults) have been selected for adaptation to larval crowding (competition at larval stage) and are maintained at a density of ~600 eggs in 1.5 mL food per vial. The MCUs have been derived from their respective ancestral controls i.e. MBs with each subscript denoting ancestry. As opposed to MBs, MCUs are maintained in 12 glass vials at larval stage (to avoid adult crowding after being transferred to cages), and at the adult stage are collected in Plexiglas cages every day from 8th day after egg lay till day 18 as the eclosion is spread out over many days due to larval crowding. The food plate is changed every alternate day till the 18th day, and the wet cotton ball is changed at every alternate food change. On day 18 day adults from both sets of populations (i.e. MBs and MCUs) are given live acetic-acid yeast supplement till day 20, and are then allowed to lay eggs for around 18 hours. On day 21 from egg lay, eggs laid by these flies are collected in their respective densities (selected or control) to start the next

generation. All populations are maintained at an adult density of about 1800 to 2000 adults. Full details of the origin and maintenance of these populations are given in Sarangi *et al* (2016). The MCU populations had undergone over 160 generations of selection prior to the population dynamics experiments.

Population dynamics experiments

We conducted population dynamics experiments in destabilizing food environments (LH food regime: Mueller and Huynh 1994). A stabilizing environment, for example an HL food regime (High food for larvae and Low food for adults i.e. absence of yeast: Mueller and Huynh 1994), induces stable dynamics in *Drosophila* populations due to a combination of relatively high larval survivorship and low adult fecundity; therefore, any differences in population stability in selected and control populations might not be detected. However, in the LH food regime (low food for larvae and high food for adults i.e. presence of yeast: Mueller and Huynh 1994) induces large fluctuations around the mean population size due to intense larval competition for food coupled with reduced sensitivity of fecundity to adult density due to the presence of yeast; therefore, evolved stability differences between different populations are much more likely to be detected in such environments (Mueller and Huynh 1994, Prasad *et al* 2003). Consequently, to look for any differences in population stability between MCUs and MBs, we set up a 31-generation long population dynamics experiment in a destabilizing (LH) food regime. We carried out this experiment at two food amounts at the larval stage i.e. one regime containing 1 mL and another containing 1.5 mL of cornmeal food for the larvae. Previously, 1 mL LH regime was shown to induce frequent extinctions (Vaidya 2013) in vial populations, and therefore, is useful to study evolution of persistence stability. We chose to also use a 1.5 mL LH regime as it parallels the food amount in the larval maintenance regime under selection for MCUs, and also to see whether food level within an LH regime interacted with selection.

From each of the four MB and four MCU populations, we derived 10 small vial populations each, after all MB and MCU populations had been reared at low density (~70 eggs/~6 mL food) for one generation to eliminate maternal effects. We started each vial population with 8 mated females which were allowed to lay eggs in 1 mL or 1.5 mL food respectively, for 24 hours and labeled this generation as generation 0. We started transferring the eclosing flies from egg vials after day 8 from egg lay, to the matched adult collection vials containing around 4 mL of cornmeal food. Since fly eclosion is spread over several days due to

competition at the larval stage, we transferred the eclosing flies daily from egg vials to their respective adult collection vials till day 18 from egg lay. We maintained vial correspondence between egg vials and adult collection vials to ensure the population identity and we did fly-transfers extremely carefully to avoid losing any flies to avoid introducing additional noise into the inherent dynamics. We moved adults to fresh adult collection vials every alternate till day 18 post egg lay. On day 18, we discarded the egg vials and gave a dab of live acetic-acid yeast on the adult collection vial wall to boost adult fecundity. On day 20 from egg collection, we transferred flies from the adult vials into new egg-laying vials with 1 mL or 1.5 mL food for next 16 hours to lay eggs for next-generation (after generation 0 the egg-laying window was decreased to 16 hours for all subsequent generations). Later, we transferred these adults into empty vials for the census counts of males and females after freezing. We also counted in the census any fly found dead during the 16-hour egg-laying phase.

The eggs laid by the flies in each vial became the next generation, i.e. density was not controlled. In parallel with the population dynamics experimental vials described above, we maintained a set of five backup vials per population whose maintenance was similar to the experimental vial populations except that backup vial populations were maintained at a low larval density to avoid the effects of crowding. Each generation, we randomly chose 5 females from each backup vial population to lay eggs for 16 hours in 6 mL of food to start the next backup generation, while the rest of the flies were discarded. Following Dey and Joshi (2006), we maintained these backup vials to reset the experimental populations (with 4 males and 4 females) in case of extinction (absence of even one male-female pair) in a vial population on day 20 post egg lay. We carried out the population dynamics experiments for 31 generations for both 1 and 1.5 mL food amounts and each generation was 21 days long. A total of 160 single-vial populations (2 selection regimes × 4 replicate populations × 2 food regimes × 10 single-vial populations) were, thus, censused over the 31 generation long population dynamics experiment. These 160 population size time series, along with the number of times each population went extinct over the 31 generations, constituted the primary data for further analyses.

Population stability measures

Constancy: We compared constancy stability (*sensu* Grimm and Wissel 1997) in MBs and MCUs using 2 indices: coefficient of variation (CV) in population size and fluctuation index

of population size (FI) for both 1 and 1.5 mL regimes separately. Coefficient of variation (CV) in population size measures population dispersion around the mean population size, scaled by mean population size (CV = standard deviation in population size/mean population size). We also measured constancy through FI which measures the mean one-step absolute change in population size, scaled by the mean population size (Dey and Joshi 2006), as

$$FI = \frac{1}{TN}\sum_{t=0}^{T-1}|(Nt+1-Nt)|$$

where T is the number of generations, N is the average population size, and N_t and N_{t+1} are the population sizes at generations t and t+1, respectively. Constancy was interpreted as being the inverse of CV or FI, respectively.

Persistence: We compared persistence stability between MBs and MCUs using the frequency of extinctions in 1 and 1.5 mL regimes separately, which was calculated by dividing the number of times a population went extinct over the course of the experiment by 31 (i.e. the number of generations). Persistence was interpreted as being reflected by the inverse of the extinction probability. We counted consecutive extinctions in the same population as one extinction event because, in experiments such as these, extinctions in consecutive generations are often not independent (Dey *et al* 2008). We also calculated mean population size of each single-vial population across the 31 generations of the population dynamics experiment.

Measuring demographic attributes

In all the single-vial populations, we examined three demographic attributes that can both respond to density-dependent selection and affect population stability: intrinsic population growth rate, equilibrium population size, and sensitivity of realized population growth rate to population density. We know from previous work that the dynamics of single-vial populations of *Drosophila* in an LH food regime are captured reasonably well by the Ricker (1954) model (Sheeba and Joshi 1998). At the same time, there is no reason to believe that the responses of *Drosophila* population dynamics to various food or selection regimes are limited by the functional form of any simple population growth model (Tung *et al* 2019, Joshi 2022). Consequently, we examined these attributes in different ways, some taking the Ricker model as the basis, while others were more empirical and model-free.

In the context of the Ricker model, we estimated the canonical parameters r and K, representing intrinsic population growth rate and equilibrium population size, respectively, as well as $\alpha = r/K$, reflecting the sensitivity of realized population growth rate to density. These estimations were done by (a) plotting a regression line between Ln (N_{t+1}/N_t) on the Y-axis and N_t on the X-axis, and taking the Y-intercept, X-intercept and slope as estimates of r, K and α, respectively, and (b) by non-linear curve fitting (following Dey *et al* 2008) using the Quasi-Newton method in Statistica vers. 5 (StatSoft 1995), followed by taking $\alpha = -r/K$. In addition, we also estimated realized population growth rates (N_{t+1}/N_t) at low ($N_t < 30$ for 1 mL food, $N_t < 40$ for 1.5 mL food) and high ($N_t > 60$ for 1 mL food, $N_t > 80$ for 1.5 mL food) densities, as correlates of r and K, respectively, following the approach of Joshi *et al* (2001). We also checked the realized population growth rates at different cut-off values for low and high density to assess the robustness of the result. Finally, we estimated realized population growth rates (N_{t+1}/N_t) over the entire range of population densities observed during the course of the experiment, in bin sizes of 30 for 1 mL food, and 40 for 1.5 mL food.

Statistical analyses

We used mixed model analysis of variance (ANOVA) to analyze all the response variables, with three predictor variables, two fixed, i.e. selection regime and food level, and one random, i.e. block, representing the common ancestry of each pair of MB and MCU populations with a common subscript. We performed separate ANOVAs on the coefficient of variation in population size, fluctuation index, extinction probability, average population size, intrinsic growth rate (estimated through three different methods), equilibrium population size (estimated through three different methods), and the sensitivity of realized population growth rate to population density (estimated through two methods). Separate ANOVAs on realized growth rates corresponding to different population size bins were performed for data from 1 and 1.5 mL food, because the bin sizes used differed between food levels. All analyses were performed in Statistica Ver. 5.0 (StatSoft 1995), and post-hoc comparisons used Tukey's HSD test at $P = 0.05$.

RESULTS

Constancy stability

For both measures of constancy – CV and FI – the pattern of results was similar: constancy was higher in 1.5 mL than in 1 mL, and in MCUs than in MBs (Fig. 1 a, b). However, the differences between food amounts and between selection regimes were significant only in case of CV (Table 1 a), but not FI (Table 1 b). For CV, the main effects of selection and food amount were significant, but not their interaction (Table 1 a).

Persistence stability

Persistence stability was significantly higher in MCUs than MBs, and in 1.5 mL than in 1 mL food (Fig. 2 a Table 2 a). There was also a significant interaction between selection regime and food amount (Table 2 a), driven by a much larger enhancement of persistence (much reduced extinction rate) in the MBs between 1 mL and 1.5 mL, compared to the MCUs (Fig. 2 a).

Mean population size

The mean population size was significantly higher in the MCUs than in the MBs, and at 1.5 mL food than 1 mL food (Fig. 2 b, Table 2 b) Going from 1 mL to 1.5 mL food, MCUs showed a greater increase in mean population size than the MBs (Fig. 2 b), but the difference was not enough to drive a significant interaction between selection regime and food amount (Table 2 b).

Ricker-based demographic attributes

Intrinsic rate of population growth (r), when estimated from linear regression of Ln (N_{t+1}/N_t) on N_t , did not differ significantly between MBs and MCUs, or between food amounts; neither the main effects of selection regime or food amount, nor their interaction, were significant (Fig. 3 a, Table 3 a). When r was estimated by non-linear curve fitting, there were no significant main effects of either selection regime or food amount (Table 3 b). However, the interaction between selection regime and food amount was significant (Table 3 b), and post-hoc comparisons revealed that MCUs had significantly lower estimated r than MBs at 1.5 mL, but not at 1 mL food amount. Apart from this one difference, not only were differences in r between MCUs and MBs not significant, even the magnitude of the differences was negligible (Fig. 3 a, b)

The equilibrium population size (K) of MCUs was substantially and significantly higher than the MBs at both food amounts, and for both methods of estimation: linear regression of Ln (N_{t+1}/N_t) on N_t and non-linear fitting (Fig. 3 c, d, Table 4 a, b). The main effect of food amount was also significant across both estimation methods (Table 4 a, b), with K being higher at 1.5 mL than 1 mL food for both MCUs and MBs (Fig. 3 c, d). Going from 1 mL to 1.5 mL food tended to increase K in the MCUs to a greater degree than in the MBs (Fig. 3 c, d), driving a significant interaction between selection regime and food amount interaction regardless of estimation method (Table 4 a, b).

The sensitivity of realized population growth rate to population density (α) showed that MCUs were significantly less sensitive to change in population density than MBs, regardless of the method of estimation (Fig. 3 e, f, Table 5 a, b). Moreover, both MCUs and MBs showed significantly reduced sensitivity (less negative values of α) at 1.5 mL than at 1 mL food, regardless of the method of estimation (Fig. 3 e, f, Table 5 a, b). The interaction between selection regime and food amount was not significant for either method of estimation of α.

Model-free demographic attributes

When we compared empirically estimated mean realized population growth rates at low versus high density in the MCUs and MBs at 1 mL and 1.5 mL food amount (Fig. 4), the only significant ANOVA effect was that of density (Table 6). Although the data suggested increased realized population growth rate in MCUs than in MBs at high density (Fig. 4), the magnitude (>25x between high and low density)) of the effect of density essentially rendered the effect of all other sources of variation on realized population growth rate relatively negligible (Table 6).

The picture became slightly clearer when we examined mean realized population growth rate across the full range of densities achieved in the single-vial populations, in bin sizes of 30 and 40 for 1 mL and 1,5 mL food, respectively (Fig. 5 a, b). There were significant ANOVA effects of selection regime, population size bin (density level), and their interaction, for mean realized population growth rate data at both 1 mL (Table 7 a) and 1.5 mL (Table 7 b) food. At both food amounts, MCUs had a higher realized population growth rate, on an average, than MBs, and realized population growth rates were substantially higher at lower population densities until a reasonably high density was attained (N_t > 100

for 1 mL food, N_t > 140 for 1.5 mL food), beyond which point realized population growth rates tended to level off (Fig. 5 a, b). The significant interactions, at both food amounts, between selection regime and population size bin were driven by the fact that MCUs tended to sustain significantly higher mean realized population growth rates than MBs over a range of intermediate, but not very low or very high population densities (Fig. 5 a, b). Post-hoc comparisons revealed significantly higher mean realized population growth rates in MCUs than MBs at densities between 30 and 90 individuals per vial at 1 mL food, and densities between 40 and 120 individuals per vial at 1.5 mL food (Fig. 5 a, b).

DISCUSSION

Prior to this study, two attempts to test the explanation that density-dependent selection can lead to the evolution of enhanced population stability (Mueller *et al* 2000, Prasad *et al* 2003), had yielded contradictory results. On one hand, Mueller *et al* (2000) found that populations of *D. melanogaster* subjected to chronic larval crowding experienced at relatively high food amounts did not evolve greater constancy than ancestral controls routinely reared at low larval density; persistence could not be compared as there were no extinctions observed in the study. On the other hand, Dey *et al* (2012) reported the evolution of greater constancy and persistence than controls in populations of *D. ananassae* subjected to chronic larval crowding experienced at very low food amounts. The crowding-adapted populations of Dey *et al* (2012) had evolved greater equilibrium population size, and reduced sensitivity of realized population growth rates, as compared to controls. These populations also showed considerably lower intrinsic population growth rates than controls, strongly suggestive of an *r*-*K* trade-off, but the difference was not statistically significant (Dey *et al* 2012). While it is often believed that reduced *r* is necessary for enhanced population stability, this is due to a conflation of the stability of the equilibrium population size (May 1974, Case 2000) with the stability of the observed dynamics: Dey *et al* (2012) further showed via simulations that populations could evolve greater constancy and persistence due to a higher equilibrium population size (*K*), leading to enhanced population growth rates at high densities, even in the absence of a concomitant decrease in intrinsic population growth rate at low density (*r*) due to an *r*-*K* trade-off. Dey *et al* (2012) speculated that the differences seen in these two studies with regard to the evolution, or not,

of stability were likely due to the very different food amounts at which crowding-adapted populations experienced chronic larval crowding in their respective selection regimes. However, strong inferences could not be drawn because the studies of Mueller *et al* (2000) and Dey *et al* (2012) also differed in the species of *Drosophila* used. The present study was one in a set of studies designed to test the speculative hypotheses of Dey *et al* (2012) with greater rigour, using populations of *D. melanogaster* that shared common ancestry with those used by Mueller *et al* (2000).

Our results clearly show that *D. melanogaster* populations (MCU) subjected to chronic crowding at low food amounts, similar to the *D. ananassae* populations (ACU) of Dey *et al* (2012), also showed correlated evolution of constancy and persistence. Thus, our results strongly support the speculation of Dey *et al* (2012) that the evolution of stability in the ACUs, but not in the *D. melanogaster* (CU) populations of Mueller *et al* (2000), is due to differing combinations of egg number and food amount at which those two sets of populations were subjected to larval crowding. We note that the MCU populations share ancestry with the CUs, being derived from the populations that served as ancestral controls to the CUs (details in Sarangi *et al* 2016). The major difference between the MCU and CU populations is that MCUs (like the ACUs) experienced larval crowding at 600 eggs per 8 dram vial with 1.5 mL of food, whereas the CU populations had been reared at 1000-1500 eggs in 6-7 mL of food per 6-dram vial. These differences in the details of how crowding was experienced were earlier seen to result in the evolution of different sets of traits in the ACU/MCU versus the CU populations (Nagarajan *et al* 2016, Sarangi *et al* 2016, Sarangi 2018). In this context, Dey *et al* (2012) noted that the traits that evolved in the ACU populations were closer to the canonical expectation from *K*-selection, whereas traits that evolved in the CU populations were more akin to those ascribable to α-selection: they speculated that typical *K*-selected traits were more likely to mediate the correlated evolution of population stability, especially constancy, due to density-dependent selection than traits that evolved via α-selection. Our results, taken together with the findings of Sarangi *et al* (2016) and Sarangi (2018), are also consistent with the above speculation of Dey *et al* (2012).

In our study, while MCUs clearly had substantially greater persistence than controls (Fig. 2 a, Table 2 a), the two measures of constancy (CV and FI) gave different results: MCUs had significantly lower CV of population size than controls (Fig. 1 a, Table 1 a), but their FI

values, though lower than controls on an average, did not significantly differ (Fig. 1 b, Table 1 b). The precise reason for this discrepancy is not clear at this time, but is likely to be connected to the actual distribution and sequence of population sizes in the respective time series of single-vial populations derived from the MCUs and their controls. We note that while CV reflects dispersion of population size values around the mean for a time series, the FI reflects the average one-step change in population size. Consequently, the specific sequence of population sizes in a time series can affect these two measures differently. We also note that, in Ricker-based simulations, FI does not increase monotonically with *r* beyond r ≈ 2.5 (Fig. 1 a in Sah *et al* 2013). Since the *r* values in our populations are close to that limit, at least when estimated by non-linear fitting (Fig. 3 b), it is also possible that the non-monotonic behavior of FI at high values of *r* may be playing some role here.

Compared to controls, MCUs had significantly greater mean and equilibrium population size (Figs. 2 b, 3 c, d, Tables 2 b, 4 a, b), and the values of mean and equilibrium population size were similar, suggestive of cyclic dynamics, as expected in an LH food regime (Mueller and Huynh 1994, Sheeba and Joshi 1998). There was no clear evidence of reduced intrinsic population growth rate (*r*) in the MCU populations (Figures 3 a, b, 4, 5, Table 3 a, b). Ricker based estimates of *r* showed no main effect of selection and only in 1.5 mL food was there a significantly lower *r* estimate for MCUs. Similarly, empirical estimates of realized population growth rates also indicated very similar values for MCUs and MBs at low density (Figs. 4, 5). Moreover, in the one case with the largest, and significant, difference between mean *r* in the MCUs and MBs (1.5 mL food, estimate based on non-linear fitting), the mean *r* in MBs was only about 4.6% higher than in MCUs. In the earlier study of Dey *et al* (2012), mean *r* in controls was about 12% higher than in the ACUs, even though the difference was not significant, leading the authors to conclude that there may well have been a *r*-*K* trade-off in the ACU populations, and that their study lacked the power to register it as being significant. Given the overall pattern of results for *r* in our study, we are inclined to assess the likelihood of an *r*-*K* trade-off in the MCU populations as being extremely low. We note that increased *K* could drive the observed less negative value of α (Fig. 3 e, f, Table 5 a, b), even in the absence of lower *r*. The large differences in Ricker-based estimates of *r* when using linear regression on log-transformed population growth rates (Fig. 3 a) versus non-linear fitting (Fig. 3 b) underscores the issues with

estimating parameters of exponential functions through linearization via log-transforms pointed out by Mueller *et al* (1995).

Overall, it appears that the greater constancy and persistence of the MCUs is driven not just by higher *K* per se, but by a broader ability to maintain somewhat elevated realized population growth rates over a range of medium to high densities, even beyond *K* (Fig. 5). This observation provides empirical support for an earlier theoretical argument about how high elevated realized population growth rates around *K* can result in greater constancy as well as persistence (see Fig. 1 in Dey *et al* 2012). We note that differences like those seen between realized population growth rates of MCUs and MBs across densities (Fig. 5), while clearly indicating reduced sensitivity of growth rates to density in the MCUs, will not contribute to less negative values of α in the absence of differences in *r* or *K* between the two sets of populations. Thus, the pattern of differences in realized population growth rates between MCUs and MBs (Fig. 5) also suggests that the framework of simple models of population growth, like the Ricker or logistic, may not be adequate to capture how population stability changes as a result of density-dependent selection, because the pattern seen in Fig. 5 cannot be explained by changes in parameters like *r* or *K*. Generalized three-parameter versions of these models like the θ-logistic or θ-Ricker tend to capture differences in dynamics between populations with differing histories of density-dependent selection better than their canonical two-parameter counterparts (Gilpin *et al* 1976), but even these model variants cannot accommodate the possible evolution of higher realized population growth rates at densities both below and above *K*.

In terms of the effect of food level (1 mL vs 1.5 mL) in the single-vial populations in the population dynamics experiment, our findings of higher constancy and persistence stability at higher food levels are in agreement with the trend reported previously from a much shorter 10 generation study on the dynamics of JB single-vial populations on an LH food regime with 1, 2 or 3 mL of food per vial (Vaidya 2013). The stabilizing effects of higher food levels can be attributed to how the demographic attributes respond to changes in food level. Equilibrium population size (*K*), and mean population size, were higher at 1.5 mL food, whereas the sensitivity of realized population growth rate to population density (α) was lower. Together with Vaidya's (2013) findings, our results suggest that increasing food levels from 1 mL to 1.5 mL per vial in a population dynamics experiment enhances stability

by reducing the sensitivity of realized population growth rates to density, whereas increasing the food level further from 2 mL to 3 mL per vial does not enhance stability further. This has consequences for experimental evolution studies since the evolved differences in stability are more easily detectable at lower food levels, which should be used for studying evolutionary changes in population stability. Previously, for example, Vaidya (2013) found no difference in constancy and persistence between MCUs and MBs in a 10 generation population dynamics experiment conducted under an LH food regime with 2 mL of food per vial, even though MCUs had evolved higher K and less negative α by that time.

To conclude, our findings add to growing evidence in support of the hypothesis that population stability can evolve as a correlated response to density-dependent selection, most likely through the evolution of certain life-history traits that influence the sensitivity of fitness components to high density. Our results also support the view that both persistence and constancy can increase as a correlated response to chronic crowding even without an evolutionary reduction in intrinsic population growth rate, as long as the adaptation to crowding facilitates the maintenance of higher realized population growth rates across a range of medium to high densities. This makes it likely that density-dependent selection might be a more common contributor to the evolution of population stability than previously thought. Moreover, our results suggest that a model-free heuristic framework might be more useful than relying on simple population growth models when studying the consequences of life-history evolution for population stability, whether via density-dependent selection or not.

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TABLES

Table 1: Summary results of ANOVA done on constancy stability measured as (a) coefficient of variation in population size, and (b) fluctuation index. The table shows the main effect of selection (MCUs and MBs), food amount (1 and 1.5 mL) and their interaction. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
a) Coefficient of variation in population size						
Selection	1	0.223	3	0.021	10.236	0.049
Food amount	1	0.425	3	0.007	60.647	0.004
Selection × Food amount	1	0.029	3	0.004	7.143	0.075
b) Fluctuation index						
Selection	1	0.096	3	0.030	3.159	0.1735
Food regime	1	0.264	3	0.038	6.957	0.0778
Selection×Food amount	1	0.0028	3	0.019	0.141	0.7315

Table 2: Summary results of ANOVA done on (a) persistence stability measured as probability of extinction, and (b) average population size. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
a) Probability of extinction per generation						
Selection	1	0.018	3	0.000	42.882	0.007
Food amount	1	0.017	3	0.000	101.400	0.002
Selection × Food amount	1	0.006	3	0.000	24.000	0.016
b) Average population size over 31 generations						
Selection	1	22492.07	3	243.008	92.56	0.002
Food amount	1	22653	3	63.597	356.19	<0.001
Selection × Food amount	1	1030.2	3	104.23	9.88	0.051

Table 3: Summary results of ANOVA done on the maximal rate of growth (r), based on the Ricker model, estimated from (a) linear regression of Ln (N_{t+1}/N_t) on N_t, and (b) non-linear curve fitting. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
a) r (estimated from linear regression of Ln (N_{t+1}/N_t) on N_t						
Selection	1	0.161	3	0.062	2.569	0.207
Food amount	1	0.127	3	0.023	5.413	0.102
Selection × Food amount	1	0.008	3	0.053	0.149	0.725
b) r (estimated from non-linear curve fitting)						
Selection	1	0.527	3	0.302	1.740	0.278
Food amount	1	0.779	3	0.148	5.245	0.105
Selection × Food amount	1	0.108	3	0.005	18.849	0.022

Table 4: Summary results of ANOVA done on the equilibrium population size (K), based on the Ricker model, estimated from (a) linear regression of Ln (N_{t+1}/N_t) on N_t, and (b) non-linear curve fitting. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
a) K (estimated from linear regression of Ln (N_{t+1}/N_t) on N_t)						
Selection	1	22321.6	3	271.292	82.28	0.002
Food amount	1	23116.5	3	102.506	225.51	<0.001
Selection × Food amount	1	1099.9	3	86.088	12.78	0.037
b) K (estimated from non-linear curve fitting)						
Selection	1	27322.8	3	214.519	127.367	0.001
Food amount	1	28266.5	3	197.585	143.060	0.001
Selection × Food amount	1	2213.2	3	89.084	24.844	0.015

Table 5: Summary results of ANOVA done on the sensitivity of growth rate to population density (α), based on the Ricker model, estimated from (a) the slope of the linear regression of Ln (N_{t+1}/N_t) on N_t, and (b) as $\alpha = r/K$, after non-linear curve fitting. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
a) α (estimated from slope of the linear regression of Ln (N_{t+1}/N_t) on N_t)						
Selection	1	0.001	3	0.000	285.523	<0.001
Food amount	1	0.001	3	0.000	1752.060	<0.001
Selection × Food amount	1	0.000	3	0.000	0.302	0.620
b) α (calculated by taking a ratio r/K, after r and K were estimated by non-linear curve fitting)						
Selection	1	0.008	3	0.000	125.636	0.001
Food amount	1	0.008	3	0.000	31.911	0.010
Selection × Food amount	1	0.000	3	0.000	3.717	0.149

Table 6: Summary results of ANOVA done on realized growth rate (N_{t+1}/N_t) at low and high population densities. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
Selection	1	0.0024	3	0.0351	0.070	0.808
Food amount	1	0.021	3	0.019	1.130	0.365
Population density	1	286.138	1	0.113	2524.222	<0.001
Selection × Food amount	1	0.063	3	0.059	1.055	0.380
Selection × Population density	1	0.214	1	0.063	3.499	0.160
Food amount × Population density	1	0.149	3	0.025	5.926	0.092
Selection × Food amount×Population density	3	0.018	3	0.045	0.408	0.568

Table 7: Summary results of ANOVA done on realized population growth rate (N_{t+1}/N_t) at different population density (N_t) bins in MBs and MCUs in (a) 1 mL food, with a bin size of 30, and (b) 1.5 mL food, with a bin size of 40. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
a) Realized population growth rate in 1 mL food						
Selection	1	0.515	3	0.037	13.858	0.033
Bin	6	41.902	18	0.035	1182.001	<0.001
Selection × Bin	6	0.293	18	0.015	18.852	<0.001
b) Realized population growth rate in 1.5 mL food						
Selection	1	1.336	3	0.058	22.848	0.017
Bin	5	44.104	15	0.039	1103.469	<0.001
Selection × Bin	5	0.370	15	0.047	7.739	<0.001

FIGURE LEGENDS

Figure 1: Constancy stability in MB and MCU populations in 1 and 1.5 mL food. (a) Mean coefficient of variation in population size, and (b) mean fluctuation index. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 2: Persistence stability and average population size for MB and MCU populations in 1 mL and 1.5 mL food. (a) Mean number of extinctions per generation, and (b) mean population size. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 3: Mean demographic attributes of MB and MCU populations, based on the Ricker equation, in 1 mL and 1.5 mL food. (a) Intrinsic growth rate r (estimated by taking the Y-intercept of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), (b) intrinsic growth rate r (estimated by non-linear fitting), (c) equilibrium population size K (estimated by taking the X-intercept of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), (d) equilibrium population size K (estimated by non-linear fitting), (e) sensitivity of realized growth rate to density α (estimated by taking the slope of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), and (f) sensitivity of realized growth rate to density α (estimated by non-linear fitting). Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 4: Mean empirical realized growth rates (N_{t+1}/N_t) at low (LD) and high density (HD) of MB and MCU populations in 1 and 1.5 mL food. LD: population size less than 30 or 40 for 1 mL and 1.5 mL, respectively. HD: population size higher than 60 or 80 for 1 mL and 1.5 mL, respectively. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 5: Mean empirical realized growth rates (N_{t+1}/N_t) at various population densities in the MB and MCU populations in (a) 1 mL (bin size 30 individuals) and, (b) 1.5 mL food (bin size 40 individuals). Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

FIGURES

Figure 1

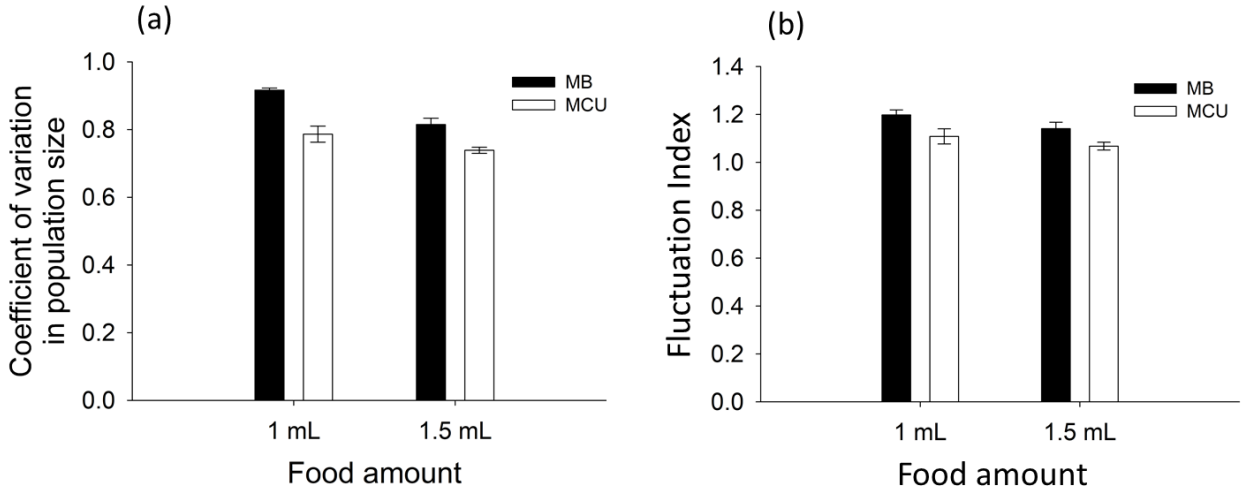


Figure 2

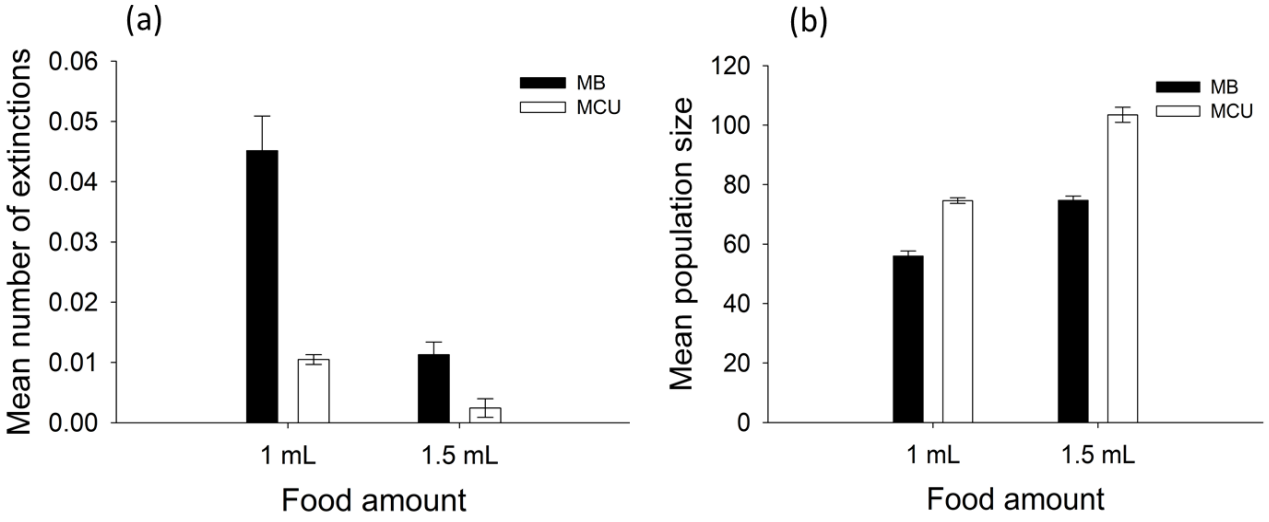

(a)
Mean number of extinctions
0.06
0.05
0.04
0.03
0.02
0.01
0.00
MB
MCU
1 mL
1.5 mL
Food amount
(b)
Mean population size
120
100
80
60
40
20
0
MB
MCU
1 mL
1.5 mL
Food amount

Figure 3

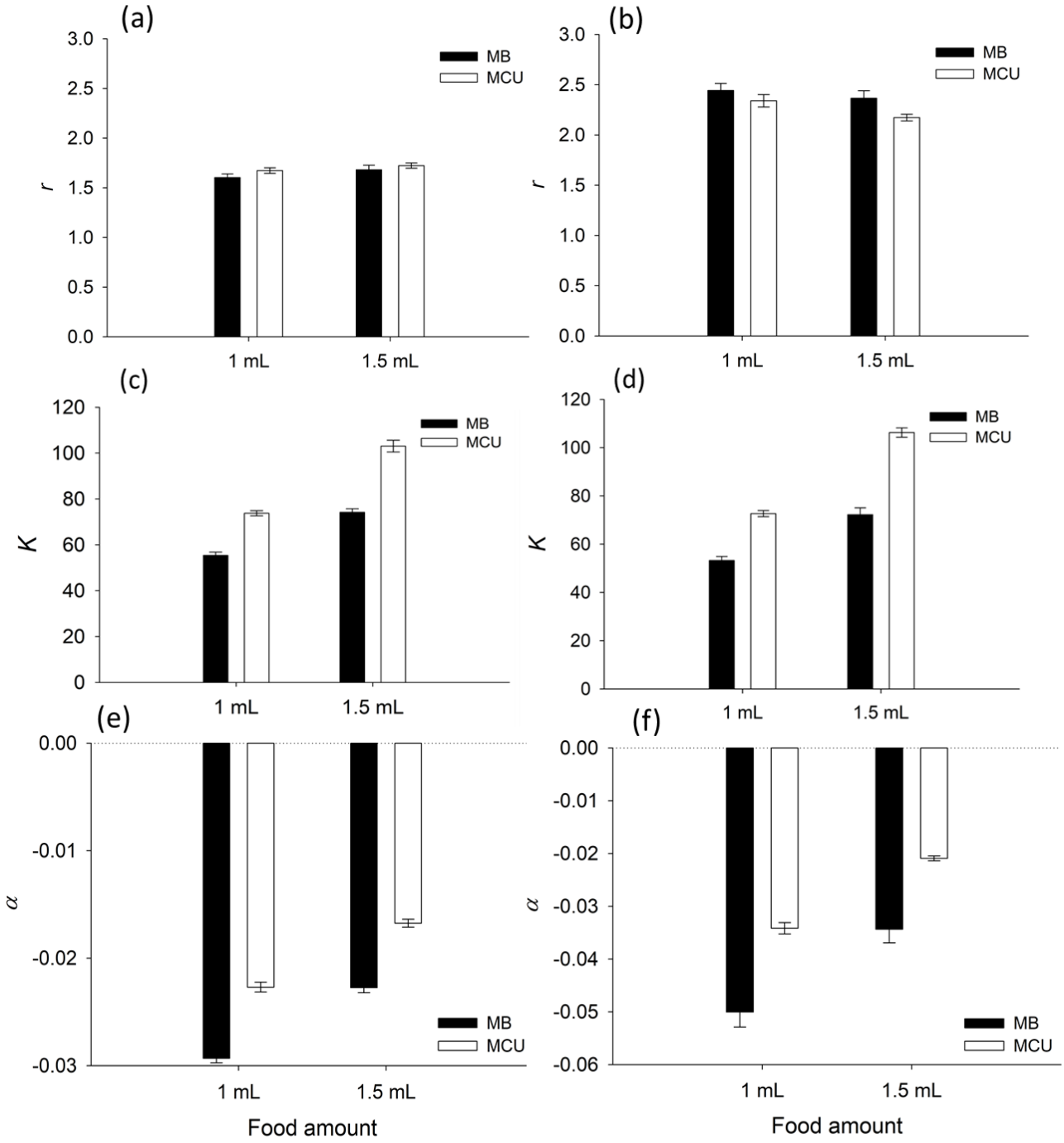


Figure 4

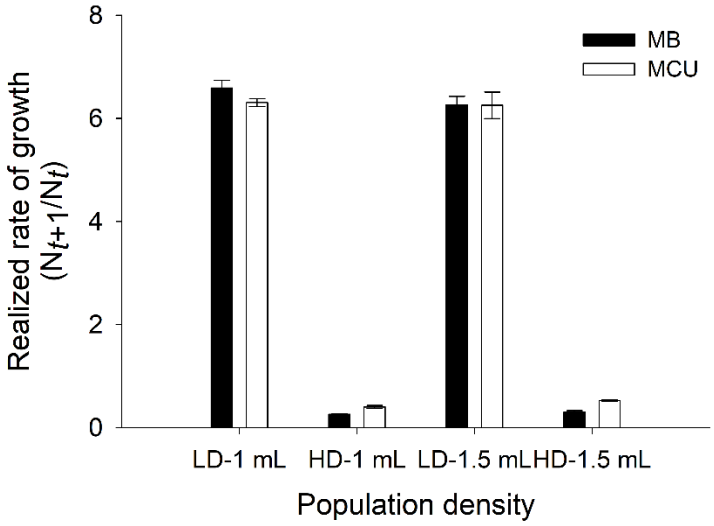
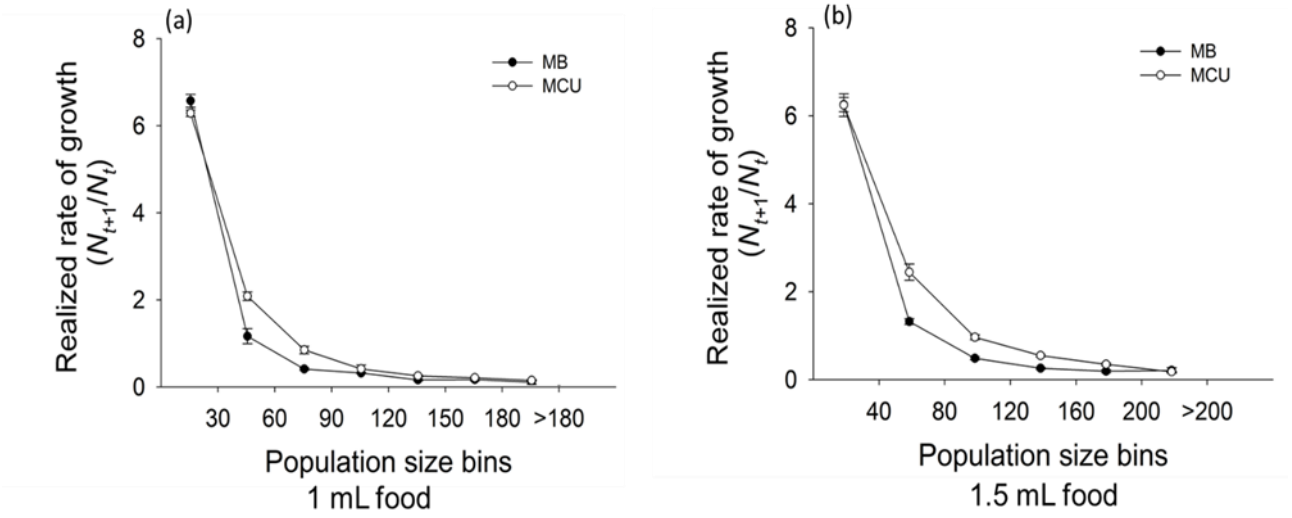

8
6
4
2
0
Realized rate of growth
(N_{t+1}/N_t)
MB
MCU
LD-1 mL
HD-1 mL
LD-1.5 mL
HD-1.5 mL
Population density

Figure 5


(a)
Realized rate of growth
(N_{t+1}/N_t)
8
6
4
2
0
MB
MCU
30
60
90
120
150
180
>180
Population size bins
1 mL food
(b)
Realized rate of growth
(N_{t+1}/N_t)
8
6
4
2
0
MB
MCU
40
80
120
160
200
>200
Population size bins
1.5 mL food

CHAPTER 3

Density-Dependent Selection at High Food Levels Leads to the Evolution of Persistence but not Constancy in *Drosophila melanogaster* Populations

Title: Density-dependent selection at high food levels leads to the evolution of persistence but not constancy in *Drosophila melanogaster* populations

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ABSTRACT

Mechanisms through which population dynamics evolve to be stable have been a subject of considerable interest in population biology. One of the ways through which population stability is likely to evolve is via density-dependent selection with or without an *r* and *K* trade-off. In this paper, we test whether the specific combination of egg number and food amount under which density-dependent selection is implemented affects the evolution of population stability attributes in *D. melanogaster* populations that have evolved under chronic larval crowding for 75 generations. Our findings show that these populations have evolved higher persistence stability than controls, although constancy stability did not evolve. Moreover, these populations did not show an *r*-*K* trade-off, and evolved persistence largely through a significant decrease in sensitivity of growth rate to population density, especially at densities ranging from medium to the equilibrium population size. Qualitative comparison of these findings with those from another set of crowding-adapted *D. melanogaster* populations, that had evolved both constancy and persistence stability, suggests that the ecology of larval crowding influences the evolution of stability attributes. We discuss previous findings on the evolution of life-history traits to argue that differences in the ecology of density-dependent selection experienced at the larval stage affects population stability differently by altering the sensitivity of population growth rate to population density.

Key words: Evolution of population stability, life-history evolution, adaptation to larval crowding, sensitivity of growth rate to population density, fruitflies.

INTRODUCTION

Following the demonstration that even simple, discrete-time, population growth models could show increasingly unstable and complex population dynamics with an increase in intrinsic population growth rate (*r*) (May 1974), it was thought that natural selection, all else being equal, would typically lead to higher intrinsic population growth rates and, therefore, to unstable dynamics. Yet, examination of population dynamics data from multiple species (Hassell *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 a) suggested that relatively stable population dynamics were quite common in nature (reviewed in Mueller *et al* 2000). This apparent contradiction between theoretical expectations and empirical data led to a growing interest in identifying evolutionary scenarios in which population would be likely to evolve to be stable. Early explanations for the evolution of population stability invoked group-selection for stable populations (Thomas *et al* 1980), as well as direct selection for reduced maximal or intrinsic growth rate (*r* in the logistic or Ricker models) (Hansen 1992, Ebenman *et al* 1996), A more plausible explanation was that of the evolution of population stability as a by-product of life-history evolution (Mueller and Ayala 1981 b), especially if the selected life-history-related traits happened to trade off with fecundity, thereby increasing population stability (Turelli and Petry 1980, Stokes *et al* 1988, Gatto 1993, Ebenman *et al* 1996, Prasad *et al* 2003).

Under density-dependent selection, population evolving at low density are expected to evolve high intrinsic growth rate (*r*) while a higher population size at equilibrium (*K*) is expected to evolve under high population density (MacArthur and Wilson 1967). While testing the hypotheses from density-dependent selection theory, Mueller and Ayala (1981 b) subjected *Drosophila* populations to low density (*r*-type) and high density (*K*-type) environments and observed evolved differences in the growth rates at low and high density and trade-offs between them. Following these observations of the effects of density on traits that influenced *r* and *K*, Mueller and Ayala (1981 b) proposed that population dynamics could evolve to be more stable if selection in chronically crowded conditions led to the evolution of traits that lowered *r* as a correlated response to selection for traits that increased *K*.

The possible role of density-dependent selection in mediating the evolution of population stability was first examined in *D. melanogaster* populations which were specifically

selected for adaptations to crowding specifically experienced at the larval stage (Mueller *et al* 2000). These CU populations faced crowding as larvae but were uncrowded at the adult stage, while their ancestral controls (UU) populations did not experience crowding in either larval or adult stage. Selection under high larval crowding in the CUs led to the evolution of traits very similar to those seen earlier in the *D. melanogaster* populations used by Mueller and Ayala (1981 b), most notably the evolution of increased larval feeding rates at the cost of efficiency of food conversion to biomass (Joshi and Mueller 1996), but an examination of their population dynamics did not show any evolved differences in constancy stability (*sensu* Grimm and Wissel 1997); in that study very large populations were used and no extinctions were observed (Mueller *et al* 2000, Mueller and Joshi 2000).

Subsequently, support for the evolution of population stability through density-dependent selection was found in *D. ananassae* populations selected for adaptation to larval crowding (ACUs: Dey *et al* 2012), which showed evolutionary increase in both constancy and persistence stability (*sensu* Grimm and Wissel 1997) as compared to their uncrowded controls (ABs). This study also suggested that the evolution of greater population stability in the ACUs was partly mediated through an *r-K* trade-off. It is worth noting that the ACU populations had evolved increased competitive ability through greater time efficiency of food to biomass conversion, without evolution of increased larval feeding rate (Nagarajan *et al* 2016), as opposed to the crowding-adapted CU populations of Mueller *et al* (2000). These differences in which traits evolved under larval crowding were finally attributed to the different combination of egg number and food amount at which the ACU and CU populations, respectively, experienced chronic larval crowding, with the ACUs being selected at very low food amounts (Nagarajan *et al* 2016, Sarangi *et al* 2016).

Based on the differences in stability evolution between the CUs and ACUs, Dey *et al* (2012) speculated that the specific traits that evolve in response to larval crowding, and whether they mostly affect the effectiveness or tolerance components of competitive ability (*sensu* Joshi *et al* 2001), could possibly help determine whether or not population stability evolved as a correlated response to density-dependent selection. To further test this idea, we studied two different sets of crowding-adapted *D. melanogaster* populations that shared common ancestry with the CU populations of Mueller *et al* (2000). One set of populations (MCUs) experienced chronic larval crowding at the same combination of egg number and food amount as the ACU populations. Another set of populations (LCU) were subjected to chronic larval crowding under egg number and food amount combination approximating

that used for the CUs. The derivation and maintenance of the MCU and LCU populations is described in detail by Sarangi (2018). When we compared the MCUs and their controls for population stability, we found that, similar to the ACUs, the MCUs had evolved greater constancy and persistence stability, but without the involvement of an *r-K* trade-off (Chapter 2). Here, we examine population stability in the LCUs and their controls (the same controls as the MCUs), specifically asking whether they show results similar to the CUs of Mueller *et al* (2000), especially since the LCUs are known to have evolved higher larval feeding rates (Sarangi 2018), like the CUs but not the MCUs. Any observed differences between how population dynamics and stability characteristics have evolved in the LCUs as compared to the MCUs would permit a rigorous experimental test of the speculative predictions of Dey *et al* (2012) about how the specific egg number and food amount combination at which larval crowding is experienced can affect whether or not population stability evolves.

MATERIALS AND METHODS

Experimental populations

We used eight large outbred lab-maintained populations (four selected and four controls) of *D. melanogaster* which are maintained on a 21-day discrete generation cycle in constant light at 25^{o}C±1^{o}C with around 80 percent humidity: four **LCU** populations (Larry Mueller CU-type, Crowded as larvae and Uncrowded as adults), and four **MB** (Melanogaster Baseline, serves as ancestral controls) populations (complete details are given in Sarangi 2018). LCUs are subjected to competition at larval stage at relatively high food amounts (hence, selected for adaptation to larval crowding), while MBs do not face larval competition for food. The LCUs are maintained in a 6-dram glass vials (9 cm height × 2-2.2 cm inner diameter) at a density of ~1200 eggs per 6 mL corn meal food, and at the adult stage at ~1800 adults in Plexiglas cages (dimension 25 × 20 × 15 cm^3). The eclosing flies from LCU culture vials are transferred to their respective cages every day after the 8th day from egg collection till day 20 post egg lay. In the cages, these flies are given corn meal food change every alternate day in a Petridish, and the cage contains a moist cotton ball which is changed every alternate food change. On the 18th day post egg lay, a Petridish containing a generous amount live acetic acid yeast paste is provided for ~2.5 days after

which a cut plate (vertical food surface) is provided (on the 20th day post egg lay) for females to lay eggs for ~18 hours, after which (on the 21st day) eggs are roughly counted to 1200 eggs and placed in vials containing 6 mL of food. The MBs are maintained similarly to the LCUs, except that they are collected at an egg density of ~70 eggs/~6 mL food in 8-dram vials (9.5 cm height × 2.2-2.4 cm inner diameter). Also, all eclosing flies from MB populations are collected at once on the 11th day after egg collection into cages, as most adults eclose by then in the absence of larval competition. Each MB population consists of 40 vials at the larval stage, as opposed to 12 vials for each LCU population, in order to maintain similar adult density (~1800 adults).

Population dynamics experiment:

We carried out the population dynamics experiment for 26 generations in a destabilizing LH food regime (L=low quantity of larval food and H=high quantity of adult food with yeast supplement: Mueller and Huynh 1994, Sheeba and Joshi 1998). This experiment was carried out with 1 mL of larval food in the LH environment, as this food regime provides a high probability of being able to detect differences in constancy and persistence (Vaidya 2013, Chapter 2 in this thesis). At the time of initiating the population dynamics experiment, the LCU populations had undergone about 75 generations of selection and had diverged from their controls in many traits relevant to fitness under larval crowding (Sarangi 2018).

We started the experiment by deriving 10 single-vial populations from each of the eight LCU and MB populations, after one generation of common rearing at low larval density to eliminate any non-genetic parental effects. We started each vial population with 8 mated females which were allowed to lay eggs in in the vial for 24 hours (counted as 16 adults in generation 0). We began transferring eclosing flies from egg vials to matched adult collection vials containing around 4 mL of cornmeal food, after day 8 from egg lay. As eclosion is spread out over several days due to larval competition, we transferred eclosing flies to their respective adult collection vials every day, till day 18 post egg lay. Correspondence between egg vials and adult collection vials was meticulously maintained, and all fly-transfers were done with extreme care to avoid losing any flies. We shifted adults to fresh adult collection vials every alternate till day 18 post egg lay. On day 18, provided flies with a dab of live acetic-acid yeast on the wall of a fresh adult collection vial to boost adult fecundity. On day 20 from egg collection, we transferred flies from the adult vials into

new egg-laying vials with 1 mL food and allowed them to lay eggs over the next 16 hours to lay eggs for next-generation. The adults were then moved into empty vials for the census counts after freezing. Any fly found dead during the 16-hour egg-laying phase was also included in the census count.

The eggs laid by the flies in each vial initiated the next generation, i.e. density was not controlled. In parallel with the vials described above, we maintained a set of five backup vials per population whose maintenance was similar to the experimental vial populations except that backup vial populations were maintained at a low larval density. Each generation, we randomly chose 5 females from each backup vial population to lay eggs for 16 hours in 6 mL of food to start the next backup generation, while the rest of the flies were discarded. Following Dey and Joshi (2006), we maintained these backup vials to reset the experimental populations (with 4 males and 4 females) in case of extinction (absence of even one male-female pair) in a vial population on day 20 post egg lay. A total of 80 single-vial populations (2 selection regimes × 4 replicate populations × 10 single-vial populations) were, thus, censused over the 26 generation long population dynamics experiment. These 80 time series of population size data, along with the number of times each population went extinct over the 26 generations, constituted the primary data for further analyses.

Stability indices

Constancy: We compared constancy stability (*sensu* Grimm and Wissel 1997) in MBs and LCUs using two indices: coefficient of variation (CV) in population size, and fluctuation index of population size (FI). Coefficient of variation (CV) in population size reflects dispersion, scaled by the mean, around the mean population size. We also assessed constancy through FI which measures the mean one-step absolute change in population size, scaled by the mean population size (Dey and Joshi 2006), as

$$FI = \frac{1}{TN}\sum_{t=0}^{T-1}|(Nt+1-Nt)|$$

where T is the number of generations, N is the average population size, and N_t and N_{t+1} are the population sizes at generations t and t+1, respectively. Constancy was interpreted as being the inverse of CV or FI, respectively.

Persistence: We compared persistence stability between MBs and LCUs using the frequency of extinction per generation in each single-vial population. Persistence was interpreted as the inverse of the extinction probability. We counted consecutive extinctions in the same population as one extinction because consecutive extinctions in experiments like these are typically not independent (Dey *et al* 2008). We also calculated mean population size of each single-vial population across the 26 generations of the experiment.

Demographic attributes

In all the single-vial populations, we examined three demographic attributes: intrinsic population growth rate, equilibrium population size, and sensitivity of realized population growth rate to population density. It is known that the dynamics of single-vial *Drosophila* populations in the LH food regime are captured reasonably well by the Ricker (1954) model (Sheeba and Joshi 1998). However, the responses of *Drosophila* population dynamics to various food or selection regimes need not necessarily be limited by any simple population growth model (Tung *et al* 2019, Joshi 2022). Consequently, we examined these attributes in different ways, some based on the Ricker model and others directly based on the empirical data.

In the Ricker-based approach, we estimated r and K, representing intrinsic population growth rate and equilibrium population size, respectively, as well as $\alpha = r/K$, reflecting the sensitivity of realized population growth rate to density. These estimations involved either (a) plotting a regression line between Ln (N_{t+1}/N_t) on the Y-axis and N_t on the X-axis, and taking the Y-intercept, X-intercept and slope as estimates of r, K and α, respectively, or (b) using non-linear curve fitting (following Dey *et al* 2008), through the Quasi-Newton method (StatSoft 1995), followed by calculating α as $-r/K$.

We also examined realized population growth rates (N_{t+1}/N_t) at low ($N_t < 30$) and high ($N_t > 60$) densities, as correlates of r and K, respectively, following the logic of Joshi *et al* (2001). We also checked the realized population growth rates at different cut-off values for low and high density to assess the robustness of the result. Finally, we estimated realized population growth rates (N_{t+1}/N_t) over the entire range of population densities observed in the single-vial populations, in bin sizes of 30.

Comparison between LCUs and MCUs

Although, our purpose in this study was to see whether populations adapted to chronic larval crowding at high versus low food amounts differed in the demographic and stability characteristics they evolved due to density-dependent selection, it was not possible to directly compare evolutionary change in the LCUs and MCUs since, for logistical reasons, the two population dynamics experiments could not be run together. Therefore, we compared them indirectly, making use of the fact that both the LCUs and the MCUs were derived from the same four ancestral control (MB) populations.

We used data from the study in this chapter, and the one described in Chapter 2, and transformed the estimated values of stability indices, mean population sizes and the three Ricker-based demographic attributes (r, K and α) into fractional deviations from control population values. For each measure from each single-vial population in the LCU and MCU population dynamics experiments, we calculated $Y^*_{ij} = (Y_{ij} - \mu_j) / \mu_j$ (i = 1...10, j = 1...4), where Y^*_{ij} was the transformed response variable, Y_{ij} was the measure of a given attribute in the i^{th} replicate single-vial population (in the population dynamics experiment) of the j^{th} replicate population (from the ongoing selection experiment), and μ_j was the mean value of that attribute in the j^{th} replicate population of the control MBs, averaged over all 10 single-vial populations within that replicate. These transformed response variables were subsequently used as input data for further analyses.

Statistical analyses

To compare the population dynamics and stability characteristics of the crowding-adapted LCUs with their controls (MBs), we used mixed model analysis of variance (ANOVA) to analyze all the response variables. The ANOVA models included selection regime as a fixed factor with two levels, crossed with random blocks (four levels) representing the common ancestry of LCU-*i* and MB-*i*. and food level, We performed separate ANOVAs on the coefficient of variation in population size, fluctuation index, extinction probability, average population size, intrinsic growth rate (estimated through three different methods), equilibrium population size (estimated through three different methods), and the sensitivity of realized population growth rate to population density (estimated through two methods). The same ANOVA design was used for the LCU-MCU comparison, using the transformed response variables (see preceding sub-section): here, the two levels of selection regime were

LCU and MCU, rather than LCU and MB, A separate ANOVA was performed on realized growth rates corresponding to different population size bins, with bin as an additional fixed factor, crossed with selection regime and block. All analyses were performed in Statistica Ver. 5.0 (StatSoft 1995), and post-hoc comparisons used Tukey's HSD test at $P = 0.05$.

RESULTS

Constancy and persistence stability

We found that constancy stability was not significantly different between the crowding-adapted LCUs and their controls, the MBs, using either the CV in population size or the fluctuation index (FI) (Fig. 1, Table 1). Indeed, both CV and FI hardly differed on average between the LCU and MB populations (Fig. 1), clearly indicating that constancy stability has not evolved in the LCU populations.

In contrast, greater persistence stability has evolved in the LCUs, as their extinction rate was significantly lower than the MBs (Fig. 2 a, Table 2 a), with LCU populations being nearly half less likely to go extinct as compared to MB populations.

Mean population size

We found that although the LCUs showing slightly higher mean population size as compared to MBs (Fig. 2 b), the difference was not statistically significant (Table 2 b).

Ricker-based demographic attributes

Intrinsic rate of population growth (r), whether estimated from linear regression of Ln (N_{t+1}/N_t) on N_t, or via non-linear fitting, did not differ significantly between MBs and LCUs (Table 3 a, b). Not only were differences in r between LCUs and MBs not significant, even the magnitude of the differences was negligible (Fig. 3 a, b).

The equilibrium population size (K) of LCUs was somewhat higher than the MBs for both methods of estimation: linear regression of Ln (N_{t+1}/N_t) on N_t and non-linear fitting (Table 3 c, d), but the differences were not significant (Fig. 3 c, d).

The sensitivity of realized population growth rate to population density (α) showed that LCUs were significantly less sensitive to change in population density than MBs, regardless of the method of estimation (Fig. 3 e, f, Table 3 e, f).

Model-free demographic attributes

When we compared empirically estimated mean realized population growth rates at low versus high density in the LCUs and MBs, the only significant ANOVA effect was that of density (Table 4). Essentially the magnitude of the difference between growth rates at low versus high density rendered the effect of all other sources of variation on realized population growth rate relatively negligible (Fig. 4 a).

The picture became slightly clearer when we examined mean realized population growth rate across the full range of densities achieved in the single-vial populations, in bin sizes of 30 (Fig. 4 b). There were significant ANOVA effects of population size bin (density level), and the interaction between selection regime and population size bin, for mean realized population growth rate data (Table 5) food. On an average, realized population growth rates were higher at lower population densities until a reasonably high density was attained ($N_t >$ 75), beyond which point realized population growth rates tended to level off (Fig. 4 b). The significant interaction between selection regime and population size bin was driven by the fact that LCUs sustained significantly higher mean realized population growth rate than MBs at densities between 30 and 60 individuals per vial; differences between LCUs and MBs at other bins were not significant in the post-hoc comparisons (Fig. 4 b).

Differences in the stability and demographic attributes of MCUs and LCUs

After transformation of various response variables pertinent to population dynamics and stability in the LCUs and MCUs, expressing their values as a fractional difference from the MB controls in the respective population dynamics experiments, ANOVAs revealed a significant difference between MCUs and LCUs only in their constancy stability as reflected by CV in population size (Table 6). For all other response variables, differences between MCUs and LCUs were not significant (Table 6).

DISCUSSION

Our results essentially confirmed the insight of Dey *et al* (2012) that *Drosophila* populations adapting to chronic larval crowding at high versus low food amounts are likely to differ in whether or not they also evolve greater population stability attributes as a correlated response to density-dependent selection. In terms of demographic attributes and population stability characteristics, we found that the LCUs, adapted to larval crowding at relatively high food amounts, had evolved a different pattern of responses relative to controls than the MCUs (Chapter 2), which were adapted to larval crowding at very low food amounts. The LCUs did not evolve higher constancy than controls (Fig. 1, Table 1), but did evolve higher persistence stability (Fig. 2 a, Table 2 a), presumably largely through the evolution of lower sensitivity of growth rate to density (less negative α: Fig. 3 e, f, Table 3 e, f) and, perhaps, a slight tendency, though not significant, towards higher K (Fig. 3 c, d, Table 3 c, d) and average population size (Fig. 2 b, Table 2 b) than the MB controls. The evolution of persistence but not constancy in the LCUs also supports the previous view (Dey *et al* 2008) that these two stability attributes do not necessarily coevolve, although they can in some circumstances (e.g. Dey *et al* 2012, Chapter 2). The empirical estimates of realized population growth rates in the LCUs across densities also revealed a difference from what was seen in the case of the MCUs (Chapter 2). The MCUs exhibited elevated realized population growth rates, compared to MB controls, across a wide range to medium to high densities, spanning both below and above the equilibrium populations size (Fig. 5 in Chapter 2). As noted in Chapter 2, this kind of change cannot be accommodated within the framework of even the θ-Ricker or θ-logistic models. The LCUs, on the other hand, showed higher realized population growth rates than MBs across a narrower range of medium to high densities, mostly spanning densities less than or up to the equilibrium population size (Fig. 4 b). This pattern of evolution could perhaps be explainable, in principle, by different degrees to which the sensitivity of various fitness components to density has evolved in the MCUs versus the LCUs, and is something that needs to be investigated further. We also note that the kind of change in realized population growth rates at medium to high densities below K seen in the LCUs can be modeled via evolutionary change in θ, using models like the θ-Ricker or θ-logistic.

We discuss these results in the context of the mechanisms through which population stability can evolve, especially through changes in the pattern of density-specific realized

population growth rates, reflected in parameters like *r*, *K* and α in simple population growth models like the logistic or Ricker. We also discuss how ecological differences in density-dependent selection at the larval stage might influence the evolution of population stability by comparing various fitness-related traits, and the population dynamics and stability attributes of the LCUs with other populations that have experienced larval crowding at various combinations of egg density and food volume than the LCUs (Table 7).

The evolution of constancy stability is understood to depend upon either a decline in intrinsic population growth rate (*r*) or increase in equilibrium population size (*K*) (Dey *et al* 2012). Actually, while much discussion on the evolution of stability centres around changes in these familiar parameters of simple population growth models, the operative mechanism is through the effects on the return map of elevated realized growth rates at high density, spanning below and above equilibrium population size (see Fig. 1 in Dey *et al* 2012). Selection for adaptation to larval crowding at high food amounts in the LCUs did not lead to a substantial evolutionary decline in *r*, or increase in *K* (Fig 3 a, b, c, d, Table 3 a, b, c, d). What did evolve in the LCUs was an elevated realized population growth rate, roughly spanning a range of densities from medium to equilibrium population size (Fig. 4 b). We suspect that the fact that LCUs, unlike the MCUs (see Fig. 5 in Chapter 2), did not evolve higher realized population growth rates at densities above the equilibrium population size is the explanation for why LCUs evolved enhanced persistence but not constancy. Assessing this speculation will require theoretical study of how changes in density-specific realized population growth rates affect the shape of the return map.

In tandem with such studies, we also need to develop a conceptual framework for understanding how changes in different fitness components, and their sensitivity to density, results in changes in the density-specific realized population growth rates. *Drosophila* populations subjected to chronic larval crowding at different combinations of egg number and food amount show considerable variation in the underlying traits through which they evolve greater competitive ability (Table 7). However, there is as yet no clear conceptual link between changes in fitness-related traits and in density-specific realized population growth rates. For example, both the MCUs and LCUs have evolved greater competitive ability, but through different life history traits, as the MCUs evolved greater time efficiency of food-to-biomass conversion and the LCUs evolved higher feeding rate at larval stage (Table 7). A trait like greater time efficiency of food-to-biomass conversion could be

increasing pre-adult survivorship under crowding in MCUs (see Chapter 4) which could contribute to higher K and less negative α in MCUs. In contrast, the evolution of higher feeding rate in the LCUs can lead to greater mortality in population dynamics assay as compared to the MCUs, which can explain stability has evolved differently in these two populations which have experienced different types of larval crowding.

The role of larval ecology in shaping constancy stability becomes more evident when we compare the LCUs with the CU populations which had been selected at a similar combination of egg number and food volume as the LCUs. Similar to the LCUs, the CUs had not evolved constancy stability (Mueller *et al* 2000). While persistence stability evolved in the LCUs, persistence could not be calculated in CUs because no extinctions occurred due to the large population sizes at which the CUs (and UUs, the controls) were maintained (Mueller *et al* 2000). Similar to the LCUs, the CUs did not evolve any differences in the surrogates of r and K (Mueller *et al* 2000) relative to the control populations. Further, both the LCUs and CUs evolved higher pre-adult survivorship and faster development at high density; although CUs had evolved increased tolerance to metabolic waste (Shiotsugu *et al* 1997, Borash *et al* 1998) while LCUs did not (Sarangi 2018) (Table 7).

The indirect comparison of LCUs and MCUs, via transformed response variables scaled by control population values, yielded no significant differences between the two selection regimes for any of the response variables other than constancy measured as CV in population size (Table 6). This pattern is slightly discordant with a qualitative comparison of the LCU versus MB, and MCU versus MB results, which suggests that LCUs differ from MCUs not just in CV of population size, but also in estimates of r and K, and the pattern of density-specific realized population growth rates. We suspect the reason these additional differences were not picked up in the analysis of transformed response variable is due to reduced statistical power in the latter, as a result of additional error being introduced during the scaling with mean control population values.

In summary, it is clear that the impact of density-dependent selection on the evolution of population stability attributes can be quite nuanced, and seems to depend on the egg number and food amount combination at which the selection for adaptation to larval crowding was experienced, thereby validating the speculations of Dey *et al* (2012). It is clear that density-dependent selection can affect the evolution of constancy and persistence in very context-

specific manners, and that further theoretical and experimental studies linking changes in fitness components, and their sensitivity to density, to consequent changes in the pattern of density-specific realized population growth rates and return maps will go a long way in enhancing our understanding of these important phenomena linking population ecology and evolution.

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TABLES

Table 1: Summary results of ANOVA done on constancy stability measured as (a) coefficient of variation in population size, and (b) fluctuation index. The table shows the main effect of selection (LCUs vs MBs). Since we were primarily interested in fixed main effects, block effects and interactions have been omitted for brevity.

Response Variable	**Effect**	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
(a) Fluctuation index	Selection	1	0.0003	3	0.0063	0.059	0.8236
(b) Coefficient of variation	Selection	1	0.0001	3	0.0011	0.102	0.7705

Table 2: Summary results of ANOVA done on (a) persistence stability measured as probability of extinction, and (b) average population size. Since we were primarily interested in fixed main effects, block effects and interactions have been omitted for brevity.

Response Variable	**Effect**	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
(a) Probability of extinction per generation	Selection	1	0.0009	3	0.0000	12.902	0.037
(b) Average population size over 26 generations	Selection	1	159.69	3	24.956	6.3988	0.0854

Table 3: Summary results of ANOVA done on the Ricker-based estimates of intrinsic population growth rate (r), equilibrium population size (K), and sensitivity of realized population growth rate to population density (α), estimated from either linear regression of Ln (N_{t+1}/N_t) on N_t (a, c, e), or non-linear curve fitting (b, d, f). Since we were primarily interested in fixed main effects, block effects and interactions have been omitted for brevity.

Response Variable	**Effect**	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
(a) r (Linear regression)	Selection	1	0.0239	3	0.0052	4.591	0.1215
(b) r (Non-linear fitting)	Selection	1	0.0168	3	0.0190	0.89	0.4157
(c) K (Linear regression)	Selection	1	155.5	3	25.435	6.113	0.0898
(d) K (Non-linear fitting)	Selection	1	189.89	3	27.454	6.916	0.078
(e) α (Linear regression)	Selection	1	0.000	3	0.000	15.382	0.029
(f) α (Non-linear fitting)	Selection	1	0.0001	3	0.000	16.273	0.0273

Table 4: Summary results of ANOVA done on realized growth rate (N_{t+1}/N_t) in MBs and LCUs at low and high population densities. The table shows the main effect of selection, population density and their interaction. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
Selection	1	0.0668	3	0.0406	1.642	0.290
Density	1	153.7260	3	0.1022	1503.725	<0.0001
Selection × Density	1	0.1246	3	0.0878	1.418	0.3193

Table 5: Summary results of ANOVA done on realized population growth rate (N_{t+1}/N_t) at different population density (N_t) bins in MBs and LCUs with a bin size of 30. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
Selection	1	0.2084	3	0.0292	7.1161	0.0758
Bin	7	38.2231	21	0.0452	845.5378	<0.0001
Selection × Bin	7	0.2657	21	0.0537	4.9421	0.0019

Table 6. Summary results of ANOVA done on the scaled differences of MCUs and LCUs from their common controls (MBs) for various stability- and dynamics-related response variables. The table shows the main effect of population type (LCUs or MCUs). Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Response Variable	**Effect**	***df* Effect**	**MS Effect**	***df* Error**	**MS Error**	***F***	***P***
(a) Fluctuation index	Population type	1	0.0075	3	0.0055	1.358	0.328
(b) Coefficient of variation	Population type	1	0.0212	3	0.002	10.464	0.048
(c) Extinction Probability	Population type	1	0.0718	3	0.0731	0.982	0.394
(d) Average population size	Population type	1	0.0702	3	0.0182	3.858	0.1442
(e) *r* (Linear regression)	Population type	1	0.0266	3	0.0041	6.365	0.0859
(f) *r* (Non-linear fitting)	Population type	1	0.0002	3	0.0052	0.0430	0.8489
(g) *K* (Linear regression)	Population type	1	0.0702	3	0.0174	4.0176	0.1387
(h) *K* (Linear regression)	Population type	1	0.0673	3	0.0163	4.1145	0.1355
(i) α (Linear regression)	Population type	1	0.0029	3	0.0088	0.3322	0.6047
(j) α (Non-linear fitting)	Population type	1	0.0318	3	0.0046	6.849	0.0792

Table 7. Comparison of various traits relevant to fitness at high larval density, demographic attributes and population stability characteristics, in the various *Drosophila* populations selected under density-dependent selection that have been investigated for population stability across multiple studies. Entries refer to evolutionary change relative to their respective ancestral controls.

Attributes	**CU** (~1000-1500 eggs/~6-7 mL)	**ACU** (~600 eggs/1.5 mL)	**MCU** (~600 eggs/1.5 mL)	**LCU** (~1200 eggs/6 mL)
Development time (at low larval density)	Not different (Santos *et al* 1997)	Decreased (Nagarajan *et al* 2016)	Decreased (Sarangi *et al* 2016)	Not different (Sarangi 2018)
Development time (at high larval density)	Decreased (A. Joshi pers. obs. In Joshi *et al* 2001)	Decreased (Nagarajan *et al* 2016)	Decreased (Sarangi *et al* 2016)	Tendency for faster development, but not significant (Sarangi 2018)
Minimum food requirement for completing development	Increased (Joshi and Mueller 1996)	Not studied directly, but not likely to have increased (Nagarajan *et al* 2016)	Not studied directly, but not likely to have increased (Sarangi *et al* 2016)	Not studied
Time efficiency of food to biomass conversion	Not studied directly, but not likely to have increased (Joshi and Mueller 1996)	Increased (Nagarajan *et al* 2016)	Increased (Sarangi *et al* 2016)	Not studied
Pre-adult survivorship (at low larval density)	Not different (Santos *et al* 1997)	Not different (Nagarajan *et al* 2016)	Slightly higher, but not significantly different (Sarangi *et al* 2016)	Slightly lower, but not significantly different (Sarangi 2018)
Pre-adult survivorship (at high larval density)	Increased (Shiotsugu *et al* 1997)	Increased (Nagarajan *et al* 2016)	Increased (Sarangi *et al* 2016)	Increased (Sarangi 2018)

Dry body-weight at eclosion	Not different (Joshi and Mueller 1996)	Not different (Nagarajan *et al* 2016)	Decreased (Sarangi *et al* 2016)	Not different (Sarangi 2018)
Waste tolerance	Increased (Shiotsugu *et al* 1997, Borash *et al* 1998)	Not increased (Nagarajan *et al* 2016)	Not increased (Sarangi *et al* 2016)	Not increased (Sarangi 2018)
Larval feeding rate	Increased (Joshi and Mueller 1996)	Not increased (Nagarajan *et al* 2016)	Not increased (Sarangi *et al* 2016)	Increased (Sarangi 2018)
Pupation height	Increased initially but then became the same (Mueller *et al* 1993, Joshi and Mueller 1996)	Increased (Nagarajan *et al* 2016)	Not increased (Sarangi *et al* 2016)	Not studied
Foraging path length	Increased (Sokolowski *et al* 1997)	Increased (Nagarajan *et al* 2016)	Not increased (Sarangi *et al* 2016)	Not studied
Demographic attributes	No difference in surrogates of r and K (Mueller *et al* 2000)	Evolved slightly decreased r and increased K, and decreased α (Dey *et al* 2012)	No difference in r, increased K, and decreased α (chapter 2)	No difference in r, slight increase in K, and decreased α (this chapter)
Population stability	No difference in constancy; persistence not examined (Mueller *et al* 2000)	Evolved increased constancy and persistence (Dey *et al* 2012)	Evolved increased constancy and persistence (chapter 2)	Evolved increased persistence, but not constancy (this chapter)

FIGURE LEGENDS

Figure 1: Constancy stability in MB and LCU populations: (a) mean coefficient of variation in population size, and (b) mean fluctuation index. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 2: Persistence stability and average population size for MB and LCU populations: (a) mean number of extinctions per generation, and (b) mean population size. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 3: Mean demographic attributes of MB and LCU populations, based on the Ricker equation: (a) intrinsic growth rate r (estimated by taking the Y-intercept of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), (b) intrinsic growth rate r (estimated by non-linear fitting), (c) equilibrium population size K (estimated by taking the X-intercept of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), (d) equilibrium population size K (estimated by non-linear fitting), (e) sensitivity of realized growth rate to density α (estimated by taking the slope of the regression line between Ln (N_{t+1}/N_t) on Y-axis and N_t on X-axis), and (f) sensitivity of realized growth rate to density α (estimated by non-linear fitting). Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

Figure 4: Mean empirical realized growth rates (N_{t+1}/N_t) of MB and LCU populations: (a) at low ($N_t < 30$) and high ($N_t > 60$) density, and (b) across the range of densities seen in the single-vial populations, in bins of 30. Error bars around the means are standard errors based on variation among the means of the four replicate populations within each selection regime.

FIGURES

Figure 1

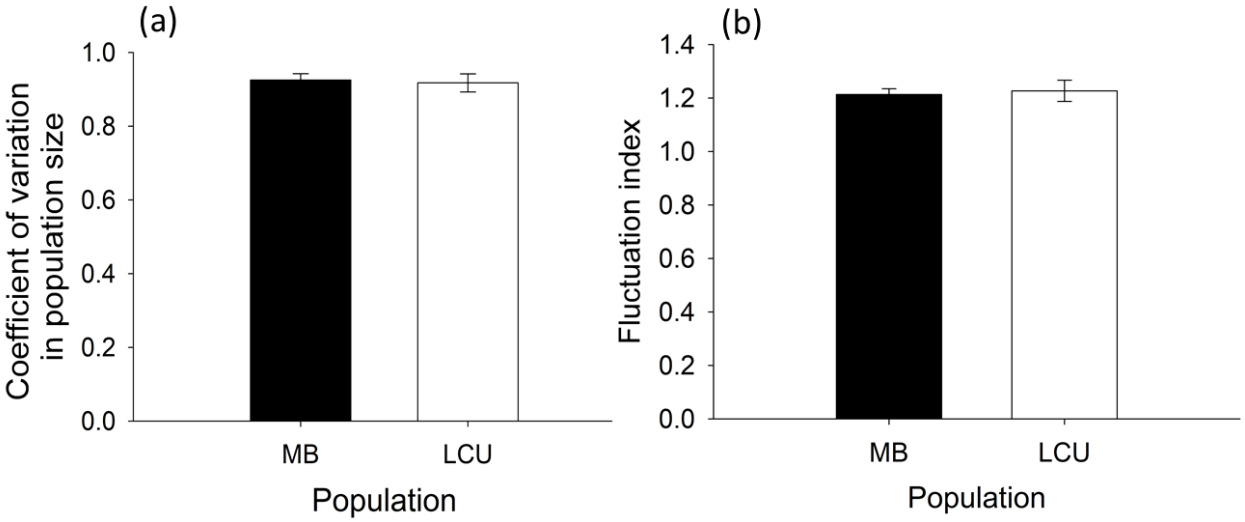


Figure 2

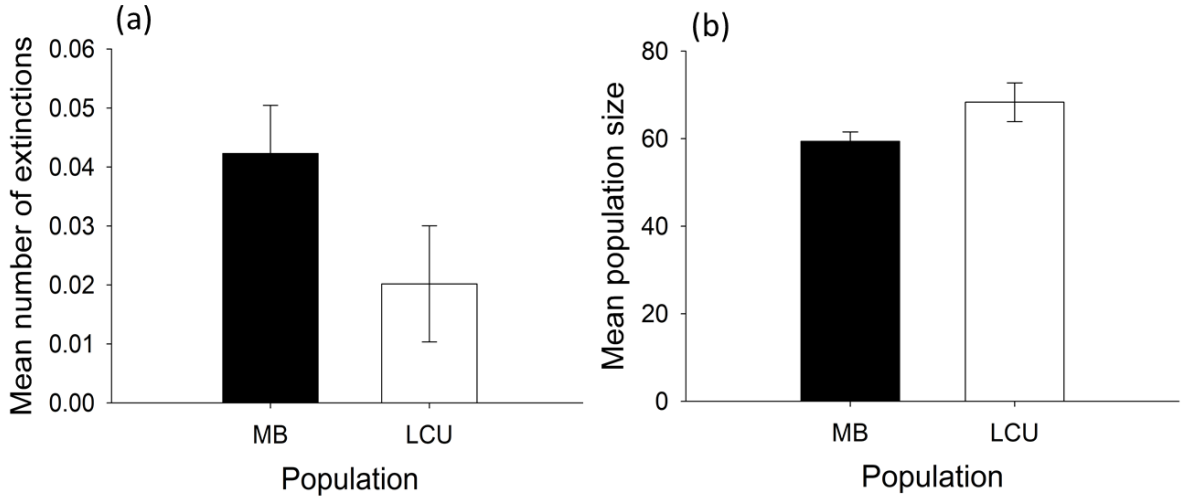

(a)
Mean number of extinctions
0.06
0.05
0.04
0.03
0.02
0.01
0.00
MB
LCU
Population
(b)
Mean population size
80
60
40
20
0
MB
LCU
Population

Figure 3

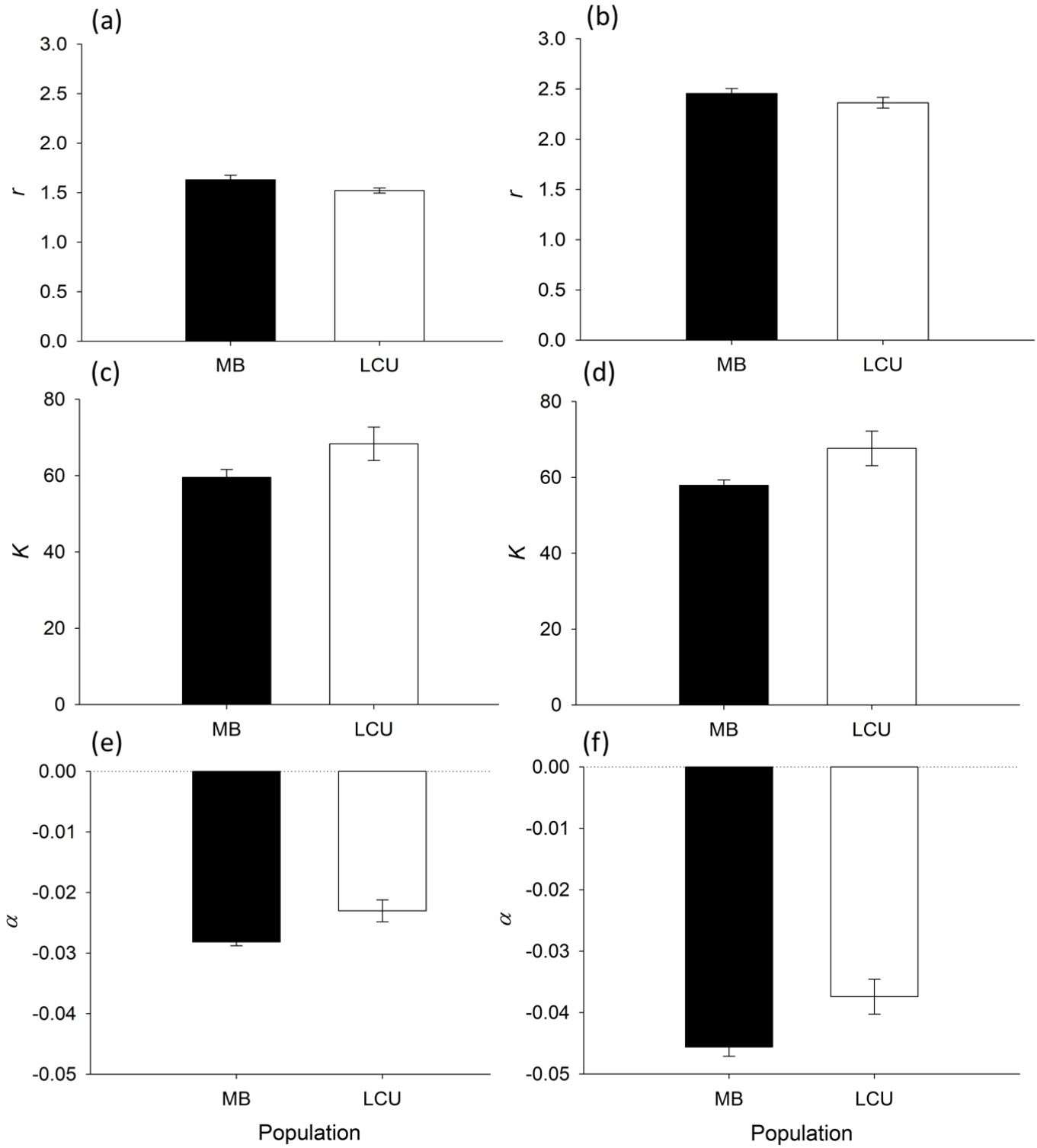
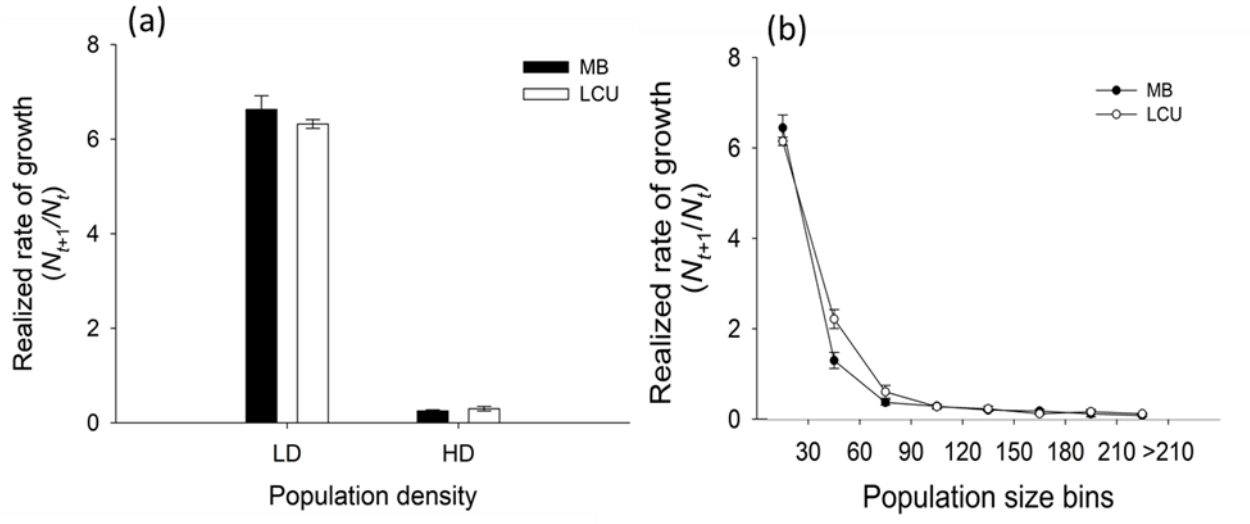


Figure 4


(a)
Realized rate of growth
(N_{t+1}/N_t)
8
6
4
2
0
MB
LCU
LD
HD
Population density
(b)
Realized rate of growth
(N_{t+1}/N_t)
8
6
4
2
0
MB
LCU
30
60
90
120
150
180
210
>210
Population size bins

CHAPTER 4

Effects of Larval and Adult Crowding on Fitness Components in *Drosophila* Populations Adapted to Larval Crowding Experienced under Different Combinations of Food Amount and Egg Number

Title: Effects of larval and adult crowding on fitness components in *Drosophila* populations adapted to larval crowding experienced under different combinations of food amount and egg number

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ABSTRACT

Since the realization in the 1970s that simple discrete-time population growth models can show complex unstable dynamics of population size, many explanations were proposed for the evolution of enhanced population stability. The most plausible of these was density-dependent selection, suggested to favour greater stability due to *r-K* trade-offs. However, the first experiment aimed at testing this prediction revealed that *Drosophila melanogaster* populations adapted to larval crowding did not evolve greater constancy stability than their ancestral controls. A subsequent study showed that *D. ananassae* populations adapted to larval crowding had evolved greater constancy and persistence than ancestral controls. These *D. ananassae* populations had experienced chronic larval crowding in conditions of very low amounts of food, whereas the earlier studied *D. melanogaster* populations had experienced chronic larval crowding at fairly high food amounts. Further theoretical work also suggested that populations adapting to crowding could evolve greater stability even in the absence of *r-K* trade-offs. Most recently, studies in our laboratory showed that two sets of crowding adapted *D. melanogaster* populations, derived from a common ancestral lineage, which differed in the food amounts at which they experienced larval crowding, evolved different patterns of constancy and persistence stability. These two sets of populations also differed in the traits, e.g. larval feeding rate, that evolved as they became more competitive. Here, we examine the response of key fitness components to larval and adult densities in these two sets of populations, to see whether differences in their stability attributes can be explained by variation in how their life-histories respond to crowding at different life stages. Of all traits examined, only pre-adult survivorship responded differently to larval density across the two sets of populations. The populations that adapted to larval crowding at low food amounts showed reduced sensitivity of pre-adult survivorship to larval density, compared to those that adapted to larval crowding at high food amounts. We discuss our results in the context of different ways in which density-dependent selection may facilitate the evolution of greater constancy or persistence, depending on the ecological details of how crowding was experienced.

Keywords: density-dependent selection, density-dependent feedback loops, population stability, life-history evolution, experimental evolution, *Drosophila melanogaster*.

INTRODUCTION

The recent focus on eco-evolutionary dynamics (e.g. Romero-Mujalli *et al* 2019, but see also Hendry 2019) notwithstanding, population ecology and evolutionary biology developed rather independently of one another during the first six to seven decades of the twentieth century (Kingsland 1995). Despite the early attempts of Elton (1927) to bridge the nascent fields of evolutionary biology and population ecology, pointing out that population cycles could have an effect on evolutionary dynamics because selection at high or low densities, respectively, might favour different sorts of traits, these two strands of density-dependent selection of varying traits and its possible effects on population dynamics and stability, began to come together only in the 1980s (reviewed in Mueller and Joshi 2000). On the one hand was the development of the formal theory of density-dependent selection (MacArthur 1962, MacArthur and Wilson 1967, Anderson 1971, Charlesworth 1971, Roughgarden 1971, Asmussen 1983), focussing attention on *r-K* trade-offs and their role in mediating adaptation to chronic crowding (Luckinbill 1978, Mueller and Ayala 1981, Mueller *et al* 1991, Vasi *et al* 1994); on the other, was the realization that simple population growth models could show varied and unstable dynamics of population size as long as intrinsic growth rates were high, and there was a time-lag in the density-dependent feedback (May 1974, May and Oster 1976), leading to investigations of proximal and ultimate causes of population stability (discussed in Jaggi and Joshi 2001, Mueller 2009, Dey and Joshi 2013, 2018).

Among the various proposed mechanisms for the evolution of population stability, the most plausible was that density-dependent selection could facilitate the evolution of stability, via an *r-K* trade-off, through promoting an evolved increase in *K* (reviewed by Mueller and Joshi 2000, Dey *et al* 2012). However, the first experiment aimed at testing this prediction did not support the notion that density-dependent selection would promote the evolution of greater stability in populations adapted to chronic crowding (Mueller *et al* 2000). Populations of *D. melanogaster* that had evolved traits indicating greater competitive ability than ancestral controls, as a result of having been subjected to high larval density for many generations, nevertheless showed no evidence of greater constancy stability (*sensu* Grimm and Wissel 1997) than controls, when reared for 68 generations in a food regime known to induce large and somewhat regular fluctuations in population size (first 45 generations reported by Mueller *et al* 2000, full study reported in Mueller and Joshi 2000). In this study,

which involved very large cage populations (population size in the thousands), there were no extinctions and, hence, persistence could not be assessed. The lack of evolution of constancy was, however, not due to a general lack of evolutionary change, as other traits related to fitness under crowding did evolve in the populations studied (Mueller and Joshi 2000, Mueller *et al* 2000, Joshi *et al* 2003).

A subsequent study, using populations of *D. ananassae* adapted to larval crowding experienced under different ecological conditions than the populations used by Mueller *et al* (2000), on the other hand, showed clear evidence for density-dependent selection resulting in the evolution of enhanced stability (Dey *et al* 2012). The crowding adapted populations of *D. ananassae* exhibited significantly greater constancy and persistence than their ancestral control populations, but whether or not this was due to an underlying *r-K* trade-off was unclear (Dey *et al* 2012). While the crowding adapted populations had evolved both significantly higher average population size and estimated *K* than the controls, their estimated *r*, although substantially lower, was not significantly different from controls (Dey *et al* 2012). The sensitivity of realized per capita population growth rate to density was also significantly lower than controls in the crowding adapted populations (Dey *et al* 2012). The principal difference between the high larval crowding experienced by selected populations in the studies of Mueller *et al* (2000) and Dey *et al* (2012) was in the combination of food level and egg number in the culture vials. The populations of Mueller *et al* (2000) experienced larval rearing densities of ~1000-1500 eggs in 6-7 mL of banana-molasses food in 6-dram vials, whereas those of Dey *et al* (2012) were reared at larval densities of ~600 eggs in 1.5 mL cornmeal food in 8-dram vials. The larval crowding adapted populations of *D. ananassae* also evolved greater competitive ability via a different set of traits than the *D. melanogaster* populations of Mueller *et al* (2000): notably, the *D. ananassae* populations evolved greater time efficiency of larval food conversion to biomass, rather than an increased larval feeding rate (Nagarajan *et al* 2016). These differences led Dey *et al* (2012) to speculate that perhaps the precise suite of traits that evolves in response to chronic larval crowding may affect whether or not population stability evolves as a correlated response to density-dependent selection. However, any explanation for the differences between the results of Mueller *et al* (2000) and Dey *et al* (2012) could not be unequivocal, since the two studies also differed in species used, in addition to the food level and egg number combination at which they experienced larval crowding.

Subsequent selection experiments using a set of *D. melanogaster* populations (MCU populations: see Materials and Methods), derived from the same ancestors as those used by Mueller *et al* (2000), and adapted to larval crowding at an egg number and food level combination similar to that of the *D. ananassae* populations of Dey *et al* (2012), indicated that the specific suite of traits that evolved in response to chronic crowding was largely determined by egg number and food level combination, not by species identity (Sarangi *et al* 2016). Consequently, another set of *D. melanogaster* populations (LCU populations: see Materials and Methods), derived from the same ancestral controls as the MCU populations, and adapted to larval crowding at an egg number and food level combination similar to that of the *D. melanogaster* populations of Mueller *et al* (2000), was found to have evolved to adapt to chronic larval crowding via traits such as increased larval feeding rate, similar to those evolved by the populations used by Mueller *et al* (2000) (Sarangi 2018). As a result of these various studies, it became clear that the LCU and MCU populations, which shared ancestral controls, evolved to adapt to chronic larval crowding via different suites of traits as a result of the different combinations of egg number and food level at which they experienced crowding (Sarangi 2018, Venkitachalam *et al* 2022). These studies, thus, paved the way for testing the speculative predictions made by Dey *et al* (2012) about whether the specific traits that evolve in response to chronic larval crowding could affect whether or not population stability evolves as a correlated response to density-dependent selection.

To this end, the dynamics and stability of the LCU and MCU populations, relative to their ancestral controls, were assessed in two separate multi-generation population dynamic experiments that revealed that the MCU populations, like the *D. ananassae* populations of Dey *et al* (2012) had evolved greater constancy and persistence than controls, whereas the LCU populations, like those of Mueller *et al* (2000) did not show greater constancy than controls, but did exhibit higher persistence (N Pandey and A Joshi, *unpubl. mss*.). The MCU populations also exhibited greater average population size and estimated *K*, and reduced sensitivity of realized per capita population growth rate to density, as compared to controls (N Pandey and A Joshi, *unpubl. ms*.). The LCU populations, on the other hand, did not differ from controls in average population size and estimated *K*, but did show significantly lower density-sensitivity than controls of realized per capita population growth rate (N Pandey and A Joshi, *unpubl. ms*.). It was this observed difference in the dynamics and stability characteristics of the MCU and LCU populations that motivated the present study

which examined whether the MCU and LCU populations differed in major life-history traits and their sensitivity to density.

MATERIALS AND METHODS

In this study, since we were interested in examining the density-responses of life-history traits in the context of their role in mediating population dynamics, we used food amounts and egg numbers that approximate the conditions in typical population dynamics experiments in our laboratory. Consequently, the larval density conditions in the present assays are different from previous assays of life-history traits in these populations under low and high larval densities (e.g. Sarangi *et al* 2016, Sarangi 2018, Venkitachalam *et al* 2022).

Experimental populations

We used eight large outbred laboratory populations of *D. melanogaster* for this study, belonging to two selection regimes, consisting of four replicate populations each, subjected to larval crowding at different combinations of egg number and food amount. Complete details of the derivation and maintenance of these populations can be found in Sarangi (2018); we reiterate the essential details here.

MCU 1-4

Each MCU population was derived from an independent ancestral population, and they were maintained at a high density of around 600 eggs in 1.5 mL of cornmeal food, in cylindrical Borosilicate glass vials of 2.2-2.4 cm inner diameter and 9.5 cm height. The MCUs had been maintained on a 21-day discrete-generation cycle, under constant light (LL), at $25^{o} \pm 1^{o}$C temperature and around 80% humidity for about 185 generations at the time of this study. To maintain a breeding population size of around 1800 adults per vial, 12 vials per population were set up for each generation. Since larval crowding prolongs development, eclosing adults were collected into cages every day till day 18 from egg-lay, once eclosions began. Eclosing adults from all vials of a population were transferred to a Plexiglas cage (25 × 20 × 15 cm^3) containing a food plate (Petridish with cornmeal food), and a wet cotton ball to help maintain humidity. The old food plate was replaced with a fresh food plate every alternate day till day 18 from egg-lay, and the cotton ball was changed at every alternate

food plate change. On the 18th day, adults were given live acetic-acid-yeast supplement, in addition to the regular food, till day 20, and on the 20th day these adults were allowed to lay eggs for around 18 hours on vertically cut sterile cornmeal food. On the 21st day, eggs laid by these flies were collected into vials to start the next generation.

LCU 1-4

Each LCU population was derived from an independent ancestral population, the same set of four ancestors as the MCUs, and they had been maintained at a high larval density of around 1200 eggs in 6 mL of cornmeal food per vial for about 64 generations at the time of this study. During the pre-adult stage, LCUs were maintained in slightly shorter and narrower vials (9 cm height × 2.0-2.2 cm inner diameter) than MCUs. The egg number, food amount, and vial dimensions of the LCUs were chosen to approximate the maintenance regime of the CUs at the larval stage (first described in Mueller *et al* 1993) used in earlier studies. Except for the difference in egg number and food amount, and vial dimensions, the maintenance protocol for LCUs was the same as for MCUs.

Trait assays

Prior to assays, we subjected all populations to one generation of common rearing at low larval density (roughly 70 eggs in 6 mL cornmeal food per vial), corresponding to that used for the ancestral control populations for both the MCU and LCU populations, to avoid non-genetic parental effects contributing to differences among selection regimes. After a generation of common rearing, we allowed females to lay eggs for 12 hours on a sterile double agar plate, and these eggs were used to initiate the various assays. Due to logistical constraints, only two replicate populations per selection regime were assayed at a time: MCU 1-2 (184 generations of selection) and LCU 1-2 (63 generations of selection) were assayed together, and MCU 3-4 (185 generations of selection) and LCU 3-4 (64 generations of selection) were assayed together.

Pre-adult survivorship

We examined the pre-adult survivorship for all the selected and control populations in the following egg number × food amount combinations: (i) 75 eggs in 1 mL and 1.5 mL cornmeal food (10 vials each); (ii) 150 eggs in 1 mL and 1.5 mL cornmeal food (5 vials each); (iii) 300 eggs in 1 mL and 1.5 mL cornmeal food (5 vials each). We counted all the eclosing adults to calculate pre-adult survivorship in each vial.

Female fecundity

We collected flies eclosing between days 9-11 after egg-lay in the vials kept at different population × egg number × food amount combinations for the pre-adult survivorship assay. These flies were kept in groups of five males and five females per vial, containing 4 mL cornmeal food), with 5 such vials set up for each selection regime × egg number × food amount combination on days 9, 10 and 11 from egg-lay (following Vaidya 2013). These collected flies were shifted to fresh food vials every alternate day till day 17 from egg-lay. Prior to assaying fecundity on day 21 post egg-lay, the flies were given live acetic-acid-yeast paste supplement in the food vials from day 18 post egg-lay. On day 20 from egg-lay, we mixed all flies from all the 15 vials for each selection regime × egg number × food amount combination, and from this pool of flies that varied in adult age from 10-12 days old, we haphazardly chose 15 males and 15 females for assaying fecundity. Individual male-female pairs were placed into a sterile food vial containing a thin layer of food for egg laying over the next 16 hours, after which we removed the flies and counted the number of eggs under a stereo-zoom microscope. Thus a total of 720 females (8 populations × 6 treatment combinations × 15 replicates) were assayed for fecundity on day 21 post egg-lay.

Dry body-weight at eclosion

We thoroughly mixed the flies eclosing in vials kept at different population × egg number × food amount combinations for the pre-adult survivorship assay, and then haphazardly chose 25 males and 25 females per population × treatment combination for weighing. We refrigerated these flies at 5^{o}C, and later dried them for 36 hours at 70^{o}C in a hot air oven before weighing them to the nearest 10^{-5} g on a Sartorius CP225D microbalance, in single-sex groups of five flies each. Each batch of five flies was weighed thrice, and the mean of those readings taken as the dry body-weight of that batch.

Adult survivorship till egg-lay

We examined the effect of adult crowding on adult survivorship from eclosion till day 21 post egg-lay, the last day of effective adult life in three-week discrete-generation cultures, by subjecting the adults to two densities. We reared larvae at a low larval density of ~70 eggs in 6 mL cornmeal food per vial. On day 11 from egg-lay, we shifted eclosing adults into one of two adult density treatments: low (50 adults per vial) and high (150 adults per vial), with a 1:1 sex ratio in each vial. We had exactly 5 mL of cornmeal food in all the vials and used cotton plugs of similar size to plug the vials so as to offer equal space for flies in

all vials. Each adult density treatment had 7 replicate vials per population. Flies were moved to fresh food vials every day to avoid confounding effects of larval activity. The number of flies dying in each vial per day, till day 21 post egg-lay was recorded.

Statistical analyses

Every replicate larval crowding adapted population shares ancestry with an MB population with the same replicate subscript i.e. replicate population *i* in the MCU, and LCU regimes is derived from replicate *i* of MB (*i* = 1..4). This permits the use of a completely randomized block design in our statistical analysis, with replicate populations bearing the same subscript treated as blocks. In the mixed model analyses of variance (ANOVAs), block was treated as a random factor crossed with the fixed factors selection regime, egg number, and food amount. An additional fixed factor, sex, was also added while analyzing dry body-weight data. For testing the effect of adult density on adult survivorship, block was treated as random factor, and selection regime, adult density, and sex were treated as fixed factors. We performed all analyses on Statistica Version 5.0 (Statsoft 1995), and used Tukey's HSD at $P = 0.05$ for all post-hoc multiple comparisons.

We also analyzed the data on pre-adult survivorship, female fecundity at day 21 post-egg lay, and male and female dry body-weight at eclosion via linear regression against larval density (eggs per unit volume food) as the independent variable, utilizing the fact that the six combinations of egg number and food amount corresponded to six different larval densities. Linear regression for each replicate MCU and LCU population was carried out separately, and then the eight values obtained for slope, intercept and R^2, respectively, were subjected to mixed model ANOVA with selection regime as a fixed factor and block as a random factor. All these analyses were also implemented in Statistica Version 5.0 (Statsoft 1995).

RESULTS

Pre-adult survivorship

As expected, pre-adult survivorship was lower at higher egg numbers, in both 1 mL and 1.5 mL food, for both LCU and MCU populations (Figure 1 A, B). For both sets of populations, the drop in survivorship was greater between 150 and 300 eggs per vial, compared to from 75 to 150 eggs (Figure 1 A, B). There was slightly lower survivorship in 1 mL than in 1.5 mL (Figure 1 A, B), but there were no significant effects of food amount or any interaction involving food amount (Table 1). The only significant ANOVA effects were that of egg number and the selection × egg number interaction (Table 1), driven by significantly higher survivorship of MCU over LCU populations, by about 20%, at the highest egg number of 300 per vial (Tukey's HSD, $P < 0.05$), in both the 1 mL and 1.5 mL food amount treatments (Figure 1 A, B).

Female fecundity at day 21 post egg-lay

As expected, female fecundity on day 21 post egg-lay was lower at higher egg numbers, and in 1 mL compared to 1.5 mL food, for both LCU and MCU populations (Figure 1 C, D). ANOVA revealed that only the main effects of egg number and food amount were significant (Table 2). For both sets of populations, the drop in fecundity was greater between 150 and 300 eggs per vial, compared to from 75 to 150 eggs (Figure 1 C, D). Fecundity of LCU females (mean: 35.6 eggs) was visibly greater than that of MCU females (mean 28.6 eggs) in the combination of 300 eggs in 1 mL food (Figure 1 C), but it was not possible to make anything of it in the absence of any significant interaction of selection × egg number × food amount.

Dry body-weight at eclosion

Male (Figure 1 E, F) and female (Figure 1 G, H) dry body-weights at eclosion showed the expected prominent effects of sex (females heavier), egg number and food amount (weight decrease with increase in egg number and reduction in food), but there was no clear indication of any difference in how the two sets of populations responded to increasing egg number or decreasing food amount (Figure 1 E, F, G, H). Correspondingly, in the ANOVA, the main effects of sex, egg number and food amount were significant (Table 3). Male dry weights (Figure 1 E, F) were less severely affected by increasing larval density than female dry weights (Figure 1 G, H), driving significant egg number × sex and food amount × sex

interactions (Table 3). There was no main effect of selection regime, and only one interaction involving selection regime (selection × egg number × food amount) was significant (Table 3). However, this interaction was due to small haphazard differences in the response of how dry body-weights changed in LCU and MCU populations across egg number and food amount combinations, and these changes did not suggest any clear pattern of differences between selection regimes in the sensitivity of dry body-weight to larval density (Figure 1 E, F, G, H).

Adult survivorship till egg-lay

As expected, adult survivorship from eclosion to day 21 post-egg lay declined sharply as density increased from 50 flies per vial (mean survivorship: 0.94) to 150 flies per vial (mean survivorship: 0.48) (Figure 2 A, B). Female (Figure 2 B) and male (Figure 2 A) survivorship was somewhat similar at 50 flies per vial (mean survivorship females: 0.90, males: 0.97), whereas at 150 flies per vial, females had markedly lower mean survivorship (0.29) than males (0.67). These results were reflected in significant ANOVA effects of sex, adult density, and the sex × adult density interaction (Table 4). There was no evidence for any difference between selection regimes in the response of adult survivorship to adult density (Figure 2 A, B), and neither the main effect of selection regime, nor any interaction involving selection regime, was significant (Table 4).

Regression analyses

The results from the regression analyses were concordant with those described above in Sections 3.1 – 3.3 (Figure 3). There were no significant differences seen between MCU and LCU populations, on an average, for either intercept or goodness of fit (R^2) for pre-adult survivorship, female fecundity on day 21 post-egg lay, or male/female dry body-weight at eclosion (ANOVA results not shown). Only one of the ANOVAs on slope showed a significant difference between selection regimes ($F_{1,3}$ =15.91, P = 0.028); the MCU populations (Figure 3 A) had a significantly less negative slope than the LCU populations (Figure 3 B) for pre-adult survivorship. Thus, MCU populations had a higher pre-adult survivorship than LCU populations over a wide range of larval densities. For female fecundity (Figure 3 C, D), and dry body-weight of males (Figure 3 E, F) and females (Figure 3 G, H), the slopes did not significantly differ between selection regimes (ANOVA results not shown).

DISCUSSION

We undertook this study to examine whether differences between the MCU and LCU populations in the sensitivity of key life-history traits to larval or adult density might explain the fact that greater constancy evolved in the MCU, but not the LCU populations (N Pandey and A Joshi, *unpubl. mss*.), as a consequence of density-dependent selection due to chronic larval crowding. In *Drosophila* cultures, there are four major density-dependent negative-feedback loops that affect population growth: larval density-dependent larval mortality, larval density-dependent adult size and, therefore, female fecundity, adult density-dependent adult mortality, and adult density-dependent female fecundity (Figure 4; Mueller 1988, Joshi *et al* 1998). In LH food regimes, the sensitivity of female fecundity to adult density is considerably weakened due to the provision of supplementary yeast past, resulting in cyclic dynamics (Mueller and Huynh 1994), and detailed individual based simulations indicate that this feedback loop has relatively little effect in further modulating the dynamics of cultures in an LH food regime (Tung *et al* 2019). Consequently, we examined only the sensitivity to density of pre-adult survivorship, female fecundity at day 21 post-egg lay, male and female dry weight at eclosion, and adult mortality from eclosion to day 21 post-egg lay in the MCU and LCU populations.

To briefly recapitulate, earlier studies (N Pandey and A Joshi, *unpubl. mss*.) showed that both the MCU and the LCU populations showed significantly greater persistence, and significantly lower sensitivity of realized per capita growth rate to density, than their common controls. In addition, the MCU populations also showed significantly greater constancy, equilibrium population size, and average population size than controls. The LCU populations had very similar constancy to controls, and their average population size and equilibrium population size were higher than controls, but not significantly so. In terms of the magnitude of difference from controls, the MCU populations differed from controls, on an average, to a degree greater than the LCU populations did, for both persistence and the sensitivity of realized per capita growth rate to density (N Pandey and A Joshi, *unpubl. mss*.).

In the present study, the only life-history trait that differed significantly in its sensitivity to density between the MCU and LCU populations was pre-adult survivorship (Figures 1 A, B and 3 A, B). This particular density-dependent feedback loop is a large contributor to stability, with decreased sensitivity of pre-adult survivorship to larval density tending to stabilize the dynamics with regard to constancy (Mueller 1988; Mueller and Joshi 2000). In discrete-generation populations, greater pre-adult survivorship at a wide range of larval densities can potentially enhance both constancy and persistence by raising the population size at troughs in populations undergoing large fluctuations in population size due very strong time-lagged density-dependent feedback. It is therefore likely that the significantly and markedly lower sensitivity of pre-adult survivorship to larval density in the MCU populations is the major contributor to the evolution of enhanced constancy in the MCU but not the LCU populations. We speculate that the evolution of greater persistence than controls in both the MCU and LCU populations is again driven by a lower sensitivity of pre-adult survivorship to larval density, as reflected in the earlier observed ability of both MCU and LCU populations to sustain a higher realized per capita growth rate across medium to high adult densities (N Pandey and A Joshi, *unpubl. mss.*).

We know that adaptation to larval crowding at a much lower amount of food as compared to the LCU populations has led to the MCU populations evolving to attain their minimum critical size faster than controls; the LCU populations, on the other, evolved a higher feeding rate than controls (Sarangi *et al* 2016, Sarangi 2018). We speculate that during the population dynamics experiment, the faster attainment of minimum critical size may be driving increased pre-adult survivorship in the MCU populations, as larval cultures in such experiments are likely to quickly become toxic due to accumulation of metabolic waste in very low food amount (1/1.5 mL LH food in population dynamics experiments). On the other hand, the LCU populations, which have evolved a higher feeding rate, may be undergoing greater mortality due to metabolic waste buildup in the food; faster feeding is likely to be harmful under high concentrations of metabolic waste (Mueller and Barter 2015).

Overall, the present study, together with earlier population dynamics studies on these populations (N Pandey and A Joshi, *unpubl. mss.*), provide empirical support for the speculation by Dey *et al* (2012) that the differing suites of traits that evolve in response to chronic larval crowding experienced at different combinations of egg number and food

amount are likely to differentially affect equilibrium population size and the sensitivity of realized per capita growth rate to density, thereby potentially affecting population stability. What ultimately links individual traits like feeding rate, minimum food requirement for development, or metabolic waste tolerance to demographic attributes like realized density-dependent per capita growth rates under competitive conditions is the sensitivity of key life-history traits to density. This study is the first attempt to connect trait evolution during density-dependent selection, experienced under differing ecological contexts, to the dynamics and stability of populations via the sensitivity of life-history traits to density. We suggest that these linkages need to be explored further, both empirically and theoretically, in a model-free framework, in order to achieve a better understanding of when and how density-dependent selection affects the evolution of population dynamics and stability attributes.

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TABLES

Table 1. Results from ANOVA done on pre-adult survivorship. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df***	**MS**	***F***	***P***
Selection	1	0.224897	4.438	0.125
Egg number	2	1.446999	34.781	<0.001**
Food amount	1	0.018818	2.852	0.190
Selection × Egg number	2	0.102034	5.373	0.046*
Selection × Food amount	1	0.000556	0.047	0.842
Egg number × Food amount	2	0.005316	1.068	0.401
Selection × Egg number × Food amount	2	0.006928	1.640	0.270

*: $P < 0.05$; **: $P < 0.01$

Table 2. Results from ANOVA done on female fecundity on day 21 post egg-lay. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df***	**MS**	***F***	***P***
Selection	1	657.4	0.528	0.520
Egg number	2	38135.0	27.681	<0.001**
Food amount	1	6008.9	12.802	0.037*
Selection × Egg number	2	684.5	3.611	0.093
Selection × Food amount	1	309.4	0.388	0.578
Egg number × Food amount	2	377.8	0.733	0.519
Selection × Egg number × Food amount	2	215.4	0.430	0.669

*: $P < 0.05$; **: $P < 0.01$

Table 3. Results from ANOVA done on dry body-weight per fly (in mg) at eclosion. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df***	**MS**	***F***	***P***
Selection	1	0.0090	5.525	0.100
Egg number	2	0.4257	46.574	<0.001**
Food amount	1	0.1256	98.176	0.002**
Sex	1	0.0918	28.001	0.013*
Selection × Egg number	2	0.0050	5.046	0.052
Selection × Food amount	1	0.00004	0.014	0.912
Egg number × Food amount	2	0.0012	0.961	0.435
Selection × Sex	1	0.000008	0.008	0.934
Egg number × Sex	2	0.0206	20.092	0.002**
Food amount × Sex	1	0.0057	11.334	0.044*
Selection × Egg number × Food amount	2	0.0025	5.536	0.043*
Selection × Egg number × Sex	2	0.0004	0.400	0.687
Selection × Food amount × Sex	1	0.000008	0.046	0.844
Egg number × Food amount × Sex	2	0.0034	17.528	0.003**
Selection × Egg number × Food amount × Sex	2	0.0002	0.382	0.698

*: $P < 0.05$; **: $P < 0.01$

Table 4: Results from ANOVA done on adult survivorship from eclosion to day 21 post egg-lay. Since we were primarily interested in fixed main effects and interactions, block effects and interactions have been omitted for brevity.

Effect	***df***	**MS**	***F***	***P***
Selection	1	0.0172	1.634	0.291
Adult density	1	11.8435	2086.225	<0.0001**
Sex	1	2.7428	84.928	0.003**
Selection × Adult density	1	0.00001	0.000	0.988
Selection × Sex	1	0.0055	0.665	0.475
Adult density × Sex	1	1.3558	405.599	<0.001**
Selection × Adult density × Sex	1	0.0075	1.061	0.379

*: $P < 0.05$; **: $P < 0.01$

FIGURE LEGENDS

Figure 1: Mean pre-adult survivorship, averaged over the four replicate populations each, of the crowding-adapted MCU and LCU populations at different egg numbers and larval food volume in 1 mL (A) and in 1.5 mL (B). Mean fecundity, averaged over the four replicate populations each, of the crowding-adapted MCU and LCU populations at different egg numbers and larval food volume in 1 mL (C) and in 1.5 mL (D). Mean dry body-weight at eclosion for males, averaged over the four replicate populations each, of the crowding-adapted MCU and LCU populations at different egg numbers and larval food volume in 1 mL (E) and in 1.5 mL (F). Mean dry body-weight at eclosion for females, averaged over the four replicate populations each, of the crowding-adapted MCU and LCU populations at different egg numbers and larval food volume in 1.5 mL (G) and in 1 mL (H). Error bars around the means are standard errors based on the means of four replicate populations within each selection regime.

Figure 2: Mean adult survivorship, averaged over the four replicate populations each, of males (A) and females (B) from the crowding-adapted MCU and LCU populations at low (50 adults) and high (150) adult density. Error bars around the means are standard errors based on the means of four replicate populations within each selection regime.

Figure 3: Pre-adult survivorship regressed on egg density for replicates of the crowding-adapted MCU (A) and LCU (B) populations. Fecundity regressed on egg density for replicates of the crowding-adapted MCU (C) and LCU (D) populations. Dry body-weight for males regressed on egg density for replicates of crowding-adapted MCU (E) and LCU (F) populations. Dry body-weight for females regressed on egg density for replicates of crowding-adapted MCU (G) and LCU (H) populations.

Figure 4: Life cycle of *Drosophila melanogaster*, and the four density-dependent feedback loops operating at different life-stages: (1) Effect of larval crowding on pre-adult survivorship, (2) Effect of larval crowding on female fecundity, (3) Effect of adult crowding

on adult survivorship, and (4) Effect of adult crowding on female fecundity. The grey arrows show the effects of larval density and the black arrows show the effect of adult density.

FIGURES

Figure 1

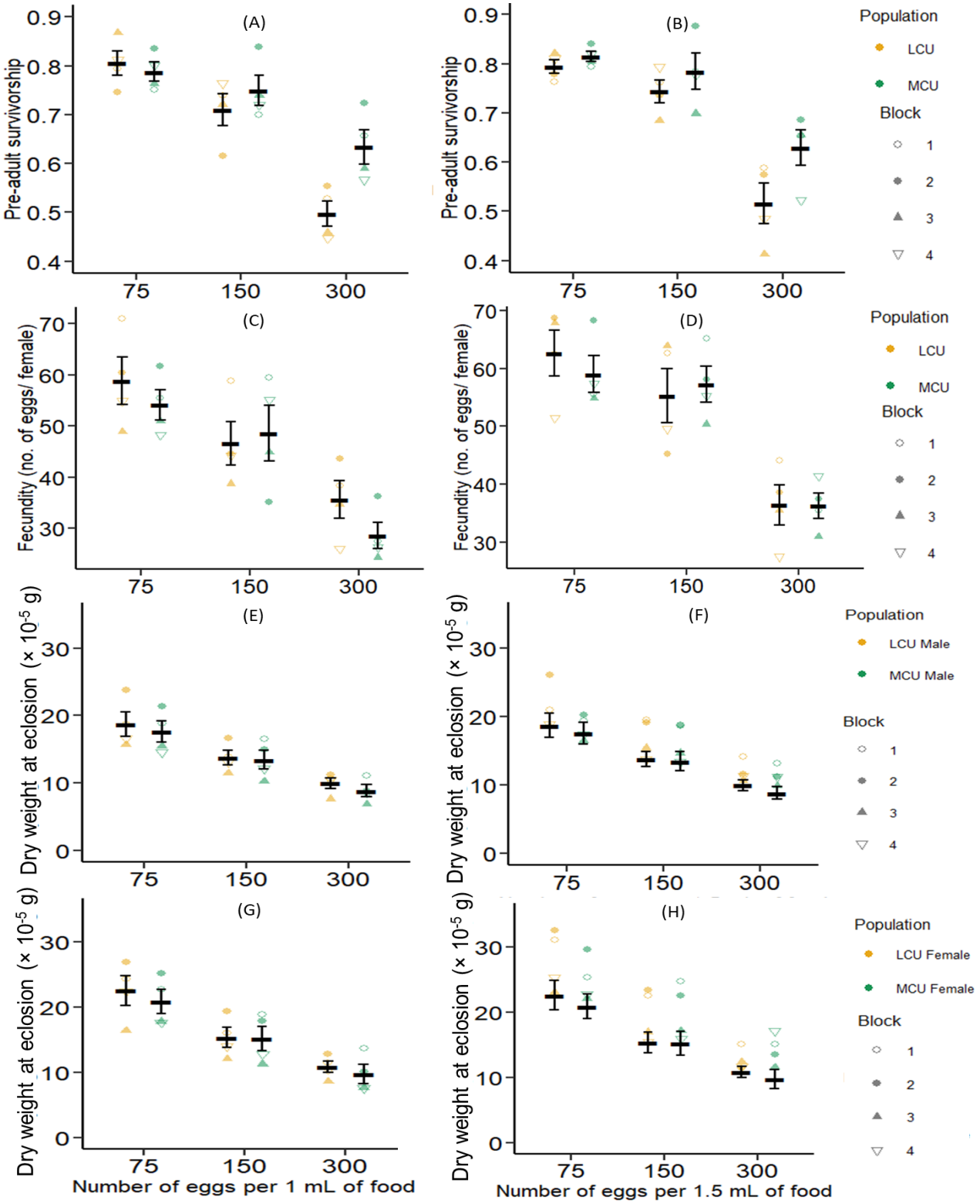


Figure 2

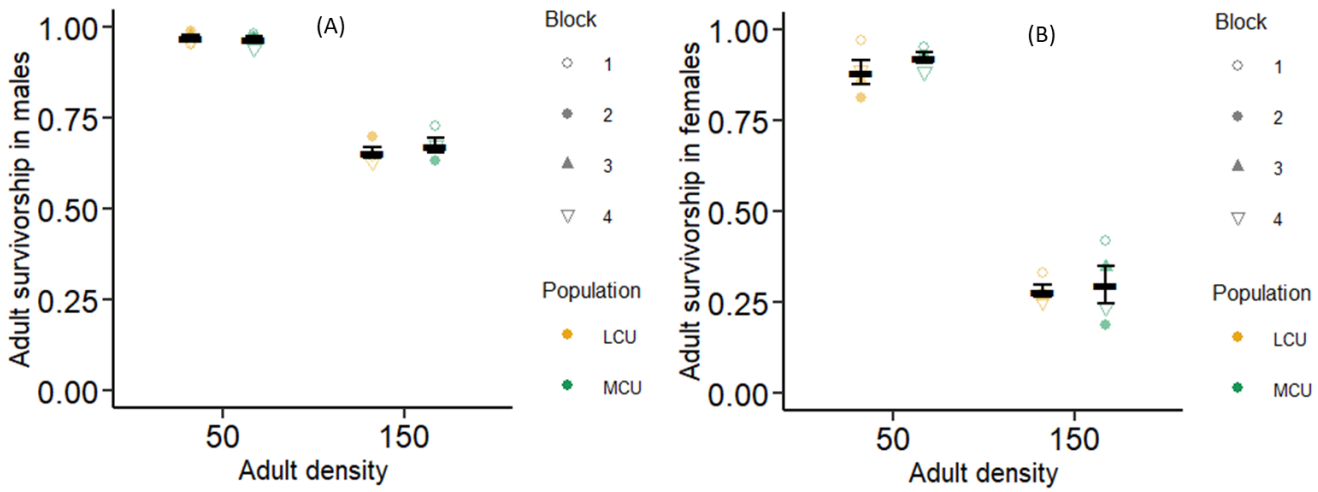

(A)
Adult survivorship in males
1.00
0.75
0.50
0.25
0.00
50
150
Adult density
Block
1
2
3
4
Population
LCU
MCU
(B)
Adult survivorship in females
1.00
0.75
0.50
0.25
0.00
50
150
Adult density
Block
1
2
3
4
Population
LCU
MCU

Figure 3

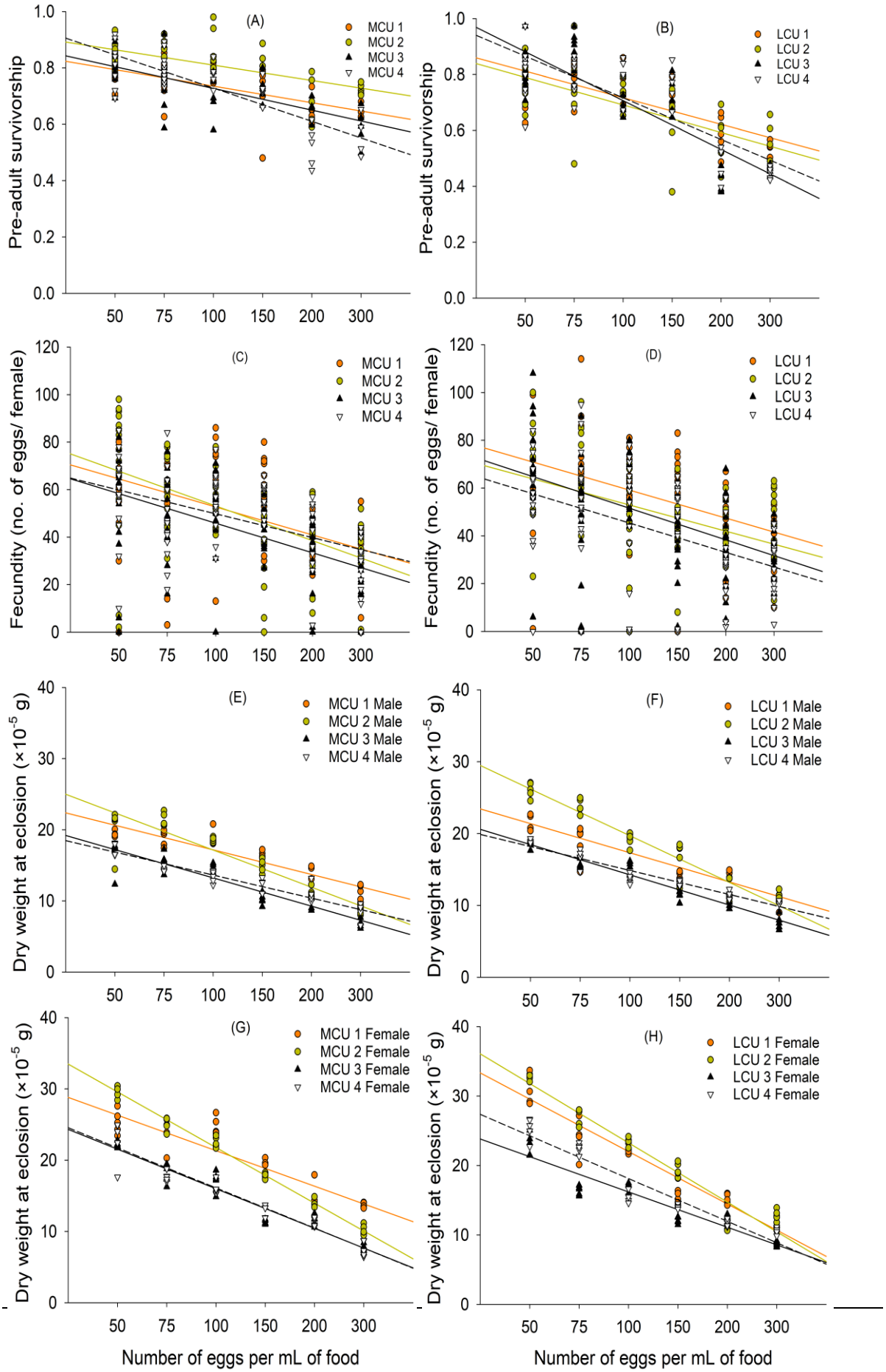
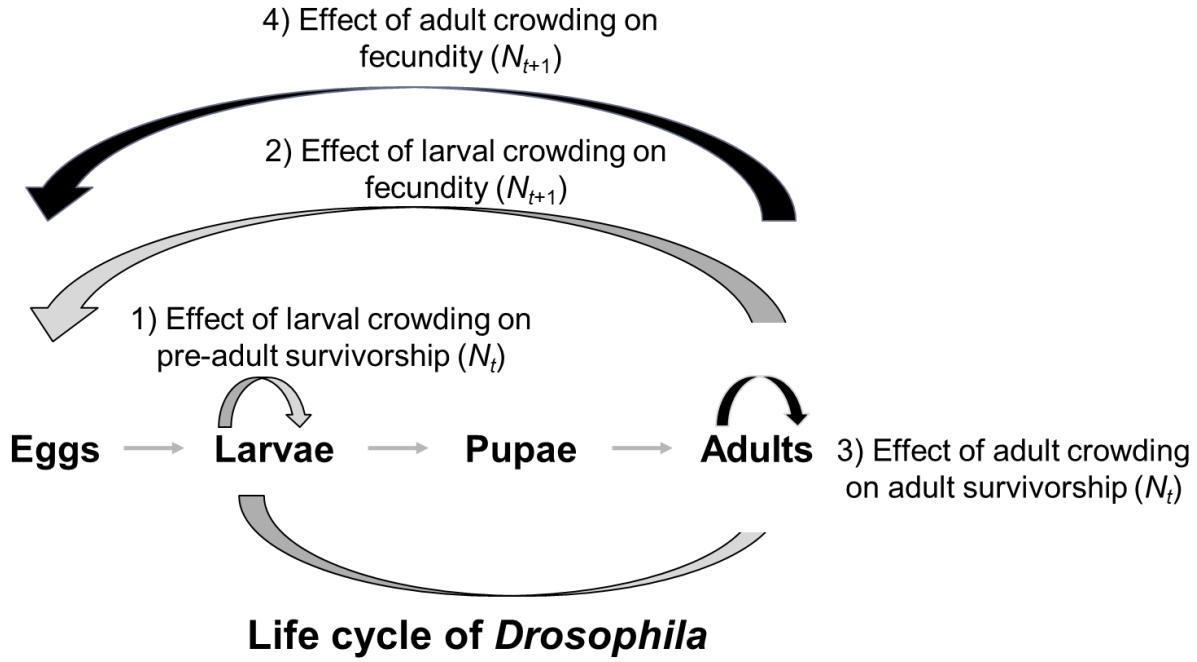

(A)
MCU 1
MCU 2
MCU 3
MCU 4
Pre-adult survivorship
(B)
LCU 1
LCU 2
LCU 3
LCU 4
Pre-adult survivorship
(C)
MCU 1
MCU 2
MCU 3
MCU 4
Fecundity (no. of eggs/ female)
(D)
LCU 1
LCU 2
LCU 3
LCU 4
Fecundity (no. of eggs/ female)
(E)
MCU 1 Male
MCU 2 Male
MCU 3 Male
MCU 4 Male
Dry weight at eclosion (×10-5 g)
(F)
LCU 1 Male
LCU 2 Male
LCU 3 Male
LCU 4 Male
Dry weight at eclosion (×10-5 g)
(G)
MCU 1 Female
MCU 2 Female
MCU 3 Female
MCU 4 Female
Dry weight at eclosion (×10-5 g)
(H)
LCU 1 Female
LCU 2 Female
LCU 3 Female
LCU 4 Female
Dry weight at eclosion (×10-5 g)
50 75 100 150 200 300
Number of eggs per mL of food
Number of eggs per mL of food

Figure 4


4) Effect of adult crowding on fecundity (N_{t+1})
2) Effect of larval crowding on fecundity (N_{t+1})
1) Effect of larval crowding on pre-adult survivorship (N_t)
Eggs
Larvae
Pupae
Adults
3) Effect of adult crowding on adult survivorship (N_t)
Life cycle of *Drosophila*

CHAPTER 5

Evolution of Faster Development Leads to the Loss of Larval Stop in *Drosophila* Populations Selected for Adaption to Larval Crowding or Rapid Development and Early Reproduction

Title: Evolution of faster development leads to the loss of larval stop in *Drosophila* populations selected for adaption to larval crowding or rapid development and early reproduction

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ABSTRACT

Larval crowding in *Drosophila* populations has been shown to induce a phenomenon called larval stop, i.e. developmental arrest, after which the larvae can resume development when abundant food becomes available again. As very few studies have investigated larval stop, we revisit larval stop in two different sets of populations, one of the populations has been selected for adaptation to larval crowding (MCU) and the other population has been selected for faster development and early reproduction (FEJ). While their respective control populations show the expression of larval stop, our findings from the selected populations suggest that both these populations have lost larval stop expression, possibly because of faster pre-adult development which has evolved in response to the two different selection pressures, and because larval stop is expressed in late larval stages. Density-dependent selection could be another reason for the loss of larval stop expression in MCU populations which have been selected for adaptation to larval crowding. In addition, expression of larval stop seems to be lower in these populations which were maintained under discrete generation cycles, as opposed to overlapping generations cycle used in earlier studies.

Key words: Density-dependent selection, arrested development, metabolic waste, development time, larval crowding, fruitflies.

INTRODUCTION

In *Drosophila*, as in many organisms with complete metamorphosis, the traits that enhance competitive ability during pre-adult life-history stages are key contributors to an individual's fitness as they also affect adult traits, such as body weight, that have fitness consequences. Since resource acquisition in the pre-adult stage is directly related to its duration in *Drosophila*, pre-adult development time is positively related to adult body weight at eclosion (Bakker 1959), which in turn influences fecundity, male mating success and adult survival (review in Prasad and Joshi 2003, Than *et al* 2021). Consequently, experimental studies have explored conditions that affect pre-adult development time in *Drosophila* on ecological and evolutionary time scales and have also examined the effects of altered development time on fitness-related traits (Zwaan *et al* 1995 Al-Saffar *et al* 1996, Nunney 1996, Chippindale *et al* 1997, Prasad *et al* 2000). Pre-adult development time in *Drosophila* can plastically reduce as an immediate ecological response to environmental variations such as high temperature (Al-Saffar *et al* 1996), while scarcity of the essential nutrients and waste accumulation in food can prolong pre-adult development time (Sang 1956, Geer and Vovis 1956, Barker and Podger 1970, Botella *et al* 1985). An evolutionary increase in pre-adult development time can be seen when populations face high extrinsic mortality at the early life stage (review in Reznick *et al* 2002), while it can reduces in response to selection pressures involving high extrinsic mortality at later life stages (review in Reznick *et al* 2002), or high larval crowding, in some cases (Nagarajan *et al* 2016, Sarangi *et al* 2016).

One interesting phenomenon seen in the pre-adult stage in *Drosophila* is that moderate to high larval competition can lead to developmental delay through 'larval stop' wherein the larvae undergo arrested development in response to high levels of crowding, after which they may either die due to food scarcity, or might resume development when food is made available again (Ménsua and Moya 1983, Botella and Ménsua 1986, Moya *et al* 1988). Ménsua and Moya (1983) detected such a developmental delay in highly crowded larval cultures by employing the overfeeding technique, wherein abundant food was given to the larvae (after they had faced extreme competition for some amount of time): such larvae were found to often resume their development, whereas other larvae died due to scarcity of food. Ménsua and Moya (1983) found that the overall pre-adult development time was greater when overfeeding was done at later larval stages, and inferred that there was

developmental arrest or "larval stop" under crowding conditions in the third-instar larvae, and that development resumed from the time when food was re-provisioned in their overfeeding experiments. The developmental delay attributed to larval stop under high levels of crowding was a minimum of ~340 hours in third-instar larval stage as compared to the usual ~48 hours duration of the third-instar stage in the absence of crowding (Ménsua and Moya 1983). Such stopped development is also seen in *Tribolium* larvae at moderate larval density (Botella and Ménsua 1986). Possible explanations for larval stop under very crowded conditions invoke the disturbance of normal metabolism due to the accumulation of nitrogenous waste, or the scarcity of nutrients necessary for successful larval development (Botella *et al* 1985). The ability of larvae to stop development under food scarcity and resume it upon re-supply of food can have fitness consequences under high population densities, as larval stop may improve the chances of survival or enhance fecundity through an increase in body weight at eclosion, in contrast with individuals who do not express larval stop and thus are highly prone to mortality under high larval density, or eclosion at relatively small adult size. One objective of our study was to revisit the phenomenon of larval stop, specifically to examine its contribution to survival if food is made available again to the larvae facing competition in laboratory populations that have experienced different selection histories.

As developmental delay through larval stop is expressed in the later larval stage, it is possible that larval stop may trade-off with the evolution of faster development. To examine such an evolutionary scenario, we examined larval stop by assaying development time in two sets of *Drosophila melanogaster* populations that have evolved faster development but through different evolutionary routes due to the differences in the selection pressure implemented in our laboratory. One set of populations, the MCUs, had been selected for adaptation to larval crowding at very low food amounts for over 160 generations at the time of the experiment. In nature, adaptation to larval crowding can be advantageous as the larvae often face ephemeral resources and high-density environments (Bakker and Nelissen 1963). The ability to suspend development in response to resource scarcity and resume when food becomes available again, as seen in larval stop, may be helpful in crowding conditions, although the crowding adapted MCUs have evolved faster development, even when assayed to low densities (Sarangi *et al* 2016), which may undermine any fitness contributions by the larval stop which is expressed in late larval stages. Moreover, in the MCU selection regime, once food has run out or become relatively non-nutritive due to

extreme crowding, there is no subsequent access to good food. The other set of populations, the FEJs, have been selected for rapid development and early reproduction for over 700 generations. As a large evolutionary reduction in pre-adult development time has taken place in the FEJs (Prasad *et al* 2001, Prasad and Joshi 2003), the expression of larval stop is expected to be lost or greatly reduced. Selection for rapid development and early reproduction (Prasad *et al* 2001, Prasad and Joshi 2003) and adaptation to larval crowding can also reduce the minimum critical size for pupation (size required for completion of pupation), and the failure to achieve minimum critical size can result in increased pre-adult mortality under crowding conditions when resources become scarce and/or toxic. Given such consequences of the evolution of faster development, we wanted to compare the expression of larval stop in these two sets of selected populations, that had been either directly or indirectly (via crowding at very low food amounts) selected for rapid development, and their respective controls. In contrast to the earlier experiments of larval stop, conducted in an overlapping-generations regime (Oregon-R and the cardinal eye-color mutation (3-75.7): Ménsua and Moya 1983, González-Candelas *et al* 1990), we examined larval stop in a discrete generation regime in which these populations (MCUs, MBs, FEJs, JBs) have been maintained in the laboratory. A discrete-generation cycle of fixed duration can also lead to a partial loss of larval stop, as the individuals developing later than the fixed duration of the discrete generation do not become part of the breeding pool while maintaining these populations. We also assayed if larval stop enhanced pre-adult survivorship when food is made available again by facilitating larval movement from crowded culture to overfeeding vials at different time intervals in the larval stage.

MATERIALS AND METHODS

Experimental populations

MB$_{1-4}$ (*D. melanogaster* baseline): The MB populations are ancestral controls to the MCUs, and are maintained at a relatively low larval density (~70 eggs/~6 mL cornmeal food) as compared to the MCUs that are selected for adaptation to larval crowding. MBs are maintained on a 21-day discrete generation cycle at a temperature of 25^{o}C±1^{o}C, with a relative humidity of ~80 percent in an all-light environment (LL).

MCU$_{1-4}$ (*D. melanogaster* Crowded as larvae Uncrowded as adults): The selected MCU populations are derived from MBs, and are maintained at a much higher larval density (~600 eggs/1.5 mL cornmeal food) than the MBs. Like MBs, MCUs are maintained on a 21-day discrete generation cycle at a temperature of 25^{o}C±1^{o}C, with a relative humidity of ~80 percent in an all-light environment (LL).

Once the larvae become adults they are collected in Plexiglas cages (25 × 20 × 15 cm^3) on day 11 from egg-lay (day 0) in the case of MBs. In MCUs the eclosing adults are collected in Plexiglas cages (25 × 20 × 15 cm^3) from ~8-18 days from egg collection, as higher larval density can prolong the pre-adult development time. The adults are maintained at a density of ~1800, for both MBs and MCUs, in cages and are provided with a Petridish containing cornmeal food (food plate) and a damp cotton ball. The food plate is replaced every alternate day and the damp cotton ball is replaced every alternate food change. On the 18th day from egg collection, adults are given a fresh live acetic-acid-yeast paste, and on the 20th day, the yeast place is replaced with a vertically cut sterile food medium to lay eggs for the next generation for the next 18 hours. Once the egg laying is done, eggs are counted on the 21st day and placed in vials (9.5 cm height × 2.4 cm diameter) containing cornmeal food. To maintain an adult density of ~1800 adults, eggs are collected at the above mentioned density in ~40 vials for MBs (pre-adult survivorship ~75 percent), and ~12 vials for MCUs (pre-adult survivorship ~40 percent). At the time of this experiment, MCU$_{1,2}$ had been selected for 162 generations, while MCU$_{3,4}$ had been selected for 163 generations. Complete details of the derivation and maintenance of the MBs and MCUs can be found in Sarangi *et al* (2016).

JB$_{1-4}$ (Joshi baseline): The JB populations are ancestral controls for the FEJs, and are maintained at a larval density of ~70 eggs in ~6 mL of banana-jaggery medium. Similar to MBs, JBs are also maintained at a 21-day discrete generation cycle at a temperature of 25^{o}C±1^{o}C, with a relative humidity of ~80 percent in an all light environment (LL).

FEJ$_{1-4}$ (Faster developing and early reproducing JBs): The FEJs are maintained at a low larval density of ~90 eggs in ~6 mL of banana-jaggery medium. FEJs are selected for rapid development and early reproduction, and in response to such selection FEJs have evolved a shorter pre-adult development time (Prasad *et al* 2000) among other traits, as compared to

the control JB populations. Because of the shortening of the pre-adult development time the FEJs are maintained on a 10-day discrete generation cycle at a temperature of 25^{o}C±1^{o}C, with a relative humidity of ~80 percent in an all-light environment (LL).

In the case of JBs, the eclosing adults are collected in glass vials (9.5 cm height × 2.4 cm diameter) containing banana-jaggery food on the 12th day from egg-lay (day 0), and these adults are provided with fresh banana-jaggery food in vials on 14th day, 16th day from egg collection. On the 18th day from egg-lay the adults are collected in Plexiglas cages (25 × 20 × 15 cm^{3}) and provided with fresh live acetic-acid-yeast paste. On the 20th day from egg-lay, the adults are provided with vertically cut sides of sterile food plates to lay eggs (~18 hours) for the next generation, and on the 21st day, these laid eggs are dispensed in the glass vials at above densities. In the case of FEJs, since only the first ~25 percent of eclosing adults (~15 adults per vial) are collected in the breeding pool, the eggs are collected in 120 such glass vials to get an final adult density of 1800 adults, similar to the JBs. The selection for rapid pre-adult development time and early reproduction is implemented in the FEJs by checking the darkened pupae (~6 days from egg collection) every two hours and transferring the eclosed adults from each vial to Plexiglas cages, when the vial has roughly 15 eclosed adults. This checking and collection of eclosed adults are continued till all the 120 vials have had ~15 eclosed adults. Further, these adults are given a fresh live acetic-acid-yeast supplement for the next ~3 days, after which the adults are provided with a fresh sterile banana-jaggery food plate with vertically cut sides to lay eggs for ~1 hour on day 10th from the egg collection in previous generation. Thereafter, these eggs are dispensed in the glass vials at the respective densities. At the time of this experiment, $FEJ_{1,2}$ had been selected for 715 generations, while $FEJ_{3,4}$ had been selected for 714 generations. Complete details of the derivation and maintenance of the JBs and FEJs can be found in Prasad *et al* (2000).

The FEJs were tested for inbreeding (after 700 generations of selection) prior to this assay, to rule out the possibility of inbreeding effects on the larval stop. Since the FEJs have evolved lower body weight than the control JBs and require a lower resource amount to complete larval development, we performed a pilot study to know at what larval density JBs and FEJs could face roughly equal larval competition, and at what stage the third instar metamorphosis starts in FEJs and JBs at the examined density. In this pilot, we used three larval densities i.e. 70 eggs/1.5 mL, 135 eggs/1.5 mL, and 200 eggs/ 1.5 mL food, and

among these three densities, 200 eggs/1.5 mL was chosen, since the pre-adult survivorship reduced by ~40 percent in both JBs and FEJs at this density.

Assaying pre-adult development time and survivorship

We studied pre-adult development time and survivorship in crowding conditions by employing the overfeeding technique of Ménsua and Moya (1983), wherein the larval competition is interrupted at different stages in different vials by providing abundant fresh food (i.e. overfeeding) to competing larvae. High crowding at the larval stage results in developmental arrest in some larvae (i.e. larval stop), and such resupply of food through overfeeding can help larvae to resume feeding and development; thus, overall pre-adult development time increases due to stopped development or larval stop (Ménsua and Moya 1983). We moved the larvae at different time points in the larval stage to the overfeeding vials (with fresh food) and observed pre-adult development and pre-adult survivorship. If the larvae are alive and have not pupated but have undergone suspension of development (i.e. larval stop), they may resume feeding on this extra food in the overfeeding vial, and can ingest enough food to survive to the next stage and possibly also become heavier adults. The overfeeding protocol for the different sets of populations we studied is described in detail below.

Overfeeding in MBs and MCUs: We set up crowded larval cultures in 40 vials with a larval density of 200 eggs/1.5 mL of cornmeal food for each replicate population of MCUs and MBs. These vials would be used for overfeeding treatment at nine different points of time and for one control. After the larvae reached the 4th day from egg-lay (onset of third instar), four replicate crowding vials from each population were transferred into bigger bottles called 'overfeeding vials' that contained ~80 mL of slanted cornmeal food for overfeeding (Fig. 1) for the next 24 hours. Overfeeding was done, on alternate days i.e. 4th day, 6th day…and so on till 18th day, for a 14 day period (from the 4th day from egg collection and till 18th day, following Ménsua and Moya (1983). This treatment was termed 'overfeeding', for which there were two controls: high-density controls (200 eggs in 1.5 mL food) and low-density controls (70 eggs in ~80 mL food), both of which did not receive the overfeeding treatment. During the overfeeding treatment, the overfeeding bottles containing crowding vials were kept horizontally to facilitate larval movement out of the vials and into the excess food, and prevent the crowding vial food from dropping into the overfeeding vial (Fig. 1). After overfeeding treatment, the crowding vials along with the larvae that were

inside them were taken out, thus separating the original group of individuals into two subgroups: the inner subgroup which consisted of individuals that had already pupated before overfeeding treatment was started, or did not move to the overfeeding vial, and the outer subgroup which contained larvae that had moved out into the overfeeding vial during overfeeding and possibly had undergone developmental arrest, followed by subsequent feeding. Both the inner vial subgroup and outer vial subgroup were then observed for eclosion and pre-adult survivorship every six hours till eclosion stopped. During the period of overfeeding, i.e. 24 hours, the vials that were undergoing overfeeding treatment could not be observed for eclosion. Thus, after the overfeeding treatment, the pre-adult development time for those adults that had eclosed during the overfeeding treatment was calculated according to the next time check. Similarly, assays of pre-adult development and survivorship were also done for low-density and high-density controls for which overfeeding treatment was not given.

Overfeeding in FEJs and JBs: We set up 56 vials at a density of 200 eggs/1.5 mL of banana-jaggery food for each replicate population of FEJs and JBs. The overfeeding treatment was started after two hours from the onset of the 3rd instar larval phase (studied in the pilot) i.e. from 72 hours in JBs and 40 hours in FEJs after egg lay. Overfeeding was done at different intervals from the onset of the 3rd instar larval phase for next 13 days in the case of JBs and 6.5 days in the case of FEJs. To account for the much reduced pre-adult development time in FEJs as compared to JBs, overfeeding was done at an interval of 24 hours in JBs and at an interval of 12 hours in FEJs. For each interval, 4 replicate crowding vials (200 eggs/1.5 mL) of Jbs or FEJs were transferred into 4 overfeeding vials that contained ~80 mL of slanted banana-jaggery medium. For the next 24 hours, the vials were kept in this position (Fig. 2) after which the inner crowding vials were taken out from the overfeeding vial. We separated the individuals into two subgroups i.e. inner subgroup which consisted of the individuals that committed to pupation before/during overfeeding, and the outer subgroup which consisted of the larvae that moved out in the overfeeding vial within 24 hours. After the overfeeding treatment, the inner and outer subgroup individuals were observed for adult eclosion and pre-adult survivorship every 24 hours. The vials that were undergoing overfeeding during eclosion checks could not be examined and the eclosed adults in that interval were counted in the next check for pre-adult development time calculation. From the 48 crowding vials of Jbs and FEJs, 4 replicate vials each were used as high density controls and thus were not treated with overfeeding, while the low-density

controls were maintained at a density of 70 eggs in ~80 mL food. Assays of pre-adult development and pre-adult survivorship were also conducted on these high and low density controls.

Statistical analyses

To analyse pre-adult development time in MB and MCU populations, we used mixed model analysis of variance (ANOVA), treating selection, day of overfeeding, vial type (inner or outer) and sex as fixed factors, and blocks representing the common ancestry of MB_i and MCU_i as random factors. For the JB and FEJ populations, we treated selection, time of overfeeding, and vial type (inner or outer) as fixed factors, and blocks representing ancestry as a random factor. The eclosion in JB and FEJ populations were observed at 24-hour interval instead of 6-hour interval used in MB and MCU populations. Sex was not used as an effect in the analysis because sex-specific eclosions were not checked. The difference in pre-adult development time of males and females are not very large and were unlikely to be detected in 24-hourly checks. We kept a time interval of 24 hours duration between two overfeeding points in the control JBs, and of 12 hours duration in the faster eclosing FEJs, to adjust for the differences in their maintenance regime, wherein all the eclosed adults of JBs are collected in cages on the 12^{th} day from egg collection while the FEJs are collected ~6.5 days from egg collection. Therefore, the day-3 of overfeeding was equated to 40 hours from egg lay in FEJs for overfeeding, and day-4 of overfeeding was equated to 52 hours from egg lay in FEJs for overfeeding, and so on. We performed ANOVA on pre-adult development time in JB and FEJ populations only till day-6 of overfeeding. This is because after day-6 of overfeeding some of the blocks did not have any eclosion for FEJ populations, as there was no movement of larvae to the outer feeding vials. Therefore, data till day 6 were statistically analysed, while we present qualitative findings for the subsequent overfeeding days. For pre-adult survivorship, all the days of overfeeding were utilised in the analysis. Similarly, for MB and MCU populations there were no eclosions (due to no movement of larvae in outer feeding vials) in some of the blocks for MBs or MCUs after day-4. Thus, for pre-adult development time in MBs and MCUs, the statistical comparisons were done only till day 4 of overfeeding, and we only did qualitative comparisons beyond this day. To analyze pre-adult survivorship in MBs and MCUs, we used data from all the days of overfeeding. All these analyses were performed in Statistica Version 5.0 (Statsoft 1995). We performed Tukey's HSD at P=0.05 for post-hoc comparison of multiple means in case of a significant interaction effect.

RESULTS

Larval stop and pre-adult survivorship in MCU and MBs

Pre-adult development time and larval stop

In the analyses of pre-adult development time till day 4 of overfeeding, pre-adult development time was shorter for the not-overfed control vials than for the outer vials ($F_{1,3}$=65.377, P=0.003). Also, the pre-adult development time was shorter in the overfed outer vials than in the inner vials ($F_{1,3}$=79.700, P=0.002), suggesting the absence of larval stop. MCUs showed significantly shorter pre-adult development time than the control MB populations ($F_{1,3}$=103.045, P=0.002). The crowding-adapted MCU population did not differ significantly from control MB populations in pre-adult development time in the inner or outer vials (Selection × vial type interaction effect: $F_{1,3}$=9.603, P=0.053, Table 1, Fig. 3).

Qualitative differences in pre-adult development time

The findings on average pre-adult development time are summarised in Table 5, and we give additional data on maximum pre-adult development time here. In the MCUs, the average pre-adult development time prolonged up to ~295 hours in outer vials in comparison to inner vials after overfeeding treatment on day 6. After day 6 of overfeeding, since there was no movement of larvae to the outer overfeeding vials, no eclosion happened in the outer overfeeding vials. In high-density control (200 eggs/1.5 mL) vials where no overfeeding treatment was given, the average pre-adult development time was ~222 hours, and it went up to 482 hours in one of the replicates (data from 1 individual). In the outer vials, the maximum increase in pre-adult development time was up to 332 hours from egg-lay (data from 2 individuals). Therefore, the MCU populations did show a prolonged pre-adult development in response to crowding, but did not show an increase in the pre-adult development time in the outer vials compared to the inner vials. Thus, the MCUs appear to have lost larval stop as a result of evolution of faster pre-adult development via density-dependent selection.

In comparison, the control populations (MBs) had a prolonged pre-adult development time in the outer overfeeding vials as a result of overfeeding treatment given on different days,

thus indicating the presence of larval stop expression in MBs. After the overfeeding treatment in MBs on day-6, 8, 10 and 12, the average pre-adult development time prolonged up to ~333 hours, ~425 hours, ~560 hours and ~548 hours (Table 5, Fig 3), respectively in the outer vials in comparison to inner vials. After the day-12 of overfeeding treatment, there was no movement of larvae from crowding vials to the outer feeding vials. In the high-density control (200 eggs/1.5 mL) vials wherein no overfeeding treatment was given, the pre-adult development time went up to 518 hours (data from 1 individual) in MB_1. In comparison, in the overfeeding vials, the maximum increase in development time went up to ~560 hours (data from 9 individuals) who were overfed on day-12 in MB_1. Thus, the MB populations showed larval stop expression because the pre-adult development time increased in the outer vial as compared to the high-density control vials. In some of the inner vials in MB_1, the pre-adult development time extended beyond ~560 hours but these vials were overfed at different intervals than the vials which showed an extension of development time in the outer vials.

Pre-adult survivorship

The effect of the interaction between selection and overfeeding day was not significant ($F_{1,3}$=1.895, P=0.085). There was also no significant effect of overfeeding day ($F_{1,3}$=0.349, P=0.958) on pre-adult survivorship. Additionally, there was no significant effect of selection on pre-adult survivorship ($F_{1,3}$=0.679, P=0.470).

Larval stop and pre-adult survivorship in FEJs and JBs

Pre-adult development time and larval stop

JBs had a longer pre-adult development time in outer vials, compared to inner vials, as a result of overfeeding treatment on different days ($F_{4,12}$=27.803, P<0.001), suggesting the presence of larval stop expression. The post-hoc analysis (Tukey's HSD at P=0.05) revealed that the JB populations that were overfed on day-5 had a longer pre-adult development time than when overfeeding was given on day-3, 4 and low-density controls (70 eggs/~80 mL food) while overfeeding given on day-6 made pre-adult development time longer than overfeeding on day-3, 4, 5 and the low-density controls. In the inner vials also, the development time in JBs was higher for overfeeding given on day-5 than overfeeding given on day-3, 4 and 5 and the high-density controls. FEJs did not differ in the development time in the inner and outer vials when overfeeding was given at different intervals. Furthermore,

overfeeding on later days/intervals from egg-lay led to an increase in development time ($F_{4,12}$=9.988, P<0.001, Table 3, Fig. 4).

As expected JBs had a longer pre-adult development time than the FEJs ($F_{1,3}$=56.502, P=0.004). The development time was longer in the inner vials than the outer vials, although this effect was not significant ($F_{1,3}$=8.831, P=0.0589). The longer pre-adult development in inner vials than in outer vials could be due to abundant food available in the outer vials and the inclusion of earlier intervals of overfeeding in the analysis.

Qualitative differences in pre-adult development time

The findings on average pre-adult development time are summarised in Table 6, and we give additional data on maximum pre-adult development time here. In the FEJs, the average pre-adult development time extended in the outer vials with a maximum of ~209 hours in comparison to inner vials. In the high-density controls (200 eggs/1.5 mL) the maximum pre-adult development time went up to 272 hours (data from 2 individuals). In the outer vials, the maximum pre-adult development time went up to 248 hours (data from 5 individuals) after egg-lay. Therefore, there does not seem to be an extension of pre-adult development time in the outer vials as a result of overfeeding.

JBs had a prolonged pre-adult development time in the outer vials as a result of overfeeding on different overfeeding days in comparison to inner vials. On the day 9 and day10 of overfeeding, the JB vials on average had a pre-adult development time of ~374 hours and ~382 hours, respectively (Table 6, Fig. 4). Also, in one of the outer vials that were overfed on day 7 in JB_3 the maximum pre-adult development time went up to 440 hours (data from 1 individual) from egg-lay till eclosion. In the control high-density cultures (200 eggs/1.5 mL) the maximum pre-adult development time prolonged up to 368 hours (data from 7 individuals) from egg-lay, thus JBs show larval stop. There is an extension of pre-adult development time in the JBs as compared to the high-density controls.

Pre-adult survivorship

There was also a significant effect on the interaction between selection and overfeeding day for pre-adult survivorship ($F_{1,3}$=3.308, P=0.001) such that for JBs it was higher for low-density control than the vials that were overfed on days 8, 9, 10, 11, 12, 13, 14 and 15, suggesting that overfeeding on early days increase pre-adult survivorship. In comparison, in

FEJs, the low-density controls had higher pre-adult survivorship than the vials which were given overfeeding on day7, 8 and 12. Overfeeding treatment also affected pre-adult survivorship ($F_{1,3}$=8.7748, P<0.001) such that it was higher in the low-density non-overfed controls than vials which received overfeeding treatment on days 7, 8, 9, 10, 11, 12, 13, 14, 15 and high-density controls which suggests that overfeeding on initial days can increase pre-adult survivorship. As expected pre-adult survivorship ($F_{1,3}$=57.350, P=0.004) was higher for JBs than FEJs (Table 4, Fig 6).

DISCUSSION

'Larval stop', or the developmental arrest exhibited by *Drosophila* larvae in high crowding conditions, has been largely unexplored after it was first described in the 1980s (Ménsua and Moya 1983, Botella *et al* 1985, Moya *et al* 1985, Botella and Ménsua 1986). Here, we revisit the phenomenon of larval stop to examine the evolutionary changes in this larval trait in two different sets of populations that have evolved faster development through two different evolutionary route; one through selection for adaptation to larval crowding at very low food amounts (MCUs) and the other through selection for rapid development and early reproduction (FEJs). As both sets of selected populations had evolved faster pre-adult development than their respective controls, our comparisons with controls examined whether the expression of larval stop, a trait exhibited by third-instar larvae, declines as a correlate of the evolution of faster pre-adult development. We also examined if larval stop played a role in enhancing pre-adult survival in crowded conditions if the larvae exhibiting larval stop are re-provisioned with fresh food. We examined larval stop in a discrete-generation system, as opposed to earlier studies conducted in the overlapping-generations system (Ménsua and Moya 1983), as our experimental populations (MCUs, FEJs, and their controls) have been maintained under a discrete-generation regime.

Larval stop in the selected populations (MCU and FEJ)

We find a loss of the expression of larval stop in both sets of populations that had evolved faster development, as their pre-adult development time in the outer overfeeding vials was not greater than their non-overfed high-density control vials, whereas their respective control populations do show larval stop expression (see below). The maximum development

time, which was 272 hours from egg lay in the non-overfed high-density control (inner) vials and was 248 hours in the overfeeding outer vials, for the FEJs. Similarly, in the MCUs, the maximum pre-adult development time in high-density control vials was 482 hours and was 333 hours from egg lay in the outer vials. This suggests that the greater maximum development time in both FEJ and MCU in outer vials is due to an ecological effect of larval crowding which increases the mean development time (Borash *et al* 1998, Sarangi 2018) rather than the development arrest i.e. larval stop. The expression of the larval stop has been evolutionarily lost in the MCUs, perhaps either because they are already adapted to high competition crowding, or because they have evolved faster development in response to larval crowding. The larvae in the MCUs have already been facing high larval competition during their evolutionary history and thus the crowding conditions introduced to them in the experimental treatment are not novel to them. Moreover, as their development time has also shortened due to their maintenance regime while implementing selection for adaptation to larval crowding, the individuals who had this trait would have been excluded from the breeding pool in the early generations of selection history, which could explain why the MCUs did not show larval stop expression while their control populations do show this trait. FEJs also do not show larval stop as a response to the evolution of faster development. Larval stop is induced in response to hostile environments like high larval crowding (Ménsua and Moya 1983) or the presence of nitrogenous waste in food (Botella *et al* 1985). As the FEJ populations have faced directional selection primarily for faster pre-adult development, they have evolved faster development and low tolerance to metabolic waste, among other life-history changes (Joshi *et al* 2001). FEJs have also become more canalized in pre-adult development time in response to density than their controls (JBs) which show greater plasticity (Ghosh *et al* 2019). Thus, it is possible that such canalization of pre-adult development time with respect to larval density and low tolerance to waste in crowding conditions have contributed to the loss of larval stop in the FEJs.

Pre-adult development and larval stop in control populations (MBs and JBs)

In contrast with the selected populations, both the control populations (MBs and JBs) showed the expression of larval stop, as the pre-adult development time in the outer overfeeding vials was greater than the non-overfed high-density control vials, as well as their respective inner vials. In the JB populations, the maximum development time in outer vials was 440 hours in the overfeeding vial on day 7. In the MBs, the maximum development time in the outer vial extended up to 560 hours after overfeeding treatment on

day 10 from egg lay. However, the number of larvae moving from the inner vial to the outer vial declined steeply for both JB and MB populations, as we saw that for MBs, after day 6 of overfeeding only larvae from MB_1 moved out into outer vials, while in the JBs, on day 11 of overfeeding only JB_3 larvae moved out for overfeeding. The small number of larvae moving out from the inner vials into outer vials, and no further movement after these days, suggests that the larval stop phenomenon is not as highly expressed in our populations as seen in Ménsua and Moya' experiments (1983), in which the maximum pre-adult development was ~624 hours. One explanation for these differences could be that while Ménsua and Moya (1983) used an overlapping generation cycle (Moya *et al* 1988), which also incorporates the larvae developing late into the breeding pool, whereas our experiments used a discrete-generation regime where each generation has a fixed duration beyond which the later eclosing individuals are not included in the breeding pool for the next generation. Due to the overall shortening of development time in response to multiple generations of selection under a discrete-generation regime, the expression of larval stop could have weakened.

The difference in the expression of larval stop in our populations and those used by Ménsua and Moya (1983) could also come from the food medium (Lewis medium) used in their study. The difference we saw between the two sets of control populations (MB and JB) in the movement of larvae to the overfeeding vial could be due to JBs having higher moisture levels in the banana-jaggery food in their culture vials than the MBs, whose cornmeal food has lower moisture content. Higher levels of crowding and lower water content in the food could probably lead to higher mortality in the MBs, possibly leading to fewer larvae moving out for overfeeding. Despite a very small number of larvae moving into the outer vials for overfeeding in MB_1 vials the overall increase in pre-adult development time was higher in MB population than the increase in development time of JB populations.

Impact of larval stop on pre-adult survivorship

Contrary to the expectation of increased survivorship for the larvae that exhibit the stopped development in response to crowding, and resumption of development when fresh food is re-provisioned, we did not see increased survivorship when provided with overfeeding outer vials (into which the larvae showing stopped development can move and resume feeding and development) as compared to the larvae in the high-density controls, except for the JB populations. The JB populations showed an increase in pre-adult survivorship from high-

density controls (200 eggs/1.5 mL) only till day-6 of overfeeding, we speculate that it is probably due to more larvae being alive till that day of overfeeding than later, when the food culture becomes more toxic and larvae die. The difference between the two sets of control populations (MB and JB) in pre-adult survivorship could be due to more larvae moving out in the outer vials in the case of JBs, whereas in the case of MBs only a few larvae from MB_1 moved out for overfeeding. We speculate that higher levels of moisture in the food used for the JB populations can explain the greater survival of the larvae as compared to MB populations whose food has less moisture. Also, the number of larvae moving out on later days was probably not large enough to cause a significant difference in pre-adult survivorship.

To summarise, our study suggests that the evolution of faster development in both sets of selected populations (FEJ and MCU) has led to the loss of larval stop expression, whereas their respective control populations do show larval stop. In addition, selection for adaptation to larval crowding in the MCUs may have also contributed to the loss of larval stop, as the crowding environment is not novel to them. The control MB and JB populations do exhibit a certain degree of larval stop in the moderate crowding levels but not to the extent seen in the earlier studies, which could be because we have been using a discrete-generation regime as opposed to the overlapping generations regime used in the earlier studies. Finally, our findings do not show much support for the expectation that larval stop can enhance the survival of larvae under crowded conditions if food is re-provisioned, at least for the kinds of populations we used.

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TABLES

Table 1: Summary results of ANOVA done on pre-adult development time (hours) in MBs and MCUs. The table shows the main effects of selection, overfeeding day, vial type (inner, outer, low-density controls, and high-density controls), sex and the fixed interaction terms. The block effects and the interactions of block with fixed factors have been omitted for brevity.

Effect	***df*** **Effect**	**MS** **Effect**	***df*** **Error**	**MS** **Error**	***F***	***P***
Selection	1	12239	3	118.772	103.045	0.0020
Overfeeding day	1	1545	3	38.067	40.591	0.0078
Vial type	1	2247	3	28.195	79.700	0.0029
Sex	1	19	3	29.004	0.646	0.4802
Selection × Overfeeding day	1	39	3	0.593	65.377	0.0039
Selection × Vial type	1	131	3	11.466	11.418	0.0431

Overfeeding day × Vial type	1	624	3	10.459	59.674	0.0045
Selection × Sex	1	43	3	5.920	7.246	0.0742
Overfeeding day × Sex	1	55	3	17.376	3.173	0.1729
Vial type × Sex	1	22	3	15.512	1.424	0.3185
Selection × Overfeeding day × Vial type	1	37	3	3.823	9.603	0.0533
Selection × Overfeeding day × Sex	1	2	3	18.516	0.093	0.7801
Selection × Vial type × Sex	1	6	3	7.830	0.771	0.4444
Overfeeding day × Vial type × Sex	1	21	3	23.795	0.889	0.415
Selection × Overfeeding day × Vial type × Sex	1	0	3	28.568	0.007	0.9393

Table 2: Summary results of ANOVA done on pre-adult survivorship in MBs and MCUs. The table shows the main effects of selection, overfeeding day, and fixed interaction terms. The block effects and the interactions of block with fixed factors have been omitted for brevity.

Effect	***df*** **Effect**	**MS** **Effect**	***df*** **Error**	**MS** **Error**	***F***	***P***
Selection	1	0.0195	3	0.0288	0.679	0.4704
Overfeeding day	10	0.0009	30	0.0028	0.349	0.9588
Selection × Overfeeding day	10	0.0063	30	0.0033	1.895	0.0858

Table 3: Summary results of ANOVA done on pre-adult development time (hours) in JBs and FEJs. The table shows the main effects of selection, overfeeding day, vial type (inner, outer, low-density controls, and high-density controls) and the fixed interaction terms. The block effects and the interactions of block with fixed factors have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
Selection	1	131130	3	2320.814	56.502	0.0048
Overfeeding day	4	675	12	67.613	9.988	0.000
Vial type	1	994	3	112.606	8.831	0.0589
Selection × Overfeeding day	4	350	12	48.771	7.183	0.0034
Selection × Vial type	1	1331	3	84.798	15.694	0.0287
Overfeeding day × Vial type	4	886	12	30.376	29.182	0.0000
Selection × Overfeeding day × Vial type	4	681	12	24.499	27.803	0.000

Table 4: Summary results of ANOVA done on pre-adult survivorship in JBs and FEJs. The table shows the main effects of selection, overfeeding day, and the fixed interaction terms. The block effects and the interactions of block with fixed factors have been omitted for brevity.

Effect	**df Effect**	**MS Effect**	**df Error**	**MS Error**	***F***	***P***
Selection	1	1.0732	3	0.0187	57.3505	0.004
Overfeeding day	14	0.0853	42	0.0097	8.7448	0.0000
Selection × Overfeeding day	14	0.0328	42	0.0099	3.3082	0.0013

Table 5: Pre-adult development time (mean and standard error) of MCUs and MBs in the inner, outer and control vials, after overfeeding at different stages in the crowding vials. The control vials were not given overfeeding treatment. The standard error and mean were calculated across the four replicate populations within each selection regime.

	Day-4	**Day-6**	**Day-8**	**Day-10**	**Day-12**	**Day-14**	**Day-16**	**Day-18**	**Day-20**	**Control**
MCU (Inner subgroup	226.085 ∓5.529	226.448 ∓6.687	232.172 ∓5.794	230.265 ∓6.600	226.387 ∓7.174	224.736 ∓6.592	225.861 ∓6.616	223.582 ∓6.278	222.758 ∓7.010	222.545 ∓5.761 (High density)
MB (Inner subgroup	256.645 ∓4.976	254.219 ∓1.802	255.689 ∓3.279	256.520 ∓3.758	256.301 ∓4.910	255.708 ∓4.453	255.388 ∓4.348	255.799 ∓3.400	254.732 ∓5.600	253.022 ∓4.464 (High density)
MCU (Outer subgroup	221.826 ∓ 5.791	295 ∓3.605	-	-	-	-	-	-	-	208.824 ∓6.349 (Low density)
MB (Outer subgroup	249.694 ∓ 3.170	333.869 ∓14.170	425.166	560	548	-	-	-	-	230.550 ∓3.875 (Low density)

Table 6: Pre-adult development time (mean and standard error) of FEJs and JBs in the inner, outer and control vials, after overfeeding at different stages in the crowding vials. The control vials were not given overfeeding treatment. The standard error and mean were calculated across the four replicate populations within each selection regime.

	d-3 JB/ 40 hrs FEJ	**d-4 JB/ 52 hrs FEJ**	**d-5 JB/ 64 hrs FEJ**	**d-6 JB/ 76 hrs FEJ**	**d-7 JB/ 88 hrs FEJ**	**d-8 JB/ 100 hrs FEJ**	**d-9 JB/ 112 hrs FEJ**	**d-10 JB/ 124 hrs FEJ**	**d-11 JB/ 136 hrs FEJ**	**d-12 JB/ 148 hrs FEJ**	**d-13 JB/ 160 hrs FEJ**	**d-14 JB/ 172 hrs FEJ**	**d-15 JB/ 184 hrs FEJ**	**Control**
FEJ (Inner subgroup)	173.743 ∓ 2.648	173.783 ∓ 2.340	175.009 ∓ 2.192	176.034 ∓ 2.128	175.222 ∓ 2.373	172.426 ∓ 3.268	174.325 ∓ 1.638	175.222 ∓ 1.149	172.825 ∓ 3.281	175.455 ∓ 2.872	176.674 ∓ 1.959	176.370 ∓ 2.719	173.208 ∓ 2.036	173.514 ∓ 2.669 (High density)
JB (Inner subgroup)	266.210 ∓ 8.480	262.950 ∓ 12.745	281.491 ∓ 14.691	248.298 ∓ 12.680	256.121 ∓ 10.127	257.638 ∓ 8.942	260.318 ∓ 9.529	261.343 ∓ 10.916	257.848 ∓ 10.549	259.067 ∓ 9.665	253.758 ∓ 13.003	257.337 ∓ 11.858	254.229 ∓ 14.818	258.782 ∓ 13.073 (High density)
FEJ (Outer subgroup)	172.433 ∓ 4.102	173.952 ∓ 3.422	177.440 ∓ 3.364	180.202 ∓ 2.358	197.358 ∓ 7.510	209 ∓ 6.363	-	-	-	-	-	-	-	173.588 ∓ 7.664 (Low density)
JB (Outer subgroup	234.340 ∓ 10.713	238.572 ∓ 10.369	257.992 ∓ 9.208	282.582 ∓ 11.270	315.626 ∓ 13.261	350.219 ∓ 11.651	374.055 ∓ 12.449	382.666 ∓ 18.1475	326	-	-	-	-	228.203 ∓ 9.571 (Low density)

FIGURE LEGENDS

Figure 1: Timing of overfeeding intervals in MBs and MCUs, and the separation of individuals into inner and outer vials after overfeeding.

Figure 2: Timing of overfeeding intervals in JBs and FEJs, and separation of individuals into inner and outer vial subgroup after overfeeding.

Figure 3: Pre-adult development time in the inner and outer vials in the (a) MB and (b) MCU populations that were overfed on different days after egg-lay, and in control vials. The black bar in the control (con) is the development time in the high-density control, while the white bar in control (con) is the development time in the low-density control. Error bars around the means are standard errors based on the four replicate populations.

Figure 4: Pre-adult survivorship in the (a) MB and (b) MCU populations that were overfed on different days after egg-lay and in the control vials (HD and LD). The HD column is high-density control while the LD column is low-density control. Error bars around the mean are standard errors based on the four replicate populations.

Figure 5: Pre-adult development time in the inner and outer vials in the (a) JB and (b) FEJ populations that were overfed on different days after egg-lay and in control vials. The black bar in the control (con) is the development time in the high-density control while the white bar in control (con) is the development time in the low-density control. Error bars around the mean are standard errors based on the four replicate populations.

Figure 6: Pre-adult survivorship in the (a) JB and (b) FEJ populations that were overfed on different days after egg-lay and in the control vials (HD and LD). The HD column is high-

density control while the LD column is low-density control. Error bars around the mean are standard errors based on the four replicate populations.

FIGURES

Figure 1

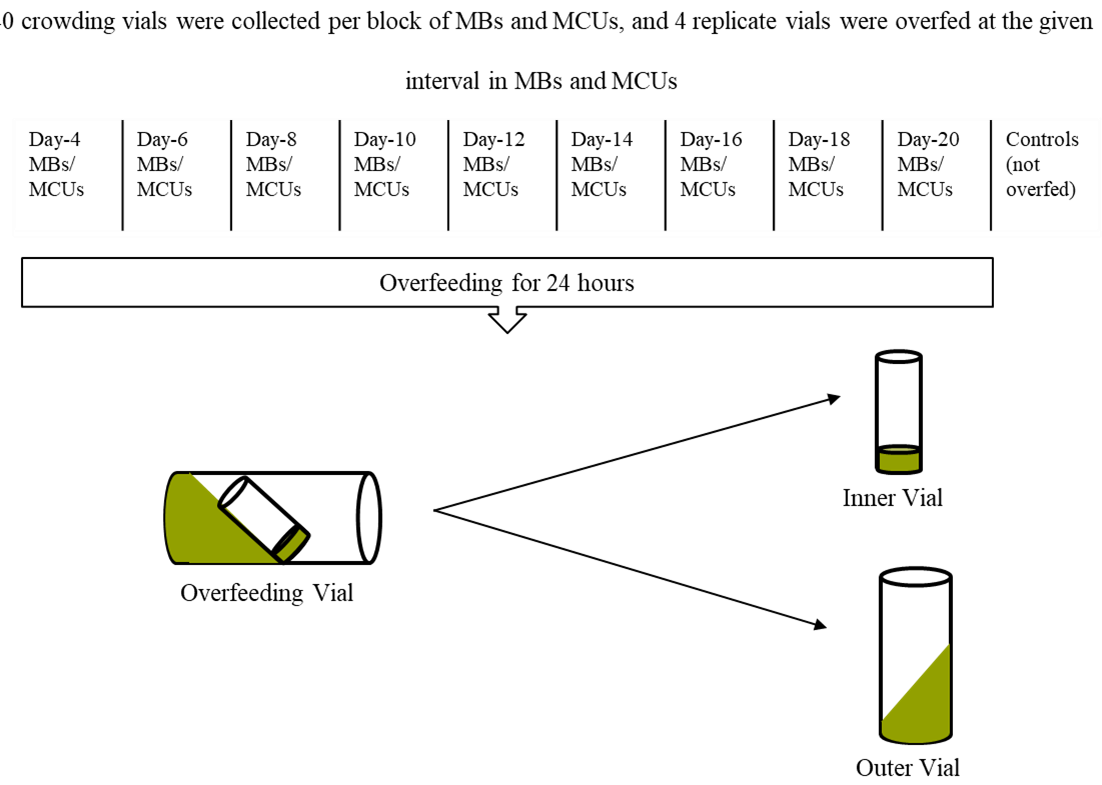


Figure 2

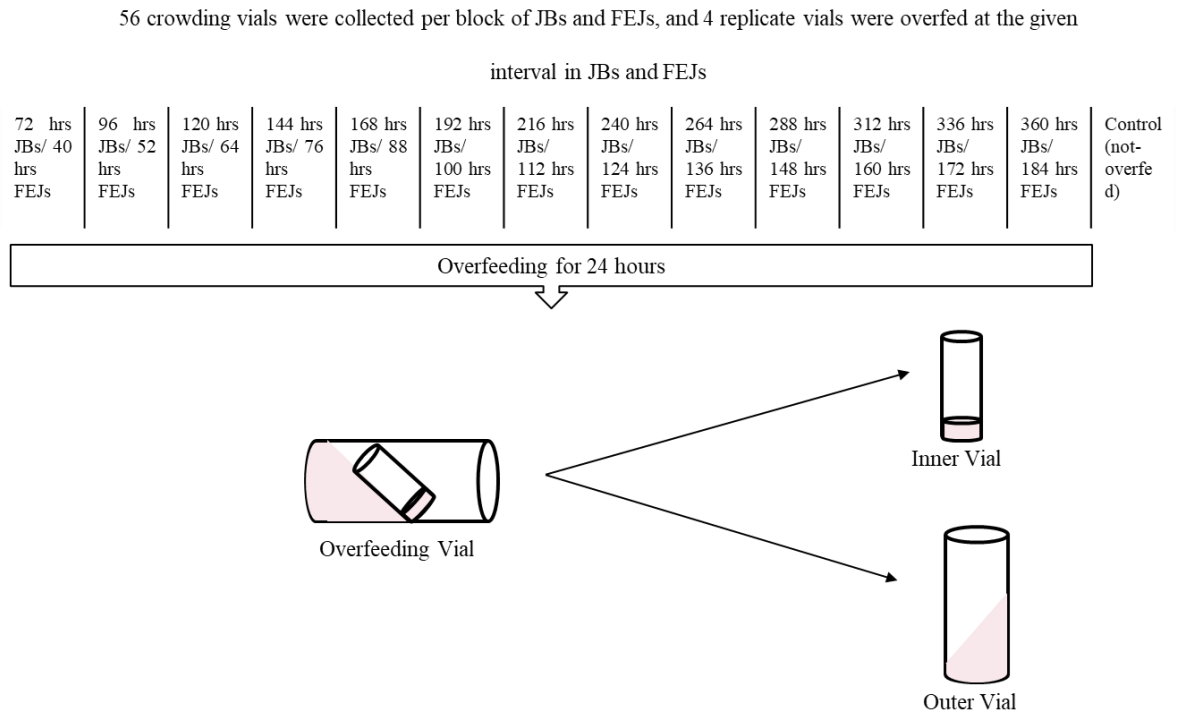

56 crowding vials were collected per block of JBs and FEJs, and 4 replicate vials were overfed at the given interval in JBs and FEJs
72 hrs JBs/ 40 hrs FEJs
96 hrs JBs/ 52 hrs FEJs
120 hrs JBs/ 64 hrs FEJs
144 hrs JBs/ 76 hrs FEJs
168 hrs JBs/ 88 hrs FEJs
192 hrs JBs/ 100 hrs FEJs
216 hrs JBs/ 112 hrs FEJs
240 hrs JBs/ 124 hrs FEJs
264 hrs JBs/ 136 hrs FEJs
288 hrs JBs/ 148 hrs FEJs
312 hrs JBs/ 160 hrs FEJs
336 hrs JBs/ 172 hrs FEJs
360 hrs JBs/ 184 hrs FEJs
Control (not-overfed)
Overfeeding for 24 hours
Overfeeding Vial
Inner Vial
Outer Vial

Figure 3

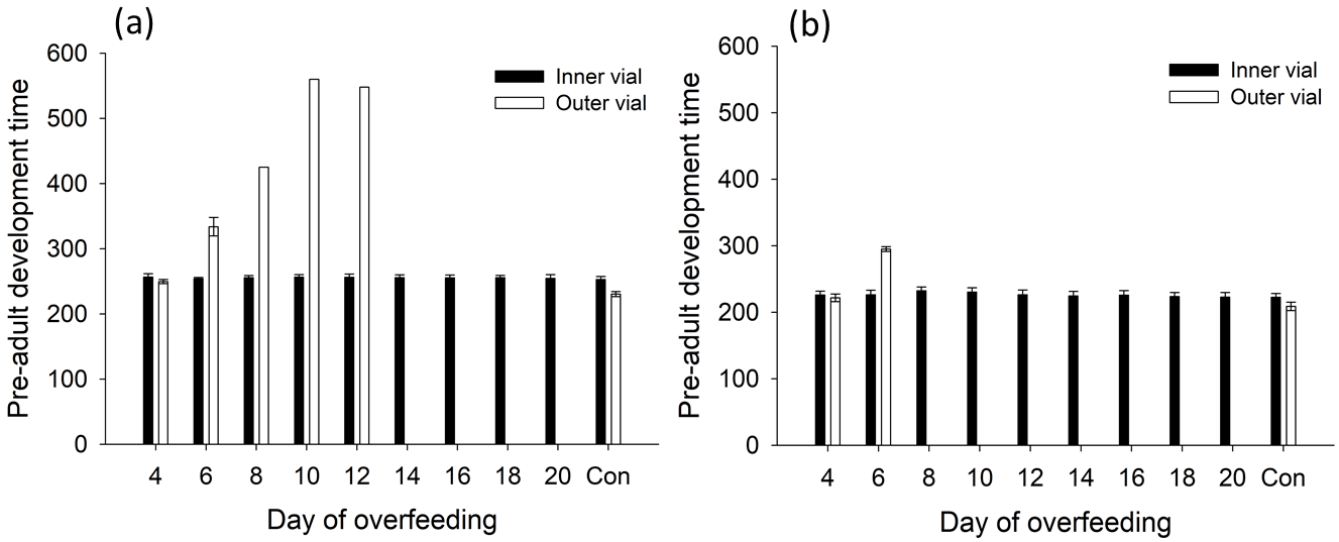

(a)
Pre-adult development time
600
500
400
300
200
100
0
Inner vial
Outer vial
4
6
8
10
12
14
16
18
20
Con
Day of overfeeding
(b)
Pre-adult development time
600
500
400
300
200
100
0
Inner vial
Outer vial
4
6
8
10
12
14
16
18
20
Con
Day of overfeeding

Figure 4

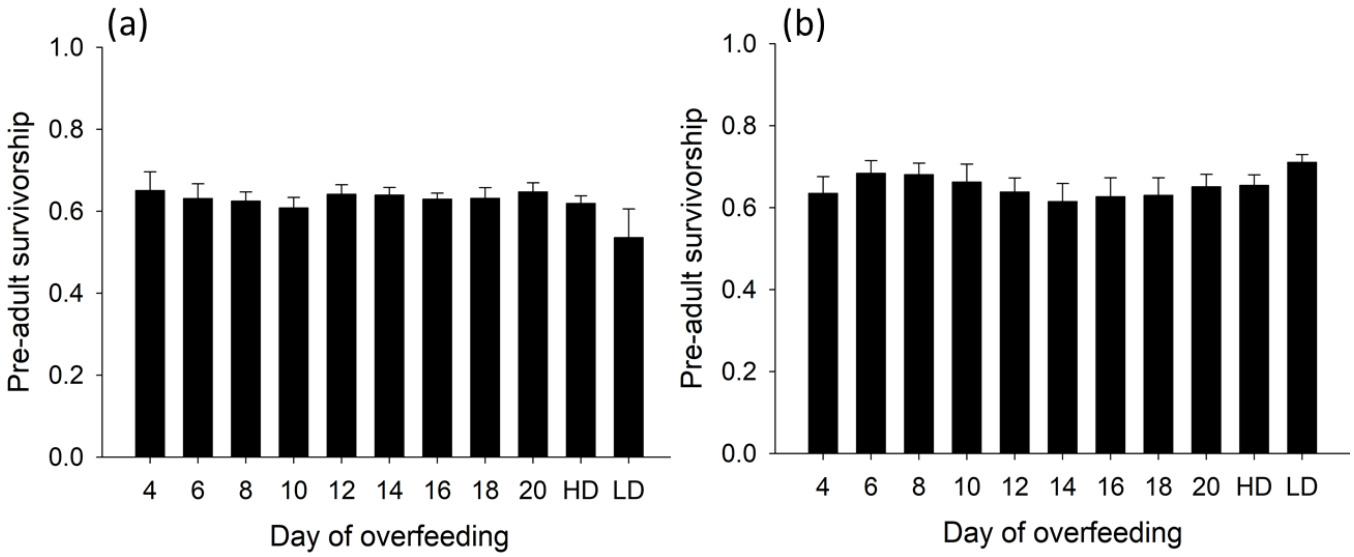

(a)
Pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
4 6 8 10 12 14 16 18 20 HD LD
Day of overfeeding
(b)
Pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
4 6 8 10 12 14 16 18 20 HD LD
Day of overfeeding

Figure 5

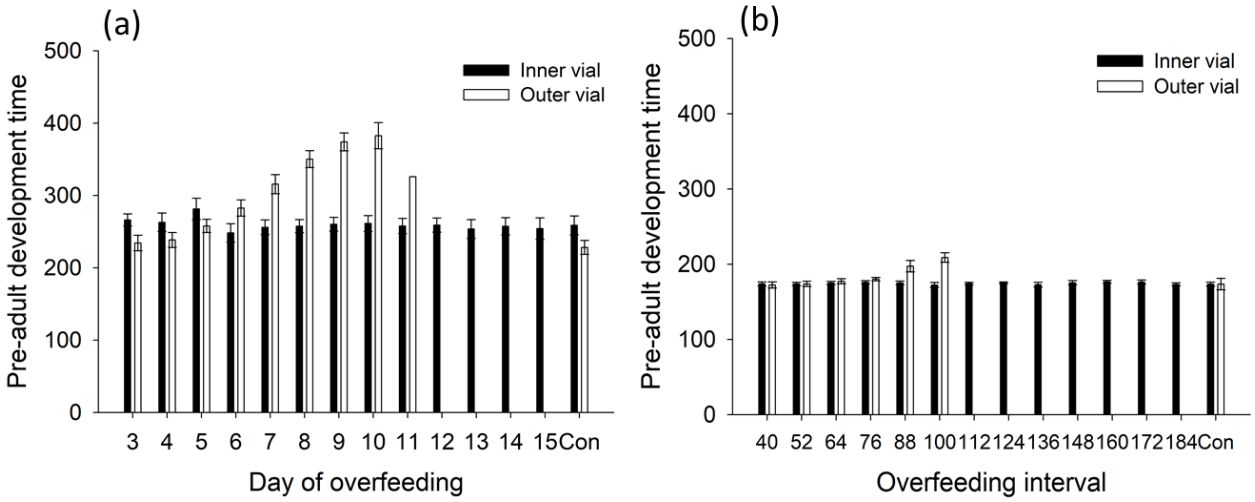
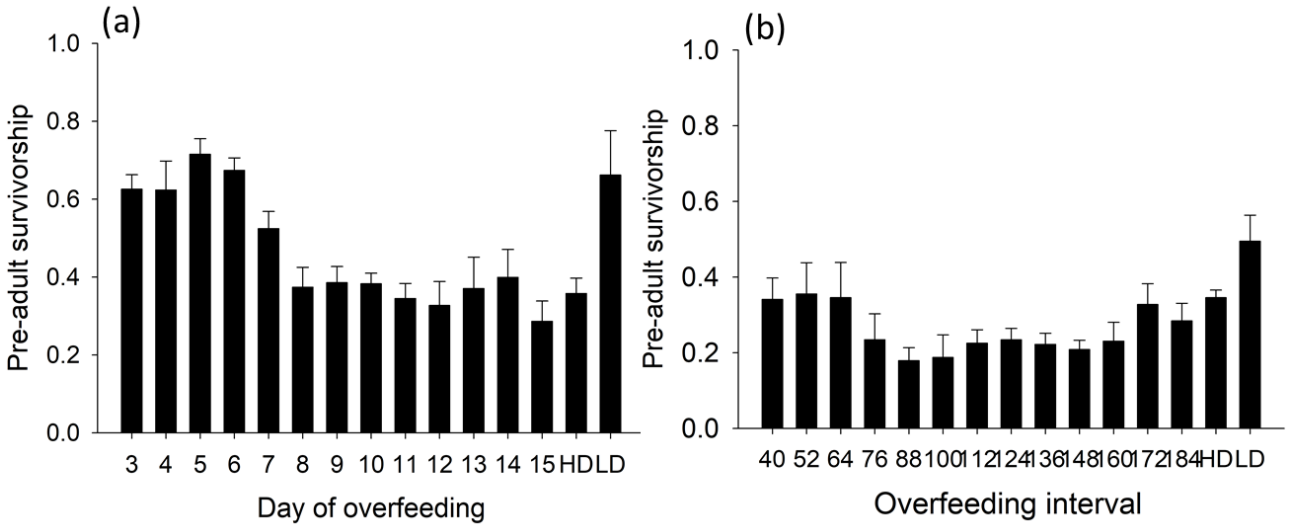

(a)
Pre-adult development time
500
400
300
200
100
0
Inner vial
Outer vial
3 4 5 6 7 8 9 10 11 12 13 14 15Con
Day of overfeeding
(b)
Pre-adult development time
500
400
300
200
100
0
Inner vial
Outer vial
40 52 64 76 88 100112124136148160172184Con
Overfeeding interval

Figure 6


(a)
Pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
3 4 5 6 7 8 9 10 11 12 13 14 15 HD LD
Day of overfeeding
(b)
Pre-adult survivorship
1.0
0.8
0.6
0.4
0.2
0.0
40 52 64 76 88 100 112 124 136 148 160 172 184 HD LD
Overfeeding interval

CHAPTER 6

Conclusions and Future Directions

CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, I have empirically examined whether density-dependent selection leads to an evolutionary increase in the constancy and persistence stability of populations and if stability is affected by variation in food abundance in the population dynamics studies. Furthermore, I examined whether differences in the implementation of density-dependent selection though differences in egg number and food amount can affect population stability attributes and if variation in key life-history traits could be linked to differences in population stability attributes, as suggested earlier (Mueller 1988, Mueller and Huynh 1994, Tung *et al* 2019). Thus, the main focus of the thesis has been examining the aspects of ecology and evolution under density-dependent selection that can affect population stability. In addition, I have also revisited the phenomenon of larval stop (Ménsua and Moya 1983), wherein the larvae in the third instar stage show developmental arrest under crowding conditions that can be reversed later if food subsequently becomes available. I examined if the evolution of faster development affects the expression of such stopped development in response to crowding. I briefly summarise below my key findings and their contributions to the existing understanding of the evolution of population stability in response to density-dependent selection.

Density-dependent selection and the evolution of population stability

One interesting question in population biology has been whether and how population stability can evolve, and one plausible explanation has been that population stability could evolve in response to density-dependent selection through an increase in the equilibrium population size (*K*) and a decrease in the maximal rate of growth (*r*), especially through a trade-off between *r* and *K* (Mueller and Ayala 1981 a). I examined this by conducting population dynamics experiments on *D. melanogaster* populations that had been selected for adaptation to larval crowding (MCUs and LCUs) and on their ancestral controls (MBs). My findings of more stable population dynamics in the crowding-adapted populations than in controls (chapters 2 and 3) suggest that density-dependent selection can indeed lead to an evolutionary increase in constancy (lower amplitude of fluctuations in population size across generations) and persistence (lower chances of extinction over generations) stability. Interestingly, my findings show that this increased constancy and persistence stability can come about even without an *r*-*K* trade-off (chapter 2), since the MCUs evolved a higher *K*

but no difference in *r* from controls, consistent with previous findings in a theoretical study (Dey *et al* 2012). It is important in this context to differentiate between the stability of the population size equilibrium, which in the logistic or Ricker models is determined entirely by *r*, and the stability (constancy) of the dynamics of population size which can be affected by both *r* and *K* (Dey *et al* 2012). Most notable was the finding that the evolution of lower sensitivity of growth rate to population density (corresponding to a less negative α in the context of the logistic or Ricker models), seems to enhance both constancy and persistence stability, as found in the MCU populations (chapter 2). The growth rate in other set of crowding-adapted populations, LCUs, was relatively more sensitive to population density which probably explains why their constancy stability did not increase with adaptation to crowding, unlike the MCUs (chapter 3). Life-history traits that contribute to greater *K* are related to the tolerance component of competitive ability, an interesting exercise for future studies could be to experimentally select populations for increased tolerance in ammonia/urea-rich food and investigate if the evolutionary increase in competitive tolerance enhances *K* and makes α less negative, thereby also enhancing constancy and persistence stability.

In my study, I found that food amount present at the larval stage could also affect population stability (proximally), such that population dynamics became more stable with an increase in food amount even in the control populations that had relatively higher *r* and lower *K* than crowding-adapted populations (chapter 2). This is because, with an increase in the food level, the density-dependent effects on life-history traits and population dynamics weaken as higher food amounts at the larval stage can sustain a higher growth rate at high density due to an increase in pre-adult survivorship (chapters 2 and 4). As population stability has been commonly observed in both natural and laboratory environments (Hassel *et al* 1976, Thomas *et al* 1980, Mueller and Ayala 1981 b, Turchin and Taylor 1992, Ellner and Turchin 1995), largely in an overlapping-generations cycle, it could be interesting to examine if the differences in population stability persist when the dynamics of the crowding-adapted and control populations are studied in an overlapping-generations cycle.

The evolution of greater stability in the crowding-adapted MCU populations, as reported in chapter 2, support the hypothesis that population stability can evolve as a by-product of life-history evolution in response to different selection pressures that populations face in their environment, such as density-dependent selection (Mueller and Ayala 1981 a, Dey *et al*

2012) or even regular life-history evolution in different environments not involving changed density (Prasad *et al* 2003, Dey *et al* 2008). Since differences in the way density-dependent selection is experienced are expected to result in differences in the precise suite of traits that populations may evolve (Sarangi *et al* 2016, Sarangi 2018), I also examined population stability in the LCU populations that are also selected for adaptation to larval crowding, but at a different combination of egg number and food amount than the MCUs. I found that the LCUs have evolved higher persistence stability but they have not evolved higher constancy than the controls (chapter 3). The evolution of higher persistence stability, possibly came through the evolutionary increase in *K* (although statistically not significant) and more likely through lower sensitivity of growth rate to population density (less negative α) than seen in the control populations. The stability attributes of LCUs are different than the MCUs which evolved both enhanced constancy and persistence stability. The difference in the stability attributes that evolved in the MCUs and LCUs is most likely due to these two sets of populations having faced different types of density-dependent selection at the larval stage, as they were subjected to chronic larval crowding at different combinations of egg number and food amount. These findings highlight that while density-dependent selection can lead to an evolutionary increase in population stability, the nature of density-dependent selection experienced can affect which population stability attributes are evolved.

This conclusion is further supported by an earlier study on another set of crowding-adapted *D. melanogaster* populations, the CUs, which were also selected in similar larval competitive conditions as the LCUs and did not evolve enhanced constancy stability (Mueller *et al* 2000). In contrast, another earlier study found the evolution of both higher constancy and persistence stability in a set of *D. ananassae* populations (ACU) that were selected at the same egg number and food amount as MCUs (Dey *et al* 2012). These multiple lines of evidence suggest that the evolution of population stability attributes in *Drosophila* is shaped by the nature of density-dependent selection experienced at the larval stage, and both constancy and persistence stability evolve when an extreme form of larval crowding is experienced as in MCUs and ACUs (~600 eggs/1.5 mL) as opposed to higher egg numbers but at high food amounts as in the CUs (1000~1500 eggs/6-7 mL) and LCUs (~1200 eggs/6 mL).

One possible caveat to these inferences is that stability was possibly higher in the MCUs because I carried out the population dynamics experiment in a food regime similar to the

maintenance regime of the MCUs (i.e. low amount of food for larvae: 1/1.5 mL). In such a regime, the MCUs are likely to be better adapted than the LCUs, which were selected at relatively higher amounts of food at the larval stage. As the food level influences traits such as pre-adult survivorship which enhance *K*, it should be interesting to see whether the observed differences between stability attributes of MCUs and LCUs sustain when the population dynamics experiments are conducted at a food level similar to the maintenance regime of LCUs. However, one will have to be wary of the food level in the population dynamics experiment because at high food amounts the density effects will weaken for both MCUs and LCUs which may prevent the detection of differences in the constancy stability between MCUs and LCUs.

Since I had inferred the evolution of population stability in MCUs and LCUs by comparing their population dynamics with the controls (MBs), I scaled their difference with the common control MB populations, to further make inferences about the differences between MCUs and LCUs in their stability and demographic attributes. Interestingly, I found that, in terms of percentage change relative to controls, the MCUs and LCUs differed only in constancy stability, whereas there was no difference in their persistence stability, *r*, *K*, the sensitivity of growth rate to population density (α), and average population size. However, upon a further comparison of realized growth rates in MCUs and LCUs at different population bin sizes to understand how the sensitivity of growth rate changed with an increase in density, I found that the MCUs had a higher rate of growth than controls at a wider range of population density than the LCUs, whose growth rate was higher than controls only in a narrow zone of population density. This is also reflected in evolution of somewhat lower sensitivity of pre-adult survivorship to larval density in MCUs as compared to LCUs (chapter 4). Thus, it appears that a higher rate of growth at wider range of population densities can explain why MCUs have evolved higher constancy stability than the LCUs populations (see chapter 3). A higher realized growth rate across a wide range of population densities can lower the amplitude of fluctuations by raising the troughs of fluctuations in population size. Such evolution of lower sensitivity of growth rate to density (less negative α) could result in the evolution of enhanced constancy without a change in *r* and *K*, which seems to be explaining the differences between MCUs and LCUs in constancy stability. A lot of work on population growth rate models like logistic and the Ricker has tended to focus on the effects of *r* and *K* on stability, but my findings suggest that populations may also evolve higher constancy stability through differences in the sensitivity

of growth rate (analogous to α in the logistic and Ricker models) at different population density, independently of changes in r and K. Therefore, future studies could examine if population stability can evolve through evolutionary changes in the sensitivity of growth rate to population density without evolutionary changes in maximal growth rate or equilibrium population size, preferably in a model-free framework, although the θ-logistic (Gilpin *et al* 1976) does permit modelling such variations.

Evolution of population stability and density-dependent feedback loops

Since the difference in population stability attributes of MCUs and LCUs could come from differences in their life-history traits and their sensitivity to population density, I also examined if MCUs and LCUs differed in the sensitivity of different life-history to population density. Specifically, I aimed to investigate key density-dependent feedback loops that shape the dynamics of populations in the LH food regime (Mueller and Huynh 1994, Joshi *et al* 1998, Tung *et al* 2019), namely the sensitivity of pre-adult survivorship to larval density, sensitivity of fecundity to larval density, sensitivity of dry body-weight to larval density, and sensitivity of adult survivorship to adult density. My findings indicate that the MCU populations have evolved higher pre-adult survivorship than the LCU populations at a wider range of larval densities (chapter 4) while the sensitivity to other life-history traits to density did not differ between them. Since higher pre-adult survivorship is a major factor affecting population stability, it is possible that because of this higher pre-adult survivorship at a wide range of larval densities in MCU populations, the MCU populations have evolved both higher constancy and persistence stability. This was also observed in the demographic attributes between MCUs and LCUs because MCUs had lower sensitivity of growth rate to population density (less negative α) and high rate of growth at medium to high population densities whereas the sensitivity of growth rate to population density was somewhat higher in the LCUs. Since higher constancy and persistence stability could come from the interaction of multiple life-history traits, future studies could examine how these sensitivities of life-history traits to density interact with one another and govern differences in population stability attributes.

Larval stop in populations that have evolved faster pre-adult development

I also explored the phenomenon of larval stop in different sets of selected populations in chapter 5. I looked at how the evolution of faster development influences the expression of larval stop which is seen in the later larval stages. My findings suggest that the larval stop is lost in the populations that are selected for faster development and early reproduction (FEJ) and in populations selected for adaptation to larval crowding (MCU), which have also evolved reduced development time relative to controls, but to a much lesser degree than in the FEJs (Sarangi *et al* 2016, Ghosh *et al* 2019). This could be because FEJ populations have evolved a faster development in response to the direct selection for faster development and early reproduction, due to which they have evolved a very small body size as compared to the controls. Interestingly, such crowding leads to high pupal mortality in the FEJs (personal observation), leading to a decrease in pre-adult survivorship due to crowding, which might be because FEJs have a lower tolerance of metabolic waste (Joshi *et al* 2001) than controls. On the other hand, the MCU populations are selected for adaptation to larval crowding thus for them the crowding regime is not a novel environment as it is for the other populations, thus loss of the larval stop trait in MCUs could come either from faster development (correlated response to selection for adaptation to larval crowding) or due to the density-dependent selection. The control populations (JB) and MB also do not show the extension of the development time to a degree as found in the earlier populations in the Ménsua and Moya study (1983). I speculate that the difference with Ménsua and Moya's study could come from their populations being maintained in an overlapping generation cycle. The populations maintained in an overlapping generation cycle incorporate the larvae that are also eclosing late in the breeding pool for the next generation, whereas the populations that are maintained in the discrete generation cycle do not incorporate the larvae that may develop later. Since the expression of larval stop trait is linked with longer development durations at the larval stage, the populations maintained in a discrete generation cycle may not show the expression of larval stop trait to a large degree as has been shown by the populations used by Ménsua and Moya (1983). In future, it will be interesting to study larval stop in the LCU populations as they have a later component of adult eclosion due to maintenance in high amounts of food.

To conclude, my PhD thesis research shows that density-dependent selection seems to lead to an evolutionary increase in persistence stability in a fairly robust manner, but whether density-dependent selection leads to the evolution of constancy stability depends upon the

type of life-history traits and their sensitivity to population density that evolve in response to density-dependent selection. It appears that selection in a very high egg number at low food amount can lead to an evolutionary increase in both constancy and persistence while selection in a very high egg number but at high food amount can only increase persistence. Additionally, the expression of larval stop is lost if populations evolve faster development, and the maintenance of the populations in a discrete generation cycle could also affect the degree to which larval stop is expressed. From a broader perspective, my work also suggests that our understanding of density-dependent selection and its effects on population stability would be greatly enhanced by pursuing model-free theoretical investigations sequentially linking the ecology of how exactly crowding was experienced to specific changes in life-history traits and their sensitivity to density, and those in turn to how realized population growth rates change with increasing density, eventually affecting the return map of the system and, therefore the dynamics and stability of population size.

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